INTERACTION OF PHORBOL ESTERS WITH CHRONIC LYMPHOCYTIC LEUKAEMIA B CELLS: EFFECTS OF CALCIUM, GOLD AND ZINC

A thesis submitted to the University of Adelaide as a requirement for the degree of Doctor of Philosophy

by

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ABSTRACT

The human B cell subset, identified by co-expression of the CD5 antigen and a phospholipid-protein complex with receptor activity for mouse erythrocytes (MER), is implicated in the pathogenesis of rheumatoid arthritis and is monoclonally expanded in B-type chronic lymphocytic leukaemia (B-CLL). Little is known concerning the early biochemical events which convey differentiation signals in these and other B cells. It is also unclear whether drugs and other biologically active substances modify these biochemical pathways. A useful model for these studies is the activation of B-CLL cells by plant-derived phorbol esters, specific ligands and activators of protein kinase C (PKC). Phorbol esters induce plasmacytoid differentiation, in vitro, in B-CLL cells. A convenient marker of an early response of B-CLL cells to phorbol ester is loss of expression of MER. One aim of the experiments described in this thesis was to investigate some of the steps leading to inhibition of MER.

Inhibition of MER did not involve change in its phospholipid moiety, which is responsible for specific binding of the mouse red cell, but did involve loss of some tightly associated protein that is essential for the rosette bond.

Specific high affinity phorbol ester receptors mediated the inhibition of MER. An essential event was the translocation of the phorbol ester receptors (and PKC activity) from cytosol to the particulate fraction of the cells.

A rise in free cytosolic calcium enhanced inhibition of MER by phorbol esters and enhanced the translocation of phorbol ester receptors resulting in their increased affinity. Activation of surface immunoglobulin by anti-Ig, or of G proteins by fluoride,

induced similar changes in phorbol ester binding as well as inhibition of MER.

However, the loss of expression of MER induced by phorbol esters was not blocked by inhibitors of PKC-dependent phosphorylation and only weakly induced by diacylglycerol, the putative endogenous analogue of phorbol esters. Certain other events associated with action of phorbol esters did not appear to be involved in loss of MER expression.

During these studies it was observed that the anti-rheumatic gold drug auranofin, which modulates some actions of phorbol esters in other cell types, also rapidly inhibited MER at concentrations which are relevant therapeutically. Zinc which has a similar ligand-binding specificity to gold also inhibited MER when given to the cells in the presence of a zinc ionophore, pyrithione. The inhibition of MER by phorbol esters was blocked by the zinc chelating agent phenanthroline.

Both auranofin and zinc pyrithione caused a two or three fold increase in binding of phorbol dibutyrate (PDBu) to cells, with a large increase in the apparent total number of receptors. Similar effects occurred in cell-free homogenates. Gold and zinc greatly enhanced translocation of PDBu receptors to a detergent-insoluble compartment of the cell which may be the cytoskeleton or nucleus. Some of these effects of gold and zinc may be mediated by the recently-described cysteine-rich sites in the regulatory domain of PKC. Various actions of calcium, gold and zinc on phorbol ester receptors in B-CLL cells were confirmed in other types of normal cells.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and to the best of my knowledge and belief contains no material previously published or written by any other person, except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan.

PETER DAVID ZALEWSKI

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Zalewski PD, Hurst NP, Valente L and Forbes IJ (1989). Modulation of the mouse erythrocyte receptor in human B cells by auranofin in a thiol-dependent manner. Submitted for publication.

Giannakis C, Forbes IJ and Zalewski PD (1989). Affinity of protein kinase C for a Zn chelate affinity column. Submitted for publication.

Parts of this work were communicated at

- The Australian Society for Medical Research, Canberra (December 1985 and 1986)
- The 4th Federation of Asian and Oceanic Biochemists Congress, Singapore (December 1987)
- The ISU Signal Transduction Symposium, Ames, Iowa, (September 1988)

ABBREVIATIONS

Anti-Ig	Antibody to immunoglobulin
B	Bound ligand
B-CLL	B cell type chronic lymphocytic leukaemia
BSA	Bovine serum albumin
$[Ca^{++}]_i$	Intracellular free calcium ions
cAMP	Adenosine cyclic monophosphate
cGMP	Guanosine cyclic monophosphate
cIg	Cytoplasmic immunoglobulin
CLL	Chronic lymphocytic leukaemia
DMSO	Dimethylsulphoxide
DOG	1,2-sn-dioctanoylglycerol
EC_{50}	Concentration of stimulus causing 50% inhibition of rosetting
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol-bis-(beta-aminoethylether)- N, N, N' N'-tetra-acetic acid
F	Free ligand
FCS	Foetal bovine serum, heat-inactivated
fmol	Femtomole
GlcNac	N-acetylglucosamine
IP_3	Inositol $_{1,4,5}$trisphosphate
Kd	Equilibrium dissociation constant
kDa	Kilodalton
2-ME	2-mercaptoethanol
MER	Receptor for mouse erythrocytes
ml	Millilitre
ul	Microlitre
uM	Micromolar
nM	Nanomolar

NP40	Nonidet P40
OAG	1-oleoyl-2-acetyl glycerol
PBS	Phosphate buffered saline, pH 7.4
PBS/ALB	PBS containing 1mg/ml albumin
PDBu	Phorbol 12,13 dibutyrate
^{3}H-PDBu	Tritium-labelled phorbol dibutyrate
PDD	Phorbol 12,13 didecanoate
PE	Phosphatidylethanolamine
PIP_2	Phosphatidylinositol$_{4,5}$bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMNL	Polymorphonuclear leukocyte
pmole	Picomole
PMSF	Phenylmethylsulphonylfluoride
PS	Phosphatidylserine
quin-2	Quin-2 acetoxymethyl ester
Rf	Relative migration on chromatography gels
RPA	12-O-retinoyl phorbol 13-acetate
RPMI/FCS	RPMI culture medium containing 10% FCS
Rt	Total number of PDBu receptors
sem	Standard error of mean
sIg	Surface immunoglobulin
Tf	Transferrin
TLCK	N-tosyl-L-lysyl-chloromethylketone
TMB-8	3,4,5- trimethoxybenzoic acid 8-(dimethylamino) octyl ester
TPA	12-O-tetradecanoyl phorbol 13-acetate
TPEN	Tetrakis (2-pyridylmethyl)ethylene diamine

CHAPTER ONE

HISTORICAL INTRODUCTION

1.1 INTRODUCTION

Plant-derived phorbol esters have been extensively studied because of their potent tumour-promoting activity in experimental carcinogenesis [reviewed in Blumberg 1980]. More recently, it has been recognized that these substances interfere with the process by which cells are activated to proliferate or differentiate [Rovera et al. 1979].

In 1980, Tötterman and colleagues reported that 12-O-tetradecanoyl phorbol acetate (TPA), the most potent of the phorbol esters, was a very efficient inducer of plasmacytoid differentiation in B-type chronic lymphocytic leukaemia (B-CLL) cells. This finding indicated that B-CLL cells were capable of being activated, contrary to previous views that most populations of B-CLL cells were poorly responsive to polyclonal B cell activators. The observation that phorbol esters induce differentiation in B-CLL cells has been confirmed in many laboratories and has stimulated new research into the biochemical events that mediate activation of normal and malignant B cells. A major aim of the experiments reported in this thesis was to further our understanding of the early actions of phorbol esters on B-CLL cells.

B-CLL cells are the malignant counterpart of a minor subset of B cells identified by expression of a receptor for mouse erythrocytes (MER) and the CD5 antigen. This normal subset is thought to be involved in the production of small amounts of natural autoantibodies as well as over-production of these antibodies in autoimmune disorders [reviewed in Lydyard et al. 1987]. Because these normal CD5+ve B cells are relatively scarce, study of the biochemistry of their activation is difficult. In

contrast, the peripheral blood of patients with B-CLL contain monoclonal, relatively homogeneous populations of these cells in large numbers.

Study of the early actions of phorbol esters in B-CLL cells may provide new information both on early activation events in CD5+ve B cells and on general cellular actions of phorbol esters. A limitation with such studies is that B-CLL cells may be abnormal in the properties being tested. Abnormalities may be attributed to the malignancy of the cells, the prior exposure of these cells to cytotoxic drugs in vivo or the fact that they are almost always cells from elderly people. For this reason, some experiments reported in this thesis were also performed on some normal cells including peripheral blood mononuclear cells, polymorphonuclear leukocytes or platelets. It was beyond the scope of this thesis to attempt to compare the responses to phorbol esters in normal and malignant CD5+ve B cells although such studies may reveal abnormalities that are associated with the malignant cells and which provide clues to the process(es) that are involved in the accumulation of these cells in vivo. Methods are required that will provide highly enriched populations of normal CD5+ve B cells in sufficient quantity for biochemical analysis

The main phenotypic changes in phorbol ester-treated B-CLL cells, including increased production of secretory immunoglobulin M and expression of MHC class II antigens and growth factor receptors, occur only after several hours to days in culture [Tötterman et al. 1981a,b] and are not easily correlated with early, often transient events in the activation such as a rise in cytosolic free calcium.

In earlier work from our laboratory, it was observed that

there was a rapid loss of expression of MER in B-CLL cells and normal MER+ve B cells during exposure to phorbol esters [Forbes et al. 1981]. Loss of expression of MER in phorbol ester-treated cells is readily quantified by a rosette inhibition assay, occurs at nanomolar concentrations of phorbol esters and is apparently irreversible. Loss of expression of MER also occurs during human B cell activation, in-vivo, as well as in response to a variety of stimuli, in vitro, indicating that it is not an artifact peculiar to phorbol ester action.

There has been partial characterization of the receptors involved in the binding of mouse red cells. Two receptor-ligand interactions mediate the binding of mouse red cells to the B lymphocyte surface [Forbes et al. 1982b]. One of these receptors is released from B-CLL cells, by mild trypsinization, in a form which retains its receptor activity [Zalewski et al. 1982]. It is a complex of phospholipid, largely phosphatidylethanolamine, in tight association with protein [Zalewski et al. 1984a]. The phospholipid forms a binding site for the mouse erythrocyte while the protein may be important for the intercellular adhesion.

The initial aim of the work in this thesis was to identify events which are involved in the inhibition of MER rosetting by phorbol esters including (a) possible changes in the structure and red cell binding activity of MER during treatment of cells with phorbol esters, (b) the role of specific receptors for phorbol esters in the inhibition of MER and (c) the role of those biochemical events following binding of phorbol esters to the B-CLL cells which are responsible for the inhibition of MER.

Phorbol dibutyrate (PDBu) was used in most of these studies because in radiolabelled form (^{3}H-PDBu) it allows measurement of

specific phorbol ester receptors in cells. It has been more difficult to assay these receptors with the highly lipophilic TPA.

Early during these studies, work from other laboratories identified the phorbol ester receptor, from brain, spleen and other tissues, as being protein kinase C (PKC), a family of closely-related enzymes which mediate the response of cells to growth factors and hormones [reviewed in Nishizuka 1988]. Phorbol esters substitute for the second messenger diacylglycerol in activation of PKC. Activation of PKC requires two other cofactors, phospholipid and calcium. An early step in the response of cells to phorbol esters and diacylglycerol is translocation of PKC from cytosol to membrane [Kraft and Anderson 1983]. The association of PKC with membrane phospholipid allosterically activates the catalytic site. PKC regulates a large number of intracellular events, some of which are thought to be critical for cell growth.

In the light of these findings, particular emphasis was placed in this thesis on the role of calcium ions, PKC and PKC-dependent events in the mechanism of loss of expression of MER in phorbol ester-treated B-CLL cells. During a study of the effect on B-CLL cells of various agents known to modulate PKC-dependent events in cells, it was observed that the anti-arthritic gold compound auranofin caused rapid inhibition of MER and greatly augmented binding of phorbol ester to B-CLL cells. These effects were due to the gold moiety. The mechanism of action of auranofin was therefore studied in detail.

These studies with auranofin may be relevant to the action of auranofin on the putative auto-antibody producing MER+ve, CD5+ve B cells in rheumatoid arthritic patients since auranofin

is immunomodulatory for B cells both in vivo and in vitro and suppresses rheumatoid factor production in vivo.

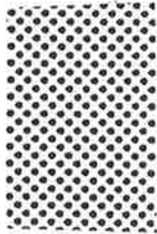
Studies with auranofin may also provide new insights into the regulation of PKC. In particular, it has recently been revealed that the regulatory domain of PKC contains two cysteine-rich motifs that also occur in some proteins which bind zinc and certain other metals [Parker et al. 1986]. In these other proteins, zinc induces conformational change which causes the cysteine-rich regions to assume a finger-like structure (zinc finger) and to interact with DNA [see chapter 1.12). Two predictions are that PKC is a zinc-containing enzyme and that some of the effects of auranofin on PKC are explained by the interaction of gold with these cysteine-rich sites. The effect of zinc on early events in B-CLL cells was studied using the lipophilic pyrithione as a zinc ionophore and phenanthrolene as a relatively specific chelator of zinc.

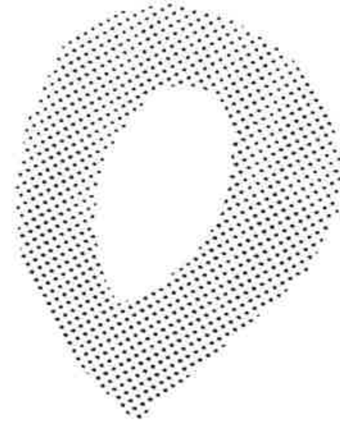
Fig 1.1 shows a model of the experimental system used to investigate the interaction of phorbol esters with B-CLL cells and their inhibition of MER.

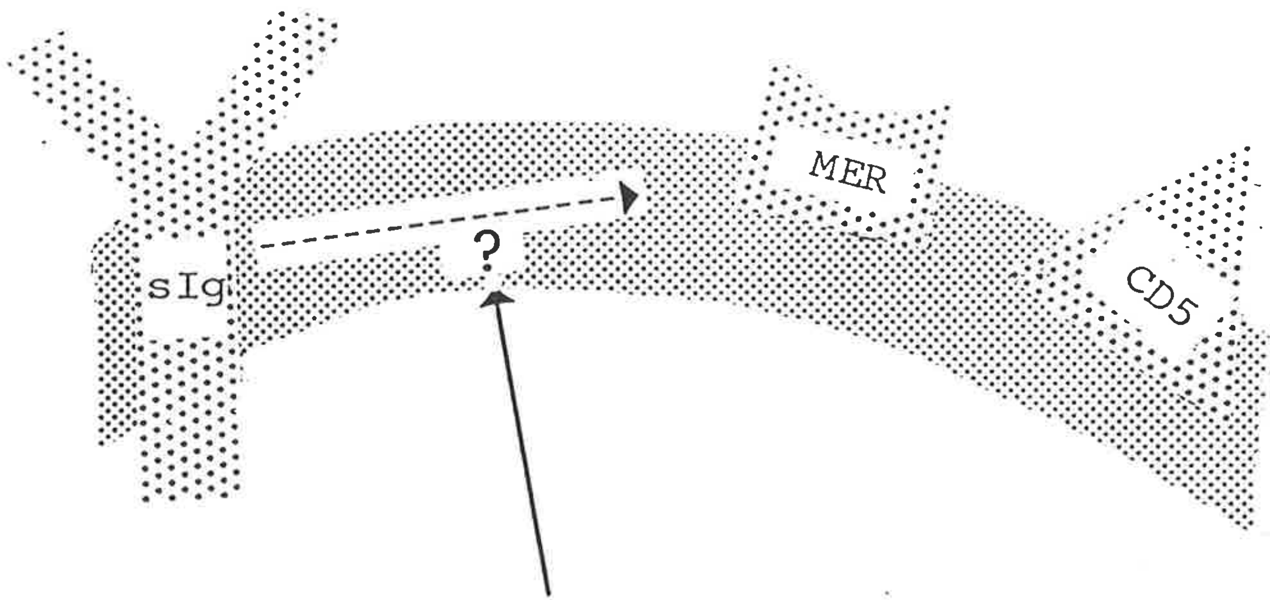
The historical introduction reviews the biology of the B-CLL cell and its relationship to normal B cell lymphopoiesis (chapter 1.2), the nature of MER (chapter 1.3), activation of B cells by phorbol esters and other stimuli (chapters 1.4 and 1.5), general biological effects of phorbol esters (chapter 1.6), the cellular receptors for phorbol esters, PKC (chapters 1.7 and 1.8), translocation and down-regulation of PKC (chapter 1.9), early biochemical events in activation of B cells (chapter 1.10) and the biological actions of auranofin and other gold compounds (chapter 1.11) and of zinc compounds (chapter 1.12).

MOUSE ERYTHROCYTE

ANTIGEN







PHORBOL ESTER CALCIUM GOLD ZINC

Legend to Figure 1.1 Model for the experiments described in this thesis

Schema showing the putative physiological pathway coupled to triggering of sIg and leading to inhibition of MER and subsequent cell activation in MER+ve, CD5+ve B-CLL cells. Phorbol esters, calcium, zinc and gold interact with this pathway at various points.

1.2 B-TYPE CHRONIC LYMPHOCYTIC LEUKAEMIA CELLS AND THEIR NORMAL TISSUE COUNTERPARTS

The B-CLL cell was used for most of the experiments described in this thesis. This section descibes some features of this type of cell. The biology of B-CLL has been recently reviewed [Freedman and Nadler 1987, Gale and Foon 1987].

1.2.1 CHRONIC LYMPHOCYTIC LEUKAEMIA, A B CELL MALIGNANCY

The disease

Chronic lymphocytic leukaemia (CLL) is a common form of leukaemia in Caucasians, affecting mainly elderly people and with a higher incidence in males [Rundles 1972]. It has been defined as "a generalized, progressive and self-perpetuating proliferative abnormality of the lymphoid tissues, affecting the small lymphocyte, particularly" [Dameshek and Gunz 1964]. The leukaemic cells are present, often in huge numbers, in blood and other lymphoid tissue and there is increasing infiltration of the cells in the bone marrow and non-lymphoid tissue as the disease progresses [Rundles 1972].

CLL is often asymptomatic although later in the disease chemotherapy may be necessary [Zacharski and Linman 1969]. The very high white cell count in some patients is lowered by leukapheresis which involves selective removal of blood leukocytes by differential centrifugation of the blood.

CLL cells

Virchow [1856] first described the leukaemic cells in the blood of patients with CLL as "small, colorless, non-granular globules." CLL cells are mostly small, quiescent lymphocytes, having diploid DNA and low RNA content [Andreeff et al. 1980].

Most populations of CLL cells are derived from bone-marrow derived (B) cells [Seligmann et al. 1973], that family of

lymphocytes which respond to antigen by maturing into antibody-producing plasma cells. The other major family of lymphoid cells, thymus-dependent T cells, mediates cellular immunity and regulates antibody production by activated B cells [Cooper et al. 1965].

CLL is thought to arise as a result of some abnormality in the development of B cells. Steps involved in the development of normal B cells are summarized below.

Development of normal B cells

Normal B cell development begins in the placenta, proceeds next in the foetal liver and finally bone marrow [reviewed in Calvert et al. 1984]. After birth, the bone marrow is the main site, producing, in mice, up to one fifth of the total number of peripheral B cells each day. Marrow stromal cells may provide the appropriate microenvironment for B cell development [Dorshkind 1987].

B cells derive from the same stem cells as other blood cells. An early step is rearrangement of several mini-gene segments on chromosome 14, forming a functional immuno-globulin mu heavy chain gene in one of the alleles. Rearrangement of the different heavy chain genes generates much of the diversity of antibody specificity [reviewed in Yancopoulos and Alt 1986]. When the mu heavy chain is formed in the cytoplasm, but not yet in the membrane, the cell is referred to as a pre-B cell [Gathings et al. 1977]. Later, pre-B cells undergo rearrangement of the three light chain Ig gene segments, first at the kappa chain gene locus on chromosome 2 and then, if this is unsuccessful in both alleles, it occurs in the lambda gene locus on chromosome 22 [Hiete et al. 1981, Korsmeyer 1985].

Successful rearrangement of both heavy and light chain genes allows expression of a complete surface IgM (sIgM) molecule containing either kappa or lambda light chain. Some early B cells also acquire MER [Gupta et al. 1976b], Fc_{gamma} receptors [Hämmerling et al. 1976], Fc_{mu} receptors [Pichler and Knapp 1977], some molecules also found on T cells, including the CD5 antigen [Bofill et al. 1985], and some B cell specific antigens defined by monoclonal antibodies [reviewed in McKenzie and Zola 1983].

Early B cells migrate to the spleen and lymph nodes where they acquire complement receptors [Calvert et al. 1984] and sIgD of the same antigenic and idiotypic specificity as the sIgM, and with the same light chain isotype [Blattner and Tucker 1984]. The delta heavy chain (in sIgD) is formed from the same RNA transcript as the mu heavy chain by alternative splicing.

Monoclonality of B-CLL cells

The monoclonality of B-CLL cells has been established by studies showing that the sIg is of a single light chain type and unique idiotype [reviewed in Freedman and Nadler 1987], that there is only one type of heavy and light chain Ig gene rearrangement [Hiete et al. 1981] and that, in patients heterozygous at the glucose-6-phosphate dehydrogenase gene locus, the CLL cells express only one of the isozymes [Fialkow et al. 1978].

1.2.2 MALIGNANCY OF THE CD5+ve, MER+ve SUBSET

While B-CLL cells have many surface markers typical of most normal B cells including sIgM and/or sIgD, Fc_{gamma} receptors, MHC class I and II antigens and several other B cell-specific antigens [see Caligaris-Cappio and Janossy 1985], they differ from most normal B cells in expression of some other surface

molecules.

Expression of CD5 and MER on B-CLL cells and some normal B cells

The B-CLL cell expresses the CD5 (cluster of differentiation #5) antigen, originally called Leul or Tl [Boumsell et al. 1980, Royston et al. 1980, Wang et al. 1980]. CD5 antigen is a 67 kilodalton (kDa) membrane protein, detected by labelling with monoclonal antibody, that is predominantly found on T cells, but also at a low density on a subset of normal B cells [Caligaris-Cappio et al. 1982, Bofill et al. 1985]. In adult lymph nodes, CD5+ve B cells are a minor subset found in or around germinal centres [Bofill et al. 1985]. They are also found in peripheral blood [Antin et al. 1986, Casali et al. 1987] and are enriched in peritoneal and pleural cavities [Antin et al. 1986]. It has been suggested that this subset is the normal tissue counterpart of the B-CLL cell [Caligaris-Cappio et al. 1982, Bofill et al. 1985]. A similar subset ($Lybl^{+}$) is found in mice [Hayakawa et al. 1985].

B-CLL cells also express a receptor for mouse erythrocytes (MER) that binds mouse erythrocytes in a rosetting assay [Stathopoulos and Elliott 1974, Forbes and Zalewski 1976, Catovsky et al. 1976, Gupta et al. 1976a]. This is distinct from the structure on human T cells which binds sheep erythrocytes [Hünig et al. 1986]. Leukaemic cells in the bone marrow and lymph nodes may express less MER [Cherchi and Catovsky 1980]. The MER rosette test is useful for identification of subtypes of CLL and for identification of a B lymphoproliferative disorder when the peripheral blood count is low or when there are large numbers of SIg-ve cells present [Forbes et al. 1979a, Cherchi and Catovsky 1980]. In some patients with CLL, sIg is lost during the course

of the disease but MER is retained [Forbes et al. 1979a]. About 20% of cases of CLL have leukaemic MER-ve cells, including uncommon cases of T cell CLL [Gupta et al. 1976a, Forbes et al. 1982b] and atypical cases of B-CLL, often with a distinct cellular morphology and a very intense staining for sIg [Catovsky et al. 1976, Gupta et al. 1976a, Forbes et al. 1978]. Transformation of MER+ve CLL to the MER-ve form, without change in sIg isotype, has been seen [Forbes et al. 1979b].

Like CD5, MER is not a leukaemia-associated marker but is found on some normal B cells [Forbes et al. 1976, Gupta et al. 1976a, Dobozy et al. 1976, Bertoglio et al. 1977] and some B lymphoblastoid cell lines including Raji, Mann, RPMI 8866 and Bristol-8 [Bertoglio et al. 1979, Forbes et al. 1982b, Youinou et al. 1984b]. T cells, monocytes, eosinophils and polymorphonuclear leukocytes (PMN) do not express MER. The expression of MER on B cells has been reviewed in detail by Irving and colleagues [1984]. The structure of MER is discussed in chapter 1.3.

Relationship between B cells expressing CD5 and MER

CD5 and MER appear to identify the same or overlapping subsets of B cells. All normal lymph node and tonsil CD5+ve B cells as well as nearly all CD5+ve B-CLL cells co-express MER [Caligaris-Cappio et al. 1982]. Early evidence suggested that few B cells in peripheral blood expressed CD5 [Plater-Zyberk et al. 1985, Caligaris-Cappio et al. 1982] whereas up to 40% of blood B cells were MER+ve. However, more recent studies have indicated that 10-30% of blood B cells are CD5+ve [Hardy et al. 1987, Cassali et al. 1987]. Isolated MER+ve B cells from blood are enriched for CD5+ve B cells [Hardy et al. 1987]. It needs to be determined whether the distinct mitogenic response of MER+ve B cells to some B cell activators [Lucivero et al. 1981, Ito et al.

1984], applies also to CD5+ve B cells. A diagram showing steps in the development of CD5+ve, MER+ve B cells is shown in Fig 1.2.

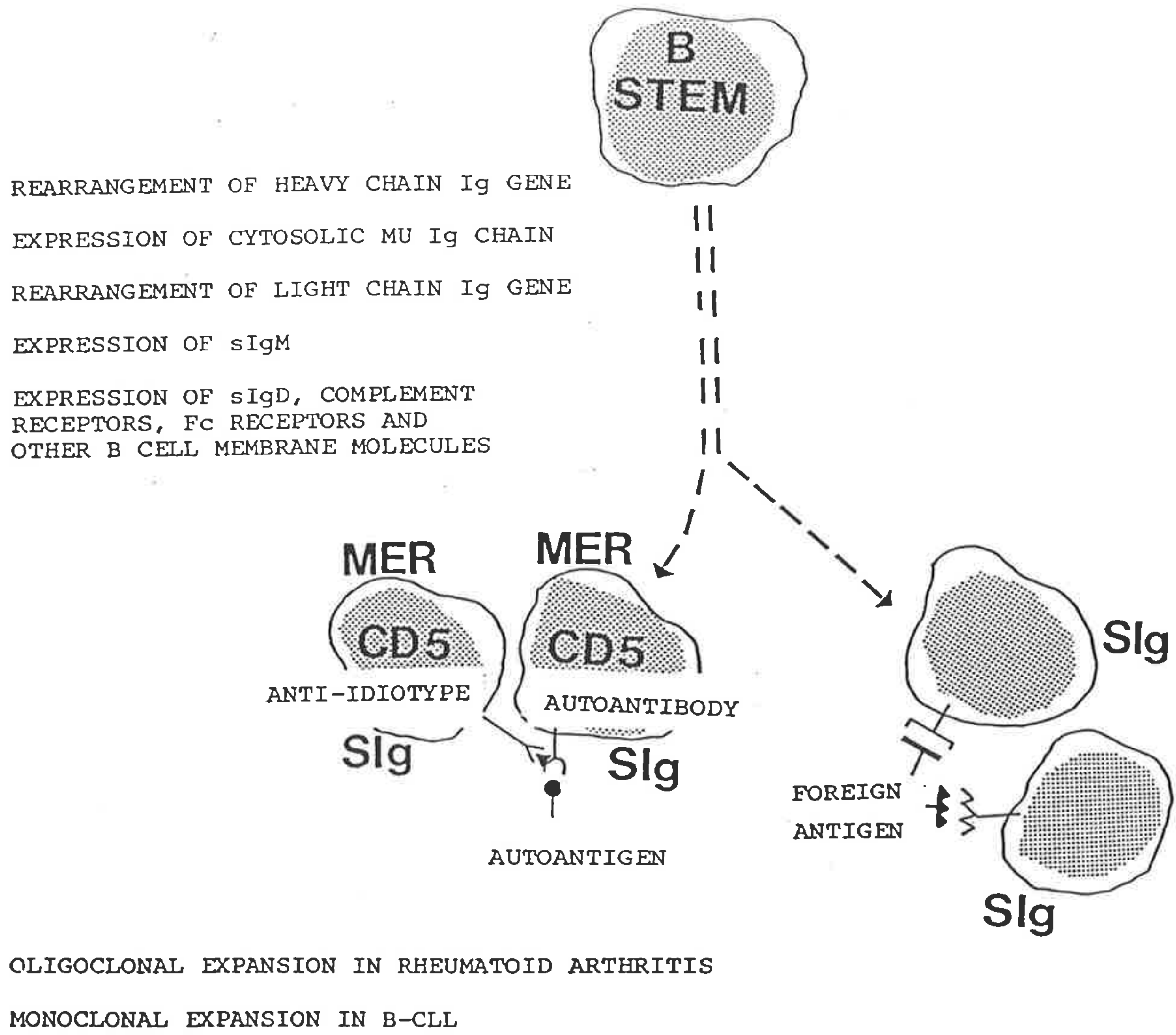
Relationship of CD5+ve, MER+ve B cells to ontogeny

B cells expressing CD5 and those expressing MER are predominant early in ontogeny. In foetal lymph nodes and spleen, an early influx of sIg+ve B cells lacking CD5 is followed by a second wave of CD5+ve B cells at about 17 weeks. At this stage, CD5+ve cells constitute the majority of the B cells in these tissues and are located in clusters around follicular dendritic cells, forming the primary follicles [Bofill et al. 1985, Antin et al. 1986]. MER+ve B cells are present in foetal liver as early as 13-17 weeks where they comprise a significant proportion of the lymphoid cells [Gupta et al. 1976b].

Relationship of CD5+ve, MER+VE B cells to autoimmunity

Both CD5+ve and MER+ve B cells are expanded in the peripheral blood of patients with rheumatoid arthritis [Room et al.1982, Plater-Zyberk et al. 1985]. Youinou and colleagues [1984a] found that in inactive rheumatoid arthritis, about 75% of the B cells were MER+ve. Only 30% of B cells were positive in patients with active disease. This may result from the loss of expression of this receptor during B cell activation (see later), since in active disease there is a higher proportion of activated B cells.

Both CD5+ve B cells and MER+ve B cells respond to some polyclonal activators by producing IgM autoantibody. Normal CD5+ve B cells appear to be responsible for producing most of the IgM autoantibodies to IgG (rheumatoid factor) and DNA after stimulation in vitro with polyclonal B cell activators [Hardy et al. 1987, Casali et al. 1987]. About 10% of the Ig secreted by



Legend to Figure 1.2 Antigen-independent development of B cells

Major steps in the antigen-independent phase of B cell development. CD5+ve, MER+ve and CD5-ve, MER-ve B cells are shown as distinct lineages of B cells. The point at which they diverge from a common precursor is unknown. This figure was adapted from Lydyard et al. [1987].

Epstein Barr virus-stimulated MER+ve B cells is rheumatoid factor [Fong et al. 1983].

That B-CLL cells are derived from this normal subset is supported by the observations that there is a high incidence of autoimmunity in B-CLL [Rundles 1972] and that the monoclonal Ig has the antibody and idiotype specificity of rheumatoid factor in one fifth of cases of B-CLL studied [Preud'homme and Seligmann 1972, Seligmann et al. 1973, Kipps et al. 1987, Kipps and Vaughan 1987].

Lydyard and colleagues [1987] have speculated that CD5+ve B cells are a distinct lineage of B cells with a restricted repertoire of V region gene segments, coding for variable regions of antibodies that preferentially recognize auto-antigens and associated idiotypes. This is supported by recent evidence that 20% of B-CLL cells express a product of the KIIIb V gene subfamily [Kipps et al. 1987] and that the analagous Lyl^+B cell subset in mice has a restricted expression of V region genes [Pennel et al. 1988].

1.2.3 <u>RELATIONSHIP OF B-CLL TO OTHER B CELL MALIGNANCIES</u>

Most B cell tumours involve cells which correspond to stages or subsets of B cell development [Salmon and Seligmann 1974, Caligaris-Cappio and Janossy 1985]. Many B type acute lymphocytic leukaemias involve pre-B cells or immature B cells. B-CLL cells correspond to the MER+ve CD5+ve subset as do cells in centrocytic lymphoma and some cases of well differentiated lymphocytic lymphoma [Caligaris-Cappio and Janossy 1985]. Malignant B cells in prolymphocytic leukaemia and non-Hodgkin's lymphoma correspond to very mature (perhaps memory or activated G_1) B cells. These cells are MER-ve, CD5-ve and have abundant sIg. "Hairy cell" leukaemia cells are thought to be early

plasma cells [Anderson et al. 1985]. Some "hairy cells" express CD5 and MER [Catovsky et al. 1976, Koziner et al 1982]. Binding of mouse red cells by "hairy cells" may be an anomaly since MER is not found on other types of normal and malignant plasmacytoid cells. Alternatively, some types of leukaemic "hairy cells" may be at a less mature stage of development.

In vitro, conversion of B-CLL cells to cells resembling "hairy cells" and prolymphocytic leukaemia cells has been induced by phorbol ester [Caligaris-Cappio et al. 1984, 1985] and lipopolysaccharide [Robert et al. 1983], respectively.

1.2.4 DEVELOPMENT OF B-CLL

Little is known about how B-CLL arises. Multiple cases may occur in the one family [Blattner et al. 1976], suggesting that there may be a genetic factor involved. In 50% or more of cases of B-CLL, there are chromosomal aberrations [Gahrton et al. 1980], the most common being trisomy 12 occurring in 5 out of the 11 cases in the study by Gahrton and colleagues. Some cases involve a translocation of genetic material from chromosome 11 to a site in the heavy chain immunoglobulin gene on chromosome 14 [Tsujimoto et al. 1984].

Mechanisms that deregulate c-*myc*, c-*ras* and b-*lym* proto-oncogenes are causative factors in certain types of B cell neoplasia although not in B-CLL [Marcu 1987].

B cell neoplasia is thought to begin at the stem cell level and to involve multiple steps [Godal and Funderud 1982] as in other cancers [Cairns 1981]. An early phase may be a polyclonal B cell hyperplasia. Patients with auto-immune disorders (where lymphoid hyperplasia occurs) have a high risk of later developing a monoclonal malignant lymphoma [Isömaki et al. 1982]. Mono-

clonality is considered a late step in the development of the neoplasm [Godal and Funderud 1982].

As in other chronic leukaemias, the accumulation of B-CLL cells results more from an increased life span of the cells than a high proliferative rate [Bierman 1967, Dormer et al. 1983]. Most mature B cells live only a few days in vivo unless they become activated [DeFreitas and Coutinho 1981]. One hypothesis is that B-CLL cells are arrested in their development at a stage prior to that which rapidly turn-overs, thus causing the cells to accumulate [Johnstone 1982]. However, as indicated by Kumararatne and Ling [1983], a maturation arrest hypothesis is inconsistent with some of the phenotypic studies, eg those showing that some B-CLL cells may express both early markers like MER and late markers such as sIgG.

1.2.5 ABNORMALITIES OF THE B-CLL CELL

Use of B-CLL cells as a model system has the reservation that these cells may have abnormalities that are related to their malignancy. It has been suggested that the B-CLL cell is aberrant, perhaps "with a temporal disruption of the co-ordinated genetic activity of the healthy cell" [Brown et al. 1985]. Some of the changes will be attributable to lesions in the genome but others will be a consequence of the altered environment of the CLL cells, as discussed by Greaves and colleagues [1986] for certain other types of leukaemias. Therefore, the conclusions drawn from studies using B-CLL cells as a model may not be applicable to normal cells. Such conclusions require confirmation in normal cells.

Phenotypic abnormalities

B-CLL cells are different from most normal B cells in that they have low amounts of sIg (estimated to be only 10-20% of

that on normal B cells) [Digherio et al. 1976], express avid Fc_{mu} receptors [Pichler and Knapp 1977], have more C3d receptors than C3b receptors [Ross and Pauley 1975], express some but not all of the MHC class II antigens, depending upon the population of cells being examined [Navarette et al. 1986, Drexler et al. 1988], have small amounts of some of the antigens usually found on activated B cells (see chapter 1.4) and lack a 185kDa macromolecular cold-insoluble globulin found in membranes of most normal B cells [Simmonds et al. 1981]. They also have a high rate of secretion of free immunoglobulin light chains like immature normal B cells [Hannam-Harris et al. 1980]. In addition to CD5, they express some other membrane molecules usually found on T cells [Brown et al. 1985].

It is not clear whether any of these differences in phenotype are also true for normal CD5+ve, MER+ve B cells.

Abnormalities in structural components

B-CLL cells are deficient in two components of the membrane lipid bilayer, cholesterol [Inbar and Shinitzky 1974] and tocopherol [Kayden et al. 1984]. Cholesterol is also deficient in other types of leukaemic cells [Inbar and Shinitzky 1974]. In addition, B-CLL cells have three fold higher contents of ascorbic acid relative to normal B cells [Liebes et al. 1981]. Since high concentrations of ascorbic acid promote lipid peroxidation and tocopherol is an anti-oxidant which protects unsaturated membrane lipids from peroxidation, lipids in B-CLL cells may have increased susceptibility to undergo peroxidation.

B-CLL cells have decreased amounts of various glycosphingolipids relative to normal B cells [Schwarting and Marcus 1979].

Metabolic abnormalities

B-CLL cells have deficiencies in alpha-fucosidase and other lysosomal glycosidases [Crockard et al. 1980], 5' nucleotidase [Conklyn and Silber 1982], leucine-favouring amino acid transport system [Woodlock et al. 1988] and irregularities, in and decreased levels of, cAMP-dependent protein kinase and cAMP [Weber et al. 1981].

Abnormalities in the cytoskeleton

Several properties involving the cytoskeleton have been found to be defective in CLL cells, including a high tendency to rupture during blood film preparation [Rundles 1972], extreme sensitivity to the microtubule poison, colchicine [Schrek 1975], decreased motility [Jarvis et al. 1976, Goldman et al. 1977], failure to cap various surface molecules [Johnstone 1982, Godal et al. 1978] and low rate of shedding of membrane receptors [Dobozy et al. 1980]. Abnormalities in the cytoskeleton that have been reported in CLL cells include lack of organized arrays of intermediate filaments [Zucker-Franklin et al. 1979], decreased actin content [Stark et al. 1982, Liebes et al. 1983] and alterations in vimentin [Stark et al. 1984]. They differ also from normal MER+ve B cells by forming membrane adhesion structures, that have been called podosomes [Caligaris-Cappio et al. 1986].

Normal B cells acquired some of these cytoskeletal abnormalities when treated with the serine esterase inhibitor di-isopropyl-fluorophosphate [Simmonds et al. 1982], suggesting that an esterase or protease is essential for expression of some normal B cell properties.

1.3 STRUCTURE OF THE RECEPTOR FOR MOUSE ERYTHROCYTES

The mechanism by which MER+ve B cells bind mouse erythrocytes is discussed in this section.

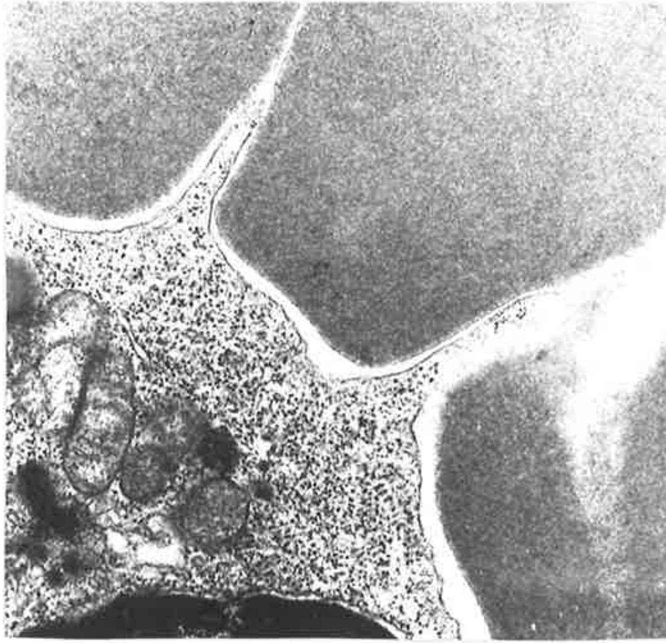
1.3.1 NATURE OF THE ROSETTE BOND

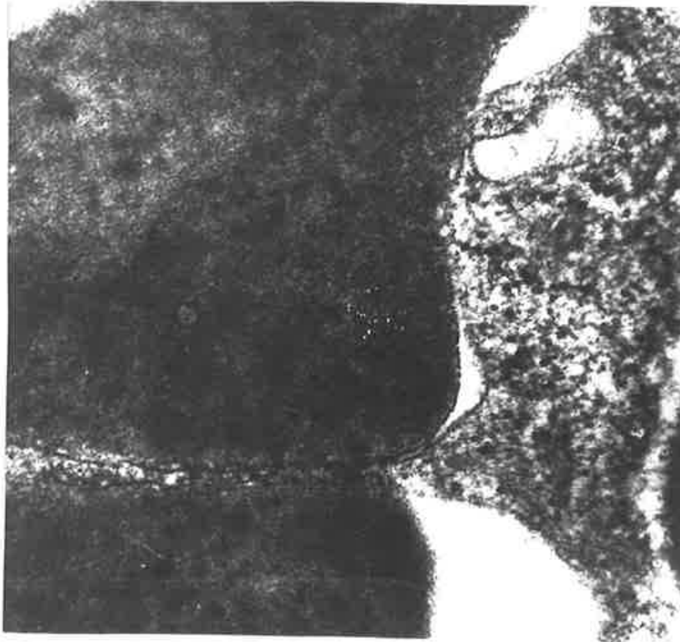
Only living cells bind mouse red cells. However, rosetting is relatively insensitive to inhibitors of cell metabolism including azide and cyanide [Forbes and Zalewski 1976].

Transmission electron micrographs show that rosetting with mouse red cells involves joining both via tips of cytoplasmic processes and via adjacent flat surfaces (Fig 1.3) [Mohanakumar et al. 1979].

Lowering of cell surface negative charge by treatment of B cells or mouse red cells with neuraminidase or by inclusion of positively charged proteins in the rosetting medium markedly increases the capacity of B cells to bind mouse red cells, increasing both the number of attached red cells and the frequency of rosetting cells [Catovsky et al. 1976, Forbes and Zalewski 1976, Gupta et al. 1976a, Hokland et al. 1981]. This treatment enhances the interaction of mouse red cells with a second type of mouse erythrocyte receptor, which is found on most normal B cells [Forbes et al. 1982b].

When MER+ve B cells are allowed to bind mouse red cells and then freed of the bound red cells by hypotonic shock, most do not reform rosettes with fresh mouse red cells [Forbes and Zalewski 1976, Zola 1977, Barboni et al. 1981] although they are still capable of binding complement-sensitized red cells [Forbes and Zalewski 1976]. The inhibition does not result from loss of viability and is not reversed during overnight culture at 37°C. Pre-rosetting may have removed, blocked or modified MER.





Legend to Figure 1.3 Ultrastructure of MER bond

B-CLL cells bind mouse erythrocytes both by long cytoplasmic processes and adjacent flat surfaces.
Final magnification: upper x 70,000; lower x 120,000

1.3.2 NATURE OF MER

Relationship of MER to other B cell surface molecules

Surface marker studies of isolated subsets, neoplastic lymphocytes and lymphoblastoid cell lines indicate that MER is distinct from other markers, including MHC class II antigens, sIg and receptors for Fc_{gamma}, Fc_{mu} and complement. Evidence that rosetting is inhibited by ant-Ig, heat aggregated gamma globulin and isolated C3b component may be due to close proximity of MER and SIg, Fcy receptors and C3b receptors or to activation of the B cells and therefore loss of MER expression [Forbes and Zalewski 1976, Mohanakumar et al. 1979, Zalewski and Forbes 1979, Dobozy et al. 1982]. Inhibition of rosetting by isolated C3b may be due to contamination with proteases, used for the preparation of this component, since proteases are potent inhibitors of rosetting [Forbes and Zalewski 1976].

The co-expression of MER and CD5 raises the question of whether these two structures are the same. Evidence against this is that CD5 is found on normal and malignant T cells which lack MER and expression of MER but not CD5 is lost with B cell activation. In fact, phorbol esters increase the expression of CD5 antigen on some leukaemic B cells [Miller and Gralow 1984], while they inhibit expression of MER (see chapter 1.5).

Mouse red cell binding is not a result of natural anti-mouse red cell antibody cytophilically adsorbed to Fc receptors on MER+ve B cells. Human serum does agglutinate mouse red cells, presumably due to natural red cell antibody, but it also agglutinates red cells of other species. There is no correlation between serum titre of mouse red cell agglutinins and frequency of rosetting cells. In addition, some populations of malignant B cells with avid Fc receptors are MER-ve. Rosetting is unaffected

by incubating B-CLL cells at pH 4 whereas cytophilic antibody is eluted at this pH [Zalewski and Forbes 1979].

Two types of mouse erythrocyte receptor

Binding of mouse red cells involves co-operation between two types of surface molecule on the B cell (receptors) and two types of surface molecules on the red cell (ligands) [Forbes et al. 1982b, Zalewski et al. 1982]. One ligand is destroyed by pronase treatment of the red cell, whereas the other ligand is resistant to this treatment.

Some B cell lines (eg Raji and Bristol-8) bind mouse red cells but not pronase-treated mouse red cells, others (eg RPMI 8392 and Daudi) bind pronase-treated mouse red cells but not untreated mouse red cells, and still others (eg RPMI8866 and Mann) have the phenotype of B-CLL cells since they bind both types of red cells [Forbes et al. 1982b]. Gentle trypsinization of B-CLL cells completely inhibits binding of untreated mouse red cells but hardly affects binding of pronase-treated mouse red cells. The supernatant of trypsinized B-CLL cells contains receptor activity against mouse red cells but not pronase-treated mouse red cells or red cells of other species [Zalewski et al. 1982].

Similarly, rosetting of T lymphocytes with sheep erythrocytes [Bernard et al. 1987] involves at least three different types of receptor-ligand binding. Presumably, the rosette bond between two cells is strengthened by multiple types of receptor-ligand interactions.

1.3.3 RELEASE OF MER FROM CELLS

Spontaneous release of MER

There have been several reports of MER-like activity released in soluble form from MER+ve B cells. Youinou et al. [1984b] reported that a 24 hour supernatant of an MER+ve B cell line induced rosetting with mouse red cells in some normal lymphocytes. Dobozy et al. [1980] found that when mouse red cells were treated with the supernatant of normal lymphocytes, prepared by incubation of the lymphocytes at 37°C in medium containing 6% FCS, they would no longer bind to MER+ve B cells. Loss of capacity to bind mouse red cells occurs when MER+ve B cells are incubated in serum-free medium at 37°C [Pegrum and Evans 1978, Zalewski and Forbes 1979, Semenzato et al. 1980]. Pegrum and Evans speculated that Ig was released from the cells during this incubation. In our study, a glycoprotein was released which did not bind to mouse red cells (suggesting that it is not MER) but did protect fresh MER+ve B cells from further loss of MER in this type of incubation. A similar substance is present in sera of a variety of species.

Release of MER by mild trypsinization of B-CLL cells

The supernatant, obtained by mild trypsinization of B-CLL cells, agglutinates mouse erythrocytes [Zalewski et al. 1982]. Specific binding of trypsin-solubilized MER to mouse red cells can also be demonstrated by binding of latex particles which have been coated with the trypsin supernatant and by binding of fluorescein-labelled trypsin supernatant. In each case, there is no binding to pronase-treated mouse red cells or other species of red cells, except those from rat.

The supernatant also induces certain types of MER-ve cells, eg Namalva B lymphoblastoid cells, to rosette (adoptive

rosetting) with mouse erythrocytes [Zalewski et al. 1982]. Treatment of Namalva cells for 30 minutes with the trypsin supernatant is sufficient to cause most of them to rosette with mouse red cells although they still do not bind pronase-treated mouse red cells or other species of red cell, except those of the rat.

The mechanism of insertion of MER into the membranes of MER-ve cells is unclear but similar examples of spontaneous insertion of membrane molecules into cells include epidermal growth factor receptor [Bishayee et al. 1982, Das and Bishayee 1984], MHC class I and II molecules [Emerson et al. 1980, Emerson and Cone 1981, 1982] and Fc_{gamma} receptor [Targowski and Milgrom 1979].

There is other evidence that the trypsin-released haemagglutinin is derived from MER. Those populations of B-CLL cells which readily lose capacity to rosette when treated with trypsin, yield supernatants with high titres of haemagglutination for mouse red cells whereas those in which rosetting is only partly inhibited by trypsinization, yield supernatants with low titres [Zalewski et al. 1982]. Little or no haemagglutinin is released by trypsin from leukaemic MER-ve T or B cells.

<u>Structure of trypsin-released MER</u>

Haemagglutinating activity and adoptive rosetting activity are lost from trypsin supernatants after passage through a wheat germ lectin - Sepharose column. Both activities are found in the eluate after elution with specific sugar, N-acetylglucosamine, suggesting that trypsin-released MER contains glycoprotein or glycolipid. The active material appears to be very large since it elutes in the void volume of an Ultrogel column, with

fractionation range up to one million daltons. It may be in the form of membrane vesicles (see later).

All of the specific mouse red cell haemagglutinating activity in preparations of MER is extracted into a lipid-containing fraction by Folch partitioning. On two dimensional thin layer chromatography, most of the haemagglutinating activity co-migrates with phosphatidylethanolamine (PE) [Zalewski et al. 1984a].

Studies with pure phospholipids and phospholipases have identified the structural features which determine whether a lipid can agglutinate mouse red cells [Forbes et al. 1983]. Phosphatidic acid is active but diacylglycerol is inactive, indicating that the phosphate group is essential. All phospholipids which do not contain a bulky choline head group are active. Removal of the choline from phosphatidylcholine by treatment with phospholipase D induces haemagglutinating activity but removal of both the choline and the phosphate, by treatment with phospholipase C, does not induce activity. Haemagglutinating activity by phospholipids is stronger if they contain unsaturated fatty acids. ^{14}C-labelled PE, but not ^{14}C-labelled phosphatidylcholine, is absorbed by mouse red cells in preference to sheep red cells and the binding is inhibited by pronase treatment of the mouse red cell [Forbes et al. 1983].

Requirement for both phospholipid and protein for rosetting

Neither the lipid extract of MER nor pure PE bind to a wheat germ lectin-Sepharose column nor do they induce adoptive rosetting in Namalva cells suggesting that in MER the lipid is associated with glycosylated material which may be essential for rosetting [Zalewski et al. 1984a].

In support of the hypothesis that rosetting is dependent

both on phospholipid and protein, both phospholipase C and proteases inhibit the capacity of MER+ve B cells to bind mouse red cells [Zalewski et al. 1984a]. Phosphatidylserine (PS), and certain other phospholipids, mediate attachment of macrophages to autologous red cells [Tanaka and Schroit 1983].

1.3.4 ALBUMIN AND MER

Albumin as putative ligand of MER

Haemagglutination of mouse red cells by either trypsin-released MER or by pure PE is inhibited by a 70kDa ligand purified from mouse red cells. This ligand behaves like albumin when subjected to phenol extraction, anion exchange chromatography and Blue Sepharose-chromatography, although formal proof that it is mouse albumin has not yet been obtained [Forbes et al. 1983].

A potent inhibitor of haemagglutination is also found in serum of all species. This inhibitor is readily detected in unfractionated foetal calf serum, which does not contain natural anti-red cell antibodies. Detection of this inhibitor in sera from other species, which do contain anti-red cell antibodies, requires partial purification of the inhibitor. The inhibitor in serum also has properties of albumin and pure preparations of serum albumin inhibit haemagglutination of mouse red cells by trypsin-released MER or by pure PE [Forbes et al. 1984]. Albumin prepared commercially by a non-denaturing technique (Blue Sepharose chromatography) also inhibits haemagglutination but albumin prepared commercially by a denaturing technique (fraction V albumin) does not inhibit haemagglutination. The harsh ethanol treatment, used in the purification, presumably destroys the inhibitory activity of albumin. Activity is restored by

treatment of the fraction V albumin with dithiothreitol or mercaptoethanol [Forbes et al. 1984]. Alkylation of the sulphydryls in thiol-treated albumin does not prevent inhibitory activity suggesting that the sulphydryls do not interact directly with PE in MER.

These results imply that one of the interactions between the B-CLL cell and the mouse red cell is through PE in the B cell membrane interacting with albumin associated with the mouse red cell. Albumin does partially inhibit rosetting. Rosetting may be restricted to a subset of B cells because proteins associated with the PE are essential for rosetting and are only found in this type of cell. This is further discussed in chapter 10.

Albumin in lymphocyte membranes

Albumin is a hidden component of mouse [Sidman 1981] and pig [Owen et al. 1978, 1980] B and T lymphocyte plasma membranes being readily radiolabelled from within the plasma membrane by a lipophilic, photoactivated reagent [^{125}I] - iodo-naphthyl-azide. The origin of membrane albumin is unknown but it is assumed to be derived by internalization of plasma albumin since lymphocytes do not synthesize albumin [Sidman 1981].

Albumin binds to many different types of cells. It binds more avidly to red cell membranes than any other plasma protein [Andersen 1982]. Lymphocytes have both specific and non-specific binding sites for albumin [Spieker-Polet and Polet 1981]. The specific binding of albumin occurs at similar concentrations to those which promote the growth of lymphocytes. Saturable albumin receptors are also found on liver cells [Lenkei et al. 1977], possibly mediating uptake of long chain fatty acids [Weisiger et al. 1981]. Uptake of fatty acids by hepatocytes has been reported to be sensitive to both proteases and phospholipases [Mahadevan

and Sauer 1974] indicating that a phospholipid-protein complex (perhaps similar to MER) is involved.

Role of albumin in lymphocyte activation

Albumin binds a broad spectrum of ligands, including fatty acids, glutathione, amino acids, inorganic anions, bilirubin, hemin, organic anions and various drugs [Brown 1977]. Albumin, is an essential component of culture media supporting lymphocyte proliferation and differentiation [Spieker-Polet and Polet 1976, 1981, Hewlett et al. 1981]. One role of albumin is as a "buffer for components of the medium in inhibitory amounts" [Guilbert and Iscove 1976]. Albumin may also serve as a source of amino acids for lymphocytes [Spieker-Polet and Polet 1976]. Another role of albumin is to provide fatty acids which are essential for cell growth [Spieker-Polet and Polet 1981]. Albumin may also carry into the cell other growth cofactors such as hormones [Krenning et al. 1979] and low molecular weight thiols [Hewlett et al. 1981]. Thiol-treated albumin is a B cell growth co-factor replacing the requirement for mercaptoethanol in B cell cultures [Hewlett et al. 1981].

1.4 B CELL ACTIVATION AND PLASMACYTOID DIFFERENTIATION

This section describes the sequence of events which accompanies B cell activation, proliferation and differentiation at a cellular level. Some of the early biochemical events that mediate B cell activation are discussed in chapter 1.10. The term B cell activation will be used to imply increased metabolic activity of the cells and progression through the cell cycle, events which are potentially reversible because they do not result from permanent pre-programmed genetic change. Differentiation includes those events which follow establishment of a new program of gene expression. Sometimes, the distinction between activation and differentiation is unclear.

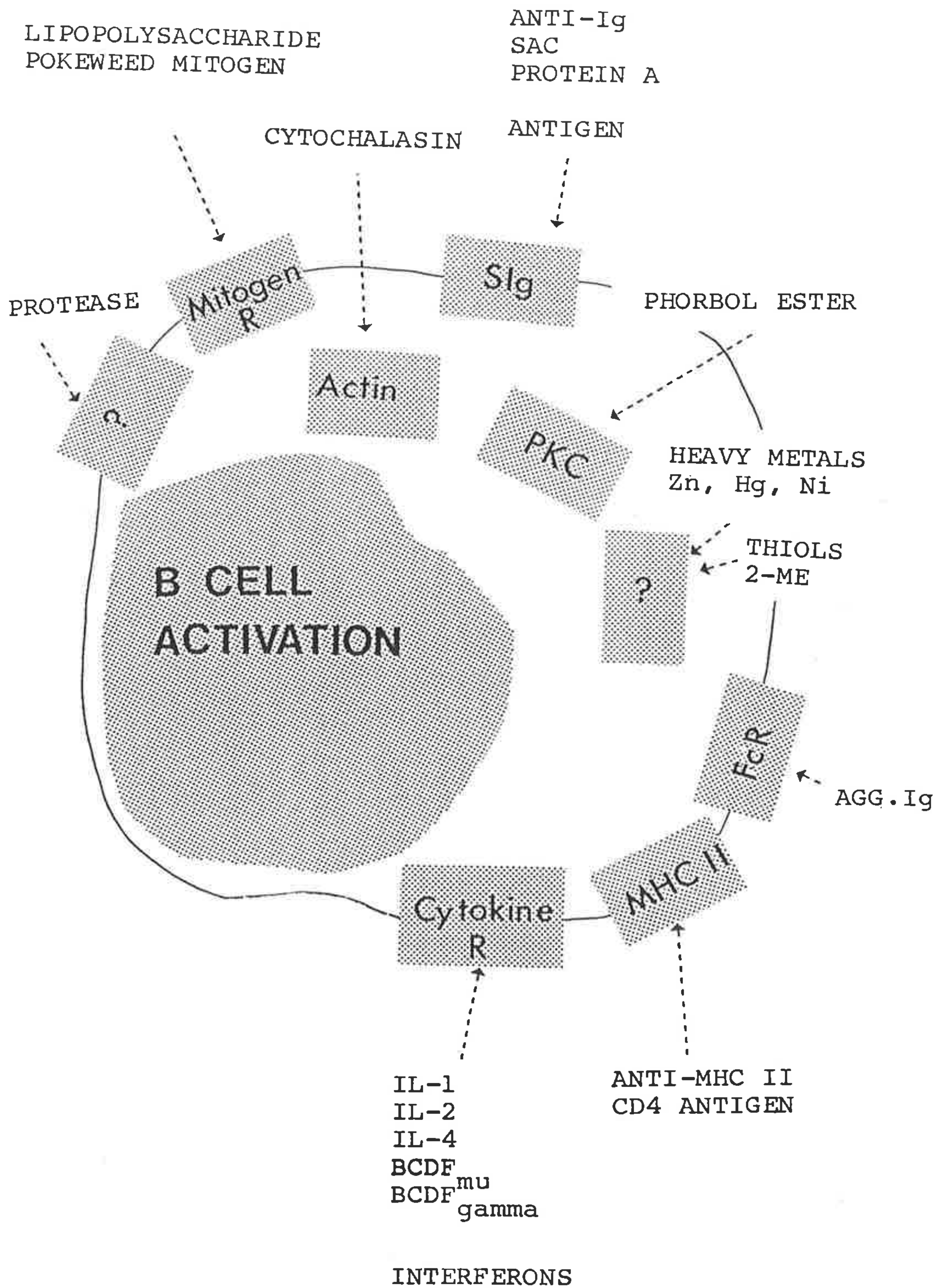
1.4.1 B CELL ACTIVATION BY DIVERSE STIMULI

Activation via sIg

B cell activation occurs within germinal centres of peripheral lymphoid tissues, in vivo, and requires antigen, T cells and macrophages and various factors derived from these cells [reviewed in Caligaris-Cappio and Janossy 1985, Klaus and Humphrey 1986]. Activation of B cells via sIg can be initiated by several types of stimuli which bind to sIg, in vitro. These include antigen, anti-immunoglobulin (anti-Ig) and protein A-containing *Staphylococcus aureus* Cowan I bacteria (SAC).

Activation by other surface molecules

B cells can be activated by a diverse range of stimuli, other than sIg (see Fig 1.4). These include the T cell-derived lymphokine interleukin-4 (IL-4), various types of interferon, bacterial lipopolysaccharide, protease, low molecular weight thiols, zinc ions and other metals, cytochalasins B and D, phorbol esters and antibody to a 35kDa membrane protein [Tötterman et al. 1980, Cunningham-Rundles et al. 1980, Cambier



Legend to Figure 1.4 Stimuli influencing B cell activation

Various stimuli acting through different types of membrane and other cellular receptors affect the process by which B cells are activated. In some cases, the site of actions are unknown.

et al. 1985, Robert et al. 1985, Justement et al. 1986, Rothstein 1986a, Goodman 1986, Finkelman et al. 1986, Clark and Ledbetter 1986]. The process of activation is modulated by ligands of MHC class II molecules and of Fc receptors [reviewed in Cambier et al. 1987a, Cambier and Ransom 1987].

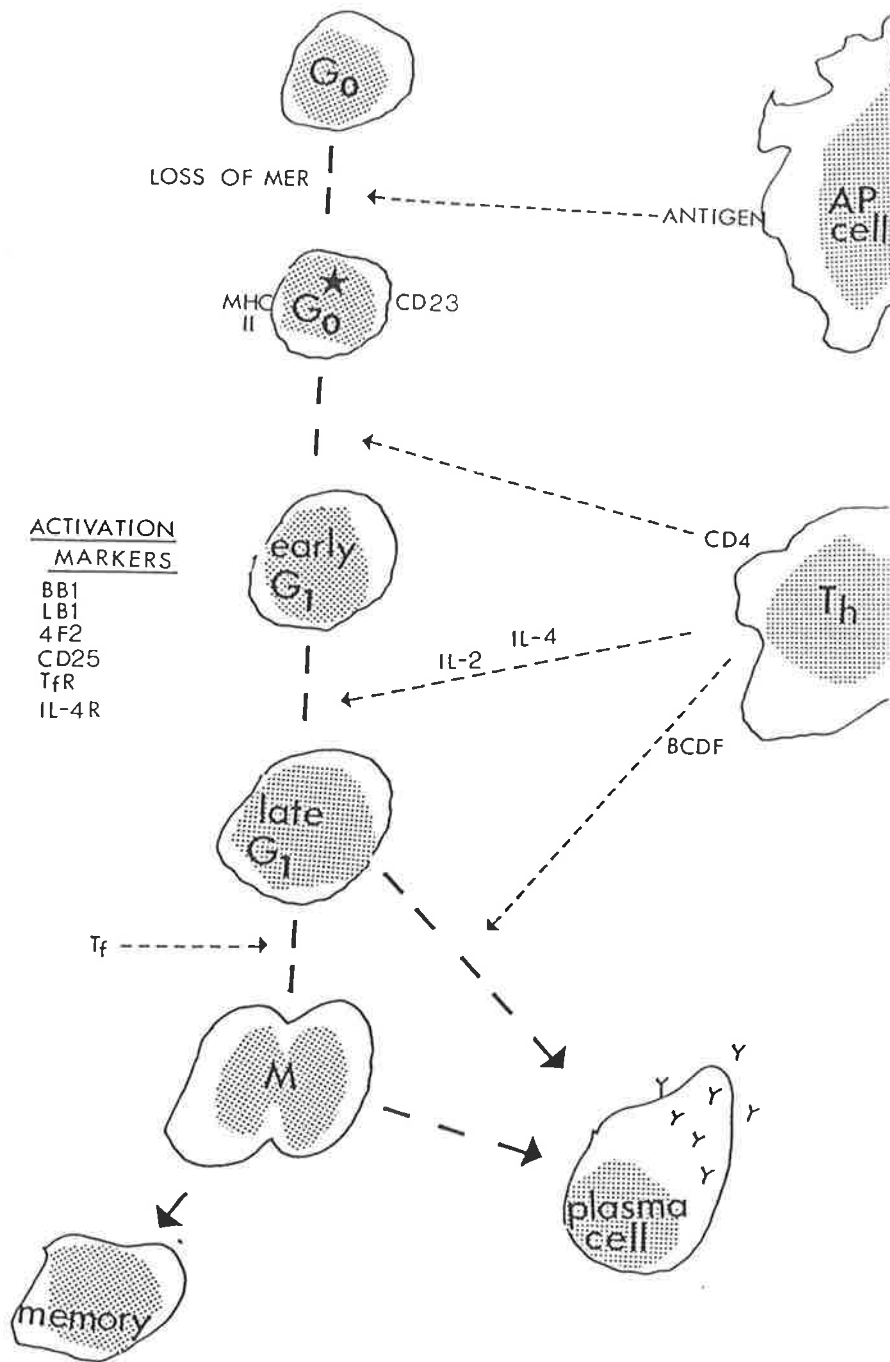
Activation of B cells by phorbol ester and by zinc ions is discussed further in chapters 1.5 and 1.12, respectively.

There is controversy as to the relative importance of these various stimuli in B-cell activation, in-vivo. The hypothesis that sIg is a triggering receptor on B cells was challenged by Coutinho and Möller [1974] who suggested that sIg merely serves to hold antigen (either soluble or associated with membranes of macrophages or T helper cells) and thereby focus polyclonal B cell activators onto the B cell. They have considered the mitogen receptor(s) to be the primary surface receptor involved in B cell activation.

It has also been argued that both models apply, sIgD being the direct signal transducer and sIgM serving a focussing role [Finkelman et al. 1986]. However, both sIgM and sIgD are coupled to the signal transduction pathway involving protein kinase C [Cambier et al. 1987a].

1.4.2 STEPS INVOLVED IN B CELL ACTIVATION

Virgin B cells are activated in a series of steps culminating in clonal proliferation and differentiation into memory cells or cells producing antibody (Fig 1.5) [reviewed in Howard and Paul 1983, Muraguchi et al. 1985]. Memory B cells may be activated by a similar mechanism although the process is greatly accelerated, possibly because of the increased numbers of antigen-specific T helper cells that are required for the



Legend to Figure 1.5 Steps involved in B cell activation

This figure indicates the putative steps in the process of B cell activation. The star indicates an "excited G_o B cell. M is mitosis. The early activation markers are described in the text. BCDF includes a variety of types of factors affecting B cell differentiation. Tf stands for transferrin. R indicates receptor. AP is antigen-presenting cell and Th is helper T cell.

process. It is presumed that a similar sequence of events accompanies B cell activation by different stimuli.

Increased expression of MHC class II molecules

At an early stage in B cell activation, both in vivo and in vitro, expression of MHC class II antigens is greatly increased. This event occurs several hours after the stimulus has acted and it requires new gene expression. At this stage, the B cells are said to be excited although not yet in cell cycle [reviewed in Cambier et al. 1985]. CD23 (B-LAST-2) is also expressed very early in B cell activation before the cells have left G_o [Thorley-Lawson and Mann 1985].

Progression to early G_1

In response to some antigens, excited B cells are driven into G_1 by a signal from T helper cells. This signal appears to be induced by interaction of CD4 molecules in the T cell membrane with MHC class II molecules on the B cell [Cambier et al. 1987a]. The increased expression of MHC class II molecules in the membranes of excited B cells may sensitize B cells to this signal from helper T cells.

Other antigens (T-independent) do not require this second signal to activate B cells. The reason for this may be that they possess additional moieties which induce a polyclonal signal. Also, a strong signal such as high concentration of anti-Ig, SAC or bacterial lipopolysaccharide will bypass the requirement for T helper cells and drive most B cells into cycle including at least one round of cell division [reviewed in Muraguchi et al. 1985].

Entry of excited B cells into G_1 phase is characterized by increase in cell size and RNA content [DeFranco et al. 1982, Finkelman et al. 1986]. At this stage, receptors for various growth and differentiation factors appear in the membrane. Some

other membrane molecules found only on activated B cells (including BB1, LB1, 4F2, B-LAST 1 and B5) also appear [Kehrl et al. 1984, Thorley-Lawson and Mann 1985, Finkelman et al. 1986].

Progression to late G_1

B cells respond to various T cell-derived growth factors by progressing into late G_1, where they begin to express receptors for transferrin [Neckers et al. 1985]. Transferrin acts in concert with the other B cell growth factors, stimulating the B cells to undergo DNA synthesis and proliferation [Howard and Paul 1983].

Proliferation and differentiation

Activated B cells undergo several cell divisions before becoming either short-lived antibody-secreting plasma cells (with abundant cytoplasmic Ig) or long-lived memory B cells (with abundant sIg and no cytoplasmic Ig) [Calvert et al. 1984, Klaus and Humphrey 1986]. Associated with mouse B cell proliferation is increased synthesis of a 40kDa nuclear protein, numatrin, whose function is not yet known [Feuerstein and Mond 1987].

Various factors derived from T cells and macrophages are essential for plasmacytoid differentiation, including $BCDF_{mu}$, $BCDF_{gamma}$ and interferons [reviewed in Hamaoka and Ono 1986]. They directly induce late G_1 or proliferating B cells to become antibody-forming cells [Melchers and Andersson 1986].

During the process of clonal expansion there is often a switch in expression of the isotype of the heavy chain of Ig so that most memory B cells now express sIgG, A or E and most plasma cells secrete antibodies of these isotypes. If no switch occurs the plasma cells are committed to secrete IgM. The

switching involves deletion of genetic material within the rearranged heavy chain gene [reviewed in Yancopoulos and Alt 1986].

Differentiation involves production of Ig destined for secretion rather than for membrane incorporation. This is regulated by alternative splicing of a single RNA transcript. Both increased gene transcription and increased stability of the mRNA are responsible for the increased Ig production. Production of J chain and polymerizing enzymes, required for formation of the pentameric form of secretory IgM and IgA, also occurs during plasmacytoid differentiation [Koshland 1983].

1.4.3 LOSS OF EXPRESSION OF MER DURING B CELL ACTIVATION

Expression of MER is lost when MER+ve B cells are exposed to various polyclonal B cell activators including anti-Ig, SAC, lipopolysaccharide, pokeweed mitogen and phorbol ester [Tötterman et al. 1981b, Forbes et al. 1981, McGraw et al. 1982, Caligaris-Cappio et al. 1984, Robert et al. 1985]. This suggests that loss of expression of MER is an event which is common to B cell activation via a number of surface signal-transducing molecules. It is not known whether the change in MER is essential for the ensuing steps of B cell activation or whether it is a consequence of some event in B cell activation.

Loss of MER expression is induced by TPA very early in culture (see chapter 1.5), indicating that the loss may be one of the earlier events in B cell activation. Circumstantial evidence suggests that MER is lost with activation of B cells, in vivo. MER is never expressed on the low density normal B cells [Lucivero et al. 1981] which have properties and markers of activated B cells [Caligaris-Cappio and Janossy 1985]. MER+ve B cells are less frequent in patients with very active rheumatoid

arthritis, where there is a higher proportion of activated B cells [Youinou et al. 1984a]. MER+ve leukaemic B cells (eg B-CLL cells) generally have properties and markers of resting B cells whereas MER-ve leukaemic B cells in prolymphocytic leukaemic and related disorders express features of activated B cells (increased content of class 11 major histocompatibility complex antigen, expression of receptors for growth factors and activation antigens) [Caligaris-Cappio and Janossy 1985]. Intra-clonal transformation of B-CLL cells to cells resembling prolymphocytes during the course of the disease, is accompanied by loss of expression of MER [Forbes et al. 1979b].

Occasionally cells have been found which express MER and sIgG or sIgA [reviewed in Kumararatne and Ling 1983]. Since these cells must have undergone isotype switching, a relatively late event in B cell activation, MER cannot be restricted only to resting or immature B cells. It is necessary to propose either that the loss of MER with B cell activation is reversible or that these sIgA/G+ve, MER+ve B cells have undergone isotype switching without activation.

1.4.4 ACTIVATION OF B-CLL CELLS

It was thought, initially, that CLL cells were relatively anergic to activation by polyclonal activators, including various lymphocyte mitogens (phytohaemagglutinin, pokeweed mitogen, concanavalin A, protein A, anti-Ig and anti-beta$_2$ macroglobulin). Since the B-CLL cell appears to derive from a functionally distinct B cell subset, its poor responsiveness to some activators may reflect lack of the receptors or signal pathways for these substances rather than a leukaemia-associated abnormality.

Another factor contributing to the relative anergy of these

cells is a defect in T cell function in B-CLL. The absolute number of peripheral blood T cells is increased in B-CLL [Fernandez et al. 1980] and many have immature features [Matutes et al. 1981]. Alterations in the relative proportions of T helper cells and suppressor cells [Herrmann et al. 1982] as well as adherent accessory cells [Waldmann and Broder 1982] may determine whether the B-CLL cells receive sufficient "help" in their activation.

Early studies indicated that some populations of B-CLL cells, but not most, could respond to various B cell activators including anti-Ig, mitogens and T cell-derived factors [Maino et al. 1977, Godal et al. 1978, Autio et al. 1979, Fu et al. 1979, Robert et al. 1980, Bloem et al. 1982, Lantz et al. 1985]. Often these were populations with more mature phenotype and showing in-vivo activation.

Studies with phorbol esters indicated that most B-CLL cells were capable of being activated to early plasma cells [Tötterman et al. 1980]. Greater understanding of the precise requirements for B cell activation in vitro has resulted in increased success in the activation of B-CLL cells [Yoshizaki et al. 1982, Robert et al. 1985, Calvert et al. 1987].

B-CLL cells proliferate in response to a signal via sIg (anti-Ig or anti-idiotype), in the presence of T cell derived growth factors (eg IL-2) [Yoshizaki et al. 1982, Kishimoto et al. 1984]. In the presence also of T cell-derived differentiation factors, B-CLL cells are induced by anti-Ig to differentiate to Ig secreting cells.

Higher concentrations of anti-Ig are required for stimulation of B-CLL cells than for normal B cells [Perri 1986]. One reason may be that these cells contain lower densities of

sIg than normal B cells. SAC, which also acts via sIg, is able to stimulate only those B-CLL cells which have sufficient sIg for it to bind to [Romagnani et al. 1981].

The morphology, RNA content, presence of MER, lack of classical activation markers such as BB1, LB1 and 4F2 and low content of MHC class II antigens suggest that B-CLL cells are quiescent G_o B cells. However, there is some indication that they may be partly induced in the activation process. First, many populations of B-CLL cells express at least three of the activation markers, CD23, B5 and CD25 (IL-2 receptors) [Freedman et al. 1987]. A few cases also express B-LAST-1 antigen, which is normally expressed in G_1. However, the density of these antigens on B-CLL cells is much less than that on the normal activated B cells and the IL-2 receptor is of lower affinity.

Secondly, B-CLL cells respond differently to some activators compared with normal B cells. B-CLL cells respond to alpha and gamma interferon with differentiation, and in some cases, proliferation [Robert et al. 1985, Östlund et al. 1986, Tötterman et al. 1988] whereas normal B cells do not directly respond to interferon but have enhanced response to other activators in the presence of interferon [Blomgren et al. 1981]. Similarly, cytochalasin B directly induces proliferation in B-CLL cells [Larson and Yachnin 1983] but is only a costimulator (with anti-Ig) for normal B cells [Rothstein et al. 1986a,b].

1.5 EFFECT OF PHORBOL ESTERS ON HUMAN B CELLS

Phorbol esters are plant-derived tetracyclic terpenes which have potent effects on a variety of cellular functions, including proliferation and differentiation, in many different types of cells. The effects of phorbol esters on normal and leukaemic B cells are described in this section and the general biological actions of these compounds are described in section 1.6. Phorbol esters induce a sequence of changes in human B cells consistent with activation and plasmacytoid differentiation of these cells. Some other effects of phorbol esters on B cells may be distinct from those induced by other B cell activators (see below).

1.5.1 EFFECT ON MER

One of the earliest effects of phorbol esters on B-CLL cells, and normal MER+ve B cells, is inhibition of capacity to rosette with mouse erythrocytes [Forbes et al. 1981, 1982a, Tötterman et al. 1981b, Shawler et al. 1984, Caligaris-Cappio et al. 1985]. Inhibition occurs within minutes in B-CLL cells treated with nanomolar concentrations of TPA [Forbes et al. 1981]. All TPA-treated B-CLL cells undergo inhibition of rosetting, even infrequent populations which are resistant to TPA-induced plasmacytoid differentiation [Tötterman et al. 1981b].

Other biologically active phorbol esters also inhibit rosetting with orders of potency paralleling their activities in other biological systems. The second stage promoter mezerein is nearly as active as TPA [Forbes et al. 1981].

Little is known about the mechanism by which phorbol esters inhibit rosetting but the effect on MER may be like their effects on other receptors, for example the receptors for epidermal growth factor and insulin (see chapter 1.6). TPA does not disrupt

pre-formed rosettes and has no effect if only the mouse red cells are treated [Forbes et al. 1981]. There is no similar rapid inhibition of capacity of B-CLL cells to bind antibody-sensitized bovine red cells (by Fc receptors) and complement-coated zymosan particles (by complement receptors). Binding of sheep erythrocytes by T cells is not inhibited by TPA [Forbes et al. 1981]. However, TPA does reduce the expression of the CD4 antigen on helper T cells [Jagielski et al. 1983].

As may be expected from the rapidity with which TPA inhibits MER, the action of TPA is not antagonized by inhibitors of protein or RNA synthesis [Forbes et al. 1981]. The effect of TPA is not reversed by washing the cells and culturing overnight in fresh medium, suggesting that the loss of expression of MER is irreversible. However, because of the lipophilicity of TPA it is difficult to remove it completely from cells by washing.

Further studies on the mechanism of action of phorbol esters on MER are described in the experimental section of this thesis.

1.5.2 EFFECT ON OTHER PHENOTYPIC MARKERS

Consistent with the hypothesis that the changes induced by TPA in B cells are a consequence of activation and differentiation, TPA increases synthesis and membrane expression of MHC class II antigens in B-CLL cells [Tötterman et al. 1981a, Okamura et al. 1982b] and rapidly increases expression of c-*myc* and c-*fos* proto-oncogenes in normal human B cells and B-CLL cells, particularly in the presence of calcium ionophore [Roifman et al. 1987, Norton et al. 1987, Larsson et al. 1987]. Levels of mRNA are increased at 1 and 4 hours of culture but not at 24-48

hours. The products of these genes have been implicated in the regulation of differentiation of HL60 cells [Müller et al. 1985]. There is loss within the first few hours of the 45kDa CD37 antigen [Carlsson et al. 1988].

TPA induces expression of the B cell activation antigens BB1, 4F2 and LB1 and of the receptors for IL-2 and transferrin [Åman et al. 1984, Carlsson et al. 1988]. Accompanying this is a decrease in expression of sIg, Fc and complement receptors, CD20 and CD21 antigens [Tötterman et al. 1980,1981b, Forbes et al. 1982a, Shawler et al. 1984, Gordon et al. 1984, Åman et al. 1984, Roifman et al. 1987]. Surface IgM is lost more slowly than sIgD. In cases of sIg-ve B-CLL, TPA causes an increase in sIg consistent with induced differentiation [Shawler et al. 1984].

Loss of Fc_{gamma} receptors in TPA-treated B-CLL cells occurs within 24 hours of culture while Fc_{mu} and complement receptors are lost at a later stage [Forbes et al. 1982a]. This is also thought to be the sequence of events which occurs during differentiation in vivo [Biberfeld and Nilsson 1980]. In the study of Tötterman and colleagues [1981b], only those B-CLL populations which responded to TPA with plasmacytoid differentiation showed loss of Fc_{gamma} receptors.

TPA induces or increases expression of CD5 on some CD5-ve and CD5+ve normal and malignant B cells, apparently resulting from increased synthesis of the CD5 antigen rather than altered expression [Miller and Gralow 1984, Shawler et al. 1984]. The significance of this is not known.

1.5.3 MORPHOLOGICAL CHANGES

Phorbol esters induce changes in the morphology of B cells that are consistent with plasmacytoid differentiation [Tötterman et al. 1980, 1981a, Okamura et al. 1982a, Cossmann et al. 1984, Caligaris-Cappio et al. 1984].

There is increase in cell size, consistent with entry into G_1 [Carlsson et al. 1988], marked change in cell shape as well as moderate aggregation of the cells, in the first few hours of culture. By 24 to 48 hours, the TPA-treated cells have abundant basophilic cytoplasm and a prominent nucleolus and, by 72-96 hours, the cytoplasm is very basophilic and vacuolated and the nucleus is larger, eccentrically located and contains less condensed heterochromatin (typical of plasmacytoid cells). The cells develop a prominent Golgi apparatus and extensive smooth endoplasmic reticulum. The number of mitochondria increases and occasional lysosome-like granules form. Between 90 and 100% of the cells are lymphoblastoid or plasmacytoid but there is only weak staining for acid phosphatase and beta-glucuronidase in a few cells, in contrast to the strong granular staining seen in mature plasma cells [Tötterman et al. 1980].

The changes in shape induced by TPA include formation of extensive cytoplasmic processes, similar to those seen in the malignant B cells of "hairy cell" leukaemia [Caligaris-Cappio et al 1984]. Like "hairy cells", TPA-treated B-CLL cells have tartrate-resistant acid phosphatase and have high density expression of CD22 and CD25 antigens [Caligaris-Cappio et al. 1985, Ziegler-Heitbrock et al. 1985]. TPA did not appear to cause formation of these membrane projections in normal MER+ve B cells, in one study [Caligaris-Cappio et al. 1982], although it did in another study [Crow and Kunkel 1985]. Associated with these

changes were alterations in the distribution of F-actin-associated membrane adhesion structures (podosomes) [Caligaris-Cappio et al. 1986].

The formation of cytoplasmic projections appears to be a response characteristic of phorbol esters rather than a step in the activation of B cells. When B-CLL cells are treated with TPA in the presence of calcium ionophore, the cells do not elongate and attach to the sides of the culture vessel and they do not acquire the hairy-like protrusions. Instead, they remain round or oval, form clusters, and acquire a deeply basophilic cytoplasm, eccentrically-located nucleus and plasma cell-like perinuclear clear zones [Drexler et al. 1987]. Calcium ions may regulate formation of these protrusions.

1.5.4 IMMUNOGLOBULIN PRODUCTION

Cytoplasmic immunoglobulin

After one day of culture with TPA, most B-CLL cells express abundant cytoplasmic Ig (cIg) of the same light chain type as the sIg on the original untreated cells [Tötterman et al. 1980, 1981a]. The average amount of cIg per cell increases five fold and there is a twenty-fold increase in frequency of cIg+ve cells with TPA treatment.

TPA increases synthesis of both heavy and light chains of Ig as shown by ^{35}S-methionine labelling and immunoprecipitation [Tötterman et al. 1981a]. Other biologically active phorbol esters, and the related compound mezerein, also induce plasmacytoid differentiation in B-CLL cells with relative potencies in agreement with their activity as inflammatory substances. Biologically inactive phorbol derivatives do not induce differentiation [Forbes et al. 1981].

Increased synthesis of cIg in TPA-treated B-CLL cells is regulated at the level of translation of mRNA. There is a switch from synthesis of sIgM to synthesis of pentameric secretory IgM, within minutes of exposure to TPA [Tötterman et al. 1988].

Tötterman and colleagues [1981a] found three (out of seventeen) cases of B-CLL in which the leukaemic B cells did not respond to TPA with plasmacytoid differentiation. In these three cases, the disease was relatively inactive. Okamura and colleagues [1982b] found that the most typical B-CLL populations, characterized by low amounts of sIg and MHC class II antigens and small cell size, responded much better to TPA than the atypical populations with high amounts of sIg and MHC class II antigens and larger cell size. Thus, the responsiveness may correlate with degree of cell maturation or state of activation.

Immunoglobulin secretion

Although TPA-treated B cells accumulate cIg, they may not always secrete the Ig. Secretion of Ig was seen in some studies [Ralph and Kishimoto 1981, Roth et al. 1982, Sugawara 1982, Okamura et al. 1982a, Cossman et al. 1984, Gordon et al. 1984, Åman et al. 1984, Murphy et al. 1987, Tötterman et al. 1988] but not in others [Tötterman et al. 1980,1981a, Weetman et al. 1982, Jagielski et al. 1983, Falcioni et al. 1985].

Falcioni's group reported little or no Ig secretion induced by TPA in peripheral blood or tonsil B cells that had been highly purified and in another study it was reported that TPA inhibits both spontaneous and mitogen-induced Ig synthesis in human blood B cells. Inhibition required at least 12 hours of exposure to TPA [Weetman et al. 1982] and may have been mediated by TPA-activated suppressor T cells.

In contrast, a strong response with tonsil B cells was

attributed to the increased number of pre-activated cells in tonsil preparations [Åman et al. 1984]. Cossman and colleagues [1984] reported that cells from all of ten cases of B-CLL were induced to secrete IgM of the same light chain type as the sIg on the original cells. This was preceded by a rapid increase in the level of mRNA specific for the secretory form of the mu heavy chain. TPA also increased secretion of Ig from several B lymphoblastoid cell lines [Polke et al. 1986].

The findings that recombinant IL-2 promotes Ig secretion by TPA-treated B-CLL cells [Kabelitz et al. 1985] and that increased secretion of Ig and $beta_2$ microglobulin in TPA-treated cells is T cell-dependent [Danersund et al. 1985, Tötterman et al. 1986, 1988] may indicate that in those systems where secretion has been found there has been production by contaminating T cells of IL-2 or other factors influencing differentiation. In support of this, normal B cells, treated with TPA and calcium ionophore, do not secrete Ig unless B cell differentiation factor is present [Clevers et al. 1985b]. The introduction of a serum-free culture system for study of TPA-induced differentiation of B-CLL cells may also help to clarify the mechanisms involved [Tötterman et al. 1988].

TPA-treated B-CLL cells only produce IgM [Tötterman et al. 1980, 1981a, Cossmann et al. 1984, Gordon et al. 1984, Forsbeck et al. 1987]. This may indicate that B-CLL cells cannot undergo isotype switching or that the factors required for isotype switching are not present in these cultures. In a case of lymphosarcoma, the malignant B cells appeared to undergo isotype switching from IgM to IgG, late in culture with TPA [Efremidis et al. 1985].

1.5.5 PROLIFERATION

There have been conflicting reports as to whether TPA is a mitogen for human B cells. Walker and colleagues [1986] found that TPA readily activated normal B cells, as determined by entry into G_1, but only a proportion underwent DNA synthesis. Falcioni et al. [1985] could find no evidence of mitogenesis induced by TPA in highly purified normal B cells. Similarly, no effect of TPA on DNA synthesis in B-CLL cells was found, in most studies [Tötterman et al. 1980, Herrmann et al. 1985, Kabelitz et al. 1985, Ziegler-Heitbrock et al. 1985, Norton et al. 1987],

Others have reported that TPA is a potent mitogen for normal B cells [Sugawara 1982, Åman et al. 1984] and B-CLL cells [Nel et al. 1985]. Cossman and colleagues [1984] found that TPA caused an increase in DNA synthesis but not cell division.

These conflicting results on proliferation, like those on secretion of Ig, may be explained by presence of IL-2, IL-4 and other factors derived from contaminating T cells. In support of this, IL-2 synergizes with TPA in induction of IL-2 receptors and DNA synthesis in a proportion of normal and B-CLL cells [Ando et al. 1985, Kabelitz et al. 1985]. Antisera to IL-2 receptor reduce the level of DNA synthesis in these cells to that of cells treated only with TPA [Ando et al. 1985]. Some proliferation may also be due to mitosis in contaminating T cells.

Calcium ionophore, ligands of sIg and various mitogens also synergize with TPA in induction of DNA synthesis in normal human B cells and B-CLL cells [Bertoglio 1983, Suzuki et al. 1985, Clevers et al. 1985b, Walker et al. 1986, Drexler et al. 1987, Roifman et al. 1987].

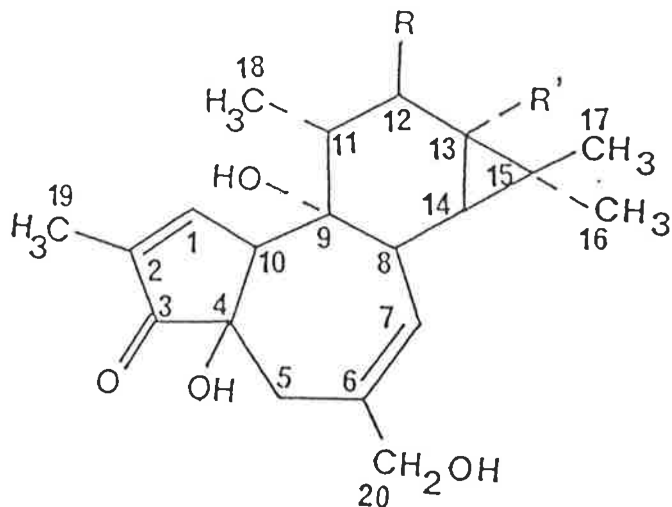
1.6 BIOLOGICAL EFFECTS OF PHORBOL ESTERS

Phorbol esters were initially studied because of their potent inflammatory and tumour promoting activity. However, subsequent studies showed that they affect a wide variety of cellular processes including proliferation and differentiation, cell adhesion, intercellular communication, gene expression, membrane receptor expression and metabolism. Phorbol esters cause some alterations in normal cells, characteristic of malignant transformation, and enhance some of these abnormalities in partially transformed cells. Many aspects of phorbol esters have been reviewed extensively [Weinstein et al. 1979, Blumberg 1980, 1988, Blumberg et al. 1983, 1984, Ashendel 1985, Aitken 1986, Evans 1986a, Evans 1986b, Kinsella 1986].

1.6.1 TUMOUR-PROMOTING ACTIVITY AND IRRITANCY

Phorbol esters are widely distributed in two families of plants, the Euphorbiaceae and the Thymelaeaceae. These plants are toxic to animals and man but certain extracts have previously been used in medicine [Evans 1986b]. For example, croton oil from the seeds of *Croton tiglium*, has been used as a purgative. In the early 1940s, croton oil was recognized as a potent tumour promoting agent in the mouse skin model [Berenblum and Shubik 1947]. The most potent of the co-carcinogens from croton oil were identified as phorbol esters [Van Duuren and Orris 1965, Hecker 1968]. TPA is the most active of these agents and is the most potent promoter known. Phorbol dibutyrate, the phorbol ester used in most of the experiments in this thesis, is a relatively weak tumour promoter [Blumberg 1980]. The structures of some of the phorbol esters are shown in Fig 1.6.

The best studied model of tumour promotion is in mouse skin. Sarcomas form when a carcinogen such as 7,12 dimethyl-



Phorbol ester	Substituent at C12	Substituent at C13
TPA	MYRISTATE	ACETATE
PDBu	BUTYRATE	BUTYRATE
PDD	DECANOATE	DECANOATE
RPA	RETINOATE	ACETATE

Legend to Figure 1.6 Structures of some phorbol esters

The common phorbol ring structure is shown and beneath are the groups esterified at carbons 12 (R) and 13 (R') for some of the phorbol esters used in the experiments in this thesis. The 4-OH is in the beta configuration.

benzanthracene is given in a single sub-threshold dose and this is followed by multiple doses of phorbol ester. Neither treatment alone is effective although higher doses of the carcinogen do produce tumours [Blumberg 1980].

Tumour promotion has also been shown in other tissues such as muscle, liver, bladder, lung and forestomach and is believed to be the rate-limiting factor in the development of cancer [reviewed in Kinsella 1986]. Therefore, a greater understanding of the mechanism of action of tumour promoters is essential. Phorbol esters are thought to be involved in the high incidence of esophageal cancer in inhabitants of the island of Curacao, where chewing of the roots of _Croton flavens_ is practiced [Weber and Hecker 1978].

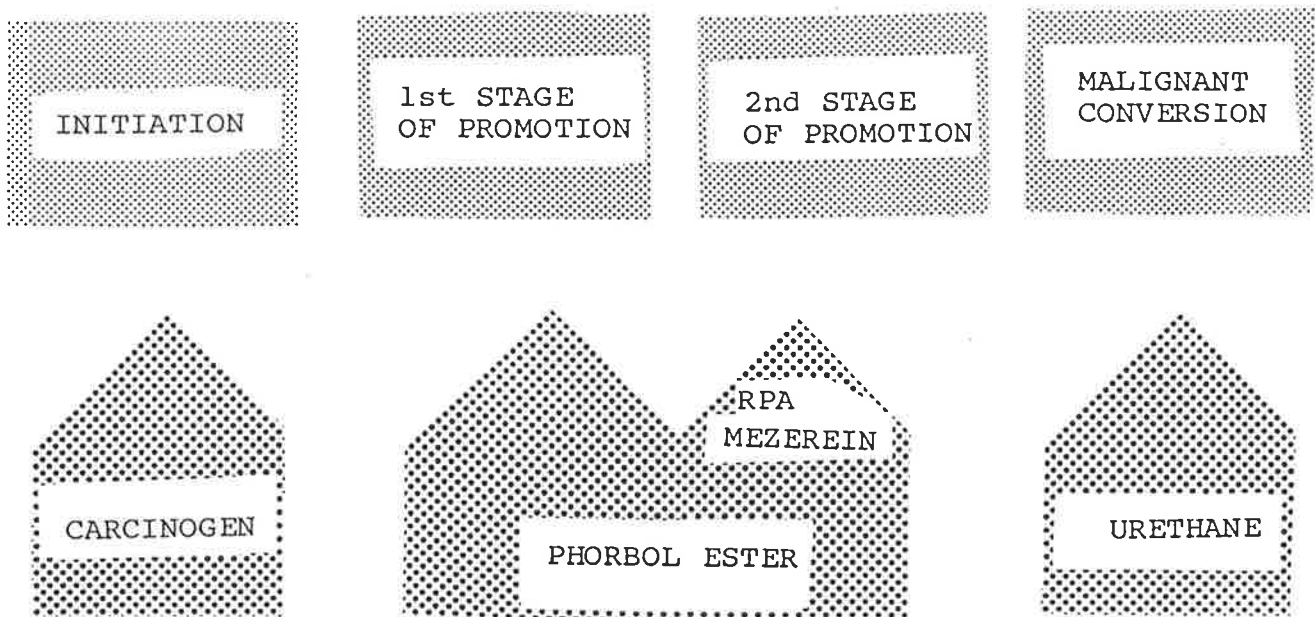
Multiple stages of carcinogenesis

Initiation is an irreversible step caused by the carcinogen that is thought to involve inheritable changes, as a result of covalent binding to DNA of highly reactive electrophiles generated from the carcinogen. In contrast, promotion is a reversible step allowing expansion of the initiated cell [Blumberg 1980] (Fig 1.7). A third stage may be involved in development of malignant tumours since initiation followed by promotion only results in benign tumours [Hennings et al. 1983]. Urethane is an effective inducer of this third stage.

Multiple steps also are required for malignant transformation of primary cell cultures, in vitro, involving at least two proto-oncogenes [Land et al. 1983] and distinct initiation and promotion phases [Mondal et al. 1976].

Two stages of promotion

It has been proposed that promotion consists of two stages



Legend to Figure 1.7 Multiple steps in carcinogenesis

The diagram shows the separate steps of initiation (by carcinogen), two stages of promotion (by phorbol ester or other tumour promoter) and malignant conversion (by other agents including urethane). Some phorbol esters (eg TPA) induce both stages of promotion whereas others like RPA and the phorbol ester-like compound mezerein induce largely or only the second stage.

with distinct mechanisms [Slaga et al. 1980, Fürstenberger et al. 1981] although Hennings and Yuspa [1985] have suggested that this occurs in only some strains of mice and is not a general feature of tumor promotion by phorbol esters. TPA and phorbol didecanoate induce both stages (ie they are complete promoters), high doses of A23187 and 4-O-methyl TPA are first stage promoters and mezerein and retinoyl phorbol acetate (RPA) are strong second stage promoters [Slaga et al. 1980, Fürstenberger et al. 1981]. RPA is almost without activity as a complete tumour promoter while mezerein is about 50 times less active than TPA. Second stage promoters often have unsaturated substituents esterified to the phorbol backbone.

The first stage is thought to involve formation of primitive skin stem cells recognized by their dark appearance [Klein-Szanto et al. 1980]. Since the first stage is blocked by esterase inhibitors, it may involve the action of an esterase or protease [Troll et al. 1970]. Chromosomal alterations are probably also important in the first stage [Petrusevska et al. 1988].

The second stage is thought to consist of events leading to expansion of the clone of initiated cells [Blumberg 1980] and to involve PKC (see later). Expansion of this population may be mediated by inhibition of differentiation in initiated stem cells [Weinstein et al. 1979]. Phorbol esters inhibit terminal differentiation of mouse epidermal cells in culture and they induce changes in mouse epidermis consistent with inhibition of differentiation or dedifferentiation.

1.6.2 EFFECTS ON CELL PROLIFERATION, IN VITRO

Phorbol esters are potent mitogens or co-mitogens for fibroblasts and lymphocytes, often acting synergistically with other mitogens including serum, platelet-derived growth factor,

fibroblast growth factor, epidermal growth factor, insulin and lectins in fibroblasts [reviewed in Weinstein et al. 1979]. Phorbol esters eliminate the requirement for macrophages in mitogen-stimulated lymphocyte cultures [Koretzky et al. 1982] and enhance IL-2 production in EL4 thymoma cells [Farrar et al. 1980].

Maximal stimulation of proliferation by phorbol esters in various types of cells is seen when the cells are in sub-optimal growth conditions eg because of lack of growth factor or calcium [Blumberg 1980]. This suggests that phorbol esters are not mitogens as such but rather sensitize cells to factors required for growth.

1.6.3 EFFECTS ON CELL DIFFERENTIATION IN VITRO

Phorbol esters are considered to be amongst the most active compounds affecting the process of differentiation [Abrahm and Rovera 1980]. In some cell types, differentiation is enhanced or induced by phorbol esters whereas in others it is suppressed.

Phorbol esters inhibit spontaneous- and chemically-induced erythroid differentiation in murine erythroleukaemia cells [Yamasaki et al. 1977], myogenesis in chick embryo myoblasts [Cohen et al. 1977] and chondrogenesis in chick embryo chondroblasts [Pacifici and Holtzer 1977]. Differentiation is suppressed by TPA in mouse neuroblastoma cells [Ishii et al. 1978] and adipose cells [Diamond et al. 1977].

On the other hand, phorbol esters induce B-CLL cells to differentiate into plasmacytoid cells (see chapter 1.5), acute lymphocytic leukaemia pre- B cells to a more mature B cell stage [Cossman et al. 1982] and promyelocytic leukaemia cell lines (eg HL60), mouse and human myeloid leukaemia cells and normal bone

marrow cells to a macrophage-like stage [Rovera et al. 1979, Huberman and Callahan 1979]. They also induce differentiation in human melanoma cells [Huberman et al. 1979] and in Rauscher virus-transformed murine erythroleukaemia cell lines [Miao et al. 1978].

Therapeutic value of phorbol esters and related compounds in leukaemia

The capacity of phorbol esters to induce differentiation of leukaemic cells in vitro has suggested their possible use as therapeutic agents in treatment of leukaemias, at least in experimental animals [Rovera et al. 1979, Weinstein et al. 1979, Lotem and Sachs 1979, Perussia et al. 1982, Koeffler 1983]. Extracts of various euphorbiaciae have been used in treatment of cancers and warts since ancient times [see Kupchan et al. 1976]. Phorbol 12-tigliate 13-decanoate and mezerein inhibit the growth of the transplantable mouse lymphoma P388, in vivo [Kupchan et al. 1976]. Mezerein and phorbol didecanoate, but not TPA, prevent formation of chloroma-like tumours in nude mice inoculated with K562 promyelocytic leukaemia cell line. Treatment of the cells with mezerein prior to inoculation also prevents tumour formation [Perussia et al. 1982].

That phorbol esters are tumour promoters has not been seen as a serious objection to their use in therapy [see Perussia et al. 1982], since promotion of mouse skin tumours, at least, requires repeated administrations of phorbol esters at specific time intervals [Weinstein et al. 1979].

A more serious objection however is the toxicity of these agents. A dose of five micrograms of TPA kills 50% of mice within 12 hours [Perussia et al. 1982]. Intravenous infusion in

rabbits causes acute pneumonitis and interstitial pulmonary fibrosis [O'Flaherty et al. 1980].

1.6.4 EFFECT ON CELL METABOLISM IN POLYMORPHONUCLEAR LEUKOCYTES

Diverse effects of phorbol esters on cell metabolism have also been described. This has been well characterized in polymorphonuclear leukocytes (PMN). Phorbol esters cause a marked stimulation of cellular oxidative metabolism in PMN, including increased superoxide anion and hydrogen peroxide production, increased glucose oxidation via the hexose monophosphate shunt and increased cellular chemiluminescence [DeChatelet et al. 1976, Goldstein et al. 1981, Lehrer 1982]. The increased oxidative metabolism is a consequence of activation of the membrane-bound flavoprotein oxidase, NAD(P)H oxidase [reviewed in Badwey and Karnovsky 1986] .

This oxidative burst normally accompanies phagocytosis; the superoxide anion, hydrogen peroxide and more toxic substances derived from them, including hydroxy radicals and oxidized halogens, are potent anti-microbial agents.

Phorbol esters also cause a partial and selective degranulation of the secondary or specific granules in PMN [Lehrer 1982].

1.6.5 EFFECTS ON CELL MEMBRANES

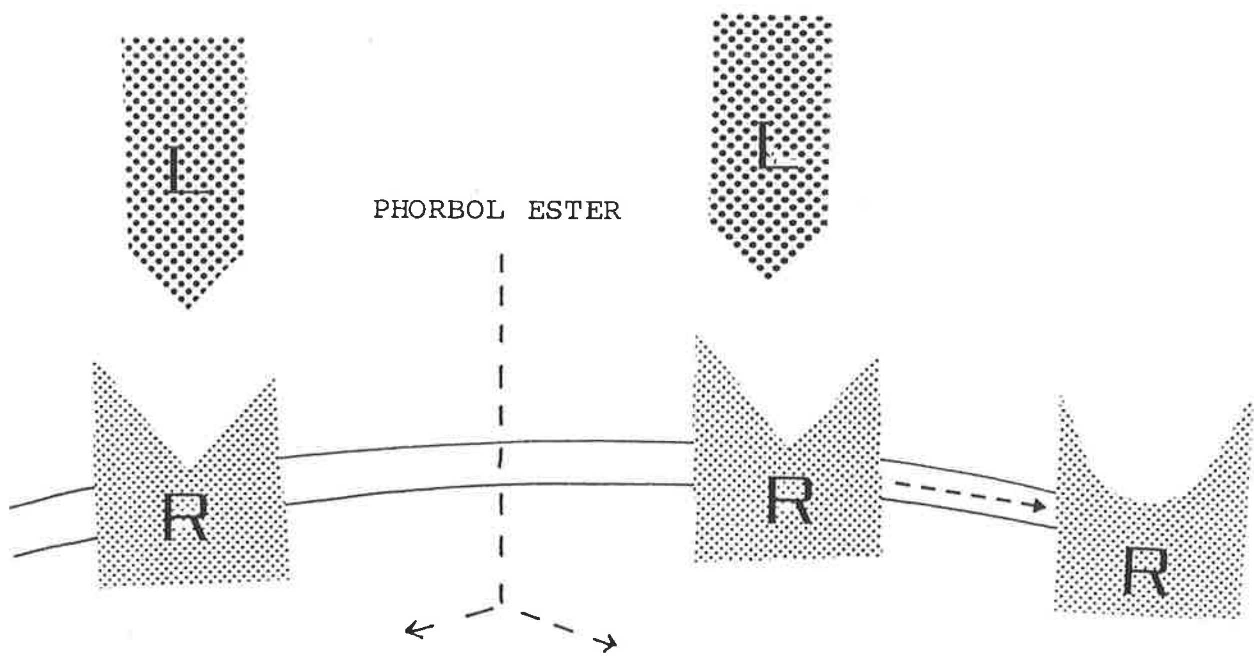
Phorbol esters alter membrane structure [Van Duuren et al. 1976], membrane fluidity [Fisher et al. 1979, Castagna et al. 1979], cell adhesion [Yamasaki et al. 1979] and uptake of various substances [reviewed in Weinstein et al. 1979]. These rapid effects on cell membranes are either a direct result of insertion of phorbol esters in the lipid bilayer or a result of a secondary process triggered by phorbol esters.

1.6.6 EFFECTS ON MEMBRANE RECEPTORS

A major action of phorbol esters on cells is the alteration of responses to extracellular signals. One mechanism is the modification of cell membrane receptors, particularly those for growth factors or hormones. Phorbol esters inhibit binding of ligands to several types of cell surface receptors (Fig 1.8). Phorbol esters uncouple beta-adrenergic receptors from adenylate cyclase [Kelleher et al. 1984]. The effect of phorbol esters on a particular receptor is often tissue-specific. For example, phorbol esters inhibit insulin binding in HL60, B lymphoblastoid cells, U937 cells and embryonic carcinoma cells but not in fibroblasts, HeLa cells and hepatoma cells [Grunberger and Gorden 1982, Thomopoulos et al. 1982].

Inhibition of the receptor for epidermal growth factor by phorbol esters has been studied in detail. Phorbol esters rapidly inhibit this receptor without affecting several other types of receptors [Weinstein et al. 1979, Shoyab et al. 1979]. Inhibition is not a result of increased degradation of epidermal growth factor nor increased internalization of epidermal growth factor-receptor complexes. The mechanism differs from that by which epidermal growth factor down-regulates its own receptor [Lin et al. 1986] and is accompanied by a loss of the tyrosine kinase activity of the epidermal growth factor receptor and phosphorylation of the receptor by PKC (see chapter 1.8) [Friedman et al. 1984]. The inhibition of binding has been attributed both to internalization of unoccupied receptors [Weinstein et al. 1979] and to decreased affinity of the receptors [Shoyab et al. 1979] (Fig 1.8).

Phorbol esters cause a decrease in affinity and phosphorylation of insulin receptors in a B lymphoblastoid cell



Internalization of receptors

Receptors for

Epidermal growth factor
Transferrin
Somatostatin
Thyrotropin-releasing hormone

Decrease in affinity of receptors

Receptors for

Epidermal growth factor
Insulin
Asialoglycoprotein

Legend to Figure 1.8 Effects of phorbol ester on receptors

The binding of ligand (L) to cell surface receptors (R) is affected by phorbol esters in two ways, a decrease in affinity of the receptor for the ligand (eg receptors for insulin and asialoglycoprotein) and a decrease in total numbers of receptors (eg receptors for transferrin, somatostatin and thyrotropin-releasing hormone). Epidermal growth factor receptors appear to be affected in both ways. Decrease in receptor number may be due to internalization of the receptors.

line [Thomopoulos et al. 1982, Jacobs et al. 1983] and a decrease in affinity of receptors for asialoglcoprotein in a hepatoma cell line [Fallon and Schwartz 1986]. In contrast, phorbol esters cause a decrease in the apparent total number of receptors (perhaps due to internalization of the receptors) for transferrin in promyelocytic leukaemia and hepatoma cells [May et al. 1985a, Fallon and Schwartz 1986] and for thyrotropin-releasing hormone and somatostatin in pituary and pancreatic acinar cells [Matozaki et al. 1986]. Transferrin receptors are phosphorylated in phorbol ester-treated cells [May et al. 1985a].

1.6.7 EFFECTS IN THE NUCLEUS

Chromosomes

TPA damages chromosomes by causing DNA strand breakage [Birnboim 1982], sister chromatid exchange [Emerit and Cerutti 1981] and chromosome aberrations [Emerit and Cerutti 1981, Fusenig and Dzarlieva 1982]. It has been suggested that superoxide anions that are generated in response to phorbol esters mediate these effects [Emerit and Cerutti 1981].

Gene expression

Phorbol esters alter the expression of genes for many different types of proteins. In many cases, new gene expression is secondary to phorbol ester-induced change in cell-cycle or stage of differentiation. Examples of this are phorbol ester-induced secretory immunoglobulin synthesis in B lymphocytes [Tötterman et al. 1980] and keratin protein synthesis in epidermal cells [Laskin et al. 1981].

Some of the new gene expression results from gene derepression. Examples include induction by TPA of genes of bovine papilloma, Epstein Barr virus, Simian sarcoma virus 40

and herpes virus in latently infected cells [Zur Hausen et al. 1978, Amtmann and Sauer 1982, Imbra and Karin 1986] and induction of c-*myc* proto-oncogene [Kelly et al. 1984].

In some cases, increased expression of a protein in phorbol ester-treated cells results from increased gene transcription (eg metallothionein) [Angel et al. 1987]. Direct induction of gene transcription by phorbol esters is mediated by changes at the level of regulatory proteins which bind to oligonucleotide sequences in the enhancer region of genes and promote gene transcription [Jones et al. 1987, Lee et al. 1987, Kadonga et al. 1987, Imagwa et al. 1987]. It is not clear whether there is an increase in the intracellular concentration of the regulatory factors or an increase in their activity (eg as a result of phosphorylation) [Imbra and Karin 1986, Angel et al. 1987].

In some other cases, there is increased stability of the messenger RNA (eg for granulocyte and macrophage colony stimulating factor) [Shaw and Kamen 1986]. The mRNA is unstable because of a poly adenosine-uracil sequence in the 3' untranslated region. A newly synthesized protein is involved since inhibition of protein synthesis by cycloheximide stabilizes the mRNA. Phorbol esters may cause post-translational modification of this protein or they may inhibit its production.

1.7 RECEPTORS FOR PHORBOL ESTERS

Many of the results in this thesis are in the form of binding plots. This section introduces some general concepts about receptors and then describes the receptors for phorbol esters. Evidence that the receptors are protein kinase C is presented in section 1.8.

1.7.1 GENERAL FEATURES OF RECEPTORS

Early concepts of receptors

Hormones and growth factors, as well as many drugs, act via receptors. These are sites which bind ligands with high affinity and in a saturable and reversible manner, and which trigger a characteristic biological response [Limbird 1986]. The biological effects occur at ligand concentrations which occupy the receptors and with a time course consistent with the kinetics of binding. Ligands which induce a response are referred to as agonists. For a series of agonists, there is correlation between affinity for the receptor (as determined by direct binding or competition binding studies) and capacity to induce the functional response. Other types of ligands, referred to as antagonists, cannot induce the conformational change in the receptor that triggers the response even though their affinities for the receptor are often higher than that of agonists.

The concept of a receptor emerged from the studies of Paul Ehrlich [1913] and John Langley [1909] on the interaction of drugs and toxins with tissues and cells. Mathematical interpretation of binding data followed the realization that drugs and hormones (ligands) combine with receptors according to mass-action law, that is association of receptors and ligands occurs at a rate dependent on the concentration of the ligand and receptor and the dissociation of the ligand-receptor complex is

at a rate proportional to the concentration of receptor complexes [Clark 1937].

Binding assays

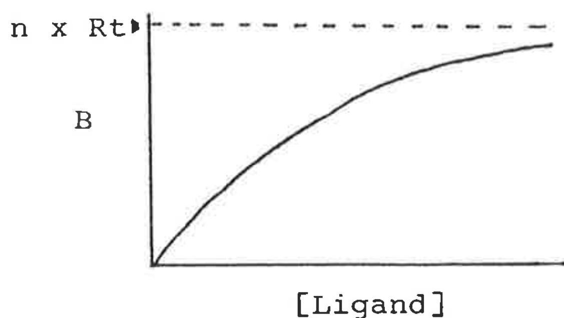
Binding assays are often done with radiolabelled ligand and bound and free ligand separated rapidly by centrifugation, vacuum filtration, precipitation, column filtration or other means. Non-specific binding, taken as binding in the presence of a large excess of unlabelled ligand, is subtracted from the total binding to give the specific binding. Non-specific binding usually increases linearly but at high concentrations of hydrophobic ligands there may be considerable partitioning of the ligand into membrane lipid and this complicates the analysis of binding.

A convenient way to analyze binding of radio-labelled ligand to receptor is to assay specific binding (at equilibrium) over a wide range of ligand concentrations. A plot of bound ligand versus concentration of ligand (saturation plot) will reach a plateau as the receptors begin to be saturated (Fig 1.9a). The horizontal asymptote will approach the maximum binding capacity.

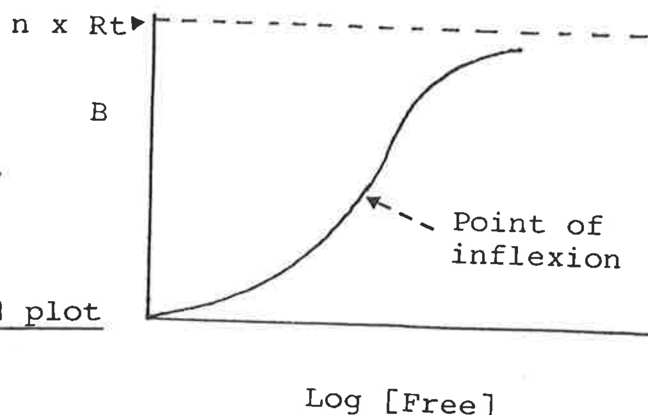
Scatchard plot

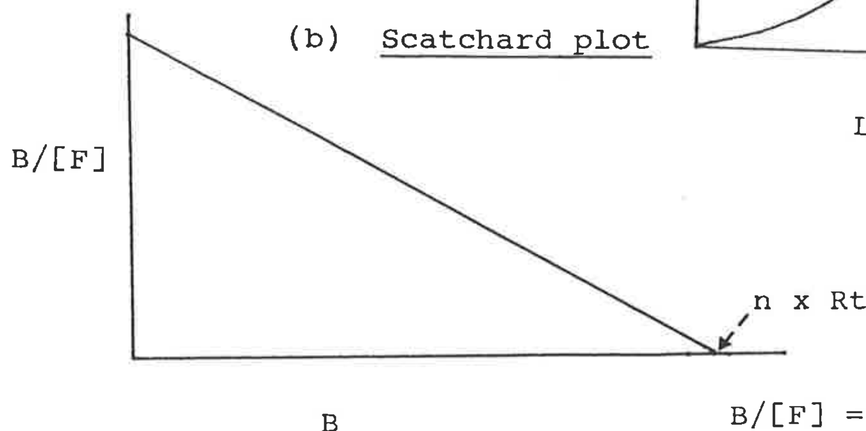
The equilibrium dissociation constant (Kd), a measure of the receptor's affinity for the ligand, and the total receptor number (Rt), can be derived from linear transformation of the binding data. The Scatchard (or Rosenthal) plot, initially used in a slightly different form for study of binding of small ligands to soluble proteins [Scatchard 1949], is the most commonly used transformation. For a system in which there is one form of the ligand and the receptors are homogeneous, a plot of [bound ligand]/[free ligand] versus [bound ligand] is a straight line whose slope is - 1/Kd and whose intercept with the abscissa is

(a) Saturation plot



(c) Klotz plot





$$B/[F] = -1/Kd\ ((n \times Rt) - B)$$

where

B = Amount of ligand bound
[F] = Conc. of free ligand
Kd = Equilibrium dissociation constant
Rt = Total number of receptors
n = Number of binding sites per molecule of receptor

Legend to Figure 1.9 Binding plots

Three types of binding plots used in this thesis are shown.

(a) Saturation plot is a plot of bound (B) versus ligand concentration. Horizontal asymptote of plot approaches the maximum binding capacity (n x Rt) as ligand concentration approaches infinity.

(b) Scatchard plot is a plot of B/ free ligand concentration versus B. Intercept with abscissa is n x Rt and slope is the negative reciprocal of the dissociation constant.

(c) Klotz plot is a plot of B versus log. free ligand concentration. Horizontal asymptote approaches n x Rt as free ligand concentration approaches infinity. Plot is an S-shaped curve symmetrical about the point of inflexion.

n x [Rt], where n= number of binding sites per molecule of receptor. This can be simplified to bound ligand/ [free ligand] versus bound ligand or B/[F] versus B. The intercept with the abscissa is n x Rt (Fig 1.9b). This form of the plot was used in the analysis of binding data in this thesis.

Correct determination of the intercept with the abscissa relies upon there being data over a sufficiently wide range of ligand concentrations. Often sufficient data at high ligand concentrations cannot be obtained because the non-specific binding increases non-linearily. A plot of bound ligand versus [free ligand] (Fig 1.9c, Klotz plot [Klotz 1982]) is useful for determining whether there are sufficient binding data to extrapolate the Scatchard plot to the abscissa. The Klotz plot should extend beyond the point of inflexion.

Validity of Scatchard analysis for ligand binding to cells

A number of assumptions are made in the derivation of the linear transformation [reviewed in Limbird 1986]: (1) that ligand-receptor interactions involve a simple, reversible bimolecular reaction between one class of ligand and one class of receptor, (2) binding has attained full equilibrium, not just steady state, (3) bound and free can be determined accurately and the relationship between the two is not altered by the separation procedure and (4) the concentration of receptor is much less than the Kd for the ligand and therefore the quantity of bound radioligand is negligible relative to the amount added.

Some of these assumptions may not be applicable when the receptors are being assayed in intact cells under steady state conditions or when the receptor is a complex containing two or more different components [Boeynaems and Dumont 1975]. In these situations, the interpretation of the parameters derived from the

Scatchard plot may be different than when they are derived from plots of ligand binding to soluble protein. This is discussed further in the Results section. A steady state model for analyzing binding in cells has been developed for some polypeptide ligands [Wiley and Cunningham 1981]. If there are more than one class of receptor or if the receptors interact, the Scatchard plot will be curvilinear. This is discussed further in chapter 4.

Regulation of receptor numbers

The number of receptors in cells usually decreases when cells are exposed to the ligand for a prolonged period. Several mechanisms may be involved in this down-regulation. Other substances may also induce down-regulation of the receptor. Sometimes there is an increase (up-regulation) of receptors in cells exposed to ligand or other substance.

Relationship between receptor occupancy and biological effect

The biological effect caused by a ligand is proportional to the concentration of ligand-receptor complexes or, in some cases, to their rate of formation [Limbird 1986]. Where the effect is proportional to the concentration of occupied receptors, half-maximal response is obtained when half of the receptors are occupied. This will occur for a ligand concentration equal to the Kd. An increase in receptor number will cause a corresponding increase in the maximum response to the ligand.

Sometimes, the maximal effect is elicited when only a proportion of receptors is occupied. Under these conditions, an increase in receptor number will cause the response to occur at a lower ligand concentration [Lefkowitz 1979]. This will facilitate receptor systems in which there is a rapid onset and termination

of signal [Lefkowitz 1979].

1.7.2 DISCOVERY OF CELLULAR RECEPTORS FOR PHORBOL ESTERS

Metabolism of phorbol esters by cells

Accurate measurement of specific cellular receptors requires there to be no significant metabolism of the ligand during the course of the experiment.

TPA and PDBu are not metabolized by normal peripheral blood lymphocytes [Estensen et al. 1980], Hela cells [Kreibich et al. 1974], human fibroblasts [O'Brien and Diamond 1978], cultured thymoma cells [Sando et al. 1982] and the particulate fraction of mouse skin [Delclos et al. 1980] during short-term culture. However, TPA does undergo deacylation in cultures of mouse L cells and hamster embryo fibroblasts [O'Brien and Diamond 1978] and mouse skin explants [Kreibich et al. 1974]. Fatty acids are readily removed from phorbol esters by esterases, including one found in liver cytosol of mice [Shoyab et al. 1981] as well as the non-specific esterase in plasma of some species [Saito and Egawa 1984, Kadner et al. 1985].

Metabolites of TPA containing only one fatty acid are thought to be biologically inactive [O'Brien and Diamond 1978]. The actions of phorbol esters on cells are not mediated, therefore, by a metabolite of the phorbol ester but result from interaction of phorbol ester with its specific receptor.

Detection of specific phorbol ester receptors

Because of its long chain myristic acid, TPA is very lipophilic and appears to be primarily taken up by cells by partitioning into the cell membrane. In early studies, it was not possible to detect saturable, specific binding of ^{3}H-TPA to cells [Lee and Weinstein 1978]. Specific binding was seen in one study using normal lymphocytes [Estensen et al. 1980].

Later, Ashendel and Boutwell [1981] and Hergenhahn and Hecker [1981] detected specific binding of ^{3}H-TPA in various cell types by washing the cells with cold acetone. Acetone removes TPA that is not bound to its receptor.

PDBu is much less lipophilic than TPA and is preferred for detection of phorbol ester receptors. Specific, saturable, reversible high-affinity receptors for ^{3}H-PDBu have been demonstrated in nearly all mammalian cell types examined, as well as in cells of some vertebrates and invertebrates although not in at least one type of bacterium [reviewed in Blumberg et al. 1984].

Specificity of receptors has been confirmed by showing inhibition of binding of ^{3}H-phorbol ester by biologically active phorbol esters and related compounds. Those analogues which lack biological activity (including phorbol, 4-alpha-phorbol didecanoate and 4-O-methyl TPA) do not inhibit even at very high concentrations.

The receptors detected with ^{3}H-PDBu are the same as those detected with ^{3}H-TPA. Excess unlabelled PDBu inhibits the specific binding of ^{3}H-TPA and vice versa. The Kds for binding of PDBu and TPA to cellular receptors agree with those derived by competition inhibition studies. TPA has a much greater affinity of binding (Kd less than 1nM) than PDBu (Kd between 10 and 100nM) [Hergenhahn and Hecker 1981, Ashendel 1985, Nishihira and O'Flaherty 1985]. The number of receptors detected is of the order of several hundred thousand per cell.

Curvilinear Scatchard plots have been seen in some studies of binding of ^{3}H-PDBu to cells. These have generally been interpreted as evidence for multiple classes of the receptor

resulting from different environments of phospholipid and/or divalent cations or resulting from different isoforms of the receptor [Sando et al. 1982, Dunn and Blumberg 1983, Dunn et al. 1983]. A non-linear Scatchard plot may also result from a technical artifact in the binding assay [Limbird 1986].

Other examples of non-linear plots include binding of oxytocin, glucagon, catecholamines and insulin to cells [reviewed in Limbird 1986]. The question of whether non-linearity of the Scatchard plot results from interaction between the receptors [DeMeyts et al. 1976] or from multiple classes of receptors with different affinities [Kahn et al. 1974] has been discussed in detail for binding of insulin to cells. Curvilinear Scatchard plots describe the binding of substrates and cofactors to some enzymes [Limbird 1986].

Specific low affinity binding sites for phorbol esters

A second class of specific, saturable phorbol ester binding sites of very much lower affinity (Kd for TPA of 100nM, Kd for PDBu of 710nM) and very high numbers (several million sites per cell) has been detected [Shoyab and Todaro 1980, Horowitz et al. 1981].

These low affinity sites may be membrane phospholipid. Biologically active analogues of phorbol ester, but not the inactive stereoisomer 4-alpha-phorbol didecanoate, alter the properties of phospholipid monolayers and liposomes [Deleers et al. 1981]. It has been reported that ^{3}H-TPA binds in a saturable manner to liposomes of sphingomyelin with a low affinity (Kd 13nM) and bound ^{3}H-TPA is displaced by biologically active phorbol esters and related compounds [Esumi and Fujika 1983].

1.7.3 DISTRIBUTION OF RECEPTORS FOR PHORBOL ESTERS IN DIFFERENT TISSUES

The abundance of phorbol ester receptors in different tissues varies greatly. Brain has most, comprising about 0.2% of the particulate protein, suggesting that each cell contains several million binding sites [Blumberg et al. 1984]. The cortex has the highest density of receptors [Nagle et al. 1981, Nagle and Blumberg 1983]. Lymphoid tissue contains the second highest amount of the receptor. Other tissues and organs have much less receptor. Red cells lack phorbol ester receptors [Blumberg et al. 1984] although nucleated chicken red cells do bind phorbol ester [Gschwendt and Kittstein 1983]. Spermatozoa (at least from the ram) lack phorbol dibutyrate receptors [Roldan and Harrison 1988].

Serum inhibits binding of phorbol ester to various cell types [Horowitz et al. 1981, Shoyab and Todaro 1982]. Different types of inhibitor have been isolated from serum, including a 60kDa protein which inhibits phorbol ester binding, non-competitively, suggesting that it does not bind to the receptor [Horowitz et al. 1981], and a 70kDa non-glycosylated, heat and acid-labile protein which inhibits binding competitively [Shoyab and Todaro 1982]. The latter protein binds ^{3}H-PDBu in a specific, rapid, reversible and saturable manner. Since it does not degrade PDBu it is unlikely to be a serum esterase [Kadner et al. 1985].

1.7.4 REQUIREMENT FOR PHOSPHOLIPID AND CALCIUM FOR BINDING TO SOLUBLE RECEPTOR

Early studies suggested that phorbol ester binds only to the particulate fraction of cells and tissues [Dunphy et al. 1980]. It was subsequently shown that a large pool of aporeceptors was

present in cytosol and detergent soluble extracts of the particulate fraction and these required exogenous phospholipid and calcium ions for detection [Ashendel et al. 1983a,b].

In order to assay soluble PDBu receptors by centrifugation, Blumberg and colleagues precipitated the receptors with 15% poyethylene glycol [König et al 1985]. However, in the presence of phosphatidylserine (PS), polyethyleneglycol is not required [Sando and Young 1983]. An assay utilizing micelles composed of both Triton X-100 and phospholipid for assay of soluble phorbol ester receptors has been described [Hannun et al. 1985]. Separation of free and bound ^{3}H-phorbol ester has also been achieved by filtration [Uchida and Filburn 1984], high performance liquid chromatography [Kikkawa et al. 1983b] and gel filtration [Niedel et al. 1983]. Methodologies involved in assay of phorbol ester receptors in subcellular fractions have been reviewed recently [Jaken 1986].

Phospholipid

PS and other anionic phospholipids greatly augment capacity of solublized receptor to bind phorbol esters [König et al. 1985, Ashendel 1985]. The choline-containing phospholipids and PE do not augment binding.

The dependence on phospholipid is related to the purity of the receptor. König and colleagues [1985] reported that there is some binding of ^{3}H-PDBu to cytosol of rat brain cells in the absence of added phospholipid. Maximum binding is increased five-fold and the Kd is decreased about ten-fold by addition of phosphatidylserine. Binding to cytosol in the absence of added phospholipid can be explained by the presence of small amounts of endogenous phospholipid, perhaps membrane vesicles, in the

cytosol.

With partially-purified receptor, there is no binding without addition of phospholipid. In one study using receptor purified from brain cytosol, half-maximal effect was seen at 5-8ug/ml of phosphatidylserine, and maximum response at concentrations greater than 100ug/ml. The Kd for binding of ^{3}H-PDBu was 0.8nM [König et al. 1985].

In intact cells, the Kd is often much higher than with soluble PKC that has been reconstituted with PS, probably because the phorbol ester receptor in cells is exposed to a membrane comprised of a mixture of phospholipids. When the aporeceptor was reconstituted with phospholipid from red blood cell membranes, the Kd was similar to that of the receptor in whole cells [Blumberg et al. 1984].

Extracellular phospholipid apparently influences binding of ^{3}H-PDBu to whole cells since the affinity of ^{3}H-PDBu for its receptor in intact rat pituitary cells is increased by addition of PS [Blumberg et al. 1984]. This may indicate that PDBu binding is limited by the amount of anionic phospholipid in the cell membrane.

Divalent cations

Binding of ^{3}H-PDBu to soluble receptor increases with the concentration of calcium [Ashendel 1985, König et al. 1985]. At the same time, the requirement for phospholipid decreases, although even at millimolar concentrations of calcium some phospholipid is essential for the binding. Once the receptor-phospholipid complex has formed there is no further requirement for calcium in binding of ^{3}H-PDBu [König et al. 1985].

Magnesium (10mM) also augments binding of ^{3}H-TPA to rat brain receptor, increasing the affinity of binding three fold

over that induced with 0.1 mM calcium [Ashendel 1985]. In the presence of both calcium and magnesium, the Scatchard plot is curvilinear.

Binding of ^{3}H-PDBu to cells is inhibited by chelators of divalent cations, EGTA and EDTA, [Shoyab and Todaro 1980] and by TMB-8 (8-(N,N-diethylamino)-octyl 3,4,5 trimethoxy benzoate) [Dougherty and Niedel 1986], an agent which interferes with calcium-dependent pathways at an intracellular level [Malagodi and Chiou 1974]. In one study, increased extracellular concentrations of calcium, manganese and zinc increased binding of ^{3}H-PDBu while magnesium, cobalt, sodium and potassium had no effect [Shoyab and Todaro 1980]. Effects of a rise in intracellular calcium and zinc on PDBu binding are described in the experimental section.

1.7.5 ROLE OF PHORBOL ESTER RECEPTORS IN BIOLOGICAL RESPONSES

Role in effects on cells, in vitro

The receptors detected with ^{3}H-phorbol esters appear to mediate the effects of phorbol esters on cells. At concentrations of phorbol ester which partially occupy the receptors, a partial functional response is induced in the cell. In most studies, the Kd is of the same order of magnitude as the concentration of phorbol ester inducing half of the maximum functional response [Driedger and Blumberg 1980, Estensen et al. 1980, Sando et al. 1982, Nishihira and O'Flaherty 1985]. For a variety of phorbol esters, there is excellent correlation between the concentration which inhibits binding of ^{3}H-phorbol ester by 50% and the biological potency of the analogue.

Teleocidin and some other compounds, which differ structurally from phorbol esters but are potent tumour promoters

and induce phorbol ester-like responses in cells, also inhibit binding of phorbol ester [Umezawa et al. 1981]. Certain essential groupings in these molecules occupy the same relative positions as in the phorbol esters and are likely to be involved in binding and activity.

Role in tumour promotion

It is not known whether separate receptors mediate the two stages of tumour promotion. Solanki and Slaga [1981] showed that inhibition of ^{3}H-PDBu binding correlated with the first stage since the complete promoter TPA inhibited binding much more efficiently than the second stage promoter, mezerein. Dunn and Blumberg [1983] found a correlation between activity of a variety of phorbol esters and related compounds as complete tumour promoters and their capacity to compete with ^{3}H-phorbol ester for binding to a lower affinity receptor in mouse skin particulate fraction. Both complete and second stage promoters inhibited binding to the high affinity receptor component with potencies paralleling their relative activities as inflammatory agents. Heterogeneity amongst a single class of PDBu binding sites has been shown by competition experiments with mezerein [Jaken et al. 1983]. Two second stage promoters RPA and mezerein are potent inhibitors of binding of phorbol ester to cells or soluble receptor [Gschwendt et al. 1983a].

A variety of tumor promoters, unrelated to phorbol esters, have no effect on ^{3}H-PDBu binding, including phenol, iodoacetic acid, bile acid, iodoacetamide, barbiturate, oleate, limonene, saccharin and cyclamate [Shoyab and Todaro 1980]. This indicates that they do not act at the phorbol ester-binding site.

Various inhibitors of tumour promotion, including retinoic acid, dexamethasone, fluocinolone acetonimide and indomethacin do

not affect binding of ^{3}H-PDBu [Shoyab and Todaro 1980, Solanki and Slaga 1981].

1.7.6 STRUCTURE OF PHORBOL ESTER RECEPTORS

Effect of treatments which modify protein and lipid

Binding of phorbol esters to particulate preparation and to detergent-solubilized receptor is inhibited by treatment with both protease and phospholipase C [Schmidt et al. 1983]. Detergent-solubilized receptor is also inactivated by low pH and boiling, typical for a protein. Enzymes which cleave sugar-linkages do not affect receptor activity [Perchellet et al. 1982, Schmidt et al. 1983].

Delclos and Blumberg [1982] identified ascorbic acid as an endogenous inhibitor of ^{3}H-PDBu binding in homogenates of calf brain particulate fraction. They concluded that ascorbic acid acts by reducing Fe^{+++} to Fe^{++}. The latter then catalyzes lipid peroxidation of the phorbol ester receptor or neighbouring lipid. The effect of ascorbic acid on phorbol ester binding was prevented by inhibitors of lipid peroxidation. N-chlorosuccinimide and hydrogen peroxide inhibit PDBu binding activity in cells, by oxidative inactivation of the phorbol ester receptor. The susceptibility of receptors to inactivation is increased by association with membranes [Gopalakrishna and Anderson 1987].

Effect of sulphydryl reagants

Sulphydryl reagents, N-ethyl maleimide and dithionitrobenzene, inhibit binding of ^{3}H-phorbol diproprionate to mouse epidermis [Schmidt et al. 1983] although they do not appear to inhibit binding of ^{3}H-PDBu to soluble receptor.

Low molecular weight thiols (dithiothreitol, mercaptoethanol and glutathione) also inhibit binding of phorbol ester. Delclos and Blumberg [1982] showed that this effect was blocked by manganese ions, suggesting that thiols are acting as reducing agents.

Photoaffinity labelling

As part of a study to identify the phorbol ester receptor, Blumberg's group synthesized a derivative of phorbol ester, which was radiolabelled and contained a photoactivatable grouping in one of the fatty acids [Delclos et al. 1983]. This substance, 12-p-azidobenzoate 13 benzoate, was allowed to bind reversibly to brain particulate fraction, by doing the binding in the dark. Exposure to UV light then caused much of the specifically bound phorbol ester to become covalently attached to material in the particulate fraction. The phorbol ester was found to be covalently bound to PS and PE. None was found to be associated with protein although binding was prevented by pretreatment of the brain material with protease. This supports the hypothesis that the phorbol ester receptor contains both phospholipid and protein.

1.8 PROTEIN KINASE C (PKC)

1.8.1 IDENTIFICATION OF PHORBOL ESTER RECEPTORS AS PKC

Early speculation on identity of phorbol ester receptor

The conservation of phorbol ester receptors with evolution, the occurrence of these receptors in nearly all cell types in the body and the failure to find mutant cells which have lost receptor activity for phorbol esters have all indicated that the receptor has an essential function [Blumberg et al.1984, Ashendel 1985].

The phorbol ester receptor has variously been considered to be a binding site for polyunsaturated fatty acid ester [Rohrschneider and Boutwell 1973] or endogenous growth factor [Lee and Weinstein 1978], the lysophosphatidylcholine-binding site in phospholipase A_2 [Smythies 1981] or a modulatory site on an enzyme or transport protein [Blumberg et al. 1984].

Identification of receptor as PKC

There is now conclusive evidence that the major (if not only) class of high affinity phorbol ester receptor is a complex of PKC and phospholipid [reviewed in Blumberg et al. 1984, Ashendel 1985, Aitken 1986].

Early studies suggested an identity between phorbol ester receptors and PKC: (a) like the phorbol ester receptor, PKC is found in a diverse array of animal life, in large amounts in the brain and mostly in the cytosol in a form requiring phospholipid and calcium [see Ashendel 1985]; (b) both PKC and phorbol ester receptors are translocated from cytosol to membrane in phorbol ester-treated cells (see chapter 1.9); (c) PKC and phorbol ester receptors co-purify from a variety of different tissues [Niedel et al. 1983, Kikkawa et al. 1983a, Ashendel 1985] so that preparations of homogeneous PKC, in the presence of phospholipid

and calcium, bind ^{3}H-PDBu with Kds similar to those seen with the whole cells and subcellular fractions [Kikkawa et al. 1983a]; (d) various biologically active analogues of phorbol esters and related substances activate solubilized PKC [Castagna et al. 1982], with an order of potency identical to the order of potency as inhibitors of ^{3}H-PDBu binding [Ashendel 1985]; (e) drugs which interact with phospholipids inhibit binding of phorbol esters, responses induced by phorbol esters and activation of PKC [Mori et al. 1980, Ashendel 1985].

Diacylglycerol, as the endogenous ligand

Like phorbol esters, diacylglycerols lower the calcium requirement of PKC and activate the enzyme [Castagna et al. 1982], inhibit PDBu binding competitively [Sharkey et al. 1984] and induce phorbol ester-like biological responses in various cell types [Fujita et al. 1984, Nishizuka 1984, Ebeling et al. 1985]. This indicates that diacylglycerol is the endogenous ligand and that phorbol esters mimic diacylglycerol by activating PKC.

1.8.2 ISOZYMES OF PKC

Two families of PKC have been identified by cDNA studies [Knopf et al. 1986, Coussens et al. 1986, Ono et al. 1986, Ohno et al. 1987, Ono and Kikkawa 1987, Nishizuka 1988]. Type I PKC (alpha, beta 1, beta 2 and gamma) are calcium-dependent and have a high degree of structural homology. Type II PKC (delta epsilon and zeta) share about 30% homology with Type I PKC. Most of the difference is in the regulatory domain and, in particular, Type II PKC are calcium-independent.

The four members of Type I PKC are encoded by three genes. In humans, alpha is encoded by a gene on chromosome 17 (band q22-

24), gamma is encoded by a gene on chromosome 19 (band q13.2-13.4) and beta 1 and beta 2 are products of alternative splicing of messenger RNA derived from a gene on chromosome 16 (band p12-q11.1) [Kubo et al. 1987]. Both alpha and beta have 671 amino acids, and delta and beta 2 have 673 amino acids, gamma has 697 amino acids, epsilon has 737 amino acids and zeta has 592 amino acids. The beta 2 isozyme predominates in most tissues including brain and lymphocytes. Both alpha and beta isozymes are abundant in B lymphocyte-rich areas of the spleen [Brandt et al. 1987]. Gamma is largely restricted to brain. That the type II PKC isozymes exist as functional enzymes in cells has not yet been proven. However, a phorbol ester- and phospholipid-dependent, calcium-independent kinase occurs in rat brain [Malviya et al. 1986].

The different isozymes show subtle differences in properties [Huang et al. 1986a,b, Jaken and Kiley 1987, Kosaka et al. 1988, Nishizuka 1988]. Alpha, beta and gamma have been separated by hydroxyapatite chromatography. Gamma appears to be more hydrophobic since it is found to a greater extent than alpha or beta in the particulate fraction of tissues. Gamma appears to be more susceptible to proteolytic separation of the regulatory and catalytic domains. The isozymes may also differ in sites of autophosphorylation.

All isoforms bind and respond to phorbol ester and diacylglycerol although the beta isozymes appears to be more dependent on these cofactors for activation and translocation than alpha or gamma. In contrast, gamma is more readily activated by arachidonic acid than alpha or beta. It has been proposed that the different isozymes transduce different signals [Ono and Kikkawa 1987].

1.8.3. STRUCTURAL DOMAINS OF PKC

PKC consists of a single polypeptide. It undergoes post-translational modification shortly after synthesis, increasing its apparent molecular weight from 76,000 to 79,000 [Young et al. 1987].

The enzyme comprises both regulatory and catalytic domains (Fig 1.10). The catalytic site, in the C-terminal half of the molecule, is homologous in parts of its sequence with the active sites of other types of kinases. Substrates are phosphorylated exclusively at serine and threonine.

Comparison of the primary structure of the different isozymes of PKC shows that there are four highly conserved regions designated C_1, C_2, C_3 and C_4 which are separated by short stretches of highly variable sequences V_1-V_5 [Nishizuka 1988]. Conserved regions may determine functions of PKC common to the group while variable regions may in part determine isozyme-specific functions. Type II PKC differs from Type I in lacking the C_2 domain and having a very large V_1 domain (of about 140-160 amino acids) preceding the first conserved region.

Hinge Region

The catalytic domain is entirely contained within a 50 kDa fragment derived by proteolytic cleavage [Hoshijima et al. 1986]. The site of cleavage is in a hydrophilic "hinge" region (V_3 segment) which separates the catalytic domain from the N-terminal regulatory domain.

Catalytic Sites

The catalytic domain of PKC contains the nucleotide binding site motif glycine-X-glycine-X_2-glycine-X_{17}-lysine (where X is any amino acid), in common with the catalytic sites of other

Legend to Figure 1.10 Structure of PKC isozymes

This figure was drawn from published sequences and is based on the subdivision of the sequence into conserved (C) domains as described by Nishizuka [1988]. The amino acids indicated are those described for the alpha and delta isozymes.

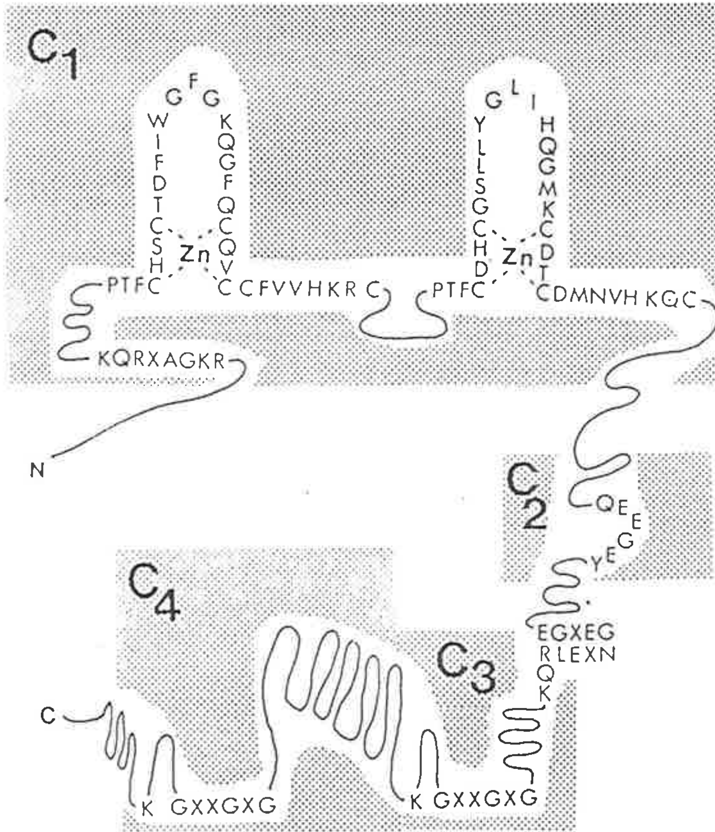
On the left is the structure of the alpha, beta and gamma isozymes. The amino acid sequence is highly conserved between the isozymes in the C_1, C_2, C_3 and C_4 domains. Separating these are short variable sequences. C_1 contains the KQRXAGKR pseudo-substrate sequence and the two cysteine-rich domains proposed to bind zinc and form zinc fingers. C_2 contains a putative calcium-binding site. Another in alpha and beta is in the variable hinge region between C_2 and C_3. C_3 and C_4 contain the glycine-rich ATP-binding sites in the catalytic site.

On the right is the deduced structure of the delta, epsilon and zeta isozymes. They differ from the first group in lacking C_2, having a large variable sequence preceding C_1 and having only one typical ATP-binding site (in C_3). Zeta may lack the first zinc finger.

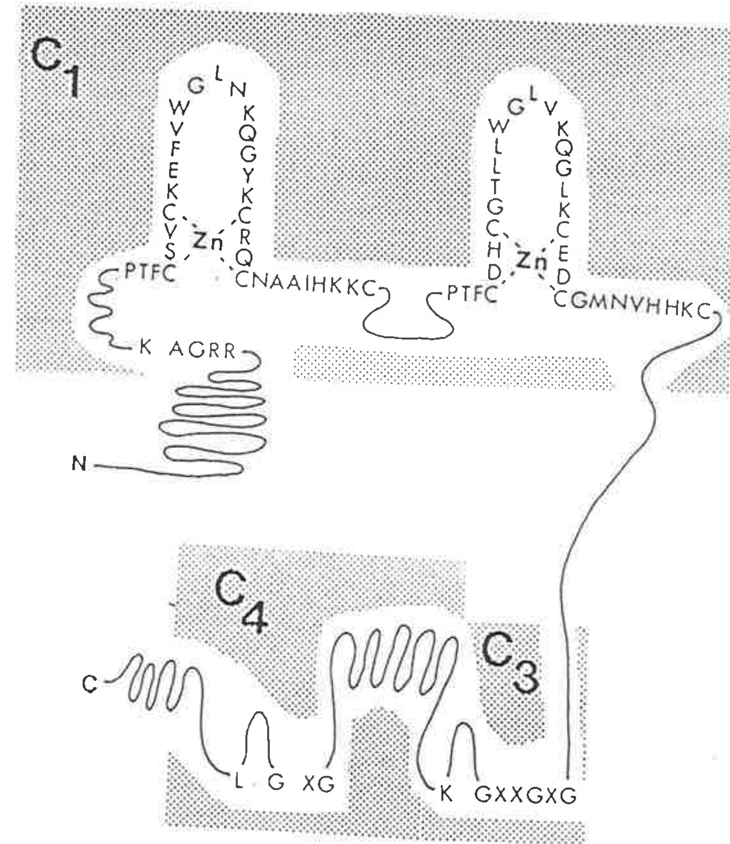
The amino acid code is : A alanine, C cysteine, D aspartate, E glutamate, F phenylalanine, G glycine, H histidine, I isoleucine, K lysine, L leucine, M methionine, N asparagine, P proline, Q glutamine, R arginine, S serine, T threonine, V valine, W tryptophane.

PKC ISOZYMES

Alpha, Beta, Gamma



Delta, Epsilon, Zeta



protein kinases. Based on studies with src kinase it has been suggested that the first glycine contacts the ribose moiety of ATP, the second contacts the terminal phosphate and the lysine mediates the phosphotransferase by proton transfer [Edelman et al. 1987]. The alpha, beta and gamma isozymes have two of these motifs one in each of the two conserved regions C_3 and C_4. It is not known whether both of these sites are involved in the catalytic activity. In contrast, the second nucleotide binding site (in C_4) is modified or lost in the Type II PKC. This may explain why the maximal kinase activity of epsilon PKC is only about 10% of that of alpha PKC [Ono et al. 1987].

Calcium-binding sites

Two putative calcium-binding sites have been described in PKC. Both contain a sequence rich in acidic amino acids which may form a coiled structure that binds calcium. One occurs in the hinge region of the alpha isozyme, but is modified in beta and absent in the other isozymes. Another site occurs in the second conserved region (C_2) in the regulatory domain. It is highly conserved in the calcium-dependent isozymes alpha, beta and gamma. The C_2 region is absent from the calcium-independent type II PKC isozymes suggesting that this site may determine the calcium-dependence of the isozymes [Nishizuka 1988].

Pseudosubtrate Sequence

Within the first conserved region (C_1) of the regulatory domain is a short sequence of amino acids postulated to be a "pseudosubstrate" [House and Kemp 1987]. In isolated peptide form, this is a potent inhibitor of the catalytic activity of PKC. Moreover, if the central alanine in the peptide is converted to a serine, the peptide becomes an extremely good substrate for PKC. This sequence may function to bind to the catalytic site(s)

of the apoenzyme form of PKC and prevent catalytic activity. Following binding of phospholipid to the regulatory domain, the pseudosubstrate sequence may be removed from the vicinity of the catalytic site permitting full enzyme activity.

Cysteine-rich zine finger motifs

Downstream of the pseudosubstrate sequence in C_1 are two cysteine-rich motifs of the type cysteine-X_2-cysteine-X_{13}-cysteine-X_2-cysteine [Parker et al. 1986]. These motifs are highly conserved in each of the PKC isozymes except that zeta appears to have only the first of the two motifs [Nishizuka 1988].

Similar motifs are found in steroid receptors and yeast transcriptional activators (see chapter 1.12). Studies with these proteins have shown that each chelates one zinc ion (in tetrahedral coordination between the four cysteines) [Freedman et al. 1988]. Zinc induces a conformational change, presumed to be a folding of the central 13 amino acids into a finger-like structure (zinc finger). These zinc fingers are essential for binding of the protein to transcriptional regulatory sites upstream of some genes.

It has been speculated that these cysteine-rich domains are involved in binding of phospholipid, phorbol ester and diacylglycerol [Weinstein 1988]. The phorbol ester-binding site appears to be in the C_1 domain since this is the only region in the regulatory domain common to all of the isozymes. However, the precise binding site of phorbol esters in PKC has not yet been determined (see chapter 4 for further discussion).

Although PKC has two putative zinc-fingers, there is no evidence that PKC contains zinc. Recently, evidence was presented that PKC interacts with DNA [Testori et al. 1988].

Phospholipase A_2-like site

In the regions just upstream of the two zinc fingers of PKC are the sequences cysteine-X_3-valine-histidine-lysine-X-cysteine. An identical sequence is found in the phospholipase A_2 of certain snake venom [Maraganore 1987]. Since these phospholipases also interact with phospholipid, it has been suggested that this region may be a part of the site in PKC which binds anionic phospholipid and that PKC may have evolved by fusion of a gene for a kinase and a gene for phospholipase A_2.

Most types of phospholipase A_2 have aspartate instead of lysine in the active site. The histidine is at the centre of the active site and the aspartate is involved in chelation of calcium which is essential for the activity of the enzyme. Conversion of aspartate to lysine inactivates the enzyme. Moreover, the naturally-occurring lysine phospholipase A_2 from snake venom has recently been shown to be inactive [Van Den Bergh et al. 1988]. Therefore this phospholipase A_2-like sequence in PKC probably lacks any lipase activity, although it may mediate interaction of PKC with phospholipid.

1.8.4 ACTIVATION OF PKC

Activation by phospholipid and calcium

In a cell-free system, PKC is fully activated by anionic phospholipid in the presence of micromolar concentrations of calcium [Kaibuchi et al. 1981]. Although the neutral phospholipids do not appear to activate PKC, it has recently been shown that PE will activate the gamma isozyme of PKC [Asaoka et al. 1988].

Phospholipid may also regulate PKC-dependent phosphorylation by modifying the structure of the substrate. Different substrates show different requirements for phospholipid [Bazzi

and Nelsestuen 1988, Nakadate et al. 1987a].

Calcium may expose hydrophobic regions in PKC, thus facilitating its interaction with phospholipid [Schatzman et al. 1981, Kikkawa et al. 1983a,b]. Another possible mechanism is that calcium cross-links PKC to carbonyl groups in the phospholipid [Ganong et al. 1986]. Of other divalent cations, lead, strontium and barium (the latter two at very high concentrations) can replace calcium [Nishizuka 1984, Markovac and Goldstein 1988]. Magnesium ions are also essential, being required for the catalytic activity.

Activation by diacylglycerol

Diacylglycerol is formed in cell membranes during receptor-linked polyphosphoinositide turnover and acts as a second messenger to activate PKC, particularly when the cytoplasmic free calcium concentration is raised [reviewed in Berridge 1987]. Addition of exogenous membrane-permeable analogues of diacylglycerol also induces PKC-dependent responses in cells. Diacylglycerol is not essential for activation of PKC but increases its sensitivity to activation by the low concentrations of free calcium that are present in cells [Kishimoto et al. 1980].

The mechanism of activation by diacylglycerol is unclear. Both carbonyl groups, the 3-OH and at least one long chain fatty acid of diacylglycerol are essential. The carbonyl and hydroxyl groups may bind to calcium in the PKC-phospholipid-calcium complex [Ganong et al 1986, Brockerhoff 1986]. An alternative view is that diacylglycerol modulates the structure of the phospholipid [Nishizuka 1984]. Thus, diacylglycerol induces formation of the hexagonal phase in membrane lipid bilayers

[Das and Rand 1984].

Activation by phorbol esters and related compounds

Phorbol esters and other diterpene tumour promoters mimic diacylglycerol in lowering the calcium requirement for activation of PKC. It is believed that the spatial arrangement of certain essential groups in these compounds is similar to that in diacylglycerol although their overall structures are quite different [Ganong et al. 1986, Wender et al. 1986, Jeffrey and Liskamp 1986].

Because phorbol esters are poorly metabolized by cells, whereas diacylglycerols are rapidly converted to phosphatidic acid by diacylglycerol kinase or to monoacylglycerols by lipase, stimulation of PKC by phorbol esters may be more prolonged and lead to events not seen with diacylglycerol. Also, phorbol esters bind to the PKC-phospholipid complex with affinities very much greater than seen with diacylglycerol [Blumberg et al. 1984]. These differences may explain why diacylglycerols and phorbol esters often do not induce entirely the same responses in cells [Kreutter et al. 1985, Yamamoto et al. 1985, Kiss and Luo 1986, Morin et al. 1987].

Activation of PKC by proteolysis

When PKC is treated with trypsin, a fragment of about 30 kDa with properties of the regulatory domain is separated from a fragment of about 50 kDa which has the kinase activity [Hoshijima et al. 1986]. The 30 kDa piece retains phorbol ester binding activity, dependent upon calcium and phospholipid, while the catalytic fragment is now independent of calcium, phospholipid, diacylglycerol and phorbol ester. This form of the kinase is referred to as PKM [Murray et al. 1987].

A physiological role for proteolysis in PKC activation has

been suggested [Murray et al. 1987]. Cells contain a calcium-dependent thiol protease capable of cleaving PKC to form various-sized fragments possessing PKM activity. Increase in PKM occurs in some cells treated with various stimuli including TPA [Tapley and Murray 1984] and A23187 [Hoshijima et al. 1986]. PKM differs from PKC in choice of substrates [Nishizuka 1986, Pontremoli et al. 1986b].

Activation of PKC by other agents

A variety of lipophilic reagents can activate PKC. These include 2-mercaptoethanol [Zabrenetzky et al. 1981], lipid X of lipopolysaccharide [Wightman and Raetz 1984] and unsaturated fatty acids [McPhail et al. 1984, Dreher and Hanley 1988], all of which may substitute for anionic phospholipid, and chloroform [Roghani et al. 1987], bryostatin, a macrocyclic lactone from algae [Kraft et al. 1986] and melittin, a peptide component of bee venom [Ashendel 1985] which may act by modifing the structure of the phospholipid or of the PKC.

1.8.5 INHIBITORS OF PKC

PKC is inhibited by a variety of substances, both in cell-free extracts and intact cells. These include trifluoperazine, chlorpromazine and related phenothiazine antipsychotic drugs [Mori et al. 1980, Feuerstein and Cooper 1984], dibucaine and related anasthetics [Schatzman et al. 1981], polyamines [Wise et al. 1982], quercetin [Gschwendt et al. 1983b, Blackburn et al. 1987], palmitoylcarnitine [Wise et al. 1982], alkyl-lysophospholipid [Kiss et al 1987], staurosporine [Tamaoki et al. 1986], polymyxin B [Wise et al. 1982] and H-7 (1-(5-isoquinoline-sulphonyl)-2-methylpiperazine) [Sha'afi et al. 1986, Hidaka and Hagiwara 1987]. These substances also inhibit other kinases.

Inhibition by palmitoylcarnitine and trifluoperazine is overcome by increased calcium or phospholipid [Wise et al. 1982]. Many of the inhibitors possess a hydrophobic region and a cationic hydrophilic group, suggesting that they interact with the regulatory domain of PKC [Ashendel 1985]. H-7 and quercetin inhibit the catalytic site of PKC, by competing with ATP [Hidaka and Hagiwara 1987, Nakadate et al. 1988].

In several types of cells, histone kinase C activity is not detectable until the cytosol has been fractionated on an anion exchange column, suggesting that cytosol contains an inhibitor of PKC.

1.8.6. SUBSTRATES OF PROTEIN KINASE C

Many proteins are substrates of PKC. Histone is the one commonly used to assay PKC. Protamine is phosphorylated without addition of calcium and phospholipid [Wise et al. 1982]. PKC shows a preference for serine in basic regions of proteins, like the cyclic AMP-dependent protein kinase [Ashendel 1985].

PKC undergoes autophosphorylation at sites in both the regulatory and catalytic domains [Huang et al. 1986a]. Autophosphorylation appears to stabilize the enzyme, increase its affinity for calcium and phorbol ester and decrease its affinity for histone.

An 80kDa protein is a common substate of PKC in a variety of types of cells [Witters and Blackshear 1987].

Membrane receptors

Many cell membrane receptors are phosphorylated by PKC in intact cells and cell-free fractions. In the receptor for epidermal growth factor, a major site of phosphorylation is at a threonine near the transmembrane domain in the portion of the receptor which is exposed to the cytoplasm [Davis and Czech 1984,

Hunter et al. 1984]. Phosphorylation is accompanied by loss of binding of epidermal growth factor and loss of the tyrosine kinase activity of its receptor. Phosphorylation of this receptor in intact cells is stimulated by TPA and diacylglycerol [Cochet et al. 1984, Iwashita and Fox 1984, McCaffrey et al. 1984]. Receptors for IL-2 and transferrin are also substrates for PKC [Klausner et al. 1984, Shackelford and Trowbridge 1986, Davis et al. 1986]. Phosphorylation may play a role in the internalization of some receptors although transferrin receptors, at least, appear to be internalized without requiring to be phosphorylated [Rothenberger et al. 1987].

Cytoskeletal proteins

Major substrates of PKC in the platelet are 20kDa and 40-47kDa proteins [Kaibuchi et al. 1981]. Phosphorylation of the latter may modulate actin polymerization [Hashimoto et al. 1987]. The 20 kDa protein is myosin light chain. After phosphorylation by PKC, it has less ATPase activity [Nishikawa et al. 1983, Ikebe et al. 1987].

PKC phosphorylates vinculin, a cytoskeletal protein which links termini of actin to the membrane [Werth et al. 1983], tau, a microtubule-associated protein [Hoshi et al. 1987], vimentin, a component of the intermediate filaments [Huang et al. 1988], troponin [Katoh et al. 1983] and actin-binding proteins [Kawamoto and Hidaka 1984].

Nuclear proteins

Although it is still not clear whether PKC enters the nucleus (see chapter 1.9), PKC phosphorylates many nuclear proteins in model systems including histones [Iwasa et al. 1980],

other chromosomal proteins [Ramachandran et al. 1984], RNA polymerase II, DNA topoisomerase II and other nuclear enzymes [Sahyoun et al. 1986, Chuang et al. 1987], nuclear lamins which regulate nuclear membrane dissolution during mitosis [Fields et al. 1988, Hornbeck et al. 1988] and proteins involved in transport of mRNA out from the nucleus [Schröder et al. 1988a,b]

Other substrates

PKC phosphorylates and alters the activity of various enzymes involved in metabolism (eg glycogen synthase [Roach and Goldman 1983]), signal transduction (eg adenylate cyclase [Yoshimasa et al. 1987]) and gene expression (eg RNA polymerase [Chuang et al. 1987]).

PKC-dependent phosphorylation of ribosomal protein S6 [Trevillyan et al. 1984] may be responsible for increased protein synthesis while phosphorylation of the Na^{+}/H^{+} exchanger appears to regulate the pH of the cytoplasm [Moolenaar et al. 1983, 1984].

1.8.7 ROLE OF PKC IN TUMOUR PROMOTION

Transfection of plasmids or retrovirus-derived vectors, containing the genes for gamma or beta PKC, into fibroblast cell lines causes an overexpression of PKC in these cells and a partial transformation of the cells to a malignant phenotype [Persons et al. 1988, Housey et al. 1988].

Activation of PKC is probably an essential event in the second stage of tumour promotion since second stage promoters bind to PKC and activate it almost as efficiently as TPA [Gschwendt et al. 1983a]. Two other types of tumour promoting agents, chloroform [Roghani et al. 1987] and oleic acid [McPhail et al. 1985], are activators of PKC.

It is not yet clear whether PKC plays a role in the first

stage of promotion.

1.9 TRANSLOCATION AND DOWN-REGULATION OF PKC

PKC exists in several discrete pools within the resting cell (Fig 1.11), as shown by phorbol ester binding, histone kinase C activity and immunocytochemistry. During cell activation, there are gross changes in the distribution of PKC between these pools. The redistribution of PKC to different compartments appears to be regulated by distinct signals and probably determines which substrates in the cell are phosphorylated. There is also a down-regulation of the PKC, which may be involved in terminating the activation.

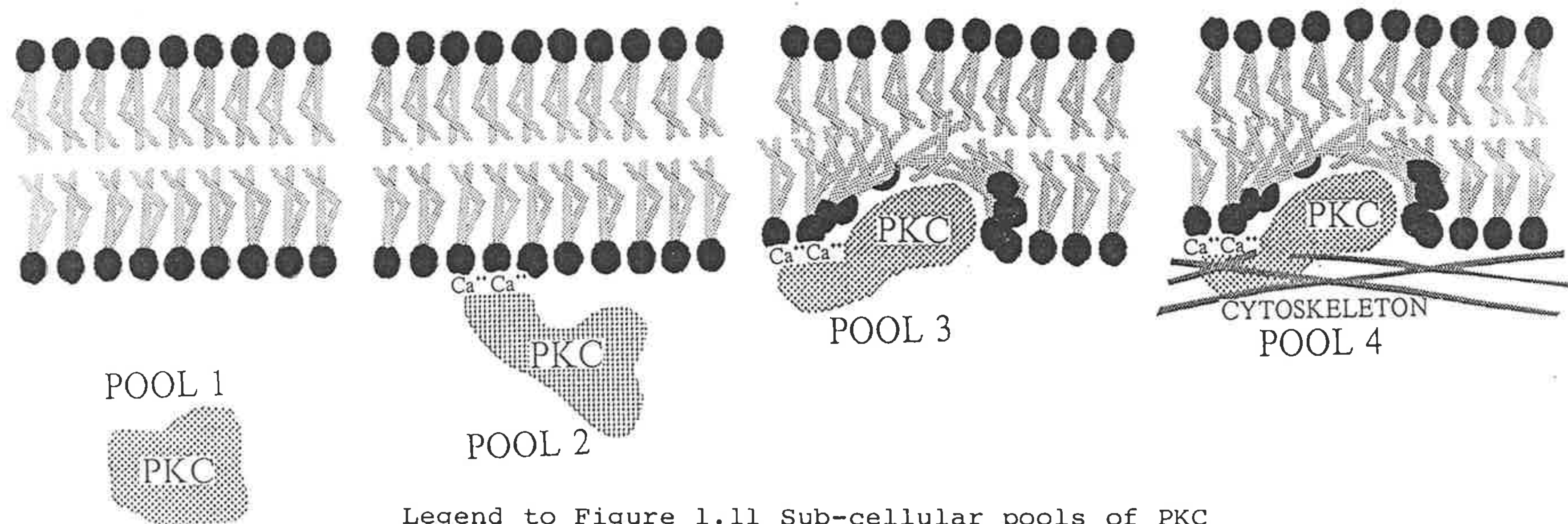
1.9.1 SUB-CELLULAR POOLS OF PKC

Cytosolic pool

The bulk of cellular phorbol ester receptors and histone kinase C activity exists in the cytosol as aporeceptors and apoenzyme. Phospholipid and calcium are required in order to detect binding and catalytic activity [reviewed in Blumberg et al. 1984]. It is presumed that cytosolic PKC does not bind phorbol ester in intact cells, until after it is translocated to the particulate fraction.

Divalent cation-chelator-labile pool

One pool of cellular PKC is loosely bound to the particulate fraction and detached by divalent cation chelators EDTA and EGTA [Niedel et al. 1983, Melloni et al. 1985]. Some of the PKC is attached to plasma membrane and other to internal cell membranes including that of the nucleus [Kraft et al. 1987]. This pool is rarely assayed since chelating agents are generally present in buffers used for cell fractionation. It is not known whether this pool of PKC is active catalytically nor whether it contributes to the intact cell binding of phorbol ester [Alkon and Rasmussen 1988].



Legend to Figure 1.11 Sub-cellular pools of PKC

Various pools of PKC exist in cells.

Pool 1 In a quiescent cell, most of the PKC is in the cytosol as an apo-enzyme and apo-receptor for phorbol ester.

Pool 2 Some PKC is attached to the particulate fraction loosely and is dissociated by EDTA. This divalent cation chelator-labile pool is thought to be attached to membrane phospholipid by calcium and perhaps other divalent cations.

Pool 3 Some particulate fraction PKC cannot be eluted by EDTA but can be solubilized by non-ionic detergent. This pool is thought to be the active form of PKC. It is unclear how it is attached to the membrane.

Pool 4 A small amount of particulate PKC is not soluble in detergent and may be attached to detertgent-insoluble structures such as the cytoskeleton or nucleus.

The relative amounts of receptor and catalytic activity recovered in cytosol and particulate fractions depend largely upon the concentration of free calcium in the extraction buffer [Niedel et al. 1983, Kikkawa et al. 1983b]. For example, in the presence of chelators, less than 3% of the phorbol ester binding activity of PMN is found in the particulate fraction, the remainder being found in the cytosol as aporeceptors [Nishihira and O'Flaherty 1985, Nishihira et al. 1986].

Some other cytosolic proteins, such as catalase and band 8 polypeptide in red cells, also bind reversibly to cell membranes in a calcium-dependent manner [Allen and Cadman 1979]. Calcium-dependent, phospholipid-binding proteins are the subject of a recent review [Klee 1988].

Detergent-soluble pool in the particulate fraction

Usually, the content of PKC in the particulate fraction is assayed in the presence of EGTA or EDTA. Most of this tightly bound PKC is solubilized by non-ionic detergent, such as Triton X-100 or Nonidet P40 (NP40). The receptors in this pool are thought to be inserted in the lipid bilayer of the cell membrane. In this form, PKC is active both as a high affinity receptor and as a histone kinase [reviewed in Ashendel 1985]. The pool of particulate PKC is increased in fast-growing cells and low in confluent cells [Miloszewska et al. 1986]. It may also be abnormally low in leukaemic cells from patients with acute and chronic myelocytic leukaemia [Sakurada et al. 1985].

It is still not clear how a hydrophilic protein such as PKC becomes tightly associated with cell membranes. PKC is often depicted as penetrating the lipid bilayer (Fig 1.11). This is unlikely for thermodynamic reasons [Chabre 1987]. One possibility

is that PKC becomes acylated during translocation and that the hydrophobic fatty acid anchors it in the membrane [Alkon and Rasmussen 1988]. In support of this, TPA causes myristylation of an 82kDa protein in HL60 cells, at concentrations at which it causes translocation of PKC to the membrane [Malvoisin et al. 1987]. However, evidence for myristylation of PKC has not yet been obtained. Other possibilities are that the hydrophobic phorbol ester or diacylglycerol anchor PKC in the membrane or that PKC associates with a memrane protein [Gopalakrishna et al. 1986].

It is likely that the different isozymes have different affinities for cell membranes since gamma PKC is preferentially located in the particulate fraction whereas alpha and beta are preferentially located in the cytosol after transfection of cell lines with genes for the isozymes [Persons et al. 1988].

Detergent-insoluble pool

A smaller pool of PKC in the particulate fraction is not solubilized by either EDTA or non-ionic detergent [Chen et al. 1986]. This detergent-insoluble fraction contains nuclear and cytoskeletal material [Osborn and Weber 1977].

There is controversy as to whether PKC exists in the nucleus of a cell. High affinity phorbol ester binding sites for TPA have been detected in nuclei of epidermis, rat brain and liver [Perrella et al. 1982a,b, Misra and Sahyoun 1987]. These receptors were solubilized by treatment with desoxyribonuclease or 0.35M sodium chloride. PKC activity was also found in nuclear extracts of testis [Kimura et al. 1984] but no kinase C activity or PDBu receptor was seen in nuclear extracts of thymoma cells, either before or after treatment of the cells with PDBu [Jensen and Sando 1987], nor was there any seen following labelling with

a fluorescent derivative of a phorbol ester [Liskamp et al. 1985]. It has been argued that PKC in nuclear extracts results from contamination with cytosol, cytoskeleton or plasma membrane [Jensen and Sando 1987]. The ability of desoxyribonuclease to solubilize this PKC does not necessarily indicate that the PKC is complexed with DNA since desoxyribonuclease also releases proteins from the cytoskeleton by disrupting the microfilaments [Gupta and Woda 1988].

Immunochemical staining has revealed the presence of PKC in the periphery of the nucleus, the nuclear membrane, nuclear matrix and in some nucleoli of brain, liver and promyelocytic cells [Girard et al. 1985, Kuo 1986, Wood et al. 1986, Capitani et al. 1987]. PKC may be particularly enriched in the nucleus in G_2 phase of the cell cycle [Kuo et al. 1986]. However, very little PKC was detected in the nuclei in studies by Nishizuka and colleagues [reviewed in Nishizuka 1986]. Some proteins associated with the nuclear matrix are phosphorylated in HL60 cells treated with phorbol ester. Other nuclear proteins are phosphorylated by PKC, in vitro (see chapter 1.8.6).

PKC in the detergent-insoluble fraction may also be associated with cytoskeleton. Other protein kinases including the calmodulin-dependent protein kinase II [Goldenring et al. 1986] and the src tyrosine kinase [Hamaguchi and Hanafusa 1987] also associate with the cytoskeleton and, at least for the src kinase, this is essential for malignant transformation of the cells. PKC phosphorylates a variety of cytoskeletal proteins in intact cells and cell-free systems (see chapter 1.8.6) but there is yet no evidence that PKC attaches to the cytoskeleton.

It is still unclear whether PKC in the detergent-insoluble

fraction requires phospholipid or some other co-factor for activity. In one study, the PKC in the nuclear fraction bound ^{3}H-PDBu in a calcium- and phospholipid-independent manner [Misra and Sahyoun et al. 1987]. Binding of PKC to some protein or other macromolecule in the nucleus or cytoskeleton may reconstitute binding and kinase activities.

1.9.2 TRANSLOCATION OF PKC

Intact cells

When cells are treated with agonist, inducing diacylglycerol formation, or with membrane-permeable diacylglycerol or phorbol ester, PKC becomes attached to the plasma membrane and other components of the particulate fraction. Translocated PKC can be detected by phorbol ester binding, histone kinase C activity and immunochemical staining [Kraft and Anderson 1983, Chen et al. 1986, Thomas et al. 1987, Deli et al. 1987].

In response to phorbol ester, most of the PKC is translocated to the detergent-soluble pool of the particulate fraction [Kraft et al. 1982, Kraft and Anderson 1983, Myers et al. 1985, Nishihira et al. 1986, Thomas et al. 1987]. Up to 30% of the cytosolic PKC is translocated to the nucleus and/or cytoskeleton in TPA-treated cells [Thomas et al. 1988]. Translocation of PKC to the membrane is rapid (occurring within 15 minutes) [Ballester and Rosen 1985] and precedes translocation of PKC to the nucleus, during TPA-induced differentiation of HL60 cells [Kiss et al. 1988].

In response to exogenous diacylglycerol or stimuli which induce diacylglycerol formation, the translocation to the particulate fraction is transient and reversible [Pike et al. 1986, Cambier et al. 1987a, Ishizuka et al. 1987] and, at least in PMN, the translocated PKC is released by divalent cation

chelators [Pontremoli et al. 1986a].

Agents which elevate cyclic AMP cause translocation of phorbol ester receptors from cytosol to a detergent-insoluble compartment, presumed to be the nucleus, in mouse B lymphocytes [Cambier et al. 1987b, Chen et al. 1987] and enhance (but not induce) translocation of PKC to the periphery of the nucleus in HL60 cells [Deli et al. 1988]. Bryostatin, acting by an unknown mechanism, also causes some translocation of the phorbol ester receptor-protein kinase C to this pool [Fields et al. 1988].

Translocation of PKC from cytosol to particulate fraction is blocked by prior treatment of cells with Concanavalin A [Patel and Kassis 1987]. The mechanism of this is unknown. Chlorpromazine inhibits the translocation induced by TPA in T cells [Isakov et al. 1987], probably by affecting the interaction of PKC with phospholipid.

In one system, treatment of adrenal zona fasciculata by adrenocorticotropin hormone, there appears to be translocation of PKC from particulate fraction to cytosol [Vilgrain et al. 1984].

Cell-free systems

In cell-free systems, TPA induces partially pure PKC to attach to membranes of PMN and red blood cells in a manner which is dependent upon calcium but not reversed by calcium chelators [Wolf et al. 1985a,b, Pontremoli et al. 1986a, Gopalakrishna et al. 1987]. Translocation, induced by phorbol ester, requires several minutes and proceeds at 25°C but not at 4°C. PKC appears to bind to a protein in the membrane since it occurs with plasma membranes but not other cell membranes, the association of PKC with the membranes is saturable at low concentrations of PKC, and is prevented by pre-treatment of the membranes with protease

[Gopalakrishna et al. 1987]. Diacylglycerol only causes part of the cytosolic PKC to translocate to a tightly bound membrane pool [Gopalakrishna et al. 1987].

In contrast, translocation induced by calcium occurs within seconds, is independent of temperature, occurs with membranes from a variety of sub-cellular fractions, is not saturable and is unaffected by pretreatment of membranes with protease. This is consistent with the studies in intact cells, indicating that calcium, alone, induces PKC to bind loosely to cell membranes.

An issue yet to be resolved is whether autophosphorylation of PKC enhances its capacity to dissociate from membranes [Wolf et al. 1985b, Gopalakrishna et al. 1987].

1.9.3 DOWN-REGULATION OF PKC

In some cells, binding of phorbol ester reaches a maximum in the first twenty minutes and then rapidly declines to about 40% to 70% of this level over the next 60 minutes. There is little further decrease with time [Shoyab and Todaro 1980, Solanki et al. 1981, Sando et al. 1982]. Down-regulation only occurs at 37°C. In one promyelocytic cell line, capacity to undergo down-regulation was lost with differentiation [Sakagami et al. 1984].

Accompanying the loss of receptors is a loss in catalytic activity. In most cell types, PKC is rapidly and completely lost from the cell following TPA treatment for several hours. Translocation of PKC to the particulate fraction is essential for this to occur. It is not a result of a decrease in the rate of transcription or translation [Young et al. 1987]. Some isozymes may be down-regulated at faster rates than others [Ase et al. 1988, A.W. Murray Personal communication]. A single application of TPA to mouse skin is sufficient to cause complete loss of

kinase C activity for up to 3 days [Fournier and Murray 1987] although down-regulation induced by PDBu is reversed during overnight culture of the cells in medium lacking PDBu [Rodriguez-Pena and Rozengurt 1984].

Several mechanisms have been proposed to account for the down-regulation. Internalization of membrane PKC may occur since, at least in one type of cell, the down-regulation was retarded by inhibitors of the internalization of receptors via coated pits [Solanki et al. 1981]. Phorbol esters cause internalization of some other receptors including the transferrin receptor [Klausner et al. 1984, Sibley et al. 1987].

Another explanation is proteolytic degradation of PKC [Ballester and Rosen 1985, Chida et al. 1986, Fournier and Murray 1987]. Proteolysis of this enzyme occurs following its association with cell membranes [Melloni et al. 1986]. Protease inhibitors block down-regulation of phorbol ester receptors [Chida and Kuroki 1983, Von Ruecker et al. 1988]. In these studies, it is difficult to determine whether the proteolysis occurs during lysis of the cells to assay for PKC rather than in the intact cell [Buday et al. 1987]. While cleavage of PKC into regulatory and catalytic fragments accompanies the loss of kinase C activity in TPA-treated mouse skin, the loss of kinase activity occurs first, suggesting that down-regulation precedes proteolysis [Fournier and Murray 1987].

Another explanation for down-regulation is that the PKC becomes inaccessible. It was claimed that the lost receptors could be detected if the cells were lysed before assay [Jaken et al. 1983]. In phorbol ester-treated mouse 3T3 cells, the lost PKC could be recovered by treatment of the soluble fraction of

the cells with non-ionic detergent or by chromatography on hydroxyapatite [Uratsuji et al. 1985, Thomas et al. 1987].

Alterations in lipid environment, intracellular concentration of divalent cations, covalent modification of receptor and translocation to other subcellular compartments have been considered as means by which the receptors may become cryptic [reviewed in Ashendel 1985].

In some cells treated with TPA, the down-regulation of catalytic activity is not a result of loss of PKC but is due to a change in the specificity of the catalytic site. There is complete loss of capacity to phosphorylate histone but capacity to phosphorylate vinculin or cytochrome P450 is unimpaired [Cochet et al. 1986, Cooper et al. 1987a]. Loss of immunoreactive PKC in these cells may indicate that the change in substrate specificity is accompanied by a change in the antigenicity of the PKC [Cooper et al. 1987a].

The existence of a variant promyelocytic leukaemia cell line, which does not undergo differentiation in response to phorbol esters and does not undergo down-regulation of its phorbol ester receptors, suggests that these two processes may be linked. However, a block in down-regulation of phorbol ester receptors was not seen in some other types of cells unresponsive to phorbol ester [Goodwin et al. 1984, Weinberg et al. 1984].

1.10 EARLY EVENTS INVOLVED IN ACTIVATION OF B CELLS

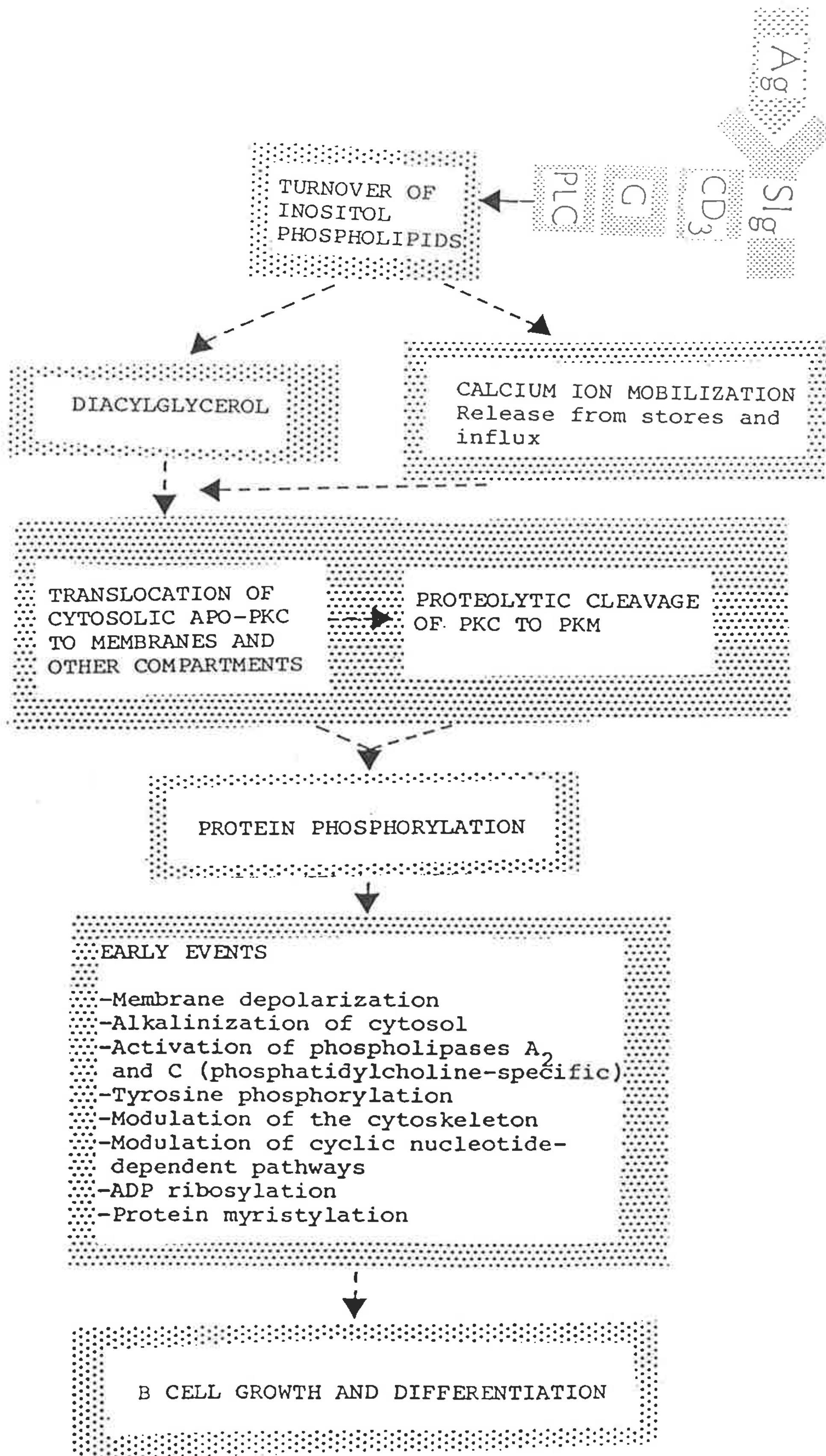
Activation of B lymphocytes is influenced by several types of membrane proteins, including sIg, MHC class II molecules, Fc receptors and receptors for mitogen, growth and differentiation factors (see chapter 1.4). Some of the biochemical and biophysical events which accompany signal transduction by these receptors have been elucidated although relatively little is known about the role of some of these processes in the activation. The pathway of events differs according to the stimulus [Cambier et al. 1985, Dugas et al. 1986b, Parker 1987]. Fig 1.12 is a schema of early biochemical events in B cell activation.

1.10.1 PHOSPHOINOSITIDE TURNOVER

Phosphoinositide turnover in B cells

Binding of ligands to some cell membrane receptors induces turnover of polyphosphoinositides, particularly phosphatidyl inositol$_{4,5}$bisphosphate (PIP_2) [Downes and Michell 1985, Berridge 1987]. PIP_2 is cleaved by a specific phospholipase C, as a result either of activation of this enzyme or changes in the distribution or concentration of PIP_2 in the membrane [Harris and Cambier 1987]. Turnover of PIP_2 results in two products which act as intracellular messengers, inositol$_{1,4,5}$ tris-phosphate (IP_3), which mediates the rise in intracellular calcium, and diacylglycerol, a specific activator of PKC [reviewed in Downes and Michell 1985].

Both of these messengers are short-lived. IP_3 is rapidly converted to inositol tetraphosphate and inositol$_{1,3,4}$ trisphosphate or cleaved by a phosphomonoesterase to form inositol bisphosphate, inositol phosphate and inositol. Diacylglycerol is phosphorylated by diacylglycerol kinase to form



Legend to Figure 1.12 Early events in B cell activation

Schema shows a model of early events induced following triggering of sIg by antigen (Ag) or other ligands (eg anti-Ig). It is unclear whether a CD3-like complex is involved in the signal transduction by sIg (as it is in T cells). G is GTP-binding protein and PLC is phospholipase C.

phosphatidic acid or cleaved by diacylglycerol lipase to form monoacylglycerol.

Inositol phospholipid turnover is an important early event in activation of B cells [Maino et al. 1975, Coggeshall and Cambier 1984, Guy et al. 1985a,b, Bijsterbosch and Klaus 1985, Bijsterbosch et al. 1986, Paul et al. 1986, Fahey and DeFranco 1987, Grupp et al. 1987]. Increased levels of IP_3, the other inositol phosphates, diacylglycerol and phosphatidic acid are detected within the first seconds or minutes of treatment of B cells with ligands of sIg such as anti-IgM, anti-IgD, SAC or antigen. Turnover of PIP_2 is also induced by anti-Ig in isolated mouse B cell membranes, in the presence of a cytosolic factor which may be phospholipase C [Harris and Cambier 1987].

The importance of the turnover of PIP_2 in the subsequent activation is shown by the capacity of exogenous phospholipase C to elicit early events in activation of mouse B cells including increased expression of MHC class II antigens [Cambier et al. 1985] and of calcium ionophore and diacylglycerol, together, to activate resting B cells [Guy et al. 1985b).

MHC class I and II antigens and receptors for lipopolysaccharide, Fc_{gamma}, IL-4 and B cell differentiation factor are not coupled to polyphosphoinositide turnover [Chen et al. 1986, Justement et al. 1986, Mizuguchi et al. 1986, Paul et al. 1986, Chartash et al. 1987, Grupp et al. 1987].

Role of G proteins in phosphoinositide turnover via sIg

A GTP-binding protein complex couples cell surface receptors to phospholipase C [reviewed in Berridge 1987]. Ligand-induced conformational change in the receptor causes the G protein to dissociate into its subunits. GTP is hydrolyzed in the process.

This type of signal transduction is blocked by pertussis toxin which causes ADP-ribosylation of the G protein. Fluoride ions increase turnover of PIP_2 and translocation of PKC in PMN and platelets by activation of the G protein [Strnad and Wong 1985, Strnad et al. 1986, Saad et al. 1987, Hauschildt et al. 1988]. It is thought that fluoride ions interact with trace amounts of aluminium in culture media and that the resulting fluoroaluminate (AlF_4^-) complex mimics the gamma-phosphate of GTP [Sasaki and Hasegawa-Sasaki 1987].

Little is known about the role of G proteins in B cell activation. GTP and GDP do not induce turnover of PIP_2 in isolated mouse B cell membranes [Harris and Cambier 1987] and pertussis toxin does not block the turnover of PIP_2 or the rise in cytosolic free calcium in mouse B lymphoma cells treated with anti-Ig [DeFranco et al. 1987].

CD3-like proteins associated with sIg

In T cells, the antigen receptor is coupled to a complex of proteins (CD_3 complex) which is important for signal transduction [Isakov et al. 1987]. Resting mouse B cells also contain a complex of proteins, comprising polypeptides of 30, 46 and 56 kDa, which are associated with sIg [Koch and Haustein 1983, Haustein and Von der Ahe 1986]. The function of these is unknown.

Feedback inhibition of phosphoinositide turnover by PKC

Brief pre-treatment of B cells with phorbol esters and exogenous diacylglycerol suppresses activation of B cells by antigen and anti-Ig as well as the increased PIP_2 turnover that is induced by these stimuli [Hawrylowicz and Klaus 1984, Cambier et al. 1985, Paul et al. 1986, Miziguchi et al. 1986]. If the treatment with phorbol ester is prolonged for several hours so that the PKC activity is completely down-regulated, there is an

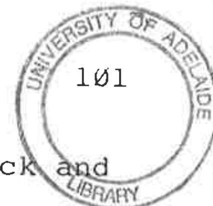
increase in the basal rate of PIP_2 turnover [Gold and DeFranco 1987]. These observations suggest that the sIg activation pathway is subject to feedback inhibition by PKC. The site at which feedback inhibition occurs is unclear. It may be at the level of sIg, the putative CD3-like proteins, G proteins or phospholipase C.

Activation of B cells via sIg also leads to feedback inhibition of sIg signal transduction. Only a part of this appears to be mediated by PKC since feedback inhibition induced by anti-Ig is only partially blocked by staurosporine, an inhibitor of PKC. Staurosporine completely blocks the feedback inhibition of sIg signal transduction induced by diacylglycerol or TPA [Cambier et al. 1988].

1.10.2 TURNOVER OF OTHER PHOSPHOLIPIDS

Phosphatidylcholine

Like some agonists of $alpha_1$ adrenergic receptors [Slivka et al. 1988], anti-Ig also stimulates turnover of phosphatidylcholine [Resch 1979], by activation either of a phosphatidylcholine-specific phospholipase C [Guy and Murray 1982] or by activation of acyl coenzyme A:1-acyl-glycero-3-phosphocholine O-acyltransferase [Resch 1979]. The latter enzyme replaces saturated fatty acids with polyunsaturated fatty acids in various types of phospholipids and is thought to cause increases in membrane fluidity and activation of the Na^+K^+ ATPase. The effects of anti-Ig on phosphatidylcholine turnover are probably mediated by diacylglycerol since exogenous diacylglycerol and phorbol ester stimulate rapid turnover of phosphatidylcholine in various cell types [Wertz and Mueller 1980, Guy and Murray 1982, Daniel et al. 1986, Kolesnick and



1980, Guy and Murray 1982, Daniel et al. 1986, Kolesnick and Paley 1987]. PKC may be involved in the activation of this phospholipase C [Muir and Murray 1987].

Phospholipase A_2

The Fc_{gamma} receptor of B-CLL cells has phospholipase A_2 activity and each mole of receptor is tightly associated with one mole of phospholipid and free fatty acid [Suzuki et al. 1981, Suzuki 1981]. Ligands of Fc receptor activate the phospholipase A_2, leading to an increase in release of arachidonic acid from membrane phospholipid. Arachidonic acid is thought to be an important intracellular messenger in cell activation [Beaumier et al. 1987, Dreher and Hanley 1988].

Phorbol esters also induce release of arachidonic acid from phospholipids, via stimulation of a phospholipase A_2 and mitogenesis of lymphocytes and other cells by phorbol esters is blocked by inhibitors of phospholipase A_2 such as quinacrine [Hirata et al. 1980a, Ganss et al. 1982, Fischer and Adams 1985]. Melittin, an activator of phospholipase A_2, mimics phorbol esters by inhibiting epidermal growth factor receptors and enhancing adenovirus transformation of rat embryo cells [Blumberg 1980, Weinstein et al. 1979]. Prostaglandin E_2, a metabolic product of arachidonic acid, modulates some events in B cell activation [Thompson et al. 1984].

Phospholipid methylation

Conversion of PE to phosphatidyl-choline by the sequential action of two methyltransferases accompanies activation of some types of cells. Whether phospholipid methylation plays a role in activation of resting B cells is not known. It has been suggested that methylation of phospholipid is essential for the capacity of B cell differentiation factor to induce differentiation in B

cells [Hirata et al. 1980b, Kishimoto et al. 1984].

1.10.3 CALCIUM MOBILIZATION

Measurement of calcium flux

A role for calcium ions in the activation of lymphocytes was indicated from early studies on ^{45}Ca uptake [Maino et al. 1974]. It is now possible to measure a rise in cytosolic free calcium ions $[Ca^{++}]_i$ using fluorescent indicators of calcium (Quin-2, Fura-2, Indo-1 and aequorin) which can be trapped in the cytoplasm of cells [Tsien et al. 1982a,b, Grynkiewicz et al. 1985].

Calcium mobilization in response to sIg ligands

Within seconds of exposure of quiescent normal and malignant B cells to anti-Ig there is a rise in $[Ca^{++}]_i$ from the resting value of about 100nM. Within three to five minutes, the $[Ca^{++}]_i$ reaches a steady state at about 200-500nM [Clevers et al. 1985a,b, Paul et al. 1986, Ransom et al. 1986]. Wilson and colleagues [1987] have described the increase in $[Ca^{++}]_i$ in B cells as "a series of repetitive oscillations between resting levels of calcium and transient peaks, that may continue for hours". The mechanism by which this occurs is unclear.

The rise in $[Ca^{++}]_i$ appears to be coupled to turnover of PIP_2 since it is also induced by exogenous phospholipase C and IP_3 releases calcium from endoplasmic reticulum of permeabilized mouse B cells [Ransom et al. 1986].

As well as release of calcium from internal stores, there is also influx of calcium across the plasma membrane [Pozzan et al. 1982, Bijsterbosch et al. 1986, Nachshen et al. 1986, Dugas et al. 1986a, 1987]. The calcium channel blockers, verapamil and nifedipine, as well as the calcium antagonist, lanthanum, inhibit

the increase in $[Ca^{++}]_i$ and also inhibit proliferation induced by ligands of sIg in human B cells [Clevers et al. 1985a, Dugas et al. 1986a]. Verapamil has no effect if given after anti-Ig suggesting that influx of calcium is an essential early event in B cell activation. The regulation of calcium influx is still poorly understood [reviewed in Neher 1987].

Effects of phorbol ester on calcium mobilization

Phorbol esters do not induce a rise in $[Ca^{++}]_i$, as measured with Quin-2 and Fura-2 [Rink et al. 1983], but they inhibit calcium mobilization induced by other stimuli by (a) inhibiting turnover of polyphosphoinositide [Cambier et al. 1987a], (b) causing sequestration of calcium to a compartment other than mitochondria [Messing et al. 1986, Yoshida and Nachmias 1987], (c) promoting efflux of calcium ions [Pollock et al. 1987] and (d) inducing PKC to phosphorylate and activate the enzyme which cleaves IP_3, thereby removing the signal for further release of calcium ions [Connolly et al. 1986].

Artificial induction of a rise in free cytosolic calcium

A rise in $[Ca^{++}]_i$ can be induced artificially by fluoride, which activates G proteins and PIP_2 turnover as well as inhibiting breakdown of IP_3 [Sasaki and Hasegawa-Sasaki 1987], and by calcium ionophores, such as A23187 and ionomycin [Reed and Lardy 1972, Liu and Hermann 1978, Pfeiffer et al. 1978].

Calcium ionophores directly induce mouse B cells to increase their content of MHC class II antigens, without inducing polyphosphoinositide turnover [Cambier et al. 1985]. Presumably, at least some activation of B cells is caused by a rise in free calcium ions alone.

Other stimuli

Lipopolysaccharide, IL-4 and antibodies to MHC class I and

II antigens and to receptors do not induce a rise in $[Ca^{++}]_i$ in B cells [Justement et al. 1986, Paul et al. 1986, Grupp et al. 1987] although the response of resting B cells to IL-4 is partly dependent upon extracellular calcium [Dennis et al. 1987].

1.10.4 TRANSLOCATION AND ACTIVATION OF PKC

Activation of PKC via sIg

PKC is abundant in lymphocytes including B cells [Ogawa et al. 1981, Coggeshall and Cambier 1985], being far more abundant than the cyclic AMP-dependent protein kinase. Little is known about which isozymes are present although alpha and beta isozymes but not the gamma isozyme are found in human T cells [Shearman et al. 1988] and mRNA for alpha and beta but not gamma is abundant in B-cell rich areas in the rat spleen [Brandt et al. 1987].

Anti-Ig, SAC and phorbol ester induce translocation of PKC to various compartments of the B cell and they cause much of the PKC to be converted to PKM [Guy et al. 1985a,b, 1986, Wickremasinghe et al. 1985, Chen et al. 1986, Cooper et al. 1987b]. Conversion of some of the membrane PKC to PKM permits phosphorylation of cytosolic and cytosleletal proteins, at least in PMN [Pontremoli et al. 1987]. In PMN, these two kinases appear to mediate separate activation events. PKC is involved in release of a neutral serine protease from the cell membrane and in stimulation of superoxide anion formation while PKM is involved in degranulation of the cells [Pontremoli et al. 1986c].

The role of PKC in activation of B cells by anti-Ig is controversial. Anti-Ig induces a pattern of protein phosphorylation similar to that induced by phorbol esters (see chapter 1.10.5) and inhibitors of PKC, palmitoylcarnitine, H-7 and polymyxin B, prevent the proliferation and increased

expression of activation antigens in human B cells treated with anti-Ig [Nel et al. 1985, Dugas et al. 1986b, Kikutani et al. 1986], although the block in proliferation can be overcome with another activator, 8-mercaptoguanosine (see later). In one study, depletion of cellular PKC by treatment of B cells with phorbol ester did not prevent anti-Ig-induced increase in expression of MHC class II antigens [Mond et al. 1987].

In one population of prolymphocytic leukaemia B cells, TPA induced translocation to the particulate fraction, while anti-Ig did not, suggesting that the sequence of events leading from triggering of sIg to translocation of PKC is blocked in these cells [Cooper et al. 1987b].

Other stimuli

Cyclic GMP may also regulate translocation of PKC to the membranes of mouse B cells [Cambier et al. 1987a]. By contrast, antibodies to MHC class II antigens appear to induce a rapid, transient translocation of PKC from cytosol to the nucleus of mouse B cells, mediated by a rise in the intracellular level of cAMP [Cambier et al. 1987b].

Translocation of PKC in lipopolysaccharide-treated B cells was seen in one study [DeFranco et al. 1987] but not in another [reviewed in Cambier et al. 1987a]. Lipopolysaccharide is an activator of PKC, apparently by mimicking phospholipid [Wightman and Raetz 1984]. The inhibitor of PKC, H-7, also blocks activation of mouse spleen cells by lipopolysaccharide [Rush and Waechter 1987] although it cannot be excluded that H-7 is inhibiting another type of kinase.

IL-4 does not induce translocation and activation of PKC [Justement et al. 1986] but gamma interferon and IL-1 induce translocation in a pre-B cell line [Ostrowski et al. 1988] as

does IL-2 in T cells [Farrar and Anderson 1985].

1.10.5 PROTEIN PHOSPHORYLATION

Protein phosphorylation is detectable soon after stimulation of B cells with anti-IgM [Nel et al. 1984, Paul et al. 1986, Hornbeck and Paul 1986]. This phosphorylation is mediated by PKC since phorbol esters and anti-Ig induce phosphorylation of similar sets of proteins in B cells and at similar sites in the proteins, as shown by tryptic peptide mapping [Paul et al. 1986, Hornbeck and Paul 1986]. Depletion of PKC from B cells, by prolonged treatment with phorbol ester, prevents anti-Ig induced phosphorylation.

Tyrosine phosphorylation is implicated in growth of cells [Hunter 1987]. PKC is thought to play a role in the activation of tyrosine kinases since exogenous diacylglcerol and phorbol esters induce tyrosine phosphorylation in various types of cell [Moon et al. 1984, Grunberger et al. 1984].

Normal B cells and B-CLL cells have a high basal level of tyrosine kinase activity [Harrison et al. 1984, Nel et al. 1985, Kuratsune et al. 1985]. Anti-Ig, phorbol esters and vanadate increase tyrosine phosphorylation in B cells [Earp et al. 1983, Nel et al. 1984,1985]. There is no evidence that sIg has tyrosine kinase activity.

Identity of phosphoproteins in B cells

Much of the protein phosphorylation in anti-Ig-treated B cells is in the cytoskeleton (see below). HLA antigen is a major substrate for phosphorylation in phorbol ester-treated lymphocytes [Shackelford and Trowbridge 1986]. Phosphorylation of HLA may be involved in its attachment to the cytoskeleton [Feuerstein et al. 1985]. A nuclear protein, lamin B, is also

phosphorylated in B cells treated with anti-Ig or TPA [Hornbeck et al. 1988]. This is associated with a decrease in the amount of detergent-insoluble lamin B and may be a signal for dissolution of the nuclear membrane in mitosis [Fields et al. 1988].

TPA stimulates phosphorylation of some proteins associated with sIg (115 and 250 kDa, on non-reducing SDS-PAGE, and 83, 76, 50 and 24 kDa, after reduction) [Newell et al. 1987]. These proteins are phosphorylated constitutively in at least one B cell lymphoma. Other substrates are CR2, the receptor for C3d and Epstein Barr virus [Barel et al. 1986] and CD20, a 35 or 37kDa protein [Valentine et al. 1987]. Phosphorylation of CD20 is accompanied by its internalization.

In the study of Nel and colleagues [1985], there was phosphorylation of tyrosine in substrates of 75, 66, 47 and 28 kDa in the detergent-soluble extracts and 56 and 61 kDa in the detergent-insoluble extracts. Using labelling with ^{32}P-ATP, in cell-free extracts, Kuratsune and colleagues [1985] reported major bands containing tyrosine phosphate of 79, 72, 68, 59 and 56 kDa in total cell extracts of B-CLL cells.

Phosphorylation associated with activation by other stimuli

Lipolysaccharide does not induce phosphorylation of proteins in mouse B cells [Johnstone et al. 1980]. IL-4 induces phosphorylation of a 44kD protein which is not phosphorylated in response to anti-Ig [Justement et al. 1986], suggesting that IL-4 activates a distinct type of kinase.

1.10.6 CHANGE IN MEMBRANE POTENTIAL

Membrane depolarization is thought to be an important early event in cell activation [Rosenthal and Shapiro 1983], thought to result from activation of the Na^+K^+ATPase of membranes, either through phosphorylation by PKC [Cambier et al. 1985] or from

increased incorporation of unsaturated fatty acids into neighbouring phospholipids [Resch 1979]. Membrane potential can be monitored by changes in the uptake of fluorescent carbocyanine dyes [Waggoner 1979, Seligmann et al. 1980].

Via sIg

Depolarization occurs within minutes of treatment of mouse B cells with anti-Ig [Monroe and Cambier 1983a] or antigen [Monroe and Cambier 1983b]. The K^+ ionophore valinomycin blocks both membrane depolarization and the increase in expression of MHC class II antigens on B cells treated with anti-IgM [Monroe and Cambier 1983a]. In contrast, anti-Ig causes a rapid hyperpolarization in human B cells preceding depolarization [MacDougall et al. 1988]. The hyperpolarization is triggered by an influx of calcium ions which opens a K^+ channel, allowing efflux of K^+.

In contrast to mouse B cells, membrane depolarization does not appear to cause increased expression of MHC class II antigens in normal and malignant human B cells [Godal et al 1985].

Other stimuli

A high concentration of K^+ in the medium also causes membrane depolarization and induces activation of the B cells, as shown by increase in expression of MHC class II antigens on mouse B cells [Monroe and Cambier 1983a].

Exogenous phospholipase C, diacylglycerol, phorbol ester, A23187 and lipopolysaccharide also induce membrane depolarization in mouse B cells while Epstein Barr virus induces rapid hyperpolarization followed by depolarization [Monroe et al. 1984, Coggeshall and Cambier 1985].

1.10.7 ALKALINIZATION OF THE CYTOPLASM

The intracellular pH regulates cell metabolism, activation of phospholipase A_2, intercellular communication and cytoskeletal organization [Grinstein et al. 1985, Sweatt et al. 1986]. A rise in intracellular pH is induced by various stimuli affecting cell growth and is thought to be an essential early event in cell activation. The increase in cell size during activation partly results from increased uptake of water to counterbalance the increased uptake of Na^+ and other ions. It is presumed that the rise in intracellular pH results from activation of the Na^+H^+ antiport by PKC-dependent phosphorylation. An inhibitor of the antiport, amiloride, blocks stimulus-induced rise in intracellular pH and later events in cell activation [Berkow et al. 1987], although recent evidence indicates that amiloride also inhibits PKC [Besterman et al. 1985].

The intracellular pH appears to be important in B cell activation since anti-Ig induces a rise in the intracellular pH of resting mouse B cells [Cambier and Ransom 1987] and phorbol ester and lipopolysaccharide induce a rise in intracellular pH as an early event in the differentiation of pre-B cell lymphoblastoid cells into sIg+ve B cells [Rosoff et al 1984]. Ouabain, an inhibitor of the Na^+K^+ ATPase, and monensin, a Na^+ ionophore, increase intracellular Na^+ ions and also induce differentiation in these cells [Rosoff et al. 1984].

1.10.8 MODIFICATION OF THE CYTOSKELETON

The cytoskeleton is involved in the activation of a variety of types of cells including PMN [Jesaitis et al. 1985], T cells [Mookerjee and Jung 1983] and B cells [Rothstein 1986a,b].

Attachment of sIg to the cytoskeleton

Various sIg ligands (anti-Ig, SAC or antigen) cause a rapid,

energy-independent redistribution of sIg into small clusters, followed by aggregation of these clusters into a cap in an energy-dependent step. Accompanying the clustering of sIg is its association with various enzymes including 5'-nucleotidase and ATPase [Raz and Bucana 1980] and with cytoskeletal proteins, including actin, alpha-actinin, alpha-spectrin and tubulin [Braun et al. 1982, Gupta and Woda 1988]. It is believed that the attachment of sIg to actin is mediated by the vitamin D_3-binding protein, group specific component [Petrini et al. 1983]. It is not clear whether the attachment of sIg to the cytoskeleton is a necessary event in the signal transduction or whether it is involved in internalization of sIg. Attachment of alpha-interferon receptors to the cytoskeleton of lymphoblastoid cells does appear to be important since it occurs in interferon-sensitive but not -resistant cells [Pfeffer et al. 1987].

Much of the phosphorylation induced by anti-Ig and phorbol ester in B cells is in the cytoskeleton [Hornbeck and Paul 1986]. The effect of this phosphorylation on the properties of the cytoskeleton is not clear although phorbol esters cause a rapid redistribution of actin, vinculin and other cytoskeletal proteins accompanied by changes in adhesion in B-CLL cells [Marchisio et al. 1988] and a kidney cell line [Schliwa et al. 1984]. In PMN, phosphorylation of a 48kDa cytoskeletal protein causes its translocation to the cytosol and phosphorylation of a 20kDa protein (by PKM) signals its digestion by protease [Pontremoli et al. 1987].

Rothstein has proposed that the cytoskeleton is involved in transducing growth promoting signals for B lymphocytes. Mouse B cells are induced to undergo DNA synthesis when stimulated with a

combination of cytochalasin (B or D), which interferes with actin polymerization, and anti-Ig [Rothstein 1986a,b]. It was proposed that the cytochalasin acts at a later stage than the anti-Ig and may mimic B cell growth factors, although more recent evidence indicates that cytochalasin enhances turnover of PIP_2, apparently by interacting with the cytoskeleton [Van Haelst and Rothstein 1988]. Although the cells synthesize DNA they do not divide. Cytochalasin also activates B-CLL cells [Larson and Yachnin 1983].

1.10.9 CYCLIC NUCLEOTIDES

Cyclic AMP (cAMP) and cyclic GMP (cGMP) are intracellular messengers which modulate many cellular processes. Their synthesis is catalyzed by adenylate cyclase and guanylate cyclase, respectively [reviewed in Alberts et al. 1983]. Adenylate cyclase is coupled to beta-adrenergic receptors via a GTP-binding protein. Occupancy of the receptor activates adenylate cyclase. Fluoride and cholera toxin increase levels of cAMP in cells by activatating the GTP-binding protein. Cyclic AMP-dependent events are effectively induced by a combination of the membrane-permeable non-hydrolyzable form of cAMP, dibutyryl cAMP, and theophylline, an inhibitor of the phosphodiesterase which degrades cAMP.

A major mechanism of action of these agents is via protein kinases dependent on cAMP (protein kinase A) or cGMP (protein kinase G) [Sibley et al. 1987].

B cells have high resting levels of cAMP and adenylate cyclase activity [Bach 1975, Mendelsohn and Nordberg 1979]. Lymphocytes contain several distinct pools of cyclic nucleotides (membrane, cytosolic and nuclear) which are selectively increased by various stimuli. Prostaglandins mainly increase the

cytoplasmic pool, isoproterenol increases both the cytoplasmic and nuclear pools and phytohaemagglutinin increases the membrane pool [Steiner et al. 1976, Wedner and Parker 1977].

TPA increases guanylate cyclase activity and cGMP levels [Coffey and Hadden 1981] and decreases adenylate cyclase activity [Heyworth et al. 1984], in lymphocytes, probably by phosphorylation of these enzymes [Zwiller et al. 1985, Yoshimasa et al. 1987].

Lipopolysaccharide lowers cAMP levels in B cells by inhibiting adenylate cyclase [DeFranco et al. 1987]. Antibodies to MHC class II antigens and to Fc receptors in B cells increase cAMP [Cambier et al. 1987a,b].

A rise in cAMP is thought to be associated with B cell differentiation rather than B cell mitogenesis [Cambier et al. 1987a]. Related to this may be the finding that agents which increase the intracellular concentration of cyclic AMP inhibit PIP_2 turnover and subsequent events in cell activation induced in B cells by anti-Ig [Coggeshall and Cambier 1984, Dennis et al. 1987], as well as causing translocation of PKC to the nucleus.

1.10.10 OTHER EARLY EVENTS

ADP-ribosylation

It has been suggested that a rise in intracellular Na^+ stimulates DNA repair in lymphocytes, and that this is an essential early event in lymphocyte activation [Johnstone and Williams 1982]. The repair involves ADP-ribosylation of proteins. Possibly related to this is the finding that inhibitors of ADP-ribosyltransferase interfere with plasmacytoid differentiation of Daudi B lymphoblastoid cells induced by phorbol esters and by interferon [Exley et al. 1987].

Myristylation

Myristylation of some proteins allows their insertion into cell membranes. Myristylation of an 82kDa protein occurs in TPA-treated HL60 cells but not in a variant cell line which does not undergo differentiation in response to TPA [Malvoisin et al. 1987].

Thiol-dependent events

Low concentrations of 2-ME or alpha-thioglycerol in culture media promote B lymphocyte activation by anti-Ig and antigen, particularly in monocyte-depleted cultures [reviewed in Hewlett et al. 1981]. Controversy exists as to whether 2-ME replaces macrophages or enhances the response of reduced numbers of macrophages.

One way in which thiols promote lymphocyte growth is by generation of an active factor in serum. This thiol-activated factor has properties of albumin. Pure albumin which has been treated with 2-ME is a cofactor for B cell activation [Claësson et al. 1979, Hewlett et al. 1981]. Since monocytes and other feeder cells secrete thiols and modify albumin in their environment [Bannai and Ishii 1982], one role of monocytes in lymphocyte cultures may be to promote albumin uptake.

Thiols increase the intracellular concentration of glutathione, which is essential for lymphocyte activation [Fischman et al. 1981].

Proteolysis

Proteolysis is thought to be important at an early stage of B cell activation [Arora et al. 1981]. Mouse B cells are induced to undergo limited cell division in the presence of trypsin [Vischer 1974]. Trypsin is required for at least 24 hours. In the B lymphoblastoid cell line CESS, activation of a serine esterase

is essential for B cell differentiation induced by B cell differentiation factor. Inhibition of the esterase by di-isopropyl-fluoro phosphate prevents differentiation [Miki et al. 1982].

Esterases are thought to mediate some other effects of phorbol esters on cells [Blumberg 1980, Patarrayo et al. 1982].

Guanosine ribonucleotides

C8-substituted guanine ribonucleotides (eg 8 bromoguanosine and 8 mercaptoguanosine) induce proliferation and plasmacytoid differentiation in mouse B cells by acting at an intracellular site. They are thought to act only on pre-activated B cells [Goodman 1986].

1.11 AURANOFIN AND OTHER GOLD COMPOUNDS

The lipophilic gold (I) compound auranofin has effects on early events in cell activation, particularly those mediated by PKC. Some of these may be involved in its therapeutic action in rheumatoid arthritis.

1.11.1 GOLD THERAPY IN RHEUMATOID ARTHRITIS

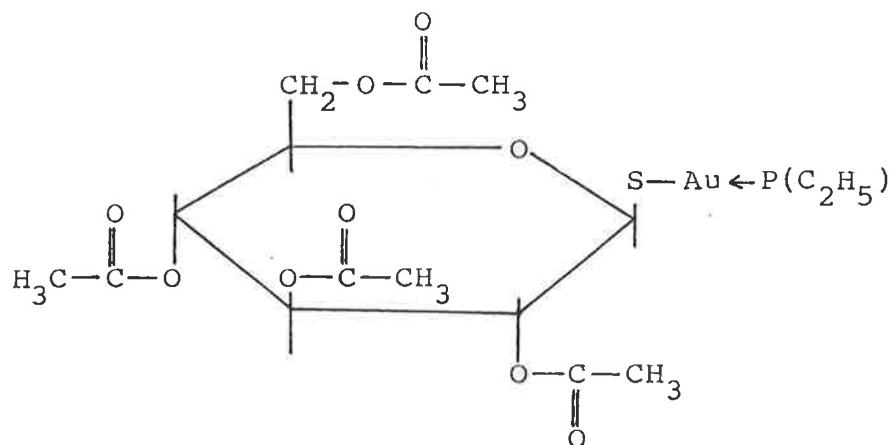
Gold therapy (chrysotherapy) has been used in many types of diseases since the early ages [reviewed in Kean et al. 1985]. In 1890, Robert Koch found that gold inhibited growth of tubercle bacilli. However, treatment of tuberculosis with gold was not very effective. Gold was used in the treatment of bacterial endocarditis and rheumatic fever and later in the treatment of rheumatoid arthritis. Clinical trials in the 1960s and 1970s showed the effectiveness of gold in treatment of rheumatoid arthritis.

Auranofin

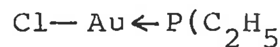
Until recently, only water-soluble, gold(I) compounds administered by intramuscular injection were used. These were mainly sodium aurothiomalate and gold sodium thioglucose. In 1972, Sutton and colleagues reported that auranofin and other lipophilic gold(I) compounds (Fig 1.13) had anti-arthritic activity when administered orally to adjuvant arthritic rats. Auranofin is now widely used in treatment of patients with rheumatoid arthritis [reviewed in Blodgett et al. 1984, Davis 1984].

Because of its lipophilicity, auranofin is readily taken up by cells. Effective therapy with auranofin is achieved at blood gold concentrations that are about four times lower than those required for aurothiomalate, resulting in less severe problems with gold toxicity. The most common side effects with auranofin

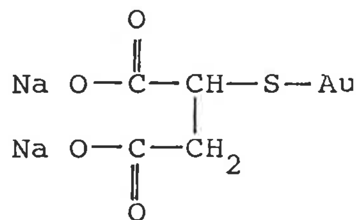
Auranofin



Chloro-triethylphosphine gold



Sodium aurothiomalate



Legend to Figure 1.13 Structures of some gold compounds

Structures of auranofin ([2,3,4,6-tetra-O-acetyl-1-thioglucopyranosato-S] [triethylphosphine] gold), chloro-triethylphosphine gold and sodium aurothiomalate are shown.

are diarrhoea and cutaneous reactions.

<u>Structure of auranofin</u>

Gold occurs most often in two oxidation states, gold (1) and gold (III). All of the anti-arthritic gold compounds are gold (I) compounds. Gold (I) forms linear complexes with two ligands. The main ligands are cyanide, thiolate and phosphine. These contain lone pairs of electrons which they share with the gold, forming a metal-ligand co-ordination bond that is thought to be intermediate between an ionic and covalent bond [Sadler 1982].

Auranofin consists of two hydrophobic ligands, triethyl-phosphine and tetraacetyl-thioglucose, attached on opposite sides of the gold (Fig 1.13). The molecule is uncharged and monomeric unlike the highly charged, polymeric, water-soluble gold salts [Sadler 1982, Grootveld et al. 1984].

<u>Mechanism of action of gold in treatment of rheumatoid arthritis</u>

Rheumatoid arthritis is an auto-immune disorder involving a persisting inflammation of the synovium in the rheumatoid joint, eventually leading to destruction of cartilage and bone. Chronic activation of inflammatory phagocytes, B cells and other types of cells, with release of toxic substances and autoantibodies, contributes to the tissue damage [Zvaifler 1983].

Abnormal activation of B cells is evidenced by the very high rate of synthesis of Ig in the patients and the high frequency of B cells expressing activation markers [Wochner 1970, Swilinski and Zvaifler 1970, Youinou et al. 1984a]. There is also expansion of the MER+ve, CD5+ve B cell subset thought to be responsible for the autoantibody production (see chapter 1.2.2). High titres of rheumatoid factor and, in some cases, anti-nuclear factor, anti-complement and anti-fibrinogen antibodies are

present in the serum and synovial fluid. Between 20% and 60% of the plasma cells in the synovium make rheumatoid factor [Natvig and Munthe 1975].

Increased activation of B cells may result from abnormalities in their regulation by helper T cells, suppressor T cells and monocytes or from persistent stimulation by a polyclonal B cell activator such as Epstein Barr virus [Paget and Gibofsky 1979, Zvaifler 1983]. Immune complexes of autoantibody and the antigen cause further activation of lymphoid cells and inflammatory phagocytes [Winchester 1975].

Infiltrating PMN, monocytes and macrophages undergo oxygen-dependent metabolism and degranulation and, in the process, release toxic oxygen metabolites (including superoxide anion, hydrogen peroxide, singlet oxygen and hydroxy radicals) and lysosomal enzymes which contribute to the tissue damage.

Suppressive effects of gold on the activation of B and T lymphocytes and phagocytes may explain the action of gold as an anti-rheumatic agent. Auranofin affects many events in cells, in vitro, at concentrations of gold which are similar to those occurring in the blood and synovial fluid of patients receiving the drug. Which of these actions are of therapeutic importance has not yet been determined [Leibfarth and Persellin 1981].

1.11.2 EFFECTS OF AURANOFIN ON LYMPHOCYTES AND PHAGOCYTES

Effects of auranofin on lymphocytes

Auranofin both enhances and inhibits lymphocyte function [Finkelstein et al. 1977, Lorber et al. 1982, Walz et al. 1982b, Gottlieb 1982, Salmeron and Lipsky 1982, Davis 1984, Griswold et al. 1985, Burns et al. 1987]. Therefore, it is has been referred to as a "condition-dependent immunoregulatory agent" [Delafuente and Osborn 1984]. Some of the effects, in-vivo, include

enhancement of cell mediated immunity, decrease in the serum concentrations of IgG, IgA and IgM, induction of hypogammaglobulinemia and significant reduction in the titre of rheumatoid factor in patients with rheumatoid arthritis. Antibody production is inhibited by auranofin in various animal models. A major difference between auranofin and aurothiomalate is that auranofin acts much more quickly in altering immune function, in vivo [Gottlieb 1982, Lorber et al. 1982].

In vitro, auranofin inhibits lymphocyte mitogenesis induced by various agents including antigen and mitogens [Finkelstein et al. 1977, Salmeron and Lipsky 1982], IL-2 production and proliferation of T helper cells [Griswold et al. 1985] and Ig secretion induced in human peripheral blood B lymphocytes by pokeweed mitogen. Inhibition of immunoglobulin production results from an action of auranofin during the first 24 hours of the culture. At least tenfold higher concentrations of aurothiomalate are required to produce the same degree of inhibition [Salmeron and Lipsky 1982].

Effects on inflammatory phagocytes

In vitro, auranofin has biphasic effects on activation of PMN and monocytes/macrophages by various stimuli including phorbol esters [Russell et al. 1982, Scheinberg et al. 1982, Davis et al. 1983, Hafström et al. 1984, Sung et al. 1984, Parente et al. 1986, Minta and Williams 1986, Hurst 1987]. High concentrations (>5uM) of auranofin inhibit whereas low concentrations (<1uM) enhance the activation. This may explain why early in the treatment of patients with auranofin there is often exacerbation of the inflammatory process whereas later there is inhibition [N.P. Hurst Personal Communication].

Auranofin affects the activation of these cells by a wide variety of agents suggesting that auranofin acts at an early, common event in the process of activation rather than at the level of binding of these substances to the cells [Hafström et al. 1984].

1.11.3 <u>CELLULAR UPTAKE OF GOLD IN AURANOFIN</u>

Gold in tissues can be measured by atomic absorption spectroscopy. Uptake of gold can also be measured by using radiolabelled gold.

<u>In-vivo</u>

About 25% of the gold in auranofin is absorbed in the upper gastrointestinal tract [Gottlieb 1982]. Ninety-five percent is excreted in the faeces and the other 5% in the urine. In animal models, the gold localizes in most tissues of the body [Intoccia et al. 1982].

In patients receiving auranofin, the gold levels in the blood and serum increase slowly. After six to twelve weeks of therapy, the total blood gold concentrations are between 0.2 and 1µg/ml [Delafuente and Osborn 1984]. About half of this is in the plasma, mostly associated with albumin, and the other half is associated with red cells, mononuclear cells and PMN [Walz et al. 1982a, Herrlinger et al. 1982, Lewis et al. 1983]. Much less gold is associated with cells when sodium aurothiomalate is used [Lewis et al. 1983].

<u>In-vitro</u>

Exposure of cells to auranofin for several minutes, in vitro, results in a level of cell-associated gold equivalent to that associated with blood cells in patients after several months of therapy with auranofin [Herrlinger et al. 1982, Gottlieb 1982, Hafström et al. 1984]. There is a linear relationship between the

concentration of ^{195}gold-labelled auranofin used to treat the cells and the amount of ^{195}gold in the cells, with no evidence of saturation up to 20uM. The uptake is temperature-dependent and complete by 10 minutes [Snyder et al. 1986].

Mechanism of uptake of auranofin by cells

Cellular uptake of gold in auranofin or sodium aurothiomalate results from thiol ligand exchange. Binding is accompanied by displacement of the tetra-acetylthioglucose and thiomalate ligands, respectively. Uptake is blocked by pretreatment of cells with the sulphydryl reagent, N-ethyl maleimide and by competing thiols such as 2-ME and glutathione or thiol-containing proteins [Hafström et al. 1984, Snyder et al. 1986]. At a concentration of ten percent (v/v), FCS reduces the cellular uptake of gold by twentyfold, a result of binding of gold to the single free sulphydryl at cysteine 34 in albumin [Coffer et al. 1986]. The effects of auranofin on various types of cells are also blocked by FCS and albumin [Snyder et al. 1986] and low molecular weight thiols [Nathan et al. 1982].

The gold in triethylphosphine gold chloride associates with cells at a faster rate than the gold in auranofin [Snyder et al. 1986] because the chloride is a better leaving group than the tetraacetylthioglucose [Sadler 1982]. Triethylphosphine gold chloride has even more potent effects on cell function than auranofin [Sung et al. 1984, Snyder et al. 1987b].

The following model has been proposed by Snyder and colleagues [1986,1987a,b] to explain the mechanism of uptake of gold by cells. Sulphydryl-containing proteins in the plasma membrane compete with the tetraacetylthioglucose for the gold in auranofin. Once bound to membrane thiols there is a dynamic

equilibrium of the gold between cell membrane, cytosol and nucleus involving a shuttling between free sulphydryls in various cellular components. This involves loss of the triethylphosphine and its oxidation to triethylphosphine oxide. It is not clear whether this oxidation occurs before or after dissociation from the gold. In red cells at least, most of the gold eventually becomes complexed with glutathione [Razi et al. 1983]. Efflux of the gold from the cell occurs by a reverse mechanism and is promoted by extracellular thiols [Crooke et al. 1986, Snyder et al. 1987a,b].

This hypothesis is supported by experiments in which auranofin, radiolabelled in different parts of the complex, is added to blood or tissue culture cells or is administered to animals [Intoccia et al. 1982, Grootveld et al. 1984, Snyder et al. 1987b].

The role of the hydrophobic triethyl-phosphine group in uptake of auranofin is not defined in the model by Snyder and colleagues. Passage of gold through the membrane may be facilitated by the presence of the triethylphosphine.

In vivo, blood cells probably never come into contact with the intact auranofin complex since auranofin is changed as it is absorbed through the wall of the intestine [Tepperman et al. 1984]. This has been interpreted as loss of the acetyl groups on the thioglucose [Tepperman et al. 1984] or loss of the whole tetraacetylthioglucose ligand [Crooke et al. 1986]. Auranofin also loses this ligand as soon as it contacts albumin or other thiol-contaning substances in the culture medium.

1.11.4 GENERAL EFFECTS OF AURANOFIN ON CELLS

Probably because of its lipophilicity and its capacity to ligate sulphydryls, auranofin has many effects on cell functions.

Cytotoxicity

Auranofin is cytotoxic when used at high concentrations for several hours. Immediately prior to lysis of cells treated with auranofin, there is blebbing and pitting of the plasma membranes suggesting that the plasma membrane may be the site of damage. Membrane blebbing also occurs in lymphocytes in vivo [Simon et al. 1979]. Blebbing may be a consequence of interaction of gold with essential sulphydryls in the cell membrane [Simon et al. 1979], calcium-dependent activation of proteases and phospholipases [Nicotera et al. 1986, Crooke et al. 1986, Lemasters et al. 1987] or peroxidation of membrane lipids as a consequence of free radical production during oxidation of triethylphosphine to triethylphosphine oxide [Snyder et al. 1987b].

Because of its cytotoxicity, auranofin has been tested for anti-tumour activity. Auranofin increases the life span of mice inoculated with the lymphocytic leukaemia cell line P388 [Simon et al. 1981, Mirabelli et al. 1985] but has no activity on several other tumours [Crooke et al. 1986]. In vitro, auranofin has potent anti-tumour activity on a variety of human tumour cells in a soft agar assay [Mirabelli et al. 1985].

Auranofin is a potent inhibitor of DNA synthesis in HeLa cells, lymphocytes and B cell lines [Finkelstein et al. 1977, Simon et al. 1979].

Cytoskeleton

Auranofin causes depolymerization of microtubules in PMN [Hafström et al. 1984]. This may result from inhibition of an intracellular glutathione-dependent pathway which is involved in microtubule polymerization.

Membrane transport

Another action of auranofin on cell membranes in human lymphocytes is inhibition of transport of thymidine and amino acids [Finkelstein et al. 1977]. Auranofin also inhibits transport of hexose, amino acids, fluid and Na^+ across the intestinal wall [Hardcastle et al. 1986]. These effects may result from inhibition of the sodium pump and may explain the high incidence of diarrhoea in patients treated with auranofin.

Membrane depolarization

Auranofin appears to cause an initial small hyperpolarization followed by pronounced membrane depolarization in PMN, perhaps by interfering with the Na^+K^+ATPase [Hafström et al. 1984].

Membrane receptors

Few studies report effects of auranofin on membrane receptors. Auranofin enhances expression of the receptor for the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine in PMN [Hafström et al. 1984], resulting from an increase in affinity of receptors rather than alteration in total number of receptors.

Auranofin mimics phorbol esters in inhibiting the epidermal growth factor receptor, decreasing both the affinity of binding and the apparent total number of receptors [Froscio et al. 1987]. These effects correlated with an increase in phosphorylation of the receptors in auranofin-treated cells [M. Froscio Personal

communication]. Tryptic peptide maps indicate that the phosphorylation induced by auranofin and TPA are similar suggesting that auranofin may also stimulate PKC. However, in cells in which most of the PKC activity has been lost, as a result of prolonged exposure to phorbol ester, auranofin still inhibits binding of epidermal growth factor [Froscio et al. 1987]. This may indicate that auranofin does not act via PKC in stimulating phosphorylation of this receptor.

1.11.5 MECHANISMS OF ACTION OF AURANOFIN

Several ways in which auranofin might affect cellular events are described below. Other mechanisms that are suggested by findings in the experiments described in this thesis are discussed in the Results.

Enzymes

Because of the preference of gold for sulphydryl residues, auranofin predominantly affects sulphydryl-dependent enzymes [reviewed in Crooke et al. 1986] including proteases [Lewis et al. 1980], DNA polymerases from various sources [Allaudeen et al. 1985] and Na+K+ ATPase [Hardcastle et al. 1986].

In contrast to its general inhibitory effects on enzymes, auranofin enhances phospholipase C activity up to 40 fold in both cell lysates and with pure bacterial phospholipase C [Snyder et al. 1988]. Since this is a zinc-requiring enzyme [Ottolenghi 1965], it has been proposed that auranofin modulates the zinc-binding site.

Protein phosphorylation

In addition to stimulating phosphorylation of the epidermal growth factor receptor, auranofin also stimulates phosphorylation of 20 and 40 kDa proteins in human platelets [Froscio et al.

1988]. Tryptic peptide maps show that the pattern of phosphorylation of both proteins is similar in cells treated with auranofin or TPA. The effects of TPA and auranofin are additive rather than synergistic.

Phosphorylation of 40 kDa protein by PKC is coupled to aggregation of platelets. However, auranofin does not induce platelet aggregation. Rather, it inhibits platelet aggregation induced by phorbol esters and other stimuli [Nathan et al. 1982, Sengelov et al. 1986]. This may indicate that auranofin inhibits an event which is essential for platelet aggregation and which is distal to activation of PKC.

It has not been excluded that the increase in phosphorylation in auranofin-treated cells is a result of inhibition of phosphatase rather than by stimulation of PKC. The effects of auranofin on PKC are discussed in some detail in chapters 8 and 9.

Cyclic nucleotides

One mechanism by which auranofin might act on cellular events is via cAMP. Auranofin increases cyclic AMP in monocytes and platelets [Scheinberg et al. 1982, Sengelov et al. 1986] but not in lymphocytes [Russell and Miller 1984]. The increase in cAMP may explain the inhibitory effects of auranofin on function of natural killer cells [Pedersen and Abom 1986], superoxide anion formation in PMN [Lehmeyer and Johnstone 1978] and aggregation of platelets [Sengelov et al. 1986]. The mechanism by which auranofin increases cAMP is not known.

Free radicals

Another mechanism of action of auranofin early in cell activation may be quenching of singlet oxygen and other oxygen-dependent radicals formed from superoxide anion [Corey et al.

1987]. Singlet oxygen can initiate peroxidation of unsaturated fatty acid derivatives and may be involved in tissue damage in rheumatoid arthritis.

Effect on metallothionein and zinc homeostasis

Zinc is an important co-factor in cell activation (see chapter 1.12). Auranofin may act by interfering with zinc homeostasis. Most of the zinc in the cell is complexed with metallothionein. Metallothionein is a low molecular weight protein which, because of its high cysteine content, binds metals including zinc and protects cells from the toxic effects of metals. It is thought that there are seven metal binding sites in metallothionein containing 3 to 4 sulphydryls per site [reviewed in Dunn et al. 1987]. Gold binds to metallothionein with higher affinity than zinc and readily displaces the latter metal. Cadmium and copper are displaced at a lower rate [Schmitz et al. 1980].

Like the other heavy metals, gold is a potent inducer of metallothionein in cells [Butt et al. 1986]. There is rapid induction of transcription of the metallothionein gene. Increased transcription of the metallothionein gene in variant cell lines has been correlated with increased resistance to cytotoxicity by auranofin [Butt et al. 1986].

1.12 ZINC

Zinc is essential for many cellular events such as proliferation, gene expression, stabilization of macromolecules and cellular structures, signal transduction and cell metabolism. Zinc is a cofactor for many vital cellular proteins including enzymes, transcription factors and hormones. Some of these contain the zinc finger structures. As discussed in chapter 1.8, PKC has two zinc finger-like structures although it is not yet known whether it is dependent on zinc for function.

One of the hypotheses in this thesis is that some of the effects of gold on early events in cell activation result from interference with zinc-dependent processes.

1.12.1 BINDING OF ZINC

Chemistry

Zinc belongs to the IIB transition series of elements. It combines with ligands in the +2 oxidation state and forms co-ordination complexes usually with tetrahedral configuration. It does not undergo redox reactions like copper and iron [Chvapil et al. 1972, Riordan 1976].

Binding to proteins

Zinc has a high affinity for compounds which contain sulphur and nitrogen [Williams 1984]. Cysteine and histidine are major binding sites for zinc in proteins. Zinc has particularly high affinity for certain regions in proteins with particular spacings of four cysteines or histidines (see below). By cross-linking cysteines in protein, zinc takes the place of a disulphide bond. The cysteine-zinc-cysteine coordinate bond is more flexible than a disulphide bond and it is also more stable than a disulphide bond [Williams 1984], particularly in the interior of cells where there is a strong reducing environment [Fahey et al. 1977].

In plasma, 50% to 60% of the zinc is bound to albumin and the rest is bound to $alpha_2$ macroglobulin, transferrin and amino acids. Within cells, most of the zinc is bound to metallothionein, a family of low molecular weight proteins which contain repeated cysteine residues [Dunn et al. 1987]. Like other metals, zinc binds in clusters to metallothionein [Williams 1984], causing the protein to fold and to bind to RNA and DNA through its basic amino acid side chains. The binding of metallothionein to its gene regulates transcription [Williams 1984].

Zinc also complexes with phosphates, because of their negative charge, and thereby binds to and stabilizes double-stranded DNA.

Zinc chelators

1,10 phenanthroline and histidine are strong chelators of zinc and will lower the concentration of intracellular zinc [Ferry and Donner 1984]. EDTA and EGTA bind zinc strongly but less selectively.

1.12.2 ZINC CONTENT OF TISSUES AND CELLS

Measurement of free and total zinc

Total (free and bound) zinc is usually measured by atomic absorption spectroscopy [Whitehouse et al. 1982], although more sensitive techniques are being developed [Vallee and Galdes 1984]. Electron microscopic X-ray analysis [Yarom et al. 1976] and X-ray fluorescence spectroscopy [Csermely et al. 1987a] are useful for detecting zinc at a sub-cellular level.

It is also important to know the free ionized zinc concentration since most of the cell-bound zinc is not freely exchangeable with that pool of zinc which participates in

metabolic reactions [Williams 1984]. Extracellular free zinc has been measured using an assay involving the enzyme phosphoglucomutase [Magneson et al. 1987]. Fluorescent probes capable of measuring the free ionized intracellular zinc concentration are not yet available. Free zinc is sometimes estimated from the affinity constants of zinc for proteins in its environment.

Zinc in tissues

Relatively large amounts of zinc occur in the neocortical gray matter, pineal and hippocampus of brain [Danscher et al. 1976, Prohaska 1987], in the pancreas [Williams 1984] and in the male reproductive tract [Chvapil et al. 1972]. The high concentration of zinc in sperm tails is thought to prevent autoxidation of keratin [Williams 1984]. Because of its large size, the liver contains more zinc than other tissues or organs.

It has been suggested that the concentration of zinc in the spleen is regulated by a polygene complex, which may code for proteins involved in uptake or efflux of zinc [Henrotte et al. 1987].

Plasma and serum contain between 8 and 25μM zinc [Heyns 1985]. In horse plasma, using the phospho-glucomutase enzyme assay, the concentration of free zinc was estimated to be 0.2nM (total zinc was 8μM) [Magneson et al. 1987].

There is a marked decrease in the plasma concentration of zinc in infection, severe burns, chronic liver disease, rheumatoid arthritis and malignancy [Williams 1984, Svenson et al. 1985].

In cells, the concentration of total zinc is of the order of 1mM [Williams 1984]. Based on a Kd for binding of zinc to metallothionein of about 10^{17}, it has been estimated that the

intracellular free zinc concentration in hepatocytes is about one picomolar [Williams 1984]. Zinc is complexed with insulin in the islets of Langerhans and with digestive enzymes in vesicles in the pancreas. These proteins have much lower binding affinities for zinc than metallothionein and, consequently, the concentration of free zinc in these compartments is much higher (about 1μM) than in cytosol [Williams 1984].

Zinc content of CLL cells

In CLL cells, the content of zinc but not most other metals, is reduced by up to 60% in both cytosol and nucleus compared with normal lymphocytes [Yarom et al. 1976]. Zinc content was assayed by electron microscopic X-ray analysis.

The concentration of zinc in serum and plasma of CLL patients is also low while the concentration in red cells is increased suggesting that there is an abnormality in zinc homeostasis in this disease [Dennes et al. 1960, Fredricks et al. 1964, Rosner and Gorfien 1968].

Subcellular distribution

Electron microscopic X ray microanalysis indicates enrichment of zinc in the nucleus and especially the nucleolus of lymphocytes [Yarom et al. 1976]. Zinc in the nucleus serves as a cofactor for many enzymes and DNA binding proteins and is essential for DNA synthesis in prokaryotes and eukaryotes [Vallee and Falchuk 1981, Vallee and Galdes 1984].

Zinc is also abundant in microtubules [Morisawa and Mohri 1972, Chvapil 1976] where it appears to regulate the assembly of microtubules [Nickolson and Veldstra 1972, Gaskin and Kress 1977]. Zinc binds to tubulin and causes it to be assembled in sheets of between 15 and 60 protofilaments.

Membranes are also stabilized by zinc [Chvapil 1976]. By interacting with sulphydryls, zinc may interfere with lipid peroxidation or enzymes affecting membrane structure [Chvapil et al. 1975].

1.12.3 ZINC FLUXES IN CELLS

Uptake of zinc by cells

In hepatocytes and fibroblasts incubated with zinc chloride, there is a rapid early phase of uptake of zinc (within the first 10 minutes) followed by a slower phase [Ferry and Donner 1984, Ackland et al. 1988]. In PMN, the uptake of zinc levels off at 10 minutes [Chvapil et al. 1977].

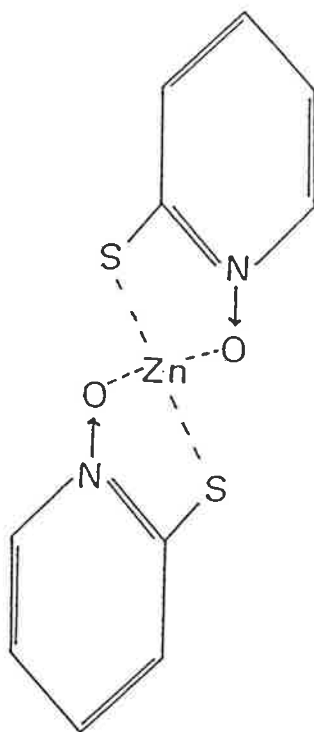
It has been proposed that uptake of zinc by cells occurs in the form of zinc complexes with amino acids and plasma proteins, including transferrin, albumin and alpha$_2$-macroglobulin [Williams 1984, Henrotte et al. 1987]. Uptake of zinc by cells may also be influenced by hormones [Cox and Ruckenstein 1971].

Alternatively, zinc may be taken up by cells by thiol ligand exchange in a manner analagous to that proposed for uptake of gold. Saturable carriers or "receptors" for zinc were detected in cell membranes in the study by Ackland and colleagues. This model is supported by the observation that uptake of zinc, like that of gold, is inhibited by plasma and serum [Schwarz and Matrone 1975, Chvapil et al. 1977].

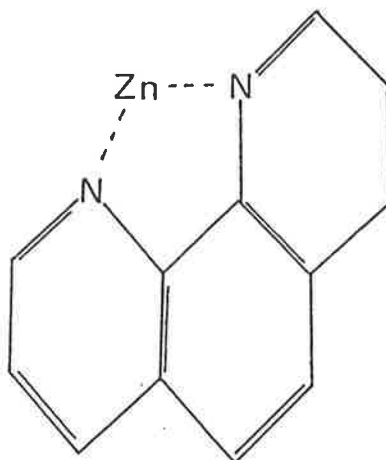
Zinc pyrithione

Uptake of zinc by various tissues in mice, in vivo, is greatly facilitated when zinc is administered in the form of zinc pyrithione [Jasim and Tjälve 1986], an anti-seborrheic agent in shampoos [Black and Howes 1979, Gibson et al. 1985a,b]. Sodium pyrithione forms a lipophilic complex with zinc, nickel and cadmium (Fig 1.14) and is likely to promote uptake of zinc into

Zinc pyrithione



1,10 Phenanthroline



Legend to Figure 1.14 Structures of zinc pyrithione and phenanthroline

Zinc is presumed to bind to the O- and S-ligands in pyrithione forming a dimer in which one zinc is bound between two pyrithiones. Zinc binds to N-ligands in phenanthroline.

cells in the same way that triethylphosphine promotes uptake of gold.

Fate of internalized zinc

A major difference between the fate of internalized zinc and gold is that most of the intracellular zinc complexes with metallothionein while most of the gold complexes with glutathione [Schmitz et al. 1980].

Movement of zinc within the cell

Treatment of rabbit lymphocytes with TPA for 90 minutes causes redistribution of zinc from nuclei and mitochondria to cytosol and microsomes, as determined by X-ray fluorescence spectroscopy [Csermely et al. 1987a].

1.12.4 EFFECT OF ZINC ON LYMPHOCYTES AND OTHER CELLS

Lymphocytes

Zinc-deficient animals have fewer T and B cells and impaired cellular and humoral immunity and natural killer cell function [Fernandes et al. 1979, Beach et al. 1980, Frost et al. 1981].

In acrodermatitis enteropathica, where there is a defect in the absorption of zinc, there is severe immunodeficiency [Barnes and Moynahan 1973]. Administration of zinc restores immune responsiveness in these patients.

It has been proposed that deficiency of zinc in cells is a primary cause of the immunodeficiency that occurs with ageing [Garfinkel 1986]. Addition of zinc to lymphocytes from aged mice restores their capacity to produce anti-red cell antibody. Zinc is required during the first 24 hours of the culture suggesting that is acts on a relatively early event [Winchurch et al. 1987].

High concentrations of zinc (200μM) induce polyclonal proliferation in T lymphocytes from humans and other species in a monocyte-dependent manner [reviewed in Kirchner and Salas 1987]. Mercury, and to a lesser extent nickel, are also mitogenic for lymphocytes but other metals including gold are inactive. Zinc has to be present continously during the culture for increased DNA synthesis to occur [Reardon and Lucas 1987].

In other systems, zinc may act as a cofactor for lymphocyte mitogenesis. Zinc activates mouse thymocytes only in the presence of 2-ME and lipopolysaccharide [Reardon and Lucas 1987]. Responsiveness of lymphocytes to phytohaemagglutinin is increased in animals on a diet rich in zinc. Chelation of zinc in lymphocytes with phenanthroline inhibits mitogenesis induced in vitro by phytohaemagglutinin. The inhibition is reversed by zinc or nickel but not other metals [Williams and Loeb 1973].

Zinc is also a polyclonal activator for B cells, being as effective as pokeweed mitogen in induction of proliferation and anti-sheep red cell antibody synthesis in human B cells [Cunningham-Rundles et al. 1980], although whether zinc acts directly on the B cell or via helper T cells is not clear.

Zinc may stimulate mitogenesis by activating zinc-dependent enzymes involved in DNA synthesis or, as shown by Grummt et al. [1986], it may promote the formation of diadenosine tetraphosphate, a co-factor for DNA synthesis.

Other cells

Like gold, zinc modulates the activation of a variety of types of cells including PMN [Chvapil et al. 1977], mast cells [Kazimierczak and Maslinski 1974] and platelets [Chvapil et al. 1975], often with enhancement at concentrations less than 70uM and inhibition at higher concentrations. In the presence of

magnesium ions, zinc inhibits activation of PMN at all concentrations [Chvapil et al. 1977].

Zinc and tumours

Zinc has both inhibitory and augmenting effects on tumor formation [Poswillo and Cohen 1971, reviewed in Kasprzak and Waalkes 1986]. Zinc inhibits carcinogenesis induced by dimethylbenzanthracene in the hamster cheek pouch, growth of transplantable lymphosarcomas in rats and development of adenocarcinomas in the breast of a highly susceptible strain of mice. A deficiency of zinc in the diet inhibits growth of the Walker 256 carcinsarcoma [Petering et al. 1967].

1.12.5 FUNCTIONS OF ZINC

Enzyme cofactor

That zinc is a cofactor for enzymes was first demonstrated for red cell carbonic anhydrase. Now more than 200 enzymes are known to contain zinc [reviewed by Vallee and Galdes 1984]. Many of the enzymes involved in DNA synthesis, repair and transcription are dependent upon zinc [Slater et al. 1971]. Sometimes, zinc has biphasic effects. For example, low concentrations of zinc enhance DNA polymerase activity while high concentrations inhibit it. Phospholipase C has an absolute requirement for zinc [Ottolenghi 1965] whereas phospholipase A_2 is inhibited by zinc [Wells 1973].

Zinc is probably added to the fully-formed enzyme rather than during the synthesis of the enzyme [Harris and Coleman 1968]. Some zinc-dependent enzymes such as liver alcohol dehydrogenase and aspartate carbamoyl transferase contain a cysteine-rich sequence cys-X_2-cys, known to bind zinc [Eklund et al. 1974, Berg 1986]. The zinc in enzymes appears to be very

tightly bound and not readily exchangeable. Phenanthroline will bind to the zinc in some enzymes and inhibit enzymatic activity [Williams 1984].

Zinc has several functions in enzymes, stabilizing them by protecting essential sulphydryls against autoxidation, playing an essential role in the catalytic site eg for thermolysin and playing a regulatory role. Because zinc can displace iron and copper, which are involved in redox reactions, zinc may inhibit some enzymes [Chvapil et al. 1972].

Growth factors

Zinc stabilizes insulin [Williams 1984] and serves to join subunits in nerve growth factor [Dunn et al. 1980].

Zinc fingers

Another role for zinc is in formation of DNA-binding zinc fingers [reviewed in Klug and Rhodes 1987]. Zinc is essential for initiation of transcription of the 5S RNA gene of Xenopus laevis by transcription factor IIIA [Miller et al. 1985]. This factor contains an abundance of cysteines and histidines and about 7 to 9 zinc atoms per molecule. Structural studies suggest the presence of 9 repetitive zinc binding domains (zinc fingers) each of approximately 30 amino acids. In each domain, one atom of zinc is coordinated tetrahedrally to one pair of cysteines and one pair of histidines. These pairs are separated by 12 amino acids. Binding of zinc is thought to pull the ends of the domain together and produce a loop or finger of amino acids [Parraga et al. 1988]. The finger structure has been confirmed by X-ray absorption spectroscopy [Diakun et al. 1986].

The fingers are rich in those amino acids (lysine, histidine, asparagine, glutamine and threonine) which can bind to phosphate in DNA [Miller et al. 1985]. Binding of zinc induces

folding of the zinc fingers and protects them from proteolysis [Frankel et al. 1987].

Zinc fingers are also found in gene products of adenovirus ElA, receptors for estrogen, glucocorticoid and progesterone, the v-erb A gene product of the avian erythroblastosis virus and a portion of the thyroid hormone receptor [Weinberger et al. 1985, Berg 1986, Green et al. 1988]. The presence of two zinc fingers in PKC was described in chapter 1.8.3.

Zinc enhances the binding of steroid receptor complexes to isolated nuclei of cells and to DNA [Colvard and Wilson 1984, Freedman et al. 1988], probably by acting through the zinc finger domain of these receptors.

<u>Neurotransmission</u>

Zinc is thought to play a role in neurotransmission at two levels [Peters et al. 1987]. Electrical stimulation provokes a calcium-dependent release of zinc into the synaptic clefts and may increase firing of some cortical neurones [Wright 1984]. Zinc also interacts with glutamate, a transmitter used in the hippocampus [Bere and Helene 1979].

<u>Calcium-dependent events</u>

Zinc enhances greatly the uptake of calcium into human red cells through the normal influx pathway [Plishker 1984]. It was postulated that zinc acts via membrane sulphydryl-groups which are involved in cation permeability.

Zinc affects calmodulin-dependent cellular events [Brewer et al. 1979]. The effect of zinc on calmodulin is biphasic, stimulating it at low concentrations and inhibiting it at higher concentrations. In the presence of calcium, zinc elicits a structural change in calmodulin, consistent with its activation

[Mills and Johnson 1985].

Other calcium-binding proteins affected by zinc are alpha-lactalbumin [Murakami and Berliner 1983] and beta-S100, a protein involved in the regulation of microtubule assembly [Baudier and Gerard 1983]. Calcium and zinc, acting at different sites, induce different structural changes in S100. Binding of calcium exposes two highly reactive sulphydryls normally buried in S100. Zinc increases the affinity of this protein for calcium.

Calcium binds to alpha-lactalbumin and induces a conformational change. Zinc and some other metals bind at a second site and reverse the effects of calcium [Murakami and Berliner 1983].

1.13 OUTLINE OF THESIS

Chapter two describes materials and methods used in the experimental section.

Chapters three to six are concerned with the mechanism by which phorbol esters inhibit expression of MER in B-CLL cells. Three types of studies were performed: (a) identification of changes in structure of MER from B-CLL cells before and after treatment with phorbol ester, (b) the role of specific, high affinity phorbol ester receptors in the effects of phorbol ester on MER and (c) the capacity of various inhibitors and activators of biological processes to interfere with phorbol ester-mediated inhibition of MER. Chapter six describes the effects of various metals on MER.

Chapters seven to nine decribe the effects of the metals on binding of phorbol ester to B-CLL cells and translocation and activation of PKC.

Chapter ten is a general discussion of the results, including new questions posed from these studies.

The appendix contains the cited references and copies of published manuscripts derived from the experimental data.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

Substances other than those described below were obtained from regular commercial sources.

Phorbol esters and related substances

12-O-tetradecanoyl phorbol-13-acetate (TPA), phorbol 12,13 dibutyrate (PDBu) and phorbol 12,13 didecanoate (PDD) were from Sigma, mezerein and 4-alpha-phorbol didecanoate (4-alpha-PDD) from P-L Biochemicals, phorbol from Life Systems Division and 12-O-retinoyl phorbol-13-acetate (RPA) was a gift of Dr. G. Fürstenberger of the DKFZ in Heidelberg, West Germany. All phorbol esters were dissolved at 1-2 mM in dimethylsulphoxide (DMSO, Ajax) and stored at -20°C.

Metallic compounds

Auranofin and chloro-triethylphosphine gold were gifts of S.K. & F. Laboratories, Philadelphia. These lipophilic gold compounds were dissolved in DMSO at 100mM and stored at - 20°C. Water-soluble sodium aurothiomalate (Myocrisin) was purchased from May and Baker Ltd. and stored at 4°C.

Zinc pyrithione, pyrithione acid and the sodium salt of pyrithione were gifts of Dr. M. Whitehouse, Dept. of Pathology, University of Adelaide, South Australia. Zinc pyrithione was dissolved in DMSO and stored at - 20°C in DMSO at a concentration of 0.1M.

Pyrithione acid, sodium pyrithione and all other metal compounds were dissolved in water. Metal compounds were obtained from the following sources: zinc chloride and calcium chloride (Ajax), zinc sulphate (Standard Labs), manganese sulphate (By-Products and Chemicals Pty Ltd), lead nitrate, aluminium potassium sulphate and strontium chloride (BDH), ferric chloride (May and Baker Pty Ltd), cupric acetate, magnesium chloride

(Sigma) and cadmium chloride, nickel chloride and cobaltous chloride (gifts from Dr S. Lincoln Dept. Physical and Inorganic Chemistry, University of Adelaide).

Calcium-mobilizing agents

Calcium ionophores A23187 (Sigma) and ionomycin (Calbiochem) were stored in DMSO, ethanol or methanol at -20°C.

Sodium fluoride was obtained from BDH and used as a fresh aqueous solution.

Fluoroscein-labelled goat $F(ab')_2$ anti-human Ig antibodies specific for delta, mu, gamma, kappa or lambda chains, were obtained from Kallestad.

Chelating agents

Ethylene diamine tetraacetic acid, disodium salt, (EDTA) and ethyleneglycol-bis-(beta-aminoethylether) N,N,N'N'-tetraacetic acid, tetrasodium salt (EGTA), 3,4,5-trimethoxybenzoic acid 8-(dimethylamino)-octyl ester (TMB-8), L-histidine and 1,10 phenanthroline were from Sigma and tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) was from Molecular Probes.

Thiols

Low molecular weight thiols used were dithiothreitol, reduced glutathione, cysteine hydrochloride and penicillamine hydrochloride (Sigma), sodium dithionite and sodium metabisulphite (Ajax), sodium thiosulphate (BDH) and 2-mercaptoethanol (2-ME) (Merck).

Other compounds

Some other compounds used in these studies were potassium chloride, sodium azide and ascorbic acid (Ajax), colchicine, melittin, prostaglandin E_2, cytochalasin B, phenyl- methyl-sulphonyl fluoride (PMSF), quercetin, chlorpromazine, N-tosyl-L-

lysyl-chloromethyl ketone hydrochloride (TLCK), HEPES, diolein, Nonidet P40 (NP40), quinacrine, tetracaine, carbachol, polymyxin B sulphate, 1-oleoyl-2-acetyl glycerol (OAG), L-palmitoyl-carnitine hydrochloride, N^6-2'-O-dibutyryl adenosine 3':5'-cyclic monophosphate, mono-sodium salt (dibutyryl cAMP), N^2-2'-O-dibutyryl guanosine 3':5'-cyclic monophosphate, mono-sodium salt (dibutyryl cGMP) and indomethacin (Sigma), trypsin (Calbiochem), 1-(5-isoquinoline sulphonyl)-2-methyl piperazine dihydrochlorate (H-7) (Seikagaku Inc.), retinoic acid, trifluoperazine, bromophenacyl bromide and 1,2 dioctanoylglycerol (DOG) (gifts from Prof. A.W. Murray, School of Biological Sciences, Flinders University of South Australia), oxothomethoxyxanthone carboxylic acid, sodium salt (gift of Dr. C. Curtain, CSIRO Division of Chemical Technology, Victoria) and 2-deoxyglucose (gift of the late Dr. W. Korytnyk, Roswell Park Memorial Institute, Buffalo, NY).

2.2 CULTURE MEDIUM

Cells were usually washed and stored for short periods in Dulbecco's phosphate-buffered saline at pH 7.4 (PBS).

In most experiments, cells (10^7/ml) were treated with reagents in RPMI 1640 tissue culture medium (Flow Labs), buffered with Hepes (Sigma) and supplemented with glutamine, sodium bicarbonate, gentamycin (80μg/ml DB labs) and 10% foetal calf serum (FCS, Flow Labs). FCS was heat-inactivated for 30 minutes at 56°C. This complete culture medium is referred to here as RPMI/FCS. For long cultures, cells were in an humidified atmosphere containing 5% CO_2.

In some experiments, cells were treated with reagents in serum-free media, RPMI or PBS, supplemented with 1mg/ml bovine serum albumin (Sigma), referred to as PBS/ALB.

2.3 CELLS

B-CLL cells

Cells were prepared mostly from leukapheresis isolates of patients diagnosed as having chronic lymphocytic leukaemia by specialists of the Haematology Departments of The Queen Elizabeth and Royal Adelaide Hospitals. Some experiments were repeated with cells obtained from peripheral blood.

Cells were used fresh or, as in most cases, after cryopreservation. Cells, in medium containing 50% FCS, were slowly mixed with an equal volume of 20% DMSO in PBS on ice. Two ml aliquots of cell suspensions were put into Nunc cryotubes and frozen at a rate of about $1^{o}C$ a minute using a programmed freezer. Frozen cells were stored in liquid nitrogen ($-160^{o}C$).

Before use, cells were thawed and diluted slowly with PBS. Cells were washed twice with PBS and centrifuged on Ficoll-Hypaque (density 1.077, as described in [Böyum 1968]) to remove dead cells and contaminating PMN and red cells. Cell viability was decreased little by this freezing method.

Normal T cells

Normal T cells were prepared by sheep erythrocyte rosette sedimentation [Forbes and Zalewski 1976]. Normal peripheral blood lymphocytes ($5x10^{7}$/ml) were mixed with an equal volume of FCS and three volumes of a 2% suspension of neuraminidase-treated sheep red cells. After centrifugation, cell pellet was incubated at $4^{o}C$ for 60 minutes, gently resuspended and cells centrifuged on a gradient of Ficoll-Hypaque for 30 minutes at 400g. Resultant cell pellet was treated with distilled water for 10 seconds to lyse erythrocytes. The remaining cells were approximately 90-95% T cells as determined by rosetting with sheep erythrocytes.

Polymorphonuclear leukocytes (PMN)

Human PMN were prepared from venous blood collected in one fifth volume of 4.5% EDTA pH 7.4 and dextran sedimentation followed by fractionation on a Percoll gradient as described in [McColl et al. 1986].

Platelets

Human platelets were prepared from venous blood collected in one seventh volume of acid-citrate-dextrose (pH 4.5). Blood was centrifuged at 200g for 13 minutes at room temperature and the platelet-rich supernatant retained. Platelets were pelleted by centrifugation at 1000g for 17 minutes and then washed by centrifugation in the following buffer:15mM Tris-HCl, 140mM NaCl pH 7.2 containing 5.5mM glucose and 0.35% BSA.

B cell lines

MER-ve Namalva cells and MER+ve Raji, RPMI 8866 and Mann cell lines were gifts of Dr. H. Zola, Dept. of Clinical Immunology, Flinders Medical Centre, South Australia. Cells were grown in RPMI/FCS.

Sheep red cells

Sheep erythrocytes were obtained in Alsever's solution from Commonwealth Serum Labs. Red cells were washed with PBS and a 5% cell suspension incubated with neuraminidase (Wellcome Labs) for 30 minutes at 37°C. Cells were washed four times with PBS.

Mouse red cells

Mouse blood was obtained from the ophthalmic venous plexus of mice (LACA or C57 Black strains) and collected into saline containing 0.38% citrate. The erythrocytes were washed three times in PBS and 0.1ml of packed cells added to 5 ml of PBS.

2.4 CELL VIABILITY AND COUNTING

Cell viability was monitored by exclusion of trypan blue

(0.3%) dye and by phase contrast microscopy. In some experiments, viability was confirmed by showing no lactate dehydrogenase released into the culture medium. Lactate dehydrogenase was assayed on cell supernatants by staff of the Clinical Biochemistry department of The Queen Elizabeth hospital using a method dependent on automated analysis of NAD-coupled enzymes [Morgenstern et al. 1965].

Cells were counted by phase contrast microscopy (Olympus BH) in an improved Neuerbauer haemacytometer chamber. Platelets and PMN were counted in a Coulter Counter, by staff of the Haematology and Rheumatology departments, respectively.

2.5 PHENOTYPIC STUDIES

Rosetting with mouse erythrocytes

To 250µl of 0.2% mouse red cell suspension was added 100µl FCS and 25µl of lymphocyte suspension ($5x10^6$/ml). These were incubated for 5 minutes at 37°C, centrifuged at 200g for 5 minutes and incubated at 4°C overnight. The cell mixture was gently resuspended, one drop of toluidene blue (0.3%, Difco) was added to stain the lymphocytes and the rosettes were counted in a haemocytometer chamber. Lymphocytes binding three or more red cells were counted as rosette-forming. Between 100 and 200 lymphocytes were counted.

Rosetting with sheep erythrocytes

To 100µl of lymphocytes was added 100µl of FCS and 200µl of 2% red cell suspension. Cells were centrifuged at 200g for 5 minutes and incubated at 4°C for at least 60 minutes. Rosette-forming cells were counted as for mouse red cell rosettes.

Cytoplasmic Immunoglobulin

Cytosmears were made by centrifugation of 250µl of cell

suspension ($2x10^6$/ml in RPMI containing 10% FCS) onto alcohol-washed glass slides using a cytocentrifuge (Shandon). Smears were fixed for 10 minutes in ice-cold acetone. After washing with PBS, a 1/10 dilution of FITC-conjugated $F(ab')_2$ goat anti-human immunoglobulin antisera was added for 45 minutes. Antisera were centrifuged for 15 minutes at 500g to remove aggregates prior to use. Smears were washed three times with PBS and mounted in PBS containing 50% glycerol. Fluorescent cells were counted using a Zeiss fluorescence microscope.

Surface HLA-DR expression

Cells ($5x10^6$/ml) in PBS were incubated with anti-HLA-DR monoclonal antibody (FMC-14) (a kind gift of Dr. H. Zola, Flinders Medical Centre, South Australia). After 30 minutes at 4^oC, cells were washed and incubated with FITC-labelled goat anti-mouse IgG (Cappel) for a further 30 min at 4^oC, then washed and kept on ice prior to FACS analysis (Becton-Dickinson Type IV).

Giemsa staining

Cytosmears were stained with giemsa by the Haematology Department, The Queen Elizabeth Hospital.

C-mu mRNA

C-mu probe was obtained from P. Leder, Harvard; probe vector was plasmid PBr322 grown in E. Coli; probes in plasmid were labelled with ^{32}P-dCTP (Bresa, 1600 Ci/mmol) by nick translation, as in [Rigby et al. 1977].

Cells were washed after treatment with test reagents and pellets (from $5x10^6$ cells) were resuspended in 45µl of cold 10mM TRIS-HCL pH 7.0 containing 1mM EDTA. Five µl of 5% NP40 was added, the suspensions vortexed and allowed to incubate on ice for 5 minutes. A further 5µl of 5% NP40 was added, the

suspension vortexed and centrifuged at 15,000g. To 50μl of supernatant was added 50μl of (3:2) 20x sodium chloride (175gm/litre) /sodium citrate (88 gm/litre), pH 7.0 buffer and formaldehyde. After 15 minutes at 60°C, samples were stored at -70°C. These were applied to nitrocellulose using a slot blot suction manifold (Schleicher and Schuell). Filters were air-dried for 20 minutes at room temperature and baked for 2 hours at 80°C in a vacuum oven.

Hybridization was performed at 42°C in the presence of 50% formamide and filters were washed at room temperature under non-stringent conditions. Autoradiographs were developed after 7 days.

2.6 ASSAYS FOR TRYPSIN-RELEASED MER

Haemagglutination

Haemagglutination was assayed in titertek trays (Linbro). To 25μl of serial two-fold dilutions in PBS were added 25μl of 2% mouse red cell suspension. Plates were incubated for 2 hours at 37°C. Haemagglutination end-point titres were taken as last well not showing button-like settling of red cells. For haemagglutination inhibition assays, 25μl of an aqueous solution of substance to be tested for inhibitory activity was added to the wells before addition of red cells.

Adoptive rosetting

10^5 Namalva MER-ve cells, volume 50μl, were incubated with 200ul of trypsin supernatant or other preparation and 50μl of FCS for 45 minutes at room temperature. Cells were washed once and resuspended in 80μl of FCS. One hundred microlitres of a 2% mouse erythrocyte suspension was added and the cells centrifuged at 800g for 5 minutes. After an incubation at 4°C of at least

120 minutes, rosetting was assayed.

2.7 PARTIAL PURIFICATION OF MER

Trypsin extract

MER was solubilised from 10^8 cells by mild trypsinisation with 20µl trypsin (0.5mg/ml, Calbiochem) for 30 minutes at 37°C, in the presence of 100ul bovine pancreas deoxyribonuclease (1mg/ml, Type 1, Sigma) to prevent clumping, and the reaction terminated by addition of 100µl egg yolk or soybean trypsin inhibitor (1mg/ml Sigma). Cells were removed by centrifugation and supernatants (trypsin extracts) stored at - 20°C.

Folch partitioning

Two ml of trypsin extract was shaken for 5 minutes with 12ml of 2:1 mixture of chloroform (Ajax) and methanol (Merck). After centrifugation (25 minutes at 200g) the upper layer was removed and its volume measured. The lower layer was then extracted with chloroform : methanol : 0.9% NaCl (1 : 10 : 10) using the same volume as for the upper layer. After centrifugation, lower layer was removed, evaporated to dryness and taken up in PBS containing 5% ethanol for haemagglutination assay or stored in chloroform at -20°C. The lower layer (organic solvent phase) is referred to as the Folch extract. The upper layer (aqueous phase) was dialyzed and concentrated by rotary evaporation.

Wheat germ lectin chromatography

Samples were incubated for two hours at room temperature on columns (1.0 x 15 cm) of wheat germ lectin Sepharose (Pharmacia), pre-equilibrated with PBS containing 0.2% azide. Unbound material was eluted with 100 ml of the same buffer. Bound material was eluted with 50 ml of a 0.2M solution of N-acetyl-glucosamine

(Calbiochem) in PBS containing 0.2% azide.

Hot aqueous phenol extraction

Supernatants were mixed with equal volumes of 50 per cent phenol (Faulding) in water. Mixtures were stirred at $4^{O}C$ for 15 minutes and centrifuged at 800g for 20 minutes. The upper aqueous phase was dialyzed against several changes of distilled water at $4^{O}C$.

Precipitation with anti-albumin.

Trypsin supernatant (200µl) was incubated for 60 minutes at $30^{O}C$, then overnight at $4^{O}C$ with varying volumes (300-1000µl) of rabbit anti-human albumin (Pharmacia) or whole rabbit immunoglobulin (Sigma). Precipitates and supernatants were recovered after centrifugation. Precipitates were resuspended in PBS to a volume of 200µl and tested for haemagglutinating activity or analyzed by SDS-PAGE.

Anti-albumin chromatography

Rabbit anti-human albumin, 2ml, was conjugated with 3g cyanogen bromide-activated Sepharose 4B (Pharmacia) according to Pharmacia specifications. One ml of crude trypsin extract or aqueous phase of phenol extract was incubated for 60 minutes at room temperature on the column (150 x 9mm). The column was washed with 30ml of 0.2M Tris-HCl containing 0.5M NaCl pH 8.0 and bound material eluted with 30ml of 0.2M glycine in Tris-HCl containing 0.5M NaCl pH 2.8. Wash and eluate were dialyzed, concentrated to 1ml and tested for haemagglutinating activity.

Preparation of serum albumin

Fresh serum albumin was prepared by the following method (modified from that in Travis et al. 1976]: To 5ml of a 1 in 5 dilution of normal human serum in PBS was added 20ml of a 40%

aqueous ethanol solution. Precipitate was extracted with N-butanol (Ajax) and the aqueous phase was chromatographed on a column (K9/15) of Blue Sepharose CL-6B (Pharmacia). Flow rate was 45 ml/hour. Column was pre-equilibrated with 0.05M Tris-HCl containing 0.1M KCl. One ml fractions were collected. Beginning at fraction 38, bound material was eluted with 0.05M Tris-HCl containing 1.5M KCl. Fractions were assayed for absorbance at 280nm.

2.8 PROTEIN ANALYSIS

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in a vertical slab gel unit (LKB) using the discontinuous method of King and Laemmli [1971]. 2-ME was used as reducing agent unless otherwise indicated. Separating gel was 10% acrylamide and stacking gel 3% acrylamide. Markers for detrmination of molecular weight were phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000) and alpha-lactalbumin (14,400).

After electrophoretic separation, gels were dried on Whatman paper using a gel-drier (LKB). Gels were autoradiographed at -70°C using Kodak X-OMAT AR film.

Protein estimation

Protein concentrations were determined by the method of Bradford [1976]. Fifty microlitres of sample was added to 1 ml of Bradford reagent (0.01% Coomassie Blue G250, 4.7% ethanol and 8.5% phosphoric acid). After 30 minutes, absorbance at 620nm was determined using a Varian Series 634 spectrophotometer.

In some column runs, protein concentration was estimated by absorbance at 280nm, rather than by the Bradford method.

Albumin estimation

Albumin concentrations were determined by radial immuno-diffusion [Ouchterlony and Nilsson 1973] using rabbit anti-human albumin antibody.

Radio-iodination

Proteins were radio-iodinated by the chloramine T method. Five microlitres of carrier-free ^{125}I-sodium iodide was added to 1 ml of protein solution. Reaction was started with 50µl of 10mM chloramine T. After 5 minutes at 0°C, reaction was terminated by addition of 50µl of 20mM sodium metabisulphite and 50µl of 0.2M sodium iodide. Free ^{125}I was removed from protein sample by dialysis.

2.9 PHOSPHOLIPID ANALYSIS

Samples were chromatographed on silica gel 60 aluminium-backed plates (20 x 20cm, Merck). Solvent one was chloroform: methanol: 2.5N ammonium hydroxide (70 : 20 : 3); solvent two was chloroform : methanol : acetone : acetic acid : water : 30 : 10 : 40 : 10 : 5). Lipids were visualized by charring with sulphuric acid or iodine vapour. Amino-containing phospholipids were detected by staining with ninhydrin (Pierce Chemicals) [Skipski et al. 1962]. Lipids were eluted from the gels by soaking in chloroform : methanol (2 : 1). Phosphate content of eluted lipids was determined using the method described in Zilversmit and Davis [1950].

2.10 SUB-CELLULAR FRACTIONATION

Preparation of membranes and cytosol

Cells were resuspended in 25mM Tris-HCl pH 7.4 (containing 1 mM EDTA) and disrupted by ultrasonication (Heat Systems Ultrasonics, 4 x 10 second pulses,) at 0°C. In some experiments, a more complex sonication buffer was used (25 mM Tris-HCl pH 7.5,

a more complex sonication buffer was used (25 mM Tris-HCl pH 7.5, containing 2mM EDTA, 5mM EGTA, 250mM sucrose, 0.01% leupeptin, 2mM PMSF and 50mM 2-ME). Particulate fraction was collected by centrifugation at 30,000g or 100,000g for 60 minutes and supernatant used as cytosol. Particulate fraction was resuspended by sonication in PBS for assay or further extracted with 0.2% Triton X-100 containing 1mM EDTA. Triton-soluble material was separated from insoluble material by centrifugation at 30,000 g for 30 minutes.

Preparation of crude nucleus-cytoskeleton complex

A detergent-insoluble fraction of the cells, containing the nucleus and probably some cytoskeletal material, was prepared by the method used in the laboratory of Dr. J. C. Cambier, slightly modified from their original method [Cambier et al. 1987b]. Cells (up to 5 x 10^7) were resuspended in 1ml of a buffer containing 25mM Tris-HCl pH 8.0, 25mM KCl, 7.5mM $MgCl_2$, 30% sucrose (w/v) and 0.2% NP40. In some experiments, buffers containing 0.1% or 0.5% NP40 were used. Cell lysates were layered on a cushion of 60% sucrose and centrifuged at 2000g for 15 minutes.

The pellet is the detergent-insoluble complex and the supernatant, above the sucrose cushion, is the detergent-soluble fraction, a complex fraction containing both cytosolic proteins and detergent-solubilized membrane proteins and lipids.

DEAE-cellulose anion exchange chromatography

Cytosol was applied to a 1.5 x 25cm DE-52-cellulose anion exchange column, pre-equilibrated with 20mM Tris-HCl pH 7.5 containing 50mM 2-ME, 2mM EDTA and 5mM EGTA. Unbound proteins were washed off the column with 350ml of 20mM Tris-HCl containing 50mM 2-ME, 1mM EGTA and 1mM EDTA. PKC was eluted with a 500ml linear concentration gradient of 0-0.3M NaCl in the washing

buffer. Five ml fractions were collected and assayed for protein, phorbol dibutyrate binding capacity and histone kinase C activity.

2.11 PHORBOL DIBUTYRATE BINDING STUDIES

PDBu binding to whole cells or particulate fractions.

The [20-^{3}H (N)]- phorbol 12,13-dibutyrate (^{3}H-PDBu) used in these studies derived from one of three sources : Dr. P. Borchert (CCRI, Minnesota, USA), New England Nuclear and Amersham. Specific activities ranged from 6.5 to 20Ci/mmol. Stock ^{3}H-PDBu (5-20μM) in ethanol or acetonitrile was stored at -20°C.

Aliquots of ^{3}H-PDBu were diluted in the binding medium, PBS/ALB or RPMI/FCS. ^{3}H-PDBu was added to the cells in the presence of the test reagents in a total volume of 200μl in flat bottomed tissue culture wells (Linbro or Falcon). The sequence of addition of ^{3}H-PDBu and reagents varied in some experiments. In some experiments, cells were incubated with ^{3}H-PDBu for 30 minutes at 37°C to achieve steady state binding before addition of test substances. In other experiments, substances and ^{3}H-PDBu were added simultaneously to the cells or substances were added before ^{3}H-PDBu. The precise details are given in the results. For each concentration of ^{3}H-PDBu, replicate wells containing a 50 times molar excess of unlabelled TPA were set up to determine non-specific binding. Specific binding of ^{3}H-PDBu equals total binding minus binding in the presence of TPA. Most binding assays were done in triplicate.

For Scatchard plots, 12 different concentrations of ^{3}H-PDBu, ranging from 0.5-200nM, were used. The precise concentrations of ^{3}H-PDBu in the wells were determined by applying 20μl of the binding mixture in selected wells to filter papers using a

pipette and determining the concentration of ^{3}H-PDBu in the well from the number of counts and the specific activity of the ^{3}H-PDBu.

At the end of the incubation period (at 37°C), bound and free ^{3}H-PDBu were separated by two cycles of washing of cells with saline onto glass fibre filters using a cell harvester (Titertek). The filters were prewashed with a 250nM solution of TPA in PBS/ALB in order to minimize direct binding of ^{3}H-PDBu to the filter papers. Filter papers were dried and added to scintillation vials containing 2ml of a scintillation cocktail (decribed below).

Analysis of curvilinear Scatchard plots

Curvilinear Scatchard plots were resolved into two plots corresponding to receptor subclasses of different affinity, as follows (method in Kahn et al. 1974): the lower affinity portion of the curve was extended to intersect the Y-axis; the Y values along this line were subtracted from the Y values of the points in the high affinity portion of the curve. The new Y values were then joined by a line to intersect the X-axis. This intercept (Xh) indicates the maximum binding capacity of the higher affinity PDBu receptor subtype in the cells. The slope of the line is the negative reciprocal of the Kd. The maximum binding capacity of the lower affinity receptor subtype (Xl) was derived by subtracting Xh from the intercept of the original binding plot. A second line was drawn from Xl parallel to the low affinity portion of the curve. The slope of this line gives the Kd of the lower affinity receptor subtype.

Binding of PDBu to soluble receptor

Soluble PKC preparations were assayed for binding of ^{3}H-PDBu by one of two methods.

(a) Column method: method of Niedel et al. [1983].

Extracts (50µl) were incubated with 10nM ^{3}H-PDBu in the presence of PS (20µg/ml) and calcium 5mM, for at least 30 minutes at room temperature. Bound and free ^{3}H-PDBu were separated on a 1.8ml AcA44 (LKB) column collecting 4 drop fractions. Bound ^{3}H-PDBu elutes in the first 7 fractions (about 1.2ml). Void volume is determined with Blue dextran 2000 and bed volume with phenol red.

(b) Vacuum filtration: method of Uchida and Filburn [1984].

To 50µl of extract in wells was added 150µl of binding buffer : Tris-HCl 20mM pH 8.0, Hepes 12mM, $MgCl_2$ 2.7mM, $CaCl_2$ 5mM, PS 100µg/ml and bovine serum albumin, 1mg/ml. Binding buffer contained 10nM ^{3}H-PDBu. After 30 minutes at 37°C, bound and free ^{3}H-PDBu were separated by filtration as for whole cells. Binding in presence of PS and calcium but absence of cell extract was negligible.

Liquid scintillation counting

The scintillation fluid consisted of 10g of 2,5-diphenyloxazole and 0.75g of 1,4-di-[2-(5-phenyloxazolyl)]-benzene, both from Koch-Light Labs, in 2.5 litres of toluene (Ajax). In the histone kinase C assays, the scintillation fluid also contained 25% Triton X-100.

In early experiments, counting was done in a Tricarb liquid scintillation counter (Packard) but subsequent assays were counted in a series LS 2800 liquid scintillation counter (Beckman). Efficiencies of counting of ^{3}H of these two machines were about 16% and 60%, respectively.

Metabolism of PDBu

B-CLL cells were incubated with ^{3}H-PDBu (100nM) in RPMI/FCS

for 4 hours at 37°C. Aliquots were then freeze-dried and extracted with methanol and the supernatant was spotted on aluminium-backed silica gel 60 thin layer chromatography sheets (Merck). Plates (2x10 cm) were developed with ethylacetate, cut into 1 cm slices and radioactivity counted after addition of 2ml of scintillation fluid. Control incubations contained ^{3}H-PDBu but no cells.

2.12 PROTEIN PHOSPHORYLATION STUDIES

Whole cells: (method of Garrison [1983]).

CLL cells, 10^8 per ml in Hepes-buffered saline , were incubated for 60 minutes at 37°C with ^{32}P-labelled orthophosphoric acid (3000 Ci/mmol, Amersham) at a final concentration of 0.1mCi/ml. Various agents were then added for the time indicated after which the cells were washed three times with PBS and lysed with 0.5% NP40. One hundred microlitres of extract was run on SDS-PAGE (7.5% gels). Autoradiographs were developed after 72 hours and densitometer tracings made using a Quick Scan densitometer (Helena Labs).

Histone kinase C activity

Method 1 was used in most of the experiments. Method 2 was performed in collaboration with Mr. S. Hardy in the laboratory of Prof. A. Murray, Dept. of Biological Science, Flinders University of South Australia.

Method 1

To 30μl of extract was added 90μl of the following assay buffer: Hepes 20mM pH7.5; $MgCl_2$ 5mM; disodium ATP (Sigma) 5μM; gamma-[^{32}P]-adenosine 5'-triphosphate, tetra (triethylammonium salt) (^{32}P-ATP, Bresa, Adelaide, 1420Ci/mmol), 10^6 cpm per 90μl; $CaCl_2$ 5mM; PS 20μg/ml; histone type 111-S (Sigma) 20μg/ml. After 10 minutes at 37°C, reaction was stopped by addition of 2ml of

ice-cold 10% trichloracetic acid. Precipitate was collected by filtration on glass fibre filters (Whatman) and washed twice with 2ml of trichloroacetic acid. Replicate tubes lacking PS and calcium and containing 1mM EGTA were set up to determine phospholipid- and calcium-independent activity. This was subtracted from total phosphorylation to give PKC activity.

Method 2: (method derived from that of Glass et al. [1978]).

Sample (20µl) was added to 70µl of the following cocktail: 20mM Hepes, pH 7.5, 10mM $MgCl_2$, 200µg/ml histone type IIIS, 200µM ATP (containing ^{32}P-labelled ATP (10mCi/mmmol, 200cpm/pmol), 90µg/ml PS and 300µM calcium chloride. After 10 minutes at 37°C, 50µl was spotted on phosphocellulose papers (P31 Whatman). Papers were immersed in 30% acetic acid, washed once with 30% acetic acid, twice with 15% acetic acid, once with acetone and after drying were placed in scintillation tubes, scintillation fluid added and radioactivity determined. As in method one, controls lacked PS and calcium and contained 1mM EGTA.

2.13 MEASUREMENT OF INTRACELLULAR FREE CALCIUM

These determinations were done in the laboratory of Dr. G. Barritt, Dept of Clinical Biochemistry, Flinders Medical Centre using the method of Hesketh et al. [1983] with modifications.

B-CLL cells (10^8/ml) in RPMI/FCS were incubated with quin-2 acetoxymethylester (Sigma, final concentration 62.5µM) or solvent DMSO (0.25%) for 25 minutes at 37°C and then were diluted to 10^7/ml with RPMI/FCS and incubated a further 40 minutes at 37°C. Cells were washed twice and resuspended to 10^7/ml in the following salt solution : 145mM NaCl, 5mM KCl, 1mM Na_2HPO_4, 0.5mM Mg_2SO_4, 5mM glucose, 1mM $CaCl_2$, 100µM diethyltriamine penta-acetic acid (to chelate heavy metals) [Arslan et al. 1985] and

10mM Hepes, pH 7.4. It was not possible to perform these experiments in RPMI/FCS as in most of the other experiments because the protein interfered with the fluorescence signal. Fluorescence was measured at $37^{o}C$ in an Aminco-Bowman spectrofluorimeter with stirring facilities. Reagents were added directly to the cuvette via a syringe.

Excitation was elicited at 340nm and emission monitored at 490nm. Intracellular free calcium $[Ca^{++}]_i$ was determined from the equation $[Ca^{++}]_i$ = Kd (F-Fmin)/(Fmax-F), where Kd (for calcium binding to quin-2) = 115 nM as calculated by Tsien et al. [1982a], F is the fluorescence of the sample, Fmax is the fluorescence after lysis of the cells with 25µM digitonin and Fmin is fluorescence after subsequent addition of 500µM $MnCl_2$ to quench the calcium-dependent fluorescence. All readings were corrected for autofluorescence of unloaded cells.

2.14 MEASUREMENT OF CELL-ASSOCIATED GOLD AND ZINC

Gold and zinc associated with cells were assayed by staff of the Clinical Biochemistry Department of The Queen Elizabeth Hospital using flame atomic absorption spectroscopy as described in James et al. [1982] for gold and in Cheek et al. [1984] for zinc.

Uptake of ^{65}zinc was determined by the following method. Cells (10^7/ml) were incubated with ^{65}zinc chloride (1.7Ci/g, Amersham) at an isotopic dilution of 1/10. Cells were washed once at $4^{o}C$ with PBS containing 1mM phenanthroline to remove extracellular zinc and, then, three times with PBS containing 100µM unlabelled zinc in place of phenanthroline. The pellet was counted for gamma irradiation in a 1282 Compugamma Universal Gamma Counter (LKB Wallach).

CHAPTER THREE

EFFECTS OF PHORBOL ESTERS ON DIFFERENTIATION OF B-CLL CELLS AND EXPRESSION OF MER

3.1 INTRODUCTION

The aim of the experiments described in the first part of this thesis was to investigate further the action of phorbol esters on B-CLL cells, in particular the binding of phorbol ester to these cells and the early events following this binding which lead to loss of expression of MER.

Most of the experiments were done on cells which had been removed, in large numbers, from patients undergoing leukapheresis as part of their therapy (Fig 3.1) and which had been cryopreserved in the presence of 10% dimethylsulphoxide (DMSO). Since fresh B-CLL cells have most often been used in studies of the activation of B-CLL cells by phorbol esters, it was necessary first to confirm that the cells which had been cryopreserved were still capable of being activated by phorbol ester, particularly since other studies have shown that cryopreservation may interfere with the activation of platelets [Dullemond-Westland et al. 1987] and lymphocytes [Lopez Karpovitch et al. 1980].

TPA has generally been used as a stimulus for induction of differentiation in B-CLL cells. In most of the experiments here, the less potent analogue PDBu was used since it partitions less into cell membranes and is therefore preferred for studies correlating specific binding of phorbol ester to the cells and induction of functional response.

Inhibition of MER was detected by a rosette inhibition assay [Forbes et al. 1981]. The significance of this event is unknown but appears to be a very early event common to activation by a variety of stimuli (see chapter 1.4.3). Study of the mechanism by which phorbol esters cause loss of expression of MER may also throw light on the function of this receptor and the significance

of its down-modulation during activation.

There are some limitations in the use of rosette inhibition as a functional response. It may occur via a number of different mechanisms including changes in the receptors, changes in the components of the membrane or cytoskeleton which are required for cell adhesion, inhibition of metabolism and cell death. Alternative means of detecting the cell membrane MER eg by antibodies or radiolabelled ligand are not yet available. In addition, there is probably no simple relationship between effects on the individual receptors for mouse red cells and the rosette inhibition since rosetting involves co-operation between many of these receptors. An added complexity is that there are two different types of MER in the membrane of the B-CLL cell and these bind different types of ligand on the red cell [Forbes et al. 1982b].

Despite its limitations, the rosette assay is simple, rapid and highly reproducible. One of the two types of MER can be released from cells by trypsinization and tested for ligand binding activity, (a) by agglutination of mouse red cells (but not red cells of other species) and (b) by conversion of certain types of MER-ve cells, such as Namalva B lymphoblastoid cells, into cells capable of rosetting with mouse red cells (adoptive rosetting). It is therefore possible to determine whether rosette inhibition induced by a particular agent is due to destruction of the receptor.

Trypsin-released MER is a complex of phospholipid, protein and carbohydrate of very large size, indicating that it is not soluble. Some of the properties are reminiscent of membrane vesicles shed by cells (see discussion). The phospholipid,

principally PE, forms a binding site for mouse erythrocytes. In contrast, adoptive rosetting requires at least one component other than phospholipid, possibly the associated protein(s) (see chapter 1.3.3).

It is proposed that the natural ligand for MER is a form of albumin (chapter 1.3.4). The putative mouse erythrocyte ligand and a component of serum, both of which inhibit haemagglutination of mouse erythrocytes by trypsin-released MER, have properties of albumin.

The aims of the experiments described in this chapter were (a) to show that PDBu and other phorbol esters induce plasmacytoid differentiation and rapid inhibition of rosetting in the B-CLL cells used in these experiments and (b) to investigate possible changes in the activity and structure of MER as a result of treatment of the isolated receptor or intact B-CLL cells with phorbol ester.

3.2 RESULTS

3.2.1 ACTIVATION OF B-CLL CELLS BY PHORBOL ESTER

Plasmacytoid diffentiation

After overnight culture with TPA or PDBu (100nM), B-CLL cells had a basophilic cytoplasm which was often vacuolated. The cells had a pleiomorphic morphology, many containing a uropod. There was abundant cIg (Fig 3.1) which increased further over the next 72 hours. These changes were not seen when the cells were cultured with the control solvent DMSO (0.1% v/v).

In each of two cases tested, the cells cultured with TPA had cIg of the same heavy chain (mu) and light chain (lambda in one, kappa in the other) as the sIg on the untreated cells. In one of these cases, levels of mRNA specific for the mu chain were investigated, at 24 hours of culture, by hybridization of

cytoplasm with ^{32}P-labelled probe specific for the constant region gene encoding the mu heavy chain. The amount of mu chain-specific mRNA was considerably enhanced in TPA-treated cells (Fig 3.1). No increase in mRNA was seen after 120 minutes of exposure to phorbol ester (not shown).

The effect of TPA on expression of MHC class II antigens was analyzed in two different populations of B-CLL cells, by indirect immunofluorescence with a monoclonal mouse antibody to the framework region of the HLA-DR antigen. The expression of MHC class II antigens was greatly increased in both populations of B-CLL cells after treatment overnight with 100nM TPA. There was no further increase during the next three days.

Inhibition of mouse erythrocyte rosetting

Phorbol esters induced rapid loss of expression of MER (Figs 3.2 and 3.3). More than 90% of rosetting cells were inhibited by TPA and between 40% and 90% were inhibited by PDBu, when the cells from each of nine populations of B-CLL cells were treated with 100nM of the phorbol esters for 60 minutes. Biologically inactive analogues, phorbol and 4-alpha-phorbol didecanoate, did not inhibit even at 10µM. Fifty per cent inhibition of rosetting (EC_{50}) occurred with approximately 1nM TPA and 50nM PDBu (Fig 3.3A). Solvent DMSO alone had no effect on rosetting up to a final concentration of 1%. The concentration of DMSO was nearly always 0.5% or less in the experiments described in this thesis.

Little difference was observed in the relative sensitivity to phorbol ester of cryopreserved and fresh B-CLL cells. In 8 populations of cryopreserved B-CLL cells treated with 100nM TPA, rosetting was inhibited by 95.5% (sem 1.6) and, in 6 populations

of fresh B-CLL cells, it was inhibited by 87.0% (sem 7.8).

TPA and PDBu also inhibited rosetting in normal MER+ve B cells and in two MER+ve B cell lines Mann (Fig 3.3B) and RPMI 8866 (not shown).

At concentrations up to 10uM, TPA and PDBu did not cause any loss in cell viability as determined by phase contrast microscopy and exclusion of trypan blue.

3.2.2 EFFECT OF PHORBOL ESTER ON ISOLATED MER

Phorbol esters may inhibit rosetting by interacting with MER. Therefore, the effect of direct addition of phorbol ester to trypsin supernatants on the haemagglutinating and adoptive rosetting capacities was determined.

B-CLL cells (10^9 in 10ml of PBS) were trypsinized in the presence of deoxyribonuclease for 30 minutes at 37°C, the supernatant (after centrifugation at 1000g) was collected, trypsin inhibitor was added, and the supernatant was centrifuged at 20,000g for 60 minutes at 4°C. The pellet, containing MER activity, was resuspended in PBS. To half of the suspension was added 100nM TPA and to the other half was added control solvent DMSO (0.1% v/v). After 30 minutes at 37°C, the preparations of MER were washed twice at 20,000g, resuspended in PBS and tested for receptor activity.

Addition of 100nM TPA to trypsin-released MER did not affect its haemagglutinating activity or capacity to induce rosetting in Namalva cells (Table 3.1). Similar results were obtained with MER from two different populations of B-CLL cells. This indicates that inhibition of MER is not a result of a direct effect of phorbol esters on the ligand binding site.

3.2.3 ACTIVITY OF MER FROM PHORBOL ESTER-TREATED CELLS

To determine whether, in intact cells, phorbol esters cause destruction of MER or its loss from the membrane, trypsin supernatants from untreated- and phorbol ester-treated cells were compared for receptor activity. Cells were pre-treated with 100nM TPA or control solvent DMSO (0.1%) for 30 minutes at 37°C, washed five times with cold PBS and trypsinized. Crude trypsin supernatants were assayed for receptor activity.

Haemagglutinating activity

There was no difference between supernatants from control- and phorbol ester-treated cells in their haemagglutination of mouse red cells (Table 3.1). Lipid extracts prepared from these crude trypsin supernatants, by extraction with chloroform/methanol, also had the same haemagglutinating activity (not shown).

This suggests that the phospholipid component and ligand binding site of MER are not lost from the membrane or destroyed during treatment of cells with phorbol ester.

Adoptive rosetting

In contrast, the preparations of MER from phorbol ester-treated cells were inactive in adoptive rosetting assays (Table 3.1). Similar results were obtained in several experiments using cells from 2 different cases of CLL.

Although the cells were washed thoroughly to remove TPA prior to trypsinization it was possible that a small amount of TPA contaminated the trypsin supernatant and was inhibiting adoptive rosetting. However, addition of 100nM TPA to the adoptive rosette assay did not affect the capacity of MER from control-treated B-CLL cells to induce rosetting in Namalva B cells (Table 3.1). The reason why adoptive rosetting is

insensitive to TPA is not known. It may be that the passively-adsorbed receptor is in a different environment than the natural receptor and as a consequence is inaccessible to signals which normally inhibit this receptor.

3.2.4 STRUCTURE OF MER FROM CELLS TREATED WITH PHORBOL ESTER

The capacity to induce rosetting in Namalva cells is dependent upon a component of MER in addition to phospholipid, since phospholipid derived from MER, or pure PE, did not adoptively transfer capacity to rosette. This component may be affected during treatment of the cells with phorbol ester. To further investigate this, studies were done on the protein and lipid components of MER, prepared from untreated- and phorbol ester-treated B-CLL cells.

(a) Crude trypsin supernatants

There was no difference between crude trypsin supernatants from untreated cells and from phorbol ester-treated cells in the profile of lipids (as detected by one dimensional thin layer chromatography) and proteins (as detected by SDS-PAGE) (not shown). Since MER is probably a minor component of the crude trypsin supernatant these studies need to be done on preparations which are enriched for MER.

(b) Enrichment of MER

MER is enriched in the 20,000g pellet of the trypsin supernatant and further enriched (about tenfold by weight of protein) in the eluate of a wheat germ lectin Sepharose column (Fig 3.4A). Phospholipid extracts of this eluate contain the agglutinating activity. The whole eluate but not the phospholipid extracts of it also contain adoptive rosetting activity.

The eluate contained only a small amount of protein. In

order to detect the protein on SDS-PAGE it was necessary to label the eluate with ^{125}I using the chloramine T method. Autoradiography of the SDS-PAGE revealed the presence of major components of 68kDa and 53kDa and a diffuse region between 25kDa and 40kDa in MER from one population of B-CLL cells (Fig 3.5A). The same polypeptides were seen in several different chromatographs. However, MER from another population of B-CLL cells had a slightly different profile consisting of up to five major components, 68, 53, 45 and 30kDa and a diffuse region less than 25kDa (Fig 3.5B).

(c) Profile of polypeptides associated with MER after treatment of cells with TPA

Autoradiographs of preparations of MER from cells treated with 100nM TPA showed much decreased intensity of labelling with iodine compared with those of preparations from control-treated cells (Fig 3.5), suggesting that very little protein is associated with the MER from TPA-treated cells. All of the bands were decreased in intensity but there appeared to be a particular loss of the 68kDa polypeptide. This was seen in preparations of MER from two different populations of TPA-treated cells. In one case, the enriched preparation of MER from TPA-treated cells had a twofold reduction in haemagglutinating activity and, in the other, there was a fourfold reduction suggesting that less MER from TPA-treated cells bound to the wheat germ lectin column.

3.2.5 ALBUMIN IN MER

The significance of these changes in the MER from the TPA-treated cells will only be understood when the proteins associated with MER are identified. Since MER binds to a wheat germ lectin column it may contain a glycoprotein. An attempt was made to purify this component using extraction with hot aqueous

phenol. Glycoproteins usually partition into the aqueous phase [Howe et al. 1972].

Hot aqueous phenol extraction

When crude trypsin extract was extracted with hot aqueous phenol, some of the haemagglutinating activity partitioned into the aqueous phase. This haemagglutinating activity was still associated with lipid since it partitioned completely into the organic phase when it was extracted with chloroform/methanol and, on one-dimensional thin layer chromatography, contained a single component which co-migrated with PE (Rf 0.47 in chloroform/methanol/2.5N ammonium hydroxide of 70 :20 :3).

Since free PE and other phospholipids partition into the phenol phase of a hot aqueous phenol extract [Howe et al. 1972], the PE in the aqueous phase of the phenol extract must be attached to hydrophilic protein. Apparently, this is not a glycoprotein since haemagglutinating activity in the aqueous phase of the phenol extract did not bind to wheat germ lectin Sepharose (Fig 3.4B). On SDS-PAGE, the aqueous phase was greatly enriched for the 68kDa polypeptide (Fig. 3.6 lane B).

The aqueous phase of the phenol extract contained 2.7nmol of phospholipid per microgram of protein (as determined by phosphate analysis). Assuming a molecular weight of the protein of 68,000, the aqueous extract contains about two hundred moles of phospholipid per mole of protein.

SDS-PAGE under non-reducing conditions

The 68kDa protein had an apparent molecular weight of 55,000 on SDS-PAGE under non-reducing conditions (absence of 2-ME in the SDS-PAGE buffer) as shown in Fig 3.6 (lane C), typical of albumin [Owen et al. 1980].

Reactivity of 68kDa protein with anti-albumin

About 2% of the protein in a crude trypsin supernatant and 75% of the protein in the aqueous phase of the phenol extract was albumin by radial immunodiffusion with rabbit anti-human antibody. All of the haemagglutinating activity was removed by passage of the extracts through a column of Sepharose conjugated with rabbit anti-human albumin antibody, but not by passage through a column of unconjugated Sepharose. The 68kDa polypeptide was entirely precipitated by anti-albumin antibody (Fig 3.6D,E).

Binding of haemagglutinating activity to albumin

Haemagglutinating activity in the aqueous phase of a phenol extract also bound to a column of Sepharose conjugated with pure human serum albumin (not shown). It was not possible to elute the haemagglutinating activity by lowering the pH of the elution buffer (to pH 2.5) or by increasing the salt concentration (to 2M NaCl). This supports the hypothesis that a component of MER, presumably the phospholipid, has affinity for albumin.

Non-denatured albumin inhibits the haemagglutination of mouse red cells by trypsin-released MER or by pure PE [Forbes et al. 1984]. The 68kDa protein in the aqueous phase of a phenol extract also inhibited haemagglutination after it was delipidated by extraction with chloroform/methanol (2/1). When added to an equivalent concentration of egg yolk PE, the delipidated 68kDa protein inhibited the capacity of the PE to agglutinate mouse red cells (reciprocal haemagglutination titre decreased from 2^6 to 0).

Effect of 68kDa protein and albumin in adoptive rosetting assays

It appears unlikely that albumin is the component of MER which is required for adoptive rosetting since both the aqueous phase of the phenol extract and various mixtures of serum albumin

(1mg/ml) and PE were inactive in adoptive rosetting assays. These complexes included a simple mixture of albumin and PE, a sonicated mixture of albumin and PE and a complex of albumin and PE formed using PE adsorbed onto the surface of celite to present the phospholipid to albumin with its fatty acids facing outwards [Spector and Hoak 1969].

3.3 DISCUSSION

B-CLL cells (fresh or previously stored in liquid nitrogen) responded to both PDBu and TPA with activation and plasmacytoid differentiation as evidenced by formation of cIgM, increased levels of mu chain-specific mRNA, increased expression of MHC class II antigens and morphological changes. These populations were almost entirely composed of leukaemic B cells and the cIgM that was induced by phorbol ester had the same type of light chain as the sIg of the original cells, indicating that is the B-CLL cells themselves that are responding. These findings are consistent with other reports [Tötterman et al. 1980, 1981a,b, Forbes et al. 1981, 1982a, Cossmann et al. 1984, Gordon et al. 1984].

Although phorbol esters may directly increase transcription of the Ig gene, or the stability of the mRNA for Ig, it is more likely that they induce other gene products which by causing the cells to differentiate turn on the programme for secretory Ig production. No increase in mRNA for mu chain was detectable in B-CLL cells after two hours of culture with TPA. Others have shown that several hours are required for TPA-induced increase in synthesis of Ig although the cells rapidly become committed to producing secretory Ig [Töttermann et al. 1988].

Both PDBu and TPA caused a rapid loss of capacity of B-CLL

cells to bind mouse erythrocytes. Higher concentrations of PDBu were required than of TPA, as is expected from the relative activities of these substances in other biological systems [Blumberg 1980].

Knowledge of the structure of MER is a pre-requisite for understanding the mechanism of its modulation by phorbol esters and the functional significance of this event. MER is a unique type of structure which will bind to and agglutinate mouse red cells through its phospholipid and will insert into carrier cells in an adoptive rosetting assay, apparently by binding to a trypsin-sensitive membrane protein.

There are similarities between the structure and properties of trypsin-released MER and vesicles which are spontaneously shed from some types of cells. Lymphocytes shed membrane vesicles which stimulate erythyroid proliferation in culture [Dainiak and Cohen 1982]. Macrophages release PE and phosphatidyl inositol (presumably in vesicle form). These lipids are then capable of binding to trypsin-sensitive structures on OKT8 T cells and activate them to suppress lymphocyte mitogenesis [Wadee and Rabson 1983]. MHC class I and II antigens, largely complexed with PE, are shed from mouse lymphocytes in vesicular form and are taken up by responder cells in mixed lymphocyte cultures and cytotoxic T cell-mediated reactions [Sachs et al. 1980, Emerson and Cone 1981, 1982]. These vesicles appear to be released by endogenous proteases during cell activation [Black 1980] and may exchange phospholipids with cells in culture [Peterson and Rubin 1969].

Both MER and the shed vesicles contain phospholipid and protein, both are retained by a wheat germ lectin column and eluted by the sugar, N-acetylglucosamine, indicating that they

contain glycoprotein or glycolipid, both are pelleted at 20,000g and both are taken up by other cells, apparently via trypsin-sensitive membrane protein(s). However, it needs to be established by ultrastructural and enzymatic studies that the trypsin-released MER is in the form of membrane vesicles.

Actin is a component of some types of shed vesicles and it is presumed that these vesicles are derived from the microfilament-rich microvilli. An enriched preparation of MER contained a 45kDa polypeptide (fig 3.5), which may be actin, although a preparation of MER from another population of cells appeared to lack a 45kDa polypeptide. It is important to show with specific antibodies whether actin is a component of MER and therefore whether MER is associated with the cytoskeleton of B-CLL cells. An interaction between receptors and cytoskeleton is necessary for some other types of cell adhesion, including that of fibroblasts binding to their substrate [Rees et al. 1977] and T cells binding to sheep erythrocytes [Mookerjee and Jung 1983].

B-cells contain adhesion structures (podosomes), which are rich in actin [Caligaris-Cappio et al. 1986] and may also contain other cytoskeletal proteins vinculin, talin and integrin [Marchisio et al. 1988]. It needs to be determined whether these structures are involved in the binding of mouse red cells. TPA causes these podosomes to become clustered in defined areas of the cell [Caligaris-Cappio et al. 1986].

The 53, 45 and 25 to 30kDa polypeptides in MER may also be related to a complex of 55, 45 and 30 kDa polypeptides that copurify with sIg in mouse lymphocytes [Koch and Haustein 1983]. Another candidate for the 45kDa polypeptide is CD37, an antigen on B-CLL cells which is down-modulated by TPA [Carlsson et al.

1988].

One of the proteins associated with MER is albumin, as indicated by the characteristic shift in apparent molecular weight in the presence and absence of reducing agents and by its reaction with anti-albumin. The albumin co-purified with MER during 20,000g sedimentation, phenol extraction and wheat germ lectin-Sepharose chromatography. Albumin is non-glycosylated [Brown 1977] and is not responsible for the binding of MER to wheat germ lectin-Sepharose.

Since the aqueous phase of a phenol extract of MER appeared to contain only PE and albumin, the albumin is probably directly complexed with the PE. This supports the hypothesis (see chapter 1.3.4) that the endogenous ligand of MER is a form of albumin. MER may function in uptake of albumin. Alternatively, the albumin may become trapped in the cluster of PE and other proteins that form the MER complex.

Although albumin-phospholipid interactions have been described previously, these have involved an interaction between the fatty acids of the lipid and the fatty-acid binding site in albumin [Brown 1977]. The interaction of the non-choline phospholipid with albumin is unique in that it apparently also involves the phosphate and the head group [Forbes et al. 1983].

That a phospholipid can mediate specific ligand binding has been shown in other systems. PS is a high affinity binding site for the vesicular stomatitis virus [Schlegel et al. 1983]. Vesicles of phospholipid also interact with trypsin-sensitive, saturable receptors in some types of cells [Pagano and Takeichi 1977, Wadee and Rabson 1983].

The mechanism by which phorbol esters inhibit expression of MER is not yet clear. There does not seem to be a direct

interaction between phorbol esters and MER at least in cell-free extracts. Presumably, the inhibition is a consequence of an activation event induced in the cell by the phorbol ester. The change appears to be in the protein rather than the lipid since MER from TPA-treated B-CLL cells had undiminished haemagglutinating activity and content of phospholipid but was inactive in an adoptive rosetting assay and there was a marked reduction in the protein components of MER. The most likely hypothesis is that protein in MER is phosphorylated by PKC. Even the albumin may be a target since thiol treatment converts albumin into a substrate for one type of kinase [Chen and Kim 1985]. If albumin is phosphorylated by PKC this would presumably have to occur following internalization of the albumin. The disappearance of albumin from MER of phorbol ester-treated cells is compatible with the hypothesis that the albumin is translocated to the cell interior where it releases fatty acids and other bound substances that are required for later events in the activation.

Some of the results in this section have been published in [Zalewski et al. 1984a, 1984b].

Table 3.1 Effect of TPA on recovery of functional MER from B-CLL cells

PREPARATION*	ACTIVITY OF MER	
	HAG titre	Adoptive Rosetting++ % Namalva cells rosetting (sem)
Buffer alone	0	2.4 (1.1)
Trypsin supernatant		
- from control-treated B-CLL cells	32	30.0 (7.8)
- from TPA-treated B-CLL cells	32	5.5 (1.9)
- from control-treated B-CLL cells + TPA**	32	26.0 (1.4)
- from MER-ve T-CLL cells	0	6.1 (1.5)
Control supernatant	0	4.1 (2.4)

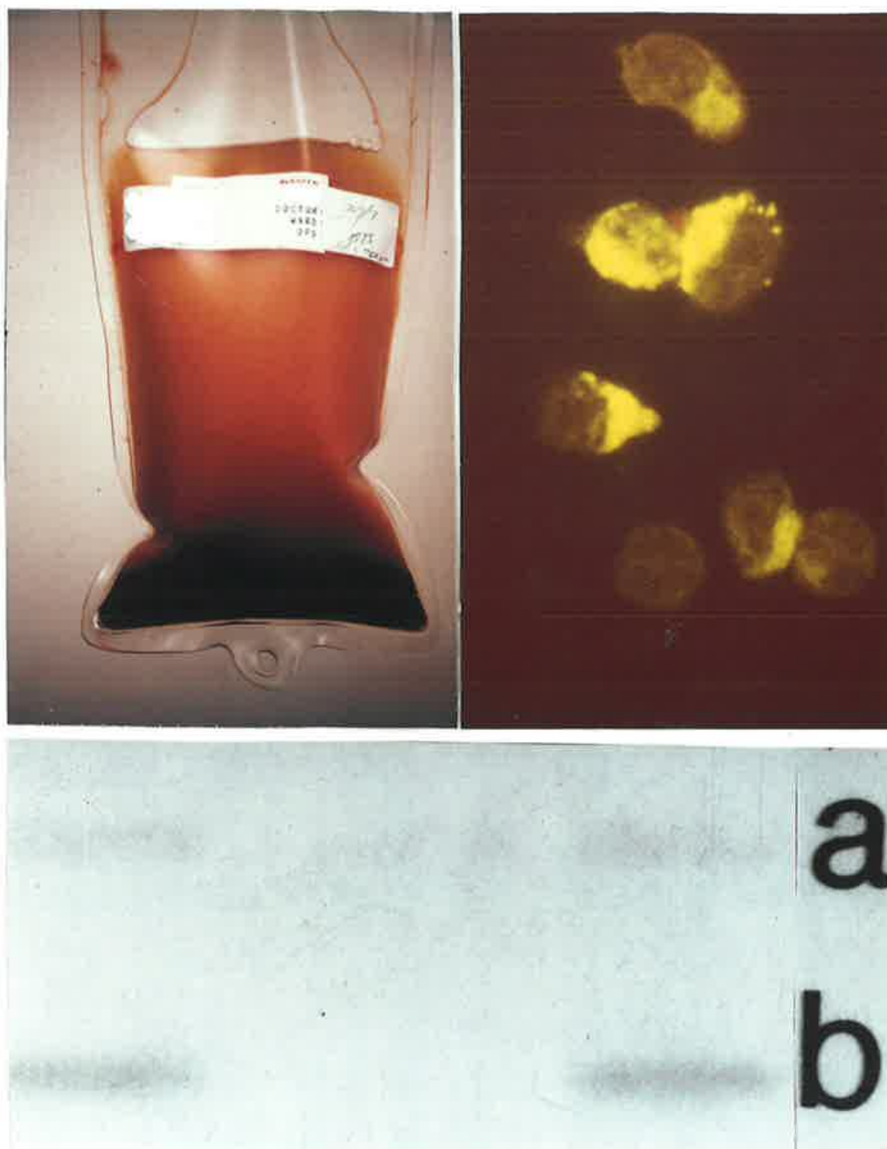
Legend

* B-CLL cells were treated with 100nM TPA or 0.1% DMSO (control) for 30 minutes at 37°C, washed four times and trypsin supernatants prepared as in methods. Control supernatant was prepared from control-treated B-CLL cells in the same way but without addition of trypsin. Trypsin supernatant from MER-ve T cell CLL was also assayed.

** 100nM TPA was added to the trypsin supernatant from control-treated B-CLL cells.

\+ Haemagglutination (HAG) was assayed by incubating serial dilutions of the trypsin supernatant with mouse erythrocytes, for 60 minutes at 37°C. Haemagglutination is expressed as the reciprocal of the dilution of trypsin supernatant which just produces haemagglutination.

++ Adoptive rosetting was assayed by incubating Namalva B cells with trypsin supernatants for 60 minutes at room temperature, washing the cells once, and then rosetting with mouse erythrocytes. Data were pooled from experiments with two populations of B-CLL cells.



Legend to Figure 3.1 Source and activation of B-CLL cells

Top left. Most of the B-CLL cells used in these experiments were obtained by leukapheresis during treatment of patients.

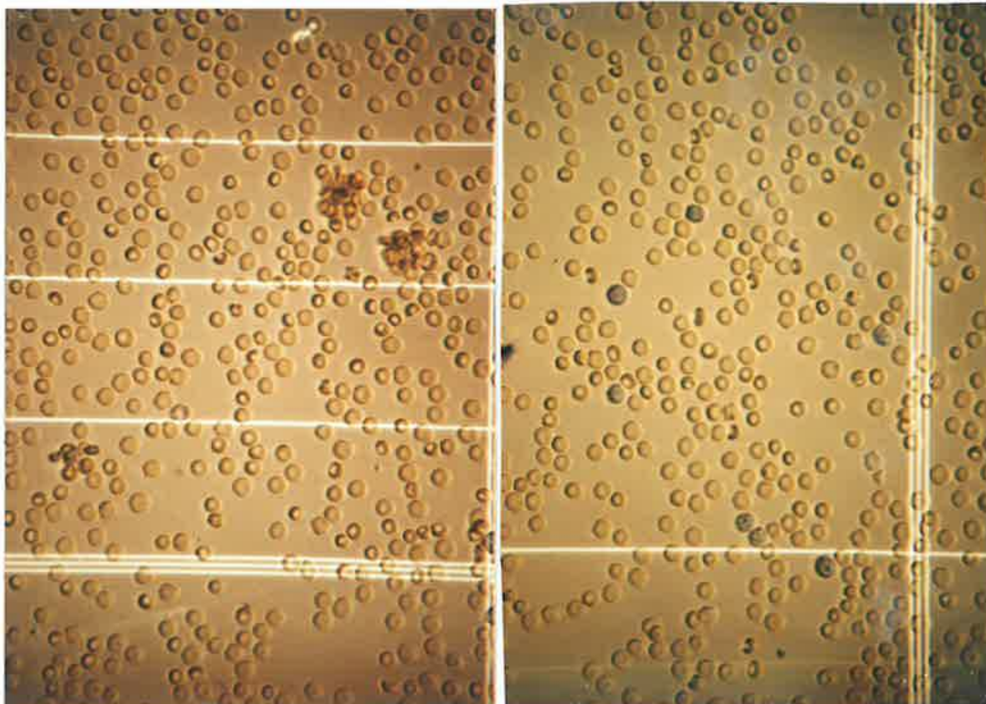
Top right. Immunofluorescence labelling of cytoplasmic mu heavy chain in B-CLL cells which have been cultured overnight with PDBu (200nM)

Bottom.

(a) mu heavy chain specific mRNA in control-treated B-CLL cells (tested in duplicate)

(b) mu heavy chain specific mRNA in B-CLL cells treated with 100nM TPA overnight (tested in duplicate)

Mu chain specific mRNA was assayed by hybridization of cytoplasms with ^{32}P-labelled probe to the constant region of mu chain gene (see chapter 2.5). Autoradiographs were developed after 7 days.

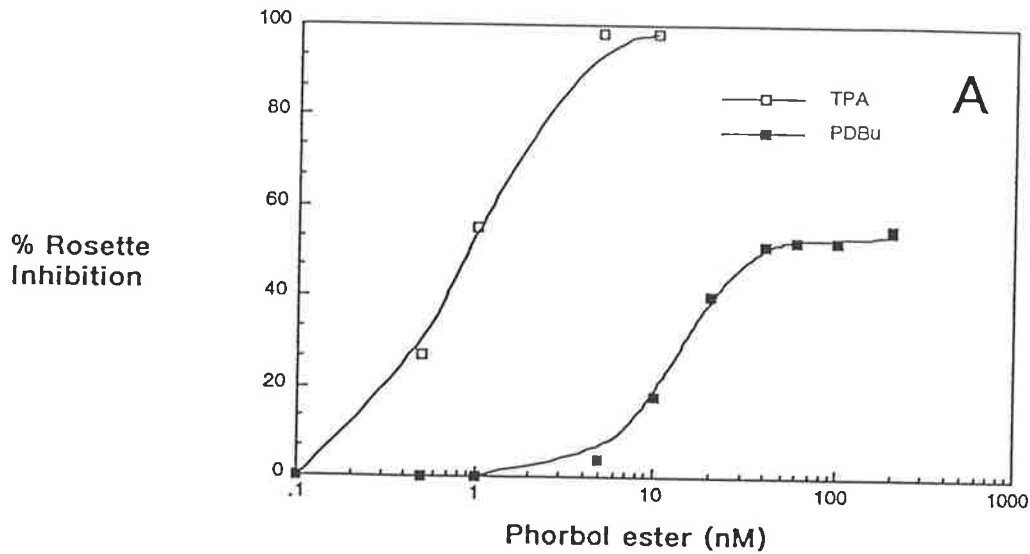


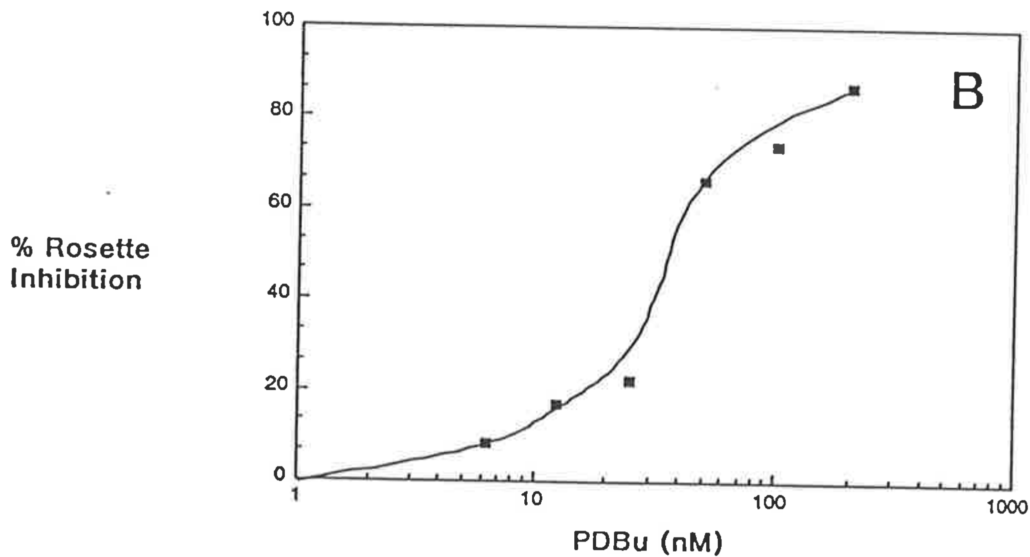
Legend to Figure 3.2 Inhibition of MER by phorbol ester

Left   Rosetting of mouse erythrocytes with control B-CLL cells.

Right   Lack of rosetting of mouse erythrocytes with B-CLL cells which have been treated with PDBu (200nM) for 60 minutes at 37°C.

Cells were stained with 0.3% toluidene blue placed in a haemocytometer chamber and photographed, using an Olympus PM10A photomicrographic system camera.





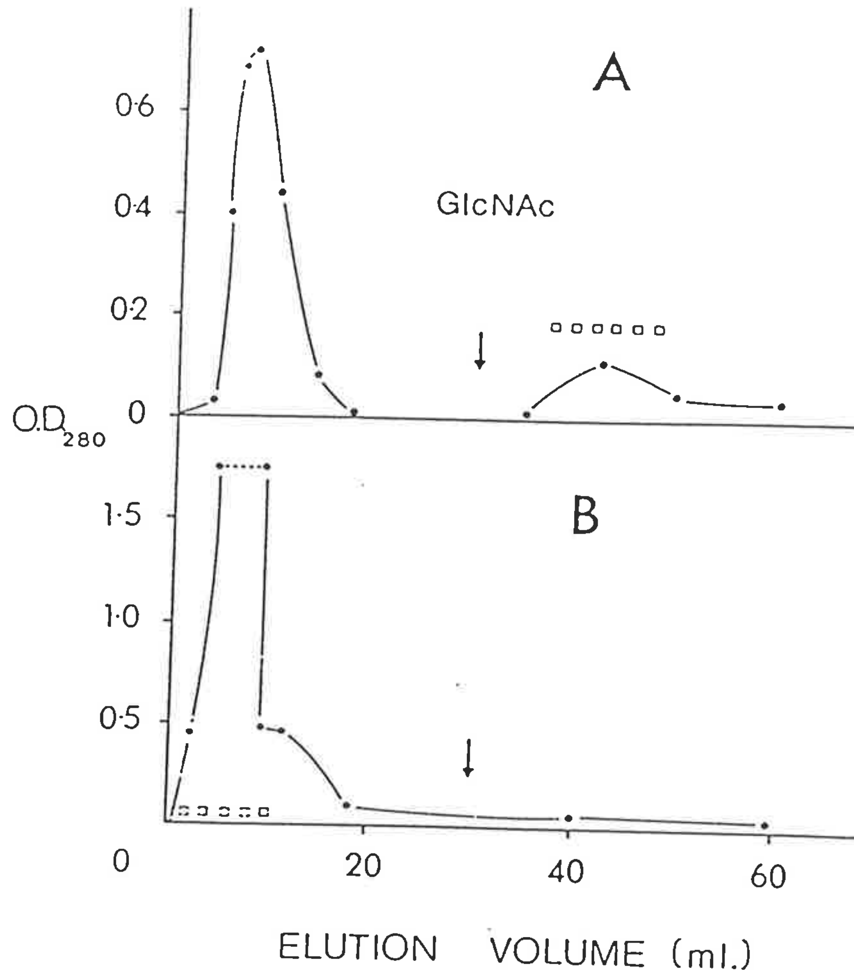
Legend to Figure 3.3 Effect of phorbol ester on rosetting

Figure shows inhibition of rosetting by phorbol esters in B-CLL cells and Mann cells.

Cells were incubated with varying concentrations of TPA or PDBu in RPMI containing 10% FCS. After 60 minutes at 37°C, cells were washed and rosetted with mouse erythrocytes. Percent inhibition of rosetting (mean of duplicates) is shown.

A. B-CLL cells.

B. Mann lymphoblastoid cells.

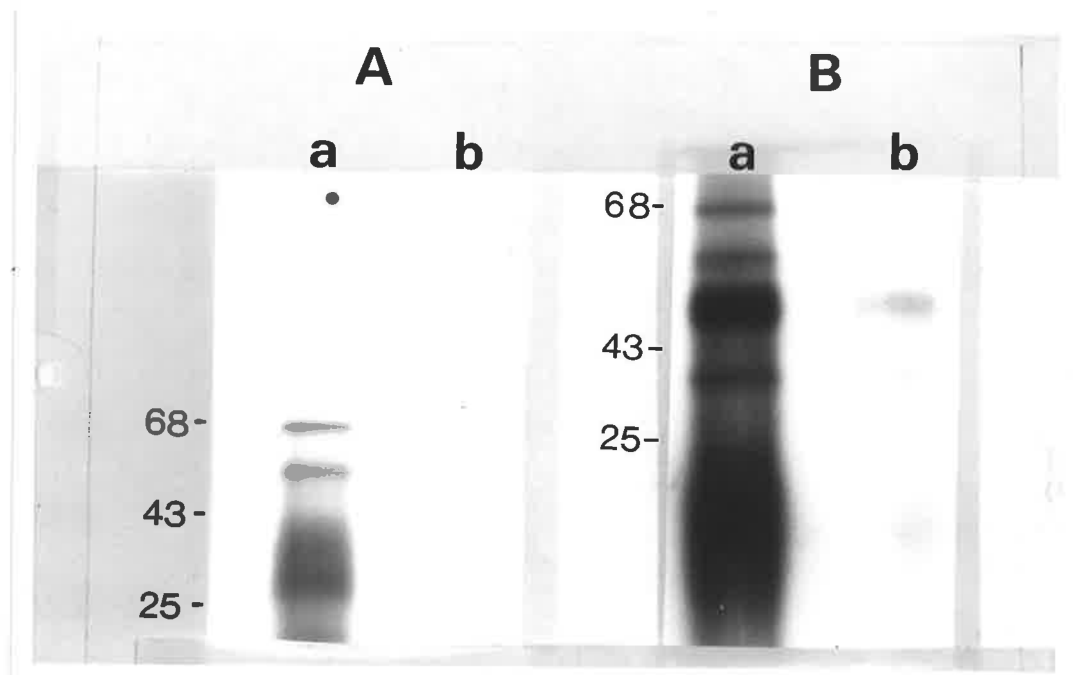


<u>Legend to Figure 3.4</u> <u>Wheat germ lectin chromatography of MER</u>

Figure shows binding of trypsin-released MER to a wheat germ lectin column, as determined by mouse erythrocyte haemagglutinating activity. In contrast, the haemagglutinating activity in the aqueous phase of the phenol extract of this trypsin supernatant did not bind to the column.

Extracts of MER were chromatographed on wheat germ lectin Sepharose. Column wash and eluate (with 0.2M N-acetylglucosamine, GlcNAc) were each 30ml. Arrow indicates addition of sugar. Protein concentration was estimated by absorbance at 280 nm (O). Haemagglutinating activity was assayed on individual fractions without removal of sugar and where present is indicated by square.

A. Trypsin supernatant
B. Aqueous phase of phenol extract of trypsin supernatant



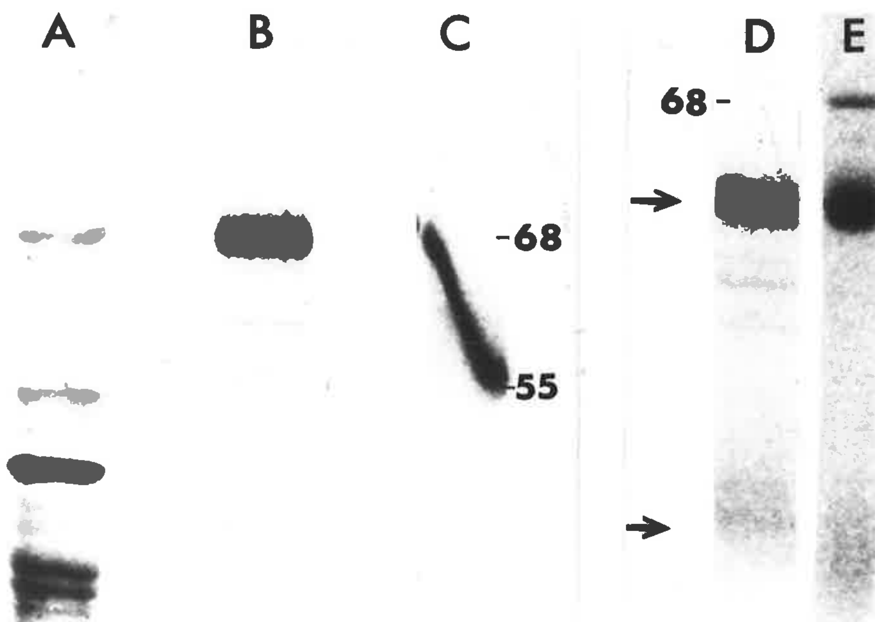
Legend to Figure 3.5 Change in MER from TPA-treated cells

SDS-PAGE autoradiography of ^{125}I-labelled MER prepared by wheat-germ lectin chromatography, showing much decreased intensity of bands in MER from two different populations (A and B) of TPA-treated cells.

(a) MER from B-CLL cells treated with control solvent DMSO (0.1% v/v)

(b) MER from B-CLL cells treated with 100nM TPA.

Cells were treated for 60 minutes, washed and trypsin extracts prepared and chromatographed on wheat germ lectin Sepharose columns (as in Fig 3.4). Eluates were radioiodinated by the chloramine T method and run on SDS-PAGE. Autoradiographs were developed after 3 days.



Legend to Figure 3.6 Identification of albumin in MER

Presence of albumin in MER is shown by shift in apparent molecular weight on SDS-PAGE under reducing and non-reducing conditions and by immunoprecipitation. Proteins were stained with Coomassie Blue.

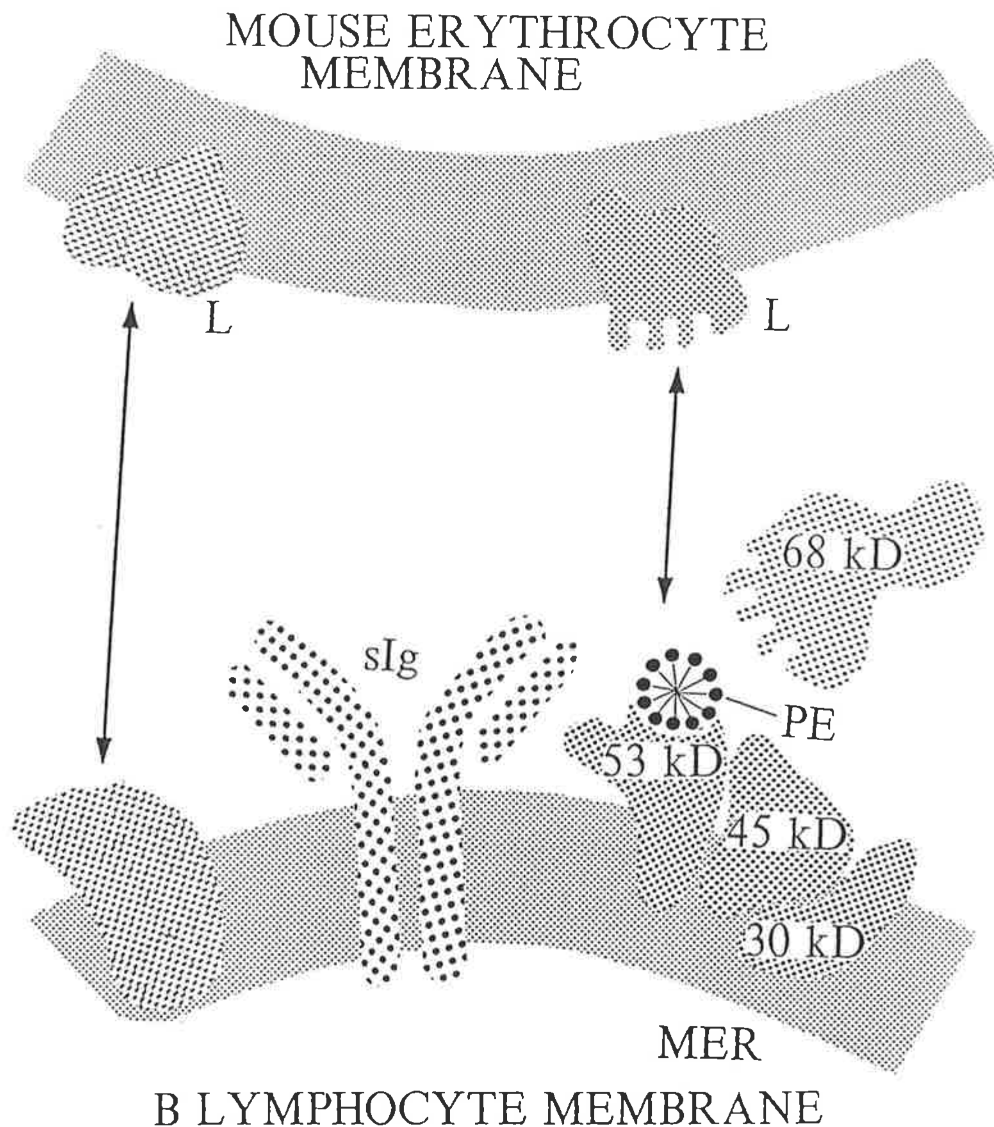
Track A: Crude trypsin extract of B-CLL cells on 10% SDS-PAGE.

Track B: Aqueous phase of a phenol extract of the material in track A on 7.5% SDS-PAGE.

Track C: The same sample as in B but 2-mercaptoethanol was omitted. B and C were run in adjacent lanes. Diffusion of 2-mercaptoethanol from track B has caused the slant of the band in C.

Track D: Supernatant following immunoprecipitation of the sample in B with rabbit anti-human albumin (IgG fraction). Arrows indicate heavy and light chains of Ig. 10% SDS-PAGE.

Track E Pellet after immunoprecipitation. 10% SDS-PAGE.



Legend to Figure 3.7 Model of binding of mouse red cells

Figure depicts a model of the rosetting. Binding of mouse erythrocytes to B-CLL cells results from cooperation between at least two types of receptor on the B cell, binding to two types of ligand on the red cell. One of the receptors, MER is released from B cells by trypsinization. It is a high molecular weight complex of phosphatidylethanolamine (PE), albumin and other proteins which may include polypeptides of 53, 45 and 30kDa, at least one of which is a glycoprotein. The putative natural ligand is plasma albumin. For simplicity, the PE is shown as sitting on one of the proteins although the arrangement of PE in MER and its relationship to membrane phospholipid is unknown.

Two explanations for the effect of phorbol esters on MER are that these agents stimulate phosphorylation of protein within the complex and thereby alter the arrangement of the complex, or that they cause some disturbance of the local membrane environment of MER.

CHAPTER FOUR

HIGH AFFINITY RECEPTORS FOR PHORBOL ESTERS IN B-CLL CELLS

4.1 INTRODUCTION

Specific, high affinity receptors for phorbol esters have been detected in many different types of cells using ^{3}H-PDBu and, to a lesser extent, ^{3}H-TPA (chapter 1.7). While the experiments in this thesis were in progress, binding of phorbol ester to cells from various types of human leukaemia including CLL was reported [Goodwin et al. 1984]. However, no detailled study of the binding of ^{3}H-PDBu to B-CLL cells has been published.

The major receptor for phorbol esters is PKC, in association with phospholipid (chapter 1.8.1). Other types of phorbol ester receptor have not been isolated from cells although a high affinity binding protein for phorbol ester has been isolated from serum (chapter 1.7.3). The relationship between this protein and PKC is unknown. Activation of PKC is an essential step in the second stage of tumour promotion by phorbol esters and related compounds. It is still not clear whether PKC also mediates events required for the first stage or whether another type of phorbol ester receptor is involved.

Phorbol esters and diacylglycerol cause a rapid translocation of PKC from cytosol to particulate fraction (chapter 1.9.2). Most of the translocated PKC is recovered in detergent-soluble extracts of the particulate fraction suggesting that it is in the cell membrane. PKC is translocated to detergent-insoluble compartments in response to other stimuli. Following translocation to the membrane, some of the PKC is lost either because of proteolytic cleavage to PKM or because it is masked in some way. Much remains to be learnt concerning the regulation of binding of phorbol esters to PKC in cells and sub-cellular fractions.

The aim of the experiments described in this chapter was to investigate the binding of ^{3}H-PDBu to B-CLL cells. The structure of PDBu is shown in Fig 1.6; the compound is labelled with tritium in the phorbol ring structure at C-20.

4.2 RESULTS

4.2.1 BINDING OF PDBu TO B-CLL CELLS AND OTHER TYPES OF CELLS

The binding assays (see chapter 2.11 for details) were performed in 96 well tissue culture trays usually for 25 minutes at 37°C. Bound and free ^{3}H-PDBu were separated by vacuum filtration on glass fibre filters using a cell harvester (Fig 4.1). This technique could accommodate large numbers of individual binding assays in a short space of time.

Non-specific binding

Non-specific binding is defined as binding which is not inhibited by a fifty-fold molar excess of unlabelled phorbol ester [Jaken 1986]. For economic reasons, TPA was used for determination of non-specific binding. However, similar results were achieved when a large molar excess of unlabelled PDBu was used instead of TPA. Specific binding of ^{3}H-PDBu is defined as total binding (in absence of TPA) minus non-specific binding (in presence of TPA).

At concentrations of ^{3}H-PDBu in the range 1-50nM, non-specific binding increased linearily (Fig 4.2A) and was about 10% of total binding. At high concentrations of ^{3}H-PDBu (250nM), non-specific binding constituted between 20% and 30% of total binding. In contrast, specific binding was saturable (Fig 4.2B).

If cells were omitted from the binding assay, some apparent binding was still detected. The amount of this binding was almost

identical to that measured in the presence of both cells and unlabelled TPA suggesting that most of the non-specific binding results from binding of ^{3}H-PDBu to something other than the cells. This may represent direct binding of ^{3}H-PDBu to the glass fibre filter papers. In all experiments, filters were pre-wet with a solution of 250nM TPA and 1 mg/ml BSA to minimize their binding of ^{3}H-PDBu.

Kinetics of binding

Specific binding of 10nM ^{3}H-PDBu peaked at between 10 and 25 minutes (Fig 4.3A). For cells in the absence of FCS, there followed a rapid down-regulation of binding; after 60 minutes, binding was 71% of maximum and at 90 minutes it was 56% of maximum. In contrast, in the presence of FCS, peak binding was maintained at least up to 90 minutes (Fig 4.3A). Down-regulation of cellular binding (by about 20%) did occur in the presence of 10% FCS when a much higher concentration (200nM) of ^{3}H-PDBu was used (not shown).

In normal PMN, down-regulation of PDBu binding following maximum binding was marked even for a low concentration of ^{3}H-PDBu (10nM) in the presence of FCS (Fig 4.3B).

Reversibility of binding

The reversibility of binding of ^{3}H-PDBu to B-CLL cells was tested in two ways. Cells were incubated with ^{3}H-PDBu (50nM) for 25 minutes in RPMI/FCS, washed and incubated for further time at 37°C in the same medium lacking ^{3}H-PDBu. Ninety percent of the total bound radioactivity was lost within 10 minutes. Those incubated for a further 10 minutes in the presence of ^{3}H-PDBu had only a small reduction (about 10%) in their binding.

In the second method, the capacity of unlabelled phorbol ester to displace bound ^{3}H-PDBu was tested. More than 90% of

bound ^{3}H-PDBu was displaced within 5 minutes by addition of 1μM PDBu or TPA to cells pre-incubated with ^{3}H-PDBu (50nM) for 25 minutes.

Saturability of binding

Binding of ^{3}H-PDBu to B-CLL cells in the presence or absence of FCS was saturable, reaching a plateau at concentrations greater than 50nM. The total binding was slightly less in the absence of serum (Fig 4.4A).

The amount of specifically bound ^{3}H-PDBu at saturating ligand concentrations varied between different populations of B-CLL cells (Fig 4.5). In particular, cells of J.T. bound two to three times as much ^{3}H-PDBu as cells from the other cases.

More than 85% of the cells in most of the populations of B-CLL cells used in these experiments were leukaemic B cells. Other cells which may be present include T cells, normal B cells and monocytes. The amount of ^{3}H-PDBu bound by B-CLL cells was comparable to that bound by normal mononuclear cells, T cells and atypical MER-ve B type leukaemia cells and exceeded that bound by platelets and PMN (Table 4.1). There was no binding to human red cells.

Receptor numbers and dissociation constants

Linear Scatchard plots were obtained if cells were assayed in the presence of FCS (Fig 4.4B). For six different B-CLL populations, the Kds varied from 18 to 57 nM and the maximum binding capacities from 300 to 750 fmoles per 10^{6} cells (corresponding to approximately 1.7 to 4.5 x 10^{5} binding sites per cell, assuming one binding site per molecule of receptor. Linear Scatchard plots were also obtained for binding of ^{3}H-PDBu to normal human PMN and platelets. Maximum binding capacities of

the cells for PDBu were less than for B-CLL cells although the affinities of the receptors were similar (Table 4.1).

When binding was assayed in the absence of FCS, the Scatchard plots were always curvilinear with upward concavity (Fig 4.6A), suggesting that some component of FCS influences the shape of the plot. Curvilinear Scatchard plots were also obtained when binding of PDBu to normal T cells was assayed in the absence of FCS. Other types of cells were not examined.

Non-linear (concave-upwards) Scatchard plots have been interpreted as showing the presence of receptors with different affinities or receptors exhibiting negative cooperativity. Assuming that the first interpretation is correct and that there are two classes of receptor, B-CLL cells contain between 0.1 and 0.4×10^5 sites per cell of the higher affinity receptor (Kd of 2 to 5nM) and between 0.8 and 3.5×10^5 sites per cell of the lower affinity receptor (Kd of 23 to 55nM) (Table 4.2).

Klotz [1982] has pointed out examples of Scatchard plots in the literature from which the derived apparent numbers of receptors were in error because of insufficient data, particularly at high ligand concentrations. For extrapolations to be reliable, a plot in the form of bound ligand versus $\log_{10}$ [free ligand] should extend beyond the point of inflexion. This criterion is satisfied in the plots from the data reported in Fig 4.6A (see Fig 4.6B) and in other plots reported in this thesis. A broad range of concentrations of ^{3}H-PDBu was always used for experiments requiring Scatchard analysis.

Metabolism of ^{3}H-PDBu by B-CLL cells

In situations where significant metabolism of ligand occurs, the binding data will not truly represent the interaction between the ligand and the cell receptor. To determine whether B-CLL cells metabolise ^{3}H-PDBu, cells were incubated with 100nM ^{3}H-PDBu for 4 hours at 37°C in RPMI/FCS. The cell suspension was extracted with methanol, aliquots run on one dimensional thin layer chromatography and gel slices were assayed for radioactivity (see methods). ^{3}H-PDBu migrated as a single component with an Rf of 0.64.

Following incubation of B-CLL cells with ^{3}H-PDBu, 66% of the radioactivity in the lipid extract co-migrated with ^{3}H-PDBu. If cells were omitted, 78% of the radioactivity co-migrated with ^{3}H-PDBu. In both cases, the remaining radioactivity was at the origin and may represent ^{3}H-PDBu that is complexed with a hydrophilic material. This experiment indicates that little metabolism of ^{3}H-PDBu occurs during the time in which binding of phorbol ester to its receptors and inhibition of MER occur.

4.2.2 PHORBOL DIBUTYRATE RECEPTORS IN SUB-CELLULAR FRACTIONS

Binding of ^{3}H-PDBu to sub-cellular fractions of B-CLL cells was determined after disruption of the cells by ultrasonication. Particulate and cytosol fractions were separated by centrifugation at 30,000g for 60 minutes at 4°C. In some experiments, the particulate fraction was further separated into detergent-insoluble and -soluble material by extraction at 4°C for 30 minutes with 0.2% Triton X-100. In most experiments, 1mM EDTA was present in the sonication buffer so that the particulate fraction will contain only the tightly bound PKC (see chapter 1.9.1).

Particulate fractions

The whole particulate material and the Triton-insoluble residue bound ^{3}H-PDBu, without need for addition of exogenous PS or calcium. Binding was assayed using the filtration assay exactly as for whole cells. About 28% of the binding activity of the particulate fraction remained in the detergent-insoluble residue.

Soluble fractions

Triton-soluble material and cytosol were assayed for binding of ^{3}H-PDBu using either a vacuum filtration assay (Fig 4.7) or a rapid column assay (Fig 4.8) in the presence of PS (20-100ug/ml) and calcium (5mM). The vacuum filtration method is the same as that used for cells or particulate fractions. It is possible to use vacuum filtration to separate free ^{3}H-PDBu from protein-bound ^{3}H-PDBu because the complexes of PKC and phospholipid are of sufficient size to be retained by the filter paper [Uchida and Filburn 1984]. In the column method, bound ^{3}H-PDBu is eluted more quickly than free ^{3}H-PDBu. Binding is quantified by the area under the first peak of radioactivity.

Binding to soluble fractions was almost totally prevented if both phospholipid and calcium were not added. Some binding did occur when only phospholipid was omitted. A similar finding was reported by König et al. [1985] for binding of ^{3}H-PDBu to rat brain cytosol. It was suggested that this was a result of the presence of some endogenous phospholipid in the cytosol. There was also some binding if calcium was not added, perhaps due to endogenous calcium in the preparations. No binding of ^{3}H-PDBu occurred with micelles of Triton X-100 or vesicles of phospholipid in the absence of PKC. In addition, there was no binding (using either assay) with bovine serum albumin,

transferrin, fetuin (type III), phospholipase D or phospholipase A_2 at 1 mg/ml, in the presence or absence of calcium and PS (not shown).

Cytosol bound between 8 and 20 times more ^{3}H-PDBu than did the Triton-soluble membrane fraction. Similar results were obtained using both the filtration and column methods to assay bound ^{3}H-PDBu. In a typical experiment with the column method, cytosol from 10^6 cells bound 17.2 fmol of 10nM ^{3}H-PDBu while the membrane fraction from 10^6 cells bound 1.9 fmol. When calcium-chelating agent (1mM EDTA) was omitted from the fractionation buffer, there was a decrease in the capacity of the cytosol to bind ^{3}H-PDBu and a 90% increase in the capacity of the particulate fraction to bind ^{3}H-PDBu. This represents loose calcium-dependent binding of cytosolic PKC to the particulate fraction. When 5mM calcium was added to the fractionation buffer, hardly any PDBu receptors were detected in the cytosol.

Anion-exchange chromatography

All of the PDBu receptor activity in cytosol of B-CLL cells eluted from DEAE-cellulose in a narrow peak at an approximate salt concentration of 0.15M (Fig 4.9). Most of the histone kinase C activity eluted in the same fraction.

4.3.3 TRANSLOCATION OF PDBu RECEPTORS AND PKC

Translocation by PDBu in intact cells

TPA causes translocation of PKC (as determined by histone kinase C activity) from the cytosol to the particulate fraction of various types of cells. To determine whether PDBu also causes translocation and whether this is accompanied by a redistribution of PDBu binding sites, B-CLL cells were treated with varying concentrations of unlabelled PDBu, for 40 minutes at 37°C, washed

to remove as much of the PDBu as possible and then the isolated cytosol and particulate fractions were assayed both for binding of ^{3}H-PDBu (10nM) and for histone kinase C activity. Removal of the unlabelled PDBu is important so that the receptors can be subsequently identified by a trace amount of labelled PDBu. It is not possible to do this experiment with TPA because this very lipophilic phorbol ester is poorly removed from cells by washing.

There was a concentration-dependent increase in PDBu-binding activity in the particulate fraction and a decrease in the PDBu-binding activity in the cytosol when cells were treated with varying concentrations (0-1μM) of unlabelled PDBu (Fig 4.10A). Pre-treatment of cells with 10nM unlabelled PDBu did not alter the content of PDBu receptors in the two fractions, whereas 100nM PDBu caused a decrease of about 30% of the PDBu-binding capacity in the cytosol. Little extra translocation was seen with 1uM PDBu.

This movement of PDBu receptors to the particulate compartment was accompanied by a translocation of histone kinase C activity (Fig 4.10B). PDBu (200nM) caused a decrease of about 30% of the histone kinase C activity and PDBu-binding activity in the cytosol and a corresponding increase in these activities in the detergent-soluble compartment of the particulate fraction. In some types of cells, it is not possible to measure the histone kinase C activity in cytosol without first partially purifying PKC on a DEAE-cellulose column. It is presumed that there are inhibitors of the kinase in the unfractionated cytosol. It is not clear from these studies whether B-CLL cells also have inhibitor in their cytosol. However, substantial kinase C activity was demonstrable in the crude cytosol of B-CLL cells. In NIH 3T3 fibroblasts, PKC activity can also be directly assayed in the

crude cytosol [Gopalakrishna et al. 1986].

Under similar conditions, pre-treatment of B-CLL cells with 200nM unlabelled PDBu (followed by washing of the cells) increased their capacity to bind 10nM ^{3}H-PDBu by up to 20%. The results of this experiment were interpreted as showing that the translocation of PKC from cytosol to membrane enhances the capacity of the cells to bind phorbol ester.

Translocation in cell-free sonicates

Translocation of PKC from cytosol to particulate fraction could also be demonstrated by direct addition of unlabelled PDBu to a sonicate of B-CLL cells. Treatment of sonicates with PDBu increased the capacity of the particulate fraction to bind 10nM ^{3}H-PDBu. This increase was proportional to the concentration of unlabelled PDBu added to the homogenate (Fig 4.11) and occurred in the presence of both low (0.1mM) and high (1mM) concentrations of calcium. It is not possible to measure the loss of PDBu receptors from the cytosol in these experiments because the presence of a high concentration of unlabelled PDBu in the cytosol interferes with the binding assay.

4.3 DISCUSSION

Binding of ^{3}H-PDBu to B-CLL cells was rapid, reversible, saturable and specific, indicative of a receptor-mediated process. Non-specific binding contributed little to the total binding and appears largely to result from binding of ^{3}H-PDBu to filter papers. There was little metabolism of the ^{3}H-PDBu by B-CLL cells as was also reported for B-CLL cells by Goodwin and colleagues [1984].

The presence of FCS in the binding assay medium affected at least two aspects of the binding. First, there was much less

down-regulation of binding following maximal binding at between 10 and 25 minutes. In the absence of FCS, about 50% of the binding was lost by 60 minutes. Similar kinetics of binding have been reported in some other cell types (see chapter 1.9.3). Inhibition of down-regulation by FCS has not previously been reported. PDBu receptors in PMN appeared to be particularly susceptible to down-regulation, even in the presence of FCS, perhaps because of their high content of proteolytic enzymes. Down-regulation is thought to result from proteolytic cleavage of PKC [Chida et al. 1986] or conversion of PKC to a state which is masked by an unidentified component [Jaken et al. 1983].

Another effect of FCS was on the shape of the Scatchard plot. In the presence of serum, a linear plot consisting of a single class of receptor was detected. The apparent total number of receptors (about 1-2 X 10^5 sites per cell) and their affinity (Kd of about 20 to 60nM) were of the order of those reported for other types of cells (chapter 1.7.2). The above calculations assume that the receptors are distributed homogeneously between the cells and that one receptor binds one molecule of PDBu, as shown by stoichiometric studies [Nishizuka 1984].

When binding was assayed in the absence of FCS, Scatchard plots were curvilinear with upwards concavity. In only a few studies, has this type of binding been reported for phorbol esters [Sando et al. 1982; Dunn and Blumberg 1983] although few studies have examined binding at concentrations of ^{3}H-PDBu between 1 and 5nM, which are required to detect the high affinity component.

Similar plots have been described in some other receptor systems, for example the binding of insulin to adipose cells. Whether this curvilinearity indicates multiple classes of

receptor with different affinities [Kahn et al. 1984] or negative cooperativity within a single class of receptor [de Meyts et al. 1976] is yet to be resolved.

Curvilinear Scatchard plots may also arise because of a technical artifact in the binding assay. A number of assumptions are made in the derivation of the plot [reviewed in Limbird 1986], some of which may not be applicable when the receptors are being assayed in intact cells under steady state conditions or when the receptor is a complex containing two or more different components. Both of these situations apply in the assay of phorbol ester receptors in intact cells.

Technical artifacts can arise if bound and free are determined inaccurately and the relationship between the two is altered by the separation procedure. In chapter 7, it is shown that for cells treated with calcium ionophore there was a linear plot of high affinity receptors, even in the absence of FCS. This suggests that calcium ions may be involved in the heterogeneity of phorbol ester receptors in intact B-CLL cells as has been shown with soluble PKC [König et al. 1985]. How FCS influences this is unclear. Serum proteins which modulate phorbol ester binding to cells have been identified (chapter 1.7).

The affinity of the PDBu receptors in six cases of CLL, studied by Goodwin and colleagues [1984], was at least five times less than that seen in the present study. This may be because, in that study, binding was assayed for 2 hours at 4°C rather than 25 minutes at 37°C. An important component of the binding is translocation of new PDBu receptors to the membrane in response to the ^{3}H-PDBu (see below). Translocation of PKC is optimal at 37°C (chapter 1.9.2).

The major class of receptor for phorbol esters and the endogenous ligand diacylglycerol is PKC in association with anionic phospholipid (chapter 1.8). Formal proof that the PDBu receptors detected in B-CLL cells were all PKC was not attempted here. However, PDBu receptors in cytosol of B-CLL cells behaved identically to PDBu receptors and PKC from other types of cells when chromatographed on DEAE-cellulose. It is still not clear what part of the PKC-phospholipid complex binds phorbol ester and diacylglycerol. Since both lipophilic and hydrophilic moieties on phorbol esters are required, the lipophilic moieties may interact with the phospholipid and the hydrophilic moieties may bind to the protein. Phorbol esters and diacylglycerol would therefore bind at the interface between lipid and PKC.

The relative distribution of PDBu receptors in various compartments of intact cells is still poorly understood because of uncertainties concerning the effects of the cell disruption and divalent cation chelators on this compartmentalization. However, it can be concluded that the bulk of aporeceptors exists in the cytosol.

Induction of translocation of PKC by PDBu in B-CLL cells was associated with an increased capacity of the cells to bind ^{3}H-PDBu. This is expected from studies with soluble PKC which indicate that cytosolic PKC requires phospholipid for binding of ^{3}H-PDBu. In an intact cell, the major source of anionic phospholipid is the inner leaflet of the membrane bilayer.

With TPA as a stimulus, there is almost complete translocation of PKC to the particulate fraction. PDBu also induced translocation of PKC (as determined by both PDBu binding and histone kinase C activities). However, even at concentrations of PDBu (200nM) known to saturate the receptors, only a small

proportion of the aporeceptors and histone kinase C underwent translocation. There was little extra translocation with 1μM PDBu. The amount of translocation of PKC, induced in B-CLL cells by PDBu, could not be determined accurately from these studies since about 30% to 40% of cytosolic PDBu receptors bound PDBu without added phospholipid and therefore may already be complexed to endogenous phospholipid or other material.

Since saturating concentrations of PDBu caused only a portion of the cytosolic aporeceptors to translocate, the apparent total number of PDBu receptors calculated from the Scatchard plots is probably much less than the number of PKC molecules in the cell. Stimuli which cause these extra aporeceptors to translocate will appear to cause an increase in the maximum binding capacity of the cell.

A second implication is that TPA and PDBu differ in their capacity to induce translocation of PKC. PDBu may induce translocation of only some of the PKC isozymes, many of the cytosolic aporeceptors may be inaccessible to PDBu or removal of the unlabelled PDBu during washing of the cells may allow much of the translocated PKC to dissociate from the membrane. It has been claimed that the continuing presence of phorbol ester is required for maintenance of the PKC-phospholipid complex [Wolf et al. 1985a,b] although this has been disputed [Golapakrishna et al. 1986]. Another possibility is that there is a factor limiting translocation of PKC in response to PDBu which is not limiting in TPA-treated cells. This question will be discussed in more detail in later chapters, in the light of effects of zinc and gold on PDBu binding.

Since these studies were done, it has become clear that

there are several different isozymes of PKC and that some may undergo translocation more readily than others [Nishizuka 1988]. Antibodies specific for the isoenzymes were not available for the experiments in this thesis. Many of the conclusions made about PKC in this thesis may not apply to all of the isozymes.

Some of the results in this chapter have been published in [Zalewski et al. 1984b,1987,1988].

Table 4.1 Phorbol ester receptors in different cell types

Cell Types	Number+	PDBu Receptors	
		Kd (nM)	Maximum binding capacities (fmols/10^6 cells)
B-CLL	6	18-57	280-750
PMN	4	16-59	200-280
Platelets	1	53	9
Red cells**	2	-	-

* Cells were prepared as in methods and assayed for specific binding over a wide range of concentrations of ^{3}H-PDBu in RPMI/FCS. Scatchard plots were linear. Binding parameters were derived as discussed in chapter 1.7.

** Dash for red cells indicates no binding.

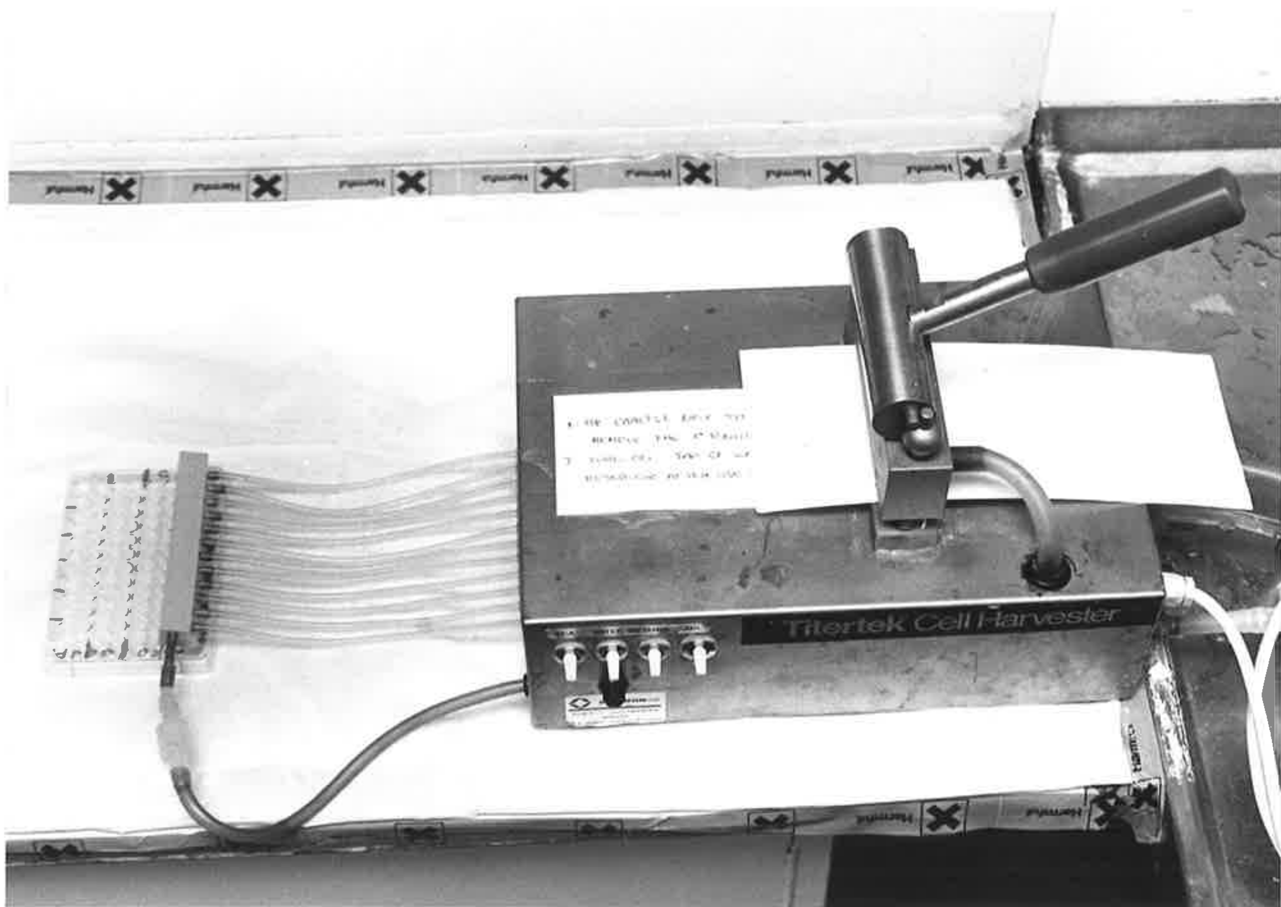
\+ Number indicates number of different samples tested.

Table 4.2 Two classes of PDBu receptors detected in cells in serum-free medium

Cells*	Maximum PDBu binding capacity (fmoles/10^6 cells)	
	High affinity	Low affinity
	Kd 1-8nM	Kd 20-60nM**
V.M.	57	277
J.T.	68	583
P.R.	50	283
P.W.	42	125
J.J.	17	167
B.L.	68	500
Normal T cells	51	150

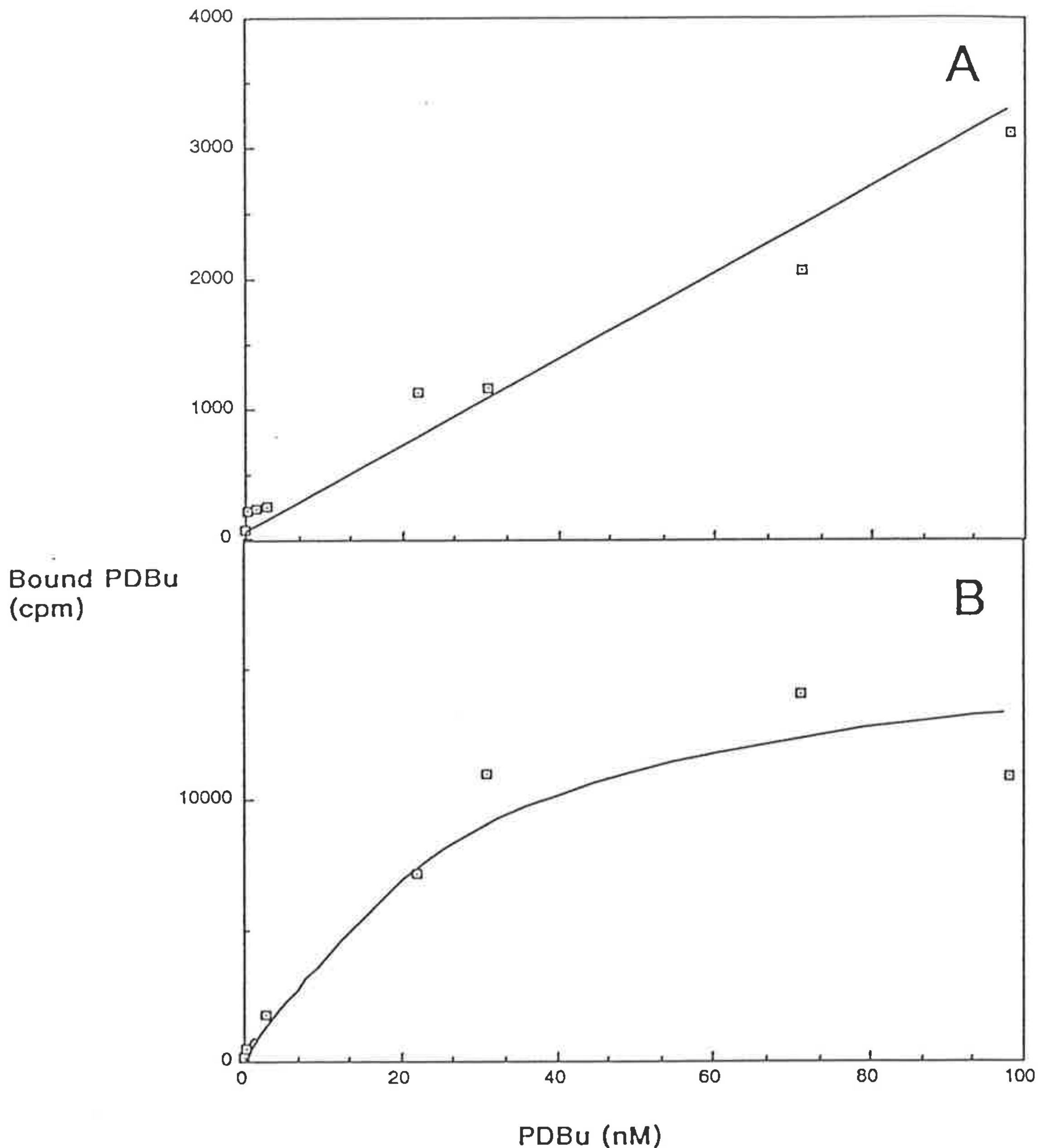
* Cells from various cases of MER+ve B-CLL (V.M., J.T., P.R., P.W., and J.J.), one case of MER-ve atypical B-CLL (B.L.) and normal T cells (prepared by rosetting with sheep erythrocytes) were assayed for PDBu receptors (binding medium was PBS/ALB).

** In all cases, Scatchard plots were curvilinear and were resolved into two classes of receptors with Kds between 1 and 8nM (high affinity class) and between 20 and 60nM (lower affinity class), by the method described in chapter 2.11.



Legend to Figure 4.1 Filtration apparatus for binding studies

Binding assays were done in 96 well microtitre plates and bound and free were separated by vacuum filtration on glass fibre filters, using a cell-harvester (see chapter 2.11).



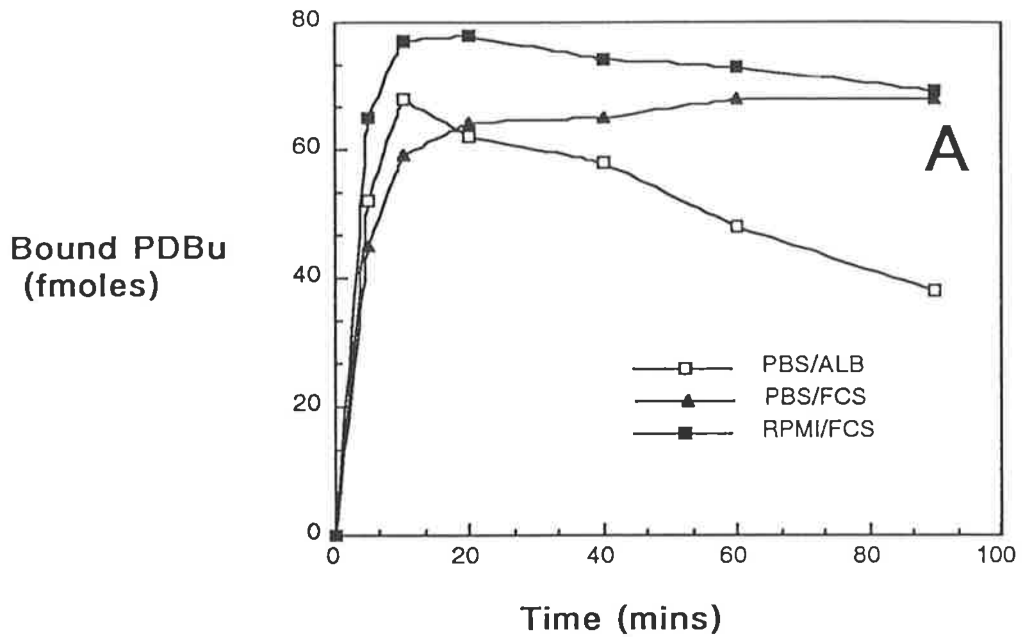
Legend to Figure 4.2 Linearity of non-specific binding plot

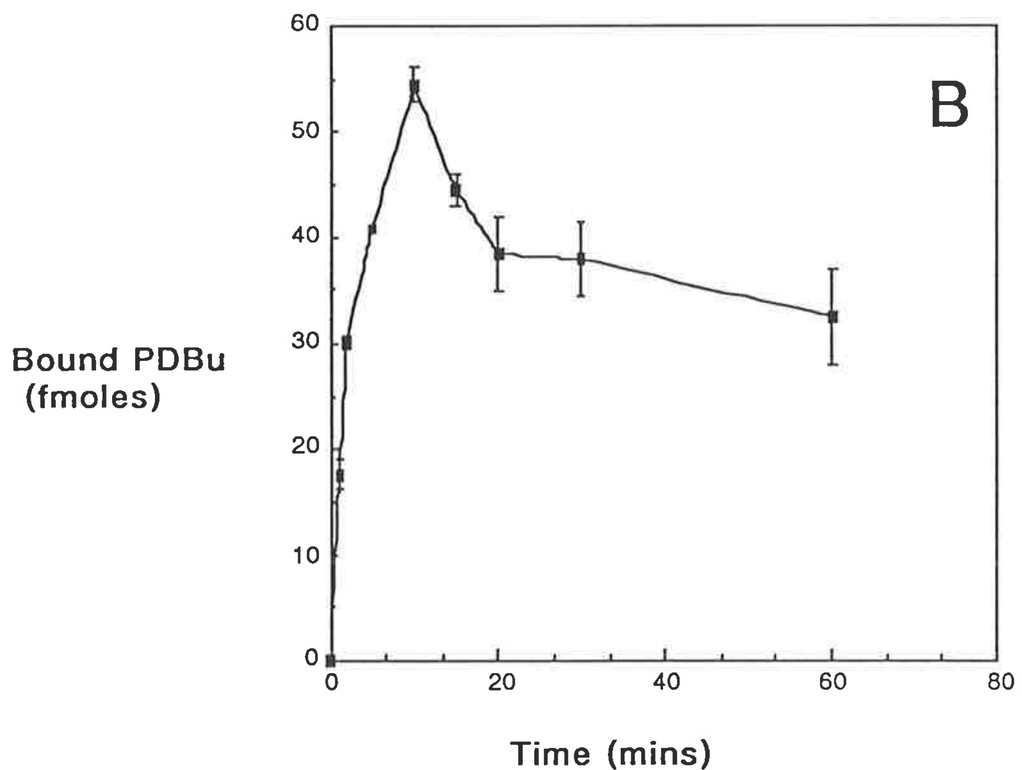
This figure shows that binding in the presence of a fifty-fold molar excess of unlabelled phorbol ester (A) was linear with ^{3}H-PDBu concentration, typical of non-specific binding. In contrast, specific binding (B) was saturable.

A. Non-specific binding (binding in presence of excess unlabelled PDBu.

B. Specific binding (total binding minus non-specific binding)

B-CLL cells were incubated with ^{3}H-PDBu (0-100nM) for 25 minutes at 37°C, in the presence or absence of 50 fold concentration of unlabelled PDBu.



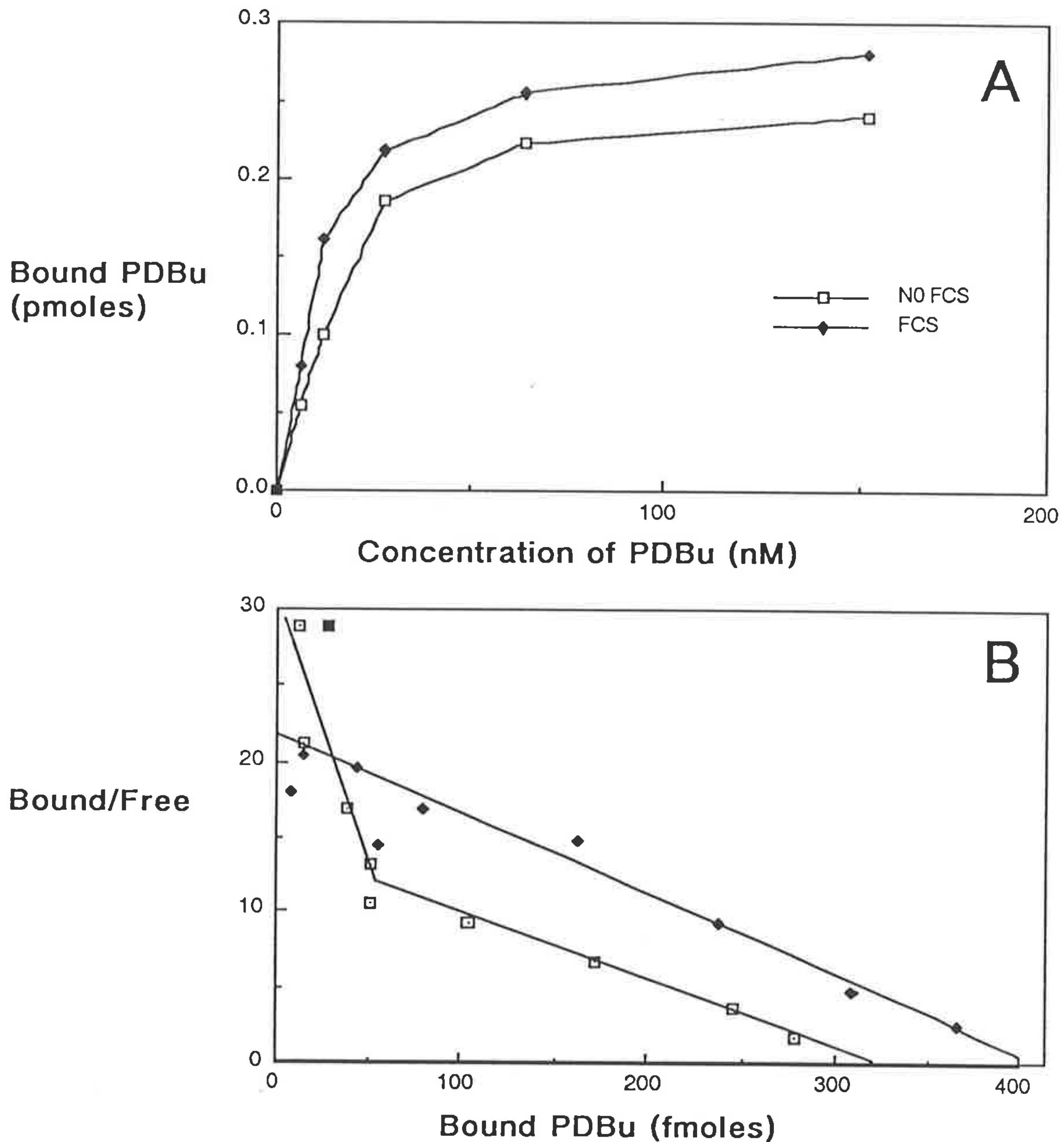


Legend to Figure 4.3 Kinetics of binding of ^{3}H-PDBu to cells

Figure shows that PDBu binding to B-CLL cells (A) and PMN (B) peaked at between 5 and 20 minutes and was followed by down-regulation of binding. This was pronounced for B-CLL cells in the absence (but not the presence) of FCS and in PMN even in the presence of FCS.

A. B-CLL cells were incubated with 10nM ^{3}H-PDBu in PBS containing 1 mg/ml albumin (PBS/ALB), PBS containing 10% FCS (PBS/FCS) or RPMI containing 10% FCS (RPMI/FCS). Specific binding was assayed at various times and is expressed as fmoles bound per 10^{6} cells.

B. PMN were incubated with 10nM ^{3}H-PDBu in RPMI/FCS.



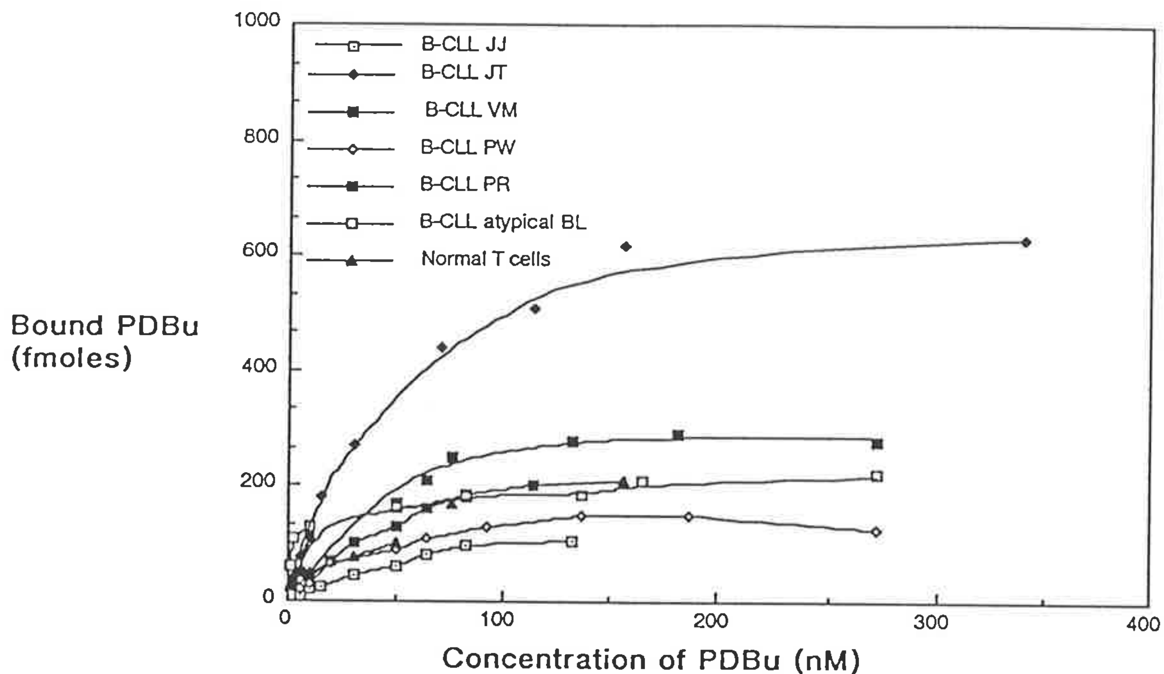
Legend to Figure 4.4 Effect of FCS on binding of PDBu

Figure shows that Scatchard plots of binding of PDBu to B-CLL cells are curvilinear when cells are assayed in the absence of FCS and linear when assayed in the presence of FCS. Binding was slightly increased in the latter. Similar results were seen in cells from other cases of B-CLL and in normal T cells.

A. Saturation plots of binding of PDBu in PBS with and without 10% FCS.

B. Scatchard plots of data in A.

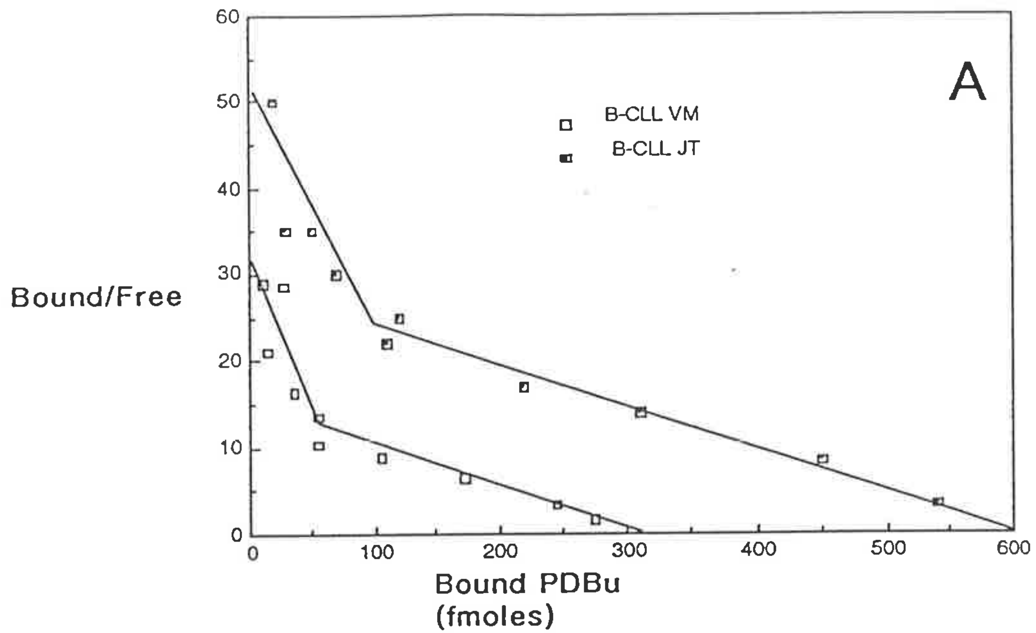
Cells were incubated with a range of concentrations of ^{3}H-PDBu in the two types of medium and specific bound PDBu (at 25 minutes) determined.

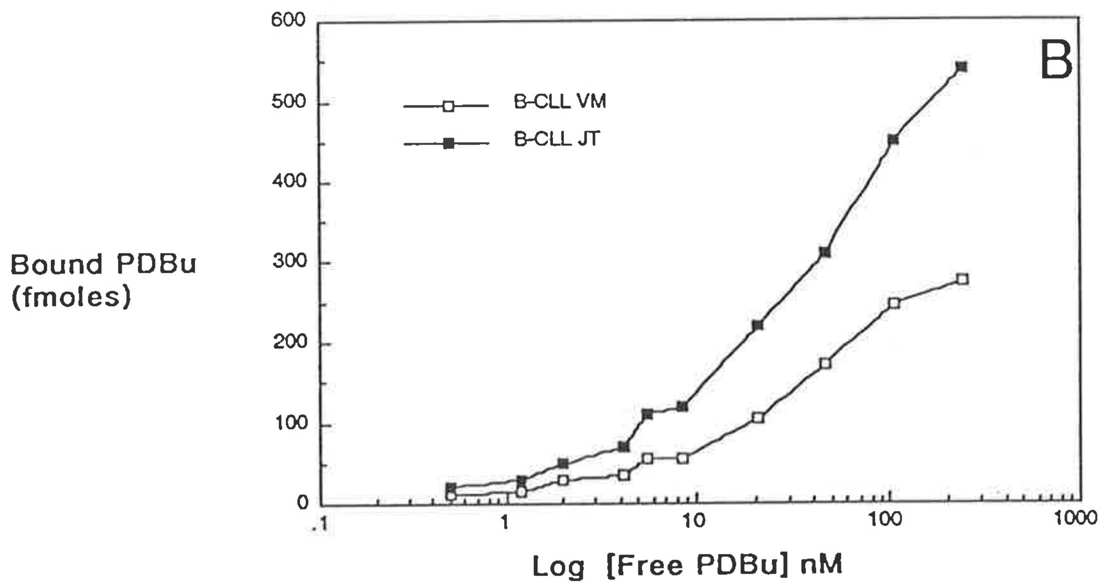


Legend to Figure 4.5 Binding of PDBu to B-CLL cells and T cells

Figure shows that different populations of B-CLL cells have different binding capacities for PDBu, in particular cells of J.T. had about twice the binding capacity of cells of the other cases. Normal T cells had similar binding capacity to most B-CLL cells.

Cells were incubated with varying concentrations of ^{3}H-PDBu for 25 minutes in PBS/ALB before assay of specifically bound ^{3}H-PDBu. Binding data were in triplicate. The standard error did not exceed 10% of means. Binding is expressed as fmol of ^{3}H-PDBu bound per 10^6 cells.





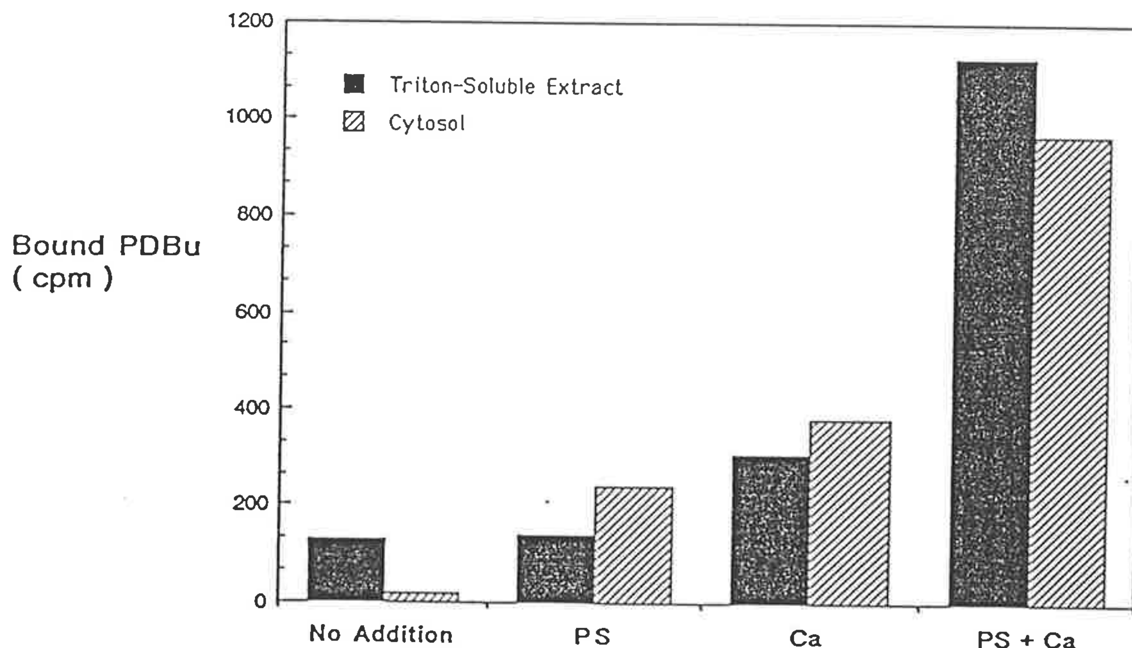
<u>Legend to Figure 4.6</u> <u>Scatchard plots of binding of PDBu to B-CLL cells in serum-free medium</u>

Scatchard plots of binding of PDBu to cells in serum-free medium were always curvilinear (A). Panel B shows binding drawn as Klotz plots.

A. Scatchard plots of binding of PDBu to cells of B-CLL cases JT and VM.

B. Klotz plots of same data.

Binding assays were performed as in Fig 4.4.

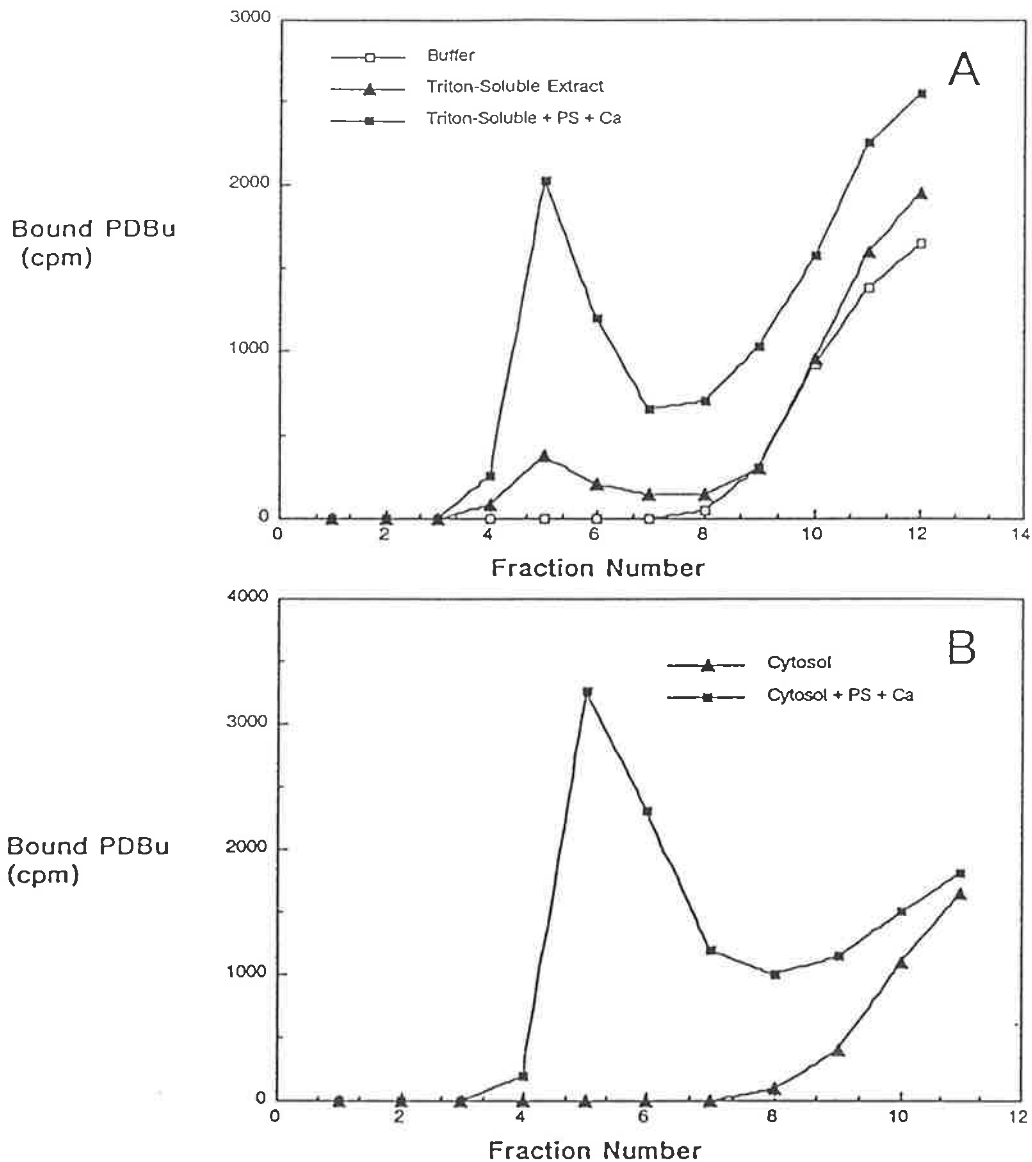


Legend to Figure 4.7 Assay of soluble PKC by filtration

In later experiments, binding of PDBu to soluble fractions was assayed by the vacuum filtration method. Binding was dependent upon addition of phosphatidylserine (PS, 20µg/ml) and calcium (Ca, 5mM) although some binding occurred when only PS or Ca were added.

Soluble extracts (50µl) were incubated with 10nM ^{3}H-PDBu in binding buffer containing the additions indicated. After 30 minutes, bound and free PDBu were separated by vacuum filtration as for cells.

PS + Ca	Extract + 20µg/ml PS + 5mM calcium
PS	Extract + 20µg/ml PS
Ca	Extract + 5mM calcium
-	Extract

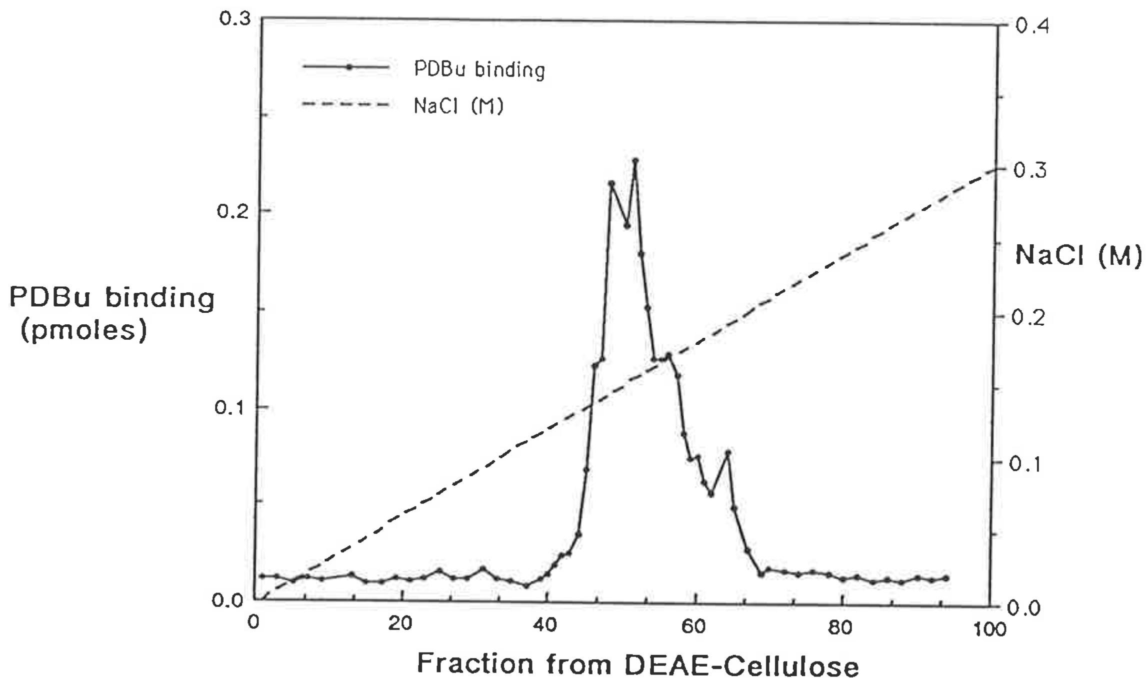


Legend to Figure 4.8 Assay of soluble PKC by column method

Early experiments used the rapid column method to assay binding of ^{3}H-PDBu to PKC in soluble extracts. Binding of PDBu was dependent upon addition of phosphatidylserine (PS, 20µg/ml) and calcium (Ca, 5mM).

A. Triton-soluble extracts of the particulate fraction
B. Cytosol

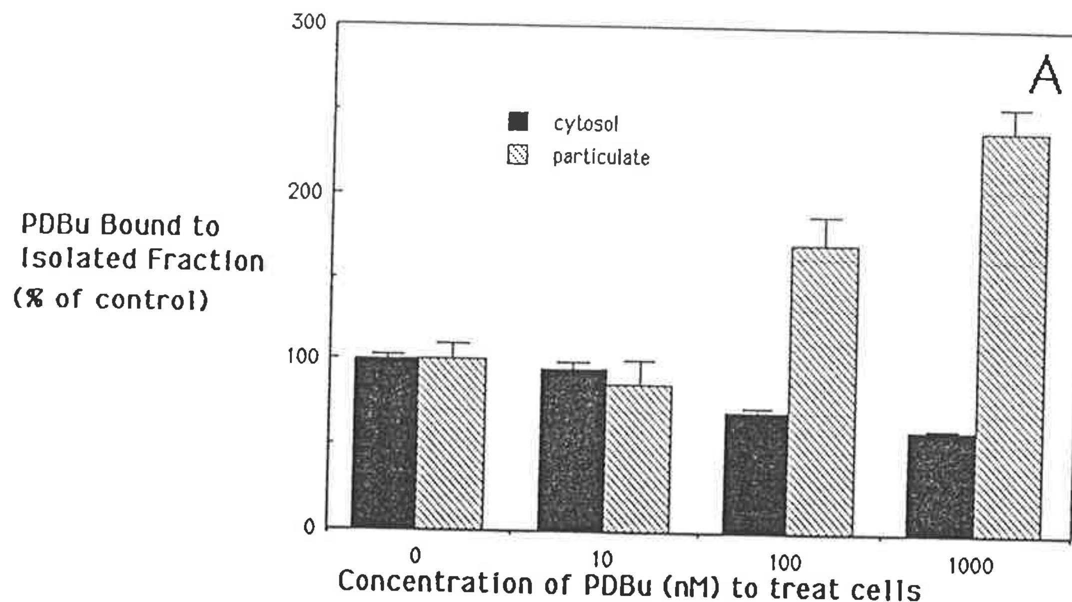
Soluble extracts (50µl) were incubated with 10nM ^{3}H-PDBu in binding buffer containing the additions indicated. After 30 minutes, mixtures were applied to AcA 44 columns (see methods) and 4 drop fractions were collected. Bound ^{3}H-PDBu eluted in the first six or seven fractions (void volume as determined using blue dextran). Free ^{3}H-PDBu eluted slightly later.

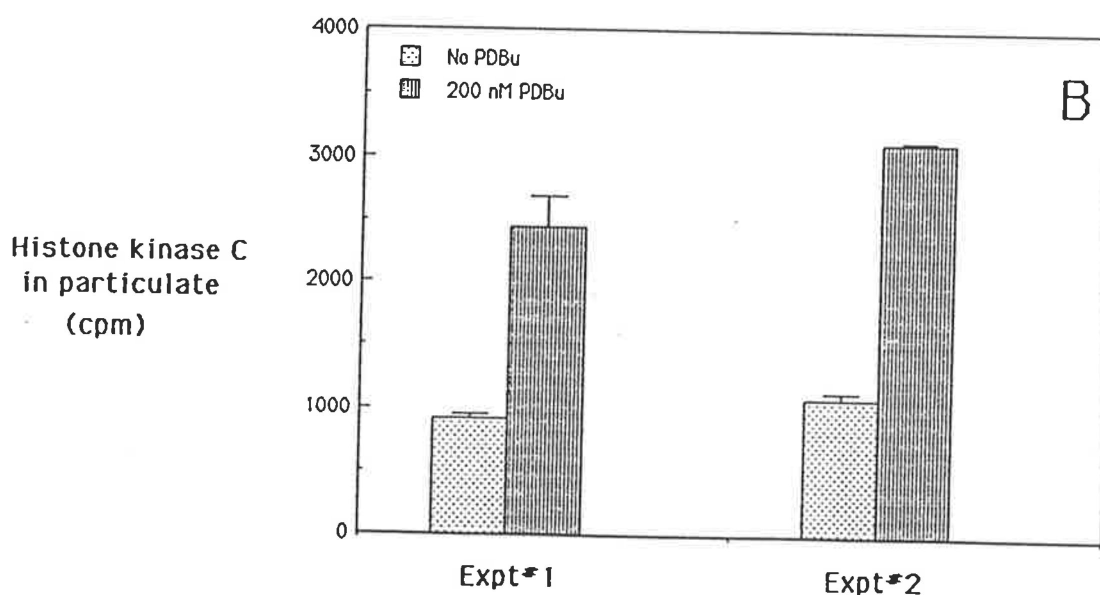


Legend to Figure 4.9 Anion exchange chromatography of receptors

Figure shows that PDBu receptors in cytosol of B-CLL cells eluted from DE-52 cellulose at salt concentrations between 0.1 and 0.15M, similar to PDBu receptors and histone kinase C activity reported for other types of cells.

Cytosol from B-CLL cells was applied to a column of DE-52 cellulose (1.5 X 25cm), pre-equilibrated with 20mM Tris-HCl pH 7.5 containing 50mM 2-ME, 5mM EGTA and 2mM EDTA. Column was washed with 350 ml of equilibration buffer and eluted with 500ml of a linear concentration gradient of NaCl (0-0.3M) in 20mM Tris-HCl pH 7.5 containing 50mM 2-ME, 1mM EGTA and 1mM EDTA. Five ml fractions were collected and assayed for binding of 10nM ^{3}H-PDBu by the vacuum filtration method.



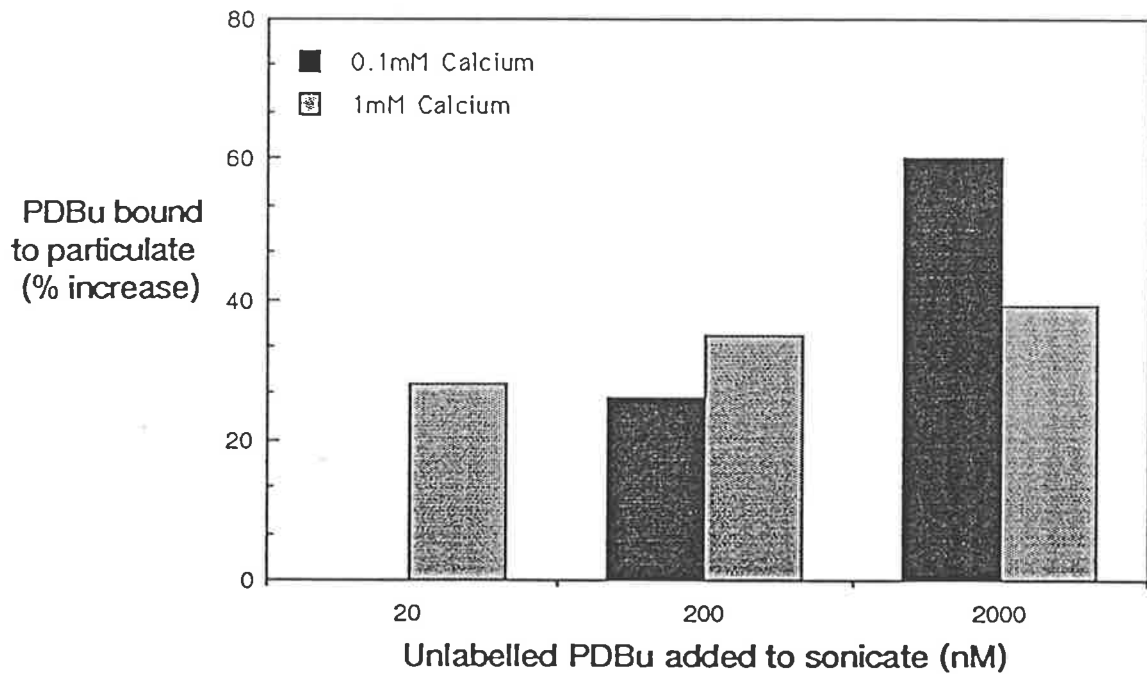


Legend to Figure 4.10 Translocation of PKC by PDBu

Figure shows that at a saturating concentration (200nM), PDBu induces a partial translocation of PDBu receptors and histone kinase C activity from cytosol to the particulate fraction in intact B-CLL cells.

A. Translocation of PDBu receptors from cytosol to particulate fraction in B-CLL cells treated with varying concentrations of unlabelled PDBu for 40 minutes at 37°C. Cells were washed before fractionation. Fractions were assayed for binding of 10nM ^{3}H-PDBu.

B. Translocation of histone kinase C activity from cytosol to detergent-soluble portion of particulate fraction. Two experiments with different populations of B-CLL cells are shown. Cells were treated with or without 200nM PDBu, and fractions were assayed for histone kinase C activity by TCA precipitation method, chapter 2). Activity in the absence of PS and calcium was subtracted from total phosphorylation.



Legend to Figure 4.11 Translocation of PKC in cell-sonicates

Translocation of PKC was induced by PDBu in sonicates of B-CLL cells. In the presence of 0.1mM calcium, translocation was only seen at high concentrations of PDBu whereas in 1mM calcium, a low concentration was sufficient to induce most of the translocation.

B-CLL cells (10^8/ml) were sonicated in Tris-HCl containing 1mM EDTA. To sonicates were added unlabelled PDBu (0, 20, 200, or 2000nM) in the presence of 0.1 or 1mM calcium. Particulate fractions were pelleted, washed with PBS and assayed for binding of 10nM ^{3}H-PDBu.

CHAPTER FIVE

ROLE OF PROTEIN KINASE C IN THE LOSS OF EXPRESSION OF MER

5.1 INTRODUCTION

The role of PKC, and various activation events induced by PKC, in the inhibition of MER are considered in this chapter.

The first experiments explore the role of the specific PDBu receptors in the effects of PDBu on MER. As well as by a receptor-mediated process, inhibition of MER by phorbol ester may result from intercalation of the lipophilic phorbol esters in membrane phospholipid surrounding or within MER. Phorbol esters modulate some properties of phospholipid monolayers and liposomes [Deleers et al. 1981,1982]. Another possibility is that phorbol esters bind to MER or to an adjacent structure and sterically inhibit rosetting. The role of phorbol ester receptors in the inhibition of MER was investigated by using ^{3}H-PDBu to assay binding and to inhibit rosetting under identical conditions.

Physiological activation of PKC in B cells is at least in part mediated through diacylglycerol formed by increased inositol phospholipid turnover in response to triggering of the antigen receptor, sIg (chapter 1.10). Ligands of sIg, including anti-Ig and SAC, activate B cells to divide and differentiate, as well as to undergo loss of expression of MER. This suggests that sIg may be involved in the normal loss of expression of MER that accompanies B cell activation and that phorbol esters usurp this pathway, in vitro. This was investigated further by testing the effects on MER rosetting of fluoride, which induces inositol phospholipid turnover by activating a G protein, and of diacylglycerol, one of the two intracellular messengers formed by cleavage of inositol phospholipid. The effect of diacylglycerols was tested by using exogenous membrane-permeable diacylglycerols 1-O-oleoyl-2-acetyl glycerol (OAG) and 1,2 dioctanoylglycerol (DOG). The role in inhibition of MER of the other intracellular

messenger calcium, which is mobilized in response to inositol phospholipid turnover, is discussed in chapter 6.

Inhibition of rosetting may result from phosphorylation of one of the proteins associated with MER, as occurs in the inhibition of the epidermal growth factor receptor [Downward et al. 1985]. This was examined by correlating induction of protein phosphorylation with inhibition of MER in phorbol ester-treated cells, looking for incorporation of ^{32}P into MER by SDS-PAGE autoradiography of trypsin-released MER and by inhibitor studies. Various substances inhibit PKC-dependent phosphorylation in whole cells or cell-free extracts. Some of these (eg palmitoylcarnitine and trifluoperazine) interfere with the regulatory domain whereas others (eg H-7) block the catalytic site. These inhibitors prevent many actions of phorbol esters in cells (chapter 1.8.5).

Rosetting may be inhibited not because MER is phosphorylated but because the phosphorylation causes a secondary event which in turn is responsible for the inhibition. Following activation of PKC there is a cascade of events during which certain events necessary for cell activation occur (chapter 1.10). The effect of some of these processes on the expression of MER or on the inhibition of MER by phorbol esters was determined by using a variety of substances known to activate or inhibit the processes. A variety of other biologically active substances were also tested including inhibitors of assembly of microtubules or microfilaments, cyclic nucleotides and low molecular weight thiols, which are known to modulate the activation of B cells (chapter 1.10).

5.2 RESULTS

5.2.1 RELATIONSHIP BETWEEN BINDING OF PDBu AND INHIBITION OF MER

Time-dependence

To determine whether the kinetics of binding of ^{3}H-PDBu parallel the kinetics of inhibition of rosetting, B-CLL cells were incubated with 30nM ^{3}H-PDBu, and bound ^{3}H-PDBu and rosetting were assayed at varying times. The experiment was done both in the presence and absence of 10% FCS since this affected the kinetics of binding (chapter 4).

In the absence of FCS, there was little inhibition of rosetting in the first 20 minutes, when binding of ^{3}H-PDBu reached a maximum. After this lag phase, inhibition occurred rapidly, being nearly complete by 120 minutes (Fig 5.1). There was considerable down-regulation of PDBu binding during this period.

In the presence of FCS, there was also very little inhibition of rosetting when binding of ^{3}H-PDBu was maximal at 20 minutes. Inhibition increased over the next three hours. The EC_{50} (concentration of PDBu that inhibited rosetting by 50%) decreased from 20nM at 1 hour to 13nM at 4 hours of culture but did not decrease further with overnight culture.

Concentration-dependence

The shapes of the curves for binding (assayed at 25 minutes) and rosette inhibition (assayed at 120 minutes) were similar, over a wide range of concentrations of ^{3}H-PDBu, regardless of whether the cells were assayed in the absence (Fig 5.2) or presence (not shown) of 10% FCS.

For cells in the presence of 10% FCS, the EC_{50} (20nM) for rosette inhibition agreed with the Kd (18-25nM) for PDBu binding.

The EC_{50} was lower (4nM) when cells were assayed in the

absence of FCS, suggesting that FCS decreases the inhibition of rosetting by PDBu. The binding also fitted a curvilinear Scatchard plot (as discussed in chapter 4). This plot could be resolved into two receptor subtypes with Kds of 1 and 50nM. The shape of the curve for rosette inhibition was very similar to that of the curve which represents binding of ^{3}H-PDBu to the very high affinity receptor (Fig 5.2).

Different phorbol ester analogues

TPA and two structurally-related compounds, mezerein and RPA, inhibited both binding of ^{3}H-PDBu and rosetting (Table 5.1). RPA and mezerein were about two to three times less active than TPA in inhibition of rosetting and PDBu binding. Mezerein is a strong second stage promoter and weak complete promoter and RPA is a very strong second stage promoter almost without activity as a complete promoter (see chapter 1.6).

The biologically inactive compounds phorbol and 4-alpha-phorbol didecanoate did not inhibit MER or compete with ^{3}H-PDBu for binding to B-CLL cells, even at a concentration of 10µM (Table 5.1).

Irrreversibility of inhibition of MER

To determine whether inhibition of MER by PDBu is irreversible, the following experiment was done. B-CLL cells were exposed to 80nM ^{3}H-PDBu for 2 hours at 37°C, at which time capacity to rosette with mouse erythrocytes was inhibited by 70%. The cells were then washed at 37°C removing 96% of the bound ^{3}H-PDBu and cultured overnight in the absence or presence of 80nM ^{3}H-PDBu. Rosetting remained inhibited by 73% in those cells incubated overnight in the absence of 80nM ^{3}H-PDBu whereas those receiving further exposure to 80nM ^{3}H-PDBu were inhibited by

100%. Thus, removal of PDBu from the cells prevents further effects on MER but does not lead to reexpression of MER.

Relative sensitivities of different B-CLL populations

Cells of patient J.T. bound twice as much ^{3}H-PDBu as cells from the other patients eg V.M., resulting from a larger maximum binding capacity of PDBu in the cells of JT rather than from a higher affinity of the receptors (see Fig 4.6 and Table 4.2). Cells of J.T. were also between 10 and 50 times more sensitive to TPA and PDBu in inhibition of MER than cells of the other cases. Thus, while cells of J.T. responded to TPA with an EC_{50} of about 0.1nM, most other B-CLL populations responded with an EC_{50} of about 1-5nM (data for 30 minutes treatment in medium containing 10% FCS). Cells of J.T. also responded much more quickly to TPA than cells of V.M. in formation of cIg. By 5 hours, 70% of the cells of J.T. expressed cIg compared to 20% of cells of V.M. By 24 hours, most of the cells in both populations were cIg+ve.

In order to determine whether the increased sensitivity to phorbol esters of cells of J.T. was a result of their increased capacity to bind PDBu, cells of J.T. and V.M. were incubated with a range of concentrations of ^{3}H-PDBu and both binding of PDBu and rosette inhibition were assayed. It was observed that under conditions in which both populations of cells bound identical amounts of PDBu, the cells of J.T. were inhibited in rosetting to a far grater extent than those of V.M. Therefore, much of the increased sensitivity of cells of J.T. to inhibition of rosetting by phorbol ester appears to result from a mechanism other than increased binding of phorbol ester.

5.2.2 ROLE OF DIACYLGLYCEROL IN EFFECTS ON MER

If PKC is involved in the inhibition of MER, the putative endogenous ligand of PKC, diacylglycerol, should inhibit MER. This was tested here by treating the cells (a) with fluoride which causes formation of diacylglycerol by stimulating PIP_2 formation and (b) with exogenous membrane-permeable diacylglycerols OAG and DOG.

Fluoride

Sodium fluoride rapidly inhibited rosetting in B-CLL cells (Fig 5.3) at concentrations (5-40 mM) which induce PIP_2 turnover in other cells. The effects of fluoride on MER cannot be explained by a rise in cyclic AMP since other agents which increase cyclic AMP did not affect expression of MER (see below).

Exogenous diacylglycerols

DOG and OAG inhibited rosetting, modestly. At a high concentration (125ug/ml) they inhibited rosetting by only 35% and 28%, respectively. The membrane-impermeable 1,2 diolein did not inhibit MER at concentrations up to 125ug/ml in a 60 minute culture.

These exogenous diacylglycerols may have only a weak effect on MER because they either do not easily reach PKC or because they are rapidly metabolized. At the same concentrations as used in the rosetting assays, OAG and DOG induced a modest amount of phosphorylation (Fig 5.6, DOG not shown) and effectively inhibited binding of ^{3}H-PDBu to B-CLL cells (by up to 63%). At concentrations of PDBu and DOG which inhibited binding of ^{3}H-PDBu to B-CLL cells to similar extents, PDBu was much more active than DOG in causing a loss of expression of MER (Fig 5.4).

5.2.3 ROLE OF PROTEIN PHOSPHORYLATION IN THE INHIBITION OF MER

To investigate the role of protein phosphorylation in the inhibition of MER, three types of experiments were performed.

First, correlation was sought between induction of total protein phosphorylation and inhibition of rosetting in phorbol ester-treated cells. Cells were labelled with ^{32}P, treated with phorbol ester and assayed for inhibition of MER. At a concentration (200nM) which strongly inhibited rosetting, PDBu stimulated little increase in phosphorylation. On the other hand, TPA inhibited rosetting and stimulated phosphorylation of several proteins in whole B-CLL cells at concentrations greater than 10nM (Fig 5.5, 5.6). Major substrates had apparent molecular weights of 58 and 48kDa (arrows Fig 5.5 track D). TPA at 0.3nM had little effect on phosphorylation and MER (not shown).

Secondly, in order to determine whether any of the proteins associated with MER are phosphorylated, MER was released by trypsinization from untreated- and TPA-treated cells that had been pre-labelled with ^{32}P. The trypsin supernatants were partially purified on wheat germ lectin Sepharose (as in chapter three) and then analyzed by SDS-PAGE autoradiography. No labelled bands were seen in preparations from cells treated with TPA (100nM) or control buffer.

Thirdly, a variety of inhibitors of PKC, including trifluoperazine, chlorpromazine, palmitoylcarnitine, H-7, quercetin and polymyxin B, had no significant effect on the inhibition of MER by TPA or PDBu (Table 5.2). The highest concentrations of inhibitors that were used, exceeded those shown to be effective in other systems.

In some experiments, the efficiency of the inhibitors in

blocking ^{32}P-incorporation into cellular proteins was determined. Palmitoylcarnitine (10μM), or a combination of this drug (1μM) with trifluoperazine (25μM), blocked much of the TPA-stimulated phosphorylation (Fig 5.6 tracks C and E) without effect on the inhibition of MER by TPA. Similar results were seen with H-7 (10-100μM) (not shown).

5.2.4 ROLE OF OTHER EARLY EVENTS IN THE INHIBITION OF MER

Change in membrane potential

Since the membrane hyperpolarizing agent, valinomycin, and membrane depolarizing agents, gramicidin and a high concentration of K^+, did not affect expression of MER or phorbol ester-mediated inhibition of MER (Table 5.3), it is unlikely that a change in membrane potential mediates the effects of phorbol esters on MER. However, these studies need to be repeated under conditions in which the membrane potential can be monitored, such as by use of fluorescent cationic dyes.

Activation of phospholipase A_2 and arachidonic acid metabolism

Melittin, which activates phospholipase A_2 and also mimics some actions of phorbol ester [Mufson et al. 1979], did not inhibit MER and inhibitors of phospholipase A_2, bromophenacylbromide and quinacrine, did not prevent the inhibition of MER by phorbol ester (Table 5.4).

One consequence of the action of phospholipase A_2 is increase in arachidonic acid, which is an intracellular messenger and is also metabolized by the cyclo-oxygenase pathway to prostaglandins (chapter 1.10). Prostaglandin E_2 has been reported to inhibit MER in normal B cells [Misefari et al. 1979]. However, arachidonic acid, indomethacin (an inhibitor of the cyclo-oxygenase pathway) and prostaglandin E_2 did not inhibit MER or interfere with phorbol ester-mediated inhibition of MER in B-CLL

cells.

Phospholipid methylation

Adenosine (500µM), homocysteine thiolactone (500µM) and deoxycoformycin (40µM), added together at concentrations up to five fold higher than those generally used to inhibit phospholipid methylation, had no effect on inhibition of MER by phorbol ester (Table 5.4).

Activation of protease or esterase

Inhibitors of protease and esterase prevent some actions of phorbol esters, including tumour promotion and aggregation of lymphocytes [Blumberg 1980, Patarroyo et al. 1982]. Proteases have also been implicated in the proliferation and differentiation of B lymphocytes [chapter 1.10.10). Calpain, a calcium- and thiol-dependent protease, is involved in the conversion of PKC to PKM [Chida et al. 1986]. Several protease inhibitors including PMSF, TLCK, soybean trypsin inhibitor, leupeptin (a specific inhibitor of calpain) and trasylol did not prevent the inhibition of MER by phorbol ester (Table 5.4).

Energy-dependence

The inhibition of MER did not appear to require energy since azide, which inhibits electron transport involved in the generation of ATP, and 2-deoxyglucose, which inhibits glycolysis and prevents TPA-induced aggregation of neutrophils [DeChatelet et al. 1976], did not affect the modulation of MER by phorbol ester (Table 5.4).

Clustering of glycosphingolipids

Clustering of glycosphingolipids accompanies lymphocyte activation by lectins [Curtain et al. 1981]. The clustering may affect the fluidity of the membrane and the activity of membrane

receptors. A specific inhibitor of this clustering, oxotho-methoxy-xanthone carboxylic acid did not prevent TPA-induced inhibition of MER (Table 5.4).

Cyclic nucleotides

Agents which raise cAMP including dibutyryl cAMP (1mM), alone or in combination with the phosphodiesterase inhibitor theophylline (1mM), prostaglandin E_2 (30μM) and adrenaline (5μM), as well as agents which raise cGMP, including dibutyryl cGMP (1mM), carbachol and bethanecol (100μM) did not inhibit MER or interfere with phorbol ester-mediated inhibition of MER (Table 5.4).

Low molecular weight thiols

2-ME, and other low molecular weight thiols, rapidly inhibited rosetting with mouse erythrocytes, without effect on cell viability, in both B-CLL cells and MER+ve RPMI 8866 B lymphoblastoid cells. The order of potency for B-CLL cells was dithiothreitol (EC_{50}, 0.7mM), sodium dithionite ($Na_2S_2O_4$, 2.1mM), sodium metabisulphite ($Na_2S_2O_5$, 3.8mM) and 2-mercaptoethanol 7.6mM). Sodium thiosulphate ($Na_2S_2O_3$), glutathione, thiourea, cysteine and penicillamine (dimethylcysteine) were inactive at concentrations up to 10mM (the highest concentration tested).

Some aspects of the inhibition of MER resembled that by phorbol esters. First, the effect of thiols on MER was not reversed by removal of the thiol and culture of the cells in medium without thiols for up to 72 hrs. Secondly, similar amounts of mouse red cell haemagglutinating activity were released by trypsinization from control and dithiothreitol-treated cells (data not shown).

Unlike phorbol esters, dithiothreitol (50μM) did not induce formation of cytoplasmic immunoglobulin in B-CLL cells in a 72

hour culture nor did it increase levels of mRNA specific for the mu chain of Ig in a 24 hour culture (not shown).

Other substances without effect

Some other substances were tested either for direct effects on MER or capacity to interfere with the inhibition of MER induced by PDBu or TPA (100nM). None of these were active at the highest concentration tested (Table 5.4). These included cytochalasin B, colchicine and vinblastine, which interfere with assembly of components of the cytoskeleton and retinoic acid, which inhibits many actions of phorbol esters including tumour promotion [Blumberg 1980] and the co-mitogenic effect of phorbol esters on bovine lymphocytes [Kensler and Mueller 1978], by a mechanism which is poorly understood.

5.3 DISCUSSION

Three pieces of evidence support the hypothesis that phorbol ester receptors mediate the effects of phorbol esters on MER. First, inhibition of rosetting occurred at nanomolar concentrations of phorbol esters, much lower than those required to affect the structure of phospholipid membranes [Deleers et al. 1981]. Secondly, the order of potency of various biologically-active and -inactive analogues in inhibiting MER was similar to that for occupancy of PDBu receptors. Finally, the shape of the binding curve was similar to that of the rosette inhibition when a range of concentrations of PDBu were employed.

The relative potencies of the second stage promoters, mezerein and RPA, both in inhibiting MER and competing for PDBu receptors, suggest that the PDBu receptor mediates the effects of second stage promoters. This is in agreement with the observation that mezerein and RPA are nearly as potent as TPA in activation

of PKC [Gschwendt et al. 1983a].

Inhibition of rosetting by PDBu was irreversible even after removal of nearly all of the bound PDBu from the cells by washing. This irreversible effect on MER is in contrast to many effects of phorbol esters in other systems. Expression of MER may also be lost irreversibly in response to other activators, in-vitro, and during B cell activation, in-vivo.

The irreversibility of the inhibition and the observation that, at the time of maximal binding, little inhibition of MER has occurred suggest that MER is not sterically inhibited by phorbol ester. Rather, inhibition occurs during a time-dependent event following binding of PDBu to its receptor.

This time-dependent process may vary in efficiency in cells from different patients as shown by studies with cells of J.T. and V.M. It needs to be determined whether normal MER+ve B-CLL cells are more efficient in this process than most populations of B-CLL cells.

In the presence of FCS, there was a five-fold increase in the EC_{50} for inhibition of rosetting by PDBu, without much effect on binding of PDBu to its receptors. Similarly, serum decreases the sensitivity of the epidermal growth factor receptors to inhibition by phorbol ester [Weinstein et al. 1979]. FCS may affect a process which follows binding such as down-regulation of PKC (see chapter 4). Down-regulation of PKC, by cleavage to PKM, has been implicated in the differentiation of HL60 cells and in the secretory response in PMN (chapter 1.9.3).

The above results suggest that PKC is involved in the inhibition of MER. Phorbol esters are thought to act by replacing diacylglycerol in the activation of PKC. In some systems, exogenous diacylglycerols are as effective as phorbol

esters while in others they are less effective or induce a different response (chapter 1.8.4).

Exogenous diacylglycerols inhibited MER to a lesser extent than phorbol esters. This did not appear to be a result of the diacylglycerols not reaching the PKC, since they effectively inhibited binding of ^{3}H-PDBu and caused some new protein phosphorylation. Possibly, these agents are less efficient than phorbol esters in inducing an essential event which follows binding to PKC. In U937 cells, both TPA and OAG induce translocation of PKC and phosphorylation of the same three substrates but OAG does not induce monocytoid differentiation of these cells [Ways et al. 1987] and did not induce differentiation of HL60 cells in the study by Yamamoto and colleagues [1985] although differentiation was induced when diacylglycerol was added continously during the culture [Ebeling et al. 1985].

Fluoride, which induces formation of diacylglycerol, in-situ, was more effective than diacylglycerols in inhibiting MER. Anti-Ig also stimulates diacylglycerol formation (chapter 1.10) and inhibits rosetting (chapter 1.3). Since these stimuli also cause a rise in $[Ca^{++}]_i$, calcium ions may play an essential role in the inhibition of MER (see chapter 6).

The inhibition of MER by fluoride supports the hypothesis that G proteins are coupled to the pathway of inhibition of this receptor. It has been argued that G proteins are not involved in coupling of sIg to phospholipase C in mouse B cell membranes [Harris and Cambier 1987]. The experiments here do not indicate whether fluoride is acting on a G protein coupled to sIg or on one coupled to another receptor in the membranes of B-CLL cells.

No evidence was obtained that protein phosphorylation was

involved in the inhibition of MER by phorbol esters. PDBu did not cause any detectable increase in total cell protein phosphorylation at concentrations which inhibited MER, no labelled band was seen in preparations of MER from ^{32}P-labelled cells that had been treated with TPA and a variety of inhibitors of PKC did not prevent the inhibition of MER by phorbol ester despite blocking much of the phosphorylation.

However, a role for protein phosphorylation in the inhibition of MER cannot be excluded. A ^{32}P-labelled band in MER may not be released from TPA-treated B-CLL cells because trypsinization only releases that portion of the protein(s) in MER which is external to the cell, while phosphorylation of these proteins by PKC is most likely to occur in the cytoplasmic tail (see chapter 1.8.6). Alternatively, the putative phosphoprotein may be lost from the complex of MER as a result of the phosphorylation.

The failure of inhibitors of PKC to block the modulation of MER could be explained by the relative insensitivity of the phosphorylation of MER to these inhibitors. These inhibitors did not block all of the TPA-induced phosphorylation in B-CLL cells. MER may be phosphorylated by PKC in a compartment of the cell inaccessible to these inhibitors. Some of the isozymes of PKC may also be less sensitive to these drugs. It has been claimed that the phosphorylation of proteins by PKM is much less sensitive to inhibitors of PKC [Pontremoli et al. 1986b,c], perhaps because many of these inhibitors are hydrophobic and localize in the cell membrane while PKM is in the cytosol. It has recently been reported that palmitoylcarnitine blocks activation of the catalytic site of PKC by phospholipid and calcium but not activation by TPA [Nakadate and Blumberg 1987b]. However, the

results shown in Fig 5.6 suggest that palmitoylcarnitine does block TPA-induced protein phosphorylation in cells.

Inhibition of MER may result from some event other than phosphorylation, which is mediated by PKC. The translocation of PKC to the inner leaflet of the plasma membrane may cause a redistribution of anionic phospholipids which in turn disturbs the association of phospholipid and protein in MER.

Membrane depolarization, subsequent to PKC activation, appears to be a critical event in mouse B lymphocyte activation (chapter 1.10.6). However, preliminary evidence suggests that it is not involved in activation of human B cells [Godal et al. 1985] and it did not appear to be involved in the inhibition of MER.

There was also no evidence for a role of activation of phospholipase A_2 and subsequent release of arachidonic acid, phospholipid methylation, proteolysis, energy-dependent steps, glycosphingolipid clustering in the membrane, alteration in the cytoskeleton or cyclic-nucleotide-dependent steps.

Inhibition of MER by thiols resembled that induced by phorbol esters in being rapid, irreversible and not involving loss or destruction of MER. However, thiols did not induce plasmacytoid differentiation.

Low molecular weight thiols have some actions in common with phorbol esters. Like phorbol esters, thiols down modulate a variety of membrane receptors including those for insulin [Böni-Schnetzler et al. 1987] and to induce responses similar to those induced by ligands of the receptors (eg for insulin [Goko et al. 1981]. Both thiols [Ali and Hynes 1978] and phorbol esters [Keski-Oja et al. 1979] cause a rapid loss of the extracellular

matrix protein fibronectin from cell surfaces. Mercaptoethanol activates PKC [Zabrenetzky et al. 1981]. Both phorbol esters and thiols are cofactors in lymphocyte activation (see chapter 1.10.10).

Whether the inhibition of MER by thiols is related to the role of thiols in B cell activation is not clear. Dithiothreitol which is the most potent of the thiols as reducing agents, was also the most potent in inhibiting MER. Membrane-impermeable thiols such as cysteine, glutathione and penicillamine did not inhibit MER, suggesting that the thiols act on a sulphydryl group which is buried in the membrane. This sulphydryl group may be in the protein of MER or it may be in some other protein involved in the inhibition of MER eg PKC.

In conclusion, although PKC appears to mediate the inhibition of MER, the time-dependent event, which follows binding of phorbol ester and leads to inhibition of MER, is not identified by these studies. In the following chapters, the role of calcium, zinc and other metals in the process is discussed.

Table 5.1 Correlation between capacity to inhibit PDBu binding and capacity to inhibit rosetting

Analogue	Concentration (nM) of analogue which inhibits by 50%	
	Binding of ^{3}H-PDBu	Rosetting by MER
TPA	15	3
Mezerein	20	10
RPA	42	11
Phorbol	>10,000	>10,000
4-alpha-PDD	>10,000	>10,000
Phorbol diacetate	>10,000	>10,000

B-CLL cells were incubated with ^{3}H-PDBu (35nM) in the presence of varying concentrations (1-10,000nM) of unlabelled TPA, mezerein, RPA, phorbol diacetate, phorbol, 4-alpha-phorbol didecanoate (4-alpha-PDD) or no addition. Binding of ^{3}H-PDBu was asayed after 25 minutes. Parallel samples lacking ^{3}H-PDBu were assayed for MER at 60 minutes.

Table 5.2 Effect of inhibitors of PKC on loss of MER induced by phorbol esters

Inhibitor	Concentration	% Rosette inhibition by TPA or PDBu (standard error in parentheses) TPA	PDBu
-		97 (2)*	62 (4)
Trifluoperazine	25μM	98 (4)	ND**
Palmitoylcarnitine	1μM	98 (2)	ND
Palmitoylcarnitine	10μM	91 (5)	68
Palmitoylcarnitine Trifluoperazine	1μM 25μM	99 (6)	ND
Palmitoylcarnitine Trifluoperazine	3μM 10μM	93 (3)	67 (3)
Palmitoylcarnitine Trifluoperazine Chlorpromazine	3μM 10μM 25μM	99 (1)	68 (6)
Quercetin	400μM	96 (3)	ND
Polymixin B	300μg/ml	97 (2)	ND
Tetracaine	500μM	95 (1)	ND
H-7	100μM	98 (5)	81 (4)

B-CLL cells ($5x10^6$/ml) were treated with various inhibitors of PKC, either singly or in combination, for 10 minutes at room temperature in RPMI/FCS before addition of TPA (200nM), PDBu (400nM) or solvent control (DMSO 0.1%v/v) for a further 30 minutes at 37°C. Cells were washed and rosetted with mouse red cells in duplicates.

* Data indicate % rosette inhibition (relative to control, in which 92% of cells formed rosettes).

** ND indicated not done

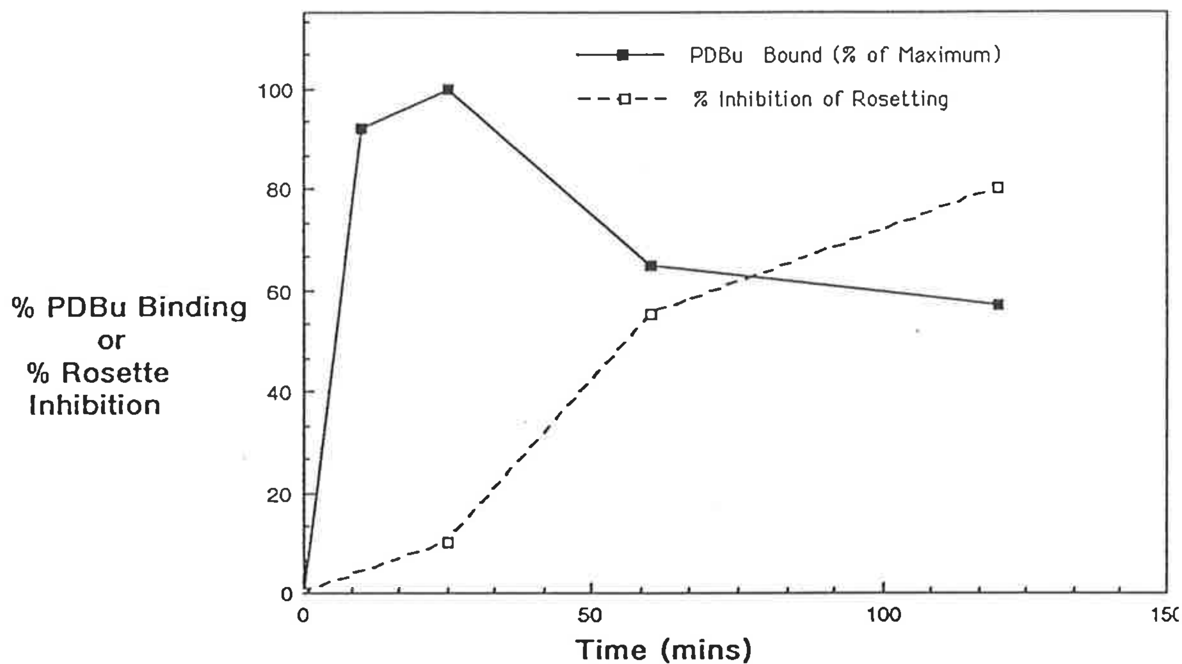
Table 5.3 Effect of agents influencing membrane potential

Agent	Concentration (μM)	% Rosette Inhibition	
		No TPA	TPA
–		0	66
Valinomycin	0.1	9	64
	1	0	51
	10	0	60
Gramicidin	0.1	9	75
	1	0	77
	10	0	57
KCl	50,000	0	72

B-CLL cells ($5x10^6$/ml were treated with valinomycin, gramicidin, KCl or control solvent (DMSO) for 10 minutes at 37°C before addition of TPA (100nM) or control solvent (DMSO 0.1%) for a further 15 minutes at 37°C. Cells were washed and rosetted with mouse erythrocytes. Means of duplicates are shown.

Table 5.4 Other substances with no effect on MER or on the inhibition of MER by phorbol ester

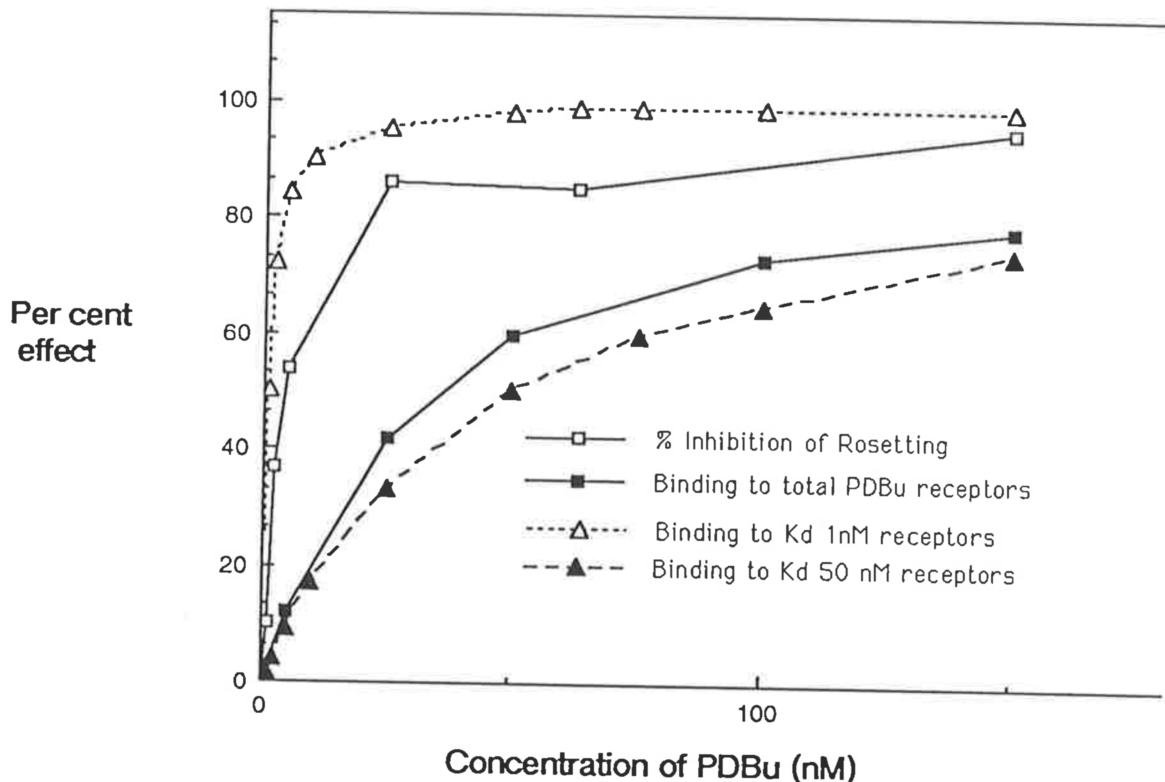
Substance	Highest concentration tested (μM)
Protease and esterase inhibitors	
Tosyl-leucyl-chloro-ketone	500
PMSF	1,000
Soybean trypsin inhibitor	1,000
Phospholipase A2 inhibitors	
Quinacrine	200
Bromophenacyl bromide	100
Phospholipase A2 activator	
Melittin	2
Metabolic inhibitors	
Sodium azide	100,000
2-deoxyglucose	10,000
Inhibitors of cytoskeleton assembly	
Cytochalasin B	10
Colchicine	2
Vinblastine	100
Increase cAMP	
Prostaglandin E_2	100
Adrenaline	5
Increase cGMP	
Carbachol	15
Bethanecol	100
Inhibitor of cyclo-oxygenase pathway	
Indomethacin	200
Inhibitor of glycosphingolipid clustering	
Xanthone carboxylic acid	1000
Inhibitor of tumor promotion	
Retinoic acid	25
Inhibitors of phospholipid methylation	
Adenosine	500
Homocysteine thiolactone	500
Deoxycoformycin	40



<u>Legend to Figure 5.1 Kinetics of PDBu binding and inhibition of MER</u>

Inhibition of MER occurred later than the time at which PDBu binding was optimal, suggesting that the inhibition occurs by a process induced by PDBu rather than by sterically hindering rosetting.

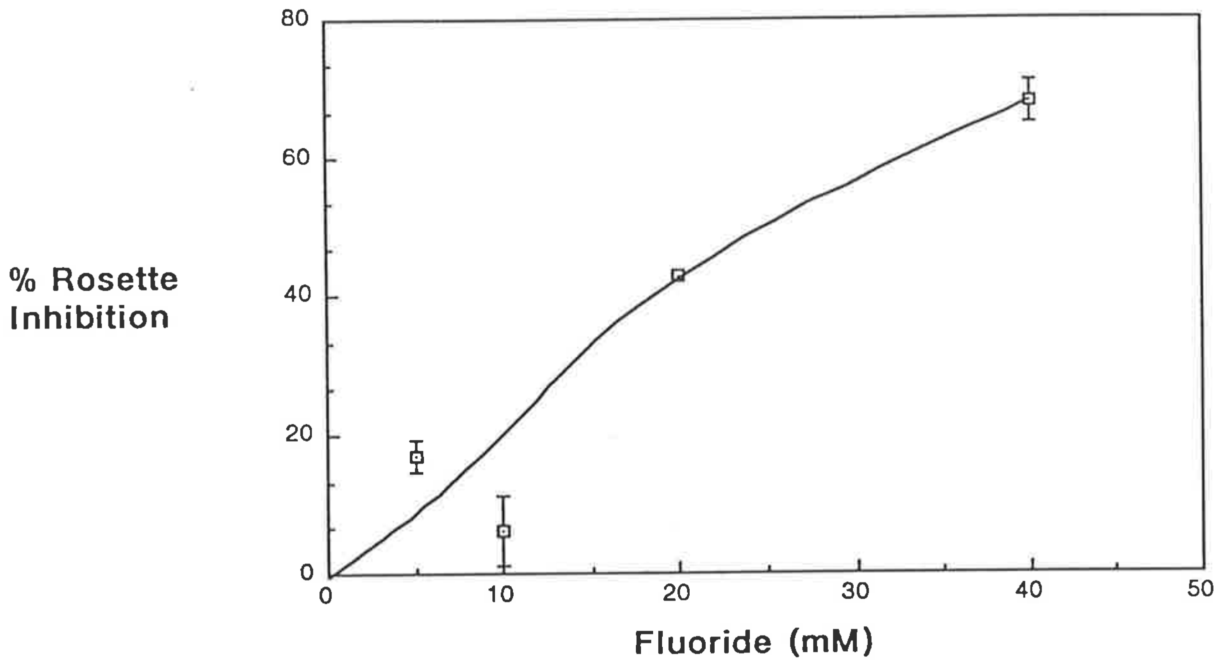
B-CLL cells were incubated with 30nM ^{3}H-PDBu in PBS/ALB and, at intervals, binding of PDBu and MER were assayed. Binding of PDBu is expressed as a percent of maximal binding (at 25 minutes).



Legend to Figure 5.2 Concentration-dependence of relationship between inhibition of MER and binding of PDBu.

Plots of inhibition of rosetting and binding of ^{3}H-PDBu to the high affinity PDBu receptor were similar in shape over a range of concentrations of PDBu suggesting that inhibition of rosetting is mediated by these receptors.

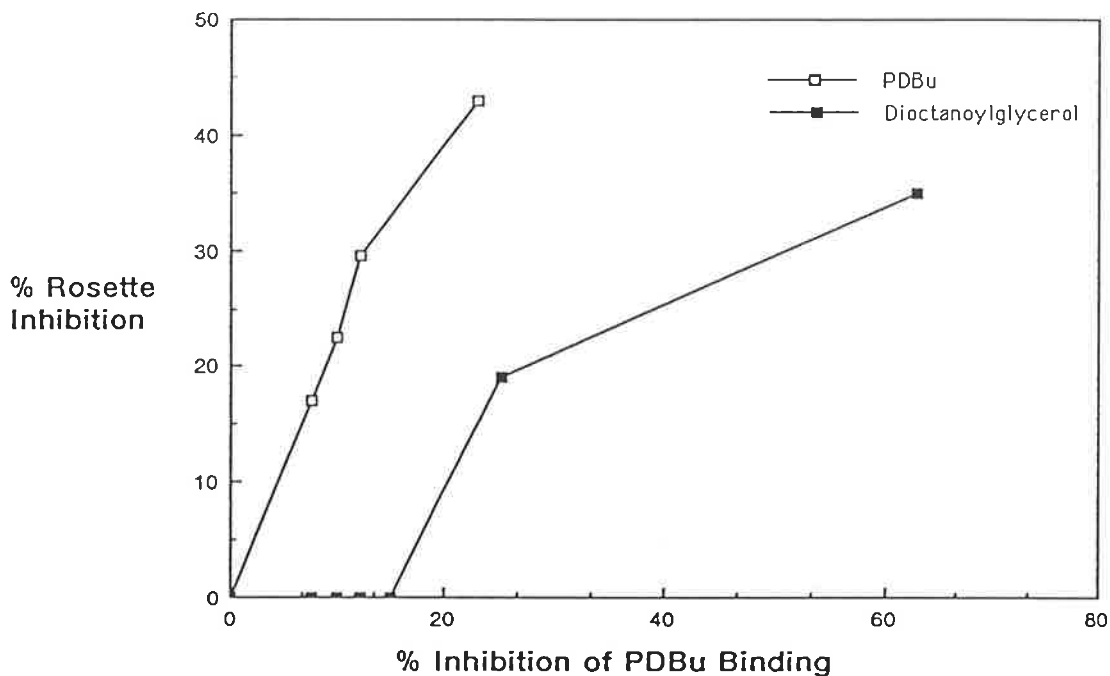
B-CLL cells were incubated with ^{3}H-PDBu (0-160nM) at 37°C in PBS/ALB, binding was assayed at 25 minutes and MER at 120 minutes. Scatchard plots were curvilinear and were resolved into two classes of receptor with Kds of 1 and 50 nM. Total binding was 390 fmol/10^{6} cells. The data show curves for inhibition of MER, occupancy of total receptors by ^{3}H-PDBu and occupancy of both high and lower affinity receptors.



Legend to Figure 5.3 Effect of fluoride on MER rosetting

Sodium fluoride was a potent inhibitor of rosetting at concentrations known to stimulate PIP_2 turnover.

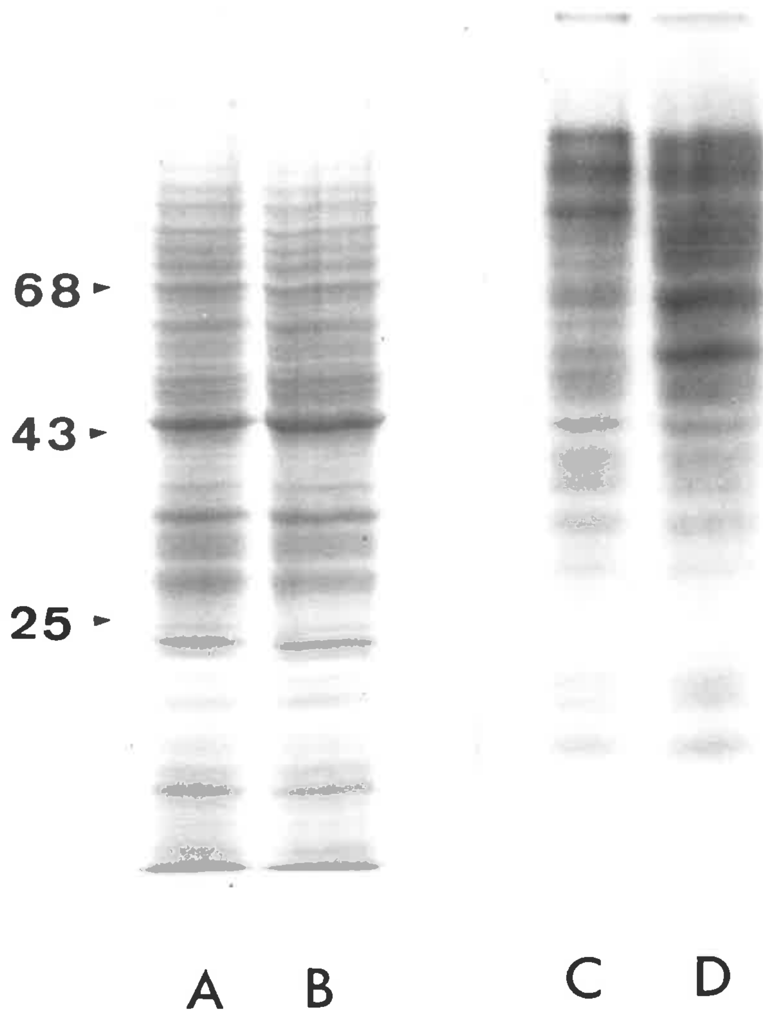
B-CLL cells were treated with varying concentrations of sodium fluoride for 40 minutes at 37°C, washed and assayed for rosetting with mouse red cells, in triplicate. Data are expressed as % inhibition of rosetting relative to control (no fluoride). Bars indicate standard error.



Legend to Figure 5.4 Relative efficiencies of DOG and PDBu in inhibition of MER

This figure shows that despite effectively competing with ^{3}H-PDBu for binding to B-CLL cells, dioctanoylglycerol (DOG) was much weaker than PDBu in inhibiting rosetting.

B-CLL cells were treated with DOG (30-125ug/ml) or unlabelled PDBu (4-250nM) for 30 minutes at 37°C. ^{3}H-PDBu (15nM) was added and after 20 minutes cells were assayed for bound ^{3}H-PDBu and capacity to rosette with mouse red cells. Each point represents the % inhibition of rosetting and % inhibition of binding of ^{3}H-PDBu for a given concentration of DOG or unlabelled PDBu relative to control-treated cells (no DOG or unlabelled PDBu). Data are means of triplicates (standard errors did not exceed 10% of means).

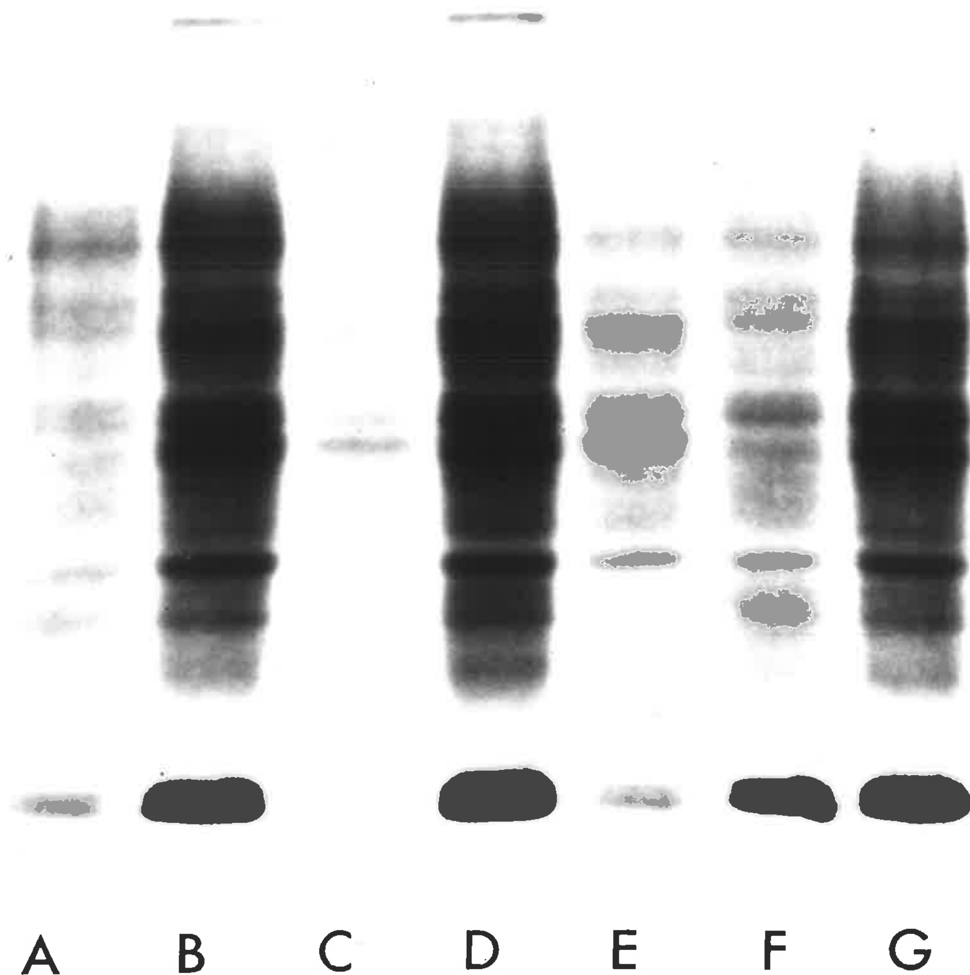


Legend to Figure 5.5 Effect of phorbol esters on protein phosphorylation in B-CLL cells

Figure shows that TPA stimulates phosphorylation of several proteins in B-CLL cells, in particular 58 and 48kDa polypeptides.

B-CLL cells were pre-incubated with ^{32}P (0.14mCi/ml) for 60 minutes at 37°C. TPA (100nM) or control solvent DMSO were then added to the cells for a further 40 minutes, the cells were washed and pellets were resuspended in 0.5% NP40 containing 1mM PMSF and 15mM $MgCl_2$. After 30 minutes at 4°C, supernatants were collected and stored at -20°C until ready to run on SDS-PAGE. Autoradiographs were developed after 2 days.

A Control - Coomassie-blue stain
B TPA - " " "
C Control - Autoradiograph of A
D TPA - " " of B



A: control
B: 100nM TPA
C: 100nM TPA + 10μM palmitoylcarnitine
D: 100nM TPA + 1μM palmitoylcarnitine
E: 100nM TPA + 1μM palmitoylcarnitine + 25μM trifluoperazine
F: 125μM OAG
G: 100nM TPA + 25μM trifluoperazine.

<u>Legend to Figure 5.6</u> <u>Effect of inhibitors of PKC on phosphorylation and inhibition of MER</u>

Figure shows that much of the TPA-induced protein phosphorylation in B-CLL cells was inhibited by 10μM palmitoylcarnitine (track C) and 1μM palmitoylcarnitine plus 25μM trifluoperazine (track E). These inhibitors of PKC did not affect TPA-induced inhibition of MER. Figure also shows a modest stimulation of phosphorylation by the diacylglycerol (OAG).

B-CLL cells were labelled with ^{32}P for 60 minutes as in Fig 5.5. Inhibitors were added 10 minutes before 100nM TPA. After a further 30 minutes, detergent-soluble proteins were extracted with 0.5% NP40 as in Fig 5.5 and samples were run on 7.5% SDS-Page. Autoradiographs were developed after 3 days.

CHAPTER SIX

ROLE OF METALS IN INHIBITION OF MER

6.1 INTRODUCTION

Calcium ions participate in many biochemical processes including those mediated by calmodulin, calcium-dependent proteases and calcium-dependent phospholipases. A rise in intracellular free calcium ions ($[Ca^{++}]_i$) also synergizes with phorbol esters and diacylglycerol in activation of a variety of types of cells including B lymphocytes (see chapter 1.10.3). A site of action of calcium may be PKC since the activation of soluble PKC by phospholipid and diacylglycerol or phorbol ester is calcium-dependent (chapter 1.8).

One way in which the $[Ca^{++}]_i$ is raised involves mobilization by IP_3 of calcium ions from intracellular calcium stores, principally the smooth endoplasmic reticulum. A further rise in the $[Ca^{++}]_i$ follows as a result of increased influx of extracellular calcium (chapter 1.10.3). $[Ca^{++}]_i$ can also be raised in cells by treatment with calcium ionophores (eg A23187 and ionomycin) in the presence of extracellular calcium. RPMI 1640, the culture medium used in these studies contains about 0.5mM calcium.

Calcium-dependent processes in cells can be partially inhibited by treatment of the cells with divalent cation-chelating agents EDTA and EGTA and with TMB-8, a substance which antagonizes the actions of calcium within the cell by a poorly-defined mechanism [Smolen 1984].

Gold compounds are potent modulators of cell activation (chapter 1.11). Auranofin is a lipophilic antirheumatic gold (I) compound which mimics phorbol esters in some cellular responses such as stimulation of phosphorylation of 40kDa and 20kDa proteins in human platelets and in the phosphorylation and inhibition of the receptor for epidermal growth factor in HeLa

and A431 cells (chapter 1.11.4). Tryptic peptide maps indicate that PKC mediates the increased phosphorylation of these proteins in auranofin-treated cells. Enhancement of superoxide anion formation by low concentrations of auranofin in PMN is accompanied by enhancement of phosphorylation of the 47kDa flavoprotein component of the NADPH oxidase which mediates superoxide anion formation [Hurst et al. 1989].

The mechanism of action of auranofin and other gold compounds as disease-remitting agents in rheumatoid arthritis is still poorly understood. A major action of auranofin, in vitro, is binding to sulphydryl groups in proteins by thiol-exchange. The gold in auranofin is internalized by cells by a similar mechanism (chapter 1.11.3). Consequently, auranofin affects the function of many thiol-dependent proteins, including enzymes.

Another reason for studying the effect of auranofin on B-CLL cells is that auranofin modulates the responsiveness of immunocompetent cells (chapter 1.11.2). Since auranofin often lowers the rheumatoid factor titre in patients with rheumatoid arthritis, it may be affecting the MER+ve, CD5+ve B cell subset which is thought to be responsible for synthesis of rheumatoid factor (see chapter 1.2). B-CLL cells are potentially a useful model for studying the effects of auranofin, in vitro, on this subset.

Zinc may have some biological effects in common with gold since it also binds to regions in protein which are rich in cysteine or histidine (chapters 1.12). Indeed, zinc compounds have been used successfully in the treatment of rheumatoid arthritis [Simkin 1982]. Other biological effects of these two metals may be different because zinc binds to its ligands in a

tetrahedral conformation whereas gold (I) binds preferentially in a linear conformation. Gold will displace zinc from some zinc-dependent enzymes. It is not known whether gold can substitute for zinc in these enzymes.

Studies of the effect of zinc on B-CLL cells may be important for several reasons. Zinc is necessary for lymphocyte activation by phytohaemagglutinin and other stimuli and zinc chloride is a potent polyclonal activator of human tonsil B cells, inducing proliferation and differentiation (chapter 1.12.4). Cells in culture are usually exposed to about 2uM total zinc (contributed by the 10% FCS) whereas in vivo they are exposed to approximately 20μM zinc from the plasma (chapter 1.12.2). Therefore, some activation events in cells, in vitro, may be limited because of a deficiency of zinc.

Zinc plays an essential role as a cofactor for some enzymes particularly those involved in nucleic acid synthesis and for some transcription factors. PKC contains two potential zinc finger structures in its regulatory domain (chapter 1.8.3). As yet there is no evidence that PKC is a zinc-containing enzyme. Phorbol esters induce a redistribution of zinc in cells (chapter 1.12.3) suggesting a possible role for zinc in some of the actions of PKC. Zinc appears to be a second messenger in the formation of diadenosine tetraphosphate [Grummt et al. 1986] which is an important factor for cell proliferation.

Water-soluble zinc chloride and zinc sulphate and the lipophilic zinc pyrithione (Fig 1.14) were used in these studies. Zinc pyrithione is an anti-dandruff agent but its mechanism of action is unclear (chapter 1.12.3). Zinc is bound between two hydrophobic pyrithione molecules which may facilitate uptake by cells. Pyrithione greatly enhanced the uptake of ^{65}zinc by

various tissues and organs in whole animal studies suggesting that pyrithione may be an ionophore for zinc.

Effects of zinc are inhibited by 1,10 phenanthroline (Fig 1.14) a relatively specific chelator of zinc and other elements of the first transition series, and histidine [Martel and Calvin 1952]. Zinc is also tightly bound by the calcium chelating agents, EDTA and EGTA.

The aim of the experiments in this chapter was to explore the role of metals in the pathway leading to loss of expression of MER.

6.2 RESULTS

6.2.1 EFFECT OF CALCIUM

Calcium ionophore

Pre-treatment of B-CLL cells with A23187 or ionomycin greatly enhanced the susceptibility of MER to inhibition by DOG (Fig 6.1) or PDBu (Fig 6.2). Neither ionophore alone inhibited MER. The calcium ionophores were most effective at low concentrations of PDBu (eg 10nM); when the PDBu concentration was 200nM, a concentration which saturates PDBu receptors, there was little or no effect of ionophore. PDBu-mediated inhibition of MER was augmented 11 fold by 0.2μM A23187 and 20 fold by 2μM A23187. The concentration of PDBu which inhibited rosetting by 50% (EC_{50}) was reduced, by pre-treatment of the cells with A23187, from 165nM to 25nM in cells from one patient (Fig 6.2) and from 58nM to 18nM in cells from another patient. A23187 (1μM) also enhanced the inhibition of MER by PDBu in Mann B lymphoblastoid cells, reducing the EC_{50} from 36 to 11nM.

In addition, the lag time for inhibition of MER was reduced by A23187. Thus, little inhibition of MER occurred in the first

40 minutes of culture with 50nM PDBu whereas 70% of rosetting was inhibited when cells were treated for 40 minutes with both PDBu and A23187 (1μM) (Table 6.1).

In contrast, there was no enhancement by A23187 (1μM) of the rosette inhibition caused by sub-optimal concentrations of dithiothreitol (100-500μM) suggesting that this thiol acts in a calcium-independent manner.

6.2.2 EFFECT OF AURANOFIN

Effect on rosetting

Auranofin inhibited rosetting with mouse erythrocytes in a time- and concentration-dependent manner (Fig 6.3). The EC_{50} was 14μM (data pooled from 21 experiments involving B-CLL cells from five patients) (Fig 6.3B). Auranofin also inhibited rosetting in the RPMI 8866 B lymphoblastoid cell line with an EC_{50} of 8μM.

Auranofin synergized with PDBu (Fig 6.4) and TPA (not shown) in inhibiting rosetting. Synergism was seen with concentrations of auranofin as low as 1μM.

Effect on ligand-binding activity of MER

The same haemagglutinating activity of MER (8 units per 10^8 cells) was recovered by trypsinization from control- and auranofin-treated B-CLL cells, even though rosetting in the auranofin-treated cells was inhibited by 75%. Therefore, in common with the modulation of MER induced by phorbol ester there was no destruction of MER or loss from membrane in auranofin-treated cells. Studies on the effect of auranofin on the protein and phospholipid components of MER and on its capacity to induce adoptive rosetting need to be done.

Effect on cell viability

Auranofin is cytotoxic. Prolonged culture of B-CLL cells with auranofin (eg 4 hours with 60μM auranofin or 22 hours with

10µM auranofin) caused membrane blebbing and considerable loss of viability.

Inhibition of rosetting was not, however, a result of cell death since cell viability was unaffected by incubation with auranofin (60µM) for up to 90 minutes as determined by phase contrast microscopy, exclusion of trypan blue (Table 6.2) and failure to release the cytosolic enzyme lactate dehydrogenase. Thus, the mean percent release of total cellular lactate dehydrogenase was 8.2 (sem 0.02, n=3) in control-treated cells and 7.7 (0.04) in cells treated for 40 minutes with 60uM auranofin. In most experiments described here, incubation with auranofin did not exceed 40 minutes.

Effect of other gold compounds

Another lipophilic gold drug chloro-triethylphosphine gold inhibited rosetting in B-CLL cells with an EC_{50} of 15µM.

In contrast, the water-soluble gold (I) complex sodium aurothiomalate did not inhibit rosetting at concentrations up to 2.5mM. Rosetting was 107% (sem 3.5%, n=3) of control value in cells treated with 2.5mM sodium aurothiomalate.

Role of thiols

At concentrations which did not affect rosetting, 2-ME almost completely blocked the effects of auranofin on rosetting (Fig 6.5). The membrane-impermeable thiol glutathione which does not affect rosetting itself at any concentration, also prevented the inhibition of rosetting by auranofin. These thiols did not prevent the inhibition of MER by PDBu (Fig 6.5).

Role of calcium

The inhibition by auranofin (0.6-60µM) was not increased in the presence of calcium ionophore A23187 (1µM) and not prevented

by 2mM EDTA or EGTA suggesting that the mechanism is independent of calcium.

Measurement of cell-associated gold

When B-CLL cells were treated with 60μM auranofin for 40 minutes at 37°C in the presence of 10% FCS, they accumulated about 0.5μg or 2.5 nmoles of gold per 10^6 cells (Table 6.3). This was within the range of the levels of cell-associated gold found in leukocytes of patients with rheumatoid arthritis who had been receiving oral auranofin therapy for a period of 1 to 6 months.

6.2.3 EFFECT OF ZINC

Effect of zinc on MER rosetting

Zinc chloride (at concentrations up to 1mM) had no effect on rosetting of B-CLL cells with mouse erythrocytes. However, 50μM of the lipophilic zinc pyrithione inhibited rosetting by 84% (sem 0.6%, n=3).

The zinc-free sodium salt of pyrithione (50μM) partially inhibited MER (by 37% sem 2.4%, n=3), possibly by forming a complex with zinc in the culture medium. Pyrithione alone did not affect rosetting when the cells were treated in the absence of FCS (and therefore in a zinc-free medium). However, when a combination of pyrithione and 100μM zinc chloride was added to B-CLL cells, rosetting was inhibited by 98% (sem 1.4%, n=3). These experiments suggest that pyrithione is enhancing uptake of zinc by the cells and that intracellular zinc is required to inhibit MER.

Fifty per cent inhibition of rosetting occurred when cells were incubated with 20μM pyrithione and 3μM zinc chloride for 40 minutes at 37°C in a serum-free medium (Fig 6.6).

Effect of zinc on cell viability

Concentrations of zinc chloride up to 1mM and zinc

pyrithione up to 100μM did not affect the viability of B-CLL cells as determined by trypan blue dye exclusion and phase contrast microscopy.

Content of zinc in cells

The content of total (free and bound) zinc in B-CLL cells was determined by atomic absorption spectroscopy. In 10^7 cells there were 2.75 nmoles of zinc. This agrees with values of about 1-2.5 nmoles of zinc per 10^7 cells for normal lymphocytes and PMN (chapter 1.12.2). Assuming a mean cell volume of about 230 femtolitres, this corresponds to about 1.2 mM total zinc, similar to the concentration estimated for other types of cells [Williams 1984].

Studies were done with ^{65}zinc to measure how much zinc is taken up by the cells, under conditions used in these experiments. Incubation of cells with ^{65}zinc, alone, resulted in very little cell-associated zinc after three washes of the cells. In the presence of 50μM pyrithione, there was a large uptake of ^{65}zinc at 37°C although not at 4°C. The amount of zinc taken up by cells exposed to 100μM zinc and 50μM pyrithione, during a 40 minute incubation at 37°C, was 2.4nmoles per 10^7 cells, corresponding to an almost doubling of the total cellular zinc. Cells incubated with 10μM zinc and 50μM pyrithione took up much less zinc, 0.38nmoles per 10^7cells. These studies do not indicate how much of the zinc is inside the cell and how much is bound to proteins on the outside.

6.2.4 EFFECT OF METAL CHELATING-AGENTS

Divalent cation-chelating agents

EDTA at 2mM completely prevented the increase in rosette inhibition induced by A23187 in PDBu-treated B-CLL cells. Data

for this is presented in the next chapter (see Fig 7.1A) as part of a larger experiment which compares the effects of EDTA on both rosette inhibition and binding of PDBu to B-CLL cells.

In addition, both EGTA (not shown) and EDTA partially prevented the inhibition of MER by PDBu (Fig 6.7) and by fluoride, although they had no effect on inhibition of MER by a high concentration (100nM) of TPA, auranofin or dithiothreitol.

Zinc chelating-agents

The zinc-chelating agent 1,10 phenanthroline (1mM) prevented the inhibition of rosetting by 20uM pyrithione and 50uM zinc chloride (Fig 6.6, 6.9).

Phenanthroline also blocked the inhibition of rosetting by TPA, PDBu and fluoride (Fig 6.8, 6.9, 6.10) but had no effect on inhibition by dithiothreitol or auranofin (Fig 6.9). The effect of phenanthroline on phorbol ester-mediated inhibition of MER could not be prevented by addition of 1mM zinc or calcium chloride (Fig 6.10). The reason for the failure of zinc to reverse the effect of phenanthroline is unclear (see discussion).

Preliminary experiments indicate that TPEN another agent which chelates zinc but not calcium [Arslan et al. 1985], also decreases the effects of phorbol ester on MER. TPEN (1mM) decreased the inhibition of rosetting induced by 100nM PDBu from 86% (sem 2%, n=3) to 60% (sem 6%) and decreased the inhibition of rosetting induced by 100nM TPA from 92% (sem 1%, n=3) to 64% (sem 5%).

6.3 DISCUSSION

These results suggest that both calcium ions and zinc ions play essential roles in the inhibition of MER by phorbol esters.

Calcium ionophores synergize with phorbol esters and diacylglycerol in many cellular events dependent on PKC,

including activation of B-CLL cells [Drexler et al. 1987], normal B cells [Ransom and Cambier 1986], T cells [Truneh et al. 1985], PMN [Dale and Penfield 1984, Robinson et al. 1984] and HL60 cells [Tyers and Harley 1986]. Consistent with the hypothesis that inhibition of MER is an early event in B cell activation, calcium ionophores also synergized with diacylglycerol and PDBu in inhibition of MER.

A rise in $[Ca^{++}]_i$ alone, induced by calcium ionophore, was insufficient to inhibit MER but sensitized B-CLL cells to inhibition of MER by both diacylglycerol and sub-optimal concentrations of PDBu. A23187 inserts into cell membranes and affects the interaction of proteins with the membrane phospholipids [Klausner et al. 1979]. It is unlikely to be affecting PDBu-mediated inhibition of MER by this non-specific mechanism since the effect of A23187 was blocked by divalent cation chelating agents.

The requirement for calcium ions in addition to diacylglycerol or phorbol ester for effective inhibition of MER explains why fluoride, which causes a rise in both $[Ca^{++}]_i$ and diacylglycerol is an effective inhibitor of MER whereas diacylglycerol alone is a poor inhibitor.

Like its actions on PKC-dependent responses in platelets and fibroblasts, auranofin mimicked phorbol esters in inhibition of MER. The effect was due to the gold moiety suggesting that metals other than calcium may also play a role in the inhibition of MER.

A major question is whether auranofin acts in the same manner as phorbol esters or by a different mechanism. Effects of auranofin on binding of ^{3}H-PDBu to B-CLL cells are discussed in

chapter 8.

Auranofin still inhibited expression of epidermal growth factor receptors in A431 cells which had been depleted of most of their PKC by chronic treatment with phorbol ester [Froscio et al. 1987]. This suggests that the mechanism of action of auranofin on this receptor is not via PKC although it cannot be excluded that the PKC involved in inhibition of epidermal growth factor is a minor pool of PKC or an isozyme which is less susceptible to down-regulation.

Similarly, the mechanism by which auranofin inhibits MER is unclear. Because the loss of MER in phorbol ester-treated cells is irreversible, it is not possible to deplete B-CLL cells of PKC by prolonged treatment with phorbol ester and then determine whether auranofin still inhibits MER. Although the inhibition of rosetting by auranofin resembled that induced by phorbol ester in that there was no loss or destruction of MER, the action of phorbol esters on rosetting was strongly dependent on calcium whereas the action by auranofin was independent of calcium. Membrane-permeable thiols also inhibited rosetting by a calcium-independent pathway.

The action of auranofin on MER was completely prevented by the presence of the membrane-impermeable thiol glutathione. Glutathione probably acts by competing with membrane protein thiols for the gold in auranofin, thereby inhibiting the uptake of the gold by the cells.

Auranofin and membrane-permeable thiols may inhibit rosetting by interacting with an intracellular sulphydryl group that is essential for rosetting. It is necessary to determine whether auranofin affects the expression of other types of receptors that are detected by rosetting assays, since auranofin

may affect the mechanism of rosetting rather than having a specific effect on the receptor.

The effects of auranofin on MER were mimicked by the lipophilic zinc pyrithione. Inhibition also occurred if sodium pyrithione and zinc chloride were given together suggesting that pyrithione can be used as a zinc ionophore.

Uptake of zinc by B-CLL cells required pyrithione but was also dependent upon the concentration of zinc. By analogy with the mechanism for uptake of gold, the first step may be interaction of zinc with membrane sulphydryls, perhaps by exchanging one of the bound pyrithione ligands. Because of its lipophilicity, the other pyrithione may then facilitate transfer of the zinc across the bilayer and into the cell.

Pyrithione should be very useful in studies of the effect of zinc on intracellular pathways and its ionophoretic activity needs to be fully characterized.

A rise in intracellular zinc ions was sufficient by itself to inhibit MER. Zinc is therefore acting in a manner distinct from that of calcium. Furthermore, chelation of zinc with phenanthroline and TPEN partially prevented the effects of phorbol esters and fluoride on MER suggesting that zinc is required for the effects of these stimuli on MER. Phenanthroline and TPEN do not chelate calcium [Martel and Calvin 1952]. The effects of phenanthroline could not be prevented by the addition of 1mM zinc chloride. It may be that more zinc than this is required to saturate phenanthroline. It appears likely that phenanthroline, like TPEN (Arslan et al. 1985), acts at an intracellular site. Phenanthroline does not transport ^{65}zinc into B-CLL cells [C. Giannakis Personal communication] suggesting that

if phenanthroline is taken up by the cells it does not go into the cells as a zinc-co-ordinated complex. Hence, even though phenanthroline is saturated with the zinc chloride outside of the cell, the cell-associated phenanthroline may be free of zinc and still capable of chelating intracellular zinc. Further experiments are underway in our laboratory to determine whether phenanthroline acts at an intracellular site in B-CLL cells.

Since EDTA and EGTA also chelate zinc more tightly than calcium [Bartfai 1979] the interpretation of the effects of these agents on inhibition of MER by phorbol esters and fluoride remains unclear. They may be acting by chelating calcium or zinc, or both. This raises questions of whether some of the effects of these agents on PKC-dependent responses in cells that have previously been interpreted as due to chelation of calcium, might not, at least in part, also be due to chelation of zinc.

Recent studies have also suggested a role for zinc in the translocation of PKC (see chapter 9) and in the attenuation of Ca^{2+} signals induced by phorbol esters [unpublished results referred to in Csermely et al. 1987b].

Phenanthroline and EDTA had no effect on the inhibition of MER by auranofin or dithiothreitol. This indicates that these agents do not inhibit MER by displacing zinc from intracellular structures which in turn inhibits rosetting. It is more likely that the gold acts similarly to zinc.

It remains to be determined whether these effects of auranofin in vitro are relevant to its therapeutic action in vivo. The MER+ve, CD5+ve B cell, helper T cells, PMN and monocytes are all potential cellular targets for auranofin, in-vivo. Chronic activation of lymphoid cells and inflammatory phagocytes in rheumatoid arthritis is mediated at least in part

by processes involving PKC [Hurst 1987]. Auranofin may suppress cell activation by interfering with PKC.

Incubation of cells with 20μM or 60μM auranofin for up to 90 minutes produced cellular levels of gold within the range found in mononuclear cells from patients undergoing treatment with auranofin. Under these conditions MER was inhibited markedly, suggesting that the findings in vitro may be relevant to the therapeutic actions of auranofin. However, it is not known whether cellular uptake of gold in vitro results in a distribution of gold amongst various thiol-containing proteins in the B-CLL cell that is similar to that occurring in-vivo. Studies of the effect of auranofin in vivo on the expression of MER and binding of ^{3}H-PDBu in blood cells of patients with rheumatoid arthritis would be of considerable interest.

Based on studies with electron microscopic X ray analysis, it has been suggested that the nuclei of B-CLL cells are deficient in zinc [Yarom et al. 1976]. However, no deficiency of zinc in B-CLL cells relative to other types of blood cells from normals was seen when zinc was assayed by atomic absorption spectroscopy.

Effects of zinc on MER were seen at micromolar concentrations of total zinc. It requires to be shown that changes are induced in MER at concentrations of free zinc ions found within the cell. In a resting cell, there is little free zinc in the cytosol because most of it is tightly bound by metallothionein [Williams 1984]. It is possible that certain signals cause a flux of zinc within cells analagous to the flux of calcium. For example, TPA induces release of zinc from the nucleus and mitochondria [Csermely et al. 1987a]. Probes for

the measurement of free intracellular zinc (analagous to quin-2 and indo-1 for free calcium ions) are required for these studies. It is important now to determine the effects of zinc pyrithione on proliferation and differentiation in normal and leukaemic B cells and other cell types. Fluxes of intracellular magnesium [Alvarez-Leefmans et al. 1987] and sodium [Severini et al. 1986] have also been implicated in early activation events in cells. The role of these metals in B cell activation needs to be determined.

Some of these results have been published in Zalewski et al. 1986a, Hurst et al. 1986.

Table 6.1 Effect of A23187 on kinetics of inhibition of rosetting

Stimulus	% Inhibition of Rosetting after		
	20	40 (minutes)	60
50nM PDBu	0	8	20
" " + A23187	20	70	75
100nM PDBu	0	35	40
" " + A23187	50	80	90

B-CLL cells were pre-treated with or without A23187 (1uM) for 20 minutes in RPMI before addition of PDBu (50 or 100nM).

Cells were harvested after a further 20, 40 or 60 minutes and assayed for rosetting. % inhibition of rosetting is indicated.

Table 6.2 Effect of auranofin on viability of B-CLL cells

Concentration of auranofin (uM)	Mean % viable cells: Incubation (hours) 1.5	5	22
0	96	97	82
0.6	97	94	82
6	93	94	60
60	92	30	10

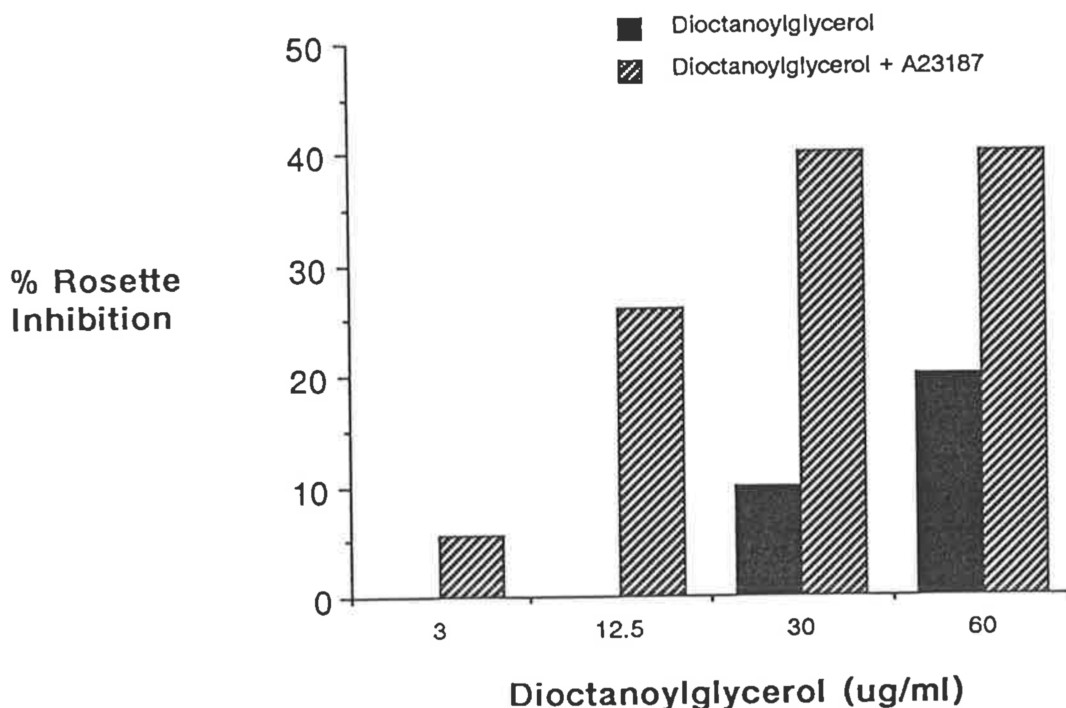
B-CLL cells (10^7/ml) were incubated in RPMI/FCS with auranofin at the indicated concentrations and at 1.5, 5 and 22 hours, cells were assayed for viability by exclusion of trypan blue dye. Data are means of duplicates.

Table 6.3 **Intracellular levels of gold after incubation of of B-CLL cells with auranofin**

Incubation time (minutes)	Cell-associated gold** ($ug/10^6$ cells)	
	20uM Auranofin	60uM Auranofin
0	0.05 (0.01)	0.06 (0.01)
30	0.21 (0.02)	0.49 (0.02)
60	0.48 (0.04)	0.53 (0.10)
90	0.46 (0.09)	0.71 (0.10)

B-CLL cells were incubated with auranofin (20 or 60uM) for 30 minutes at 37°C in RPMI/FCS. Cells were washed and gold content of pellets determined by atomic absorption spectroscopy.

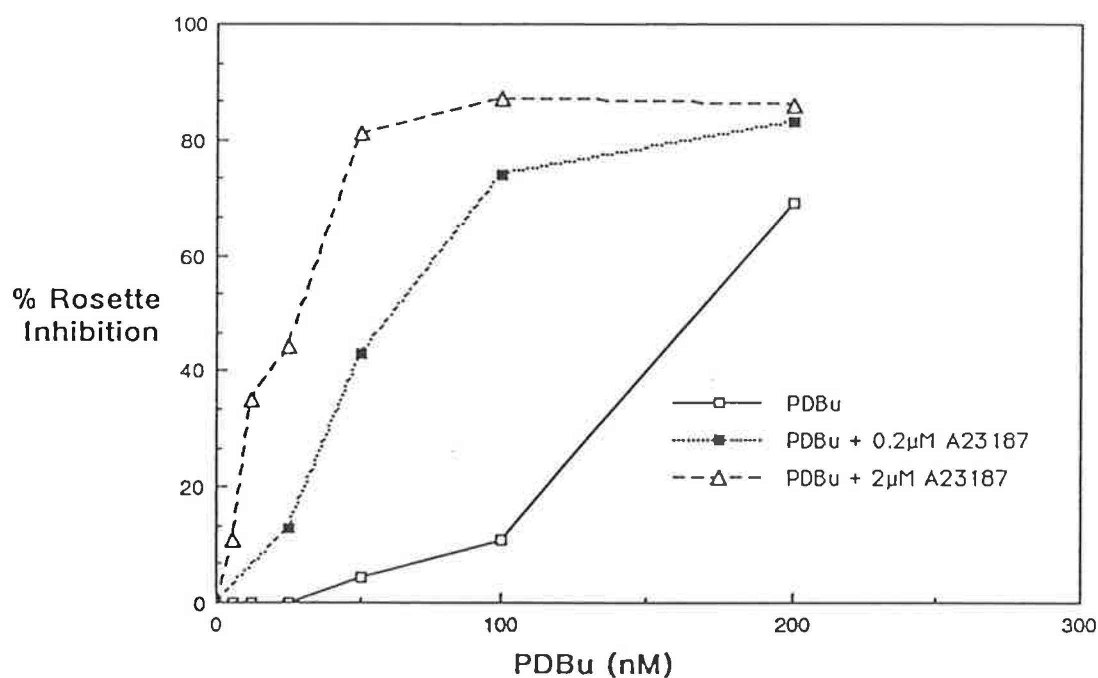
** Data (mean of triplicates with standard error in parentheses) refer to gold (ug) per 10^6 cells.



Legend to Figure 6.1 Effect of A23187 on inhibition of MER by diacylglycerol

Calcium ionophore A23187 greatly enhanced inhibition of rosetting by 1,2-dioctanoylglycerol (DOG), without itself inhibiting rosetting.

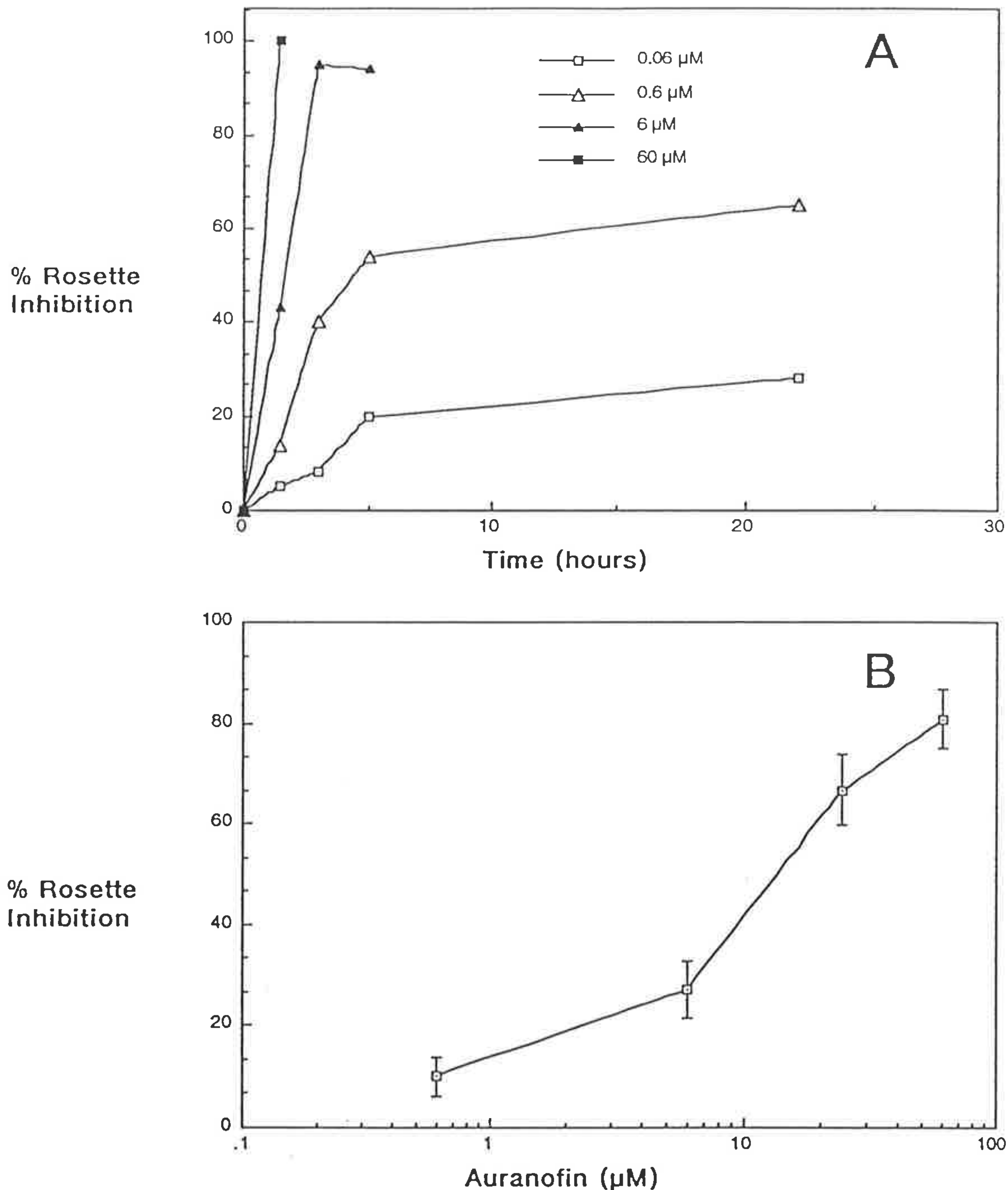
B-CLL cells were treated with varying concentrations of DOG in the presence or absence of 1μM A23187 at 37°C in RPMI. Where present, A23187 was added 20 minutes before DOG. Forty minutes after addition of DOG, cells were washed and assayed for rosetting with mouse erythrocytes, in duplicates.



Legend to Figure 6.2 Effect of A23187 on inhibition of MER by PDBu

A23187 greatly enhanced the inhibition of rosetting by sub-optimal concentrations of PDBu, without itself inhibiting rosetting.

B-CLL cells were treated with varying concentrations of PDBu in the presence or absence of 0.2 or 2μM A23187 at 37°C in RPMI. Where present, A23187 was added to the cells 20 minutes before PDBu. Forty minutes after addition of PDBu, cells were washed and rosetted with mouse erythrocytes, in duplicate. Data are expressed as mean % inhibition relative to control.



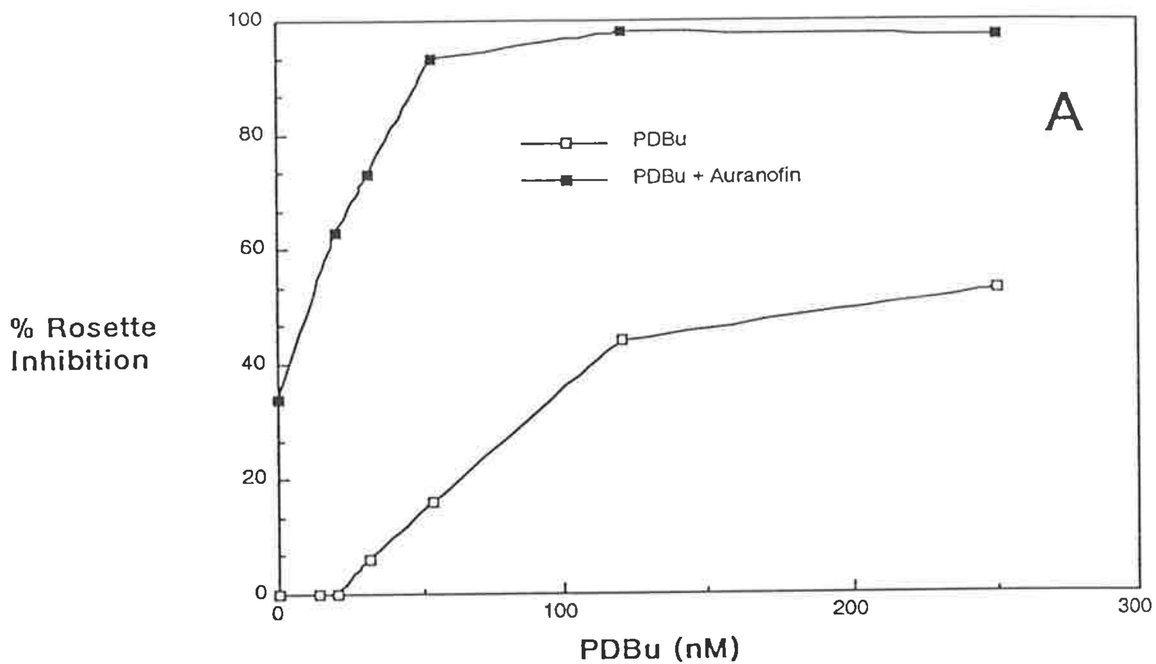
Legend to Figure 6.3 Effect of auranofin on MER rosetting

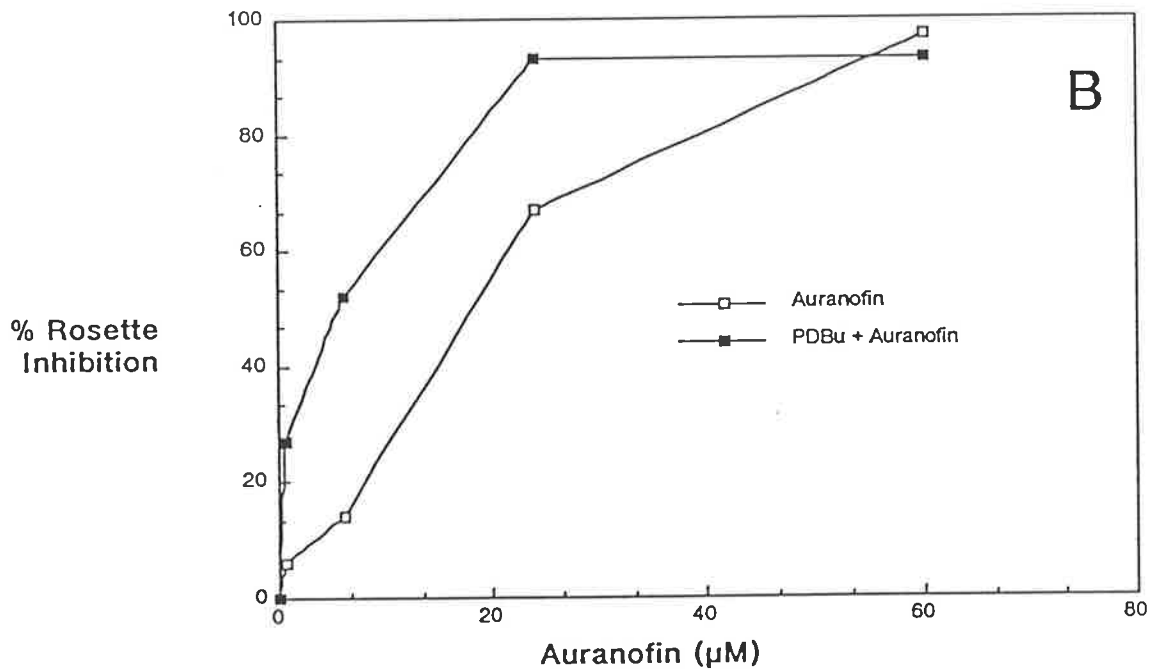
Auranofin inhibited rosetting in a time- and concentration-dependent manner.

B-CLL cells were incubated with auranofin at 37°C in RPMI/FCS, washed and assayed for rosetting with mouse erythrocytes, in duplicate. Data are expressed as % rosette inhibition relative to control (no auranofin).

A: Kinetics. Cells were incubated for varying periods of time, with concentrations of auranofin indicated.

B: Concentration-dependence. Cells were incubated for 40 minutes with varying concentrations of auranofin. Data were pooled from 12 separate experiments with cells from five different populations of B-CLL. Bars indicate standard errors.

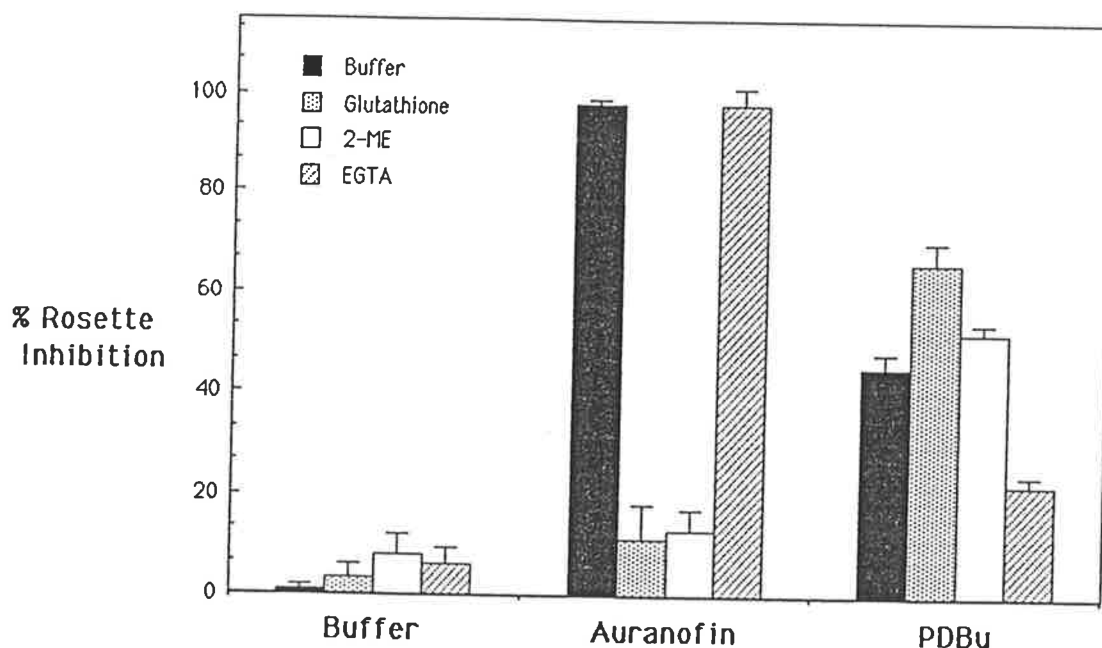




<u>Legend to Figure 6.4</u> <u>Effect of auranofin on PDBu-mediated inhibition of MER</u>

Auranofin synergized with PDBu in inhibition of MER.

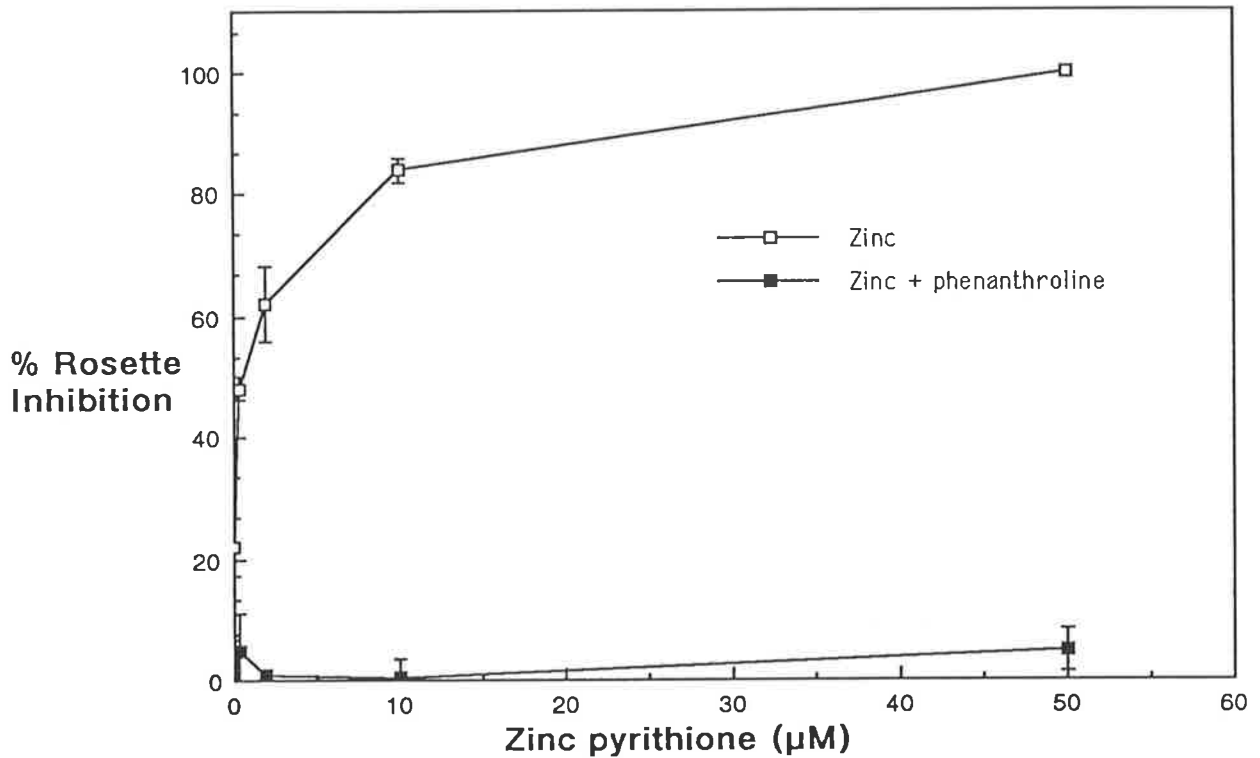
B-CLL cells were incubated with or without 6μM auranofin for 15 minutes at 37°C in RPMI/FCS and then varying concentrations of PDBu were added and cells incubated for a further 30 minutes. Cells were washed and rosetted with mouse erythrocytes, in duplicate. Data are expressed as % rosette inhibition.



<u>Legend to Figure 6.5</u> <u>Effect of thiols and EGTA on modulation of MER by auranofin</u>

The inhibition of rosetting by auranofin was prevented by competing low-molecular weight thiols, glutathione and 2-ME, but not by a divalent-cation chelator EGTA. 2-ME was used at a concentration which did not itself inhibit rosetting.

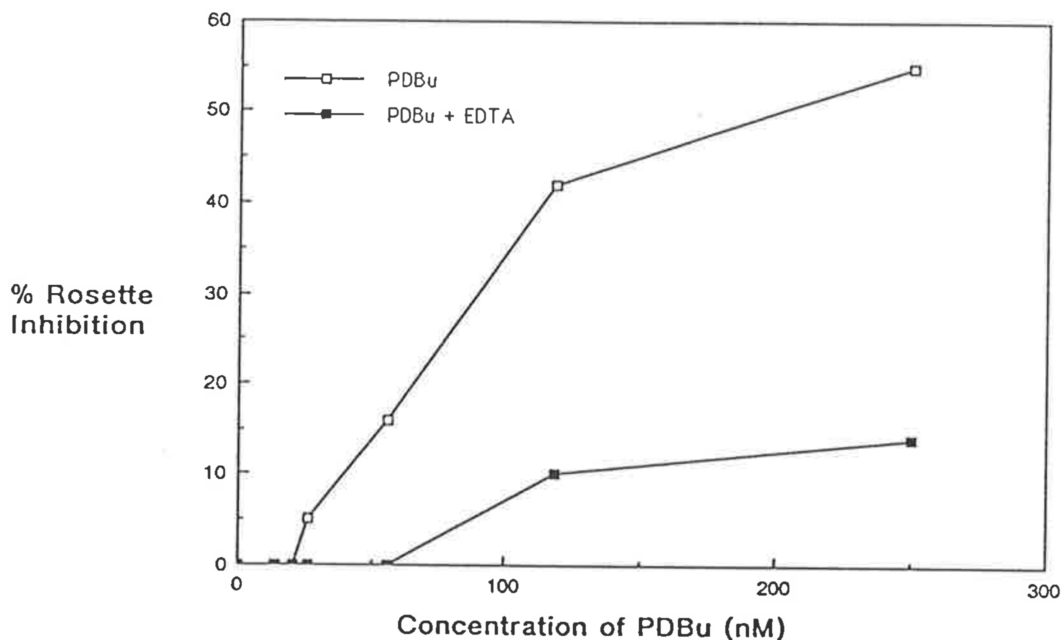
B-CLL cells were pre-incubated for 10 minutes at 37°C in RPMI/FCS with glutathione (10mM), 2-ME (1mM), EGTA 10mM or control buffer before addition of auranofin (60uM), PDBu (200nM) or control solvent DMSO (0.1%v/v). After a further 40 minutes, cells were washed and rosetted with mouse erythrocytes, in triplicate. Data are expressed as % rosette inhibition relative to control (incubated with buffer alone). Bars indicate standard errors.



Legend to Figure 6.6 Effect of zinc on rosetting

Zinc pyrithione inhibited rosetting with mouse erythrocytes and this was prevented by the zinc chelating-agent phenanthroline.

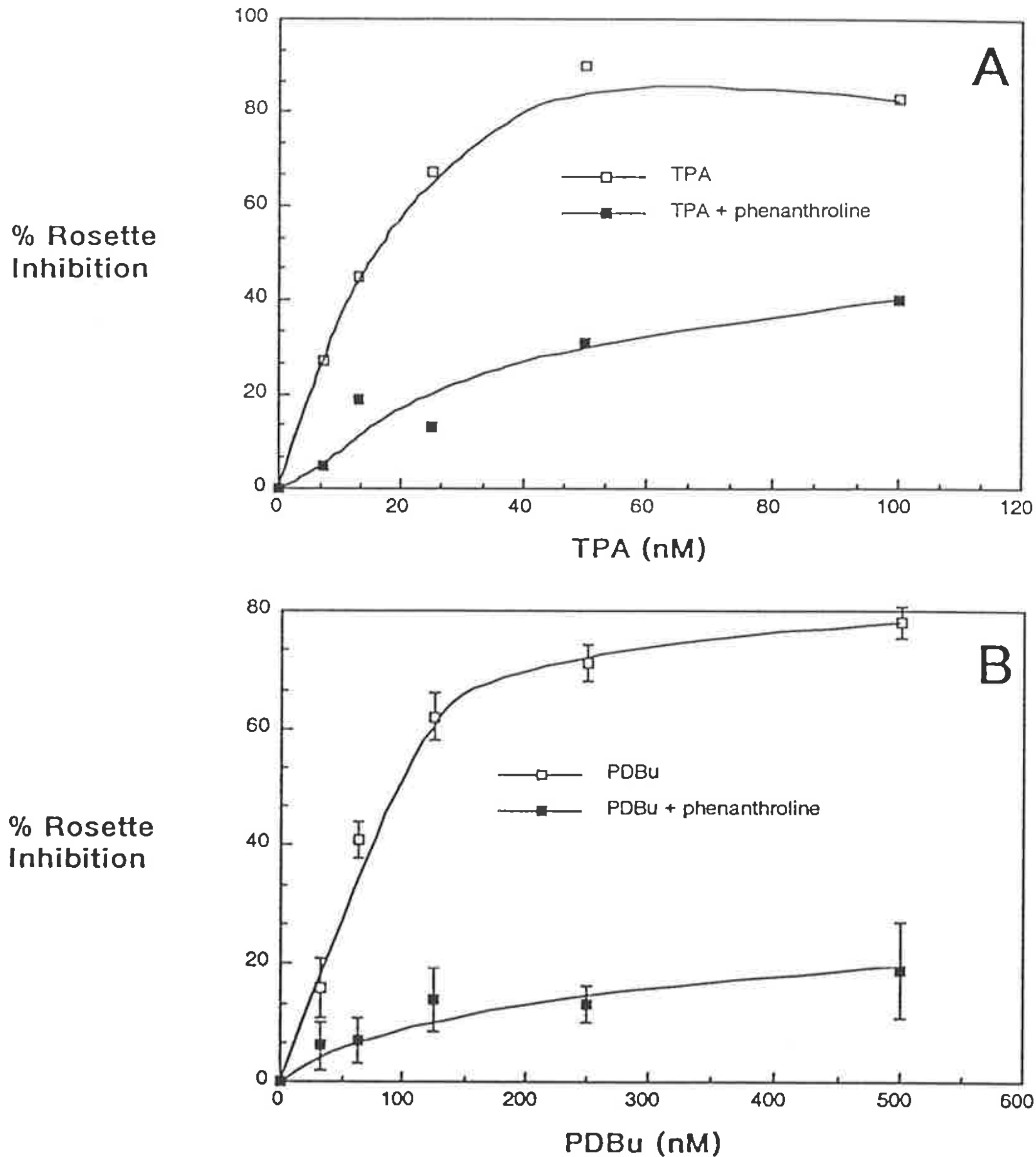
B-CLL cells were incubated with or without 1mM phenanthroline for 10 minutes at 37°C in RPMI, before addition of varying concentrations of zinc pyrithione. After 40 minutes, cells were washed and rosetted with mouse erythrocytes. Data are expressed as % inhibition of rosetting relative to control (incubation with buffer alone). Bars indicate standard errors (n = 3).



Legend to Figure 6.7 Effect of EDTA on inhibition of rosetting by PDBu

The divalent cation chelator EDTA decreased the inhibition of MER rosetting by PDBu.

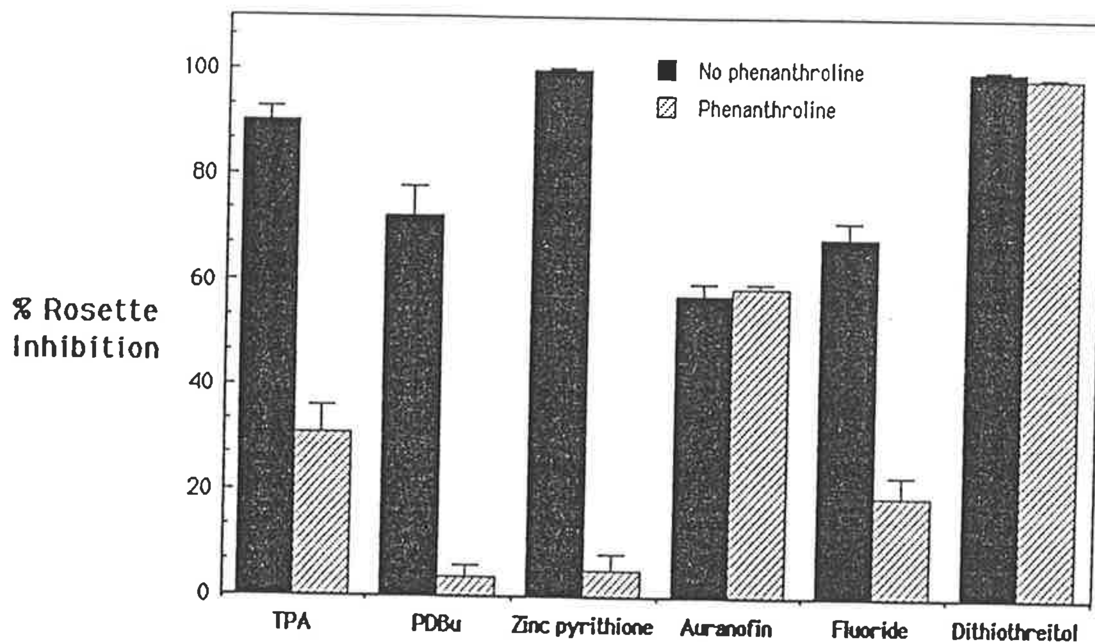
B-CLL cells were incubated with or without EDTA (10mM) for 10 minutes at 37°C in RPMI/FCS before addition of varying concentrations of PDBu or control buffer. After 60 minutes, cells were washed and rosetted with mouse erythrocytes, in duplicate. Data are expressed as % inhibition of rosetting relative to control (incubation with buffer alone).



Legend to Figure 6.8 Effect of phenanthroline on inhibition of rosetting by phorbol ester

Phenanthroline greatly decreased the effects of both TPA (panel A) and PDBu (panel B) on MER rosetting.

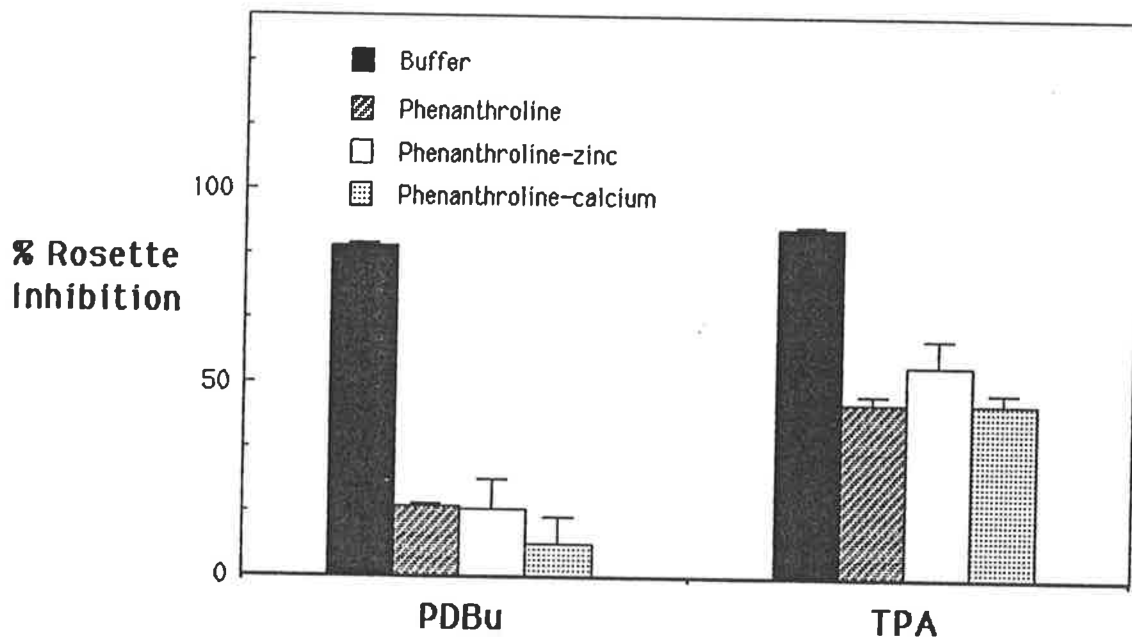
B-CLL cells were incubated with or without phenanthroline as in Fig 6.6, before addition of varying concentrations of PDBu or TPA. After 60 minutes, cells were washed and rosetted with mouse erythrocytes. Data are expressed as % inhibition of rosetting relative to control (incubation with buffer alone). In panel A, rosettes were counted in duplicates and means shown. In panel B, rosettes were counted in triplicates and bars show standard errors.



Legend to Figure 6.9 Effect of phenanthroline on inhibition of rosetting by various stimuli

Phenanthroline decreased the effects of PDBu, TPA, zinc pyrithione and fluoride on rosetting but had no effect on inhibition of rosetting by auranofin or dithiothreitol.

B-CLL cells were incubated with and without phenanthroline as in Fig 6.6 and then TPA (100nM), PDBu (400nM), zinc pyrithione (20uM pyrithione, 50uM zinc chloride), 60uM auranofin, 1mM dithiothreitol or control buffer were added. After 60 minutes, cells were washed and rosetted with mouse erythrocytes in triplicate. Data are expressed as % inhibition of rosetting relative to control (incubation with buffer alone). Bars indicate standard errors.



Legend to Figure 6.10 Effect of zinc and calcium on protection by phenanthroline

Phenanthroline decreased effects of phorbol esters on MER and pre-treatment of 1mM phenanthroline with 1mM zinc chloride or 1mM calcium chloride did not overcome this (see Discussion).

B-CLL cells were pre-treated for 10 minutes at 37°C in RPMI with or without 1mM phenanthroline, 1mM phenanthroline + 1mM zinc chloride or 1mM phenanthroline + 1mM calcium chloride. TPA (400nM), PDBu (400nM) or control buffer were added. After 60 minutes, cells were washed and rosetted with mouse erythrocytes. Data are expressed as % inhibition of rosetting relative to control (incubation with buffer alone). Bars indicate standard errors (n=3).

CHAPTER SEVEN

EFFECT OF CALCIUM ON PHORBOL ESTER RECEPTORS

7.1 INTRODUCTION

In the preceding chapter it was shown that calcium ionophores synergized with phorbol esters and diacylglycerol in the inhibition of MER. One site of action of calcium may be PKC since at least some of the isozymes are dependent upon calcium, both for binding of phorbol esters and catalytic activity (chapter 1.7, 1.8).

Although it has been established that the affinity of soluble PKC for phorbol ester is influenced by the concentration of calcium in the environment of PKC [König et al. 1985], much less is known about the effects of $[Ca^{++}]_i$ on the binding of phorbol ester to PKC in intact cells. Prior to the experiments reported here, it had been shown that A23187 increases binding of ^{3}H-PDBu in HL60 cells, apparently by increasing the affinity of the receptors [May et al. 1985b].

The aim of the experiments presented in this chapter was to study the effects of calcium ionophores (A23187 and ionomycin), calcium-mobilizing agents (fluoride and anti-immunoglobulin) and calcium-chelating agents, on PDBu binding to B-CLL cells and other types of cells. Correlation was sought between effects on PDBu binding and effects on MER.

7.2 RESULTS

7.2.1 EFFECT OF CALCIUM IONOPHORES

Augmentation of binding

A23187 (1μM) enhanced the specific binding of ^{3}H-PDBu, (Fig 7.1B), in parallel with its enhancement of PDBu-mediated inhibition of MER (Fig 7.1A). Non-specific binding was not increased. As with rosette inhibition, calcium ionophore was most effective at low concentrations of ^{3}H-PDBu and binding was not enhanced at a high concentration (200nM) of ^{3}H-PDBu.

Augmented binding was seen at concentrations of A23187 as low as 0.2μM (Fig 7.2). Fifty per cent increase in PDBu binding occurred at concentrations of A23187 of 0.45 and 0.57μM in two separate experiments with different populations of B-CLL cells. Ionomycin (0.5μM) also increased specific binding of ^{3}H-PDBu to B-CLL cells (Table 7.1).

Other cells

A23187 increased binding of PDBu in a variety of other types of cell, excluding red cells which lack the receptors for PDBu (Table 7.1). A fifty percent increase in binding of ^{3}H-PDBu to PMN was induced by A23187 at a concentration of 0.24μM (sem 0.08μM, three different populations of normal PMN). Increased binding in PMN could only be detected if 10% FCS was present during treatment with A23187. The reason for this was not investigated further.

Kinetics

The increase in binding in A23187-treated cells occurred regardless of whether cells were pre-equilibrated with ^{3}H-PDBu before addition of A23187 (Fig 7.3) or whether both agents were added together (Fig 7.4). The effect of A23187 was rapid, occurring within 5 minutes (the first time point assayed). A23187 increased the initial rate of binding of ^{3}H-PDBu but did not alter the overall shape of the curve (Fig 7.4).

In one of three populations of B-CLL cells and in both populations of PMN tested (one shown in Fig 7.4), the increase in PDBu binding was transient, decreasing to control levels with time. Thus, 20 minutes after the addition of ionophore, binding was increased by about 140% over that in control cells whereas after 60 minutes it was only increased by about 70% (Fig 7.4),

possibly because of accelerated down-regulation of PDBu receptors in the ionophore-treated cells.

Effect on receptor numbers and affinity

The increase in binding in A23187-treated cells largely resulted from an increase in affinity of PDBu receptors as shown by Scatchard plots. In the absence of FCS, the Scatchard plot changed from curvilinear to linear, interpreted as a change from a mixture of a few very high affinity receptors (Kd 5nM) and a tenfold excess of lower affinity receptors (Kd 83nM) to a single class of very high affinity receptors (Kd 9nM) (Fig 7.5A). In the presence of FCS, the single class of lower affinity receptors (Kd 57nM) was converted to a single class of higher affinity receptors (Kd 9nM) by treatment of the cells with A23187 (Fig 7.5B). Maximum binding capacity of the cells was not changed.

Similarly, increase in binding to PMN resulted from an increase in the affinity of PDBu receptors (Kd decreased from 27 to 10nM), without large change in the maximum binding capacity of the cells (Fig 7.6).

7.2.2 EFFECT OF CALCIUM-MOBILIZING AGENTS

Anti-Ig

Anti-Ig (50 to 100μg/ml) increased to a small extent (about 15%) the capacity of B-CLL cells to bind ^{3}H-PDBu (Fig 7.7). For a population of B-CLL cells expressing abundant delta heavy chains and lambda light chains and small amounts of mu and gamma heavy chains, the largest increase in binding of ^{3}H-PDBu was induced by anti-delta and anti-lambda chain antisera. Anti-mu and anti-gamma chain antisera induced small increases and anti-kappa chain antiserum was without effect (Fig 7.7). There was a small increase in binding in the first 4 minutes and a larger increase after 15 minutes in anti-IgD-treated B-CLL cells (Fig

7.7).

Fluoride

Fluoride induced a much larger increase than anti-Ig in PDBu binding in B-CLL cells. The same concentration of fluoride that inhibited MER, and which is known to increase $[Ca^{++}]_i$ in some cells, increased PDBu binding in B-CLL cells and PMN (Fig 7.8, 7.9) as well as in normal peripheral blood mononuclear cells (not shown). The increase in binding of ^{3}H-PDBu occurred after a lag period of about 10 minutes. Scatchard analysis of binding data showed that fluoride increased the affinity of binding of ^{3}H-PDBu two-fold without changing the apparent total number of receptors in both B-CLL cells and PMN (Fig 7.9).

Since fluoride also increases cAMP levels in cells, the effect of a rise in cAMP on PDBu binding was studied. No increase in PDBu binding in B-CLL cells was seen with dibutyryl cAMP (1mM) in combination with theophylline (1mM), adrenaline (5μM) or prostaglandin E_2 (30μM).

7.2.3 EFFECT OF DIVALENT CATION-CHELATING AGENTS AND TMB-8

EDTA, EGTA and TMB-8 partially inhibited specific binding of ^{3}H-PDBu to B-CLL cells and PMN and prevented the increase in binding induced by A23187 (Fig 7.1B, Table 7.2).

7.2.4 EFFECT OF CALCIUM ON TRANSLOCATION OF PKC

Effect of calcium on translocation to particulate fraction

Binding of ^{3}H-PDBu to cells involves binding to PKC that is already in the membrane as well as binding to PKC that is newly translocated from the cytosol as a result of exposure of the cell to ^{3}H-PDBu (chapter four). Pre-treatment of B-CLL cells with calcium ionophore may augment subsequent binding of ^{3}H-PDBu either by directly causing PKC to translocate to the membrane or

by enhancing the translocation of PKC from cytosol to membrane that occurs following exposure of cells to ^{3}H-PDBu.

To investigate this, B-CLL cells were treated with A23187 alone or in combination with unlabelled PDBu, the cells were washed, particulate and cytosolic fractions were prepared and the content of PKC in these was determined by binding of 20nM ^{3}H-PDBu.

Treatment of cells with A23187 (1μM) alone had no effect on the content of PKC in the cytosol or particulate fraction. Treatment of cells with a very low concentration (10nM) of PDBu induced only a small amount of translocation. However, when cells were treated with both agents together there was a marked translocation of PKC from cytosol to particulate fraction. The content of PDBu receptors in the particulate fraction increased by 67% (sem 2.8%, n=3) and the content of PDBu receptors in the cytosol decreased by 27% (sem 1%), relative to cells treated with 10nM PDBu alone. Nearly all of this increased PKC was recovered from the particulate fraction by Triton X-100 extraction.

When cells were treated with a much larger concentration (200nM) of PDBu there was significant translocation of PKC from cytosol to particulate, involving about 30% of the cytosolic aporeceptors, and this was not further enhanced by pre-treatment of the cells with A23187. This result is in agreement with the observations that calcium ionophore does not augment binding of PDBu and does not increase rosette inhibition by PDBu, when a high concentration (200nM) of PDBu is used.

Effect of calcium on translocation to chelator-labile pool

Normally, the cytosol and particulate fractions are prepared in the presence of calcium-chelating agents (EDTA and/or EGTA) to prevent degradation of PKC by calcium-dependent proteases

released during cell fractionation and to remove any PKC that is loosely bound to the particulate fraction by calcium, and therefore not true membrane PKC (chapter 1.9.1).

To investigate whether, by causing a rise in $[Ca^{++}]_i$, calcium ionophores increase the pool of PKC that is loosely attached to the particulate fraction, the following experiment was done. B-CLL cells were treated with calcium ionophore, phorbol ester or control solvent DMSO (0.1%), washed and the sub-cellular fractionation performed in the absence of calcium-chelating agents. Under these conditions, A23187 or ionomycin, alone, caused a large increase in the content of PKC in the particulate fraction (Fig 7.10). This increase did not occur if EDTA was in the sonication buffer. On the other hand, PDBu increased both the tightly bound pool and the loosely associated PKC. Thus, in intact cells, calcium ionophores by themselves induce a loose, calcium-dependent association of PKC with the particulate fraction but this PKC only becomes tightly associated in the presence of phorbol ester.

7.3 DISCUSSION

At least one site of action of calcium in the inhibition of MER is on the binding of PDBu to its receptor. The calcium ionophores increased the binding of ^{3}H-PDBu to B-CLL cells and PMN, without affecting binding at saturating concentrations of ^{3}H-PDBu.

EDTA, EGTA and TMB-8 partially inhibited binding of PDBu, consistent with their effect on PDBu-mediated inhibition of MER. Since the chelating agents do not penetrate cells very well it would appear that binding of ^{3}H-PDBu to its receptor in B-CLL cells is partially dependent upon the concentration of

extracellular cations. Since EGTA and EDTA react strongly with zinc and other divalent cations in addition to calcium, the effects of these agents on PDBu binding may not be entirely due to chelation of calcium. The role of metals other than calcium in phorbol ester binding is considered in the next chapters. TMB-8, which antagonizes calcium-dependent events at an intracellular site by a poorly-defined mechanism [Smolen 1984], inhibited binding of ^{3}H-PDBu to a greater extent than the chelating agents, indicating that intracellular calcium is required in the binding of PDBu to cells.

Other calcium-mobilizing agents, anti-Ig and fluoride, also increased binding of ^{3}H-PDBu to B-CLL cells. A small, rapid increase in binding of ^{3}H-PDBu was followed by a larger increase afer 15 minutes. The rise in $[Ca^{++}]_i$ induced by anti-Ig in normal B cells appears to have similar kinetics involving a small, rapid mobilization of calcium stores and a slower, sustained increase in the influx of calcium across the cell membrane.

Fluoride appears to stimulate a rise in cytosolic calcium in two ways. The first mechanism is by activation of G proteins coupled to inositol phospholipid turnover, resulting in formation of IP_3 which mobilizes calcium ions from the endoplasmic reticulum (chapter 1.10). Secondly, fluoride also inhibits degradation of IP_3, thereby sustaining the action of IP_3 on calcium stores. Fluoride increases cyclic AMP levels in lymphocytes [Sato et al. 1985] but this cannot explain the effects of fluoride seen here, since a variety of other agents which increase cyclic AMP did not augment PDBu binding.

A major question is how a rise in $[Ca^{++}]_i$ increases PDBu binding. Affinity of binding was increased up to two-fold without

large change in the total number of receptors as shown by Scatchard analysis of binding in both B-CLL cells and PMN, treated with A23187 or fluoride. A similar effect of calcium ions on affinity of PDBu receptors has also been shown in HL60 cells [May et al. 1985b], PMN [Dougherty and Niedel 1986] and with soluble PKC, reconstituted with phospholipid [König et al. 1985].

As discussed in chapter four, the interpretation of such concepts as affinity is not the same for PDBu receptors as it is for binding of small ligands to soluble protein molecules. In particular, binding of ^{3}H-PDBu is not a simple bimolecular reaction but rather a complex interaction between PKC, phospholipid, calcium ions and PDBu. The binding involves both binding of ^{3}H-PDBu to PKC already complexed with membrane phospholipid and binding of ^{3}H-PDBu to PKC that is newly translocated as a result of treatment of the cells with the ^{3}H-PDBu. Calcium ions may enhance either of these two processes.

There is some evidence that PKC which is already complexed with membrane phospholipid is independent of calcium both for binding of phorbol ester and histone kinase C activity (chapter 1.7 and 1.8). A rise in $[Ca^{++}]_i$ is now known to promote phorbol ester-induced translocation of PKC to membranes in various types of cells including normal human B cells [Guy et al. 1985a,b, 1986] and T cells [Isakov et al. 1987]. In a cell-free model, micromolar concentrations of calcium enhanced the binding of partially purified PKC to isolated red cell membranes [Wolf et al. 1985a]. Immunocytochemical evidence for calcium-enhanced translocation of PKC to plasma membranes has recently been reported [Ito et al. 1988]. Therefore, the effects of calcium on PDBu binding to cells are probably largely a result of

enhancement of PDBu-mediated translocation of PKC.

The experiments described here also show that calcium ionophores synergized with PDBu in translocation of PKC in B-CLL cells. As with enhancement of PDBu binding and rosette inhibition, PDBu-induced translocation of PKC from cytosol to the particulate fraction was enhanced by A23187 only at low concentrations of PDBu and not at a saturating concentration (200nM). This indicates that with optimal concentrations of PDBu, a rise in intracellular calcium ions is not required for translocation and explains why chelation of calcium had no effect on inhibition by the more potent TPA.

Optimal concentrations of PDBu only induced translocation of a portion of the cytosolic aporeceptors (chapter 4). A23187 did not enhance translocation of PKC by 200nM PDBu indicating that the remaining cytosolic aporeceptors which do not translocate when cells are treated with an optimal concentration of PDBu, also do not translocate when the $[Ca^{++}]_i$ is raised.

When the sub-cellular fractionation was performed in the absence of EDTA, both A23187 and ionomycin greatly increased the content of PDBu receptors in the particulate fraction. The interpretation of this is that the PKC translocated by calcium ionophores alone is loosely attached to the particulate fraction in a calcium-dependent manner whereas that induced by phorbol esters is more tightly associated with the membrane. Treatment of PMN with N-formyl-methionyl-leucyl-phenylalanine, a calcium-mobilizing agent, also increases the content of PDBu receptors in the calcium chelator-labile pool of the particulate fraction [Melloni et al. 1986]. This pool, presumably PKC linked by calcium to the phosphate of phospholipids, may serve as an intermediate step in the translocation process. The close

proximity of PKC and phospholipid would facilitate subsequent insertion of PKC into the phospholipid structure in the presence of phorbol ester or diacylglycerol.

The parallel effects of calcium on binding of ^{3}H-PDBu to B-CLL cells and inhibition of MER support the hypothesis that the specific receptors detected by ^{3}H-PDBu mediate the actions of phorbol esters on MER. The concentrations of A23187 (0.2-1uM) which were most effective in augmenting the binding of PDBu and inhibition of MER by PDBu were also the most effective in activation of B-CLL cells by phorbol ester [Drexler et al. 1987].

The results presented here are consistent with results obtained in PMN, indicating that A23187 increases the sensitivity of PMN to PDBu-induced superoxide anion formation by increased binding of PDBu to its receptor [Dougherty and Niedel 1986, Zalewski et al. 1986a, French et al. 1987]. However, it cannot be inferred from these studies that enhancement of PDBu binding is the only way in which calcium affects PDBu-induced functional responses, since other calcium-dependent enzymes including kinases, proteases and phospholipases may also be involved. In platelets, at least part of the synergism between calcium and phorbol ester appears to result from action at a site distal to PKC and involving calmodulin-dependent protein kinases [Nishizuka 1984].

Some of these results have been published in Zalewski et al. 1986a, Hurst et al. 1986a, Zalewski et al. 1987, French et al. 1987.

Table 7.1 Effect of calcium ionophores on binding of PDBu to different cell types

Cell	Bound ^{3}H-PDBu (fmol/10^6 cells, standard error in parentheses)		
	Control	A23187	Ionomycin
B-CLL # 1	52.2 (1.5)	101.2 (1.1)	91.4 (1.4)
" # 2	72.8 (3.1)	ND*	140.9 (4.9)
Normal mononuclear cells	58.9 (2.4)	111.5 (2.8)	ND
Red blood cells	0.0 (0.3)	0.0 (0.5)	ND
PMN	57.3 (1.7)	124.1 (4.4)	ND

Cells (5×10^6/ml) were pretreated with A23187 (1uM), ionomycin (0.5uM) or control solvent DMSO (0.5%) for 10 minutes at 37°C in complete culture medium and then ^{3}H-PDBu was added. The concentration of ^{3}H-PDBu was 10nM except in B-CLL # 2 where it was 20nM. After a further 20 minutes, bound ^{3}H-PDBu was determined. Means of triplicates (standard errors in parentheses) are shown.

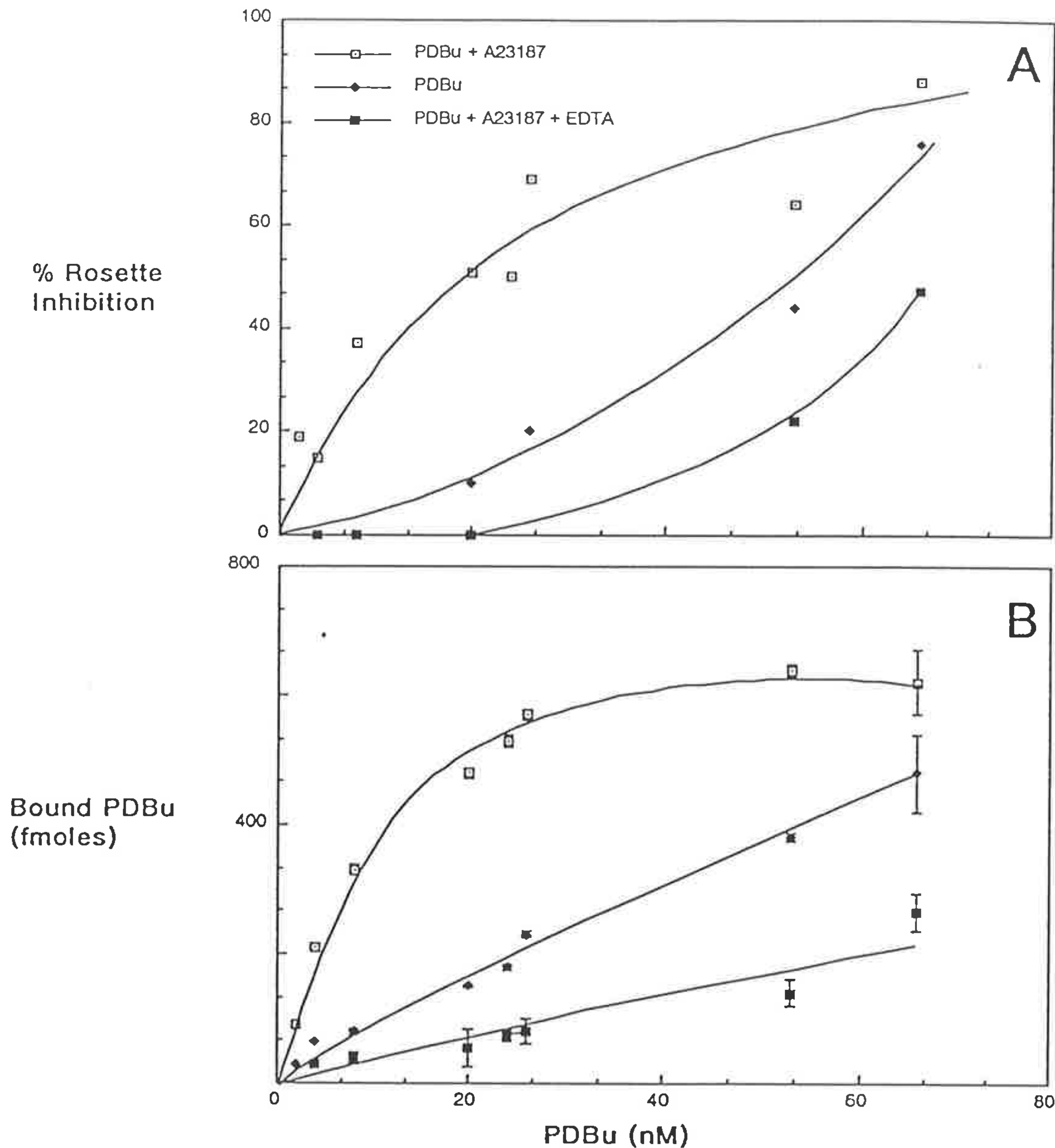
* ND indicates not done

Table 7.2 Effect of EDTA, EGTA and TMB-8 on augmentation of PDBu binding in A23187-treated cells

Treatment		Bound PDBu (% of control, standard errors in parentheses) B-CLL		PMN	
Buffer		100		100	
" " +	EGTA	83	(6)	110	(16)
" " +	EDTA	73	(2)	ND	
" " +	TMB-8	50	(3)	113	(24)
A23187	Buffer	196	(9)	173	(13)
" " +	EGTA	ND		114	(14)
" " +	EDTA	100	(2)	ND	
" " +	TMB-8	89	(5)	111	(11)

B-CLL cells or PMN (5 x 10^6/ml) in RPMI/FCS were pre-treated with buffer, EGTA (2mM), EDTA (2mM) or TMB-8 (200uM) for 10 minutes at 37°C, then with A23187 (1uM) or control solvent DMSO(0.1% v/v) for a further 10 minutes and finally ^{3}H-PDBu (10nM) for 20 minutes. Binding of ^{3}H-PDBu was assayed.

Data are expressed as % binding relative to control (cells treated with buffer alone). Data were pooled from experiments with two populations of B-CLL cells and two populations of normal PMN. Standard errors (n=6) are shown in parentheses.



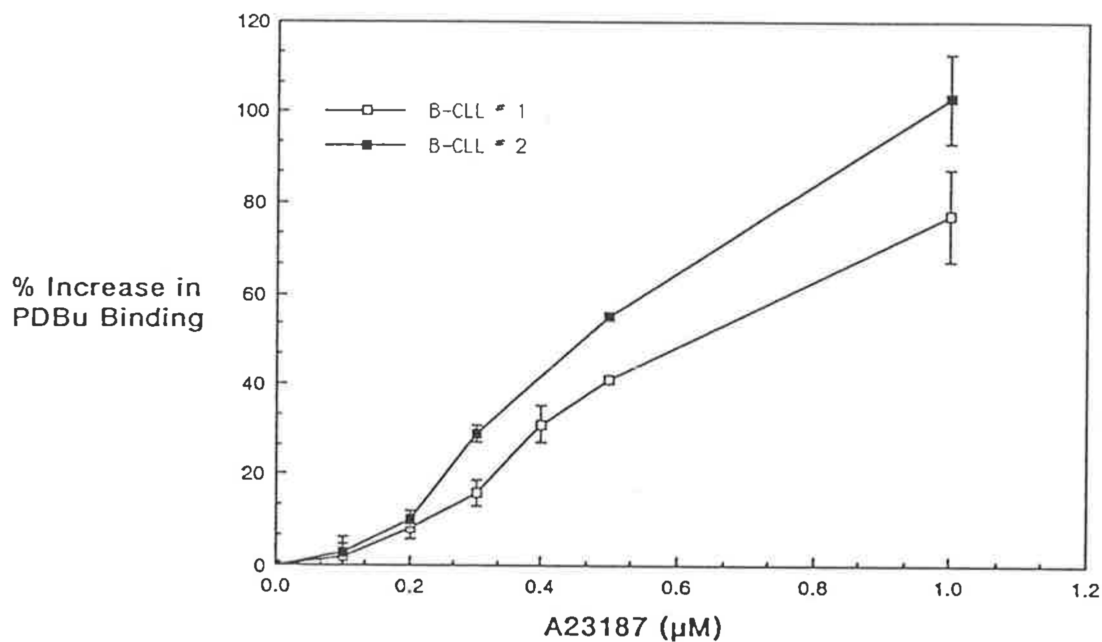
Legend to Figure 7.1 Relationship between intracellular calcium and PDBu-mediated inhibition of MER

Both PDBu binding and PDBu-mediated rosette inhibition increased with an increase in intracellular calcium (achieved by treatment of cells with A23187) and decreased with a decrease in intracellular calcium (achieved by treatment of cells with calcium-chelating agent).

A: Effects on rosetting (expressed as % rosette inhibition, mean of duplicates).

B: Effects on PDBu binding (expressed as pmoles of PDBu bound, mean of triplicates. Bars indicate standard errors.

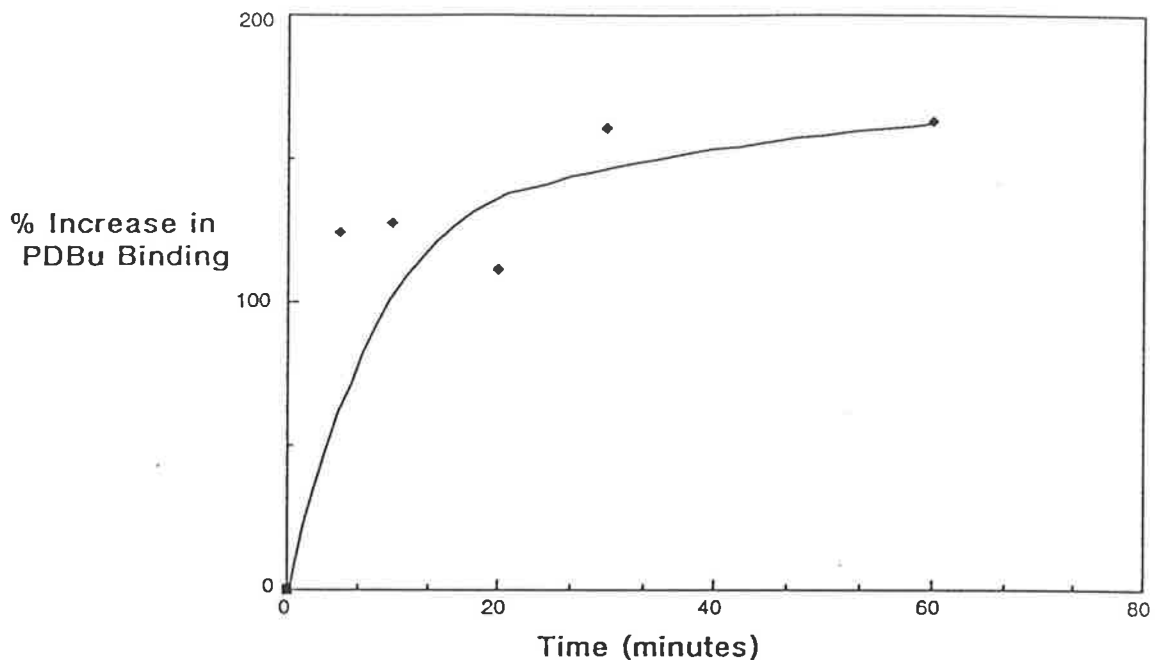
B-CLL cells were pre-treated with or without 10mM EDTA for 10 minutes at 37°C in RPMI, then with or without A23187 for a further 20 minutes, before addition of varying concentrations of ^{3}H-PDBu. Cells were assayed for bound ^{3}H-PDBu, after a further 25 minutes, and for rosetting after 60 minutes.



Legend to Figure 7.2 Concentration-dependence of effects of A23187 on PDBu binding.

A23187 augmented PDBu binding to B-CLL cells in a concentration-dependent manner.

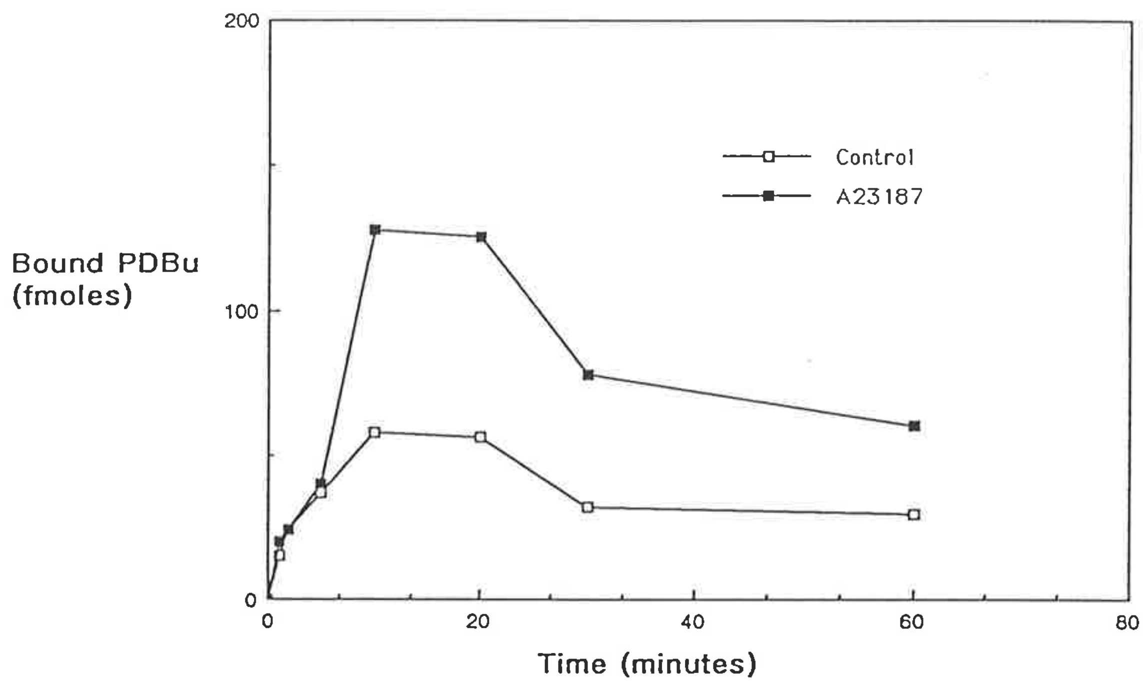
Two populations of B-CLL cells were treated with varying concentrations of A23187 for 10 minutes at 37°C in RPMI before addition of 10nM ^{3}H-PDBu. After a further 20 minutes specific binding of PDBu was assayed. Data are expressed as % increase in binding relative to control cells. Means of triplicates are shown with standard errors indicated by bars.



<u>Legend to Figure 7.3</u> <u>Effect of A23187 on kinetics of binding of PDBu in B-CLL cells</u>

Figure shows a rapid, sustained increase in PDBu binding in B-CLL cells treated with A23187. A similar kinetics was seen when cells from another case were studied (not shown) but, in a third case, the increase was transient (see Zalewski et al. 1988).

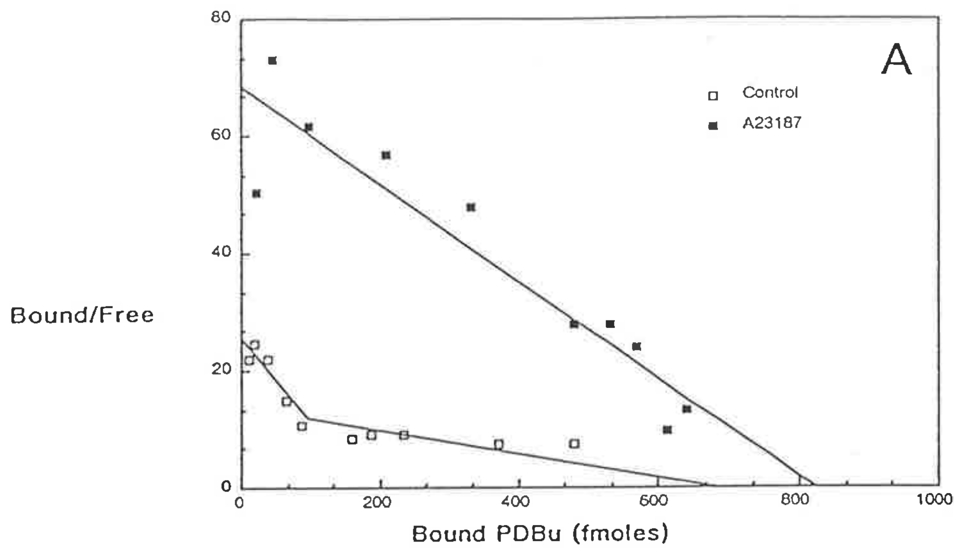
B-CLL cells were pre-equilibrated with ^{3}H-PDBu for 30 minutes at 37°C in RPMI before addition of A23187 (1uM) or control buffer. PDBu binding was assayed at various times and is expressed as % of control binding (control cells are cells treated without A23187 at each time point). PDBu binding in controls decreased by 20% during the course of the experiment. Determinations were in triplicates, standard errors did not exceed 10%.

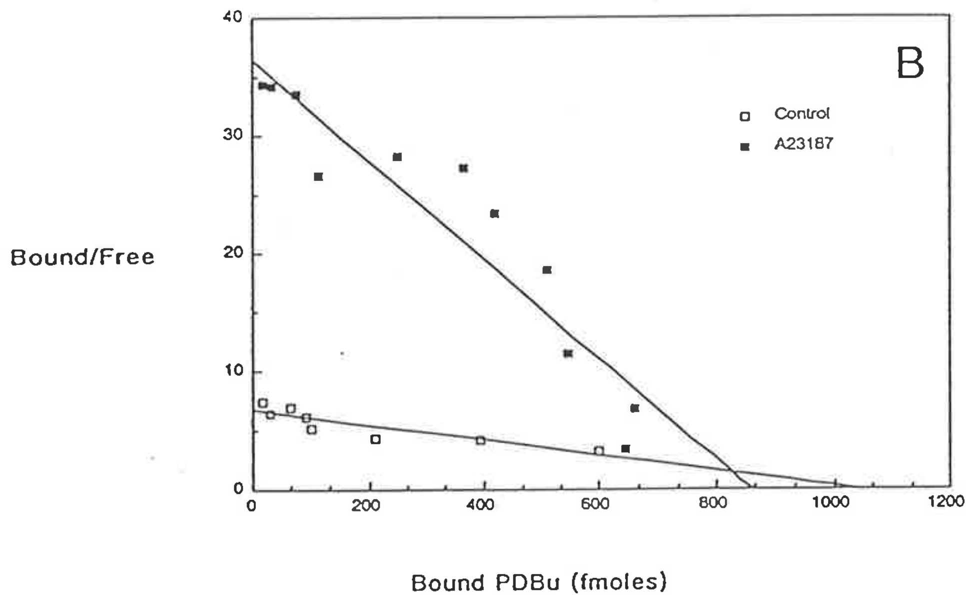


<u>Legend to Figure 7.4</u> <u>Effect of A23187 on kinetics of PDBu binding in PMN</u>

A23187 rapidly increased PDBu binding in PMN. Increase was transient. No increase occurred in the absence of FCS.

PMN were incubated with 10nM ^{3}H-PDBu in the presence or absence of A23187 (1uM). At times indicated, bound PDBu was assayed. Determinations were in triplicate and are expressed as fmoles of PDBu bound per 10^{6} cells; standard errors did not exceed 2 fmol.



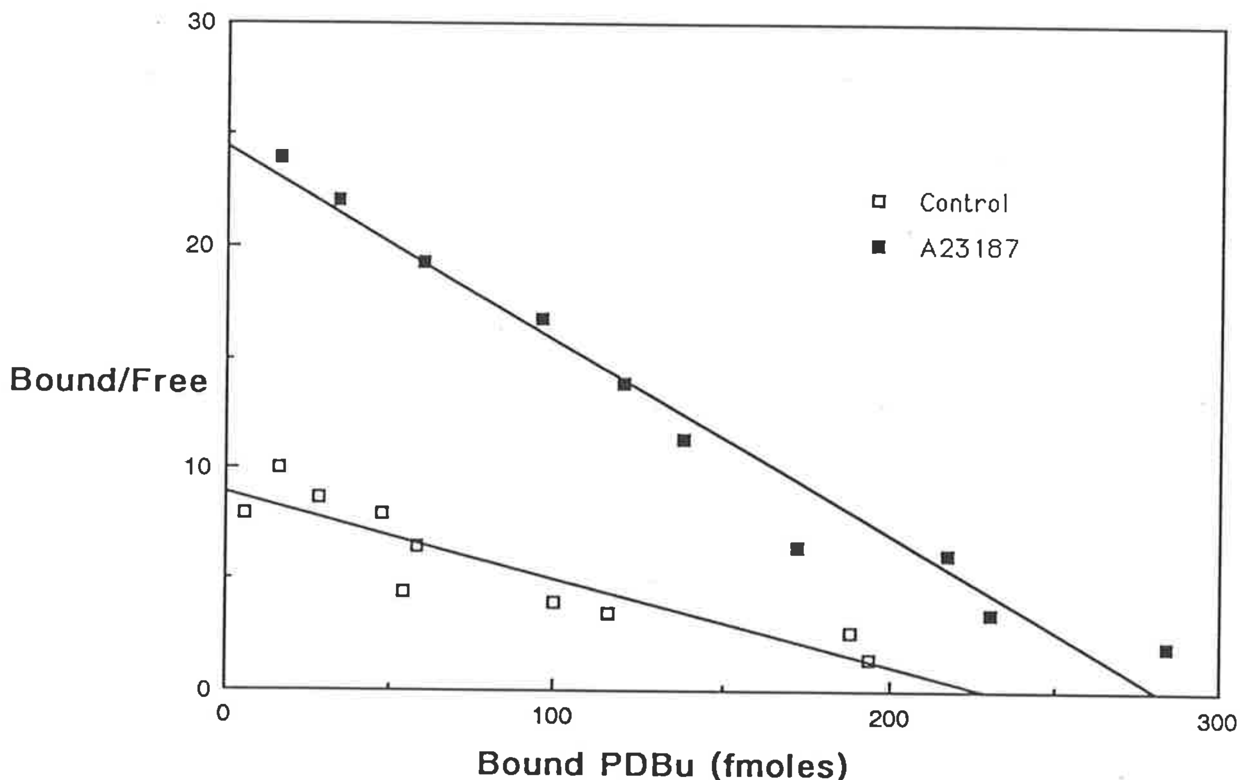


Legend to Figure 7.5 **Effect of A23187 on PDBu receptor numbers and affinities in B-CLL cells**

A23187 greatly increased affinity of PDBu receptors in B-CLL cells with little or no effect on total binding capacity (as determined by Scatchard plots), in serum-free (A) and serum-containing (B) medium. In serum-free medium, curvilinear Scatchard plot became linear when cells were treated with A23187.

A. Serum-free medium
B. Medium contained 10% FCS

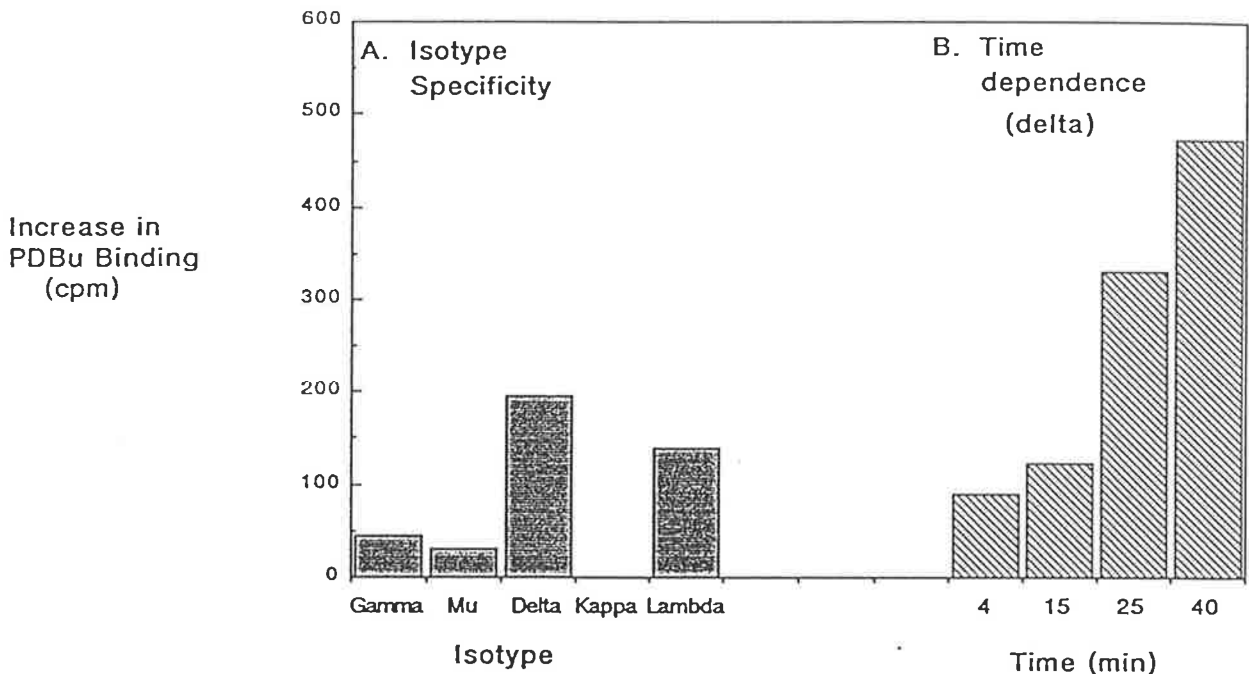
B-CLL cells were treated with or without A23187 (1uM) for 10 minutes at 37°C in (A) RPMI or (B) RPMI/FCS before addition of varying concentrations of ^{3}H-PDBu for another 20 minutes. Specific binding of ^{3}H-PDBu was determined (in triplicate) and expressed as bound PDBu (fmoles) per 10^{6} cells. Data are shown as Scatchard plots.



Legend to Figure 7.6 Effect of A23187 on PDBu receptor numbers and affinities in PMN

A23187 greatly increased affinity of PDBu receptors in PMN cells with little change in total binding capacity (as determined by Scatchard plots).

PMN were treated with or without A23187 (1μM) for 10 minutes at 37°C in RPMI/FCS before addition of varying concentrations of ^{3}H-PDBu for another 20 minutes. Specific binding of ^{3}H-PDBu was determined (in triplicate) and expressed as bound PDBu (fmoles) per 10^{6} cells. Data is shown as a Scatchard plot.



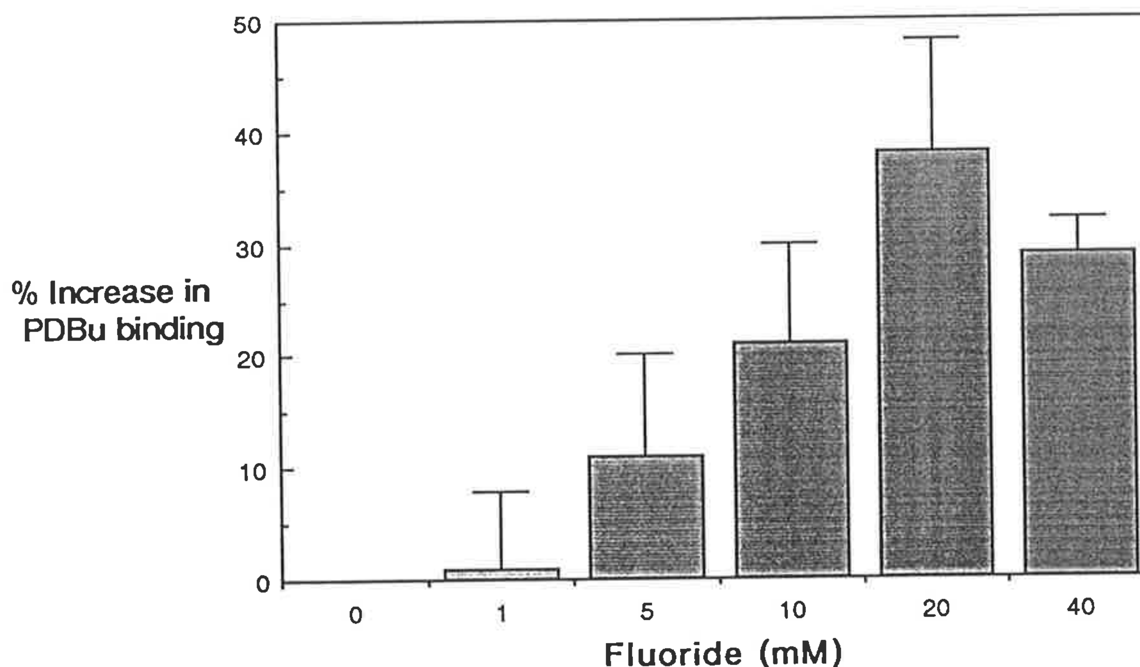
Legend to Figure 7.7 Effect of anti-Ig on binding of ^{3}H-PDBu

Anti-Ig caused a moderate isotype-specific, time-dependent increase in PDBu binding in B-CLL cells.

A : Increase in bound ^{3}H-PDBu caused by antibodies to various isotypes of Ig.

B : Kinetics of increase in bound ^{3}H-PDBu caused by anti-delta heavy chain antibody.

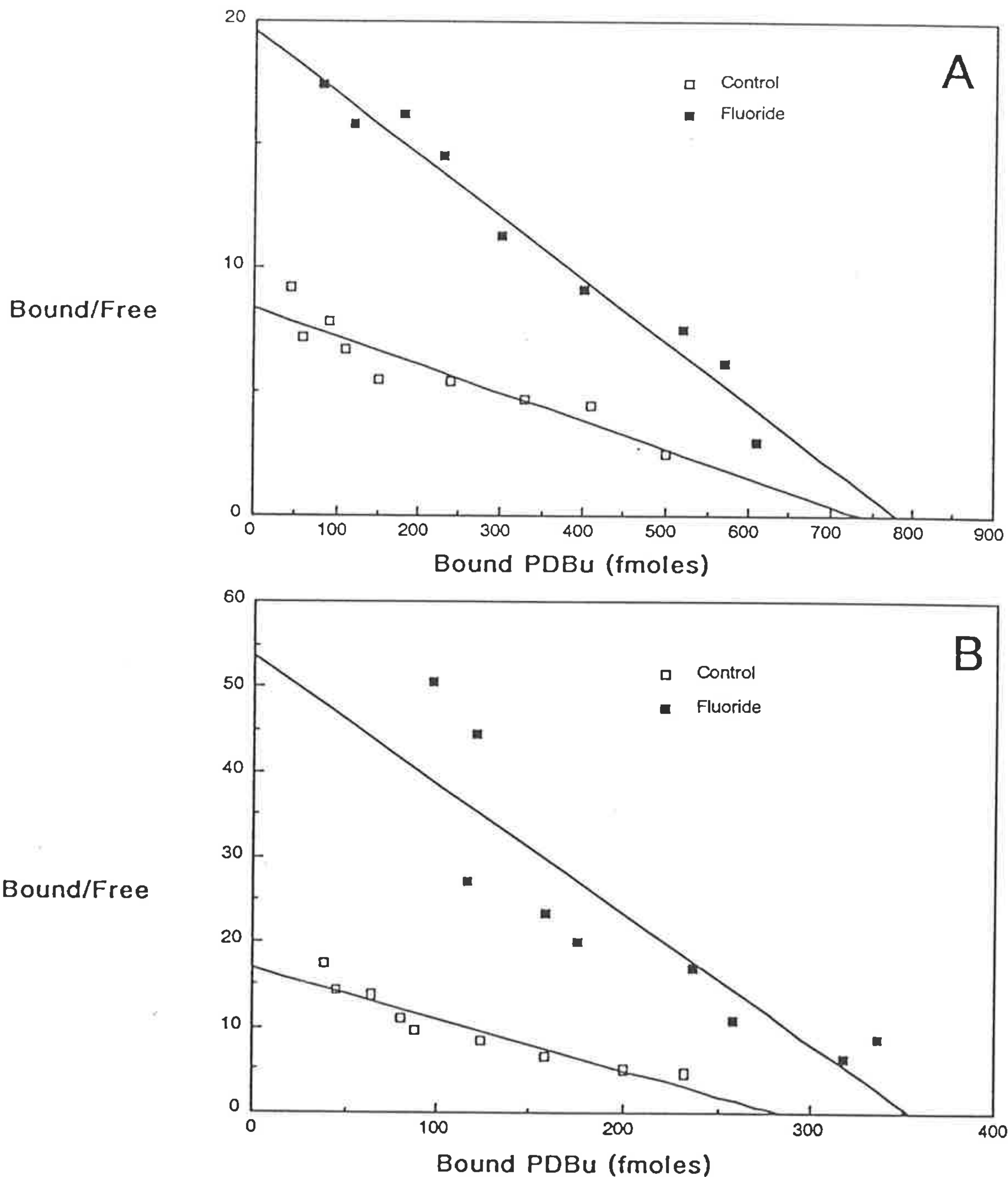
B-CLL cells were pre-equilibrated with 10nM ^{3}H-PDBu for 30 minutes at 37°C in RPMI/FCS before addition of anti-Ig (100ug/ml). Bound PDBu was determined after 30 minutes in (A) and after varying periods of time in (B). Y-axis shows increase in binding of ^{3}H-PDBu (cpm) over control (treated with buffer instead of anti-Ig). Data are means of triplicates. This population of B-CLL cells had large amounts of delta heavy chains and lambda light chains and smaller amounts of gamma and mu heavy chains (as detected by direct immunofluorescence).



Legend to Figure 7.8 Effect of fluoride on binding of ^{3}H-PDBu.

Fluoride increased specific binding of PDBu to B-CLL cells, at concentrations which are known to cause an increase in PIP_2 turnover and rise in $[Ca^{++}]_i$ in some types of cells and to inhibit MER in B-CLL cells (chapter 5).

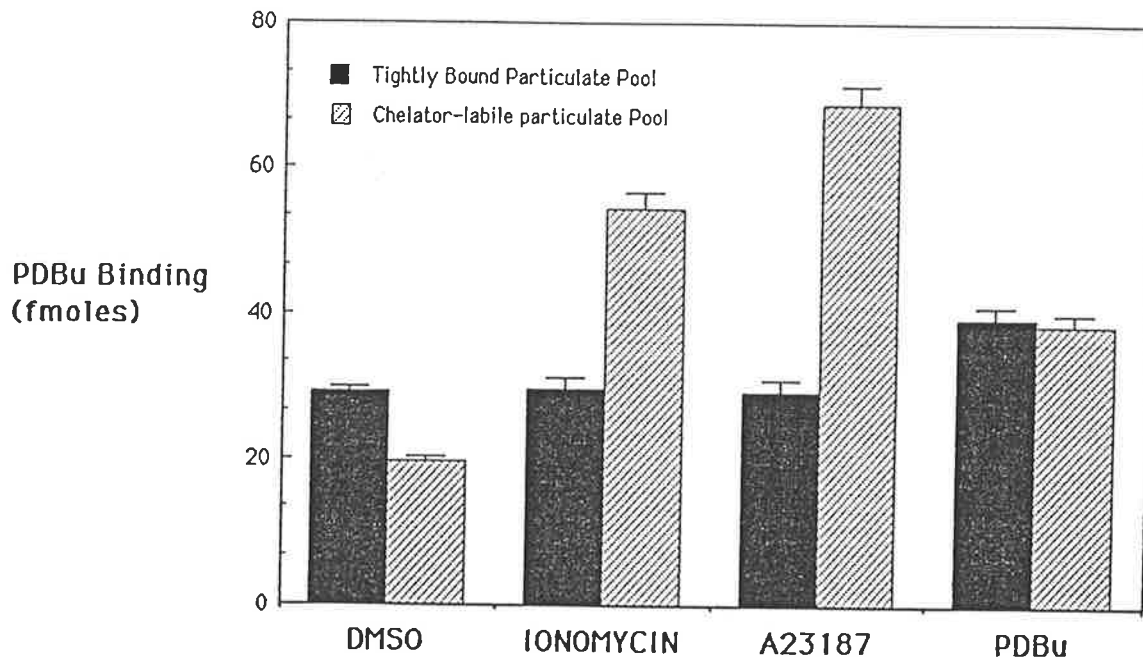
B-CLL cells were pre-equilibrated with 10nM ^{3}H-PDBu for 30 minutes at 37°C in RPMI/FCS before addition of varying concentrations of sodium fluoride. After a further 40 minutes, bound PDBu was determined. Data are expressed as % of PDBu binding in control cells (no fluoride).



<u>Legend to Figure 7.9</u> <u>Effect of fluoride on PDBu receptor numbers and affinities in B-CLL cells and PMN</u>

Like A23187, fluoride greatly increased affinity of PDBu receptors in B-CLL cells and PMN with little or no effect on total binding capacity.

B-CLL cells or PMN were treated with or without fluoride (40mM) for 10 minutes at 37°C in RPMI/FCS before addition of varying concentrations of ^{3}H-PDBu for another 20 minutes. Specific binding of ^{3}H-PDBu was determined (in triplicate) and is shown as fmoles bound per 10^{6} cells. Data are shown as Scatchard plots.



Legend to Figure 7.10 Effect of calcium ionophores on translocation of PDBu receptors

Both A23187 and ionomycin increased PDBu binding to the divalent cation-chelator-labile pool of the particulate fraction but not to the tightly bound pool.

B-CLL cells were treated with ionomycin (0.5μM), A23187 (1μM), unlabelled PDBu (200nM) or control solvent DMSO (0.1% v/v) for 40 minutes at 37°C in RPMI, cells were washed thoroughly and particulate fraction prepared by sonication in the presence or absence of 1mM EDTA. Particulate fractions were assayed for binding of 10nM ^{3}H-PDBu.

Binding of ^{3}H-PDBu to the particulate fraction that was prepared in the absence of EDTA is the total binding to both tightly bound and chelator-labile pools of PKC. Binding to particulate fraction that was prepared in the presence of 1mM EDTA is assumed to be binding to the tightly bound pool of PKC, alone. The difference between the two is assumed to be the binding of ^{3}H-PDBu to the chelator-labile pool.

CHAPTER EIGHT

EFFECT OF GOLD AND ZINC ON PHORBOL ESTER RECEPTORS

8.1 INTRODUCTION

Auranofin has several actions in common with phorbol esters, including effects on the epidermal growth factor receptors (chapter 1.11) and on MER (chapter 6). One possibility is that auranofin interacts with the same site in PKC as phorbol esters and thereby activates the enzyme. If this is correct, auranofin should compete with ^{3}H-PDBu for binding to PKC. Therefore, the effect of auranofin on PDBu binding was determined. These studies indicated that auranofin does not compete with PDBu but rather augments its binding.

The hypothesis that auranofin directly interacts with PKC was reinforced by subsequent DNA sequence studies from the laboratory of Dr Parker and colleagues [1986], which revealed the two putative heavy metal binding sites (zinc fingers) in the regulatory domain of PKC (chapter 1.8). Gold binds more avidly than zinc to cysteines in metallothionein [Butt et al. 1986]. The effects on PDBu binding of zinc and other metals were therefore also tested, both in intact cells, in the presence of pyrithione as ionophore, and in cell-free sonicates, crude cytosolic preparations and isolated particulate fraction.

In chapter nine, the effects of zinc and gold on translocation of PKC and activation of the catalytic site are discussed.

8.2 RESULTS

8.2.1 EFFECT OF AURANOFIN AND OTHER GOLD COMPOUNDS

Augmentation of PDBu binding by auranofin

In order to determine whether auranofin competes with ^{3}H-PDBu for binding to B-CLL cells, cells were pre-equilibrated for 30 minutes with 10nM ^{3}H-PDBu, then incubated with auranofin or control solvent DMSO (0.1% v/v) for varying periods of time

before assaying for bound ^{3}H-PDBu.

Auranofin caused a slow, sustained increase in binding (up to 4 fold) with a lag period of about 10 minutes (Fig 8.1). When B-CLL cells were given auranofin and 10nM ^{3}H-PDBu simultaneously, auranofin increased binding at all times tested. The shape of the binding curve was largely unchanged from that in control cells (Fig 8.1A). Some down-regulation of binding occurred in cells treated with 200nM ^{3}H-PDBu. This was not seen in auranofin-treated cells.

The binding of ^{3}H-PDBu initially increased at auranofin concentrations between 10 and 20μM (Fig 8.2). Further increases occurred with higher concentrations of auranofin. No increase in binding of ^{3}H-PDBu was seen when binding of auranofin-treated cells was assayed in the presence of excess cold phorbol ester, indicating that the increase is in specific PDBu binding.

Effect on different cell types

Auranofin augmented specific PDBu binding in all six populations of B-CLL cells tested, as well as in normal blood mononuclear cells, PMN and platelets (Table 8.1). As with B-CLL cells, binding to PMN began to increase at concentrations of auranofin greater than 10μM (Fig 8.2).

Auranofin did not induce red cells to bind ^{3}H-PDBu (Table 8.1) confirming that auranofin does not simply cause cell membranes to bind ^{3}H-PDBu, non-specifically.

Effect on affinity and receptor numbers

To determine whether the augmentation of binding by auranofin resulted from an increase in affinity of receptors or an increase in their number, the binding data were analyzed by Scatchard plots. Auranofin increased the total numbers of

receptors (by about 40%) as well as causing a two- to four-fold increase in affinity of PDBu receptors in B-CLL cells (Fig 8.3) Similar results were seen in three different populations of B-CLL cells (Table 8.2). There was also a moderate increase in apparent total numbers of PDBu receptors and a large increase in affinity of PDBu receptors in auranofin-treated PMN (Fig 8.4 and Table 8.2).

Effect of other gold compounds

Binding of 10nM ^{3}H-PDBu was increased by 41.7% (sem 4.9%, n=3) when B-CLL cells were treated with 25μM of the lipophilic gold compound chloro-triethylphosphine gold. In contrast, binding was unaffected by treatment of cells with up to 2.5mM of the water-soluble sodium aurothiomalate. Thus, binding of 10nM ^{3}H-PDBu was 102% (sem 2.4%, n=3) of control cell values in cells treated with 2.5mM sodium aurothiomalate.

Effect of thiols

PDBu binding was not increased by auranofin in B-CLL cells or PMN in the presence of competing low molecular weight thiols, 2-ME and glutathione (Fig 8.5).

Effect of calcium

Increased binding of ^{3}H-PDBu to auranofin-treated B-CLL cells was partially blocked by 2mM EGTA and 2mM EDTA and completely blocked by 500μM TMB-8 (Fig 8.5). Similarly, in PMN, the increase in ^{3}H-PDBu binding induced by 60μM auranofin was reduced by 2mM EGTA from 248% (sem 12.3, n=3) to 152% (sem 0.8) (Fig 8.5).

These results indicate that one action of auranofin is mediated by calcium or other divalent cation. The effect of auranofin on $[Ca^{++}]_i$ was determined using the calcium-sensitive indicator Quin-2. Auranofin (at concentrations above 25μM)

increased fluorecence of Quin-2-loaded B-CLL cells after a lag period of about 90 seconds (Fig 8.6). Auranofin induced a mean two-fold increase in $[Ca^{++}]_i$ in five experiments, much less than that induced by ionomycin (Table 8.3).

Other sulphydryl reagents

2-ME and dithiothreitol had little effect on PDBu binding at concentrations which inhibited MER although at a very high concentration (7mM), dithiothreitol decreased binding of ^{3}H-PDBu by 21% (sem 6%, n=3).

Role of increased binding in action of auranofin on MER

The synergism between auranofin and PDBu in inhibition of MER was not due to augmentation by auranofin of PDBu binding since concentrations (1-6μM) of auranofin which were potently synergistic in inhibiting rosetting had no effect on PDBu binding.

8.2.2 EFFECT OF ZINC

Zinc chloride

Zinc chloride, alone at concentrations up to 100μM, had no effect on the capacity of B-CLL cells to bind ^{3}H-PDBu. At 1mM, zinc chloride increased binding of ^{3}H-PDBu by 10% to 20%. As with gold in the water-soluble gold salts, ionic zinc may be taken up poorly by cells.

However, zinc chloride considerably enhanced the increase in PDBu binding caused by anti-Ig (Fig 8.7, Table 8.4), auranofin (Fig 8.8, Table 8.4), fluoride and A23187 (Table 8.4). Concentrations of zinc as low as 4μM were effective. One possibility is that these agents enhance uptake of zinc.

Effect of the lipophilic zinc pyrithione on PDBu binding

In contrast to the weak effects of zinc chloride alone on

PDBu binding, the lipophilic zinc pyrithione caused a large increase in specific binding of ^{3}H-PDBu to B-CLL cells. Zinc pyrithione (100μM) increased the binding of ^{3}H-PDBu to a level up to three times that of control cells (Fig 8.9). At 50μM, zinc pyrithione enhanced binding to 1.7 times that of control cells. At 10uM, there was a small increase in binding (about 20%).

In the absence of zinc chloride, the sodium salt of pyrithione had a small enhancing effect on ^{3}H-PDBu binding which was completely prevented by the zinc chelating agent 1,10 phenanthroline or by omission of FCS from the medium and is presumably due to the 2uM of zinc contributed by the FCS to the culture medium.

Increase in PDBu binding also occurred when a combination of sodium pyrithione and zinc chloride was added to the cells (Fig 8.10), instead of the pre-formed zinc pyrithione complex. Ten micromolar sodium pyrithione was optimal. The increase in binding was very dependent upon the concentration of zinc chloride. When the concentration of zinc chloride was 100μM, binding was increased up to 2.4 fold. Little further increase in PDBu binding occurred with 1000μM zinc chloride (not shown) suggesting that 100μM is optimal for increase in binding of PDBu. With 30μM zinc chloride, binding was increased about 50% and with 10μM zinc chloride binding was only slightly increased.

Kinetics of increase in binding of PDBu

The above studies were done by assaying binding of ^{3}H-PDBu after 40 minutes incubation with zinc. To determine the effect of the duration of the incubation with zinc on PDBu binding, cells were incubated with pyrithione and zinc chloride for varying periods of time before binding of PDBu was assayed (Fig 8.11).

Binding of PDBu increased within 10 minutes of addition of pyrithione and zinc chloride but most of the increase occurred between 30 and 75 minutes. There was little further increase in binding after 75 minutes. At this time, PDBu binding was increased three fold over that of control-treated cells. Similar results were obtained for three different populations of B-CLL cells.

Parallel studies were done on zinc uptake with ^{65}zinc. There was a very good correlation between uptake of zinc by the cells and increase in PDBu binding (Fig 8.12). Thus, uptake of zinc also reached a plateau after about 60 minutes. This indicates that the extent to which PDBu binding is increased is limited by the amount of zinc which can be taken up by the cells.

Effect on other cells

Zinc pyrithione also greatly enhanced PDBu binding in two other types of cells tested, PMN and platelets (Table 8.5).

Effect of zinc on PDBu receptor number and affinity

Zinc pyrithione enhanced binding at all concentrations of ^{3}H-PDBu tested. Scatchard plots showed that zinc pyrithione more than doubled the apparent total number of receptors in both populations of B-CLL cells tested as well as normal platelets and PMN (Fig 8.13, 8.14 and Table 8.5). In platelets and one population of B-CLL cells there was only a small or no effect on receptor affinity, in the other population of B-CLL cells there was a two-fold increase in affinity and in PMN there was a three fold increase in affinity of PDBu receptors in zinc-treated cells (Table 8.5).

Effect of washing and phenanthroline

The reversibility of the effect of zinc on binding of ^{3}H-

PDBu was investigated. The large increase in PDBu binding that occurred in cells treated for forty minutes with zinc and pyrithione, was not diminished by washing the cells three times before assaying PDBu binding. Studies with ^{65}zinc indicated that washing of the cells did not result in loss of cell-associated zinc.

Phenanthroline inhibited the effects of zinc pyrithione on PDBu binding, whether it was added to the cells before zinc pyrithione and ^{3}H-PDBu, after zinc pyrithione but before ^{3}H-PDBu or after both zinc pyrithione and ^{3}H-PDBu (Fig 8.15). Phenanthroline consistently decreased by about 10% the binding of ^{3}H-PDBu to untreated B-CLL cells.

Phenanthroline removed about 20% of the ^{65}zinc that had been transported into cells by zinc pyrithione. This was associated with a decline in PDBu binding to levels below those of control cells (not shown).

8.2.3 EFFECT OF OTHER METALS

Various other metal salts (100μM) were tested for effects on PDBu binding in intact B-CLL cells in the presence or absence of pyrithione (20μM). Serum-free RPMI was used as the medium for the assays.

No metal affected binding in the absence of pyrithione. Only zinc and cadmium increased binding significantly in the presence of pyrithione (Fig 8.16). Copper caused an almost complete inhibition of PDBu binding. Fifty per cent inhibition occurred with 15μM cupric acetate. Copper also inhibited binding in cells treated with zinc pyrithione (not shown).

8.3 DISCUSSION

Gold, zinc and cadmium, metals with similar ligand-binding properties [Schmitz et al. 1980], caused large increases (up to threefold) in the PDBu-binding capacities of several types of cells, when they were taken up by these cells in the form of lipophilic complexes. Pyrithione alone did not increase PDBu binding in zinc-free medium. Preliminary evidence indicates that another zinc ionophore, zinc diiodo-hydroxyquinoline also increases specific PDBu binding in B-CLL cells, in a manner dependent upon the zinc [C. Giannakis Personal communication].

Copper which, like zinc, gold and cadmium, interacts with cysteines, had the opposite effect on PDBu binding. The inhibition of PDBu binding by copper may result from oxidation of PKC, since other oxidizing agents inhibit PDBu binding [Gopalakrishna and Anderson 1987].

Since zinc and gold bind to sulphydryl groups in proteins, it needs to be determined whether other sulphydryl reagents also augment PDBu binding. Low molecular weight thiols did not increase PDBu binding in B-CLL cells. In one study, N-ethylmaleimide, a potent sulphydryl reagent, inhibited binding of phorbol ester to intact cells but not to soluble PKC [Schmidt et al. 1983]. The effect of gold, zinc and other metals on soluble PKC is discussed in chapter 9.

Gold, zinc and cadmium salts alone had no effect on PDBu binding in cells, presumably because they are taken up poorly by cells. It has recently been reported that zinc chloride causes a large increase in PDBu binding to cytosol of T cells [Csermely et al. 1988]. Similar effects were seen when zinc, gold and some other metals were added to cell-free preparations of B-CLL cells (chapter 9).

Zinc chloride did synergize with some other stimuli (anti-Ig, fluoride, A23187 and auranofin) in enhancing binding of ^{3}H-PDBu to intact B-CLL cells. Uptake of zinc may be promoted by these stimuli, eg by opening calcium channels in the membrane. Whether zinc can pass through calcium channels is not known. A23187 also can transport zinc. Zinc may exchange with the gold in auranofin and thereby be carried into the cell.

A rise in intracellular zinc may be an important signal in regulating PKC. Much of the zinc transported into the cells by pyrithione could not be removed by phenanthroline and may be tightly bound zinc. The zinc removed by phenanthroline may be the free and loosely bound zinc. If this is so, it suggests that the pool of free or loosely bound zinc is important in modulating PKC, since phenanthroline completely inhibited the effects of zinc on PDBu binding in zinc pyrithione-treated cells. In contrast, phenanthroline only slightly inhibited PDBu binding in untreated cells, suggesting that zinc is not involved to a great extent in the basal level of PDBu binding.

It has previously been reported that lanthanum also increases PDBu binding in PMN [Dougherty and Niedel 1986]. The mechanism was attributed to interference with calcium homeostasis. It is unclear as to whether calcium is also involved in the actions of gold and zinc on PDBu binding. Auranofin did induce a small rise in cytosolic calcium in B-CLL cells, possibly by directly activating phospholipase C [Snyder et al. 1988]. Because zinc interacts with quin-2, it is not possible to determine whether zinc also causes a rise in $[Ca^{++}]_i$ with this intracellular free calcium indicator.

The effect of auranofin on PDBu binding was partially

blocked by EDTA, EGTA and TMB-8. The significance of this is unclear. They may act by chelating gold, by interfering with the mechanism by which auranofin augments PDBu binding or by interfering with the binding of ^{3}H-PDBu to the cells.

Some differences were observed between the actions of gold or zinc and those of calcium ionophore. First, the augmentation of PDBu binding by zinc pyrithione and auranofin was greater in magnitude than that seen with calcium ionophore and yet auranofin was much less effective in augmenting intracellular calcium. Secondly, unlike calcium ionophore, auranofin and zinc pyrithione also increased binding at saturating concentrations of ^{3}H-PDBu. This increase in binding was associated with an increase in the apparent total number of PDBu receptors. In contrast, calcium ionophores only increased the affinity of PDBu receptors.

One implication of the zinc-mediated increase in number of PDBu receptors is that the total numbers of receptors may vary from one tissue or cell to another depending upon the concentration of free zinc. It has been estimated that brain contains millions of PDBu binding sites per cell [Blumberg et al. 1984], far more than are detected in other types of cells. Brain is particularly rich in zinc, possibly because zinc is involved in neurotransmission. Moreover, similar regions of the brain including the cortex, pineal and hippocampus are rich in zinc [Danscher et al. 1976, Prohaska 1987] and phorbol ester receptors [Nagle et al. 1981, Nagle and Blumberg 1983]. It would be of interest to know whether the relatively high number of PDBu receptors in the B-CLL cells of J.T. (see chapter 4) was a result of increased intracellular zinc. The hypothesis that the maximum PDBu binding capacity of cells is influenced by the intracellular free zinc ions, may be tested when intracellular free zinc

indicators become available.

It is not clear how the total number of PDBu receptors is increased by zinc. It seems unlikely that another type of protein becomes a PDBu receptor in the presence of zinc, since the Scatchard plot remained linear indicating a single class of receptors.

It has recently been shown that chloroform also causes an increase in the apparent total number of phorbol ester receptors without effect on affinity [Roghani et al. 1987]. Chloroform is a tumour promoter and activates PKC in intact platelets and cell-free systems. It was speculated that chloroform affects the structure of the phospholipid component of PKC [Roghani et al. 1987]. Zinc may interact with the phospholipid membranes causing a change similar to that caused by chloroform. Micromolar concentrations of zinc chloride greatly potentiate calcium-induced phase separation in phospholipid vesicles, important for activation of some enzymes [Deleers et al. 1986]. High concentrations (> 250uM) of zinc chloride also greatly increased the number of growth hormone receptors in adipocytes, increasing the sensitivity of the cells to the hormone [Herington 1985].

The possible role of enhanced translocation of PKC in the augmentation of PDBu binding in cells treated with gold and zinc is discussed in chapter 9.

In A23187-treated cells, the enhanced binding of PDBu was accompanied by enhanced inhibition of MER in B-CLL cells [Zalewski et al. 1986a, Hurst et al. 1986a] and enhanced superoxide anion formation in PMN [French et al. 1987]. Auranofin synergized with phorbol ester in the inhibition of MER. However, synergism was seen at low concentrations of auranofin which did

not cause an increase in binding of PDBu.

The effect of auranofin on some other early activation events induced by phorbol esters needs to be investigated in order to determine whether the enhanced binding of PDBu in auranofin-treated cells is correlated with enhanced functional responses.

In at least two systems, auranofin appears to inhibit phorbol ester-induced cell activation. At concentrations of auranofin which increased PDBu binding in PMN, phorbol ester-induced superoxide formation was inhibited. Similarly, although auranofin mimicked phorbol esters in phosphorylation of 40kDa and 20 kDa proteins and enhanced PDBu binding in platelets, phorbol ester-stimulated platelet aggregation was inhibited by auranofin and stimulated by phorbol ester [Froscio et al. 1987]. Thus, unlike the situation with calcium ionophores, there may be no simple correlation between effects of auranofin on PDBu receptors and functional response. One reason for this is that auranofin may affect events distal to activation of PKC. Similar studies which correlate the effects of zinc pyrithione on PDBu binding and on PDBu-induced functional responses need to be done.

Some of these results have been published in [Zalewski et al. 1986b, 1987, 1988, Forbes et al. 1989, Hurst et al. 1989].

Table 8.1 Effect of auranofin on PDBu binding in various cell types

Cell Type	Bound ^{3}H-PDBu (fmol/10^6 cells)	
	Control	AURANOFIN
B-CLL	54.4 (0.6)	168.1 (3.1)
Mononuclear cells	58.9 (2.4)	223.9 (4.5)
PMN	65.3 (0.8)	156.0 (7.9)
Platelets	30.2 (3.0)	83.4 (2.5)
RBC	0.0 (0.3)	0.8 (0.2)

Peripheral blood mononuclear cells, PMN, platelets and RBC were prepared as described in chapter 2.3. Cells were treated at a concentration of 5×10^6/ml except for platelets which were treated at 2×10^7/ml. Medium was RPMI/FCS.

Cells were pre-equilibrated with ^{3}H-PDBu (10nM) for 30 minutes before addition of auranofin (60μM). Specific binding of PDBu was assayed in triplicate after a further 25 minutes. Figure in parenthesis is standard error.

Table 8.2 **Effect of auranofin on affinity and maximum binding capacity of PDBu receptors in whole cells**

Cells	Auranofin	PDBu Receptors	
		Kd (nM)	Maximum binding capacity (fmoles/10^6cells)
B-CLL # 1	-	19.3	281
	+	5.0	335
" # 2	-	24.4	337
	+	10.6	507
" # 3	-	19.2	233
	+	8.2	400
PMN	-	26.3	263
	+	12.6	450

B-CLL cells from three patients and normal PMN were incubated in RPMI/FCS at 37°C with ^{3}H-PDBu, at varying concentrations, in the presence (+) or absence (-) of auranofin (as in Fig 8.3). Concentration of auranofin was 100µM with B-CLL # 1 and 60µM with # 2, # 3 and PMN. Bound ^{3}H-PDBu was assayed after 40 minutes and data were analyzed by Scatchard plots. Plots for B-CLL # 2 and PMN are shown in Figs 8.3 and 8.4, respectively.

Table 8.3 Effect of auranofin on intracellular free calcium

Expt. #	$[Ca^{++}]_i$ (μM) after treatment with		
	DMSO	Auranofin	Ionomycin
1	0.32	0.98	4.25
2	0.51	1.13	
3	0.58	1.13	
4	0.30	0.59	
5	0.36	0.40	5.4
Mean (sem)	0.41 (0.07)	0.85 (0.20)	4.8 (n/a)

B-CLL cells, preloaded with Quin-2 as in chapter 2.13, were placed in cuvettes and auranofin (final conc. 60μM), ionomycin (0.5 μM) or DMSO (0.1% v/v) were added. Fluorescence readings were made as in Fig 8.6.

Data were converted to $[Ca^{++}]_i$ using the equation $[Ca^{++}]_i = (115)\ (F-F_{min})/F_{max}-F)$. F, F_{min} and F_{max} are defined in Fig 8.6.

Data were pooled from five experiments. n/a indicates standard error (sem) was not applicable because there were only two readings.

Table 8.4. Effect of zinc chloride on PDBu binding in B-CLL cells treated with various stimuli

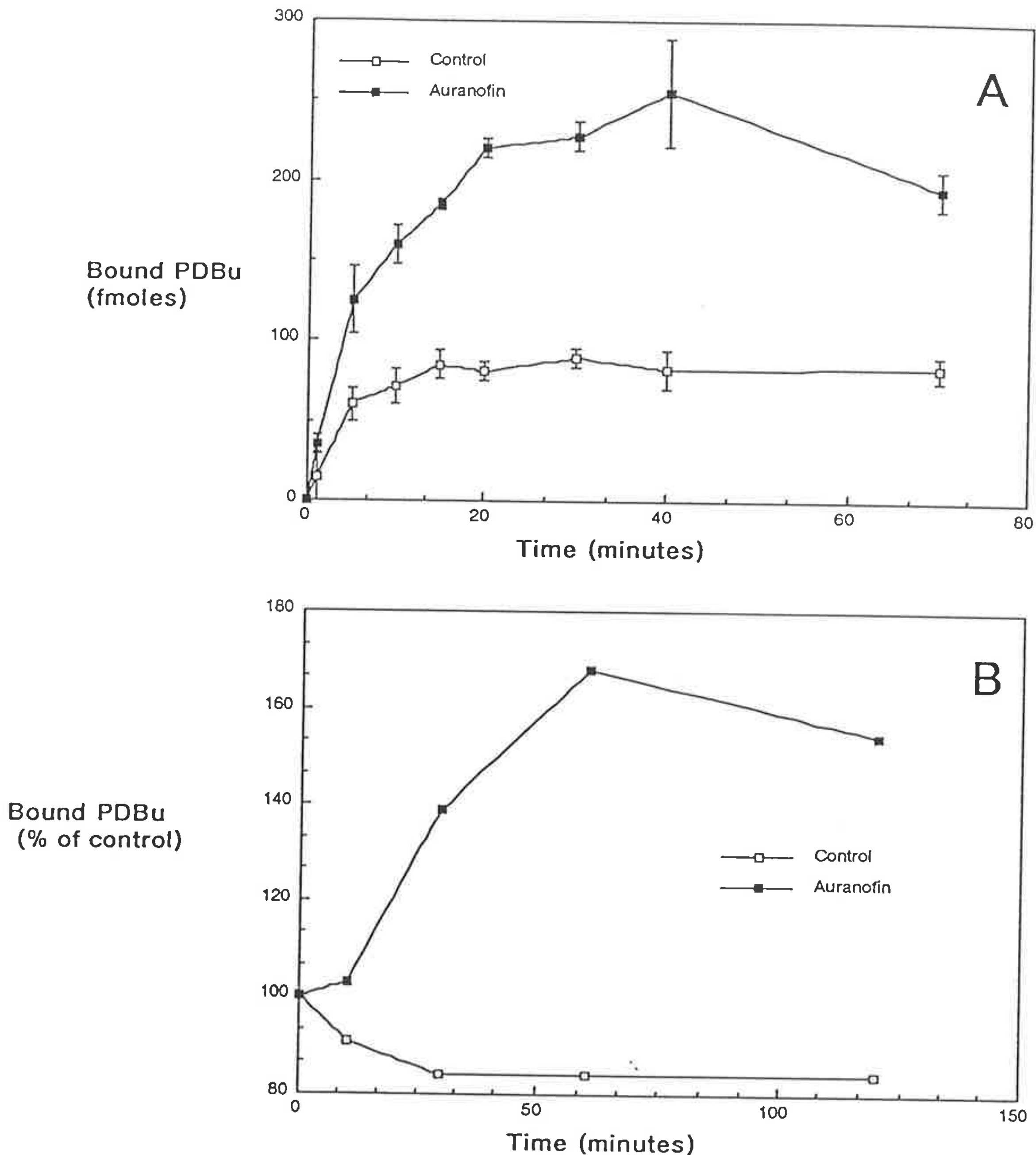
Stimulus	PDBu binding to cells % of control (sem)	
	No zinc	100μM zinc
Control	100	100 (0.5)
Anti-Ig (100μg/ml)	114 (4.3)	122 (3.7)
Fluoride (20mM)	120 (1.1)	137 (4.3)
A23187 (1μM)	168 (26.3)	213 (4.4)
Auranofin (60μM)	194 (1.6)	284 (28.7)

B-CLL cells were pre-equilibrated with 10nM ^{3}H-PDBu for 30 minutes, to achieve a steady state binding, before treatment with various stimuli in the presence or absence of 100μM zinc. After a further 40 minutes, bound PDBu was assayed. Data are expressed as a % of the binding in control cells (no stimulus). Data were pooled from two experiments, each in triplicate.

Table 8.5 Effect of zinc pyrithione on affinity and maximum binding capacity of PDBu receptors in whole cells

Cells	Zinc	PDBu Receptors Kd (nM)	Maximum binding capacity (fmoles/10^6 cells)
B-CLL # 1	-	43	740
	+	43	1400
" # 2	-	38	210
	+	20	316
PMN	-	59	205
	+	17	510
Platelets	-	51	9
	+	37	21

B-CLL cells from two patients, normal PMN or platelets were incubated in RPMI/FCS at 37°C with ^{3}H-PDBu, at varying concentrations, in the presence (+) or absence (-) of zinc pyrithione. Concentration of zinc chloride was 50μM and pyrithione was 20μM. Bound ^{3}H-PDBu was assayed after 40 minutes at 37°C. Data were analyzed by Scatchard plots.



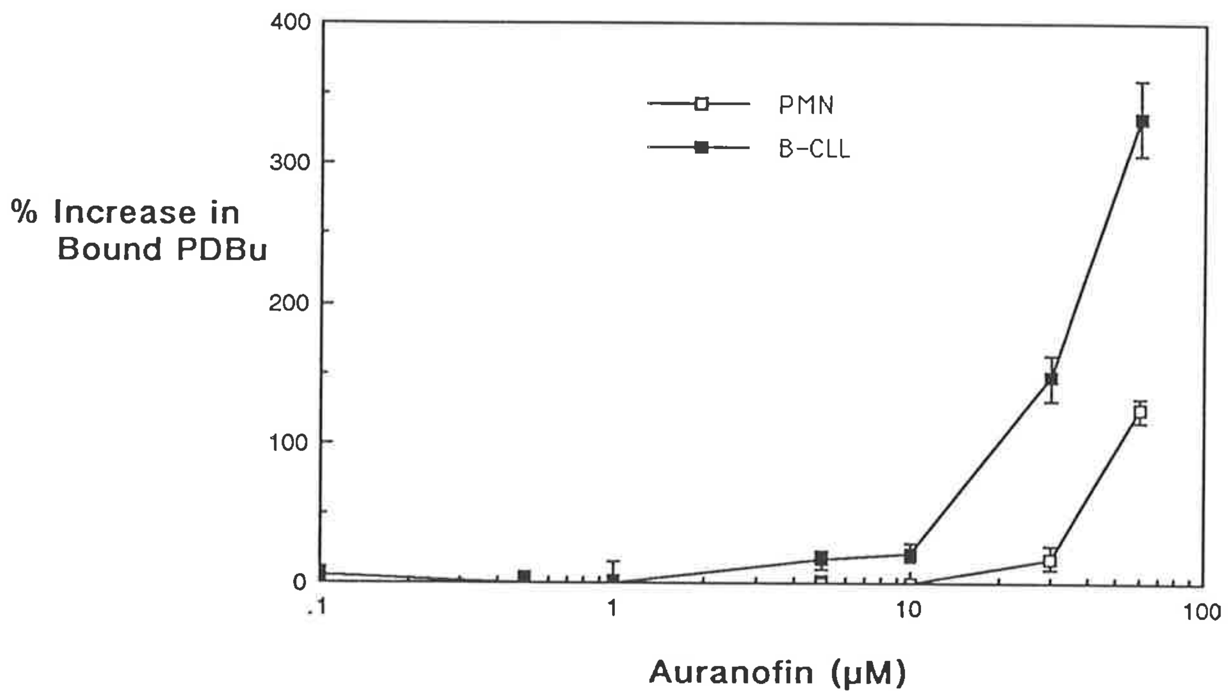
<u>Legend to Figure 8.1</u> <u>Kinetics of augmentation of PDBu binding by auranofin</u>

Auranofin caused a sustained increase in binding of ^{3}H-PDBu to B-CLL cells.

A: Auranofin and ^{3}H-PDBu added simultaneously (at time 0).

B: Cells pre-equilibrated with ^{3}H-PDBu for 40 minutes (to acheive a steady state of binding) before addition of auranofin (at time 0).

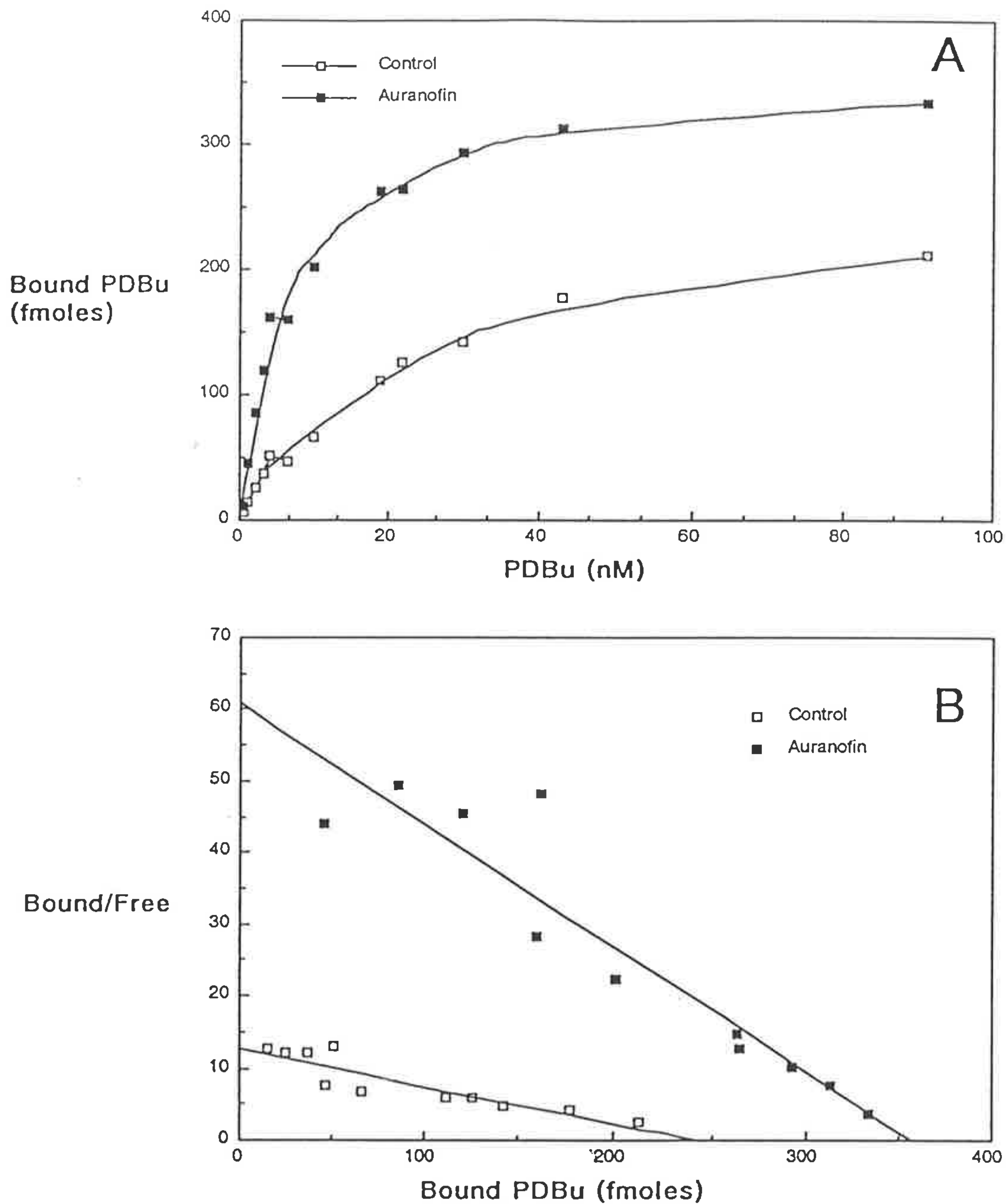
B-CLL cells were incubated at 37°C in RPMI/FCS with 10nM ^{3}H-PDBu alone or in the presence of auranofin (60μM) for varying periods of time before binding was assayed. All bindings were done in triplicate. Data are expressed as fmoles PDBu bound/10^{6} cells. Standard errors are indicated by bars.



Legend to Figure 8.2 Concentration-dependence of augmentation of PDBu binding by auranofin

Auranofin augmented PDBu binding, in B-CLL cells and PMN, at concentrations greater than 10μM.

B-CLL cells or PMN were incubated at 37°C in RPMI/FCS with varying concentrations of auranofin and 10nM ^{3}H-PDBu. Bound PDBu was assayed after 50 minutes and is expressed as fmoles of bound PDBu per 10^{6} cells. Bars indicate standard errors.

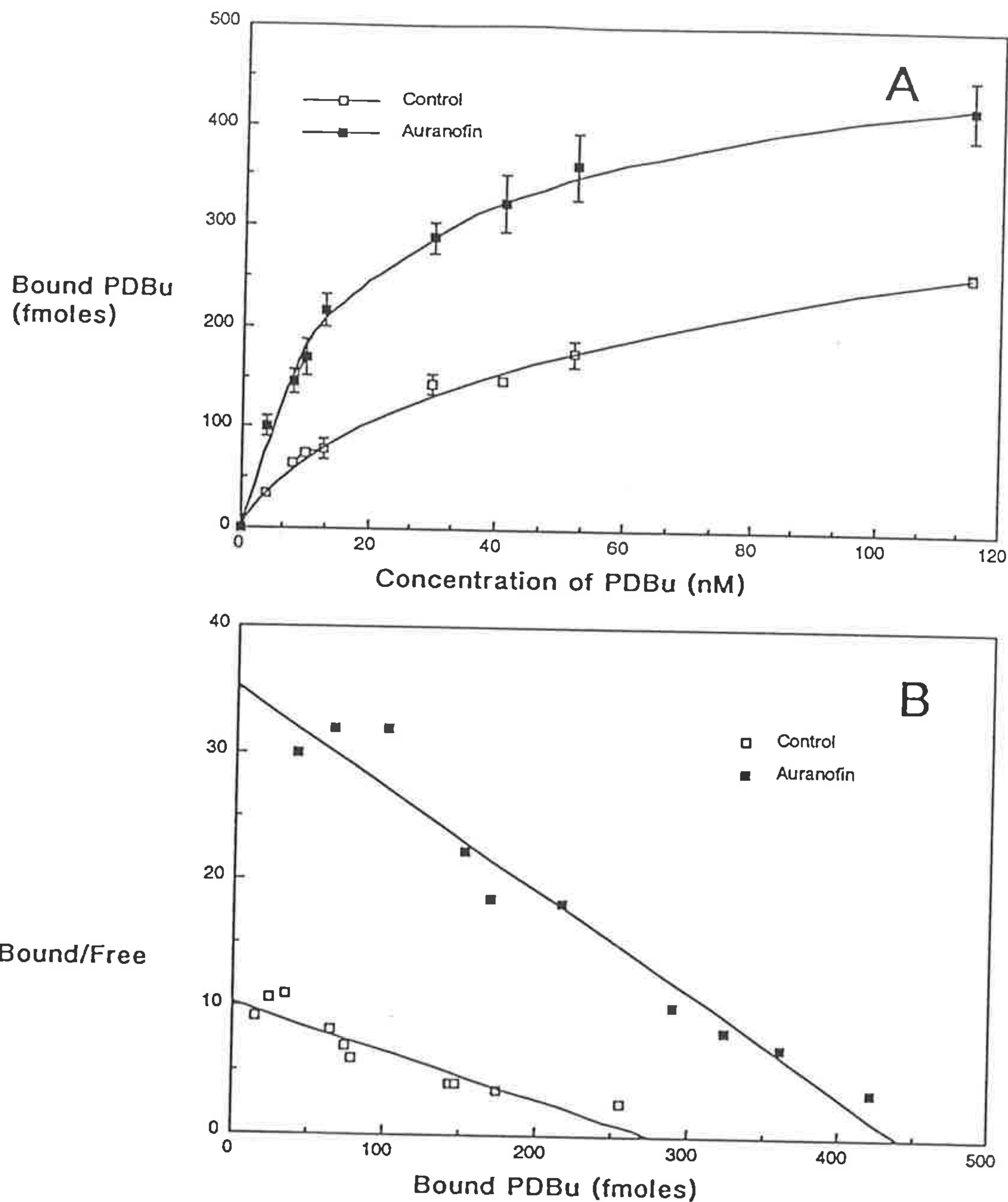


<u>Legend to Figure</u> <u>8.3</u> <u>Effect of auranofin on PDBu receptor affinity and maximum binding capacity in B-CLL cells</u>

Auranofin increased both PDBu receptor affinity and the maximum binding capacity of the cells. Similar results were seen with other populations of B-CLL cells (Table 8.4) and with PMN (Fig 8.4).

A: Saturation plot
B: Scatchard plot

B-CLL cells were incubated at 37°C in RPMI/FCS with or without 60µM auranofin and with varying concentrations of ^{3}H-PDBu. Binding was assayed after 40 minutes. Data are expressed as bound PDBu (fmoles) per 10^{6} cells and are shown as both saturation and Scatchard plots. Kds and binding capacities are shown in Table 8.4 (patient #2). Bars in the saturation plot indicate standard errors (n=3).

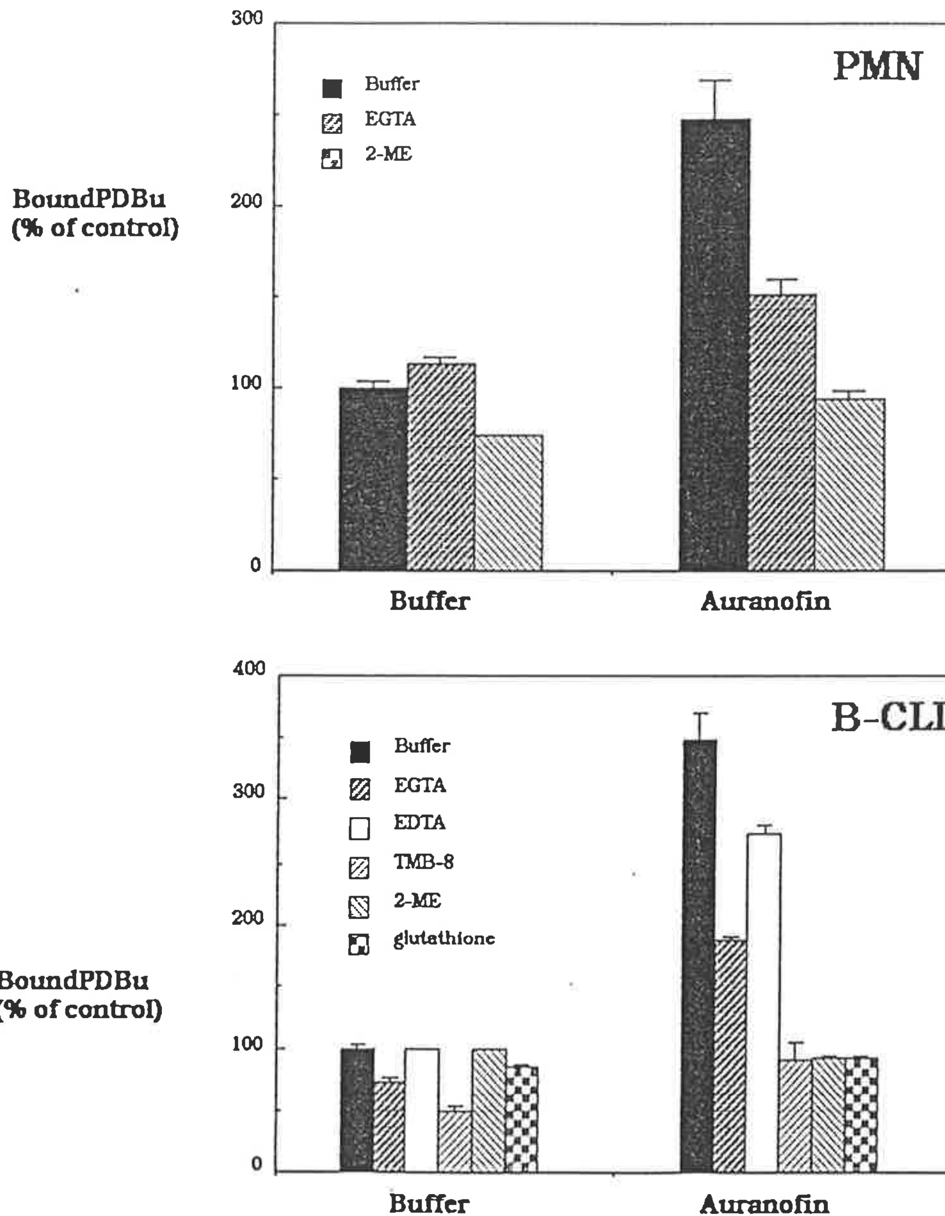


<u>Legend to Figure 8.4</u> <u>Effect of auranofin on PDBu receptor affinity and maximum binding capacity in PMN</u>

Auranofin increased both receptor affinity and maximum binding capacity of cells in PMN, as in B-CLL cells (Fig 8.3).

A: Saturation plot
B: Scatchard plot

PMN were incubated with auranofin and ^{3}H-PDBu as in Fig 8.3. Data are expressed as bound PDBu (fmoles) per 10^{6} cells. Kds and binding capacities are shown in Table 8.2 (PMN). Bars in the saturation plot indicate standard errors (n=3).

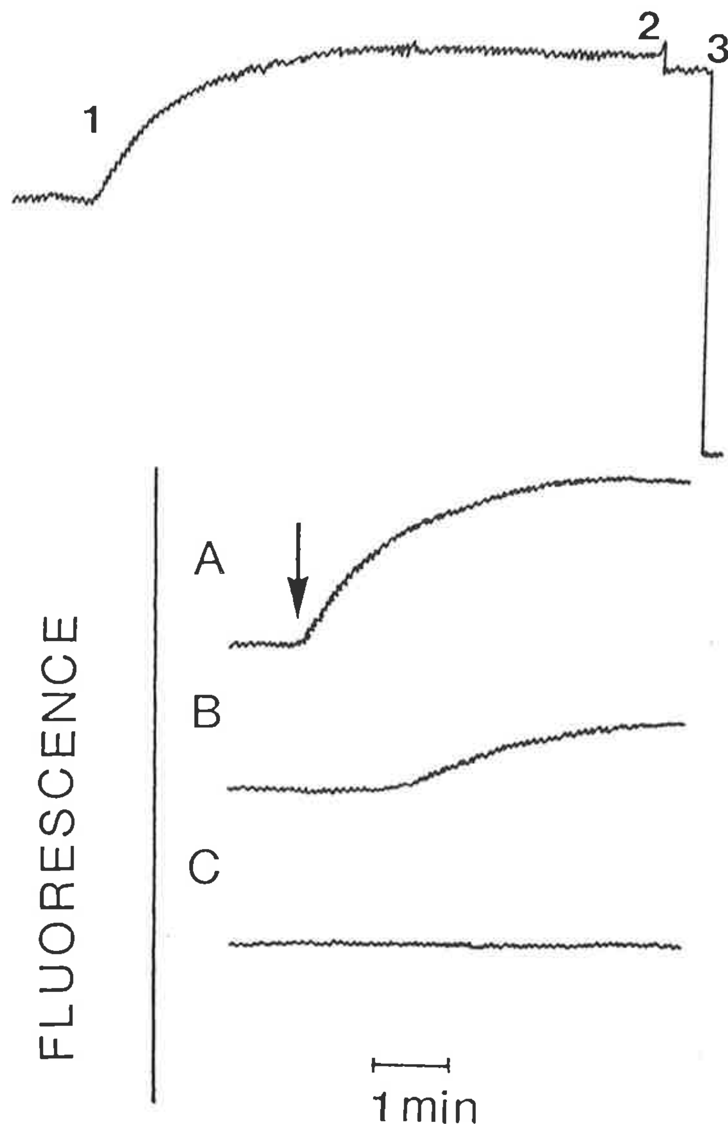


Legend to Figure 8.5 Effect of thiols, divalent cation-chelating agents and TMB-8 on augmentation of binding

Pre-treatment of cells with competing low molecular weight thiols, divalent cation-chelating agents and TMB-8 partially or completely prevented the increase in PDBu-binding caused by auranofin.

PMN
B-CLL cells

Cells were pre-treated for 10 minutes with 1mM 2-ME, 10mM glutathione, 2mM EGTA, 2mM EDTA, 250uM TMB-8 or buffer before addition of 5nM ^{3}H-PDBu and either auranofin (60μM) or control solvent DMSO. Binding was assayed after 40 minutes. Binding is expressed as a percentage of binding in controls (cells treated with ^{3}H-PDBu and buffer only). Bars indicate standard errors (n=3).



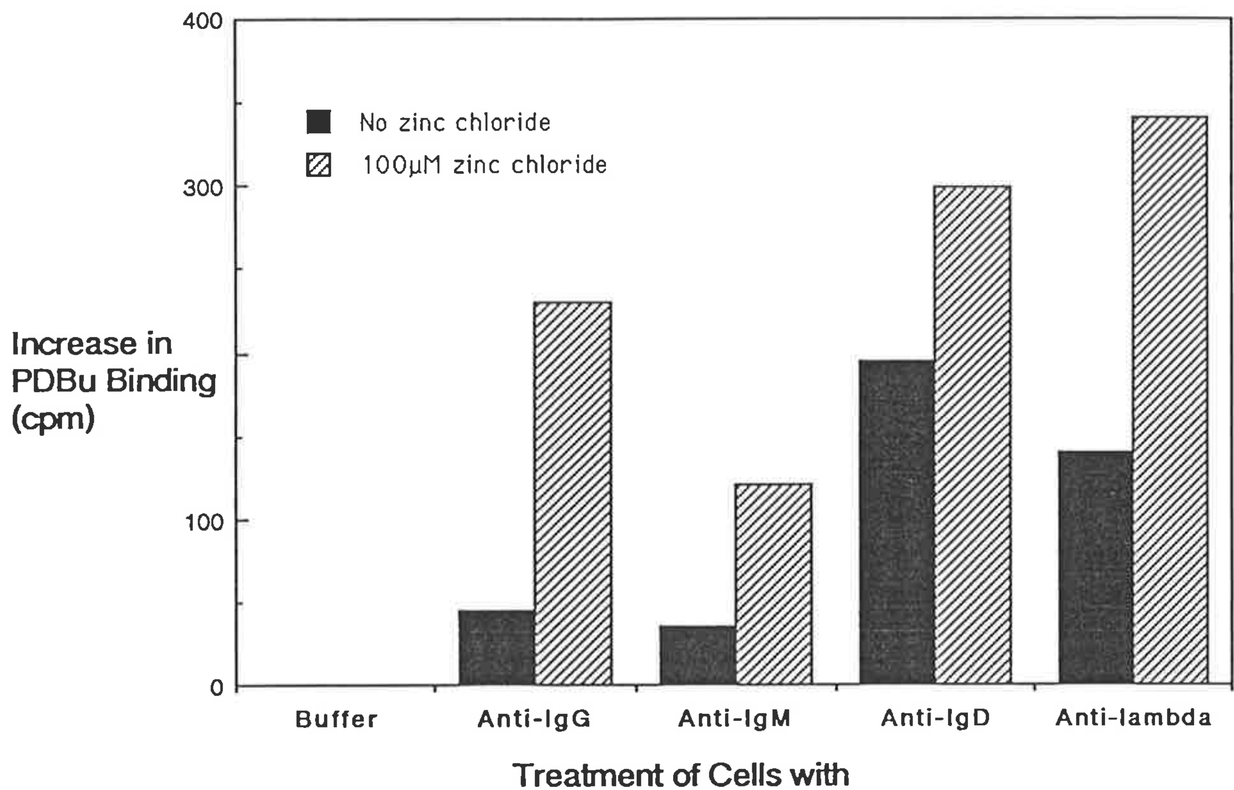
Legend to Figure 8.6 Effect of auranofin on intracellular free calcium

Auranofin caused a slow, two-fold increase in free cytosolic calcium in B-CLL cells, much less than the increase caused by calcium ionophore.

B-CLL cells were loaded with Quin-2 acetoxy-methylester as described in methods, resuspended in salt solution containing 1mM Ca^{++} and fluorescence was monitored in a spectro-fluorimeter. Excitation wavelength was 340nm and monitoring wavelength 490nm.

Upper panel: Standardization curve for auranofin trace. Auranofin (100μM) was added at (1), digitonin (25μM) at (2) and Mn^{++} (500μM) at (3). Readings were made as in chapter 2.13. Reading after addition of digitonin was taken as F_{max} and reading after addition of Mn^{++} as F_{min}. Autofluorescence of unloaded cells was subtracted from all readings.

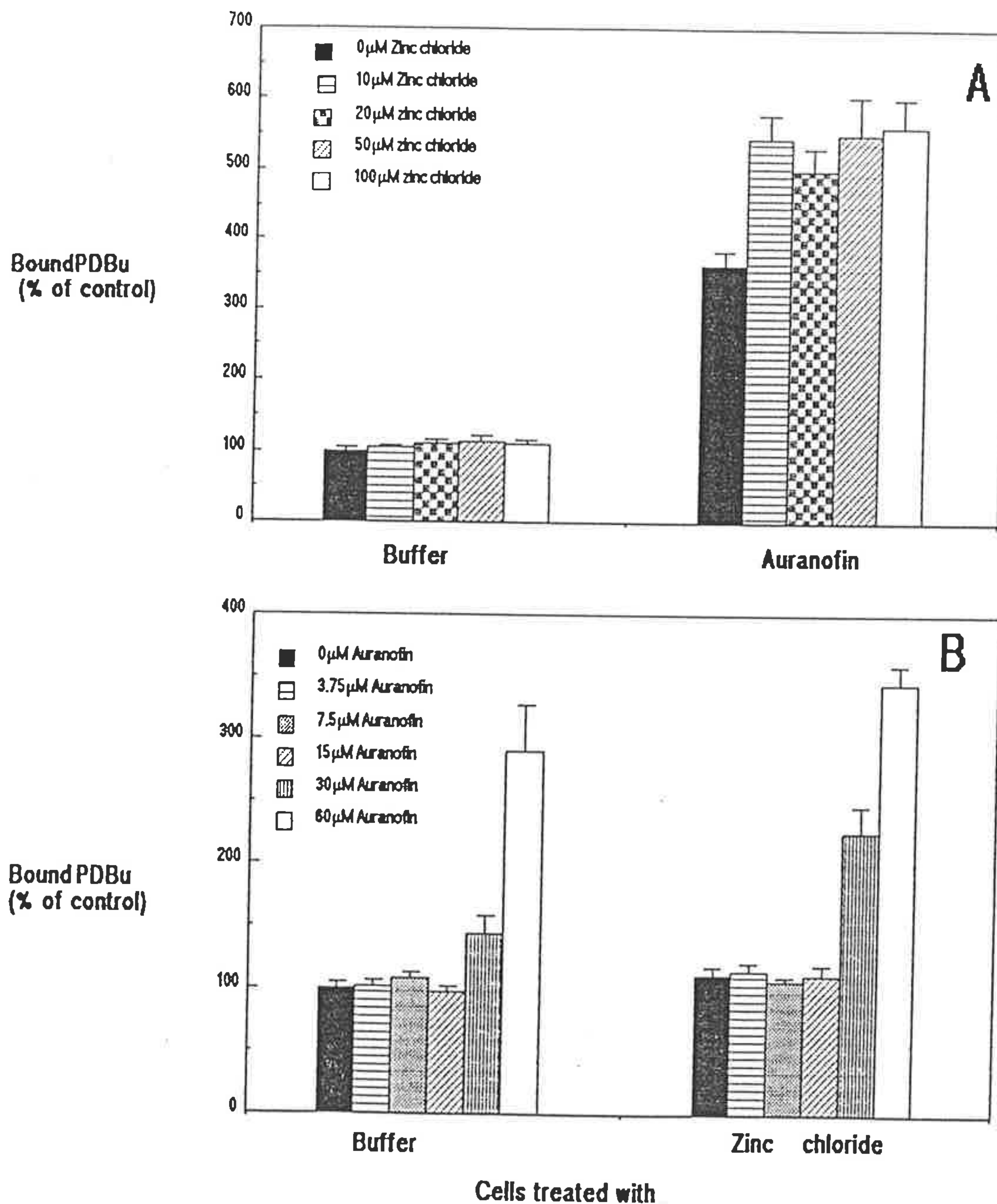
Lower panel: Reagents were added at time indicated by arrow.
A: Ionomycin
B: Auranofin
C: Control solvent DMSO (0.1% v/v).



Legend to Figure 8.7 Effect of zinc chloride on PDBu binding in cells treated with various anti-Ig isotypes

Zinc chloride alone had no effect on PDBu binding but amplified the increase in PDBu binding induced by anti-Ig.

B-CLL cells were pre-equilibrated with ^{3}H-PDBu (10nM) for 30 minutes at 37°C in RPMI/FCS before addition of zinc chloride (100μM) or buffer. After 5 minutes, various anti-Ig isotype-specific antibodies were added (100μg/ml). After a further 25 minutes, bound ^{3}H-PDBu was assayed. Increase in PDBu binding (cpm) over that of control cells (no anti-Ig or zinc) is shown.

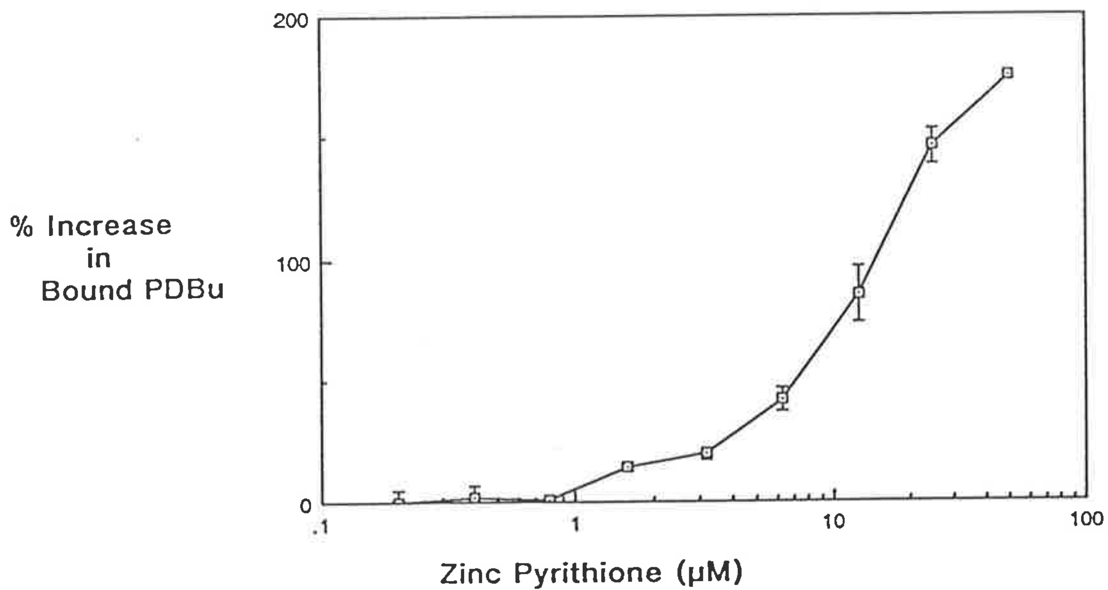


Legend to Figure 8.8 Effect of zinc chloride on auranofin-induced increase in PDBu binding

Zinc chloride amplified the increase in PDBu binding observed in auranofin-treated cells.

A: Varying concentrations of zinc
B: Varying concentrations of auranofin

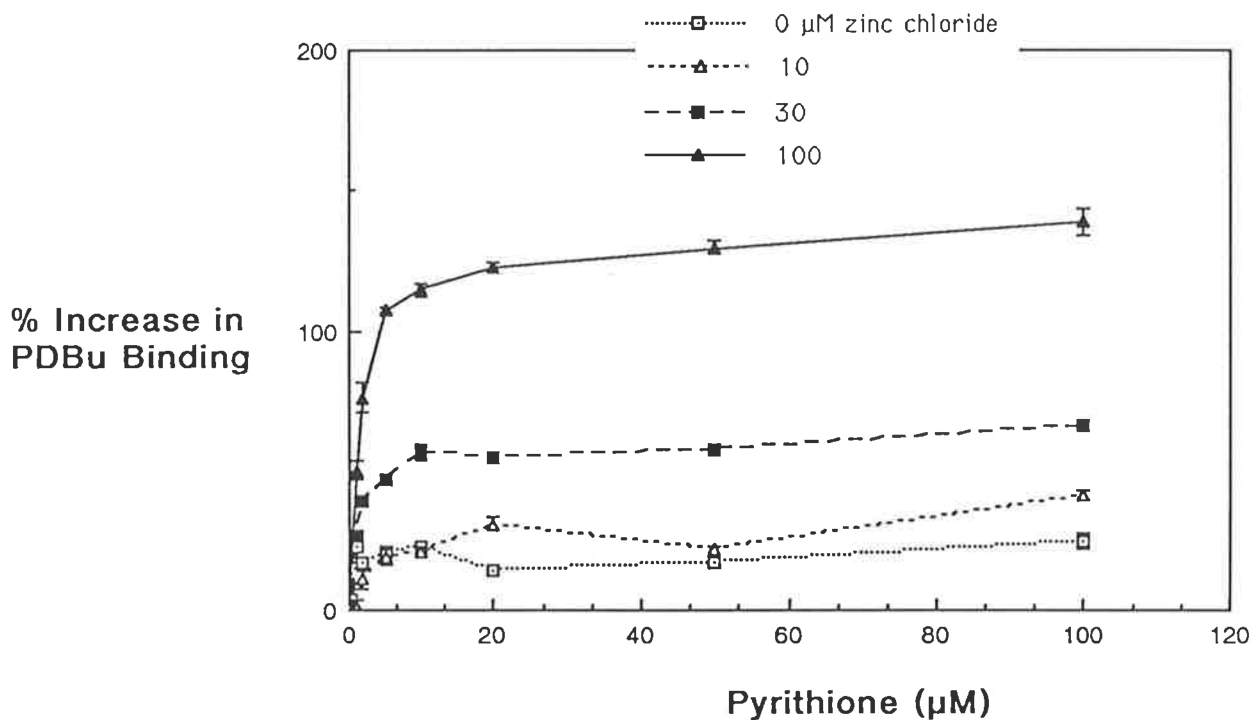
B-CLL cells were pre-equilibrated for 30 minutes at 37°C with 10nM ^{3}H-PDBu in RPMI/FCS and then incubated with varying concentrations of auranofin in the absence or presence of zinc chloride (100μM), or with varying concentrations of zinc chloride in the presence or absence of auranofin (60μM). Bound PDBu is expressed as % of binding in control (no auranofin or zinc). Bars indicate standard errors (n=3).



Legend to Figure 8.9 Effect of zinc pyrithione on PDBu binding

The lipophilic zinc pyrithione greatly increased PDBu binding in B-CLL cells.

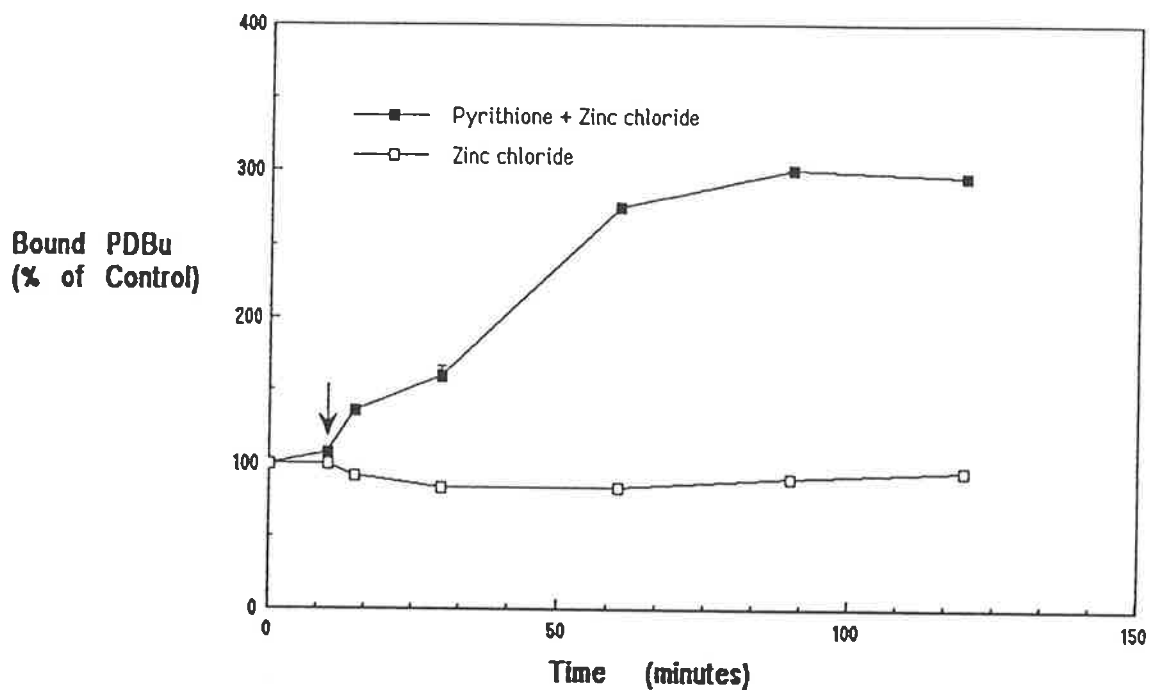
B-CLL cells were pre-equilibrated with 10nM ^{3}H-PDBu for 30 minutes at 37°C in RPMI/FCS before addition of varying concentrations of zinc pyrithione. After 40 minutes, cells were washed and bound ^{3}H-PDBu assayed. Binding is expressed as a % of binding in control (no zinc pyrithione). Bars indicate standard errors (n=3).



<u>Legend to Figure 8.10</u> <u>Effect of varying concentrations of zinc chloride and sodium pyrithione on PDBu binding</u>

Augmentation of PDBu binding by a combination of zinc chloride and sodium pyrithione increased with increasing concentrations of zinc chloride. Pyrithione was optimal at a concentration of about 10µM.

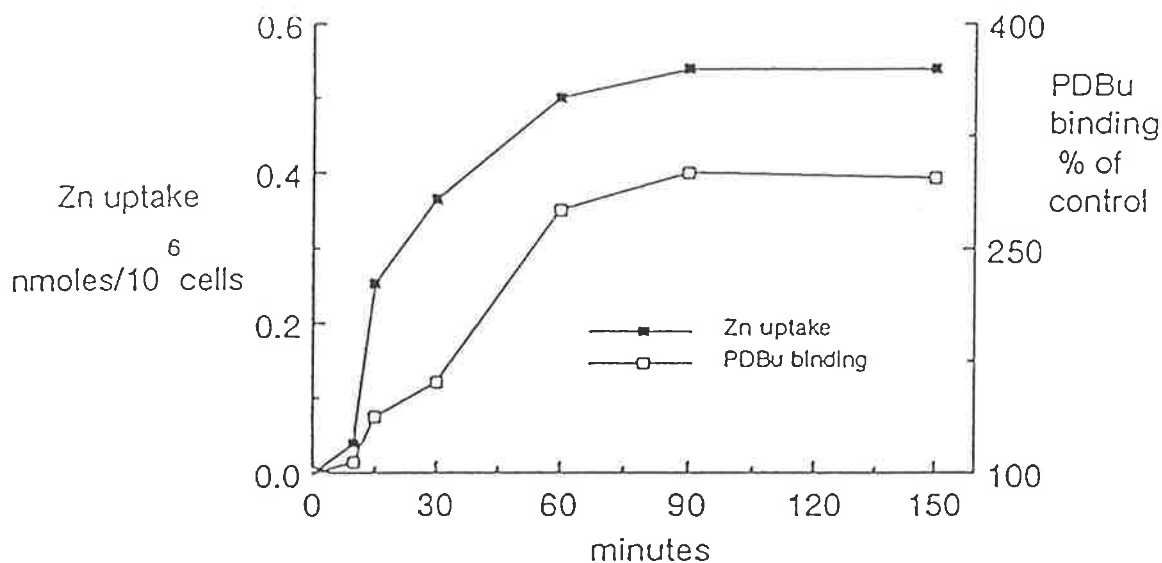
B-CLL cells were pre-equilibrated with 10nM ^{3}H-PDBu at 37°C in RPMI/FCS for 30 minutes before addition of varying concentrations of zinc chloride and sodium pyrithione. After a further 40 minutes, binding was assayed. Binding is expressed as a % of binding in control (no zinc or pyrithione). Bars indicate standard errors (n=3).



Legend to Figure 8.11 Kinetics of increase in PDBu binding in zinc-treated cells

In the presence of pyrithione, ionic zinc caused a slow increase in PDBu binding, particularly between 30 and 60 minutes.

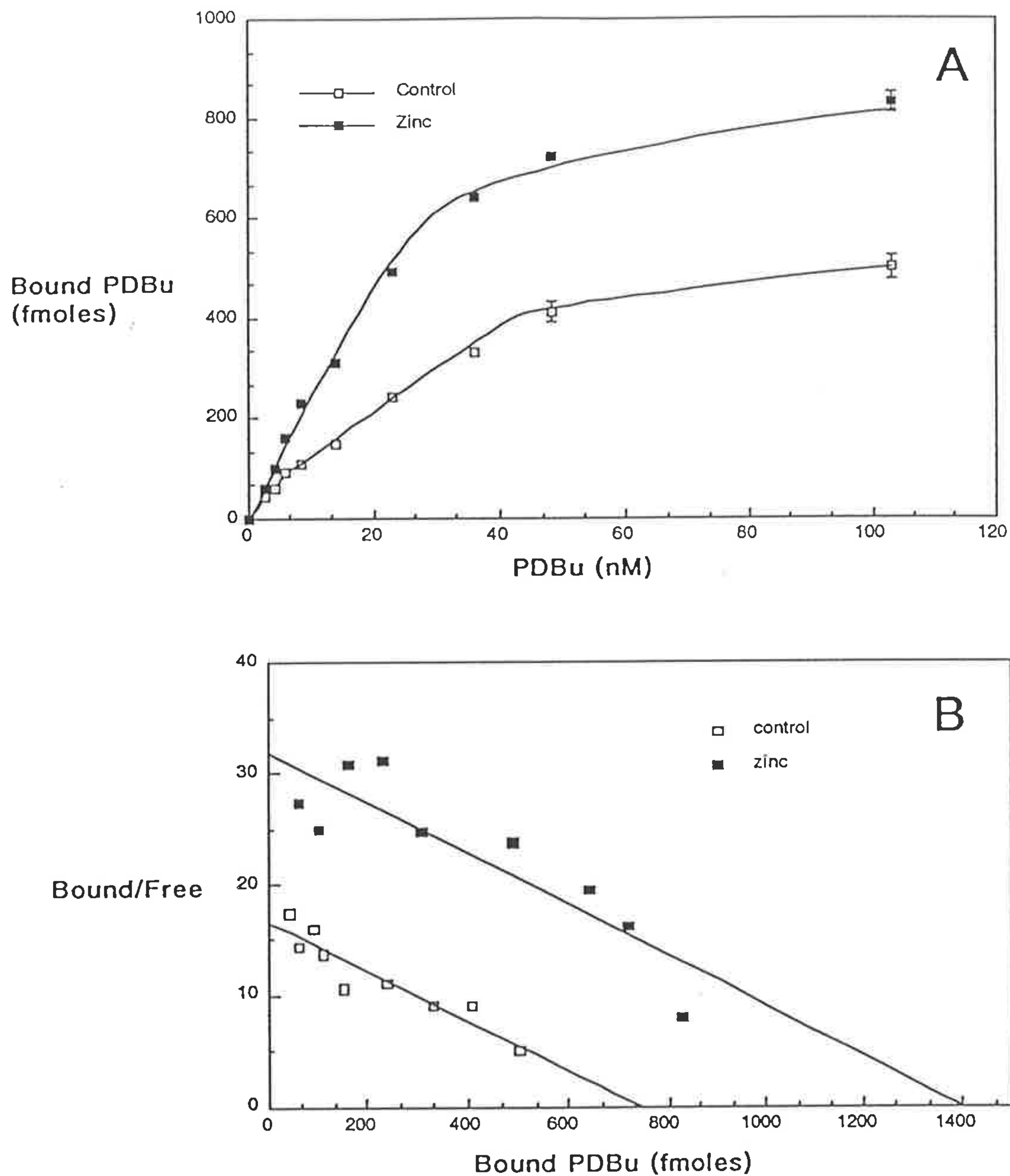
B-CLL cells were pre-equilibrated with 10nM ^{3}H-PDBu for 30 minutes before addition of 50μM zinc chloride, at time 0, and 20μM sodium pyrithione as indicated by arrow. Binding was assayed at varying times and is expressed as a % of binding in control cells (not treated with zinc or pyrithione).



<u>Legend to Figure 8.12</u> <u>Relationship between uptake of zinc and increase in PDBu binding</u>

The kinetics of increase in PDBu binding caused by zinc pyrithione paralleled that of uptake of zinc by the cells (as determined by ^{65}zinc studies).

B-CLL cells were treated with 5μM pyrithione and 50μM zinc chloride at 37°C in RPMI. One set of tubes was used for PDBu binding studies. The other contained a trace of ^{65}zinc chloride and was used for uptake studies. After 30 minutes, cells in both sets were washed by the following procedure, designed to allow estimation of intracellular ^{65}zinc. Cells were washed once at 4°C with PBS containing 1mM phenanthroline (to chelate extracellular ^{65}zinc) and three times with PBS containing 50μM unlabelled zinc chloride (to displace any other externally-bound ^{65}zinc). PDBu binding was assayed after the addition of 20nM ^{3}H-PDBu for 30 minutes. ^{65}zinc was assayed in cell pellets using an LKB gamma counter.

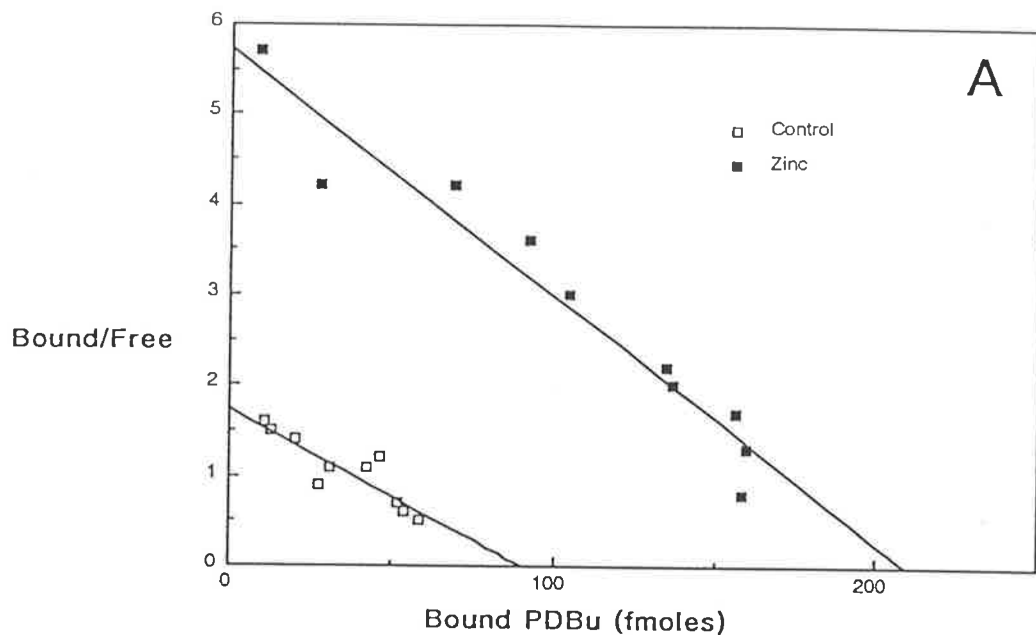


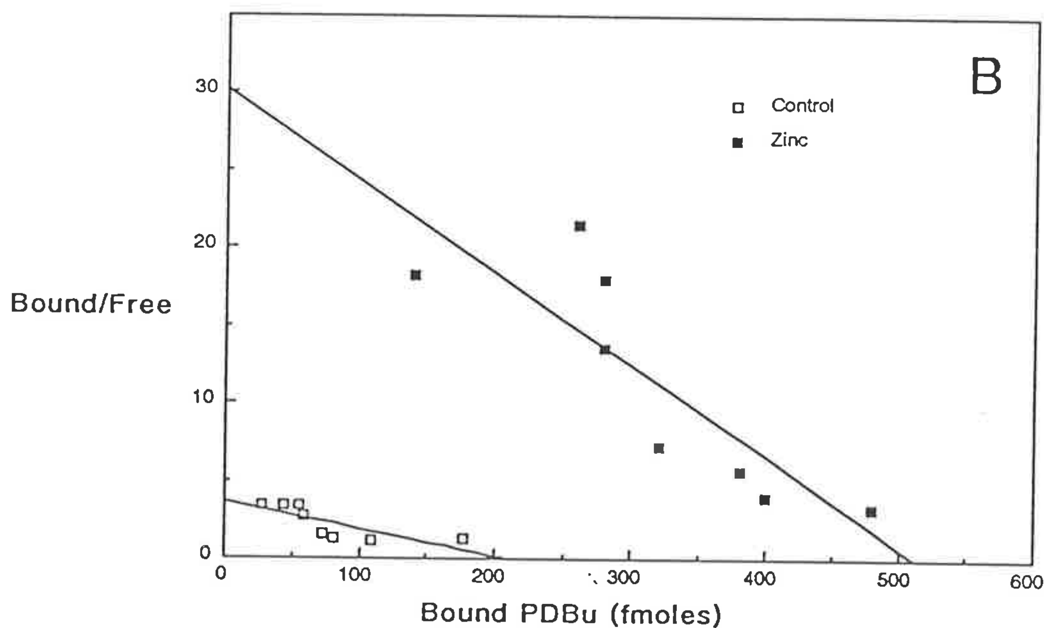
Legend to Figure 8.13 Effect of zinc on PDBu receptor affinity and maximum binding capacity in B-CLL cells

Zinc pyrithione caused a large increase in maximum PDBu binding capacity but little or no change in receptor affinity in B-CLL cells.

A: Saturation plot
B: Scatchard plot

B-CLL cells were treated with or without 50μM zinc and 20μM sodium pyrithione and varying concentrations of ^{3}H-PDBu for 40 minutes at 37°C in RPMI/FCS. Specific binding of ^{3}H-PDBu was determined (in triplicate) and is expressed as bound PDBu (fmoles) per 10^{6} cells. Data are shown as both saturation and Scatchard plots.



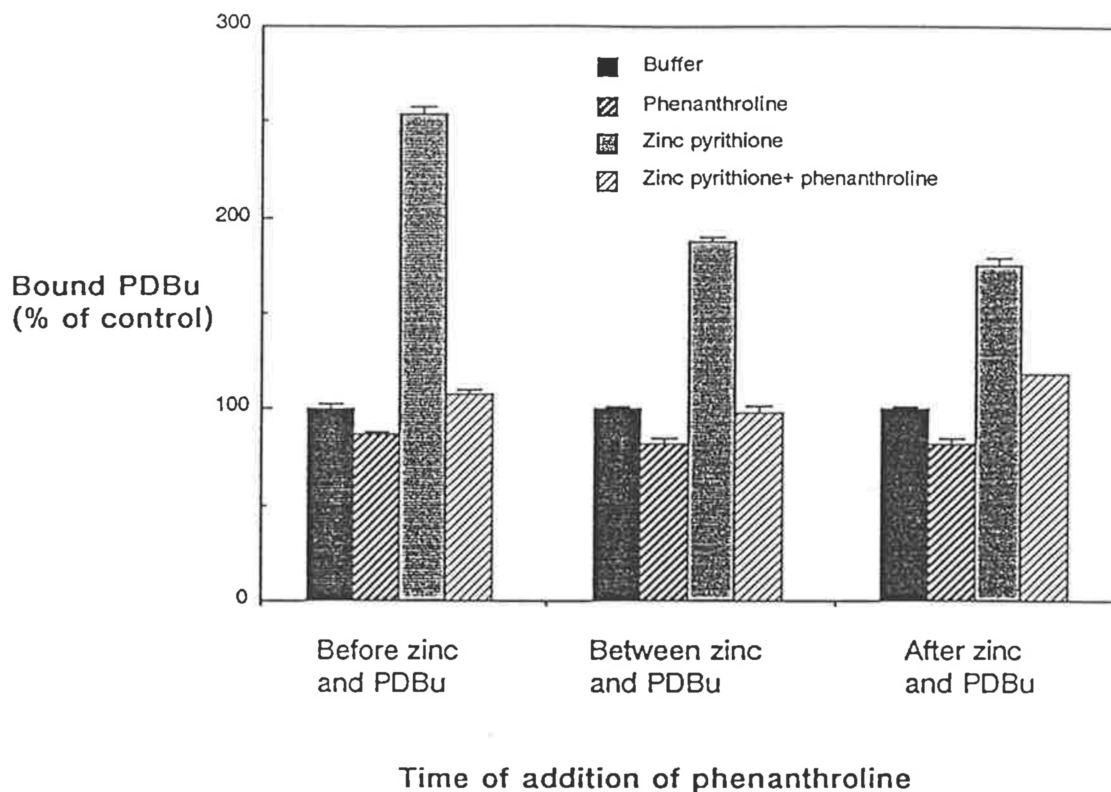


<u>Legend to Figure 8.14</u> <u>Effect of zinc on PDBu receptor affinity and maximum binding capacity in platelets and PMN</u>

Zinc pyrithione caused a large increase in maximum PDBu binding capacity but little or no change in receptor affinity in platelets (as in B-CLL cells) and a large increase in maximum binding capacity and receptor affinity in PMN.

A: Platelets
B: PMN

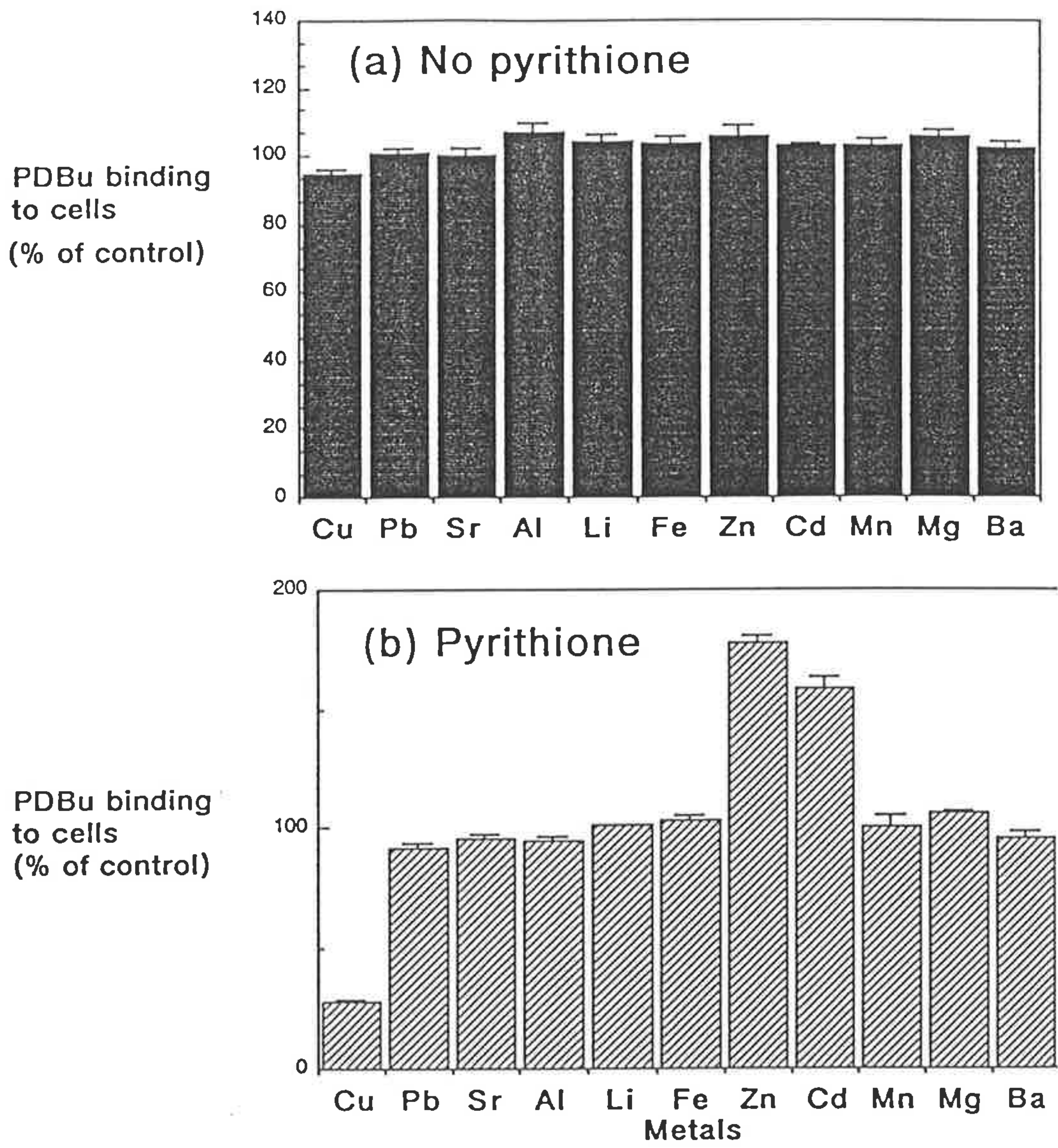
Platelets (10^8 cells per ml) and PMN (5×10^6 cells per ml) were treated with or without zinc pyrithione and varying concentrations of ^{3}H-PDBu and binding was assayed as in Fig 8.13. Specific binding of ^{3}H-PDBu was determined (in triplicate) and is expressed as bound PDBu (fmoles) per 10^{10} platelets and per 10^6 PMN. Data are shown as Scatchard plots.



Legend to Figure 8.15 Effect of phenanthroline on zinc-induced increase in PDBu binding

Phenanthroline completely prevented the effects of zinc pyrithione on PDBu binding in B-CLL cells, when added either before zinc pyrithione or after zinc pyrithione but before ^{3}H-PDBu. Phenanthroline partially prevented the increase in PDBu binding when added after both zinc pyrithione and ^{3}H-PDBu.

B-CLL cells were treated at 37°C in RPMI with 20µM pyrithione and 50µM zinc chloride (added at time 10 minutes) and 10nM ^{3}H-PDBu (added at 40 minutes). Bound PDBu was assayed at 80 minutes. Phenanthrolene (1mM) was added at 0, 20 or 50 minutes (that is, before both zinc pyrithione and PDBu, between addition of zinc pyrithione and PDBu or after both zinc pyrithione and PDBu, respectively). Bound PDBu is expressed as a % of that in control cells (incubated with only ^{3}H-PDBu). Bars indicate standard errors (n=3).



Legend to Figure 8.16 Effect of different metals on PDBu binding

None of the metals tested affected PDBu binding to B-CLL cells in the absence of pyrithione. In the presence of pyrithione, cadmium also increased PDBu binding and copper inhibited binding.

B-CLL cells were pre-equilibrated with 10nM ^{3}H-PDBu for 30 minutes at 37°C in RPMI before addition of various metals (100μM), in the absence (a) or presence (b) of 20μM sodium pyrithione. Bound PDBu was assayed after a further 40 minutes and is expressed as % of binding in control cells (untreated).

CHAPTER NINE

EFFECTS OF GOLD AND ZINC ON TRANSLOCATION AND ACTIVATION OF PKC

9.1 INTRODUCTION

This chapter considers the mechanism(s) by which auranofin and zinc pyrithione cause large increases in the maximum capacity of the cells to bind ^{3}H-PDBu. The maximum binding capacity is a product of the total receptor number and the number of binding sites per receptor (presumed to be one under normal conditions of assay). Gold and zinc may cause an increase in receptor numbers or in the number of moles of PDBu bound per mole of PKC.

The increase in binding may be a direct effect of the metals on PKC or some other component involved in the binding (eg phospholipid) or it may be mediated by other mechanisms induced in cells by metals such as a rise in $[Ca^{++}]_i$. The effect of gold, zinc and other metals on binding of ^{3}H-PDBu to cell-free extracts of PKC was determined.

An increase in total number of PDBu receptors can be explained by increased translocation of cytosolic aporeceptors, since at concentrations of ^{3}H-PDBu which saturated membrane receptors in the cells, only a proportion of the cytosolic aporeceptors was translocated to the particulate fraction (chapter 4). Gold and zinc may enhance binding by causing more of the cytosolic aporeceptors to move to the particulate fraction.

Particulate PKC exists in both detergent-soluble and -insoluble states (chapter 1.9.1). It is presumed that the latter represents PKC in the nucleus and cytoskeleton, although the existence of PKC in these pools is still debated. It has been claimed that translocation of PKC to the nuclei is induced by cAMP, bryostatin and TPA (chapter 1.9.2). Most of the translocation induced by phorbol ester and calcium is to a detergent-soluble state, thought to represent a complex between

PKC and membrane phospholipid.

The aims of the experiments in this chapter were to determine (a) whether auranofin, zinc and other metals affect PDBu binding in cell-free extracts, (b) whether they affect translocation of PKC from cytosol to the various pools in the particulate fraction, both in intact cells and cell-free homogenates and (c) whether changes in catalytic activity accompany these effects.

9.2 RESULTS

9.2.1 EFFECT OF METALS ON BINDING OF PDBu TO PKC IN CELL-FREE EXTRACTS

Direct addition to cell-sonicates

As with intact cells, addition of auranofin, zinc and cadmium increased PDBu binding in sonicates of B-CLL cells, while copper and iron (ferric ions) inhibited binding (Fig 9.1, Fig 9.2). Copper inhibited binding by 50% at a concentration of 17μM. The inhibitory effects of copper and iron may be associated with the capacity of these two metals as oxidizing agents.

Unlike intact cells, sonicates had increased PDBu binding when treated with zinc chloride in the absence of pyrithione, confirming that the role of pyrithione is only in promoting uptake of zinc by cells. Some enhancement was seen at the lowest concentration of zinc tested (10μM) and was optimal at 100μM zinc. The effects were much decreased in the presence of EDTA and EGTA and therefore these chelators were omitted from the sonication buffer.

Direct addition to the particulate fraction

Auranofin and zinc chloride had no effect on binding of ^{3}H-PDBu when added to the isolated particulate fraction of B-CLL cells.

Direct addition to the cytosol

However, auranofin (see Fig 9.5) and zinc chloride (Fig 9.3) or zinc sulphate (not shown) did increase binding to the isolated cytosol (reconstituted with PS and calcium). Augmentation of PDBu binding was seen at concentrations of zinc between 1 and 10uM and most of the increase was seen at a concentration of 100uM. Auranofin or zinc had no effect on binding if cytosol was omitted, indicating that the metals do not cause some artifactual binding of ^{3}H-PDBu to other components such as the phospholipid or the filter papers.

Both auranofin and zinc also increased binding of ^{3}H-PDBu to cytosol in the absence of exogenous PS indicating that zinc is not enhancing binding by facilitating the interaction of PKC with phospholipid. A significant portion of the PDBu binding activity in the cytosol occurred without addition of PS. The most likely explanation of these results is that the cytosol still contains some membrane vesicles or other particulate matter, not pelleted at 30,000g and that metals facilitate the binding of PKC to this material. Similar results were obtained when cytosol was prepared at 100,000g.

Zinc chloride did not enhance binding of PDBu to cytosol which had been completely depleted of PDBu receptors by absorbing it with particulate fraction in the presence of 5mM calcium (to promote association of PKC with the membrane phospholipid). This result suggests that zinc is acting on PKC (or at least something which binds to the particulate fraction in the presence of calcium). It also suggests that there is little calcium-independent PDBu receptor in the cytosol of B-CLL cells and, therefore, zinc is acting on the calcium-dependent form of PKC

and not on some other pool of PKC such as the putative, calcium-independent delta, epsilon and zeta isozymes (see chapter 1.8).

The effects of zinc on binding of ^{3}H-PDBu to the cytosol were almost completely inhibited by the zinc-chelating agents phenanthroline (1mM) or histidine (250μM) (Fig 9.4).

Scatchard analysis indicated that there was no significant change in the affinity of PDBu receptors but the apparent total numbers of receptors were increased by 40% with 60μM auranofin (Fig 9.5) and 100% with 50μM zinc chloride (Fig 9.6). Similar increases occurred if phospholipid was not added to the cytosol. Therefore, the increased binding may reflect an interaction of metals and PKC with endogenous phospholipid or endogenous membranous vesicles derived from the particulate fraction and not pelleted at 30000g.

It is unlikely that zinc and gold increase PDBu binding by blocking turnover of PKC, since PDBu binding activity was not lost in control preparations, when aliquots were assayed at various times during the 60 minute incubation. The effect of zinc was optimal by 15 minutes and did not increase further with time even up to 120 minutes.

9.2.2 TRANSLOCATION OF PKC TO THE PARTICULATE FRACTION IN CELL-SONICATES

To determine whether enhanced binding of PDBu to sonicates, treated with auranofin or zinc chloride, was accompanied by enhanced translocation of PKC from cytosol to the particulate fraction, metals were added to sonicates of B-CLL cells and then the particulate fractions were pelleted at high speed, washed and assayed for binding of 10nM ^{3}H-PDBu.

Addition of auranofin (6 or 60μM) to the sonicates increased binding of ^{3}H-PDBu to the particulate fraction by 12% and 38%,

respectively (Table 9.1). Addition of zinc chloride (100μM) to the sonicates increased PDBu binding to the particulate fraction by 54%.

9.2.3 TRANSLOCATION OF PKC TO THE PARTICULATE FRACTION IN CELLS

Auranofin

Auranofin caused a large (139%) increase in the PDBu binding activity of the particulate fraction, which was detectable only when this fraction was prepared in the absence of 1mM EDTA (Table 9.2). No increase was detectable if the particulate fraction was prepared in the presence of EDTA. Therefore, like the calcium ionophores (chapter 7), auranofin increased the divalent cation chelator-labile pool of PKC but not the pool of PKC that is tightly associated with the particulate fraction.

Although auranofin alone did not increase the pool of PDBu receptors that was tightly bound to the particulate fraction, auranofin greatly enhanced PDBu-induced translocation of PKC to this pool. Thus, while treatment of B-CLL cells with 200nM unlabelled PDBu decreased the PDBu binding activity and histone kinase C activity in the cytosol by about 30% and caused a 35% (sem 1.7%, n=3) increase in the PDBu binding activity of the particulate fraction, treatment of B-CLL cells with auranofin plus PDBu caused a much greater loss of the PDBu binding activity and histone kinase C activity in the cytosol and there was a very large (140%, sem 1.2%, n=3) increase in the PDBu binding activity of the particulate fraction.

Auranofin (60μM) synergized with 10, 100 and 1,000 nM PDBu in causing translocation of PKC to the particulate fraction (Fig 9.7). Low concentrations (0.6 or 6μM) of auranofin had no effect.

Zinc

Zinc pyrithione alone induced translocation of PKC to the tightly bound particulate fraction in B-CLL cells (Table 9.3). There was a synergism between zinc pyrithione and PDBu in causing translocation (see chapter 9.2.4).

9.2.4 EFFECT OF TREATMENT OF CELLS WITH AURANOFIN AND ZINC PYRITHIONE ON DETERGENT SOLUBILITY OF THE PKC

Effect of auranofin on translocation to detergent-insoluble portion of the particulate fraction

Consistent with other studies (chapter 1.9), PDBu caused PKC to translocate from the cytosol to the detergent-soluble portion of the particulate fraction. PDBu (200nM) increased both the PDBu binding and histone kinase C activities in the Triton-soluble fraction of the particulate fraction (Fig 9.8, 9.10). There was also a small increase in PDBu binding in the insoluble fraction. It was not possible to assay the insoluble fraction for histone kinase C activity since this assay requires soluble PKC.

In contrast to the results with PDBu, auranofin caused translocation of PKC to the detergent-insoluble fraction. When cells were treated with auranofin, alone, there was no increase in the PDBu binding activity of the particulate fraction (as shown in chapter 9.2.3) but there was a profound change in the detergent-solubility of the PKC in the particulate fraction. About 50% to 65% of the PKC in the particulate fraction, as assayed by PDBu binding or histone kinase C activity, could no longer be extracted by 0.2% Triton X-100. The detergent-insoluble residue had a modest increase (about 20%) in PDBu binding activity (Fig 9.8).

Both the PDBu-binding activity (Fig 9.9) and the histone

kinase C activity (Fig 9.10), in the detergent-soluble fraction, decreased at concentrations of auranofin of 6μM and greater. PDBu binding activity in the insoluble fraction increased at these concentrations. These results suggest that auranofin alone causes membrane PKC to become detergent-insoluble, without affecting the cytosolic PKC.

In cells pretreated with both auranofin and PDBu, there was a large increase in the PDBu binding activity of the particulate fraction, as shown in chapter 9.2.3, but most of the binding sites for PDBu were in the Triton-insoluble fraction (Fig 9.8). In agreement with this, very little PDBu-binding and histone kinase C activity was extracted by Triton X-100 from the particulate fraction of cells treated with auranofin plus PDBu (Fig 9.9, 9.10A). Calcium-and phospholipid-independent histone kinase activity was decreased also but to a lesser extent (Fig 9.10B).

These results suggest that auranofin enhances the PDBu-induced translocation of PKC from cytosol to particulate fraction but, as in cells treated with auranofin alone, most of the PKC is now in the detergent-insoluble compartment.

Effect of auranofin on translocation to the nucleus-cytoskeleton complex

The effect of auranofin on translocation to a detergent-insoluble compartment was tested in another way using a detergent lysis of the whole cell. Cells were extracted with 0.2% NP40 and centrifuged at 3,000 rpm on a 60% sucrose cushion, as described in chapter 2.10. The pellet was taken as the detergent-insoluble residue of the cell. It is claimed that this pellet contains only cell nuclei [Cambier et al. 1987b]. Examination by phase contrast microscopy of the pellet after resuspension indicated the

presence of nuclei without any intact cells. Cytoskeletal material, that has collapsed onto the nuclei, may also be present. Therefore, the pellet is referred to here as the nucleus-cytoskeleton complex. This was assayed for PDBu receptors after sonication.

Treatment of cells with 200nM unlabelled PDBu increased binding of ^{3}H-PDBu to the nucleus-cytoskeleton complex by 32%, treatment with auranofin alone increased it by 31% and treatment with a combination of PDBu and auranofin increased it by 152% (Fig 9.11). Therefore, auranofin synergizes with PDBu in translocation of PKC to the nucleus and/or cytoskeleton of B-CLL cells.

A rise in intracellular cyclic AMP causes translocation of PKC to this nucleus-cytoskeleton complex in mouse B cells [Cambier et al. 1987b]. Treatment of B-CLL cells with dibutyryl cyclic AMP and theophylline caused an increase in the binding of ^{3}H-PDBu to the nuclei by 53%. When 200nM unlabelled PDBu was added to the cells at the same time as dibutyryl cyclic AMP and theophylline, no increase of binding was seen (Fig 9.11).

Therefore, the mechanism by which auranofin induces translocation to this fraction does not appear to be mediated by a rise in cAMP alone.

Effect of zinc pyrithione on translocation of PKC to the nucleus-cytoskeleton complex

Like auranofin, zinc pyrithione synergized with PDBu in causing translocation of PKC to the nucleus-cytoskeleton complex (Fig 9.12,9.13). The insoluble material from zinc-treated cells had about twice as much PDBu binding activity as that from control cells, while that from cells treated with both zinc and

PDBu had a nine-fold capacity to bind ^{3}H-PDBu. Similar results were seen following extraction with 0.1% NP40. However, there was less binding of ^{3}H-PDBu to the nucleus-cytoskeleton complex of zinc-treated cells when extraction was performed with 0.5% NP40, suggesting that this concentration of detergent may solubilize some of the translocated receptors.

Between 25% and 35% of the PDBu receptors associated with the detergent-insoluble complex in cells treated with zinc pyrithione, or zinc pyrithione plus PDBu, were lost when this fraction was prepared in the presence of 1mM EDTA suggesting that the PKC is partly attached to the detergent-insoluble material by zinc or other divalent cation (Fig 9.13).

Accompanying the increase in PDBu receptors in the detergent-insoluble residue, in cells treated with zinc or zinc plus PDBu, there were corresponding decreases in the PDBu aporeceptors in the supernatant (Fig 9.12). These aporeceptors in the supernatant could be assayed by addition of PS and calcium. It was not possible to assay them in the supernatants of cells treated with 0.5% NP40 presumably because this high concentration of NP40 interferes with the binding assay.

Effect of A23187 on translocation of PKC to the nucleus-cytoskeleton complex

Since calcium ionophores also augment PDBu binding in cells and enhance translocation of PKC to the membrane (chapter 7), the effect of A23187 on translocation to the detergent-insoluble compartment of B-CLL cells was determined. Treatment with A23187 did not increase PDBu binding in the detergent-insoluble compartment (Fig 9.13). This was the case regardless of whether PDBu was present during the time of incubation of cells. This indicates that calcium is not involved in the attachment of PKC

to the detergent-insoluble fraction. It also indicates that translocation of PKC by calcium is to a different compartment than translocation of PKC by zinc and gold.

9.2.5 EFFECTS OF GOLD AND ZINC ON PHOSPHORYLATION

Effect on protein phosphorylation in cells

Low concentrations of auranofin (0.6 to 6μM) increased phosphorylation of some proteins in intact B-CLL cells (Fig 9.14). Enhanced phosphorylation was seen in all six experiments. The pattern of phosphorylation differed somewhat from that induced by TPA. In contrast, 60μM auranofin decreased the intensity of phosphorylation in both resting and TPA-treated B cells (not shown).

A small increase in phosphorylation of proteins in the nucleus-cytoskeleton fraction was also seen in B-CLL cells treated with auranofin (Fig 9.15).

The effect of zinc pyrithione on protein phosphorylation in B-CLL cells needs to be tested.

Histone phosphorylation in vitro

To determine whether gold directly affects the catalytic activity of PKC, auranofin and the water-soluble sodium aurothiomalate were added to cytosol and histone kinase C activity was determined.

At concentrations greater than 6μM, auranofin inhibited histone kinase C activity when added to Triton-soluble extracts of B-CLL cells or to cytosolic fractions (Fig 9.16). The calcium- and phospholipid- independent kinase activity was inhibited to a much lower extent. No enhancement of catalytic activity was seen even at very low concentrations of auranofin (0.1-1μM). Sodium aurothiomalate also inhibited histone kinase C

activity when added to cytosol of B-CLL cells, suggesting that it is the gold moiety which is responsible for the inhibition.

The inhibition of PKC by auranofin and aurothiomalate was completely blocked by 2-mercaptoethanol (Fig 9.17).

Like gold, zinc chloride inhibited the capacity of cytosolic PKC to phosphorylate histone in a calcium-and phospholipid-dependent manner (Fig 9.18). Fifty per cent inhibition of activity occurred at about 100μM zinc. The inhibition by zinc was prevented when 2-ME was present in the buffer. No enhancement of histone kinase C activity was seen even at low concentrations of zinc (1μM).

9.3 DISCUSSION

In cell-free extracts, as in intact cells (chapter 8), zinc, gold and cadmium increased PDBu binding and copper inhibited it. In cell-free extracts, but not in intact cells, ferric ions were also inhibitory. Pyrithione may not effectively transport this metal. Inhibition of PDBu binding by copper and iron may be due to their strong oxidizing activities. Zinc is a relatively poor oxidant [Riordan 1976].

One possibility is that zinc and gold increase the number of PDBu binding sites per molecule of PKC. A doubling in apparent total number of receptors may correspond to the unmasking of another PDBu binding site in PKC. That treatment of the isolated particulate fraction with auranofin or gold does not increase PDBu binding, argues against this hypothesis. Nevertheless, precise stoichiometric studies with pure PKC are required to confirm this.

Preliminary evidence not shown here indicates that zinc has no effect on PDBu binding to pure PKC, even in the presence of phospholipid and calcium, whereas it has a marked effect on a

cell-free extract containing PKC and particulate material [C. Giannakis, personal communication]. This indicates that for zinc to affect PDBu binding some component other than PKC and phospholipid is required.

Both auranofin and zinc pyrithione greatly enhanced PDBu-induced translocation of PKC from cytosol to particulate fraction in both intact cells and in cell-free sonicates. Zinc chloride-induced translocation of PKC in cell-free extracts of mouse thymocytes has also been reported [Csermeley et al. 1988].

Parente and colleagues [1987] have shown independently that auranofin (5-20μM) induces a time and concentration-dependent translocation of PKC from cytosol to particulate fraction in human PMN. This occurred with a lag period of between 10 and 15 minutes, much slower than translocation induced by TPA. Only about 25% of the PKC activity lost from the cytoplasm in auranofin-treated cells could be recovered in Triton-extracts of the particulate fraction. Parente and colleagues [1987] speculated that the loss of PKC activity in auranofin-treated PMN was a result of enhanced conversion of PKC to PKM.

The results described here indicate that the decreased PKC activity in the detergent-soluble extracts of the particulate fraction is a result of the PKC becoming resistant to extraction by detergent. By assay for binding of PDBu, some of the "lost" PKC could be detected in the Triton-insoluble fraction. It is not possible to comment on the histone kinase C activity of the whole particulate fraction or Triton-insoluble portion because the assay requires soluble PKC. Phosphorylation of endogenous PKC substrates in these fractions, needs now to be studied.

PKC may become resistant to extraction by detergent because

auranofin and zinc pyrithione are very hydrophobic and stabilize the association of PKC with the membrane. However, this cannot explain the action of zinc chloride on PDBu binding in cell-free extracts. Because they can bind more than one sulphydryl group, gold or zinc may cross-link PKC to some detergent-insoluble protein in the membrane, possibly a cytoskeletal protein. Alternatively, these metals may cause PKC to translocate to a detergent-insoluble compartment, such as the nucleus. Treatment of cells with non-ionic detergent leaves a "shell", composed largely of nucleus and cytoskeleton [Osborn and Weber 1977].

Both auranofin and zinc pyrithione induced translocation of PKC to the nucleus-cytoskeleton complex of B-CLL cells, particularly in the presence of PDBu. At least with auranofin, this was accompanied by enhanced protein phosphorylation in this fraction.

Translocation of PKC to the nucleus-cytoskeleton complex by anti-MHC class II antibodies is a consequence of a rise in cyclic AMP [Cambier et al. 1987b]. Since auranofin has been reported to raise cAMP in some cell types [Scheinberg et al. 1982], cAMP may mediate some of the effects of auranofin in this study. However, treatment of B-CLL cells with a variety of agents which elevate cyclic AMP did not cause increased cellular binding of PDBu or inhibition of MER (chapter 5). Although treatment of B-CLL cells with dibutyryl cAMP and theophylline did cause a moderate increase in binding of ^{3}H-PDBu to the nucleus-cytoskeleton complex, this increase did not occur when cells were treated with these agents and PDBu together. PDBu also blocks cyclic AMP-mediated translocation of PKC to the nucleus in mouse B cells [J. Cambier, personal communication]. In contrast, a strong synergism was seen between auranofin or zinc pyrithione and PDBu in

translocation of PKC to the nucleus-cytoskeleton complex.

Zinc and gold may cause PKC to translocate to the nucleus in the same way that zinc enhances binding of androgen receptors to isolated nuclei, by facilitating the interaction of their zinc finger with DNA [Colvard and Wilson 1984]. This hypothesis is strengthened by recent evidence that PKC binds to DNA in cell-free preparations [Testori et al. 1988]. Sequences rich in basic amino acids, and which are thought to permit some large proteins to enter the nucleus [Richardson et al. 1988], may be present in at least one of the isozymes (epsilon) of PKC [Ohno et al. 1988].

Since zinc and gold also augmented PDBu binding in platelets, which lack a nucleus but have a well-defined cytoskeleton, these metals may affect the binding of PKC to the cytoskeleton, structures which are rich in thiols and known to interact with zinc (chapter 1.12). Auranofin enhances phosphorylation of two proteins in platelets, both of which appear to be associated with the cytoskeleton [Nishizula 1984, Hashimoto et al. 1987].

Further work is needed to establish the site to which PKC is translocated in metal-treated cells, and to show whether the PKC is now attached to some other molecule than phospholipid and is catalytically active.

The mechanism by which auranofin enhances PDBu-mediated translocation of PKC is not clear from these studies. PDBu, even at very high concentrations, only induces a partial translocation of PKC from cytosol to particulate fraction. One interpretation of this is that there is something other than the PDBu which limits the translocation, eg anionic phospholipid, divalent cation or membrane protein, that is required for the

translocation (see chapter 1.9). Unlike PDBu, the more potent analogue TPA causes complete translocation of PKC from the cytosol and about 30-40% of this is to a detergent-insoluble compartment [Thomas et al. 1988].

A significant portion of the cytosolic apo-PKC may require zinc as a cofactor for translocation. This may be a particular isozyme of PKC which requires a metal cofactor or it may be a pool of PKC which is complexed with something in the cytosol that prohibits translocation. Metals may displace PKC from such a structure. It has been reported, recently, that the zinc chelator TPEN blocks TPA-induced translocation of PKC to the particulate fraction of cells and zinc reverses this [Csermeley et al. 1987b]. Furthermore, TPA caused an efflux of zinc from stores in the cell [Csermeley et al. 1987a]. These observations are compatible with the hypothesis that zinc is essential for phorbol ester-induced translocation of PKC.

Since the effects of zinc chloride on PDBu binding in cytosol were not seen when calcium-dependent PKC was depleted from the cytosol it appears likely that zinc acts on the same PDBu receptor as calcium.

Another explanation is that PDBu does induce a complete translocation of PKC but that when the cells are washed prior to fractionation, PDBu is lost from the membrane and much of the translocated PKC returns to the cytosol. Studies in a cell-free system [Wolf et al. 1985a,b] suggest that phorbol ester is required for stable association of PKC with the cell membrane. Auranofin and zinc pyrithione may stabilize PKC in the particulate fraction so that when PDBu is removed by washing, translocated PKC does not return to the cytosol. However, since the metals augment PDBu binding in whole cells, even without

washing, this explanation seems unlikely.

It is important to determine whether the effects of gold and zinc on the regulatory domain of PKC (PDBu binding) influence the catalytic domain, particularly since both auranofin (chapter 1.11) and zinc (chapter 1.12) increase phosphorylation of various proteins in intact cells. These metals may also affect the state of phosphorylation of some proteins by inhibiting phosphatases [Brautigan et al. 1981].

Addition of both gold and zinc to soluble preparations of PKC inhibited the kinase C activity even at low concentrations. It is difficult to relate this type of cell-free experiment to that in whole cells since the concentrations of metals which interact with PKC in the whole cell may be quite different from those acting on PKC in cell free extracts.

Heavy metals may inhibit the catalytic activity by binding to cysteines in PKC, either the putative heavy metal binding cysteine-rich sequences in the regulatory domain, or the essential sulphydryl in the catalytic domain of PKC (chapter 1.8.3). In favour of the latter hypothesis, auranofin inhibits the histone kinase C activity of the isolated catalytic domain of PKC [M. Froscio, personal communication]. The inhibition was evident with substrates other than histone indicating that auranofin does not alter the substrate specificity of PKC.

Parente and colleagues [1987] reported no inhibition of histone kinase C activity in soluble PKC by treatment with auranofin but their preparation of PKC was in a buffer containing 2-ME. The results described here show that thiol prevents the inhibition of PKC by gold and zinc, presumably by competing with sulphydryls in PKC for the metals.

In contrast, recent reports describe the activation of the catalytic site of PKC by zinc [Murakami et al. 1987, Csermeley et al. 1987b] and by lead [Markovac and Goldstein 1988]. In one study with zinc, the effects were dependent on the calcium concentration. A biphasic effect of zinc was seen, with activation at low concentrations (5μM) of calcium and inhibition at high concentrations (50μM). Zinc did not inhibit oleic acid-induced activation of PKC which is calcium-independent. In the other study [Csermely et al. 1987b], there was activation of PKC by a very high concentration (800μM) of zinc. That study did not investigate the effects of varying the calcium concentration. Both studies were complicated by the presence of zinc chelating agents EGTA and 2-ME in the buffers. Some of the effects may be the result of displacement of calcium from EGTA by zinc [Bartfai 1979]. These experiments need to be repeated under conditions in which the free calcium and zinc concentrations can be precisely determined.

PKC appears to be a protein which responds differently to both calcium and zinc. Distinct effects of zinc and calcium have been observed with some other proteins including calmodulin, alpha-lactalbumin and brain S-100 protein (see chapter 1.12). Zinc and calcium act at different sites in these proteins. The putative zinc- and calcium-binding sites in PKC appear also to be in different parts of the molecule, the zinc-finger being in the C_1 domain and the calcium-binding site presumed to be in C_2 or V_3 (see chapter 1.8.3).

Some of the results in this chapter have been published in Zalewski et al. 1987, Zalewski et al. 1988, Forbes et al. 1989.

Table 9.1 Effect of zinc and gold on translocation of PDBu receptors in cell-sonicates

Addition to sonicates	% Increase in PDBu binding to the particulate fraction
10μM $ZnCl_2$	19.0
100μM $ZnCl_2$	53.8
6μM Auranofin	12.4
60μM Auranofin	38.3

B-CLL cells were sonicated in Tris-HCl containing 1mM EDTA and 1mM calcium chloride. Zinc chloride or auranofin were added to the indicated final concentrations. After 40 minutes at 37°C, particulate fractions were pelleted at 30,000g and washed once with PBS. Pellet was sonicated in PBS to form a homogeneous suspension and assayed for binding of 10nM ^{3}H-PDBu.

Results are expressed as % increase in binding (to the particulate fraction) relative to that binding to the particulate fraction of control cells.

Table 9.2 Effect of auranofin on translocation of PDBu receptors to calcium-chelator-labile and resistant pools of the particulate fraction

Stimulus	Specific binding of ^{3}H-PDBu to the particulate fraction (fmols)	
	No EDTA	1mM EDTA
DMSO	48.6 (0.6)	29.2 (0.5)
Auranofin	73.8 (3.1)	26.9 (0.4)
PDBu	77.9 (1.4)	39.4 (1.5)
PDBu + auranofin	125.8 (3.1)	70.3 (2.2)

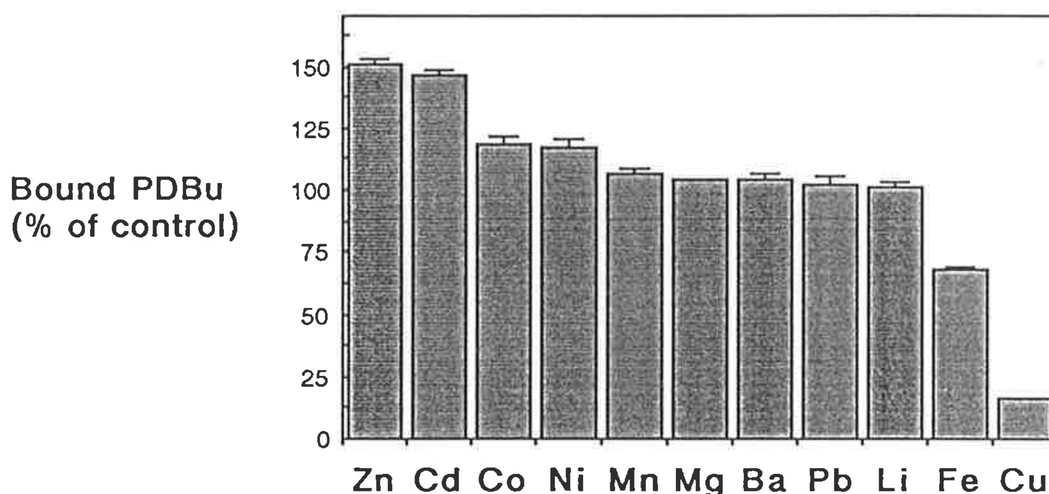
B-CLL cells (10^7/ml) were treated with PDBu (200nM), auranofin (100μM), PDBu plus auranofin or control solvent DMSO (0.1%) for 40 minutes at 37°C. Cells were washed with PBS and sonicated in 25 mM Tris-HCl with or without 1mM EDTA. Particulate fraction was collected by centrifugation (25,000g) and resuspended by sonication in PBS containing 1mg/ml of albumin. Particulate fraction was assayed for specific binding of 10nM ^{3}H-PDBu. Standard errors (n=3) are indicated in parentheses.

Table 9.3 PDBu-binding activity of the soluble and particulate fractions of B-CLL cells after treatment of cells with zinc pyrithione

Stimulus	PDBu binding to isolated fraction cpm (sem)	
	Soluble	Particulate
Buffer	5659 (62)	4384 (10)
Zinc pyrithione	3159 (46)	6346 (84)

B-CLL cells were incubated with either buffer or zinc pyrithione (50μM zinc chloride and 20μM sodium pyrithione) for 40 minutes at 37°C in RPMI. Cells were washed three times, disrupted by sonication in 25mM Tris-HCl pH 8.0 and centrifuged at 100,000g for 60 minutes at 4°C. Supernatant (soluble fraction) and pellet (particulate fraction) were assayed for binding of 20nM ^{3}H-PDBu in triplicate. Binding was assayed by the vacuum filtration method.

Figure in parenthesis is standard error.

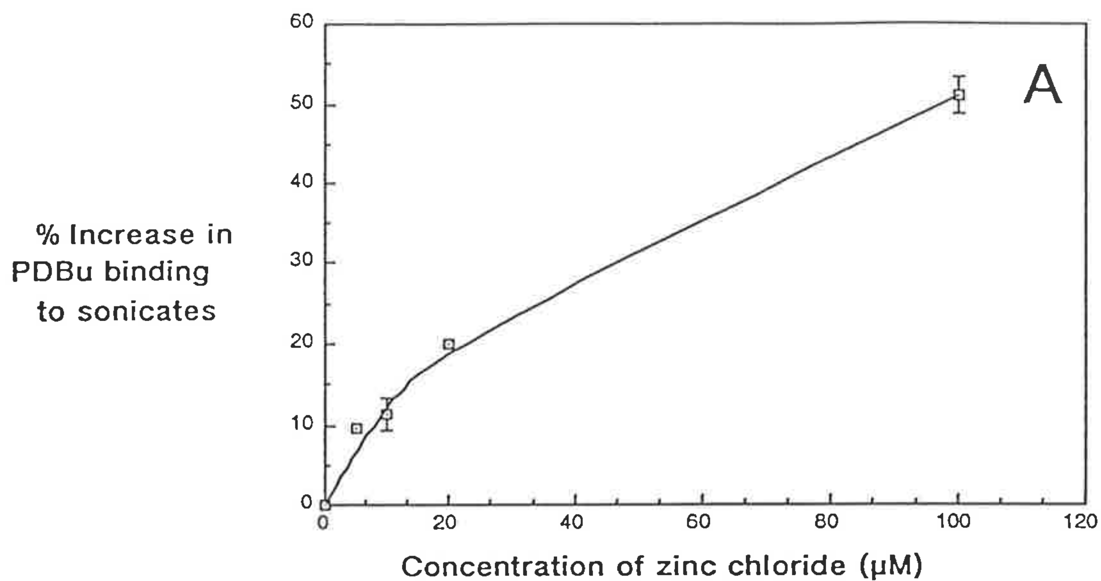


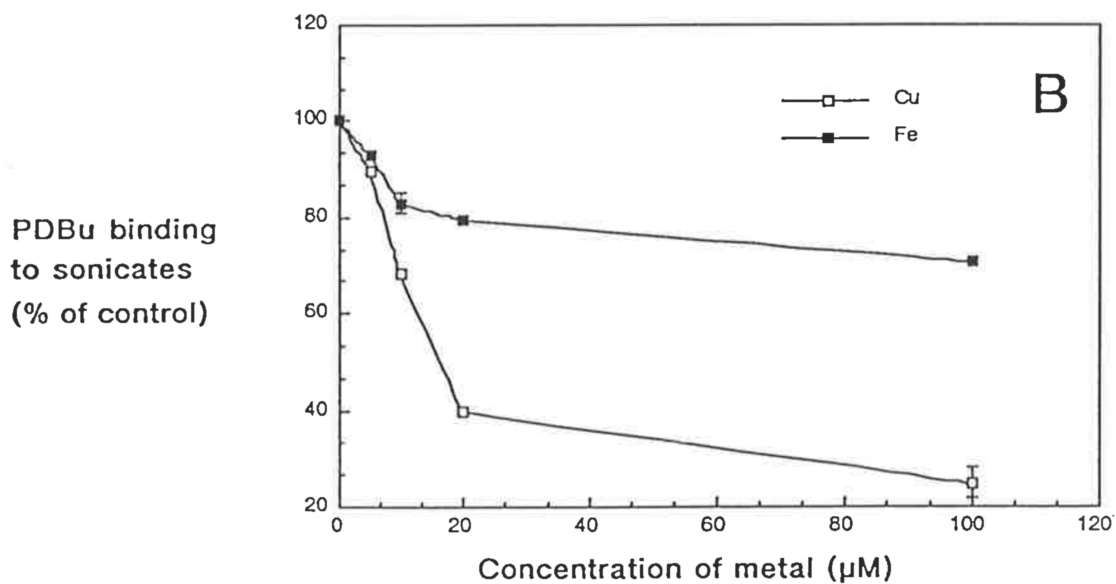
Legend to Figure 9.1 Effect of metals on PDBu binding in cell sonicates

Zinc and cadmium greatly increased PDBu binding to cell-free sonicates of B-CLL cells. Nickel and cobalt caused small increases. Copper and iron (+3) greatly decreased binding.

B-CLL cells (10^8/ml) were disrupted by ultrasonication in 25mM Tris-HCl (pH 7.5). To aliquots of 50µl of sonicate and 130µl of binding buffer (25mM Tris-HCl pH 8.0 containing 24mM Hepes, 5mM $CaCl_2$ and 10nM ^{3}H-PDBu) were added 20µl of metal compounds in H_2O to give final metal concentrations of 100µM. After 45 minutes, binding was assayed by vacuum filtration. Binding of PDBu is expressed as a % of binding to control sonicates (no addition of metal). Bars indicate standard errors (n=3).

Metals used were cupric acetate (Cu), ferric chloride (Fe), barium chloride (Ba), lead nitrate (Pb), lithium bromide (Li), magnesium chloride (Mg), cadmium chloride (Cd), zinc chloride (Zn), nickel chloride (Ni) and cobaltous chloride (Co).



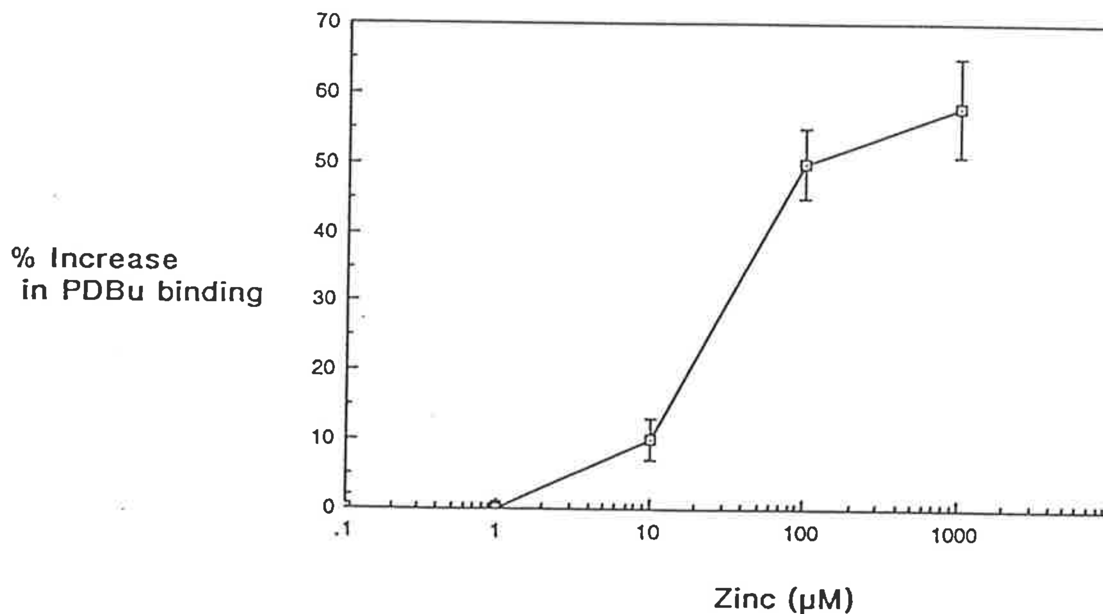


Legend to Figure 9.2 Concentration-dependence of effects of zinc, copper and iron on PDBu binding

Zinc increased PDBu binding, while cupric and ferric ions decreased PDBu binding, to sonicates of B-CLL cells, in a concentration-dependent manner.

A. Zinc
B. Cupric and ferric ions

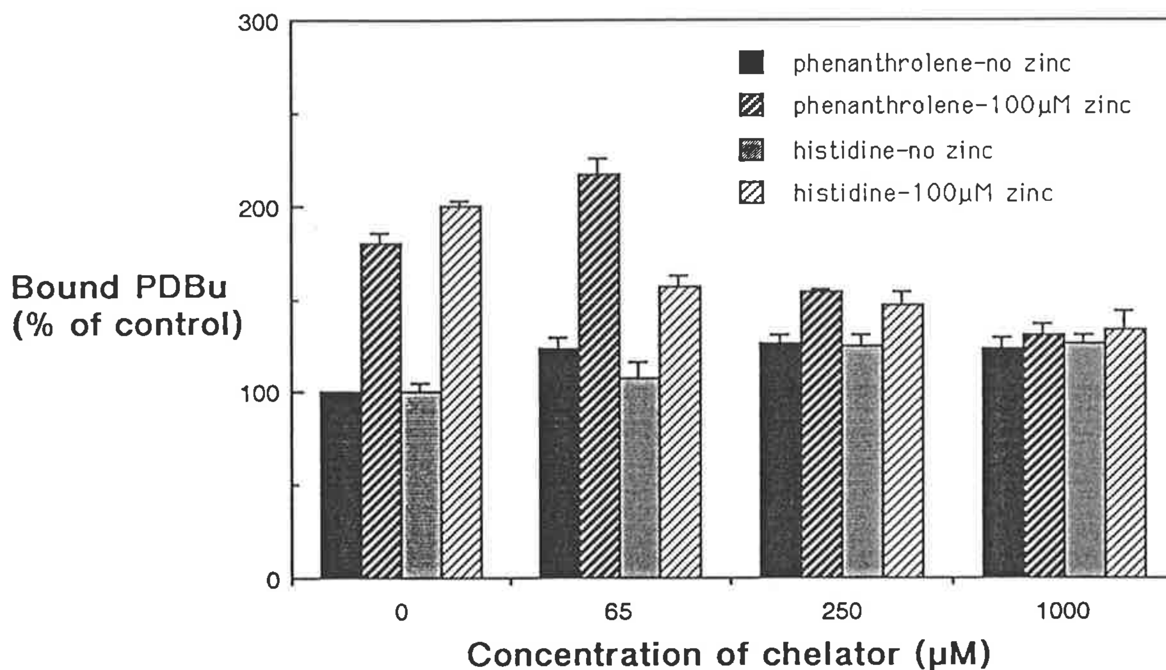
Conditions were the same as in Fig 9.1 except that varying concentrations of zinc chloride, ferric chloride or cupric acetate were added to the mixtures of sonicate and binding buffer, before assaying ^{3}H-PDBu binding.



Legend to Figure 9.3 Effect of zinc on binding of PDBu to cytosol

Zinc chloride augmented PDBu binding to cytosol of B-CLL cells, reconstituted with PS and calcium. Most of the increase was seen at concentrations of zinc between 10 and 100µM.

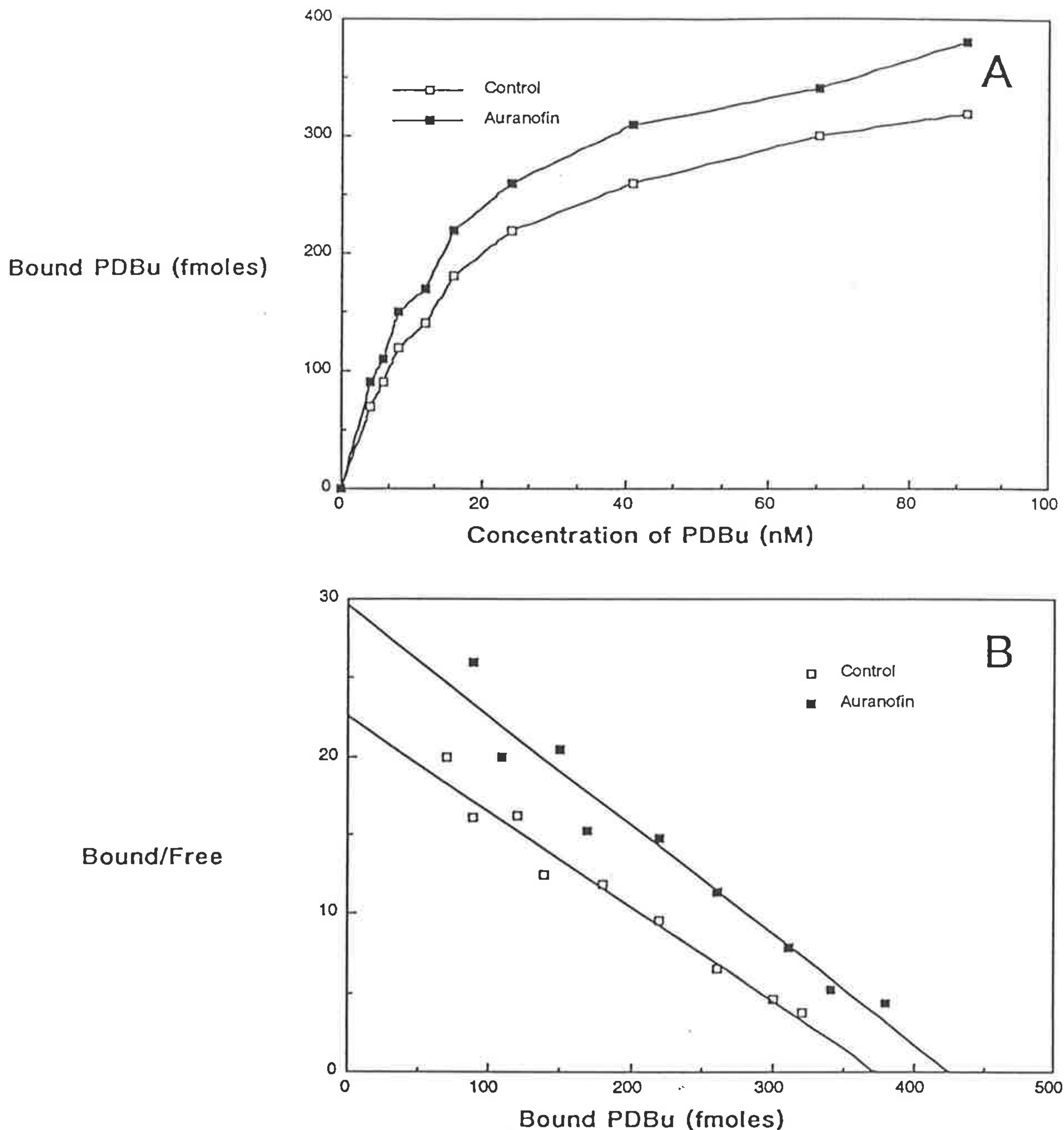
Cytosol of B-CLL cells was prepared by taking the supernatant of a 30,000g sedimentation of cell sonicate (prepared as in Fig 9.1). To mixtures of 50µl of cytosol and 130µl of binding buffer (same as in Fig 9.1 but also containing 4mM $MgCl_2$ and 100µg/ml of PS), were added varying concentrations of zinc chloride. After 45 minutes, binding of PDBu was determined by vacuum filtration. PDBu binding to cytosol is expressed as % of binding to control cytosol (no zinc). Bars indicate standard errors (n=3).



Legend to Figure 9.4 Prevention of effects of zinc on cytosol by zinc-chelating agents

Zinc-chelating agents, 1,10 phenanthroline and histidine, prevented the increase in binding of ^{3}H-PDBu to cytosol caused by 100µM zinc chloride.

Mixtures of cytosol of B-CLL cells and binding buffer (prepared as in Fig 9.3) were pre-incubated for 10 minutes at 37°C with varying concentrations of phenanthroline or histidine before addition of 100µM zinc chloride. After a further 45 minutes, binding was assayed as in Fig 9.3. Binding is expressed as a % of binding in control (no zinc chloride and no zinc-chelating agent). Bars indicate standard errors (n=3).

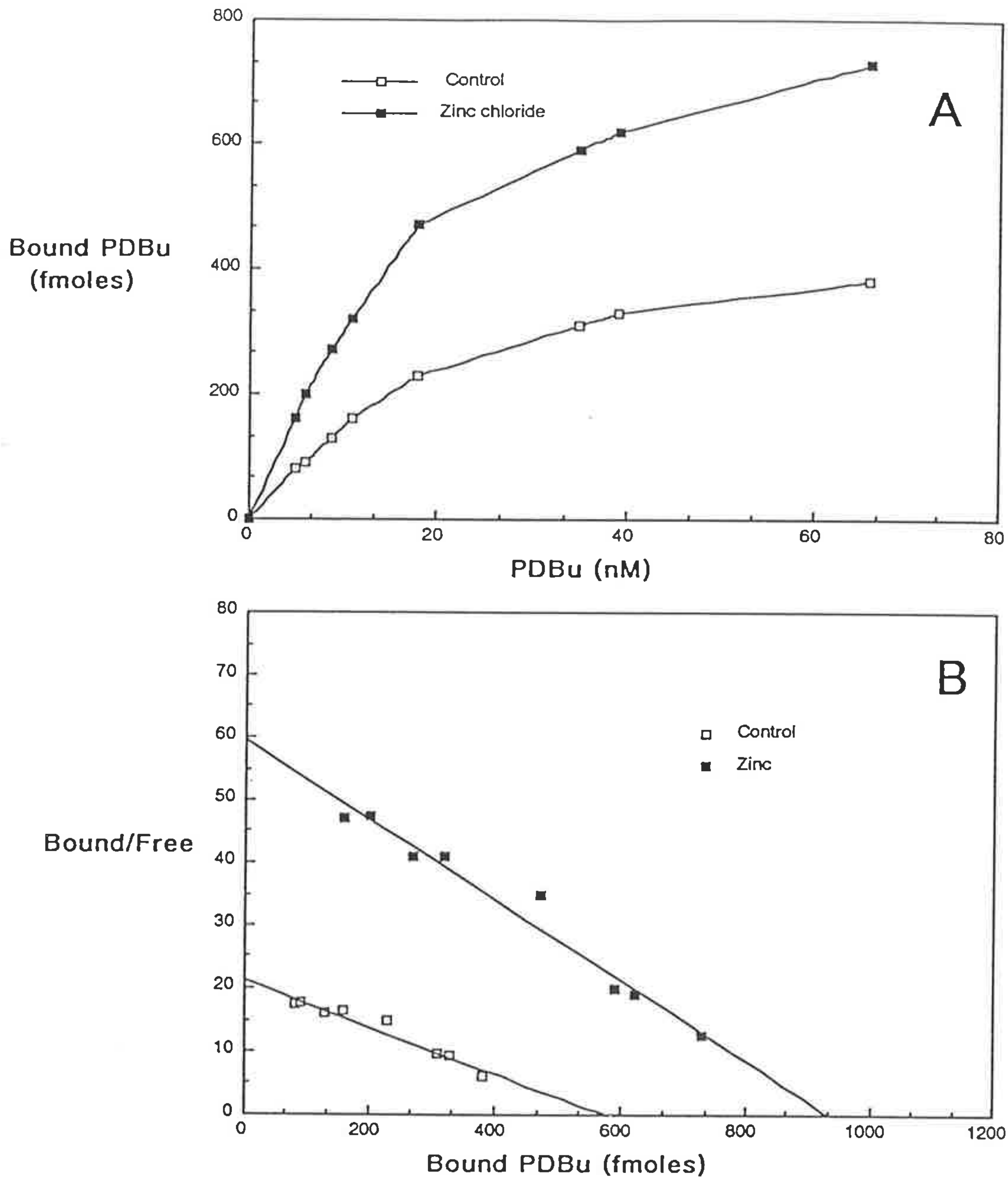


<u>Legend to Figure</u> <u>9.5</u> <u>Effect of auranofin on PDBu receptor affinity and maximum binding capacity in cytosol of B-CLL cells</u>

Auranofin caused a 40% increase in maximum PDBu binding capacity of cytosol, without change in receptor affinity.

A: Saturation plot
B: Scatchard plot

To mixtures of B-CLL cytosol and binding buffer, prepared as in Fig 9.3 but with varying concentrations of ^{3}H-PDBu, was added 60μM auranofin or control solvent DMSO (0.1% v/v). After 45 minutes at 37°C, specific PDBu binding was assayed (by vacuum filtration). Binding is expressed as fmoles of PDBu per 50μl aliquot of cytosol.

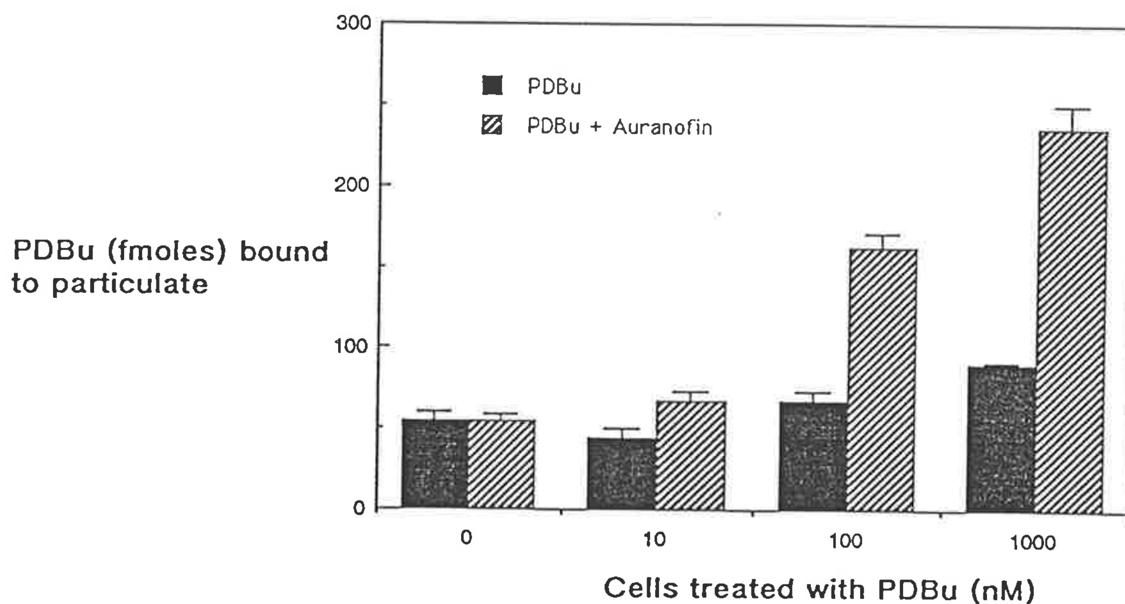


Legend to Figure 9.6 Effect of zinc chloride on PDBu receptor affinity and maximum binding capacity in cytosol of B-CLL cells

Zinc chloride caused a doubling in the maximum PDBu binding capacity of cytosol, without change in receptor affinity.

A: Saturation plot
B: Scatchard plot

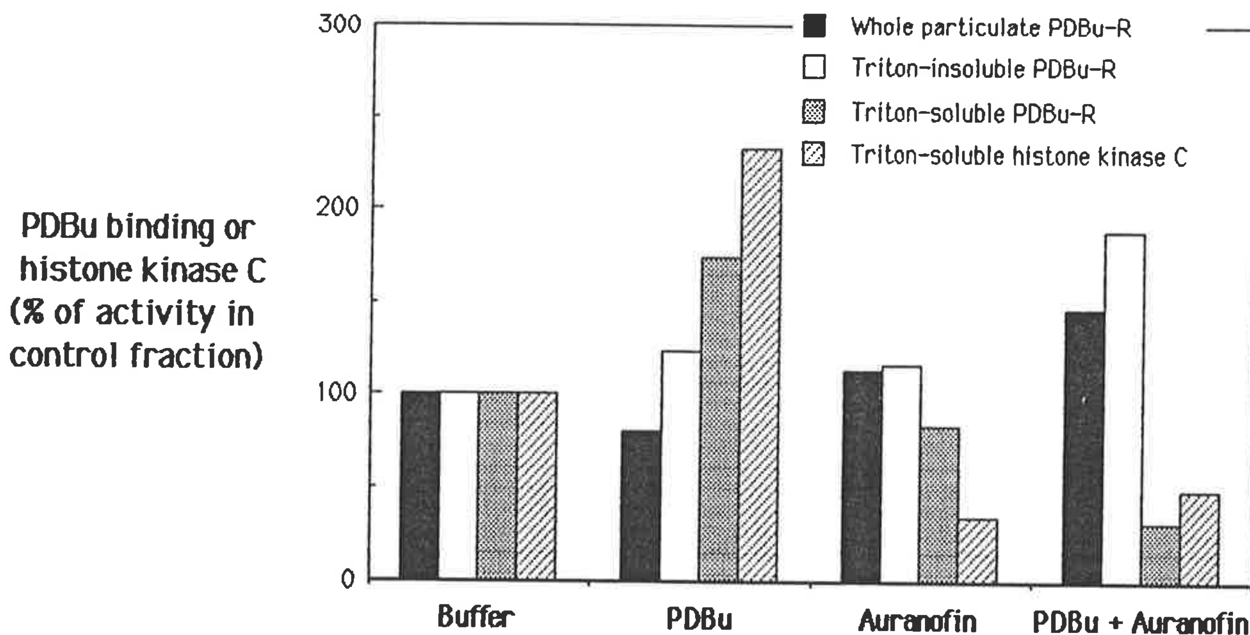
Conditions were the same as in Fig 9.5 except that zinc chloride (50μM) was used instead of auranofin.



Legend to Figure 9.7 Effect of auranofin on translocation of PDBu receptors from cytosol to particulate fraction of B-CLL cells

Auranofin alone did not cause translocation of PDBu receptors to the calcium-chelator-resistant fraction of the particulate fraction of B-CLL cells but it greatly enhanced the capacity of PDBu to induce translocation, even at saturating concentrations of PDBu.

B-CLL cells were treated at 37°C in RPMI/FCS with varying concentrations of unlabelled PDBu (shown in abscissa) in the presence or absence of 60μM auranofin for 40 minutes. Cells were then washed, sonicated in the presence of 1mM EDTA and particulate fraction was assayed for specific binding of ^{3}H-PDBu (10 nM). Binding is expressed as fmoles of PDBu bound per 30μl of particulate fraction. Bars indicate standard errors (n=3).

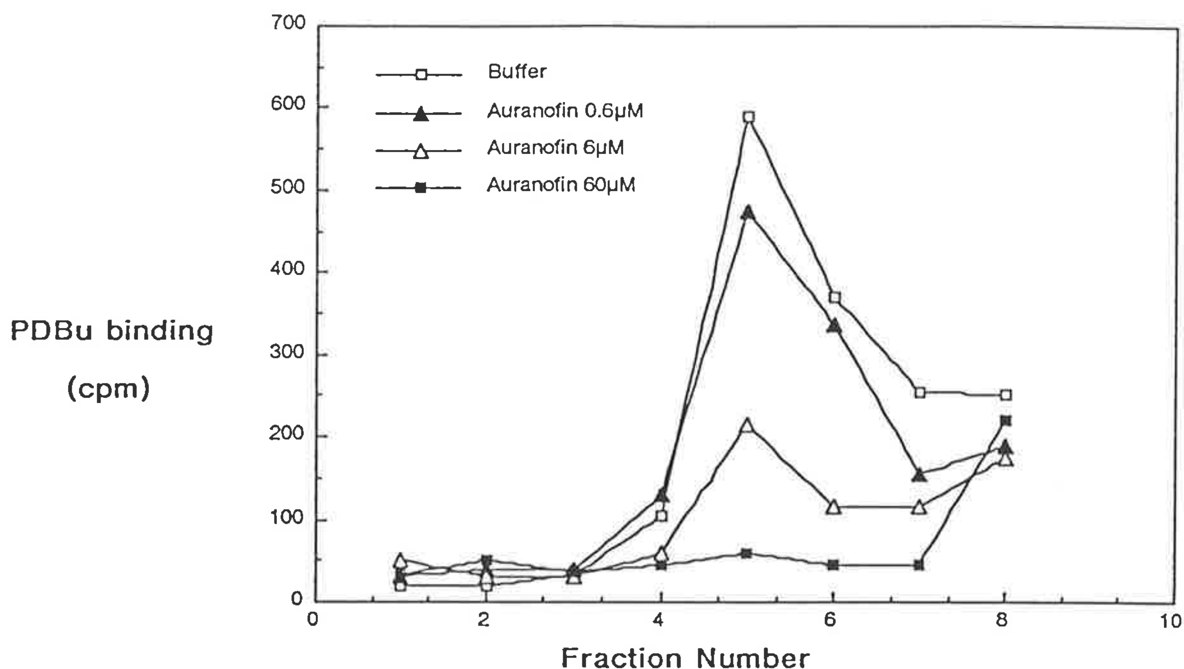


Legend to Figure 9.8 Effect of auranofin on detergent-solubility of PKC

Auranofin caused PKC, in the particulate fraction of B-CLL cells, to become detergent-insoluble.

B-CLL cells were treated with auranofin (60µM), unlabelled PDBu (200nM), auranofin plus PDBu or control buffer, for 40 minutes at 37°C in RPMI/FCS. Cells were washed with PBS and sonicated in 25mM Tris-HCl containing 1mM EDTA. Particulate fraction was prepared by centrifugation at 30,000g and extracted with 0.2% Triton X-100 in sonication buffer.

Whole particulate fraction and the Triton X-100-soluble and -insoluble fractions were assayed for binding of 10nM ^{3}H-PDBu. Triton-soluble material was assayed for binding, by vacuum filtration, and for histone kinase C activity (by method 1 chapter 2.12). Binding and kinase activities were determined in the presence of 20µg/ml PS and 5mM calcium. Activities are expressed as a % of activity in extracts from control cells (treated with buffer alone).

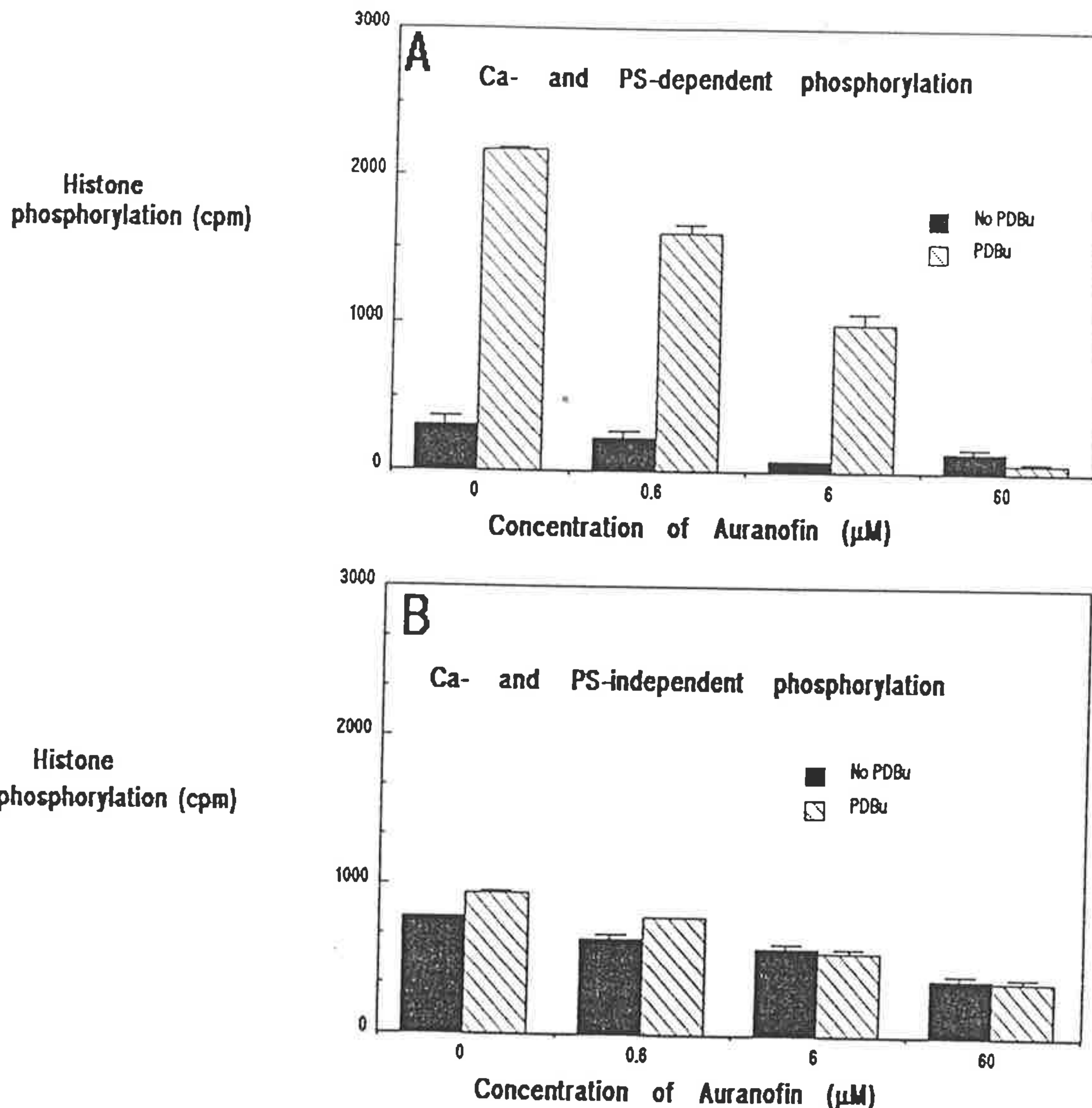


Legend to Figure 9.9 Concentration-dependence of effect of auranofin on detergent-solubility of PDBu receptors

Auranofin decreased PDBu binding activity in the Triton-soluble part of the particulate fraction of B-CLL cells, in a concentration-dependent manner. Effects were seen at concentrations of auranofin as low as 0.6μM. PDBu binding was assayed by the column method.

B-CLL cells were treated with varying concentrations of auranofin and the Triton-soluble extracts of the particulate fractions were prepared as in Fig 9.8. These were assayed for PDBu binding (by the column method, see Fig 4.8). Radioactivity eluting in the first 8 fractions is bound ^{3}H-PDBu.

PDBu binding is expressed as a % of activity in extract from control cells (cells treated with buffer, alone).



Legend to Figure 9.10 Concentration-dependence of effect of auranofin on detergent-solubility of PKC

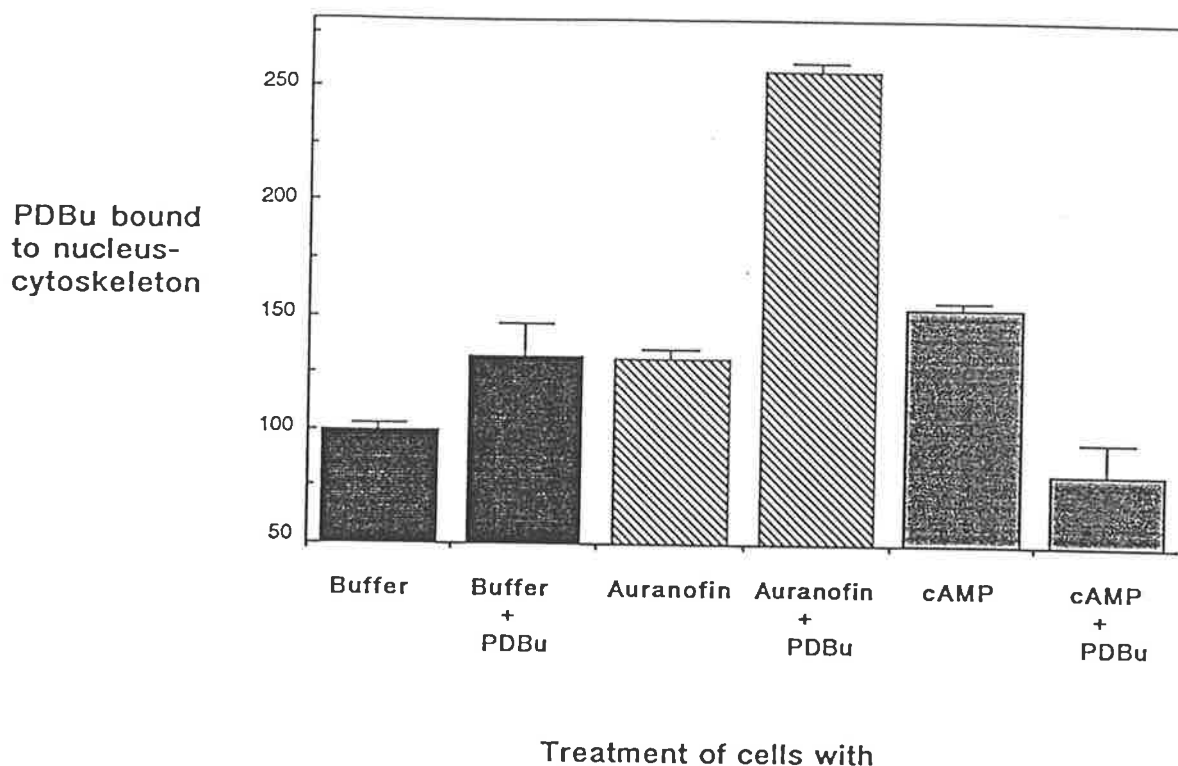
Histone kinase C activity in the Triton-soluble extracts of the particulate fractions of B-CLL cells decreased as cells were treated with increasing concentrations of auranofin, in the presence or absence of PDBu. Effects were seen at concentrations of auranofin as low as 0.6μM. Much less effect was seen when calcium-and phospholipid-independent kinase was assayed.

A. Ca- and PS-dependent phosphorylation in Triton-soluble extracts from cells treated with auranofin, in the presence or absence of 200nM PDBu.

B. Ca- and PS-independent phosphorylation in same extracts.

B-CLL cells were treated with varying concentrations of auranofin, in the presence or absence of 200nM unlabelled PDBu. Triton-soluble extracts were prepared and histone kinase C assayed by a modified method (method 2 in chapter 2.12).

Ca- and PS-dependent phosphorylation is derived by subtracting Ca- and PS-independent phosphorylation from total phosphorylation (in the presence of 5mM calcium and 20μg/ml PS). Ca- and PS-independent phosphorylation is phosphorylation with no added calcium or PS and with 1mM EGTA (to chelate endogenous calcium).

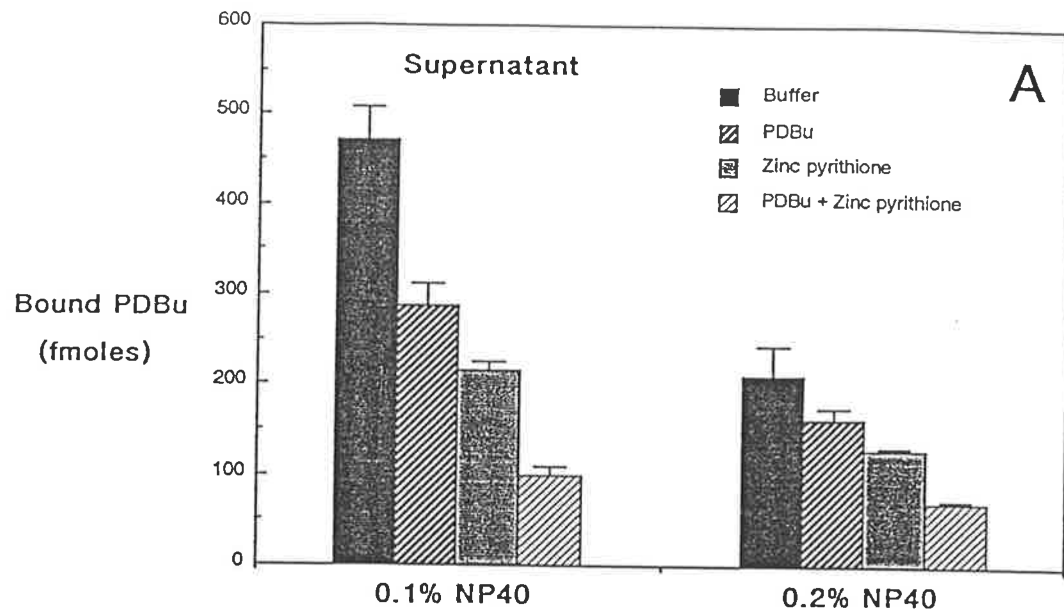


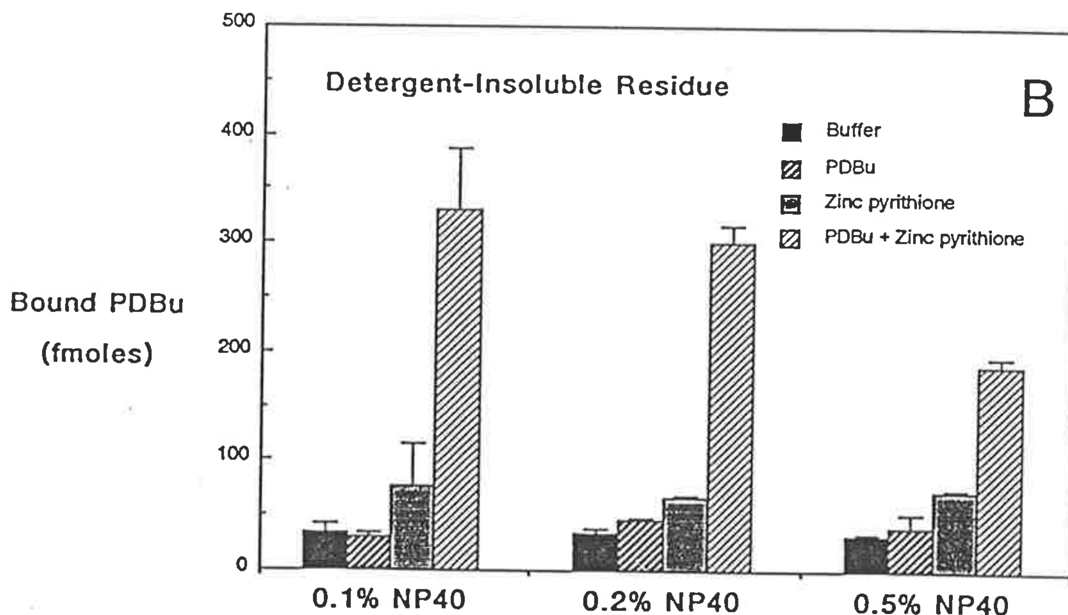
Legend to Figure 9.11 Effect of auranofin on translocation of PKC to nucleus-cytoskeleton

Auranofin induced translocation of PDBu receptors to a detergent-insoluble compartment (presumed to be the nucleus and/or cytoskeleton) in intact B-CLL cells. This was particularly marked when cells were treated with a combination of auranofin and PDBu. As shown by Cambier et al. [1987b], dibutyryl cAMP also induced translocation of PKC to this compartment but there was no synergism with PDBu.

B-CLL cells were incubated for 6 minutes at 37°C in RPMI/FCS with 60µM auranofin, 1mM dibutyryl cAMP, 200nM unlabelled PDBu, auranofin plus PDBu, dibutyryl cAMP plus PDBu or control buffer. Cells were washed and lysed with 0.2% NP40 as in methods (chapter 2.10).

Detergent-insoluble residue was sonicated to make a homogeneous suspension and assayed for binding of 10nM ^{3}H-PDBu. Binding is expressed as a percentage of the binding to the same fraction from control cells (treated with buffer, alone). Bars indicate standard errors (n=3).



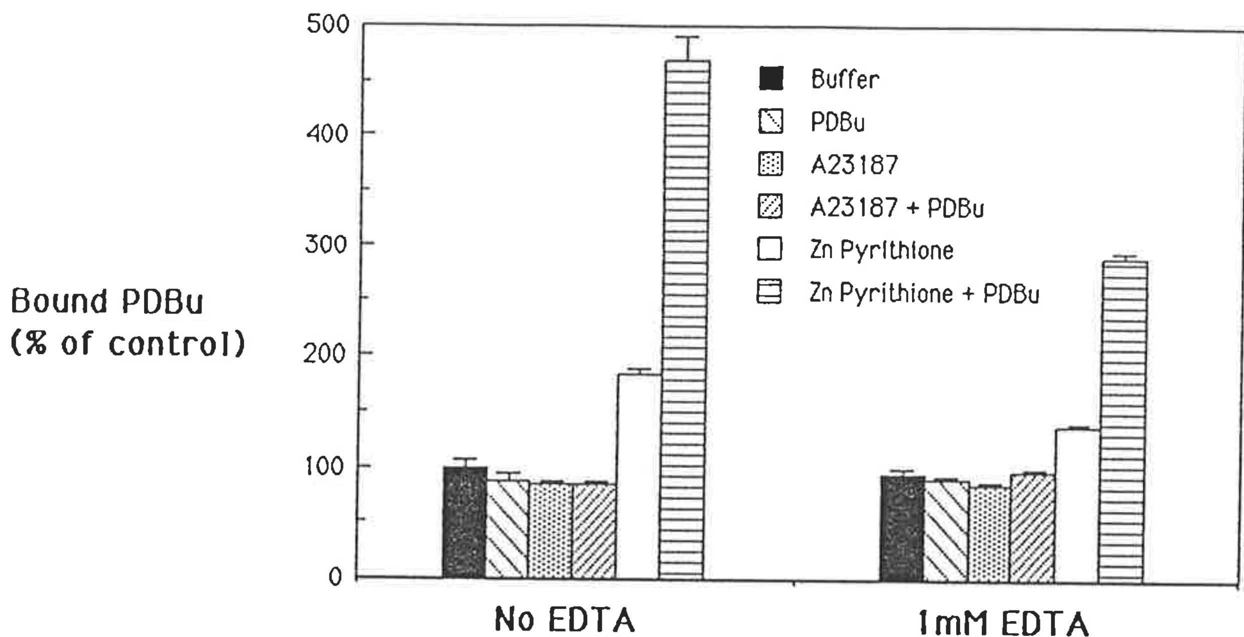


Legend to Figure 9.12 Effect of zinc pyrithione on translocation of PKC to nucleus-cytoskeleton complex

Treatment of cells with zinc pyrithione caused an increase in PDBu binding activity in the nucleus-cytoskeleton complex and this was most prominent when cells were treated with zinc pyrithione in the presence of PDBu. Similar results were seen when detergent-insoluble residue was prepared using three different concentrations of NP40. There were corresponding decreases in the PDBu binding activity of the supernatants although binding could not be detected in the 0.5% NP40 supernatant, presumably because this high concentration of detergent interferes with the binding assay.

A. PDBu binding to supernatant
B. PDBu binding to detergent-insoluble pellet

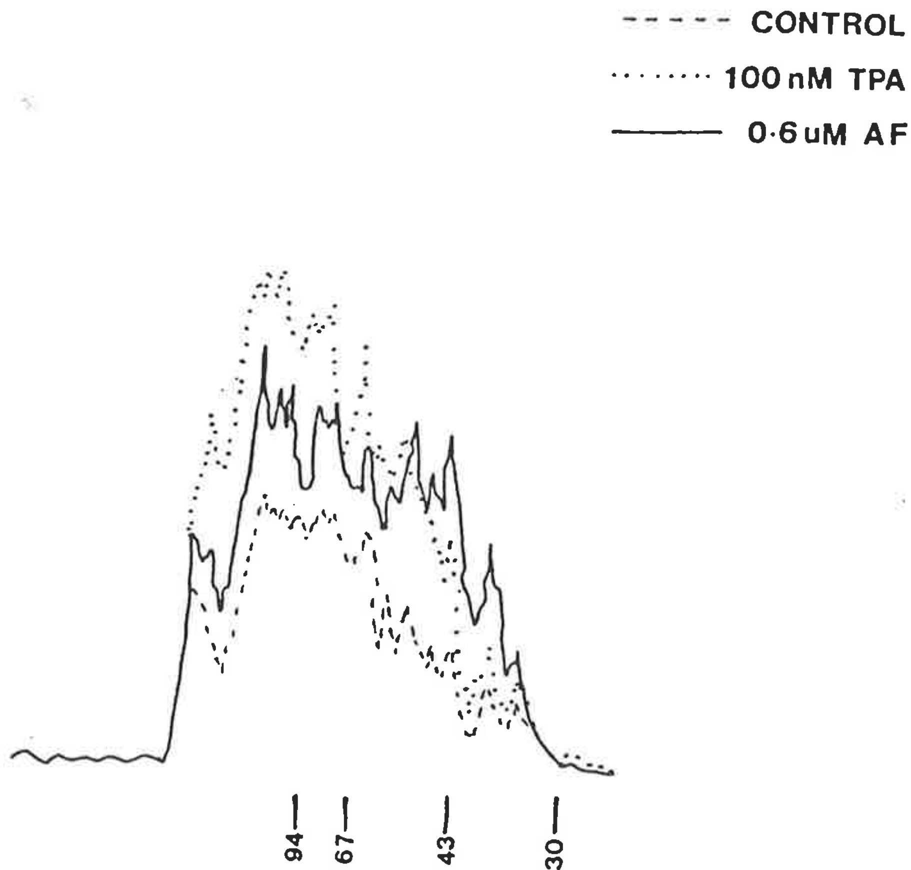
Cells were treated with reagents for 40 minutes (other conditions as in Fig 9.11, except that three concentrations of NP40 were used to prepare the insoluble nucleus-cytoskeleton and PDBu binding was assayed both in the insoluble residue (pellet) and the supernatant. Concentration of zinc chloride was 50uM, sodium pyrithione was 20uM and unlabelled PDBu was 200nM.



Legend to Figure 9.13 Relative effects of calcium and zinc on translocation of PKC to nucleus-cytoskeleton

While zinc pyrithione synergized with PDBu in causing translocation of PKC to the nucleus-cytoskeleton, calcium ionophore A23187 did not induce translocation of PKC to this compartment and did not synergize with PDBu in this effect. Zinc-induced translocation to the nucleus-cytoskeleton complex was partially reversed by preparing lysates in the presence of 1mM EDTA, suggesting that zinc or other divalent cation is involved in the attachment of PKC to this complex.

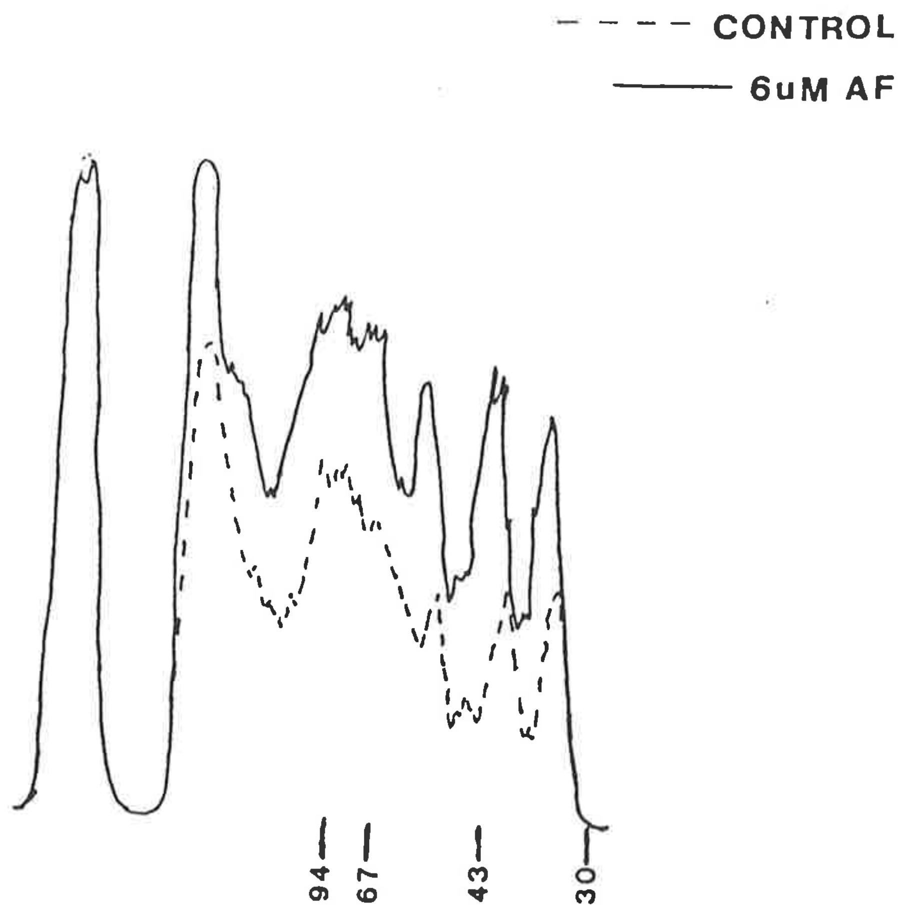
B-CLL cells were treated with reagents indicated as in Fig 9.12, except that some cells were also treated with A23187 (1µM) or A23187 plus 200nM PDBu. Nucleus-cytoskeleton complexes were prepared using 0.2% NP40, with or without 1mM EDTA.



Legend to Figure 9.14 Effect of auranofin on protein phosphorylation in B-CLL cells

Auranofin (AF, 0.6uM) enhanced phosphorylation of proteins in intact B-CLL cells. The profile of phosphoproteins differed to some extent from that seen in extracts from TPA-treated cells.

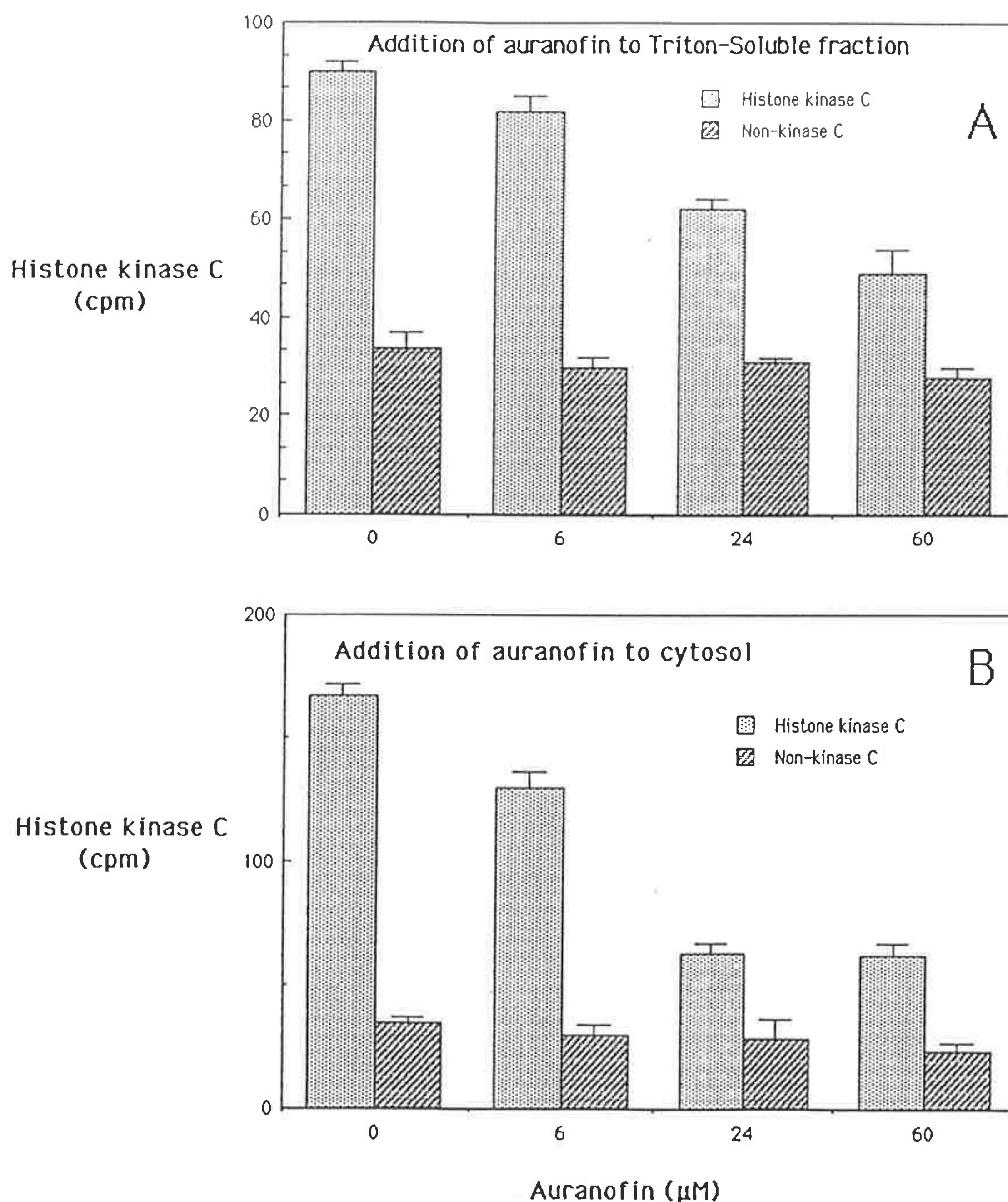
B-CLL cells (10^8/ml) were labelled with ^{32}P for 60 minutes in phosphate-free medium (Hepes-buffered saline containing 10% FCS), then washed and resuspended in RPMI/FCS (at 2 x 10^7/ml), before treatment with reagents indicated for 40 minutes at 37°C. Cell lysates were run on SDS-PAGE (10%) under reducing conditions. Densitometer tracings of the autoradiographs are shown with molecular weight markers indicated.



Legend to Figure 9.15 Effect of treatment of cells with auranofin on protein phosphorylation in the nucleus-cytoskeleton

Treatment of B-CLL cells with auranofin (AF) enhanced protein phosphorylation in the detergent-insoluble nucleus-cytoskeleton.

B-CLL cells were labelled with ^{32}P (see Fig 9.14) and then the cells were treated with or without 6uM auranofin for 40 minutes at 37°C. Cells were washed, the nucleus-cytoskeleton complex was prepared (see Fig 9.11) and extract was chromatographed on 10% SDS-PAGE. Densitometer scans of developed autoradiographs are shown. Molecular weight markers are indicated.

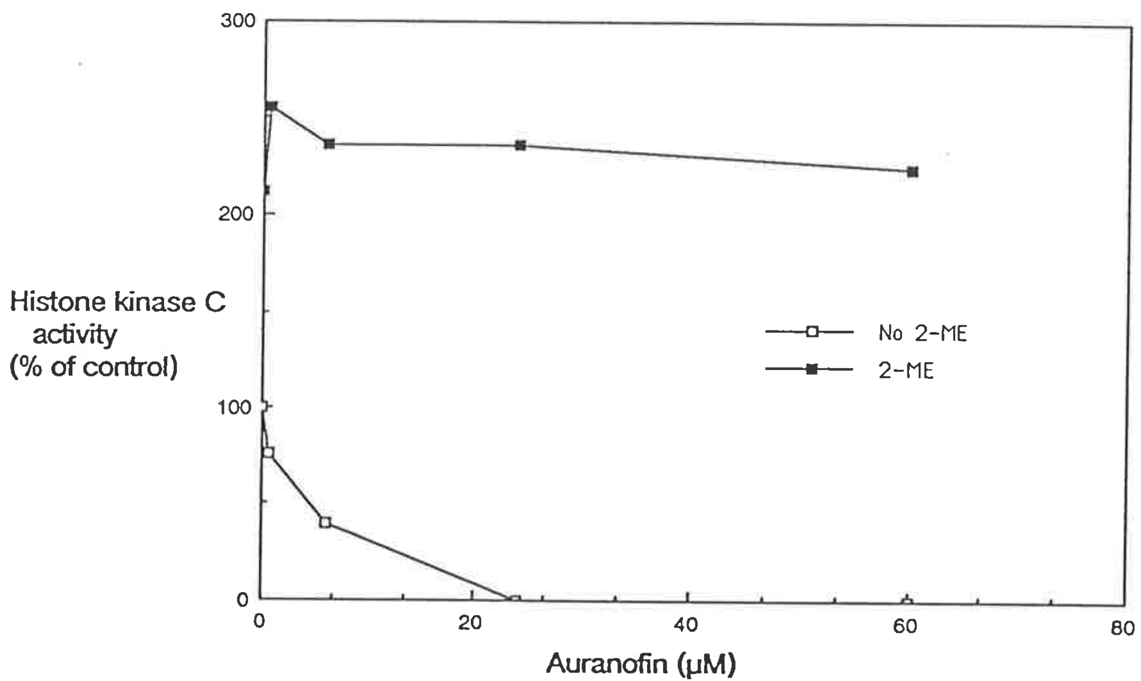


Legend to Figure 9.16 Effects of auranofin directly on histone kinase C activity.

Auranofin inhibited calcium- and phospholipid-dependent histone phosphorylation when added to Triton-soluble extracts of the particulate fraction or when added to cytosol. There was little or no effect on the calcium- and phospholipid-independent histone phosphorylation.

A. Triton-soluble extract
B. Cytosol

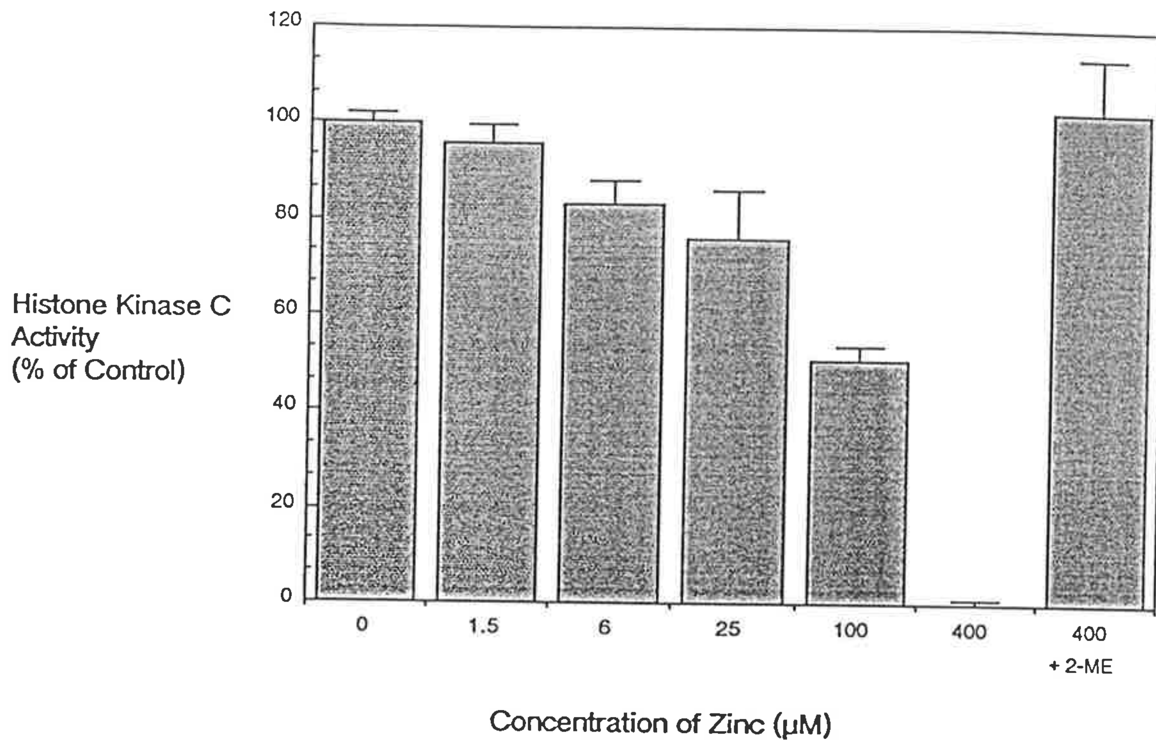
Cytosol or Triton-soluble extract of the particulate fraction from B-CLL cells were incubated with auranofin (at the indicated final concentration) for 30 minutes before assay of calcium- and phospholipid-dependent and -independent histone kinase activities, using method 1 (chapter 2.12). Bars indicate standard errors (n=3)



Legend to Figure 9.17 Prevention of effects of auranofin on catalytic activity by 2-ME

Auranofin did not inhibit calcium- and phospholipid-dependent histone kinase C activity in the presence of 2-ME.

Cytosol from B-CLL cells was treated with auranofin in the presence or absence of 50mM 2-ME. Conditions for treatment and assay of histone kinase C were as in Fig 9.16.



Legend to Figure 9.18 Effect of direct addition of zinc on histone kinase C activity in cytosol

Like gold (auranofin), zinc chloride inhibited calcium- and phospholipid-dependent histone kinase activity when added directly to cytosol of B-CLL cells. Inhibition was prevented by 2-ME.

Cytosol of B-CLL cells was incubated with varying concentrations of zinc chloride for 30 minutes before assay of histone kinase C activity (method 1 in chapter 2.12). Where present, 2-ME was at a concentration of 50mM.

CHAPTER TEN

CONCLUSIONS AND FUTURE STUDIES

The aim of the experiments described in this thesis was to investigate the mechanisms involved in the interaction of PDBu and other phorbol esters with B-CLL cells and in the subsequent loss of expression of MER. These studies have revealed actions of calcium, gold and zinc on the binding of PDBu to its receptor, PKC, and on some of the events which follow this binding.

10.1 Advantages and limitations of this model.

The studies in this thesis were greatly facilitated by having a supply of large numbers of constant populations of cells. B-CLL cells did not appear to behave abnormally, at least in the regulation of their phorbol ester receptors, since the experiments could be confirmed in other types of normal cells.

Inhibition of MER proved to be a useful early functional response for studying the actions of phorbol esters, although it would be useful to have a more direct means of identifying MER such as by specific antibody or radiolabelled ligand. These probes may not detect subtle changes in the structure or arrangement of MER that are induced by phorbol ester in the intact cell but they would facilitate studies of the solubilized receptor.

10.2 Changes in MER caused by phorbol ester

Further understanding of the significance of the loss of expression of MER in activated B cells requires characterization of the proteins associated with the complex. MER released from B-CLL cells by trypsinization had properties of those actin-rich vesicles which are shed spontaneously by lymphocytes and other cells, presumably as a result of endogenous proteolysis. This may indicate an attachment of MER to the cytoskeleton.

Treatment of cells with phorbol ester caused a loss in a

number of proteins associated with MER. These studies were done with enriched vesicular preparations of MER and need confirmation with pure soluble receptor, when the techniques become available.

The finding of albumin, tightly bound to the MER complex supports the hypothesis that at least part of the binding of mouse red cells to B-CLL cells is due to the binding of red cell-associated mouse albumin and that the natural ligand for MER is plasma albumin. This interaction of albumin with PE cannot be explained by albumin binding to fatty acids since the phosphate is essential for the interaction, the head group greatly influences the binding and fatty acids do not interfere with the haemagglutination of mouse red cells by PE.

The disappearance of albumin from the MER complex in phorbol ester-treated cells supports the hypothesis that phorbol esters promote the internalization of albumin by B cells, but definitive studies with ^{125}-labelled albumin need to be done.

Since albumin and PE are ubiquitous components of cells and plasma, it has to be asked why other types of cells do not bind mouse red cells. One reason may be that MER+ve B cells have an enrichment of PE in the outer leaflet. PE, and other non-choline phospholipids, are usually found in the inner leaflet of the cell membrane and therefore cannot participate in the binding. This asymmetry of phospholipid distribution is regulated in red cells by spectrin and other cytoskeletal proteins [Haest et al. 1980, Williamson et al. 1987], and it appears to determine whether macrophages can bind autologous red cells [Tanaka and Schartz 1983]. One or more of the proteins associated with the PE in the MER complex may control the distribution of PE and other non-choline phospholipids in the membrane. The use of membrane-impermeable reagents which label PE may determine whether there

is an enrichment of PE in the outer leaflet of B-CLL cells, relative to that in other cells. Another reason why rosetting may be specific to this subset of B cells is that the proteins associated with MER, that are essential for rosetting, are only found in this cell. The second type of MER, which binds the pronase-resistant ligand and which cooperates in the binding of the red cell, appears also to be B cell specific.

10.3 Involvement of PKC in the inhibition of MER

These studies have shown that B-CLL cells, like many other types of cells, have specific, high affinity receptors for PDBu. Although there was no formal attempt to prove that these were PKC, they behaved like PKC in some properties such as dependence upon phospholipid and calcium, chromatography on DE52 cellulose and translocation from the cytosol to the membrane. However, the assumption that all PDBu receptors in B-CLL cells are PKC must remain provisional until the receptors are purified and fully characterized, particularly in view of the apparent unmasking of new PDBu receptors in cells treated with zinc and gold.

The PDBu receptors detected by the binding assay appeared to mediate the effects of phorbol esters on MER, since the shape of the curve describing inhibition of MER at different concentrations of ^{3}H-PDBu was very similar to that describing binding of ^{3}H-PDBu to the cells, the EC_{50} for inhibition of MER by PDBu was of the same order as the dissociation constant of the PDBu receptors and the order of potencies of a variety of analogues of PDBu in inhibiting rosetting and inhibiting ^{3}H-PDBu binding were similar. Further evidence, showing that auranofin and zinc pyrithione which inhibit MER and profoundly affect PKC, is summarized later

Inhibition of rosetting is a result of a time-dependent cellular process that follows binding of phorbol ester to its receptor. The efficiency of this process may vary with different populations of B-CLL cells as shown by studies with cells of J.T. and V.M. It will be important to determine the relative efficiencies of B-CLL cells and normal MER+ve B cells in events leading to inhibition of MER and plasmacytoid differentiation. The mechanism underlying the accumulation of MER+ve cells in B-CLL is little understood, in particular whether it is a consequence of an abnormality in an early activation event.

Stimuli which induce formation of diacylglycerol via PIP_2 breakdown, as well as exogenous diacylglycerols, partially inhibited MER, supporting the hypothesis that PDBu is acting on rosetting via PKC and that PDBu is usurping a physiological pathway in the cell that is activated following triggering of sIg. Since fluoride activates G proteins and inhibits MER, it would appear that a functional G protein exists in B-CLL cells and that it is coupled to the pathway leading to inhibition of MER. Studies with inhibitors of G proteins such as pertussis toxin may determine whether the effects of anti-Ig on MER are mediated by G proteins.

As in many other PKC-dependent cellular responses, a rise in $[Ca^{++}]_i$ did not itself affect expression of MER but it greatly enhanced the capacity of diacylglycerol and PDBu to inhibit MER. Calcium-chelating agents decreased the inhibition of rosetting by PDBu.

Other receptors which are down-modulated by phorbol esters are, at the same time, phosphorylated by PKC. Direct evidence that the inhibition of MER was a result of phosphorylation by PKC was not obtained in these studies. However, further studies need

to be done to exclude this hypothesis. Since PKC inhibitors did not prevent the down-modulation of MER by phorbol esters, the pool of PKC or PKM involved in the rosette inhibition may be relatively insensitive to, or inaccessible to, the PKC inhibitors. PKM, for instance, is unaffected by inhibitors which interfere with the activation of the regulatory domain by phospholipid since this kinase contains only the catalytic domain. Other inhibitors of PKC, especially staurosporine [Tamaoki et al. 1986], which has recently become available commercially, need to be tested.

Failure to find ^{32}P in a component of MER released from ^{32}P-labelled TPA-treated cells may be because the proteases, which were used to release MER from the cells, also removed the part of the polypeptide which was phosphorylated. It is necessary to have other means of isolating MER eg by immunoprecipitation of MER from detergent lysates of B-CLL cells.

10.4 Role of other early events in the inhibition of MER

Various events including membrane depolarization, activation of phospholipase A_2, methylation of phospholipid, proteolysis and clustering of membrane glycosphingolipids did not appear to be involved in the inhibition of MER by phorbol ester. The role of other early events such as changes in intracellular pH, phospholipid turnover, metabolism of arachidonic acid and action of free radicals need to be determined. The effects of purified interleukins and interferons on MER, PDBu binding and translocation of PKC to soluble and insoluble compartments may provide additional insight into the mechanism.

10.5 Effect of membrane-permeable thiols on MER

It is unclear whether the inhibitory effects of thiols on MER are relevant to the action of thiols as macrophage-replacing agents in B cell cultures. It has been proposed that they act by increasing the concentration of intracellular glutathione which is necessary for one or more steps in lymphocyte activation. Furthermore, thiol-treated albumin could replace thiols in B cell cultures. The use of cyclohexene-1-ol, which depletes intracellular glutathione and inhibits lymphocyte activation by mitogens, may determine whether glutathione is involved in the inhibition of MER by phorbol esters and thiols.

10.6 Role of zinc and other metals in the inhibition of MER

Both auranofin and zinc pyrithione inhibited MER. These actions were mediated by the gold and zinc moieties of the compounds. Lipophilic ligands, triethylphosphine and pyrithione, are required, apparently to promote uptake of the metals by the cells. A mechanism of cellular uptake of gold in auranofin has been proposed by Crooke et al. [1986]. It involves thiol-exchange between the ligands of gold and thiol-containing cellular proteins. The hydrophobic triethylphosphine group of auranofin presumably facilitates the passage of gold across the lipid bilayer. Zinc may be taken up by cells in a manner similar to that of gold.

It is not yet known whether these compounds cause the same changes in the proteins of MER, as do phorbol esters. Auranofin, at least, did not cause loss or destruction of MER, as shown by undiminished haemagglutinating activity of the isolated receptor. However, the effect of auranofin was independent of calcium. The action of gold and zinc on MER may be similar to that of the membrane-permeable thiols, especially since heavy metals

preferentially interact with sulphydryls. Zinc may inhibit MER either by directly interfering with sulphydryls in MER or by acting via some other protein. This protein could be PKC since at concentrations of zinc and gold which inhibited rosetting, zinc and gold altered at least one property of PKC, its detergent-solubility.

The finding that phenanthroline, and to a lesser extent TPEN, prevented the effects of phorbol esters on MER suggests that zinc or other similar metal is involved in the mechanism of action of phorbol esters. This conclusion is supported by the finding that TPEN blocks some effects of phorbol esters in thymocytes [Csermely et al. 1987b]. Phorbol esters may cause a rise in intracellular zinc, perhaps in the cytosol or membrane as shown by the studies in thymocytes. Alternatively, phorbol esters may increase the sensitivity to zinc of some process involved in the inhibition of MER. This would be analagous to the increased sensitivity in phorbol ester-treated cells of some calcium-dependent processes. One way of investigating this further would be to determine whether phorbol esters synergize with zinc in the inhibition of MER, or whether the two agents act in an additive manner.

It is necessary now to examine the role of zinc and other metals in other early activation responses mediated by PKC, both in B-CLL cells and other types of cells. The relatively rapid loss of expression of the CD37 antigen in phorbol ester-treated B-CLL cells and the inhibition of epidermal growth factor receptors in fibroblasts may be good models to begin with.

There was insufficient time to study the effects of zinc pyrithione on later events in B cell activation, such as

proliferation, cIg formation and other phenotypic changes. These studies, as well as the effect of phenanthroline on phorbol ester-induced differentiation, will be major interests of our laboratory in the future.

10.7 Problems with analysis of PDBu-binding plots

Binding of ^{3}H-PDBu to cells involves both binding to receptor already in the membrane and in other pools of the particulate fraction (eg the detergent-insoluble pool), as well as binding to receptor that is newly translocated from the cytosol as a result of incubation of the cells with the ^{3}H-PDBu. The use of Scatchard plots in these studies is not entirely valid since the binding of PDBu to cells is at best a steady state situation in which there is both translocation of aporeceptors and down-regulation of occupied receptors. Nevertheless, useful information on the regulation of phorbol ester-binding to cells can be obtained using Scatchard analysis, with the proviso that the dissociation constant and total number of receptors that are derived from these plots may not have their true physicochemical meaning. For example, an increase in "affinity" of PDBu receptors as determined by Scatchard analysis may not necessarily indicate individual receptors with higher affinity but rather that the process of translocation of these receptors from the cytosol is facilitated.

10.8 Effects of FCS on PDBu binding

FCS had a number of effects on phorbol ester binding. In the absence of FCS, the Scatchard plots were curvilinear. This was not seen when cells were treated with A23187, suggesting that divalent cations may play a role in the apparent receptor heterogeneity and that FCS may affect this by chelating them. Another effect of FCS was to reduce the amount of down-regulation

of PDBu binding after maximum binding had been reached. Since down-regulation is thought to occur by calcium-dependent proteolysis of membrane PKC, FCS may also be acting here by chelating calcium. Further studies should test whether the effects of FCS are overcome by adding more calcium, zinc or other divalent cations.

10.9 Effects of calcium on PDBu binding and PKC translocation

A rise in $[Ca^{++}]_i$ caused an augmention of binding of PDBu to B-CLL cells as well as normal lymphocytes, PMN and platelets. On the other hand, treatment of cells with calcium chelating agents and TMB-8 decreased binding of PDBu. It is not clear to what extent the extracellular concentration of calcium influences PDBu binding. EGTA and EDTA do not readily penetrate cell membranes and presumably decrease PDBu binding by decreasing the extracellular calcium concentration and therefore the influx of calcium ions. An effect of these chelating agents on zinc and other divalent cations may also be responsible for their effects on PDBu binding and inhibition of MER.

As in other cell types, calcium ionophores enhanced PDBu-induced translocation of PKC from cytosol to the particulate fraction in B-CLL cells. One mechanism by which a rise in $[Ca^{++}]_i$ appears to facilitate translocation is the formation of a pool of PKC which is in loose, calcium-dependent association with the membrane phospholipid. Activation of PKC requires its further translocation by phorbol ester or diacylglycerol to a pool which is more tightly bound to the membrane and requires non-ionic detergent for solubilization.

In all three processes, namely inhibition of MER, augmentation of PDBu binding to the cells and translocation of

PKC from cytosol to particulate, calcium ionophores enhanced the effects when suboptimal concentrations of PDBu were used but had no effect at a concentration (200nM) of PDBu which saturated binding capacity of the cell. This conclusion is in agreement with the Scatchard analyses which indicate that the augmentation of PDBu binding by calcium ionophores and fluoride in both B-CLL cells and PMN is a result of an increase in affinity of the receptors for PDBu and not in the total numbers of receptors. A similar conclusion was reached by May et al [1985b] concerning the augmentation of PDBu binding to HL60 cells by calcium ionophore.

10.10 Effects of zinc and gold on PDBu binding

Both auranofin and zinc pyrithione increased PDBu binding. Although auranofin caused a slow two-fold increase in $[Ca^{++}]_i$, it is unlikely that this was sufficient to cause the large effects of auranofin on binding since calcium ionophores which augment the $[Ca^{++}]_i$ by more than ten-fold, increased PDBu binding by much less than auranofin. Auranofin may increase $[Ca^{++}]_i$ by interference with calcium homeostasis [Brewer et al. 1979] or by activation of phospholipase C [Snyder et al. 1988]. Since zinc quenches the fluorescence of Quin-2 it was not possible to do similar studies on the effect of zinc on $[Ca^{++}]_i$ although such studies may be possible with more recently developed calcium indicators [Grynkiewicz et al. 1985].

The sudies with gold and zinc indicate that the real number of phorbol ester receptors in cells may be two to three times higher than previously thought. It was possible to cause up to a three-fold enhancement in the apparent maximum binding capacity of both cells and a crude cytosolic preparation when these were treated with zinc. Brain may have a very large number of

receptors for PDBu partly because it has a very high content of zinc.

Zinc does not appear to cause a molecule other than PKC to bind ^{3}H-PDBu since the affinity of PDBu receptors in zinc-treated cells was identical to that in untreated cells. Also, the augmentation of PDBu binding in cytosol by zinc was prevented by depletion of the PKC from the cytosol, by absorption with membranous phospholipid in the presence of a high concentration of calcium ions. Studies with pure PKC are needed.

Zinc did not increase the total number of receptors by blocking receptor turnover, since little loss of PDBu binding capacity occurred in control B-CLL cells or sub-cellular fractions during the duration of the experiments. It is also unlikely that zinc and gold cause PKC to bind more than one molecule of ^{3}H-PDBu because these metals did not augment binding of ^{3}H-PDBu when added to the isolated particulate fraction of cells.

10.11 <u>Effect of zinc and gold on translocation of PKC</u>

The increase in maximum PDBu binding capacity in zinc- and gold-treated cells appears to arise by facilitation of translocation of cytosolic apo-PKC. Even at saturating concentrations, PDBu was only able to cause a partial translocation of its apo-receptors to the membrane. Zinc and gold facilitated this translocation. Zinc and gold only increased binding of ^{3}H-PDBu to cell-sonicates or to crude cytosol which contained some particulate material, indicating that binding of PDBu is augmented by enhancement of translocation from cytosol to the particulate fraction. Further studies need to be done in a model translocation system similar to that set up to

investigate the role of calcium in translocation of PKC. This system consisted of pure soluble PKC and inside-out red cell vesicles [Wolf et al. 1985a,b].

The role of calcium in the translocation and activation of PKC needs to be reassessed in the light of the pronounced effects of small concentrations of zinc on PDBu binding. This is particularly so for those studies which have employed "calcium" ionophores and chelating agents, since these agents interact even more strongly with zinc.

How zinc and gold permit full translocation has not been determined. There are several possibilities to be tested. The zinc-dependent pool may be one or more of the isozymes of PKC. Differences in the rate of translocation of some of these isozymes have been observed. Alternatively, this pool of PKC may be bound to a component in cytosol and therefore unable to translocate. For example, some cytosolic PKC can be revealed only by treatment with nonionic detergent and subsequent passage down a DEAE-cellulose column. Zinc may displace PKC from such a structure and thereby permit its translocation.

Another possibility is that translocation of PKC is limited by the capacity of the membrane to interact with PKC. There may be insufficient anionic phospholipid in the inner leaflet of the cell bilayer to accomodate binding of all of the PKC. It has been suggested that translocation of PKC to the particulate fraction is mediated by a membrane protein which binds PKC in a saturable manner [Gopalakrishna et al. 1987]. This protein may be in limited quantity in the cell membranes. By diverting PKC to another compartment of the cell, zinc and gold may enable all PKC to be translocated. Unlike PDBu, TPA induces a complete translocation of PKC to the particulate fraction and about 40% of

this is to a detergent-insoluble fraction [Thomas et al. 1988].

Zinc and gold synergized with PDBu in causing PKC to translocate from cytosol to a detergent-insoluble compartment, which may be the nucleus or cytoskeleton. It is not known whether the PKC is functionally active in this compartment although in cells treated with auranofin there was increased protein phosphorylation in the isolated nucleus-cytoskeleton complex. Whether zinc pyrithione causes similar effects on phosphorylation in this compartment needs to be determined. Localization of PKC in the nucleus or cytoskeleton of B-CLL cells and other types of cells by treatment with gold or zinc needs now to be confirmed by immunoenzymatic techniques. Antibodies to PKC have only just become available for these studies.

PDBu receptors in the detergent-insoluble material were assayed without addition of exogenous phospholipid. Phospholipid is the only substance known to convert soluble PKC into a receptor for phorbol ester. In the detergent-insoluble fraction, PKC may be in association with a molecule, other than phospholipid, which permits binding of ^{3}H-PDBu. Alternatively, this fraction may not be totally depleted of phospholipid. Identification of the substance (nuclear envelope, nuclear matrix, histone, DNA or non-nuclear component) to which PKC becomes bound in the detergent-insoluble fraction, may throw light on the significance of this translocation.

That augmentation of PDBu binding by gold and zinc also occurred in platelets, which lack nuclei, may indicate that PKC is translocated to the cytoskeleton. The inhibition of MER by zinc and other metals may be due to metal-induced translocation of PKC to the cytoskeleton. It needs to be determined whether PKC

becomes attached to components of the cytoskeleton in cells treated with zinc or gold and, if so, what these components are and whether they are associated with MER. Since gold also inhibits epidermal growth factor receptors, cytoskeletal PKC may play a general role in the regulation of receptor internalization. A role for cytoskeletal PKC in the phosphorylation of membrane components destined for internalization has been suggested [Feuerstein et al. 1985].

10.12 Effect of gold and zinc on catalytic activity of PKC

It is unclear whether these effects of zinc and gold on the regulatory domain of PKC result in activation of the catalytic site. At least with auranofin, protein phosphorylation is enhanced in intact cells in certain substrates known to be phosphorylated by PKC. It must be confirmed that auranofin is not acting by inhibiting phosphatases and that the effect of auranofin is on PKC and not other types of protein kinase. Such studies with zinc may be difficult to interpret because this metal is a potent inhibitor of phosphatases [Brautigan et al. 1981] and also activates tyrosine kinases in platelets [Findik and Presek 1988].

Both gold and zinc compounds inhibited histone kinase C activity when added directly to the cytosol of B-CLL cells (these studies) and platelets. Auranofin also inhibited the platelet protein kinase C activity directed towards other substrates, indicating that the inhibition is not simply a change in the substrate-specificity of PKC. High concentrations of gold and zinc may inactivate the sulphydryl in the catalytic domain that is known to be essential for catalytic activity. Zinc, at least, may be capable of activating the catalytic site under some circumstances, as shown by two other studies (discussed in

chapter 9). This issue needs further study.

10.13 Binding of zinc to PKC

It is important to determine whether PKC contains endogenous zinc in its zinc fingers and if so what role this zinc and the fingers play in the function of PKC. Sufficient pure PKC was not available to determine whether PKC contains zinc by atomic absorption analysis. The zinc content of different pools of PKC may vary, especially since most of the zinc is associated with the particulate fraction of cells. It will need to be determined whether gold displaces zinc from zinc fingers of PKC and, as an extension of this, whether PKC in cells from patients treated with auranofin is relatively deficient in zinc and instead contains bound gold.

Preliminary studies in our laboratory indicate that PKC binds to a zinc affinity column (which bind some other zinc-dependent enzymes [Shriner and Brautigan 1984]). Other experiments aim to determine whether ^{65}zinc will bind to PKC in western blots (as has been shown for some other zinc-binding proteins [Mazen et al. 1988]). Future studies will investigate the effect of zinc and gold on the structure of PKC.

10.14 Relevance of these studies to action of gold and zinc in vivo

A major unresolved question is whether the effects of gold and zinc described in these studies occur in vivo.

The effects of auranofin occurred at cellular levels of gold that were found in leukocytes of patients with rheumatoid arthritis who had been receiving the drug for several months. However, it is not known whether the gold localizes in the same cellular compartments and interacts with similar sets of proteins

when it is given to cells in vitro as it does when administered in vivo. The expression of MER and properties of PKC in cells from patients undergoing therapy with auranofin and other gold compounds must be compared with those of cells from patients not receiving gold and with those from normal subjects.

Similarly, it requires to be shown that the effects of zinc in vitro occur at similar concentrations to those found in vivo. Total zinc in B-CLL cells (200 pmoles per 10^6 cells) as measured by atomic absorption studies was of the order of those reported for other types of cells. This corresponds to a total zinc concentration of about 1mM. Treatment of cells with about 50uM zinc (in the presence of pyrithione) approximately doubled the total zinc concentration of the cell. More importantly, it is necessary to estimate the concentration of free zinc ions in cells since it is the free zinc rather than total zinc which will act on PKC. Free zinc is thought to be only in the nanomolar or picomolar range in the cytosol of cells because most of the zinc is chelated by the cysteine-rich metallothionein. Fluorochromes which enable measurements of intracellular free zinc ions are required for these studies. Such probes would also enable studies of the effect of various growth stimuli on fluxes of zinc ions and their involvement in cell activation.

APPENDIX

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A PHOSPHATIDYLETHANOLAMINE-CONTAINING COMPLEX ON HUMAN B CELLS THAT MEDIATES ROSETTE FORMATION WITH MOUSE ERYTHROCYTES[1]

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The maturation-associated human B cell rosette receptor (MER) for mouse erythrocytes has been solubilized from B cells by mild trypsinization. It specifically agglutinates mouse red cells. Material with hemagglutinating activity partitioned into the lipid-soluble phase of a Folch partition of the trypsin extract was sensitive to phospholipase C and alkali, and on two-dimensional thin layer chromatography, it co-migrated principally with phosphatidylethanolamine (PE). Phosphatidylcholine, the major lipid present, was inactive. The relationship of phospholipid structure to hemagglutinating activity has been described. PE in the crude trypsin extract was associated with unidentified glycoprotein and albumin. Material containing hemagglutinating lipid bound to a wheat germ lectin-Sepharose column and was released by *N*-acetylglucosamine, indicating that the PE was complexed with glycoprotein. When the crude trypsin extract or eluate from the lectin column was extracted with aqueous phenol, hemagglutinin in the aqueous phase no longer bound to wheat germ lectin-Sepharose; however, albumin was greatly enriched, indicating that some of the PE exists in a complex with albumin. The molar ratio of PE to albumin was approximately 200:1. After delipidation, this albumin (in molar excess) inhibited hemagglutination by PE in the same way as a recently described subclass of serum albumin. Studies with phospholipase-treated B cells were also consistent with PE being the MER.

We conclude that MER is PE, existing in a complex containing glycoprotein and a subclass of albumin. The capacity to form rosettes can be transferred to nonrosetting Raji B cells by the complex, but not pure PE, indicating that the proteins may be involved in orienting PE correctly for it to function as the MER.

Rosette formation with mouse erythrocytes is a specific property of human B lymphocytes restricted to an early stage of B cell maturation (1). The phenomenon is important because the capacity to form these rosettes is normally lost during maturation of B lymphocytes. This maturation step, which involves a rapid change in the membrane (2), is blocked in chronic lymphocytic leukemia (CLL)[2] (3). Loss of rosetting capacity of both normal and leukemic B cells can be induced *in vitro* by maturation-inducing agents of the phorbol ester type (2) or lymphokines. To understand the mechanism of this modulation and its abnormalities in CLL, we investigated the structure of the rosette receptor, MER. MER has been solubilized as a mouse red cell-specific hemagglutinin by mild trypsinization or detergent extraction (4). We now show that it is phosphatidylethanolamine (PE). It is released from membranes of leukemic B cells by trypsin in the form of a complex with an unidentified glycoprotein or glycoproteins and a subclass of albumin. It has been shown that pure PE and other noncholine phospholipids agglutinate mouse erythrocytes by binding to a 70 kilodalton (Kda) albumin-like protein in the mouse erythrocyte membrane (5). A subclass of albumin in serum of humans and other species interacts in this system (6), inhibiting agglutination of mouse erythrocytes by MER and PE.

MATERIALS AND METHODS

Cells. Lymphocytes were obtained from two patients with CLL undergoing leukapheresis. Cells were washed three times in Dulbecco's phosphate-buffered saline, pH 7.4 (PBS). Animal erythrocytes were prepared as described in Reference 1.

Preparations of crude trypsin extract. Trypsin supernatants were prepared by mild trypsinization of CLL cells, at 10^8/ml, followed by ultrafiltration as described (4).

Folch partitioning. Two milliliters of trypsin extract were shaken for 5 min with 12 ml of 2:1 mixture of chloroform (Ajax, Warren, OH) and methanol (Merck, Rahway, NJ). After centrifugation (25 min at 200 × G), the upper layer was removed and its volume was measured. The lower layer was then extracted with chloroform/methanol/0.9% NaCl (1:10:10) using the same volume as for the upper layer. After centrifugation, the lower layer was removed, evaporated to dryness, and taken up in PBS containing 20% ethanol for hemagglutination assay or stored in chloroform at −20°C. The lower layer (organic solvent phase) was designated the Folch extract. The upper layer (aqueous phase) was dialyzed and concentrated by rotary evaporation.

Hemagglutination and hemagglutination-inhibition assays. Hemagglutination assays with Folch extract were performed as described in Reference 5 and those with aqueous extracts were as in Reference 4. Egg yolk PE (Sigma Chemical, St. Louis, MO) and dipalmitoyl PE (Sigma) were prepared as described (5). Mouse erythrocyte membrane extract was prepared and tested in hemagglutination-inhibition assays by a published method (5).

The subclass of albumin was prepared by the following method. To 5 ml of a 1/5 dilution of normal human serum in PBS was added 20 ml of a 40% aqueous ethanol solution. Precipitate was extracted with *N*-butanol (Ajax) and the aqueous phase was chromatographed

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[1] This work was supported by Grant 830697 80 12002 from The National Health and Medical Research Council of Australia.

[2] Abbreviations used: CLL, chronic lymphocytic leukemia; KDa, kilodalton; PE, phosphatidylethanolamine; MER, receptor for mouse erythrocytes on B lymphocytes.

on Blue Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ) by a method similar to that for preparation of the mouse erythrocyte ligand (5). Mannan and fetuin, type III, were purchased from Sigma Chemical.

Thin layer chromatography. Samples were chromatographed on silica gel 60 aluminium-backed plates (20 x 20 cm; Merck). Solvent one was chloroform/methanol/2.5 N ammonium hydroxide (70:20:3); solvent two was chloroform/methanol/acetone/acetic acid/water (30:10:40:10:5). Lipids were visualized by charring with sulfuric acid or iodine vapor. Staining with ninhydrin (Pierce Chemicals, Rockford, IL) was by a published method (7). Lipids were eluted from the gels by soaking in chloroform/methanol (2:1).

Phosphate analysis. Phosphate was assayed by the method of Zilversmit and Davis (8).

Wheat germ lectin chromatography. This was performed as described (4).

Phenol extraction. Supernatants were mixed with equal volumes of 50% phenol (Faulding, Adelaide, South Australia) in water. Mixtures were stirred at 4°C for 15 min and centrifuged at 800 × G for 20 min. The upper aqueous phase was dialyzed against several changes of distilled water at 4°C.

Phospholipase treatment of CLL cells. CLL cells, 10^8/ml in PBS, were incubated with phospholipase C (*B. cereus*, Sigma), phospholipase D (Sigma) from cabbage, or peanut or phospholipase A_2 (*Apis mellifera*, Sigma) for 30 min at 37°C. After washing, cells were tested for rosette formation with mouse erythrocytes (4). Cell viability was determined by trypan blue exclusion.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This was carried out by the discontinuous method of King and Laemmli (9) with 2-mercaptoethanol unless otherwise indicated.

Protein estimation. Protein concentrations were determined by the method of Lowry *et al.* (10) or by absorbence at 280 nm.

Albumin estimation. Albumin concentrations were determined by radial immunodiffusion (11) with rabbit anti-human albumin (Pharmacia).

Anti-albumin chromatography. Rabbit anti-human albumin, 2 ml, was conjugated with 3 g cyanogen bromide-activated Sepharose 4B (Pharmacia) according to Pharmacia specifications. One milliliter of crude trypsin extract or aqueous phase of phenol extract was incubated for 60 min at room temperature on the column (150 x 9 mm). The column was washed with 30 ml 0.2 M Tris-HCl containing 0.5 M NaCl, pH 8.0, and bound material was eluted with 30 ml 0.2 M glycine-HCl containing 0.5 M NaCl, pH 2.8. Wash and eluate were dialyzed, concentrated to 1 ml, and tested for hemagglutinating activity.

Adoptive rosetting. Raji B cells (10^7), on day 2 of subculture, were incubated with PE, egg yolk (1 mg/ml), or trypsin extract as described (4) before washing and testing for rosette formation with mouse erythrocytes.

RESULTS

Solubilization of MER. Soluble MER was prepared by trypsinization of 10^{10} to 10^{11} CLL B cells. Trypsin inhibitor was added to the supernatants, which were then simultaneously concentrated and ultrafiltered (Amicon XM 100 membrane). When cells were trypsinized at 10^8/ml and the supernatant was concentrated 10-fold, dilutions up to 1/128 (protein concentrations of 10 to 20 μg/ml) agglutinated mouse erythrocytes. SDS-PAGE of this extract revealed a complex mixture of components (Fig. 1*a*). Considerable precipitation of material occurring on storage at −20°C was associated with partial loss of activity.

Folch partitioning. Hemagglutinating activity in the trypsin extract was traced to the organic solvent phase of a folch partition (chloroform/methanol 2:1). All of the activity was found in the Folch extract. The specificity of hemagglutination was the same for trypsin extract and the Folch organic phase extract of it; both specifically agglutinated mouse erythrocytes and both were inhibited by a subclass of albumin and an extract of mouse erythrocyte membranes (containing the putative ligand) and commercial preparations of mannan and fetuin (Table I).

Effect of treatment on activity. Activity in the Folch extract was not affected by boiling or freezing in water

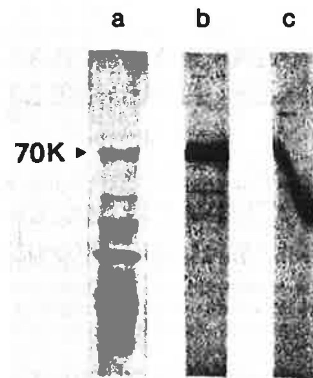

Figure 1. SDS-PAGE of MER. *a*, a 10% gel (silver stained) of a trypsin extract of CLL cells. *b*, a 7.5% gel (stained with Coomassie Blue) of the aqueous phase of a phenol extract of the material in *a*. *c*, the same as *b* but 2-mercaptoethanol was omitted. Diffusion of 2-mercaptoethanol from *b* has caused the slant of the band in *c*. The common band at 70 KDa is indicated.

TABLE I

Specificities of hemagglutinin in trypsin extract and its Folch organic phase extract

Indicator Red Cells[a]	Hemagglutination Titer[b]	
	Trypsin extract	Folch, organic phase
Mouse	6	7
Sheep	0	0
Human	0	0
Bovine	0	0
Pronase-treated mouse	0	0
Mouse + 70 KDa protein[c]	0	0
Mouse + albumin[c]	0	0
Mouse + fetuin	0	0
Mouse + mannan	1	2

[a] Pronase-treated mouse erythrocytes were prepared as in Reference 1.
[b] Trypsin extract and its Folch organic phase were prepared as in *Materials and Methods*. Titer is expressed in reciprocal form as power of 2. O, no hemagglutination.
[c] The 70 KDa protein from mouse erythrocytes was prepared as described (5). Albumin from serum was prepared as in *Materials and Methods*. Hemagglutinating extract was incubated with the proteins for 15 min before adding red cells. Final concentration of albumin, fetuin, and mannan was 2 mg/ml, and of 70 KDa protein, 0.1 mg/ml.

or 90% aqueous ethanol. Activity in the crude trypsin extract was lost completely on boiling (5 min), but this was not due to destruction of the hemagglutinin because all of the original activity could be recovered in a Folch extract of the boiled trypsin extract.

Hemagglutinating activity in the Folch extract was inhibited by alkaline hydrolysis (which cleaves fatty acid ester bonds). The Folch extract was treated with 0.1 N NaOH in methanol for 15 min at 37°C. A chloroform/methanol extract of this contained less than 1% of the original activity. Acid hydrolysis (30 min, 80°C in 0.05 N HCl), which removes sialic acid, had no effect.

Trypsin extract (10 ml), original titer 2^8, was treated with phospholipase C (1 mg *B. cereus*) at 37°C. After 1 hr, the titer was 2^5, after 2 hr, 2^2, and after 3 hr, no hemagglutinating activity remained. Incubation without phospholipase had no effect. A sample treated for 3 hr and a control sample were extracted with chloroform/methanol and the organic phase was tested for activity. The control Folch extract had a titer of 2^{10} whereas the Folch extract of the phospholipase C-treated extract had a titer of 2^9. Therefore, whereas phospholipase C inacti-

vated hemagglutinin in a trypsin extract, substantial activity was recovered from it by extraction with lipid solvents. Treatment of Folch extract with phospholipase C (1 mg) abolished agglutinating activity (titer decreased from 2^{10} to 0.) This may indicate that much of the PE in the trypsin extract is masked. Treatment of trypsin and Folch extracts for 30 min with phospholipase D (1 mg/ml, cabbage) increased activity two- to fourfold (see *Discussion*). Trypsin extracts were treated with pronase (1 mg/ml, Sigma) or neuraminidase (0.1 U/ml, Hoechst) for 30 min at 37°C. Folch extracts of these contained all of the original activity.

Thin layer chromatography. The Folch extract was analyzed by two-dimensional silica gel thin layer chromatography with the use of pure phospholipid markers. Lipids were visualized by charring with sulfuric acid (Fig. 2) or iodine vapor. Lipids were eluted in chloroform/methanol and tested after evaporation of the solvent and resuspension in PBS. Most of the hemagglutinating activity corresponded to the spot indicated (arrow) in Figure 2. This co-migrated with pure PE, was ninhydrin-positive, and contained phosphate.

Phosphatidylcholine, the major detectable lipid, was inactive after elution. Small quantities of other lipids, which have not yet been identified, were present and active as hemagglutinins. Controls showed that no detectable lipid, or hemagglutinin, was derived from the substances used in the preparation of trypsin extracts (trypsin, deoxyribonuclease, or trypsin inhibitor).

Wheat germ lectin chromatography. All of the hemagglutinating activity in the trypsin extract bound to wheat germ lectin-Sepharose and was eluted from it by the specific sugar *N*-acetylglucosamine (Fig. 3*a*). The eluate contained only about 10% of the original protein, but on SDS-PAGE was still complex. Folch extracts of the wash and eluate confirmed that all of the hemagglutinating lipid was in the eluate, suggesting that the PE was complexed with glycoprotein.

Phenol extraction. Trypsin supernatant was extracted with phenol with the intention of preparing a glycoprotein extract. As expected, hemagglutinating activity was present in the aqueous phase, but surprisingly, the material with agglutinating activity no longer bound to wheat germ lectin-Sepharose (Fig. 3*b*). On SDS-PAGE this extract was greatly enriched for a polypeptide of apparent m.w. 70,000 (Fig. 1*b*). Under nonreducing conditions, this polypeptide had a greatly reduced apparent m.w. typical of albumin (12) (Fig. 1*c*). By radial immunodiffusion, at least 75% of the protein was albumin.

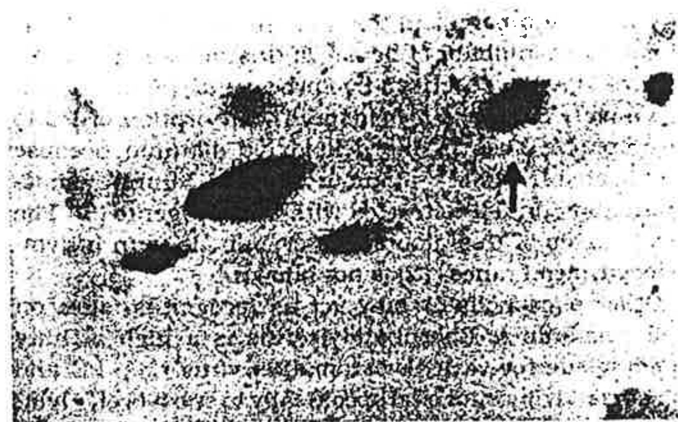

Figure 2. Two-dimensional thin layer chromatography of Folch organic phase. Chromatography was performed in two dimensions (as in *Materials and Methods*), with solvent one in the vertical axis and solvent two in the horizontal axis. Lipids were visualized by sulfuric acid charring. On identical runs, plates were stained with ninhydrin or lipids visualized by iodine vapor, eluted, and tested for hemagglutinating activity. Arrow, area contained greater than 90% of the total hemagglutinating activity of the Folch extract. It co-migrated with PE, was ninhydrin-positive, and contained phosphate.

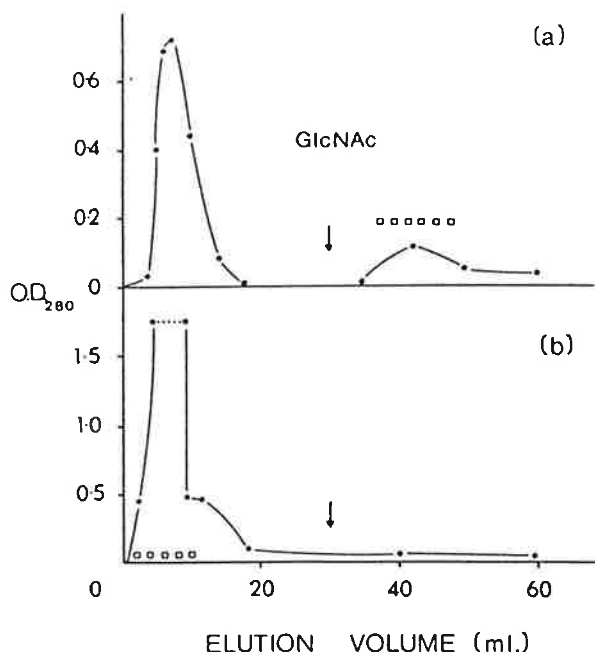

Figure 3. Wheat germ lectin-Sepharose chromatography of MER. Crude trypsin extract (*a*) or aqueous phase of phenol extract of trypsin extract (*b*) were chromatographed on wheat germ lectin-Sepharose (see *Materials and Methods*). Washes and eluates were each 30 ml. Protein was estimated by absorbence at 280 nm (●–●–●). Hemagglutinating activity (□–□–□) was assayed on individual fractions without removal of sugar.

The aqueous phase of a phenol extract of the eluate of a wheat germ lectin-Sepharose chromatograph was also enriched for this 70 KDa protein. This component could also be seen in the more complex SDS-PAGE profiles of the crude trypsin extract (Fig. 1*a*) and wheat germ-Sepharose eluate. Analysis by radial immunodiffusion indicated that 2% of the protein in the trypsin extract was albumin; however, material with hemagglutinating activity did not bind to an anti-albumin column. Bands with m.w. of approximately 70 KDa were seen in both the wash and eluate of a wheat germ lectin-Sepharose chromatograph of the trypsin extract, indicating that only some of the albumin may be in the MER complex.

A Folch extract of the aqueous phase of the phenol extract contained the agglutinating activity. On one-dimensional thin layer chromatography, a single component, co-migrating with PE (R_f 0.47 in chloroform/methanol/2.5 N ammonium hydroxide of 70:20:3) was seen. Because of the small amount of phospholipid present, it was not possible to determine whether minor phospholipid components were also present. Six hundred micrograms of protein after Folch partitioning yielded 1.6 μmol of phosphate, corresponding to an approximate protein to phospholipid molar ratio of 1:200 (assuming m.w. for phospholipid of 750 and for protein of 70,000). A sample of this Folch extract when resuspended in 20% aqueous ethanol agglutinated down to PE concentration of 60 μg/ml. Similar preparations of egg yolk PE and dipalmitoyl PE agglutinated down to 0.1 and 240 μg/ml, respectively.

Thus, PE derived from MER was less active per unit weight than egg yolk PE, but was more active than dipalmitoyl PE.

Delipidated MER. When the aqueous phase of a phenol extract of the trypsin extract (containing largely PE and albumin) was extracted with chloroform/methanol, the aqueous phase of the Folch extraction now contained a potent inhibitor of PE-mediated hemagglutination. Thus, when equal weights of the aqueous phase and egg yolk PE were pre-incubated, there was complete inhibition of hemagglutination.

Phospholipase treatment of B cells. When CLL B cells were treated with phospholipase C from *B. cereus* (15 U/ml), the percentage of rosette-forming cells decreased from 83 to 57. Higher concentrations of enzyme significantly decreased the viability of the cells. Phospholipase A_2 decreased rosetting from 83 to 6% at a nontoxic concentration (1 μg/ml). Phospholipase D from peanut and cabbage, at 500 μg/ml increased rosette-forming cells from 83 to 96 and 92%, respectively, and also increased the number of attached erythrocytes.

Adoptive rosetting. Preincubation with PE did not induce rosetting in Raji B cells, on day 2 of subculture. Preincubation with the whole trypsin extract increased the percentage of rosette-forming cells from 0 to 48.

DISCUSSION

Knowledge of the structure of MER may provide insight into the mechanism of its modulation in B cells (2, 3), apparently an essential early step of B cell development (2) and site of maturation arrest in CLL (1). The specific mouse erythrocyte binding properties of MER are retained after its solubilization by mild trypsinization or detergent extraction (4). Soluble MER is rapidly and simply assayed by agglutination of mouse erythrocytes. Retention by a wheat germ lectin affinity column led us to postulate that MER was a glycoprotein (4).

We have now established that the hemagglutinating factor in the trypsin extract is PE, with a minor contribution by other noncholine phospholipids. The presence of lipid in MER was first suggested by its pronounced tendency to aggregate (4). The hemagglutinating property was resistant to many treatments causing denaturation or destruction of protein, but was very sensitive to treatments causing destruction of lipid (alkaline hydrolysis, phospholipases). Conclusive evidence that the hemagglutinating factor is PE was provided by thin layer chromatography. Thus, the active spot was ninhydrin-positive (PE and phosphatidylserine are the only ninhydrin-positive phospholipids), contained phosphate, and co-migrated with PE. The hemagglutinating specificities of trypsin extract and pure PE were identical with respect to type of indicator red cell and inhibition by exogenous substance (Table I).

Confirmation was provided by two sets of experiments: studies relating structure to activity of a variety of pure phospholipids from commercial sources and studies of digestion of these with phospholipases (5). Phospholipase C, which removes both the phosphate and base, destroyed the hemagglutinating activity of MER and pure PE; phospholipase D, which removes only the base (leaving phosphatidic acid, a mouse red cell agglutinin) (5), did not affect its activity. The similar effect of these phospholipases on rosetting supports this concept.

The next question concerned the nature of the structure into which MER is incorporated. Studies of the binding of fluorescein-labeled MER to mouse erythrocytes indicated that it was a stable complex containing protein (4). It was also established that the amount of specific hemagglutinin released by trypsin correlated with the capacity of the B cells to form rosettes. It is unlikely that the very gentle treatment with trypsin releases PE indiscriminantly because the viability is not affected, and PE is found predominantly in the inner layer of the membrane (13). Lectin affinity chromatography suggested that one of these molecules in the complex with PE is a glycoprotein. After phenol extraction some hemagglutinin partitioned into the aqueous phase (unlike free PE (14)). This material was not retained by a wheat germ lectin column, indicating that glycoprotein had been removed from the complex by this treatment. Phenol-extracted material bound to an anti-albumin column and most of it was albumin as shown by immunoassay and the characteristic shift in m.w. on SDS-PAGE in the absence of 2-mercaptoethanol. Albumin is a nonglycosylated protein (15). Failure of the hemagglutinin in the crude trypsin extract to bind to an anti-albumin column indicates that the albumin is masked.

The ligand in this system is a hitherto unrecognized subclass of albumin. Agglutination of mouse erythrocytes by trypsin extract or PE is inhibited by a subclass of serum albumin or by 2-mercaptoethanol-treated fraction V albumin (6). We showed previously (4) that hemagglutination by trypsin extract was inhibited by an extract of mouse erythrocyte stroma and by commercial preparations of two glycoproteins, mannan and fetuin. Hemagglutination by PE is similarly inhibited. The inhibitor in fetuin was shown to have the properties of albumin (6). Similarly inhibition by a crude mannan preparation was associated with the presence of albumin, which was absent from a noninhibitory pure mannan preparation (unpublished observations).

When the albumin in MER is freed from PE, it also becomes an inhibitor of hemagglutination, similar to the type of albumin described by Forbes *et al.* (6). Inhibition is unlikely to be due to nonspecific adsorption of PE to hydrophobic sites in the delipidated albumin because delipidated fraction V albumin does not inhibit hemagglutination until it is treated with thiol reagents (6). The relationship of this albumin to cryptic albumin in lymphocyte membranes (12) is not known.

Other phospholipids may act as recognition sites on cell membranes. Phosphatidylserine is a high affinity binding site for vesicular stomatitis virus (16); PE and phosphatidylinositol bind specifically to subsets of T lymphocytes (17); and complexes of protein with phosphatidylserine and PE form the binding site for phorbol esters (18). We conclude that the MER is PE, and the proteins associated with it are evidently important for the correct disposition of PE because by itself PE will not adoptively transfer the capacity to rosette to nonrosetting Raji B cells.

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MECHANISM OF INDUCTION OF MOUSE ERYTHROCYTE RECEPTOR SWITCH IN HUMAN B CELLS[1]

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An early event in phorbol ester-induced maturation of chronic lymphocytic leukemic (CLL) B cells is a membrane change characterized by the inactivation of a mouse erythrocyte receptor (MER). This event, the MER-switch, is quantified by inhibition of rosette formation. By using [^{3}H]phorbol dibutyrate ([^{3}H]PDBu), both to stimulate MER-switch and assay binding of PDBu to CLL cells, it was shown that MER-switch was an irreversible, time-dependent event which occurred some time after maximal binding of [^{3}H]PDBu to cells. Two classes of binding sites, one of high affinity (Kd 1 to 2 nM) at low frequency (1.5 to 5 × 10^4 sites per cell), and a lower affinity site (Kd 33 to 50 nM) of higher frequency (2 to 3.5 × 10^5 sites per cell), were detected. Binding of [^{3}H]PDBu was inhibited by phorbol ester analogs that stimulated MER-switch, but not by inactive analogs. This, and the similarity in shapes of the binding and rosette inhibition curves over a range of concentrations, suggests that stimulation of MER-switch by phorbol esters is due to this specific binding. The phorbol ester receptor and MER are distinct because MER-ve T cells and MER-ve atypical B cells from a patient with CLL had both classes of PDBu receptor. Solubilized MER did not bind [^{3}H] PDBu. Time-course studies, and the irreversibility of the switch, despite removal of most of the bound [^{3}H]PDBu, indicate that inhibition of rosetting is not due to competitive or steric hindrance by phorbol esters.

Equivalent activities of soluble MER were released from fresh and phorbol ester-treated CLL cells, indicating a rearrangement of MER, rather than a loss. A supernatant of phytohemagglutinin-stimulated human spleen cells also induced MER-switch in CLL lymphocytes, suggesting that a lymphokine may be a natural inducer of this event.

Two subsets of human B cells can be distinguished by the capacity of one to form rosettes with mouse erythrocytes (1). The B cell mouse erythrocyte receptor (MER)[3] is phosphatidylethanolamine attached to a complex of membrane proteins (2). The receptor complex, when solubilized from the membrane by mild treatment with trypsin, will specifically agglutinate mouse erythrocytes (3). Pure phosphatidylethanolamine and other non-choline phospholipids also agglutinate mouse erythrocytes (4). A 70,000 dalton protein on mouse erythrocytes, presumed to be the ligand, potently inhibits this agglutination (4). A similar substance (probably the natural ligand for MER) is a subtype of serum albumin (5). The same activity is induced in non-inhibitory crystalline human albumin by treatment with 2-mercaptoethanol and other low molecular weight thiols (5).

One of the earliest events in phorbol ester-induced plasmacytoid maturation of human B cells is inactivation of MER (6, 7). This event, the MER-switch, also occurs spontaneously over a period of several hours in serum-free culture (8) or over several days in serum-containing culture (7). Recently, phorbol ester receptors have been identified on a variety of cell types (9, 10), and were shown to be associated with phospholipid-dependent protein kinase activity (11). In this paper, we report investigations of the relationship between [^{3}H]PDBu[3] binding to chronic lymphocytic leukemia (CLL) cells and loss of capacity to form rosettes with mouse erythrocytes. Active MER was sought in trypsin extracts (3) of phorbol ester-treated B cells to study the mechanism of rosette inhibition. Finally, the effect of lymphokines on MER-switch is described.

MATERIALS AND METHODS

Lymhpocytes. Normal T lymphocytes were prepared by E rosette sedimentation of peripheral blood lymphocytes from normal donors (1). CLL cells obtained from two patients and atypical CLL cells obtained from one patient, all undergoing leukopheresis, were frozen in liquid nitrogen by using a controlled freezing rate (1°C per min) and were stored at −180°C. Viability after thawing exceeded 95%. Cells were washed in phosphate-buffered saline (PBS, pH 7.4) and were stored for short times at 4°C in PBS containing bovine albumin at 5 mg/ml (PBS/ALB).

Cell culture. Cells were cultured at 10^6 cells per well in flat-bottomed tissue culture plates (Linbro Chemical Co., New Haven, CT) in PBS/ALB or in medium/FCS-medium 199, HEPES buffered, and supplemented with 15% FCS (Flow Laboratories, Rockville, MD). An equal volume of dilutions of [^{3}H]PDBu in PBS/ALB was added. Plates were incubated in a humidified CO_2 incubator.

Mouse erythrocyte rosette assay. Cultured cells (10^5) were washed twice with PBS/ALB, were resuspended in one drop of FCS and were mixed with one drop of 2% mouse erythrocyte suspension. Rosettes were counted as described (1).

EC_{50}. EC_{50} is the concentration of PDBu that inhibits rosetting to 50% of maximal response.

[^{3}H]PDBu receptor studies. [^{3}H]PDBu (specific activity 7.6 Ci/mmol) was purchased from Dr. P. Borchert (CCRI, Minneapolis, MN). Air was displaced with nitrogen, and the liquid was diluted five times with ethanol and was stored in glass vials at −20°C. 12-*O*-tetrad

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[1] This work was supported by an NH and MRC Grant 80/12002.
[2] To whom correspondence should be addressed.
[3] Abbreviations used: B, bound [^{3}H]PDBu; B_t, bound [^{3}H]PDBu at saturation; CLL, chronic lymphocytic leukemia; EC_{50}, concentration of [^{3}H] PDBu giving half-maximal response; Kd, dissociation constant; medium/FCS, medium containing 15% FCS; MER, mouse erythrocyte receptor; PBS/ALB, PBS containing 5 mg/ml bovine albumin; [^{3}H]PDBu, tritium-labeled phorbol dibutyrate; 4-α-PDD, phorbol didecanoate; PHA-super, PHA-treated spleen cell supernatant; PHA-control, same as PHA-super but spleen cells omitted; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate.

canoyl phorbol-13-actetate (TPA) was purchased from Sigma Chemical Co., St. Louis, MO. Mezerein and 4-α-phorbol didecanoate (4-α-PDD) were obtained from P-L Biochemicals, Milwaukee, WI, and phorbol was obtained from Life Systems Division, Woburn, MA. Aliquots of the [^{3}H]PDBu solution (5 × 10^{-6} M) were evaporated under a stream of air, 50 μl of dimethylsulfoxide were added to dissolve the residue, and PBS/ALB was added to give double the desired final concentration of [^{3}H]PDBu. At the final concentration, in assays of 0.5%, dimethylsulfoxide has no effect on MER-switch (6, 7) or cell viability. One hundred microliters of each dilution were added to wells containing 100 μl of cells (10^7/ml) in PBS/ALB or medium/FCS. Replicate wells containing a 50-fold molar excess of TPA in addition to [^{3}H]PDBu were set up for each concentration of [^{3}H]PDBu to determine nonspecific binding. In all harvests, the filters were pre-washed with a 10^{-6} M solution of TPA in PBS/ALB. Approximate concentrations of [^{3}H]PDBu in wells ranged from 0.5 nM to 250 nM in most experiments. Exact concentrations were determined by transferring 10-μl aliquots from selected wells to filter papers before harvesting. Remaining cell suspensions were harvested directly onto glass fiber filters by using a Titertek cell harvester with two cycles of washing with PBS. Filter papers were dried extensively and were added to scintillation vials containing 2 ml of toluene, PPO, and POPOP scintillation cocktail for counting in a Packard Tricarb liquid scintillation counter. Specific binding equals total binding minus binding in the presence of TPA.

Thin-layer chromatography (TLC) of [^{3}H]PDBu. To determine the extent of metabolism of [^{3}H]PDBu, CLL cells were incubated with [^{3}H]PDBu (100 nM) in medium/FCS for 4 hr at 37°C. Aliquots were then freeze-dried, were extracted with methanol, and the supernatant was spotted on aluminum-backed silica gel 60 TLC sheets (Merck Chemical Div. Rahway, NJ). Plates (2 × 10-cm) were developed with ethylacetate, cut in 1-cm slices, and counted after the addition of 2 ml of scintillant. Control incubations contained [^{3}H]PDBu but no cells.

Preparation and assay of protein kinase C. Crude rat brain protein kinase C was prepared and was tested with ^{32}P-ATP (3 Ci/mmol; Amersham, Arlington Heights, IL) and histone type III (Sigma) by a described method (11).

Soluble [^{3}H]PDBu binding assay. Binding of [^{3}H]PDBu to protein kinase C or MER was assayed by using a described method (11), except that free and bound [^{3}H]PDBu were separated on Biogel P10 columns (0.9 × 8-cm), equilibrated with PBS as described previously (12).

Solubilization of MER activity and hemagglutination assay. Methods for solubilization of MER activity from CLL B cells by mild trypsinization and Nonidet P-40 detergent and for assay of activity by hemagglutination in microtiter wells have been published previously (2, 3).

Prepartion of lymphokine. Splenocytes from a patient with hereditary spherocytosis were cultured at 2 × 10^6/ml in medium/FCS containing phytohemagglutinin (PHA; Wellcome, Beckenham, UK) at 1% v/v. After 20 hr, supernatant (referred to as PHA-super) was collected and was stored at −20°C until use. Two control supernatants were used; PHA-control was prepared in the same way but without cells being present, while cell-control was prepared with cells but without PHA.

RESULTS

[^{3}H]PDBu binding: specific and nonspecific binding. CLL cells were incubated with dilutions of [^{3}H]PDBu for 25 min at 37°C and were then harvested on glass fiber filters to separate bound and free [^{3}H]PDBu. Nonspecific binding (see *Materials and Methods*) varied between 15 and 25% of the total binding. Controls lacking cells were set up. At each concentration of [^{3}H]PDBu, binding in the absence of cells closely approximated binding in the presence of cells and TPA, suggesting that most of the nonspecific binding was due to binding of [^{3}H]PDBu to filters.

Reversibility of [^{3}H]PDBu binding. Reversibility of binding was tested by incubating CLL cells with [^{3}H]PDBu (50 nM) for 20 min and, after washing of the cells, incubating for varying periods at 37°C in medium lacking [^{3}H]PDBu. Ninety percent of total bound radioactivity was lost within 10 min of incubation.

Binding kinetics. At 5 min, in 25 nM [^{3}H]PDBu, specific binding was 92% of that at 25 min. At 60 min, it was 80% of binding at 25 min, and 57% at 120 min. Thus, all binding was determined at 25 min.

Saturability of [^{3}H]PDBu binding (Fig. 1). Binding of [^{3}H]PDBu to CLL cells was saturable, as shown by the flattening of the binding plot at concentrations of [^{3}H]PDBu greater than 50 nM (Fig. 1).

Determination of number and affinity of receptors (Fig. 1). Specific binding was determined over the concentration range of 0 to 350 nM [^{3}H]PDBu (12 different concentrations). Data were plotted by the method of Scatchard. In all nine experiments, a curvilinear plot (upward concavity) was obtained. A typical experiment is shown in Figure 1. This type of Scatchard plot has been interpreted variously (see *Discussion*). To determine individual parameters, data were pooled from three experiments. Assuming two binding sites, there was a high-affinity receptor (Kd 1 nm) of low capacity (0.02 pmol/10^6 cells at saturation) and a low-affinity receptor (Kd 50 nM) of high capacity (0.36 pmol/10^6 cells at saturation). The Kd and binding site capacities were calculated as described previously (13). Assuming receptors are univalent and homogeneously distributed amongst the cells, we calculate approximately 1.5 × 10^4 high-affinity and 2.15 × 10^5 low-affinity receptors.

With the cells of another patient with MER+ve CLL, a similar nonlinear Scatchard plot was obtained. There were approximately 4 × 10^5 receptors per cell. For a two-binding-site model, the distribution of receptors was 5 × 10^4 (Kd 2 nM) and 3.5 × 10^5 (Kd 33 nM).

With cells from a patient with atypical CLL, which failed to rosette and which did not release MER on treatment with trypsin (3), binding was similar to that of the two other cases of CLL. There were 4 × 10^4 sites of Kd 2 nM and 3 × 10^5 sites of Kd 55 nM. E rosette-purified T cells had 3 × 10^4 (Kd 3 nM) and 9 × 10^4 (Kd 50 nM) binding sites.

Correlation between potency of stimulators of MER-switch and capacity to inhibit [^{3}H]PDBu binding of a variety of phorbol esters and mezerein. The relative potencies of different types of phorbol esters and the related compound mezerein as competitive inhibitors of binding were determined. CLL cells were incubated with 35 nM [^{3}H]PDBu in the presence of unlabeled TPA, mezerein, phorbol, or 4-α-PDD at various final concentrations

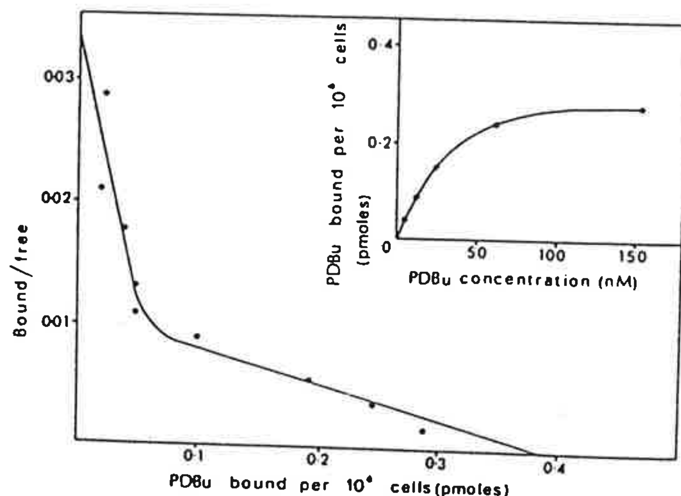

Figure 1. Scatchard plot of binding of [^{3}H]PDBu to CLL cells. Binding assay was as described in *Materials and Methods*. Inset shows the saturation curve for binding. Binding is expressed as pmol per 10^6 cells.

from 1 nM to 10 μM. At 10 μM, there was no inhibition of binding of [^{3}H]PDBu with phorbol or 4-α-PDD, but 80% inhibition of binding was seen with TPA at 1 μM and 65% inhibition at 50 nM. With mezerein, these values were 70% and 60%, respectively. At 1 nM, there was negligible inhibition of binding by any of the phorbol esters. The specific concentrations of TPA and mezerein giving 50% inhibition of specific binding of [^{3}H]PDBu were 15 and 20 nM, respectively.

TPA and mezerein are potent stimulators of MER-switch, whereas phorbol and 4-α-PDD are inactive (6). When CLL cells were cultured for 25 min under identical conditions to the binding assay, 50% loss of MER was induced by a TPA concentration of 3 nM.

Stimulation of MER-switch by [^{3}H]PDBu: dependence on concentration. CLL cells were cultured with [^{3}H]PDBu (0 to 350 nM) under the same conditions as used in binding assays. After 25 min at 350 nM, however, only 10% of the cells had lost capacity to bind mouse erythrocytes. After 120 min, more than 95% of cells had lost MER. Controls lacking [^{3}H]PDBu showed 30% loss of MER due to spontaneous MER-switch which occurs in serum-free culture (8). There is therefore a lag phase between optimal binding of [^{3}H]PDBu and effect on MER. To determine the relationship between concentration of [^{3}H]PDBu and effect on MER, rosette inhibition (corrected for spontaneous loss of MER) was plotted against concentration of [^{3}H]PDBu. In a 120-min culture, there was a maximal response of 95% inhibition. EC_{50} was 4 nM. The shapes of the binding and inhibition curves were similar over the range of concentrations of [^{3}H]PDBu (Fig. 2).

Relationship of MER switch to time of incubation with [^{3}H]PDBu. The effect of longer culture times on stimulation by [^{3}H]PDBu was determined in a medium containing 15% FCS. EC_{50} (at 120 min) was 20 nM as compared with 4 nM in PBS/ALB, suggesting that a component of the medium or FCS inhibited MER-switch. Figure 3 shows MER loss curves over a range of [^{3}H]PDBu concentrations up to 400 nM, reading the end point at 1, 4, and 22 hr. At the highest concentration (400 nM), rosette inhibition occurred in 55% of the cells during the first hour of culture; by 2 hr, 86% of the cells had lost MER (data not shown), and rosetting was completely inhibited by 4 hr. EC_{50} decreased with time.

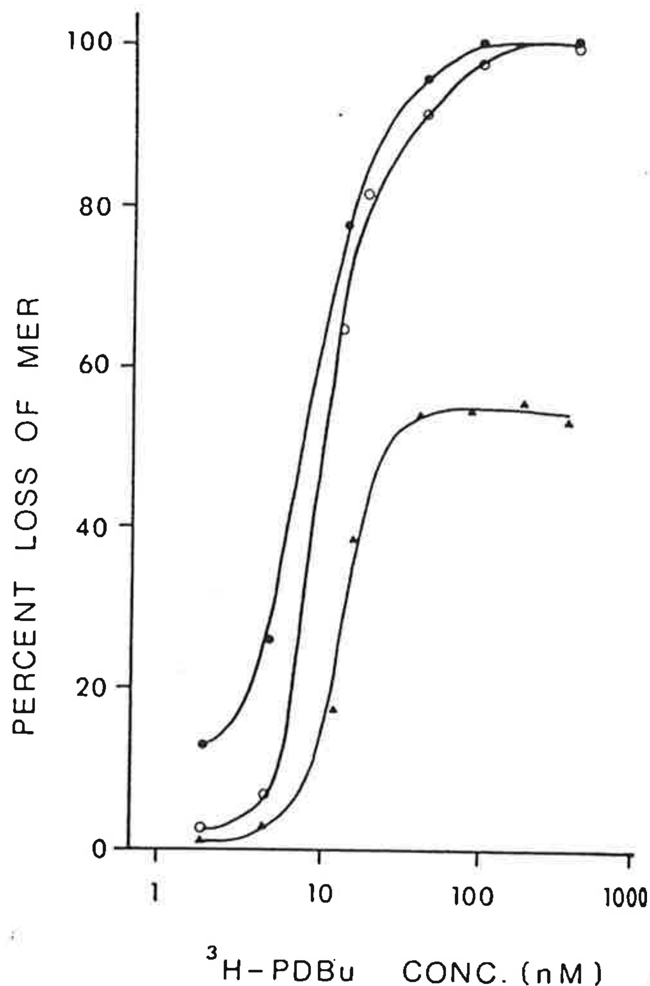

Figure 3. Kinetics of stimulation of MER-switch in CLL cells by [^{3}H]PDBu. Cells were stimulated in culture medium containing FCS at 1 (▲), 4 (○), and 22 (●) hr of culture. MER-switch is expressed as percent of cells that have lost capacity to rosette with mouse erythrocytes.

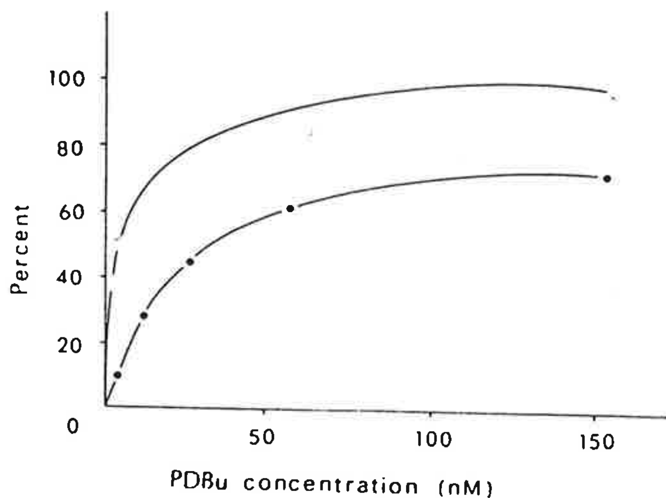

Figure 2. Comparison of percent of cells inhibited in rosetting (○) and average percent receptor occupancy (●) at different concentrations of [^{3}H]PDBu. Percent receptor occupancy is determined by the fraction B/B_t where B_t is the total binding capacity at receptor saturation (0.39 pmol per 10^6 cells from Fig. 1).

Dependence on continuing presence of PDBu. Cells released bound [^{3}H]PDBu at 37°C in medium free of [^{3}H]PDBu (see above). When cells were exposed to 80 nM [^{3}H]PDBu for 2 hr at 37°C in medium containing FCS, rosetting was inhibited by 70%. After washing and culture overnight, those incubated overnight without [^{3}H]PDBu were inhibited 73% (21% rosetting), while those incubated in the presence of [^{3}H]PDBu (80 nM) were inhibited 100%. Thus, removal of PDBu stopped further modulation but did not lead to reversal of rosette inhibition.

Metabolism of [^{3}H]PDBu by lymphocytes. CLL cells were cultured with [^{3}H]PDBu at 100 nM (in the presence of serum) for 4 hr at 37°C. Aliquots of the cultures were run on TLC (see *Materials and Methods*) and gel slices were counted for radioactivity. In control incubation (no cells), there was a major component R_f 0.64 (78% counts), while extract of [^{3}H]PDBu incubated with cells had 66% of counts in this region. Stock [^{3}H]PDBu used in these experiments had 100% of counts at this R_f. Both control and cell-incubated [^{3}H]PDBu had significant peaks of radioactivity at the origin, suggesting binding of [^{3}H]PDBu to solvent-insoluble components (e.g., serum proteins). No significant metabolism of PDBu was seen.

Binding studies with soluble MER. MER prepared by Nonidet P-40 detergent extraction failed to bind [^{3}H]PDBu

(50 nM), whereas crude protein kinase C bound 0.1 pmol. This protein kinase catalyzed the incorporation of 693 cpm of ^{32}P into histone over 10 min as compared with a blank of 140 cpm.

Effect of MER-switch on receptor activity. After 30 min exposure to TPA, when rosetting was inhibited completely, trypsin and Nonidet P-40 supernatants of 10^9 cells had the same mouse erythrocyte hemagglutinating titer (2^6 and 2^8, respectively) as those prepared from untreated cells. No hemagglutinin was detected in the supernatant of cells during the TPA treatment. Thus, MER-switch is not due to a physical loss or destruction of the receptor.

Effect of lymphokines on MER-switch. CLL cells containing 83% rosette-forming cells were cultured in medium 199 (containing 15% FCS) supplemented with 50% PHA-supernatant (see *Materials and Methods*). Rosetting decreased to 6% after 20 hr at 37°C, corresponding to 93% inhibition of rosetting (Table I). Cultures in the presence of PHA-control, cell-control, or medium-FCS contained 49 and 47% rosette-forming cells, respectively. Viability of cells was unaffected by incubation in the presence of PHA-supernatant. Significant stimulation of MER-switch was seen at 5 and 10% (v/v) concentration of PHA-super, but not at 1%. Dialyzed, ultrafiltered PHA-super stimulated MER-switch as well.

DISCUSSION

MER-switch, the irreversible loss of mouse erythrocyte rosette-forming activity in human B cells, is an obligatory B cell development which is retarded in CLL and potently stimulated *in vitro* by phorbol esters (6, 7). It is therefore relevant to B cell maturation, maturation arrest in leukemia, and tumor promotion by phorbol esters.

The MER-switch can be viewed as a disturbance of a state of equilibrium involving numerous factors, representing an excellent model of a step in B cell maturation, requiring the correct interplay of specific and nonspecific factors. In addition to the tumor-promoting phorbol esters, MER-switch is induced by contact with mouse erythrocytes and proteases (unpublished observations), incubation in plasma-free media (8), and, as shown in this paper, by a factor or factors in supernatants of mitogen-stimulated spleen cells.

TABLE I
Stimulation of rosette inhibition in CLL cells by PHA supernatant

Culture Supplement	RFC[a]	% Rosette Inhibition[b]
No culture	83	0
—	47	43
50% Cells-control	47	43
50% PHA-control	49	41
50% PHA-super	6	93
25% PHA-control	49	41
25% PHA-super	9	89
15% PHA-control	46	45
15% PHA-super	15	82
10% PHA-control	46	45
10% PHA-super	24	71
5% PHA-control	47	43
5% PHA-super	33	60
1% PHA-control	48	42
1% PHA-super	49	41
Dialyzed, ultrafiltered PHA-control[c]	46	45
Dialyzed, ultrafiltered PHA-super[c]	18	78

[a] Cells were cultured at 2 × 10^6/ml for 20 hr in culture medium supplemented with varying concentrations (1 to 50% v/v) of PHA-super, PHA-control, or cell-control (see *Materials and Methods*).
[b] % Rosette inhibition is expressed relative to control 83% rosetting. 43% rosette inhibition represents spontaneous MER-switch.
[c] PHA-super or PHA-control were dialyzed overnight against PBS and concentrated by ultrafiltration (PM10 membrane). Final concentration in cultures corresponded to 25% supernatant.

Tumor-promoting phorbol esters bind to leukemic human B cells at specific sites, as shown by the rapidity, reversibility, and saturability of binding and its inhibition by excess of unlabeled active phorbol ester. The quantity and affinity of phorbol receptors was comparable with that seen in other cell types (10). The curvilinear Scatchard plot with upward concavity indicates binding site heterogeneity, which may be interpreted (13) as two or more types of binding sites of different affinity and capacity, negative cooperativity between binding sites, or heterogeneity of binding sites among the different cells in the population tested.

The evidence indicates that MER-switch is directly induced by tumor promoting phorbol esters. The similarity of the shapes of the MER loss curves to phorbol ester binding curves (Fig. 2) suggests that the proportion of cells undergoing MER-switch is related to the quantity of bound phorbol ester. Active tumor-promoting compounds, which induce MER-switch (TPA, mezerein), displaced bound [^{3}H]PDBu, whereas nonpromoting phorbols neither induced MER-switch nor displaced bound [^{3}H] PDBu.

The kinetics of MER-switch (Fig. 3) suggest a time-dependent process with an initial lag period. Thus, at 20 min, when binding of [^{3}H]PDBu was maximal, MER-switch had occurred in less than 10% of the cells. The subsequent rate of MER-switch was dependent on the concentration of PDBu. With the most potent ester, TPA, a lag period was not detected at optimal concentrations, MER-switch occurring in more than 95% of the cells within minutes (6). The evidence suggests that a rate-limiting step distal to binding is essential in the mechanism. This step may be phosphorylation of the protein component of MER, because the phorbol ester receptor of rat brain co-purifies with a protein kinase C, which can be activated by TPA in the presence of phospholipid (11). TPA induced phosphorylation of a variety of cellular proteins, including receptors for insulin and somatomedin C (14).

No way has been found to reverse the MER-switch, including removal of most of the bound phorbol ester and further culture. It is concluded that once this rate-limiting step has occurred, the membrane modulation is irreversible. Most other cellular responses to phorbol esters have been reported to be reversible (15).

Trypsinization or detergent extraction of CLL cells release a factor that agglutinates erythrocytes from mice and rats but not from other species, and that is the soluble form of MER. The hemagglutinating activity is due to phosphatidylethanolamine. Protein complexed with the lipid appears to serve as the anchor for phosphatidylethanolamine in the B cell membrane (2). Equal quantities of MER were obtained from B lymphocytes before and after TPA-induced modulation, indicating that the receptor is not lost during MER-switch, but is rendered inactive within the membrane. The difference in lectin-agglutinability between transformed and non-transformed cells may be analogous (16). A second analogy may be the binding of cells to a ligand-coated sub-

strate in which two processes were shown to be involved—specific binding (sticking) and cross-linking of glycoproteins and cytoskeleton (gripping) (17).

A supernatant of PHA-stimulated human spleen cells, known to be rich in T cell- and monocyte-derived interleukins (18), stimulated MER-switch in an overnight culture of CLL cells, suggesting that interleukins may be natural inducers of MER-switch.

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CALCIUM IONOPHORE A23187 PRIMES HUMAN B-CELLS FOR ACTIVATION BY PHORBOL DIBUTYRATE BY CONVERTING RECEPTORS FOR PHORBOL DIBUTYRATE FROM A LOW TO HIGH AFFINITY STATE

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SUMMARY: The calcium ionophore A23187 synergised with phorbol dibutyrate-induced activation of human chronic lymphatic leukaemia B-cells, as assessed by modulation of the membrane receptor for mouse erythrocytes. Thus A23187 (1uM), which alone had no effect on expression of the receptor for mouse erythrocytes, reduced the EC50 and shortened the lag period for modulation of this receptor by phorbol dibutyrate. This action of A23187 was shown to be due to enhanced binding of [^{3}H]phorbol dibutyrate to its receptor (phospholipid/Ca^{++} dependedent protein kinase C) whose affinity was altered from a predominantly low affinity state (Kd 83nM) to high affinity (Kd 9nM). A23187 had no effect on the total number of phorbol dibutyrate receptors. EDTA abolished these actions of A23187.
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INTRODUCTION: Tumour promoting phorbol esters are potent cell activating agents which may induce differentiation or proliferation depending on the target cell [1]. The cellular receptor for phorbol esters is now known to be the phospholipid/Ca^{++} dependent protein kinase (protein kinase C),(PKC) [2]; recent evidence suggests that phorbol ester binding to PKC is heavily dependent on the presence of phospholipid [2] and that translocation and binding of cytosolic PKC to the plasma membrane may therefore be an important step in phorbol ester or diacyl glycerol-induced activation of PKC [3].

Phorbol esters induce plasmacytoid differentiation in immature chronic lymphatic leukaemia (CLL) B-cells and in their normal immature B-cell counterparts, both of which express a receptor for mouse erythrocytes (MER)

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ABBREVIATIONS: CLL - chronic lymphatic leukaemia; MER - mouse erythrocyte receptor; PDBu - phorbol dibutyrate; PKC - protein kinase C.

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[4,5]. One of the earliest activation events, occurring within minutes under optimal conditions, is the down modulation of MER [6]. Although the function of the MER and the physiological significance of this modulation is unclear it is none the less a useful model for studying early signals involved in B-cell activation.

Several recent studies have shown that calcium ionophores act synergistically with phorbol esters in a variety of physiological cell responses including B-cell proliferation [7]. One suggested mechanism for this synergism is that a rise in cytosol Ca^{++} promotes translocation and binding of PKC to the cell membrane, thereby increasing availability of phospholipid cofactor and thus the affinity of binding of PKC for phorbol ester [3,8]. We have investigated this hypothesis further by examining the effect of calcium ionophore A23187 on phorbol dibutyrate (PDBu)-induced modulation of MER and on the binding of PDBu in CLL B-cells.

MATERIALS AND METHODS: CLL cells were prepared from heparinised peripheral blood or from leukophoresis samples by Ficoll/Hypaque density centrifugation [9]. In some experiments, cells which had been stored in liquid nitrogen were used. Cells were washed in phosphate-buffered saline and resuspended in RPMI 1640 containing glutamine, HEPES, sodium bicarbonate (pH 7.2) and bovine albumin (1mg/ml) (Sigma). Viability was monitored by phase contrast microscopy and trypan blue exclusion. MER was assayed as %CLL cells rosetting with mouse red cells as previously described [5]. Cells were pretreated with A23187 (Sigma), or methanol solvent (0.1%), at indicated concentrations. After 20 minutes at 37°C, aliquots of each were incubated with PDBu (0-200nM) (Sigma). After a further period of incubation, as indicated, cells were washed and rosetted with mouse red cells. Results are expressed as % inhibition of rosetting (mean of duplicates). Assay of [^{3}H]PDBu-binding (in triplicate) was performed as previously described [10]. Non-specific binding was assayed in the presence of a 50 fold molar excess of unlabelled phorbol ester.

RESULTS AND DISCUSSION: Pretreatment with A23187, at concentrations between 0.2 and 2uM, augmented the modulating effect of PDBu on MER-rosetting by CLL cells (Fig 1). A23187 alone neither modulated MER nor affected cell viability (data not shown). Synergistic effects of A23187 were most profound at low concentrations of PDBu (<50nM), which have little or no effect on MER rosetting. The concentrations of PDBu which inhibit rosetting by 50% (EC50) was reduced from 165nM in the absence of A23187 to 25nM in the presence of 2uM A23187. At high concentrations of PDBu (>200nM) A23187 had no synergistic effect.

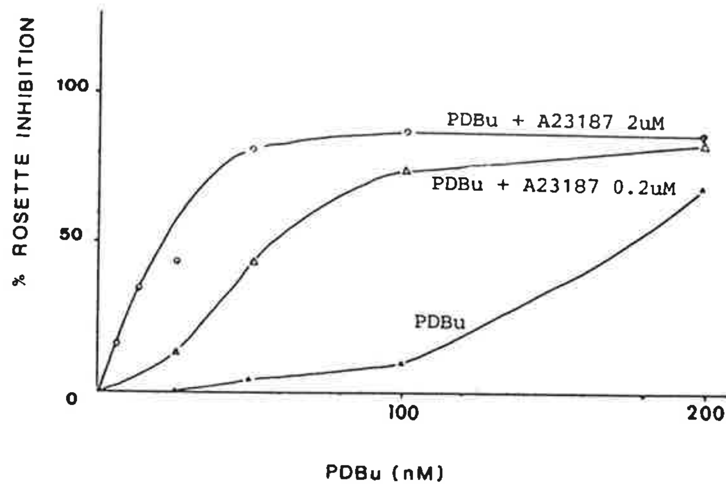

Fig 1: Modulation of MER - synergism between A23187 and PDBu:
CLL cells were preincubated with A23187 at 0, 0.2 or 2uM for 20 minutes before addition of varying concentrations of PDBu for a further 40 minutes. After washing, cells were rosetted with mouse red cells.

To determine whether A23187 promotes a rate-limiting step during cell activation by PDBu, the effect of A23187 on the lag phase for modulation of MER by PDBu was examined. At 100nM or less, PDBu does not induce significant modulation of MER in the first 20 minutes of culture, even though binding of [^{3}H]PDBu to cells is maximal at this time [10]. After pretreatment with A23187, the lag phase for modulation of MER with 50 or 100nM PDBU was significantly shortened (Table 1), confirming that A23187 catalyses a rate limiting step during cell activation. Augmentation of PDBu-induced modulation of MER by A23187 (Fig 2b) was paralleled by a similar effect on PDBu-binding (Fig 2a). Scatchard plots of [^{3}H]PDBu-binding to CLL cells were curvilinear (Fig 2c) and typical of those

Table 1
Effect of A23187 (1uM) on PDBu-induced modulation of MER

	% modulation of MER			
PDBu	50nM		100nM	
A23187	-	+	-	+
20'	0	20	0	50
40'	8	70	35	80
60'	20	75	40	90

CLL cells were pretreated with or without A23187 (1 uM) for 20 minutes before addition of PDBu (50 or 100nM). Cells were harvested at 20, 40 or 60 minutes for assay of MER rosetting.

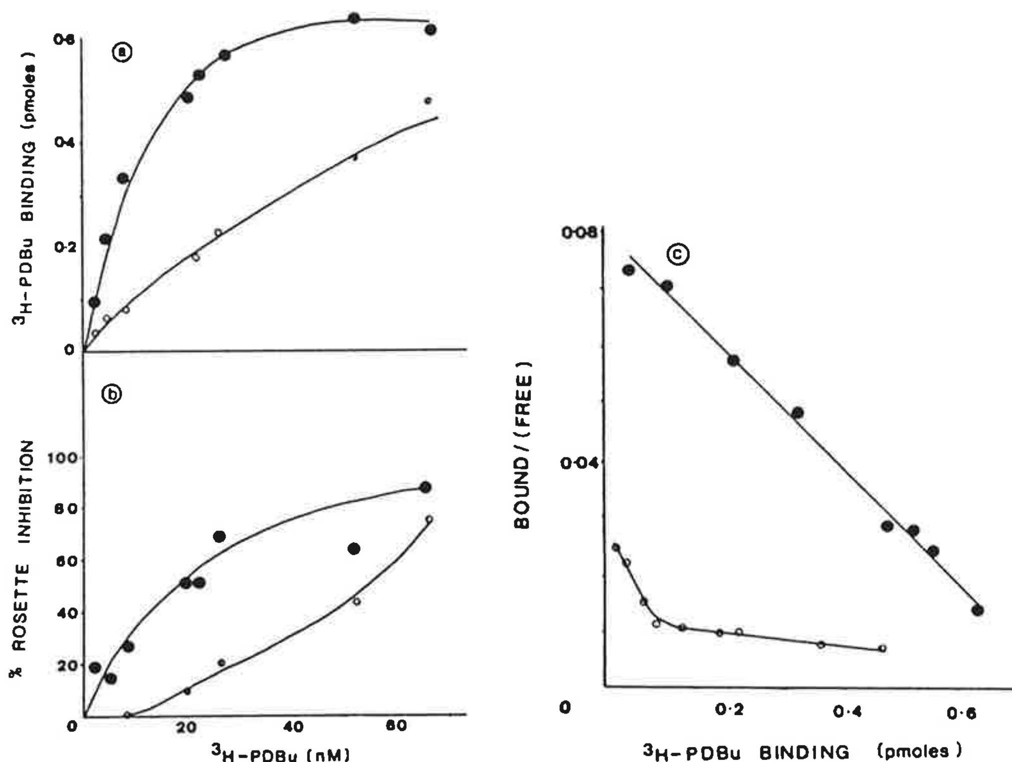

Fig 2: Effect of A23187 on PDBu binding:
CLL cells were pretreated with (●) or without (○) A23187 (1uM) for 20 minutes before addition of varying concentrations of [^{3}H]PDBu. Cells were harvested (a) after 25 minutes for binding studies and (b) after 60 minutes for rosette inhibition studies. Scatchard plots of PDBu binding in the presence or absence of A23187 are shown in (c).

we have reported previously [10]. These plots have been interpreted as showing two classes of receptor differing in affinity [10]. With the cells used in this study the Kds' of these populations are approximately 5 and 83nM, with the majority of receptors being of low affinity. In A23187 treated cells, the plot became linear showing a single class of high affinity receptors (Kd 9nM) (Fig 2c). Thus A23187 appears to convert low affinity PDBu receptors to high affinity. Similar results have been reported for HL60 cells [3] and are consistent with the hypothesis that a rise in cytosol Ca^{++} converts low affinity receptors (represented by cytoplasmic PKC, free of phospholipid) to high affinity receptors (represented by plasma membrane bound PKC).

To confirm the Ca^{++}-dependence of the effect of A23187, CLL cells were studied in the presence of EDTA. EDTA completely prevented the synergistic

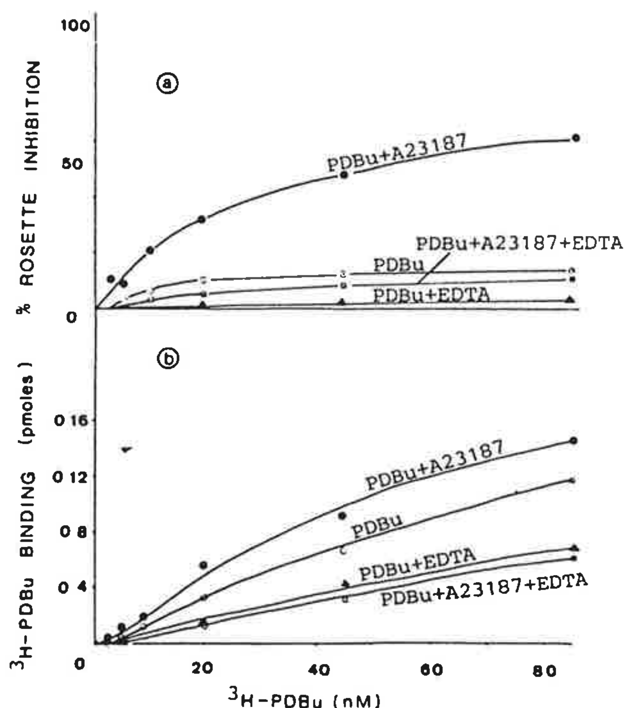

Fig 3: EDTA inhibits the effect of A23187 on modulation of MER and PDBu-binding: CLL cells were pretreated with or without EDTA (10mM) for 10 minutes, then with or without A23187 (1uM) for 20 minutes and finally with ^{3}H-PDBu at varying concentrations up to 85nM. Cells were harvested after 45 minutes for rosetting studies (Fig 3a) or after 25 minutes for PDBu binding studies (Fig 3b).

action of A23187 on PDBU-induced modulation of MER (Fig 3a), and the enhancing effect of A23187 on [^{3}H]PDBu-binding (Fig 3b), thus confirming a requirement for Ca^{++}. EDTA also partially inhibited the modulation of MER by PDBu alone suggesting that even in the absence of calcium ionophore, there may be a small contribution by extracellular Ca^{++} to the activation process. However, EDTA did not block the effect of an optimal dose (100nM) of the more biologically active, and highly lipophilic, phorbol 12,13 myristate acetate (data not shown). Thus the requirement for Ca^{++} in the activation process may depend to some extent on the relative potency of the phorbol ester.

In conclusion, these data provide further evidence for synergism between A23187 and phorbol esters during B-cell activation and demonstrate that the mechanism of this synergism is a Ca^{++}-dependent increase in binding affinity of receptors for phorbol ester. These findings are consistent with the recently

proposed hypothesis that Ca^{++} promotes translocation and binding of cytosolic PKC to the plasma membrane [3,8].

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AURANOFIN INCREASES THE AFFINITY OF PHORBOL DIBUTYRATE RECEPTORS IN CHRONIC LYMPHOCYTIC LEUKEMIA CELLS (B CELLS)

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Previous studies have shown that auranofin (AF), a lipophilic gold I complex, modulates metabolic events in leukocytes stimulated by phorbol esters, whose major cellular binding site is now known to be the Ca++/phospholipid-dependent protein kinase (protein kinase C). In these experiments we have investigated the effect of AF on the binding of phorbol dibutyrate (PDBu) to human chronic lymphocytic leukemia (CLL) B cells.

AF enhanced binding of PDBu to its receptor in CLL cells by a) causing an increase in the affinity of PDBu receptors from K_d 20.3 nM to 7.3 nM, and b) enhancing translocation of PDBu receptors to the cell membrane. The increase in PDBu binding induced by AF in whole cells was only partially reversible by EGTA or the intracellular Ca++ antagonist TMB-8. Studies performed with quin-2-labeled cells showed that 100 μM AF caused a mean (±SD) rise in cytosolic Ca++ levels from 0.41 (0.12) to 0.85 (0.33) (n = 5). Thus the mechanism by which AF increases binding of PDBu to its receptor appears to be partially dependent on Ca++.

These effects of AF occurred at cellular levels achieved in mononuclear cells during chrysotherapy of patients with rheumatoid arthritis.

Auranofin (AF)[2] ([2,3,4,6-tetra-O-acetyl-1-thioglucopyranosato-S] [triethylphosphine] gold) is a lipophilic anti-rheumatic gold I complex now widely used in the treatment of rheumatoid arthritis. It has a number of effects in vitro on activation responses in various cell types stimulated by phorbol esters (1) and agonists that stimulate hydrolysis of phosphatidyl inositol-4,5-biphosphate (PIP_2) (1, 2). Recent evidence suggests that the Ca++/phospholipid-dependent protein kinase (protein kinase C [PKC]) is a key binding site for the tumor-promoting phorbol esters, and that many of the effects of phorbol esters are mediated by activation of PKC (3). The physiological activator of PKC is diacylglycerol, which is released after phosphoinositidase C-dependent hydrolysis of PIP_2 in response to some surface receptor-dependent agonists (2). As PKC is therefore one possible site of action of this drug, we have been exploring the effects of AF on biological events mediated by phorbol esters in different systems. In these studies we have used human chronic lymphocytic leukemia (CLL) cells (B cells) as a model system for studying the effect of AF on phorbol ester binding to its receptor (PKC).

MATERIALS AND METHODS

Materials. AF (a gift from Dr. M. Whitehouse, Department of Pathology, University of Adelaide), 12-O-tetradecanoyl phorbol-13-acetate (TPA), and phorbol dibutyrate (PDBu) (Sigma), quin-2 acetoxymethyl ester (a gift from Dr. G. Barritt, Department of Clinical Biochemistry, Flinders Medical Centre, South Australia), and ionomycin (Sigma) were stored at −20°C in dimethyl sulfoxide (DMSO). ^{3}H-PDBu (New England Nuclear; 6.5 Ci/mmol) was diluted in ethanol ×5 and was stored at −20°C. A23187 (Sigma) was stored at −20°C in ethanol. Sodium aurothiomalate (ATM) (aqueous solution) was purchased from May and Baker.

Cell culture. CLL cells from two patients were removed by leukaphoresis and were stored in liquid nitrogen. After thawing, cells were centrifuged on Ficoll-Hypaque to obtain mononuclear cells. Cell viability was assayed by exclusion of trypan blue (0.3%). In all experiments, except for studies with quin-2, cells were cultured in plastic tubes, with or without AF or other agents, in HEPES-buffered RPMI 1640 (Flow Laboratories) supplemented with glutamine, sodium bicarbonate, gentamicin, and 10% fetal calf serum (FCS) (Flow Laboratories).

^{3}H-PDBu-binding assay. Samples (10 μl) of ^{3}H-PDBu (5×10^{-6} M) were evaporated under a stream of nitrogen and were redissolved in 50 μl of DMSO; RPMI + 10% FCS was added to give the desired final concentration. One hundred microliters of each dilution were added to wells containing 100 μl of cells (10^7/ml) in RPMI 1640/10% FCS. Replicate wells containing a 50-fold excess of TPA in addition to ^{3}H-PDBu were set up for each concentration of ^{3}H-PDBu to determine nonspecific binding. In all harvests, the filters were prewashed with a 10^{-6} M solution of TPA in PBS. Exact concentrations of ^{3}H-PDBu in each well were determined by transferring 20-μl samples from selected wells to filter papers before harvesting. Remaining cell suspensions were harvested directly onto glass fiber filters by using a Titertek cell harvester, with two cycles of washing with PBS. Filters were dried thoroughly and were added to scintillation vials for counting. Specific binding equals total binding minus binding in the presence of a 50-fold molar excess of TPA.

Cell fractionation and assay of PDBu-binding to cell membrane fraction. B-CLL cells (10^7/ml) in RPMI/10% FCS were incubated in the presence or absence of various agents as indicated for 30 min at 37°C. Cells were washed and resuspended to 2×10^7/ml in 25 mM Tris-HCl, pH 7.4, with or without EDTA (1 mM) as indicated. Cells were then sonicated (five 5-sec bursts) in a Heat Systems Ultrasonicator fitted with a microtip. The particulate fraction was pelleted by centrifugation at 25,000 × G for 30 min, was resuspended in the same volume of PBS, and was sonicated as before. Samples (100 μl) were put into wells of a flat-bottomed microtiter tray (Linbro), and triplicates were incubated with 100 μl of ^{3}H-PDBu (10 nM in PBS + 1 mg/ml bovine serum albumin) alone or with the further addition of TPA (500 nM). After 25 min, bound ^{3}H-PDBu was separated by using a cell harvester.

Measurement of intracellular free Ca++. Intracellular Ca++ was measured with minor modifications as described (4). CLL cells (10^6/

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[1] Correspondence to Dr. N. P. Hurst, The Queen Elizabeth Hospital, Woodville, South Australia, 5011.
[2] Abbreviations used in this paper: AF, auranofin; PKC, protein kinase C; PMN, neutrophil polymorph; PBL, peripheral blood leukocyte; TPA, 12-O-tetradecanoyl phorbol-13-acetate; PDBu, phorbol dibutyrate; ATM, sodium aurothiomalate; CLL, chronic lymphocytic leukemia cell; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(dimethylamino) octyl ester; quin-2, quin-2 acetoxymethyl ester; EGTA, ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid; DMSO, dimethyl sulfoxide.

ml) in RPMI/10% FCS were incubated with quin-2 acetoxymethyl ester (quin-2) (final 62.5 μM) or solvent (DMSO 0.25%) for 20 min at 37°C and then were diluted to 10^7/ml with RPMI/10% FCS and were incubated for a further 40 min at 37°C. Cells were washed twice and resuspended to 10^7/ml in salt solution (145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 0.5 mM Mg_2SO_4, 5 mM glucose, 1 mM $CaCl_2$, 100 μM diethyl triamine penta-acetic acid (to chelate heavy metals), 10 mM HEPES, pH 7.4). It was not possible to perform these experiments under conditions identical to those of other experiments because the use of either protein in the medium or plastic cuvettes interfered with the signal. Fluorescence was measured at 37°C on an Aminco-Bowman spectrofluorimeter with stirring facilities. Reagents were added directly to the cuvette via a syringe.

Excitation was elicited at 340 nM and emission was monitored at 490 nM. Intracellular free Ca^{++} was determined from the equation: $[Ca] = K_d(F - F_{min})/(F_{max} - F)$, where K_d = 115 nM, F = fluorescence of sample, Fmax = fluorescence of sample after lysis with 25 μM digitonin in presence of 1 mM Ca^{++}, and Fmin = fluorescence of sample after subsequent addition of 500 μM Mn^{++}. All readings were corrected for autofluorescence of unloaded cells.

Cellular uptake of AF. After incubation of CLL cells with various concentrations of AF for specified times, cells were washed twice with protein-free buffer. The centrifuged pellet was assayed for gold content by atomic absorption spectroscopy as described (5).

RESULTS

Effect of AF on PDBu receptor binding in CLL. Initial experiments were performed to determine whether AF altered binding of PDBu to its receptor. CLL cells were assayed for binding of ^{3}H-PDBu in the presence or absence of AF.

AF enhanced binding of PDBu (10 nM) to CLL cells in a concentration-dependent manner (Fig. 1*A*). This effect occurred whether AF was added at the same time or up to 40 min after addition of PDBu and was evident over a range of PDBu concentrations up to 60 nM (not shown). Results were similar in CLL cells from four separate patients. The enhancing effect of AF on binding of PDBu was partially reversed by EGTA[2] (2 mM) and was almost completely reversed by the intracellular Ca^{++} antagonist TMB-8[2] (250 μM) (Fig. 1*B*). Scatchard analysis showed that 100 μM AF converted PDBu receptors, in three separate CLL cell samples, from a single class of low affinity receptors with (mean ± SD) K_d 20.3 ± 2 nM to a single class of high affinity K_d 7.3 ± 2 nM (Fig. 1*C*). AF did not increase nonspecific binding of ^{3}H-PDBu to CLL cells in the presence of a 50-fold excess of unlabeled TPA (not shown). Consistent with its effect on affinity, AF also increased the initial rate of binding of PDBu (Fig. 1*D*). In contrast to the effect of calcium ionophore A23187 (unpublished observations) (not shown), there was no subsequent time-dependent loss of PDBu binding sites. However, if CLL cells were preincubated with AF *before* addition of PDBu, there was gradual loss of PDBu binding sites (for example, 30 to 40% inhibition of ^{3}H-PDBu binding after 90-min preincubation with 100 μM AF). The water-soluble anti-rheumatic gold I complex ATM (up to 2.5 mM) had no effect on binding of PDBu (not shown).

Effect of AF on PDBu binding in other cell types. To determine whether the effect of AF on PDBu binding was restricted to CLL cells, the action of AF on human erythrocytes (RBC), peripheral blood lymphocytes (PBL), and polymorphonuclear neutrophils (PMN) was studied. AF (100 μM) enhanced PDBu binding to PBL and PMN in the same manner as in CLL cells. PDBu did not bind to RBC either before or after treatment with AF (Fig. 2).

Effect of AF on translocation of PDBu receptors from cytosol to cell membrane. The Ca^{++}-dependent increase in affinity of the phorbol ester receptor in the presence of AF suggested that AF, like calcium ionophore (6, 7), might enhance translocation of PKC to the cell membrane. To investigate this possibility, CLL cells were incubated with either AF (100 μM), A23187 (1.0 μM), iono-

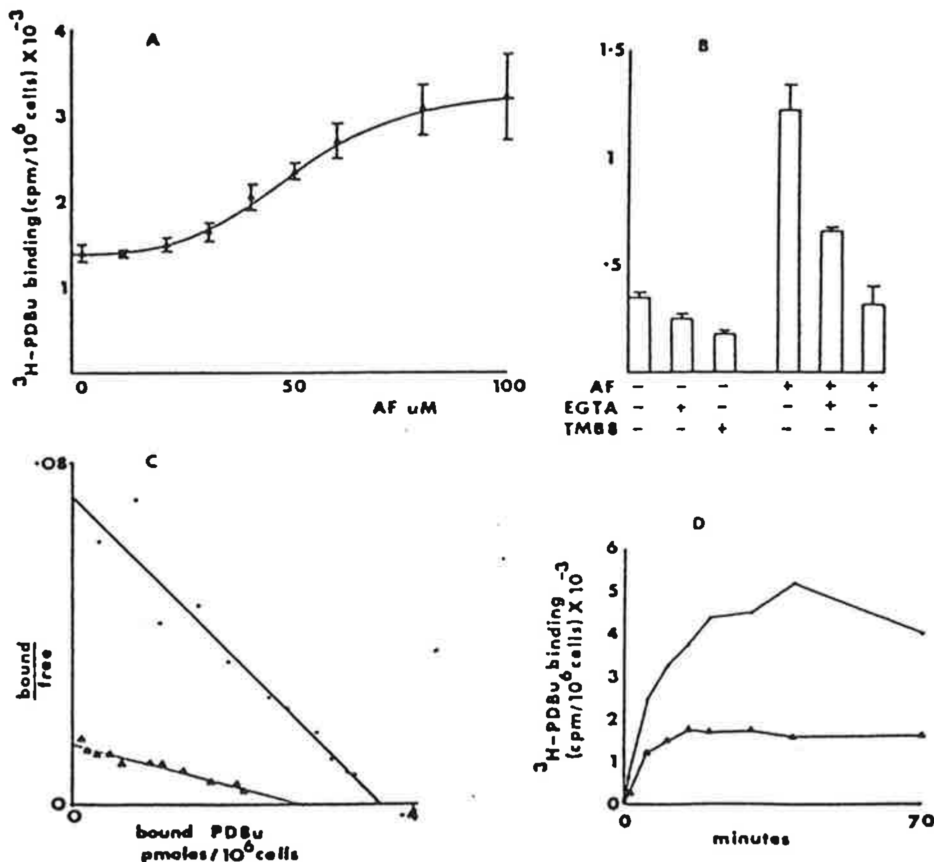

Figure 1. Effect of AF on ^{3}H-PDBu binding to CLL cells. (*A*) Concentration-dependence: CLL cells were incubated either with ^{3}H-PDBu (10 nM) alone or with the addition of AF (0 to 100 μM) for 30 min and then were assayed for bound ^{3}H-PDBu. AF enhanced PDBu binding in a concentration-dependent fashion. Each point represents mean ± SD of triplicates. (*B*) Calcium-dependence: The effect of AF (100 μM) on binding of ^{3}H-PDBu to CLL cells was examined in the presence of EGTA (2 mM) or the intracellular calcium antagonist TMB-8 (250 μM). EGTA and, to a greater extent, TMB-8 inhibited binding of ^{3}H-PDBu in the absence of AF, and similarly inhibited the enhancing effect of AF on ^{3}H-PDBu binding. Neither agent completely blocked the effect of AF. Each point represents mean ± SD of triplicates. (*C*) Scatchard analysis: CLL cells were incubated with (●) or without (▲) AF (100 μM) in the presence of varying concentrations of ^{3}H-PDBu (0 to 200 nM) for 30 min at 37°C, and then bound ^{3}H-PDBu was determined. In the presence of AF (100 μM) PDBu receptors were converted from a single class of low affinity (mean ± SD) (K_d 20.3 ± 2 nM) to a single class of high affinity (K_d 7.3 ± 2 nM) (n = 3). There was also a small increase in the apparent number of receptors. One typical set of data from three separate experiments is shown. (*D*) Kinetics: CLL cells were incubated with (●) or without (▲) 100 μM AF in the presence or absence of 10 nM ^{3}H-PDBu for varying times at 37°C before determination of bound ^{3}H-PDBu. AF caused an increase in the initial rate and extent of PDBu binding. Each point represents the mean of triplicate values (SD did not exceed 5%).

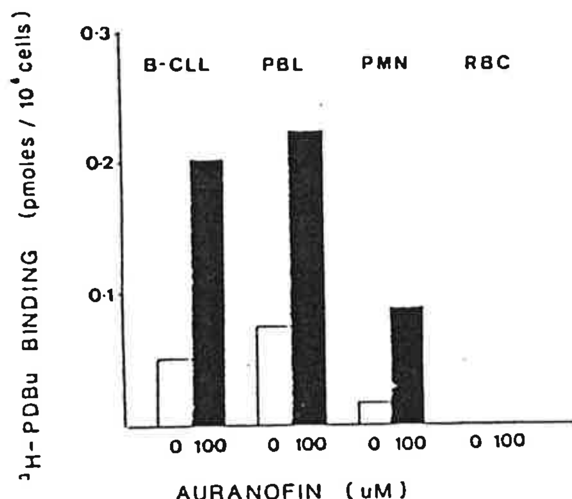

Figure 2. Effect of AF on binding of PDBu to CLL cells, normal PBL, PMN, and RBC. Enhancement by AF (100 μM) of PDBu-binding to PBL and PMN was similar to that seen with CLL cells. RBC did not bind PDBu either in the absence or presence of AF. Each measurement was performed in triplicate (SD did not exceed 5%).

TABLE I

Effect of AF on translocation and binding of phorbol ester receptors[a]

Cell Treatment	Membrane-Associated ^{3}H-PDBu (cpm ± SD) (n = 3)	
	+ EDTA	− EDTA
DMSO control	584 ± 17	972 ± 19
100 μM AF	537 ± 14	1476 ± 104
0.5 μM ionomycin	592 ± 45	1682 ± 67
1.0 μM A23187	584 ± 48	1957 ± 85
200 nM PDBu	788 ± 51	1558 ± 47

[a] CLL cells were treated for 30 min at 37°C with reagents as indicated, then were washed and sonicated in the presence or absence of EDTA (1 mM) as described in *Materials and Methods*. The particulate membrane fraction was resonicated in PBS and was assayed for ^{3}H-PDBu-binding in the absence of EDTA.

mycin (0.5 μM) or PDBu (200 nM). After 20 min the cells were sonicated, either in the presence or in the absence of EDTA, and the particulate membrane fraction was collected by centrifugation at 25,000 × G. In the presence of AF, ionomycin, or A23187, increased binding of ^{3}H-PDBu to the particulate membrane fraction was observed; this was abrogated if sonication was performed in the presence of EDTA (Table I). The increase in membrane binding after treatment with PDBu was only partially reversed in the presence of EDTA. Thus AF promotes Ca^{++}-dependent translocation of phorbol receptors to the cell membrane in a manner analogous to calcium ionophores.

Effect of AF on cytosolic calcium in CLL cells. The effect of AF on cytosolic Ca^{++} was examined by using quin-2-loaded cells. AF (100 μM) stimulated a greater than twofold rise in mean (±SD) cytosol free Ca^{++} from 0.41 (0.12) to 0.85 (0.33) μM (n = 5), compared with ionomycin, which stimulated at least a 10-fold rise (Fig. 3), and PDBu, which had no effect. Ionomycin induced a more rapid rise of cytosolic Ca^{++} concentration than AF.

Uptake of AF by CLL cells. Incubation of CLL cells for up to 90 min with 30 or 100 μM AF produced mean (±SD) intracellular gold levels of 0.46 (0.014) and 0.71 (0.18) μg/10^6 cells, respectively. These levels are comparable to those found in six patients undergoing chrysotherapy for rheumatoid arthritis (mean 0.278 μg/10^6 cells; range 0.1 to 0.79). These patients had received AF

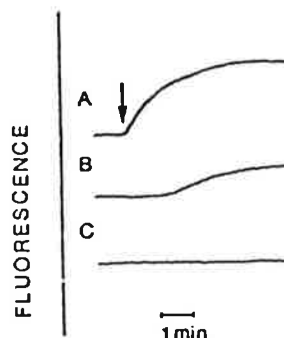

Figure 3. Effect of AF on intracellular free Ca^{++} in CLL cells. CLL cells were loaded with quin-2 as described in *Materials and Methods* and then treated (*arrow*) with A) ionomycin (0.5 μM); B) AF (100 μM); or C) solvent control (0.1% DMSO). A typical set of tracings of changes in cellular fluorescence are shown.

for between 1 and 6 mo.

Cell viability. Mean cell viability after incubation with AF (100 μM) for up to 90 min was 92% ± 1 (control 96% ± 1).

DISCUSSION

In previous studies researchers have reported modulating effects of AF on phorbol ester-stimulated cellular events; for example, high doses of AF (>10 μM) inhibit, whereas low doses (1 μM) enhance, TPA-induced superoxide anion production by monocytes (8) and PMN (1). We have also found that AF mimics and augments effects of TPA in other cell systems. For example, AF directly stimulates PKC-mediated phosphorylation of both 40-kilodalton (40-Kd) and 20-Kd proteins in human platelets (8). AF also inhibits epidermal growth factor receptor binding in HeLa cells, but this effect is apparently not mediated by PKC.[3] In contrast to its effect on events stimulated by phorbol esters, AF seems to have little or no effect on cellular responses to the Ca^{++} ionophore A23187, which activates Ca^{++}/calmodulin-dependent metabolic pathways (1).

AF, added before or after PDBu, enhanced binding of PDBu to CLL cells (EC50) (48 μM) in a dose-dependent fashion. AF also enhanced PDBu-binding to PBL and PMN. Lack of PDBu-binding to RBC, regardless of exposure to AF, was evidence against AF causing a nonspecific increase in binding of PDBu secondary to membrane perturbation or damage. Scatchard analysis showed that increased binding of PDBu was due mainly to an increase in affinity of the phorbol ester receptor rather than to an increase in the number of binding sites. Consistent with this effect on affinity, AF also increased the rate of binding of a nonsaturating dose of PDBu. Enhancement of PDBu binding is at least partially dependent on Ca^{++}, since both EGTA and, to a greater extent, the intracellular Ca^{++} antagonist TMB-8 (9) inhibited the effect of AF on PDBu-binding. The failure of the Ca^{++} antagonists EGTA and TMB-8 to reverse completely the effect of AF on binding of PDBu to whole cells suggests that AF may produce alterations in phorbol receptor binding and translocation by at least two mechanisms: one Ca^{++}-dependent, the other independent of Ca^{++}.

In contrast to the effect of adding AF with or after

[3] Froscio, M., N. P. Hurst, and A. W. Murray. 1986. Inhibition of epidermal growth factor binding to HeLa cells by auranofin. *Submitted for publication.*

PDBu, preincubation of CLL cells for up to 90 min with AF resulted in progressive loss of receptor binding. A similar effect is seen after treatment of cells with A23187. This may be attributable to Ca^{++}-dependent proteolysis of PKC (10) and is currently being investigated.

Recent studies have shown that Ca^{++} ionophores act synergistically with phorbol esters in a variety of physiological cell responses, including B cell proliferation (11). One suggested mechanism for this synergism is that a rise in cytosol Ca^{++} promotes translocation and binding of PKC to the cell membrane, increasing availability of phospholipid cofactor, and thus the affinity of binding of PKC for phorbol ester (12). We have recently confirmed that A23187 increases the affinity of binding of PDBu in human B cells (6). The Ca^{++}-dependent increase in affinity of PDBu binding in the presence of AF suggested that AF enhances translocation of PKC, the cellular receptor for phorbol esters, to the cell membrane. Subcellular fractionation of cells, after treatment with AF, A23187, or ionomycin, confirmed that AF also increases translocation and binding of phorbol receptors to the membrane fraction in a manner comparable to that of Ca^{++} ionophores.

To investigate whether translocation of cytosolic phorbol receptors in the presence of AF was associated with a change in cytosolic free Ca^{++} levels, the effect of AF on intracellular free Ca^{++} levels were investigated by using CLL cells loaded with quin-2. AF (100 μM) caused a twofold rise in cytosol free Ca^{++}. In contrast to ionomycin, the effect of AF was slow in onset. Ionomycin (0.5 μM) produced effects on phorbol receptor binding and translocation similar to those produced by 100 μM AF, but caused a rise in free Ca^{++} five times greater than 100 μM AF. Lower doses of ionophore, although still producing greater effects than AF on cytosol Ca^{++} under comparable conditions, have less effect on translocation of phorbol receptors than 100 μM AF (not shown). Because plastic and protein interfere with the fluorescent signal, the quin-2 assay was performed in glass cuvettes in the absence of protein. Thus it is not possible to make a direct comparison between these data and effect of AF on PDBu-binding. Furthermore, because AF is strongly protein bound it is likely that the quin-2 assay overestimates the effect of AF on cytosolic Ca^{++}. Nonetheless, these data are consistent with our observations made during experiments with calcium antagonists. The mechanism by which AF causes a rise in Ca^{++} is not clear, but one possibility is via an effect on membrane ion pumps. This is supported by recent studies showing selective effects on membrane Na^+/K^+-ATPase activity in enterocytes (13), and reduction of phagocyte resting membrane potential similar to that produced by A23187 (1).

The effect of AF on binding of PDBu was not shared by ATM, another anti-rheumatic gold (I) complex, which, in contrast to AF, is water soluble and oligomeric (14). In other more recent studies (not shown) we have found that chloro-triethylphosphine gold (CTG), another lipid soluble gold I complex in which a chloro ligand is substituted for the thioglucose ligand in AF, also enhances PDBu-binding. Thus it seems likely that lipid solubility and the presence of the triethyl phosphine group are important determinants of the cellular effects of these gold I complexes. CTG was much more cytotoxic than AF and caused loss of cell viability after short incubations. Loss of viability due to CTG, or prolonged treatment with high doses of AF, was associated with loss, rather than enhancement of PDBu-binding.

An important question is whether the conditions we have used, and the effects of AF we have observed, are relevant to its anti-rheumatic actions. Our data on cellular uptake of AF show that in vitro incubation of cells with 30 or 100 μM AF for up to 90 min gives cellular levels equivalent to those found in mononuclear cells from patients receiving AF therapy. Under these conditions, marked effects were seen on phorbol ester binding and translocation of phorbol receptors, suggesting that our in vitro findings may be relevant to conditions present in vivo. The time dependence of the various effects of AF we have observed presumably reflects the time required for AF to partition into or across the cell membrane, and because AF is strongly protein bound, uptake is markedly delayed by the presence of serum (1, 15): thus at higher doses the effect of AF was achieved much more rapidly.

In conclusion, AF has important effects on CLL cells in vitro that shed further light on the mechanism of action of AF. These include an increase in affinity of PDBu receptors and stimulation of PDBu receptor translocation to the cell membrane. Effects on PDBu receptors were partially Ca^{++}-dependent and may be mediated via a rise in cytosol Ca^{++}.

Acknowledgment. We gratefully acknowledge the help and advice given by Dr. G. Barritt (Flinders Medical Centre, S.A.) on intracellular calcium studies.

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Calcium ionophore A23187 enhances human neutrophil superoxide release, stimulated by phorbol dibutyrate, by converting phorbol ester receptors from a low- to high-affinity state

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The calcium ionophore A23187 acted synergistically with phorbol dibutyrate (PDBu) to stimulate human neutrophil superoxide production. A23187 shortened the lag period and markedly increased the initial rate of neutrophil superoxide production induced by suboptimal concentrations of PDBu. 1 μM A23187 reduced the EC_{50} value for superoxide release from 56 to 8 nM PDBu. This effect of A23187 was correlated with enhanced binding of [^{3}H]PDBu to its receptor and a reduction in the dissociation constant (K_d) from 27 to 10 nM, without altering the apparent total number of phorbol dibutyrate receptors. These actions of A23187 were abolished in the presence of EGTA or TMB-8, confirming a dependence on Ca^{2+}.

Ca^{2+}; Ionophore A23187; Phorbol ester; Protein kinase C; Neutrophil

1. INTRODUCTION

Tumour-promoting phorbol esters are potent stimulants of neutrophil (PMN) superoxide anion production [1]. The major cellular receptor for phorbol esters is now known to be the phospholipid/Ca^{2+}-dependent protein kinase (protein kinase C) (PKC) [2]; recent evidence suggests that phorbol ester binding to PKC is dependent on phospholipid [2] and that translocation and binding of cytosolic PKC to the plasma membrane may therefore be an important step in activation of PKC by phorbol ester or diacylglycerol [3]. Recent studies have shown that the calcium ionophore A23187 acts synergistically with phorbol esters to elicit a variety of physiological cell responses including guinea pig PMN superoxide production [4]. One suggested mechanism for this synergism is that the rise in cytosol Ca^{2+} produced by A23187 promotes translocation and binding of PKC to the cell membrane, thereby increasing availability of phospholipid cofactor and thus the affinity of binding of PKC for phorbol ester [3,5].

In the experiments described here we have investigated this hypothesis further by comparing the effect of calcium ionophore A23187 on PDBu-induced PMN superoxide anion production with its effect on the binding of PDBu in human PMN.

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Abbreviations: PDBu, phorbol dibutyrate; PMN, neutrophil polymorphonuclear leucocyte; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester

2. MATERIALS AND METHODS

All reagents were obtained from Sigma except for [^{3}H]PDBu (12.5 Ci/mmol) which was obtained

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from New England Nuclear. Human PMN were prepared from venous blood by dextran sedimentation followed by fractionation on a Percoll gradient as described [6]. PMN were washed twice and resuspended in RPMI 1640 (Flow Labs) + 10% fetal calf serum (at 10^6 or 5×10^6 PMN/ml for superoxide or PDBu-binding studies, respectively). Unless otherwise stated PMN were preincubated for 10 min at 37°C with A23187 or methanol solvent (0.2%, v/v, final) prior to incubation for 20 min with PDBu or [^{3}H]PDBu in DMSO (0.05%, v/v, final). Final concentrations of methanol or DMSO did not affect the performance of either assay (not shown). Assay of [^{3}H]PDBu binding (in triplicate) was performed as in [7]. Non-specific binding was assayed in the presence of a 50-fold excess of unlabelled phorbol ester.

Superoxide production was measured by monitoring superoxide dismutase-inhibitable ferricytochrome *c* reduction at 550 nm. For kinetic experiments ferricytochrome *c* reduction was

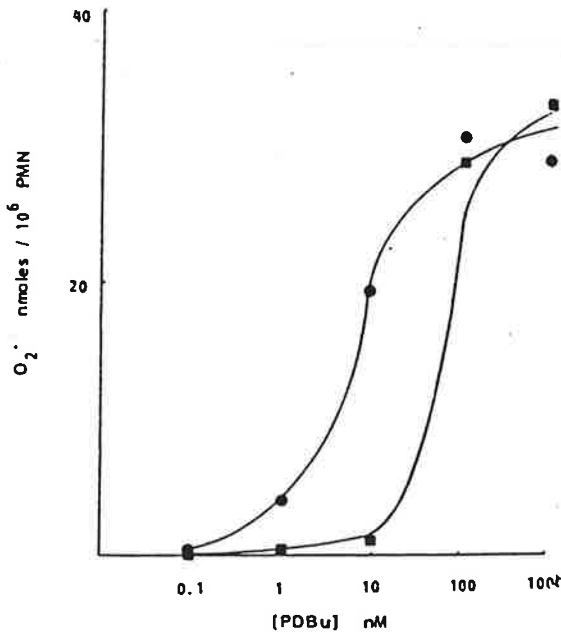

Fig.1. Effect of A23187 on PDBu-stimulated superoxide production. PMN were treated with 1 μM A23187 or an equivalent volume of methanol for 10 min, then PDBu for 20 min. In the presence of A23187 the dose response to PDBu was shifted to the left. A23187 + PDBu (●); PDBu alone (■). Data represent one typical result from three experiments. SE did not exceed 0.8 nmol/10^6 PMN.

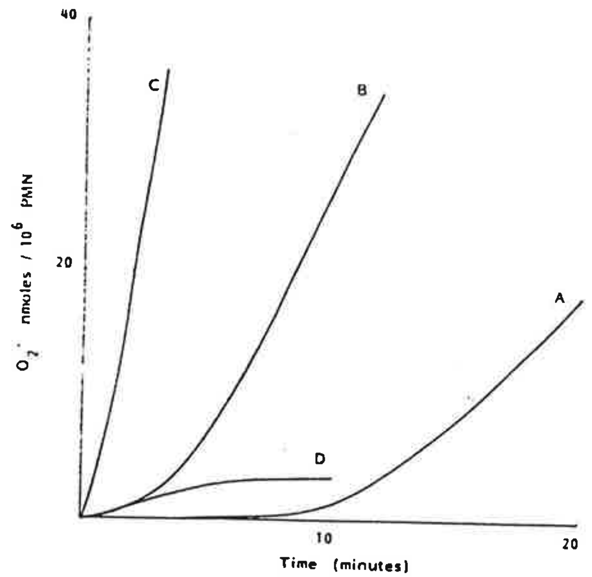

Fig.2. Effect of A23187 on kinetics of PDBu-stimulated superoxide production. The initial rate of PMN superoxide release was increased and the lag time for response to 50 nM PDBu (A) was shortened by 10 min pretreatment with 0.1 μM (B) or 1 μM (C) A23187. 1 μM A23187 alone (D) stimulated only minimal superoxide release.

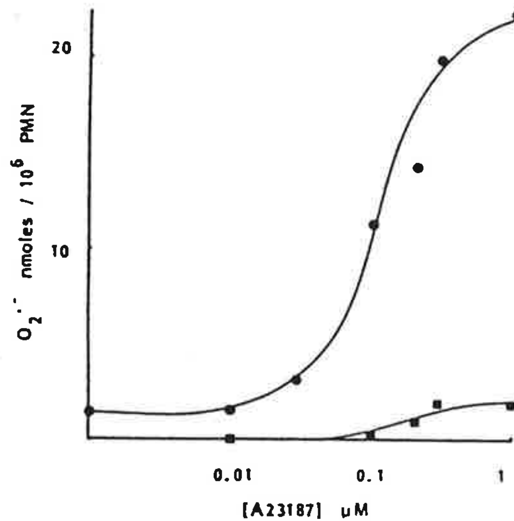

Fig.3. Dose dependence of the synergistic effect of A23187 on PDBu-stimulated superoxide production. 10 nM PDBu + A23187 (●); A23187 alone (■). Data represent one typical result from five experiments.

Table 1

Synergistic action of A23187 on superoxide release and PDBu binding

	(a) Superoxide release (nmol/10^6 PMN per 20 min)		(b) [^{3}H]PDBu binding (% of control)	
	+A23187 (1 μM)	−A23187	+A23187 (1 μM)	−A23187
PDBu	12.8 ± 2.4	1.3 ± 0.6	173 ± 13	100
PDBu + EGTA	0.3 ± 0.1	0.4 ± 0.2	114 ± 14	110 ± 16
PDBu + TMB-8	0.9 ± 0.3	0.2 ± 0.1	111 ± 11	113 ± 24
A23187	1.9 ± 0.7	0	–	–

Effect of 2 mM EGTA or 200 μM TMB-8 on the synergistic action of 1 μM A23187 on mean (± SE) (a) superoxide release elicited by 10 nM PDBu (n = 5); (b) binding of 10 nM [^{3}H]PDBu (n = 3)

monitored continuously at 37°C [8] in a spectrophotometer (LKB) linked to an Apple computer (Ultraspec reaction rate programme). Fixed time point measurements of superoxide release experiments were conducted in duplicate as described in [9].

3. RESULTS AND DISCUSSION

3.1. *Effect of A23187 on superoxide production*

After pretreatment of PMN for 10 min with A23187 (1 μM), the dose response for PDBu-stimulated PMN superoxide production was shifted to the left (fig.1). The mean (± SE) concentration of PDBu which produced 50% of the maximal superoxide response (EC_{50}) was reduced from 56 ± 8 to 8 ± 2 nM (n = 3) in the presence of 1 μM A23187. At high concentrations of PDBu (>100 nM) A23187 had no synergistic effect (fig.1). As reported in [4] the enhancing effect of A23187 was due both to a shortening of the lag time for response to PDBu and to an increase in the initial rate of superoxide release (fig.2). A23187 (1 μM) alone caused only minimal stimulation of superoxide production (fig.2).

The synergistic action of A23187 on the superoxide response elicited by a 'threshold' concentration of PDBu (10 nM) was dose-dependent (fig.3) with a mean (± SE) EC_{50} of 0.18 ± 0.07 μM A23187 (n = 5). In the presence of EGTA (2 mM) or TMB-8 (200 μM) the synergistic effect of A23187 was inhibited, thus confirming the dependence on Ca^{2+} (table 1). The response to PDBu in the absence of A23187 was also partially inhibited by EGTA or

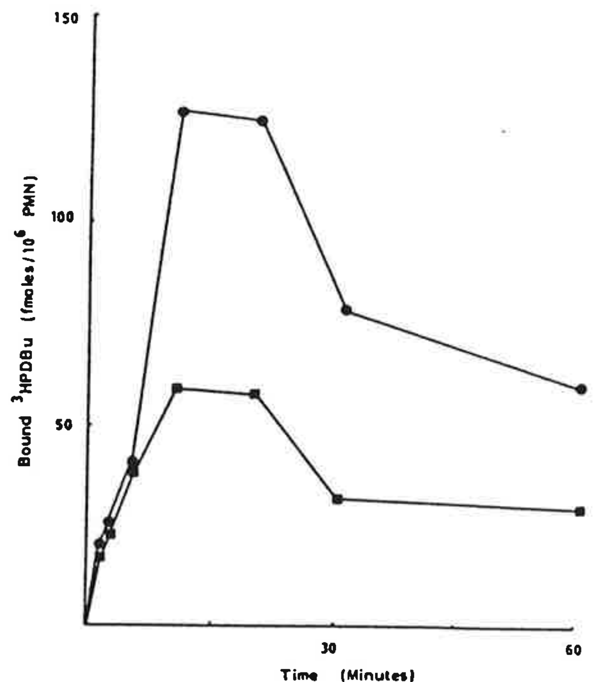

Fig.4. Effect of A23187 on the kinetics of binding of [^{3}H]PDBu to PMN. 10 min pretreatment with 1 μM A23187 increased the extent and rate of PDBu binding but did not alter the overall shape of the kinetic curve. (●) 10 nM PDBu alone; (■) 10 nM PDBu + 1 μM A23187 (SE did not exceed 2 fmol).

the intracellular calcium antagonist TMB-8 [10], emphasizing the importance of Ca^{2+} in the cellular response to PDBu alone.

3.2. *Effect of A23187 on PDBu binding*

To determine whether the synergistic effect of A23187 correlated with increased phorbol ester binding, the action of A23187 on [^{3}H]PDBu binding to PMN was examined. The time course of binding of 10 nM [^{3}H]PDBu to PMN was followed over 60 min. After an initial phase of rapid binding, which lasted 10–20 min, there was a slow decline in PDBu binding. Pretreatment of PMN for 10 min with A23187 (1 μM) increased the initial rate of PDBu binding, but did not alter the overall shape of the kinetic curve (fig.4). Similar binding kinetics have been reported for the HL60 cell line [11], and may reflect proteolytic cleavage of PKC and loss of binding sites following activation of PKC [12,13].

Pretreatment of PMN with A23187 (1 μM) also increased the affinity of binding of phorbol ester receptors for PDBu from a single class of low affinity (K_d 27 nM) to a single class of high affinity (K_d 10 nM), with no apparent alteration of total receptor numbers (fig.5). Thus, as previously reported for other cell types (3,14], A23187 appears to convert low-affinity PDBu receptors to high affinity. These data are consistent with the hypothesis that a rise in cytosol Ca^{2+} converts low-affinity PDBu receptors, believed to be cytoplasmic PKC, to high-affinity receptors representing PKC bound to plasma membrane phospholipid [3,5]. Nonetheless other indirect ef-

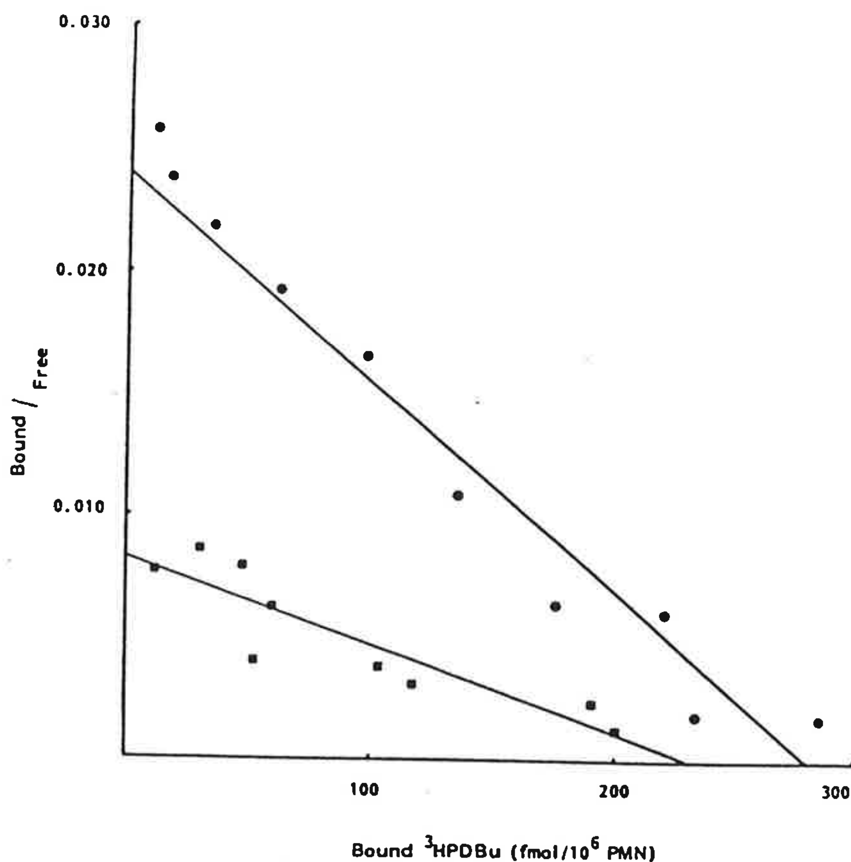

Fig.5. Scatchard plot of [^{3}H]PDBu binding to PMN with or without pretreatment with A23187. 10 min pretreatment with 1 μM A23187 increased the affinity of binding of phorbol ester receptors from a single class of low affinity (K_d 27 nM) to a single class of high affinity (K_d 10 nM). (■) PDBu alone; (●) PDBu + 1 μM A23187. Data represent one typical result from 3 experiments. Each point represents the mean of triplicates, SE did not exceed 2 fmol.

fects of Ca^{2+} on PDBu receptors cannot be excluded.

The action of A23187 on binding of PDBu (10 nM) was dose-dependent with a mean (± SE) EC_{50} of 0.24 ± 0.08 μM A23187 (n = 3), which correlated closely with the EC_{50} (0.18 ± 0.07 μM) for the effect of A23187 on PDBu-induced superoxide release. The effect of A23187 on PDBu binding was inhibited by EGTA or TMB-8 (table 1).

In conclusion these data provide further evidence that the basis for the synergistic effect of A23187 on the initial response of PMN to phorbol ester is due to a Ca^{2+}-dependent increase in binding affinity of receptors for PDBu. In view of the lack of effect of A23187 on the response to maximal doses of PDBu, these data suggest that A23187 affects early events during PMN activation and are consistent with the recently proposed hypothesis that Ca^{2+} promotes translocation and binding of cytosolic PKC to the plasma membrane [3,5].

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SHORT COMMUNICATIONS

Translocation of protein kinase C to a Triton-insoluble sub-cellular compartment induced by the lipophilic gold compound auranofin*

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The Ca^{2+}-activated, phospholipid-dependent protein kinase C (PKC)† phosphorylates substrates in cytosol, membrane, cytoskeleton and nucleus [1]. Diacylglycerol and phorbol esters in association with Ca^{2+} induce translocation of PKC from cytosol to membrane [2, 3]. Some of the translocated PKC is proteolytically cleaved and the catalytic fragment returns to the cytoplasm where it initiates further phosphorylation independently of calcium, phospholipid and diacyglycerol [4]. Another portion of PKC is translocated to a detergent-insoluble compartment [5], assumed to be the cytoskeleton and/or karyoskeleton.

Auranofin (AF), a lipophilic anti-rheumatic gold compound, mimics and synergizes with phorbol esters [6–8] in several cell models and increases both the affinity and apparent total number of PDBu receptors in human lymphocytes [9].

Here we show that AF markedly enhances the association of cytoplasmic phorbol receptors (PKC) with a detergent-insoluble subcellular fraction of B lymphocytes.

Materials and methods

AF (gift of Dr M. Whitehouse, Pathology Dept., University of Adelaide), A23187 and PDBu (Sigma) were stored in DMSO. CLL cells obtained by leukapheresis were stored in liquid nitrogen and cultured at $1–2 \times 10^7$/ml as described previously [9]. For subcellular fractionation, cells were resuspended in 25 mM Tris–HCl, pH 7.4, containing 1 mM EDTA and ultrasonicated (4 × 10 sec pulses, Heat Systems Ultrasonics), at 0°. Supernatant after 40,000 *g* centrifugation for 60 min was used as cytosol. The particulate fraction was sonicated in PBS for assay or further extracted with 0.2% Triton X-100 containing 1 mM EDTA and centrifuged at 40,000 *g* for 30 min to yield Triton-soluble and -insoluble material. Phorbol receptors were assayed by binding of ^{3}H-PDBu (New England Nuclear, 20 Ci/mmol) in cells, particulate and soluble fractions were determined as in [10, 11], the latter in the presence of phosphatidylserine (20 μg/ml) and Ca^{2+} (5 mM). Binding was <15% of total without these additions. PKC was assayed by incorporation of ^{32}P from ^{32}P-ATP into histone [4]. ^{32}P-incorporation into histone in replicates containing 1 mM EGTA and lacking phosphatidylserine and calcium, was subtracted from total phosphorylation to give PKC activity.

Results and discussion

AF causes a Ca^{2+}-dependent increase in affinity of phorbol receptors in CLL cells [9]. AF increased binding in cells pre-equilibrated for 40 min with ^{3}H-PDBu, and there was little or no subsequent time-dependent loss of this binding. This contrasts with the transient increase of binding in A23187-treated cells (Fig. 1). The apparent increase in total receptor numbers may be due to lack of time-dependent down-regulation of PDBu-binding capacity resulting from proteolytic cleavage of PKC [12], rather than to an actual increase in receptors, resulting possibly from resistance of PKC to degradation or a change in accessibility to protease. AF increases affinity of PDBu receptors without time-dependent loss of these receptors in neutrophils [manuscript in preparation].

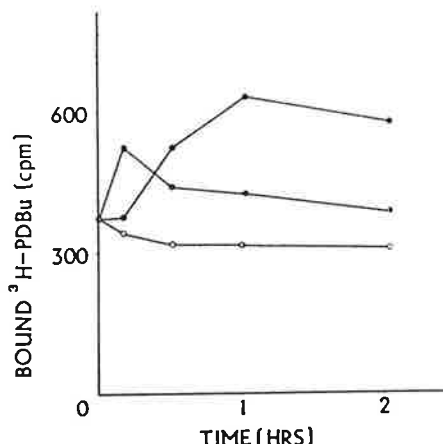

Fig. 1. Augmentation of binding of PDBu (cpm/10^6 cells) in intact CLL cells incubated with ^{3}H-PDBu (10 nM) for 40 min at 37° and then (at time 0) exposed to AF, 60 μM (●), A23187, 1 μM (◑) or control solvent dimethylsulphoxide, 0.1% v/v (○). Means of triplicates; SD did not exceed 10% of means.

Calcium ionophores increase cellular binding of PDBu [3, 13] by promoting translocation of PKC to the particulate fraction [2, 3]. B-CLL cells were treated with unlabelled PDBu for 40 min in the presence or absence of AF, washed thoroughly, then assayed for binding of 10 nM ^{3}H-PDBu in the isolated particulate fraction. PDBu increased PDBu receptor activity of the particulate fraction in a concentration-dependent manner (Fig. 2). This effect was greatly increased in the presence of AF, although AF alone (60 μM) had little or no effect. The synergism was evident within 5 min (not shown).

While binding of ^{3}H-PDBu to the particulate fraction increased in cells treated with AF plus PDBu, calcium- and phospholipid-dependent histone kinase activity was almost completely lost from the cytosol (Fig. 3). However, little kinase or PDBu receptor activity could be extracted with Triton X-100 from the particulate fraction of cells treated with AF or with AF plus PDBu, relative to control cells or cells treated with PDBu alone. In contrast, Triton-insoluble residues of particulate fractions from cells treated with AF or AF plus PDBu were considerably enriched for PDBu receptor activity (up to 88%, Fig. 3). The content of PDBu

* This work was supported by a grant from the Anti-cancer Foundation of the Universities of South Australia.

† Abbreviations used: AF, auranofin; CLL, chronic lymphocytic leukaemia; DMSO, dimethyl sulphoxide; EDTA, ethylene diamine tetra acetic acid; EGTA, ethylene glycol-bis(beta-amino-ethyl ether) *N*, *N*, *N*′, *N*′-tetra acetic acid; kD, kilodalton; PBS, phosphate buffered saline; PDBu, phorbol dibutyrate; PKC, protein kinase C; PS, phosphatidylserine.

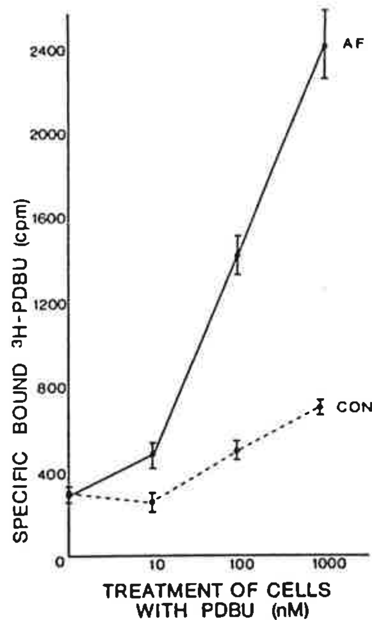

Fig. 2. Cells were treated for 40 min with varying concentrations of unlabelled PDBu in the absence (CON) or presence of AF (60 μM) and washed. Particulate membrane fractions were assayed for binding of 10 nM ^{3}H-PDBu. Means of triplicates, bars indicate SD.

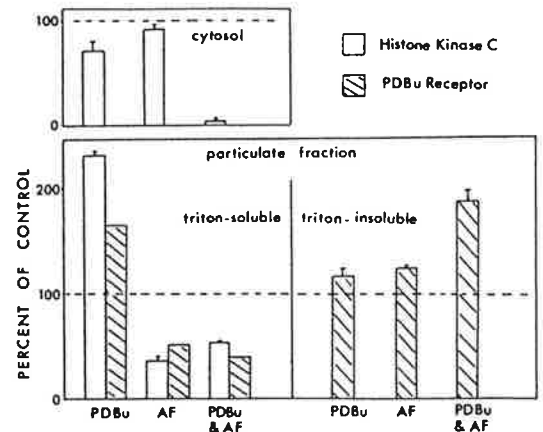

Fig. 3. B-CLL cells were treated with AF (60 μM), PDBu (200 nM), AF + PDBu or control solvent DMSO (0.1% v/v). After 40 min cytosol, Triton-soluble and -insoluble fractions were prepared and assayed for binding of ^{3}H-PDBu (10 nM) or histone kinase C activity. Activities in control cell fractions were taken as 100%. Bars indicate SD. ▧, PDBu-receptor; □, histone kinase C.

receptors recovered in the Triton-insoluble fraction increased progressively from 28% in control preparations to 43% in cells treated with AF 60 μM, and Triton-extractable receptors (Fig. 4) and histone kinase C activity (not shown) decreased with increasing concentrations of AF. It is not yet possible to determine how much histone kinase C activity is present in this Triton-insoluble fraction because the assay requires soluble PKC.

The detergent-insoluble portion of the particulate fraction consists largely of cytoskeletal and nuclear material [14]. PKC has been reported in both the cytoskeleton and nucleus of lymphocytes [5, 15]. Many cytoskeletal proteins are substrates for PKC [1] and some tyrosine kinases (which may be regulated by PKC) are located in the cytoskeleton [16]. Translocation of PKC to the cytoskeleton may mediate down-regulation by AF of EGF receptors in HeLa cells [6] and mouse erythrocyte receptors in CLL cells [manuscript submitted].

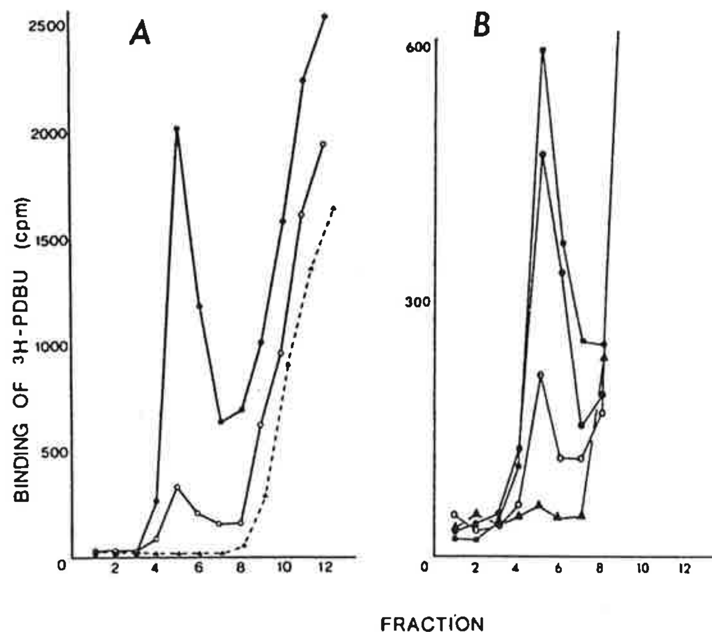

Fig. 4. Triton-soluble material was assayed for PDBu receptors by column filtration. (A) Calcium and phospholipid-dependence of binding; ●, Triton soluble material, PS and Ca^{2+}; ○, Triton-soluble material alone; ▲, PS and Ca^{2+} alone. (B) Triton-soluble materials from cells treated with AF at 0 (■), 6 (●), 24 (○) or (▲) 60 μM.

AF may induce translocation of PKC by elevating cAMP [17], by analogy with induction of translocation of PKC to nuclear fractions by cAMP in mouse lymphocytes [18]. Alternatively, AF and metals which are known to interact with thiols [19], may act via the cysteine-rich site in the regulatory domain of PKC [20]. Metals have an affinity for the cytoskeleton [21], a region rich in sulphydryls.

In summary, AF, which modulates PKC-dependent events *in vitro*, synergizes with the phorbol ester PDBu, a specific ligand of PKC, in causing translocation of PKC from cytosol to the particulate subcellular fraction. This translocation is accompanied by increased affinity of binding of phorbol ester. Translocation induced in the presence of AF, unlike that induced by phorbol ester alone, results in attachment of PKC to a detergent-insoluble compartment. In this state PKC is not subject to normal down-regulation. Studies are now needed to show whether translocation of PKC to particular subcellular compartments is mediated by particular intracellular messengers.

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Azidopine photoaffinity labeling of multidrug resistance-associated glycoproteins

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The problem of developing effective cancer chemotherapeutic regimens in humans in the presence of drug-resistant tumor cells is being approached by studying model systems in tissue culture. Mammalian cells selected for resistance to drugs often display the multidrug resistance (MDR*) phenotype which includes (1) cross-resistance to unrelated drugs, (2) a net decrease in drug accumulation, and (3) the overexpression of a MDR-associated glycoprotein (MDRG), also known as P-glycoprotein, that is found in the plasma membrane [1]. Calcium antagonists such as verapamil can partially reverse MDR by increasing intracellular drug levels [2–8]. Verapamil specifically inhibits the binding of vinblastine and its photoactive analog to the MDRG, thereby suggesting that it interacts with the MDRG [9–12].

MDR cell lines derived from the murine cell, J774.2, have been selected in our laboratory for resistance to colchicine (CLC), vinblastine (VBL) or taxol (TAX) [13, 14]. The MDRGs observed by SDS–PAGE in the CLC- and VBL-resistant cells have an approximate M_r of 130–150 kDa, whereas the MDRG from the TAX-resistant cells migrates as a doublet in a similar molecular weight range [15, 16].

The arylazide 1,4-dihydropyridine, azidopine, is a calcium antagonist which photolabels calcium channels [17]. [^{3}H]Azidopine has the advantages of being relatively stable in the absence of UV light and capable of forming covalent bonds via nitrene intermediates upon UV irradiation. In

* Abbreviations: MDR, multidrug resistance; MDRG, multidrug resistance glycoprotein; CLC, colchicine; VBL, vinblastine; TAX, taxol; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone.