THE HYDROLYSIS OF INOSITOL PHOSPHOLIPID IN MOUSE EXOCRINE PANCREAS

A thesis

submitted for the

DEGREE OF DOCTOR OF PHILOSOPHY

of

The Department of Physiology

University of Adelaide, South Australia

by

KARIN ANNE TENNES B.Sc.(Hons.)

1984

TABLE OF CONTENTS

Page

SUMMARY

1. A method was developed which allows the rapid, reproducible measurement of the hydrolysis of inositol phospholipid in mouse exocrine pancreas. The technique involves the *in vivo* labelling of pancreatic tissue with *myo*-(2-^{3}H) inositol and does not require the time-consuming extraction and chromatographic separation of lipids which has been necessary in the majority of assays of phosphoinositide turnover.

   The measurement of the release of *myo*-(2-^{3}H) inositol-labelled products from inositol phospholipid provides a direct assay of phosphoinositide breakdown and not of a combination of hydrolysis and resynthesis, since the (^{3}H)-inositol released is not re-incorporated into inositol phospholipid. In addition, the increase in (^{3}H)-inositol released is not due to agonist-activation of the phosphatidylinositol : *myo*-inositol exchange enzyme which would cause a complication in measuring lipid breakdown due to an increase in exchange of inositol between lipid-bound and free states.

   Since the method measures the (^{3}H)-inositol-labelled products of phosphoinositide breakdown the validity of the method does not depend on whether the lipid hydrolysed is phosphatidylinositol, phosphatidylinositol-4 phosphate or phosphatidylinositol-4,5 bisphosphate.

   This assay facilitates the study of inositol phospholipid hydrolysis. In particular, an improved examination of the dose-dependent

relationship between lipid hydrolysis and pancreatic function is possible.

2. The agonist-stimulated breakdown of inositol phospholipid was found to be closely linked to receptor activation. This lipid response is not dependent on the agonist-activated influx of Na^+ and does not require an intact cytoskeletal network or protein synthesis. A dependency of the agonist-stimulated lipid breakdown on ATP was identified, which supported the proposal that phosphatidylinositol-4,5 bisphosphate and not phosphatidylinositol may be the initial lipid hydrolysed following receptor activation.

3. The agonist-stimulated breakdown of inositol phospholipid is not Ca^{2+}-dependent since it occurs in the absence of extracellular Ca^{2+} and does not require the release of Ca^{2+} from intracellular stores. Inositol lipid breakdown is not activated by Ca^{2+} since an increase in intracellular Ca^{2+} with ionophore A23187 does not stimulate the lipid response.

4. The presence of Ca^{2+} in the extracellular medium potentiates inositol phospholipid hydrolysis. This potentiation is not due to the movement of Ca^{2+} through the plasma membrane since neomycin, an agent which blocks Ca^{2+} influx, does not alter the potentiation effect.

5. Two sites of potentiation by Ca^{2+} of inositol lipid hydrolysis have been identified on the external surface of the pancreatic cell membrane. One site is associated with potentiation of unstimulated

inositol lipid hydrolysis. This site is lanthanum insensitive but manganese can replace Ca^{2+} at this site. The second site is associated with potentiation of agonist-stimulated inositol lipid hydrolysis. Lanthanum and manganese compete with Ca^{2+} at this site to remove the potentiation effect.

6. Results show that at high concentrations of agonists the degree of inositol phospholipid hydrolysis correlates with the degree of inhibition of amylase secretion. Since this effect on secretion is due to Ca^{2+} the result suggests that inositol lipid breakdown and the increase in intracellular Ca^{2+} are correlated.

7. At low concentrations of agonists, when amylase secretion is stimulated, the degree of inositol lipid hydrolysis is very small but may be sufficient to cause release of Ca^{2+} from intracellular stores.

In conclusion, the results of the study support the proposal that agonist activation of inositol phospholipid hydrolysis may control the mobilization of Ca^{2+} in the exocrine pancreas.

DECLARATION

I declare that the material presented in this thesis is original work and has not been accepted for the award of any other degree or diploma in any University, and that, to the best of my knowledge and belief, contains no material previously published or written by any other person, except when due reference is made in the text.

I consent to the thesis being made available for photocopying and loan if accepted for the award of the degree.

Signed . . . . . .

KARIN A. TENNES

ACKNOWLEDGEMENTS

My sincere thanks to my supervisor, Dr. Michael Roberts, for his continued support and assistance throughout my studies.

I wish to thank Dr. Timothy Miles for many helpful discussions and Michael Crouch, my co-worker in the laboratory. Thanks to Dr. Bill Venables for his assistance with the factorial statistical analysis and Dr. Jennifer Kennedy for the studies of receptor binding.

I also wish to thank my parents and sister, Jane, for their encouragement and assistance.

Many thanks to my mother and Janet Parlett for typing the draft of the thesis and especially to Pat Draper for all the work done in the final typing of the thesis.

LIST OF TABLES

LIST OF FIGURES

Figure | Page

CHAPTER 1

INTRODUCTION

1.1 : INTRODUCTION

The binding of hormones and neurotransmitters to their receptors on the external surface of the plasma membrane is coupled to the physiological response in many cells by an increase in the cytosolic calcium concentration. Agonists can stimulate an increase in intracellular calcium by mobilizing calcium from cellular stores and by increasing the permeability of the plasma membrane to calcium. The mechanism which links receptor activation to an increase in this intracellular second messenger is not known.

A common event associated with the stimulation of receptors which activate an increase in intracellular calcium content is an alteration in the metabolism of inositol phospholipids of cell membranes. Hokin and Hokin (1953) were the first to report an acetycholine-stimulated increase in the incorporation of (^{32}P)-inorganic phosphate ((^{32}P)-P_i) into total phospholipid in pigeon pancreas slices. Subsequently, phosphatidylinositol (PtdIns) was identified as the major phospholipid to be synthesized in response to agonist-stimulation in the exocrine pancreas (Hokin and Hokin, 1955). Michell (1975) proposed that the agonist-stimulated breakdown of PtdIns was an early event in cell activation leading to an increase in intracellular calcium concentration.

Michell's proposal gained support from studies in various tissues showing that the agonist-stimulated breakdown of this phospholipid is a calcium-dependent event (Jones and Michell, 1975; Berridge and Fain, 1979; Billah and Michell, 1979). An essential argument

in support of PtdIns hydrolysis preceding calcium-gating rather than occurring as a result of calcium entering the cytosol is that PtdIns breakdown is not dependent on an increase in intracellular calcium concentration. However, the calcium dependence of the PtdIns response has been the subject of question since studies on neutrophils (Cockroft et. al., 1980) and platelets (Bell and Majerus, 1980) demonstrated stimulation of PtdIns breakdown by calcium ionophores.

Michell's initial proposal has been recently modified to suggest that the hydrolysis of phosphatidylinositol-4,5 bisphosphate (PtdIns-4,5P_2) may be the primary event in the alteration of inositol lipid metabolism mediated by receptor activation and that the decrease in PtdIns is due to its phosphorylation to refill the PtdIns-4,5P_2 pool, rather than its hydrolysis.

There is now increasing evidence showing that the cellular content of the phosphorylated derivatives of PtdIns, PtdIns-4,5P_2 and phosphatidylinositol-4 phosphate (PtdIns-4P) decrease more rapidly that PtdIns in response to agonists which cause an increase in the intracellular concentration of calcium (Kirk et. al., 1981; Weiss et. al., 1982; Billah and Lapetina, 1982). More recent studies showing the receptor activated formation of inositol-1,4,5-trisphosphate and inositol-1,4-bisphosphate (Berridge et. al., 1983; Berridge, 1983) support the proposal that PtdIns-4,5P_2 may be the initial inositol lipid hydrolysed. The mechanism by which the binding of agonists to their receptors activates the hydrolysis of inositol phospholipid is unknown and until very recently, the functional

significance of this lipid response has also been unclear. Studies reported over the last twelve months, however, have provided evidence to support a link between phosphoinositide hydrolysis and mobilization of intracellular calcium (Streb et. al., 1983; Burgess et. al., 1984; Prentki et. al., 1984; Joseph et. al., 1984).

At the time at which this study began, February 1980, most studies of phosphoinositide metabolism were concerned with PtdIns, with very few studies on the polyphosphoinositides, PtdIns-4,5P_2 and PtdIns-4P. These early studies on the polyphosphoinositides showed a calcium-dependent breakdown of PtdIns-4,5P_2 in rabbit iris smooth muscle (Akhtar and Abdel-Latif, 1978; 1980) in human erythrocytes (Allan and Michell, 1978) and in synaptosomes (Griffin and Hawthorne, 1978).

Interest in the polyphosphoinositides then waned for a number of years, and research concentrated on PtdIns. The majority of studies on PtdIns metabolism examined the synthesis of PtdIns by measuring the incorporation of radioactive precursor into PtdIns rather than the initial event of breakdown. Although the breakdown and synthesis of PtdIns do form part of a cycle of reactions causing phosphoinositide turnover, it was becoming clear at this time that resynthesis measurements do not provide a satisfactory indication of inositol lipid breakdown. Some agents, including calcium, can alter the synthesis of PtdIns (Berridge and Fain, 1979) but not its breakdown (Berridge and Fain, 1979; Jones and Michell, 1976). In addition, a stimulation of the *de novo* synthesis of PtdIns (Hokin and Hokin, 1958 a; Allan and Michell, 1975; Calderon et. al., 1979, 1980;

Chapman et. al., 1983), would result in an increase in the incorporation of precursor into PtdIns and not be due to an increase in the breakdown of PtdIns. Therefore, it was necessary to develop a method to measure hydrolysis directly and not measure a synthesis step.

In the present study, the exocrine pancreas was chosen to investigate the role of inositol phospholipid hydrolysis in controlling the intracellular calcium concentration, since an increase in calcium is a critical step in the sequence of events linking receptor activation by a number of secretagogues to exocytosis. The influx of calcium through the plasma membrane to the cytosol is a voltage-independent mechanism since depolarization of the acinar cell membrane does not stimulate secretion (Poulsen and Williams, 1977) and so the permeability properties of the plasma membrane must therefore be controlled by some mechanism other than a change in membrane potential. Inositol phospholipid breakdown was investigated as one such possible mechanism.

Exocrine gland cells being electrically inexcitable allow the study of the biochemical reactions and ionic movements linking receptor occupation with the final cell response without the complication of potential-dependent events occurring. Mouse exocrine pancreas also provided sufficient tissue for biochemical assays and the physiological cell response of release of secretory product can be easily assayed by measuring amylase secretion.

The aim of this study was to investigate the agonist-stimulated hydrolysis of inositol phospholipid in mouse exocrine pancreas in an attempt to identify the critical link between receptor activation and the increase in intracellular calcium. The specific aims were:

1. To develop a technique for measuring the hydrolysis of inositol phospholipid in mouse exocrine pancreas, which unlike the majority of methods available at the time eliminates the time-consuming requirement to extract and chromatographically separate the phospholipids for each experiment and allowed the processing of a larger number of samples in each experiment. This would reduce variance and also enable an improved examination of the dose-dependent relationship between inositol lipid hydrolysis and pancreatic secretion.

2. To examine how closely inositol phospholipid hydrolysis is linked to receptor occupation by investigating whether these events are directly or indirectly coupled. These experiments aimed to examine whether an agonist-stimulated influx of Na^+ mediated the lipid response; whether an intact cytoskeletal network was required; whether the synthesis of a protein linked the two events or if there was a requirement for adenosine triphosphate. In addition, the question of whether activation of different receptors causes the breakdown of inositol phospholipid by a common mechanism was also to be considered.

3. To study the role of extracellular calcium and calcium stored intracellularly on the agonist-stimulated hydrolysis of inositol phosphlipid in order to examine if the lipid breakdown may precede an increase in the cytosolic calcium concentration.

4. To investigate the dose-dependent relationship between agonist-stimulated breakdown of inositol phospholipid and the calcium-mediated stimulation and inhibition of amylase secretion in an attempt to identify the relationship between lipid breakdown and calcium mobilization.

The following chapter reviews the literature concerning pancreatic exocrine function. The general structure and function of the pancreas is discussed in addition to a description of the secretagogue-stimulated events that occur in the acinar cell. Since the role of inositol phospholipid hydrolysis in cell function may be to control the cytosolic calcium content, the emphasis of the literature review will be on calcium-mediated events. The stimulated movements of calcium which occur and the site(s) from which calcium is released will be discussed, followed by a review of the possible involvement of inositol phospholipid in stimulus-response coupling, particularly in the pancreas. However, those studies in other tissues which have provided additional information regarding activation of metabolism of inositol phospholipids will also be discussed.

CHAPTER 2

LITERATURE REVIEW

2.1 : PANCREATIC STRUCTURE AND FUNCTION

The pancreas consists of exocrine and endocrine tissue. The exocrine portion is comprised of acinar cells which release secretory products into the lumen. The intercalated ducts also form part of the exocrine tissue and penetrate into the lumen of the acini, this intra-acinar portion of the duct is composed of centro-acinar cells. The intercalated ducts converge into a single pancreatic duct which joins the bile duct from the liver before entering the duodenum.

A small proportion, less than 5% of the total pancreatic tissue in rat (Kempen et. al., 1977) and guinea-pig (Bolender, 1974) is endocrine tissue, which is dispersed through the exocrine pancreas as small groups of cells, the islets of Langerhans. These endocrine cells secrete hormones, for example, insulin, glucagon, somatostatin and pancreatic polypeptide.

The pancreatic acinar cell is a pyramidal-shaped cell which is highly polarized. The basolateral membrane is the site of action of neurotransmitters and blood-borne hormones and is likely to be the site of the receptors for these agonists. Williams et. al. (1982 a) demonstrated that radioiodinated cholecystokinin binds to specific receptors on the basolateral surface of the pancreatic acinar cells. The morphology of the pancreatic acinar cell has been described from transmission-electron-microscope studies in many species including guinea-pig (Palade and Siekevitz, 1956; Caro and Palade, 1964;

Jamieson and Palade, 1967 a, b); mouse (Sjöstrand and Hanzon, 1954 a, b); rat (Ekholm et. al., 1962; Bieger et. al., 1976) and man (Ekholm and Edlund, 1959; Blackburn and Vinijchaikul, 1969).

The basal region of the cytoplasm is dense with ribosomes most of which are attached to the outer surface of the flat cisternae of the rough-surfaced endoplasmic reticulum. The nucleus occupies a central or basal location and is surrounded by the rough endoplasmic reticulum. The Golgi complex, localised towards the centre of the cells, is large and elaborate. The apical cytoplasm contains large, spherical membrane-bound granules : the zymogen granules. The lateral and basal plasma membranes of the acinar cell are relatively straight whereas the apical plasma membrane has numerous microvilli which protrude into the acinar lumen. Adjacent pancreatic acinar cells are connected by junctional complexes, one of the functions being to provide a semi-permeable barrier between the acinar lumen and the intercellular space. Gap junctions are regions of low resistance in the lateral membranes joining adjacent acinar cells through which small molecules and ions move, thereby allowing electrical coupling between cells (Petersen and Ueda, 1976 a).

The protein molecules constituting the tight junction fibres appear to maintain their integrity by their associations with extracellular calcium. Meldolesi (1976) reported that after a 10 min incubation of pancreatic tissue in a Ca^{2+} -free Krebs solution containing 0.5mM EGTA, the structure of the tight junctions as examined by freeze-fracture technique became disrupted. The junctional fibres were spread along the lateral regions of the plasmalemma rather

than being ordered and localized around the secretory lumen. Extracellular calcium therefore appears to be important in maintaining the permeability barrier at the site of the tight junctions between acinar cells.

Pancreatic exocrine secretion comprises both protein and electrolyte secretions. The protein secretion is released solely from the acinar cells which synthesize over twenty digestive enzymes (Case, 1978), most of which are pro-enzymes (Clemente et. al., 1972). Extensive studies of the synthesis, storage and intracellular transport of enzymes have been described by Siekevitz and Palade (1960); Palade et. al. (1962); Caro and Palade (1964); Redman et. al. (1966); Jamieson and Palade (1967 a, b) and reviewd by Jamieson (1972) and Case (1978).

The ribosomes attached to the endoplasmic reticulum membranes are the site of synthesis of the enzymes which, once formed are released within the lumen of the rough endoplasmic reticulum cisternae and then transported throughout the cell to the Golgi complex, where sugar residues of glycoproteins are attached to the peptide backbone and condensing vacuoles form. A progressive aggregation of protein occurs within the condensing vacuoles which undergo a process of organization to form zymogen granules. The conversion of condensing vacuoles into zymogen granules does not require energy (Jamieson and Palade, 1971 a) or continued protein synthesis (Jamieson and Palade, 1968).

The transport of protein from the rough endoplasmic reticulum to the Golgi cisternae is not fully understood. Suggestions of possible mechanisms have been reviewed by Case (1978), which include transport of vesicles formed from protrusions of the rough endoplasmic reticulum to the Golgi cisternae where fusion occurs or transport via tubular connections between endoplasmic reticulum and Golgi.

The zymogen granules formed accumulate in the apex of the acinar cell, the discharge of these secretory products occurs by exocytosis which involves the fusion of the zymogen granule membrane with the apical portion of the plasma membrane, followed by the opening at the point of fusion. Although the exact mechanism of zymogen granule migration is not clear a role for microtubules and microfilaments has been suggested (Bauduin et. al., 1975; Seybold et. al., 1975; Williams and Lee, 1976). Since these organelles are composed of contractile proteins it is possible that they may provide a contractile mechanism for the movement of zymogen granules within the cell.

Microtubules were found primarily in the apical portion of the pancreatic acinar cell and in association with the Golgi region (Williams and Lee, 1976). Colchicine and vinblastine cause the disruption of microtubules by binding to the tubulin subunits (Margulis, 1973). These agents, under conditions that reduced or abolished microtubules, also inhibited the release of agonist-stimulated amylase secretion in pancreas from mouse (Williams and Lee, 1976) and rat (Seybold et. al., 1975). Seybold et. al. (1975) suggested that the antisecretory action of antimicrotubular agents may inhibit the transport of newly synthesized proteins. However, the intracellular

transport of exportable proteins from the endoplasmic reticulum toward the Golgi was not stimulated by physiological stimuli (Jamieson and Palade, 1971 b). Since antimicrotubular agents only affected the agonist-stimulated release of protein and not the spontaneous release (Williams and Lee, 1976) it is possible that the action of these agents is to inhibit the migration of zymogen granules and perhaps also release into the lumen.

Microfilaments are predominantly found constituting the terminal web lying beneath the apical plasma membrane and extend into the microvilli (Bauduin et. al., 1975; Seybold et. al., 1975). Cytochalasin B disrupts this microfilament web and causes disappearance of microvilli in pancreatic acinar cells (Bauduin et. al., 1975; Stock et. al., 1978). In the presence of this agent, the zymogen granules situated close to the acinar lumen were found not to fuse with the apical cell membrane, and stimulated secretion was inhibited (Stock et. al., 1978), suggesting a role of microfilaments in exocytosis.

Although there is a correlation between disruption of microtubule, microfilament structure and protein secretion the agents used do have other non-specific effects. Colchicine and vinblastine reduced amino-acid entry and intracellular transport in rat pancreas (Seybold et. al., 1975) and cytochalasin B inhibited monosaccharide transport in pancreas at concentrations lower than required to alter microfilaments (Bauduin et. al., 1975).

The proportion of any particular enzyme synthesized depends on diet. Grossman et. al. (1943) first reported that the synthesis of

trypsin and amylase was increased in rats fed a high carbohydrate diet whereas an increased trypsin and lipase synthesis occurred in those fed a high protein diet. These results have been supported and extended by other studies (Howard and Yudkin, 1963; Snook, 1971; Robberecht et. al., 1971). The mechanism by which diet brings about alterations in enzyme synthesis is unknown.

The single secretory pathway of exocytosis would require that the release of protein occurs concomittantly and in constant proportions in each pancreatic acinar cell - there is no selective protein secretion. This idea of parallel secretion was supported by the results of Scheele and Palade (1975) showing that the secretagogue-stimulated release of seven proteins from guinea-pig pancreas was in constant proportions at each time point up to two hours after stimulation and that the proportions of these proteins found in the tissue at the end of the experiment were identical to those discharged into the medium. A parallel discharge of enzymes in guinea-pig pancreas was confirmed by Tartakoff et. al. (1975) and in isolated rabbit pancreas by Steer and Glazer (1976).

The possibility of an additional, alternative discharge mechanism was suggested by Rothman (1975). This equilibrium model suggests that the secretory enzymes are not confined within an intracellular organelle but can move across cellular membranes and through the cytoplasmic spaces and are in equilibrium with various compartments of the cell. This would allow for non-parallel discharge of secretory proteins, that is, the transport out of the cell of different enzyme species at variable rates relative to each other

resulting in a secreted-fluid of variable rather than fixed enzyme composition.

Rothman and Wilking (1978) reported that the discharge of chymotrypsin from rabbit pancreas, *in vitro*, stimulated by cholecystokinin (CCK), (20% pure) was considerably lower than that for trypsinogen. Stimulation with acetyl-β-methylcholine or the COOH-terminal octapeptide of cholecystokinin (CCK-8) did however cause parallel secretion of these enzymes. Steer and Manabe (1979) using CCK (pure amino acid polypeptide) were unable to confirm the non-parallel discharge findings of Rothman and Wilking (1978).

Non-parallel discharge of protein occurs in some situations such as following an overnight fast (Dagorn, 1978; Rinderknecht et. al., 1978), this effect may be explained by subpopulations of zymogen granules that contain varying mixtures of secretory proteins (Scheele, 1980) rather than an alternative secretory pathway.

The subject of whether parallel or non-parallel discharge of secretory protein occurs is a controversial one and is not addressed by studies described in this thesis.

There are two distinct pathways by which secretagogues can stimulate pancreatic enzyme secretion. The majority of receptors on pancreatic acinar cells activate a pathway which has been regarded as calcium-dependent (Williams and Chandler, 1975; Petersen and Iwatsuki, 1978; Gardner, 1979; Williams, 1980; Schulz and Stolze, 1980). These receptors may be activated *in vivo* by acetylcholine,

cholecystokinin and gastrin. The muscarinic receptors may be stimulated *in vivo* by acetylcholine released from the vagus nerve since truncal and extragastric vagotomy and administration of anticholinergic drugs dramatically decreased the pancreatic enzyme and bicarbonate secretory responses to intestinal protein and fat digestion products and to hydrochloric acid (Debas et. al., 1975; Singer et. al., 1980, 1981).

The physiological role of the duodenal hormone cholecystokinin as a hormonal regulator of pancreatic secretion has not been clearly defined due to the lack of sensitive and specific radioimmunoassays for this peptide. The studies reported so far using CCK assays specific for the sulphated carboxyl terminal of the molecule (and will not therefore cross react with gastrin which is structurally similar) have shown that following a meal, plasma CCK concentrations increased five - to tenfold (Walsh et. al., 1982; Jansen and Lamers, 1983). Although exogenously administered CCK stimulated an increase in enzyme secretion (Banwell et. al., 1967; Debas and Grossman, 1973; Fölsch et. al., 1978a; Petersen et. al., 1978) it is not known whether the concentrations of CCK used are similar to the plasma concentrations of CCK obtained following a meal. It will be necessary to compare the effects of increases in CCK (measured using a specific C-terminal antibody) by exogenous infusion and endogenous release on pancreatic secretion before the role of CCK in this secretory response can be clearly defined.

The role of gastrin, released from the antrum of the stomach, in mediating pancreatic secretion in response to a meal is not

established. Gastrin has been postulated as a mediator of pancreatic secretion since analogues of gastrin administered exogenously, stimulated pancreatic enzyme secretion (Stening and Grossman, 1969). However, no studies have shown that the levels of gastrin produced after a meal and measured by radioimmunoassay are capable of stimulating pancreatic secretion.

The other pathway for stimulation of enzyme secretion involves increases in adenosine 3', 5' -cyclic monophosphate (cAMP) (Robberecht et. al., 1974; Gardner et. al., 1976; Gardner and Jackson, 1977). Recently, evidence for an adrenergic control of the exocrine pancreas has been described (Lingard and Young, 1983; Pearson et. al., 1984). In addition, vasoactive intestinal polypeptide (VIP) -containing nerves have been described in the pancreas of many species including man and rat (Larsson, 1979; Bishop et. al., 1980) and a role for VIP in controlling pancreatic secretion has been suggested (Pearson et. al., 1981 a).

Although protein secretion occurs solely from the acinar cells, electrolytes and water are released from both acinar and duct cells. Studies of pancreatic fluid secretion and the ionic transport mechanisms involved have been reviewed recently by Schulz and Ullrich (1978); Scratcherd et. al. (1981) and Petersen et. al. (1981).

The pancreas secretes a fluid which is isosmotic with blood (Case et. al., 1968), the major cations present are sodium and potassium (Rothman and Brooks, 1965; Case et. al., 1968), and the major anions bicarbonate and chloride (Case et. al., 1969; Sewell and Young, 1975).

The enzyme-containing fluid secreted from the acinar cells has a high chloride concentration and a low (plasma-like) bicarbonate content (Case et. al., 1969; Dockray, 1972; Sewell and Young, 1975; Kanno and Yamamoto, 1977; Petersen and Ueda, 1977). This neutral fluid secretion which is evoked by acetylcholine, cholecystokinin, gastrin-like peptide and bombesin required the presence of calcium but not bicarbonate in the perfusion fluid (Petersen and Ueda, 1977; Kanno and Yamamoto, 1977; Ueda and Petersen, 1977; Ueda et. al., 1980).

The duct cells secrete a bicarbonate-rich fluid which is dependent on the presence of extracellular bicarbonate but is less dependent than the acinar cell on extracellular calcium (Rothman and Brooks, 1965; Argent et. al., 1973; Petersen and Ueda, 1976 b; Kanno and Yamamoto, 1977). The secretion of this alkaline fluid is stimulated by secretin (Scratcherd et. al., 1975; Sewell and Young, 1975) and VIP (Dockray, 1973; Scratcherd et. al., 1975).

There are large species differences in the ability of hormones or neurotransmitters to stimulate fluid secretion from the pancreas. The cat pancreas secretes fluid in response to secretin (Scratcherd et. al., 1975) and not to cholecystokinin, caerulein or the octapeptide of cholecystokinin (Schulz and Ullrich, 1978), whereas the rabbit is insensitive to the hormone secretin (Scratcherd et. al., 1975). In the rat, the fluid secretion evoked by enzyme secretagogues is greater than that evoked by secretin (Sewell and Young, 1975). In the dog, the secretagogues cholecystokinin and caerulein were shown to stimulate a copious fluid secretion in addition to enzyme secretion (Stening and Grossman, 1969; Debas and Grossman, 1973).

The mechanism of secretin action on fluid secretion appears to be via an increase in cAMP since the adenylate cyclase activity in isolated membranes from duct cells was increased by secretin (Fölsch and Creutzfeldt, 1975). In addition, the effect of secretin on fluid and bicarbonate secretion was mimicked by dibutyryl adenosine 3', 5' -cyclic monophosphate (Case and Scratcherd, 1972; Wizemann et. al., 1973). Cholera toxin, a specific activator of the adenylate cyclase in many cells, stimulated an increase in cAMP and in fluid secretion in the pancreas from rat (Kempen et. al., 1975; Smith and Case, 1975) and cat (Smith and Case, 1975), whereas enzyme secretion was not stimulated. The mechanism by which cAMP induces electrolyte secretion is unknown.

The transport processes responsible for ductular secretion of a bicarbonate-rich fluid have been recently discussed by Scratcherd et. al. (1981). Possible cellular mechanisms involve the diffusion of the bicarbonate ion into the ductular lumen and the active transport of the proton back into the cell via a proton pump (Mg^{2+} -ATPase) which concomittantly transports Na^{+} into the duct lumen (Schulz, 1980 a). The proton moves across the basolateral membrane into the extracellular fluid via a hydrogen-sodium exchange carrier (Swanson and Solomon, 1972, 1975; Schulz, 1980). The energy for this latter exchange may be provided by the (Na^{+} - K^{+}) -ATPase located in the basolateral membrane. As for chloride transport - passive diffusion down its electrochemical gradient probably accounts for the majority of chloride flux, but a chloride-bicarbonate exchange carrier has also been suggested by Scratcherd and Hutson (1981).

The mechanism of action of agonists on fluid secretion from acinar cells has been reviewed recently by Petersen et. al. (1981). Agonists such as acetylcholine, cholecystokinin and bombesin stimulate a calcium-dependent uptake of Na^+ and Cl^- into the acinar cells (Iwatsuki and Petersen, 1977 a, b; Putney and Van de Walle, 1980 a). The inward Na^+ current resulting from the agonist-induced increase in the permeability of Na^+ is of sufficient magnitude to account for the acinar secretion of Na^+, however, a direct link between the influx and secretion of Na^+ has not been demonstrated (Petersen et. al., 1981). The depolarization due to Na^+ influx into the acinar cell provides the driving force for Cl^- influx. K^+ ions move out of the rat and mouse acinar cells by electrodiffusion through Ca^{2+} -activated non-discriminatory cation channels (Petersen and Maruyama, 1984). The evidence for the ion movements and their control by calcium will be discussed in detail in 2.4.

The transport processes involved in pancreatic acinar fluid secretion have not been clearly identified and so have been the subject of recent investigation and discussion (Chipperfield, 1984; Petersen, 1984; Singh, 1984; Case et. al., 1984; Seow and Young, 1984 a).

An early study by Petersen (1970) showed that agonist-stimulated K^+ efflux from perfused cat submandibular gland was followed by K^+ re-uptake. This re-uptake was blocked when Cl^- was replaced by NO^-_3 and also was dependent on external Na^+. A $Na^+/K^+/Cl^-$ cotransport system appears to be present in salivary gland and it is likely that this cotransporter may also be present in pancreatic acinar cells (Petersen, 1984). The operation of the $Na^+/K^+/Cl^-$

cotransport system is dependent on the Na^+ gradient and so on the operation of the Na^+/K^+ pump. There is at present no evidence for the localization of this cotransport system in pancreas but if present in the basolateral membrane it would provide a mechanism for uptake of Na^+ and Cl^- which could lead to fluid and electrolyte secretion at the luminal membrane. Case et. al. (1984) showed that fluid secretion in mandibular glands appears to depend on two independent transport systems located in the basolateral plasma membrane. One is a Cl^--dependent, furosemide-sensitive system, probably a Na^+/Cl^- (or $Na^+/K^+/Cl^-$) symport, as suggested earlier by Petersen (1970). The other is HCO_3-dependent, affected by carbonic-anhydrase inhibitors and likely to be a double antiport system exchanging Na^+/H^+ and Cl^-/HCO^-_3. Recently, the possible role of one or both of these transport systems in pancreatic acinar fluid secretion has been examined.

Seow et. al. (1984) reported that diuretics inhibited agonist-stimulated secretion from perfused rat pancreas and that the potency of these drugs was reduced by the presence of HCO^-_3 in the perfusate. In addition, the dose-response curves for inhibition by diuretics was biphasic. These effects are not consistent with inhibition of a chloride symport since this should not be affected by HCO^-_3, nor should the dose response curves by biphasic. It was suggested that rat pancreatic acini possess a system of double antiports for Na^+/H^+ and Cl^-/HCO^-_3 exchange and that secretion could not depend solely on a Cl^- symport (Seow et. al., 1984). In a further study, Seow and Young (1984) reported that inhibitors of the $Na^+/K^+/Cl^-$ symport (diuretics) and drugs which affect the Na^+/H^+ antiport (amiloride) and $Cl^-HCO^-_3$

antiports (SITS : 4-acetamido, 4-isothiocyanato, stilbene 2-2 disulphonic acid) each produced almost complete inhibition of caerulein-stimulated fluid secretion in a HCO^-_3 and/or Cl^- containing fluid. When all the extracellular anions except for 25mM HCO^-_3 or Cl^-, were replaced with isethionate, secretion from the rat pancreatic acini was blocked completely. If the $Na^+/K^+/Cl^-$ cotransporter was functional, a small amount of secretion might be expected in a Cl^- containing, HCO^-_3 free solution. These results suggested that a Na^+/H^+, Cl^-/HCO^-_3 double antiport system is present in rat pancreatic acini and also indicated that a chloride symport may not be present (Seow and Young, 1984).

The existence of a diuretic sensitive, cation- and anion-dependent cotransporter in mouse pancreatic acinar cells was investigated by Singh (1984) by examining the ionic dependency and diuretic sensitivity of secretagogue stimulated K^+ transport using $^{86}Rb^+$ as a tracer. The results of the study suggested that in addition to the Ca^{2+}-activated cation channel, K^+ extrusion from acinar cells may also be via a $Na^+/K^+/Cl^-$ cotransporter carrier system since the removal of chloride and the presence of the diuretics furosemide and piretanide markedly inhibited secretagogue-evoked $^{86}Rb^+$ release (Singh, 1984). The basal efflux of $^{86}Rb^+$ was also reduced by diuretics in agreement with the results of Chipperfield (1984) using mouse pancreas. There is no evidence at present as to the location of the $Na^+/K^+/Cl^-$ cotransporter. Its presence in the basolateral membrane would not aid in fluid secretion since it would cause K^+ release rather than uptake into the cell, whereas if present at the luminal membrane it could be involved in secretion of fluid into the ducts.

Although this cotransporter appears to exist in mouse pancreatic acinar cells (Singh, 1984) there is no evidence to show it has any role in agonist-stimulated fluid secretion in pancreatic acinar cells. The studies by Seow et. al. (1984) and Seow and Young (1984) do provide evidence of a functional role for a Na^+/H^+, Cl^-/HCO^-_3 antiport system in fluid secretion in rat pancreatic acini. This area is the subject of investigation at present and the contribution of the $Na^+/K^+/Cl^-$ cotransporter or the double antiport system is a controversial one.

2.2 : INTERACTION BETWEEN ENDOCRINE AND EXOCRINE PANCREAS

An interaction between endocrine and exocrine tissue function was initially proposed on the basis of results from dietary studies. Grossman et. al. (1943) showed that a glucose-rich diet was effective in inducing an increase in pancreatic amylase output. Several studies have shown that insulin-dependent diabetic patients have abnormal responses to secretin and cholecystokinin and so the secretion of pancreatic fluid and enzymes is affected (Chey et. al., 1964; Domschke et. al., 1975). In experimental animals, the destruction of β cells by toxins such as alloxan and streptozotocin induces diabetes and results in a decrease in pancreatic amylase levels which can be reversed by *in vivo* administration of insulin (Söling and Unger, 1972).

An interaction between endocrine and exocrine tissue function has also been shown by *in vitro* studies since insulin, secreted by the endocrine tissue, caused stimulation of protein synthesis (Korc et. al., 1981 a)and glucose uptake in pancreatic acinar cells (Williams et. al., 1981). In addition, insulin although unable alone to evoke pancreatic

secretion, potentiated pancreatic fluid and enzyme secretion stimulated by agonists which mobilize calcium (Kanno and Saito, 1976; Saito et. al., 1980). It is not known by what mechanism insulin produces this effect, although it appears not to be via Ca^{2+} since this hormone had no effect on $^{45}Ca^{2+}$ content of pancreatic acini (Williams et. al., 1982 b).

To further examine the effect of insulin on pancreatic exocrine function, Otsuki and Williams (1982) studied isolated pancreatic acini prepared from streptozotocin-induced diabetic rats. Two defects were observed in diabetes. The content of amylase and ribonuclease was markedly reduced leading to an altered amount of zymogen secretion in response to CCK and the cholinergic agent, carbamylcholine. In addition, the sensitivity to CCK but not to carbamylcholine was altered in acini from diabetic rats.

Both insulin and CCK stimulate the phosphorylation of 23kDa and 32.5kDa proteins in pancreatic acini (Burnham and Williams, 1982 a). When these two hormones are present simultaneously the phosphorylation of the two proteins was greater than the effect of either hormone alone. In contrast, insulin had no effect on the dephosphorylation of the 21kDa and 20.5kDa proteins that were regulated by CCK. From these results it has been suggested that the phosphorylation of the 23kDa and 32.5kDa proteins by insulin and CCK may be involved in their additive effects on glucose transport and protein synthesis, whereas the dephosphorylation of the 21kDa and 20.5kDa proteins may mediate CCK-stimulated zymogen release (Goldfine and Williams, 1983).

A capillary portal circulation between the islets of Langerhans and the acinar portion of the pancreas has been demonstrated by

measurements of blood flow which showed that 11 to 12% of total pancreatic blood flow went directly to the islets (Lipson et. al., 1980). The results supported the view that all or nearly all of the efferent islet blood flow enters capillaries surrounding the acinar cells prior to entering the systemic viens. This study suggested that insulin would reach the exocrine tissue in much higher concentrations compared with peripheral blood and so could influence the exocrine pancreas. A more recent study by Bonner-Weir and Orci (1982) investigated the islet microvasculature using methacrylate corrosion casts in rats. Their study showed that only efferent capillaries from small islets partially passed through peri-insular exocrine tissues before coalescing into venules, whereas efferent capillaries from intermediate and large islets did not.

Other endocrine-exocrine interactions in the pancreas have been reported with studies on the effects of hormones produced by the islet cells, such as somatostatin and pancreatic polypeptide. Immunohistochemically, somatostatin-producing cells have been found in the pancreatic islets of different species (Luft et. al., 1974). Recently, somatostatin binding to purified pancreatic acinar plasma membranes has been demonstrated. The somatostatin receptor appears to be a single protein with a Mr of 90,000 and the binding of somatostatin to this receptor in pancreatic plasma membranes was regulated by CCK and CCK analogues (Sakamoto et. al., 1984). This indicates that CCK may regulate any possible effect of somatostatin in pancreatic acinar cells.

The effect of somatostatin on exocrine function has not been clearly defined. An inhibitory effect of this hormone on pancreatic

secretion *in vivo* was reported in humans (Dollinger et. al.,1976), dogs (Susini et. al., 1976) and rats (Fölsch et. al., 1978 b). However, even high doses of somatostatin had only a weak inhibitory effect on pancreatic enzyme release from *in vitro* preparations of rat and cat pancreas (Albinus et. al., 1976).

Pancreatic polypeptide, released from the endocrine pancreas has been shown to both stimulate and inhibit dose-dependently secretin- and CCK-stimulated pancreatic secretion (Lin et. al., 1977). This hormone was also reported to inhibit bicarbonate secretion caused by exogenous and endogenous secretin in other studies (Greenberg et. al., 1978; Adrian et. al., 1979; Chance et. al., 1981). A recent study by Beglinger et. al. (1984) demonstrated that pancreatic polypeptide inhibited exocrine secretion in dogs *in vivo* in response to six different stimulants. It was suggested that pancreatic polypeptide is an important component of a negative feedback loop that regulates exocrine pancreatic secretion after feeding (Beglinger et. al., 1984).

Another hormone released from the islet cells is glucagon. Natural glucagon can increase enzyme secretion from fragments or dispersed acinar cells from mouse pancreas (Manabe and Steer, 1979; Singh, 1980 a). However, these studies did not eliminate the possibility that some agent contaminating the natural glucagon preparation was responsible for the increase in enzyme secretion. A recent study by Pandol et. al. (1983) using dispersed acini from guinea-pig pancreas, demonstrated that although natural glucagon stimulated enzyme

secretion, biologically active synthetic glucagon did not. When natural glucagon was subject to reverse-phase, high pressure liquid chromatography, the amylase releasing activity in natural glucagon occurred in multiple peaks, none of which coeluted with glucagon. These results indicated that amylase secretion was increased by some as yet unidentified contaminant of the natural glucagon rather than glucagon per se. Although the identity of this secretagogue is not known, the results demonstrate another example of endocrine and exocrine interaction.

2.3 : PANCREATIC SECRETAGOGUES

2.3 (a) Secretagogues which Increase the Intracellular Concentration of Calcium

The pancreatic acinar cell has four groups of receptors which mobilize calcium and lead to an increase in enzyme secretion. These receptors interact with the following agents:

Group 1 : Muscarinic cholinergic agents (Petersen and Ueda, 1976 a; Williams et. al., 1978; Ng et. al., 1979; Gardner and Jensen, 1980; Schulz and Stolze, 1980.

Group 2 : Cholecystokinin and the structurally related peptides, caerulein and gastrin (Jensen et. al., 1980; Sankaran et. al., 1982; Williams et. al., 1982 a).

Group 3 : Bombesin and structurally related peptides, litorin, ranatensin and alytensin (Deschodt-Lanckman et. al., 1976; Jensen et. al., 1978; Uhlemann et. al., 1979; Lee et. al., 1980).

Group 4 : Physalaemin and strcturally related peptides substance P, eledoisin and kassinin (Uhlemann et. al., 1979; Jensen and Gardner, 1979).

Secretagogues which cause an increase in intracellular calcium also stimulate an increase in another possible intracellular second messenger-diacylglycerol which activates protein kinase C (Kishimoto et. al., 1980). Evidence has recently accumulated to support a role for diacylglycerol in activating cell responses (Gunther and Jamieson, 1979; Gunther, 1981 b; Wooten and Wrenn, 1984 a; Wrenn and Wooten, 1984; Knight and Scrutton, 1984; Di Virgilio et. al., 1984), which has led to the questioning of the importance of calcium in mediating cell secretion and has been the subject of recent discussion (Rink et. al., 1983; Baker, 1984; Di Virgilio, 1984). The subject of the role of calcium and other possible intracellular messengers which may mediate pancreatic secretion stimulated by agonists in the above four groups is a major area of this review and will be discussed in detail in the sections to follow.

2.3 (b) Secretatogues which Cause an Increase in the Intracellular Concentration of cAMP

Activation of pancreatic receptors by the following groups of agonists results in an increase in cAMP.

Group 1 : Secretin, VIP and the heptacosapeptide PHI (peptide, histidine, isoleucine), Helodermin (Deschodt-Lanckman et. al., 1975; Robberecht et. al., 1976; Dimaline and Dockray, 1980; Pearson et. al., 1981 a; Bissonnette et. al., 1984; Raufman et. al., 1982; Robberecht et. al., 1984 a).

Group 2 : Contaminants of natural glucagon (Pandol et. al., 1983).

Group 3 : Adrenergic agonists (Lingard and Young, 1983; Pearson and Singh, 1983; Pearson et. al., 1984).

Group 4 : Cholecystokinin (Renckens et. al., 1980; Gardner et. al., 1983).

Initial studies (Christophe et. al., 1976; Gardner et. al., 1976; Gardner et. al., 1979 a), have shown that acinar cells from guinea-pig pancreas possess two classes of receptors each of which interacts with VIP and secretin. One class is VIP-preferring in that it has a high affinity for VIP and a low affinity for secretin. The other class is secretin-preferring in that it has a high affinity for secretin and a low affinity for VIP. Occupation of either class of receptors by VIP or secretin causes activation of adenylate cyclase and increased cellular cAMP (Robberecht et. al., 1976, 1977; Gardner et. al., 1976, 1979 a; Olinger and Gardner, 1979). Stimulation of enzyme secretion, however, is associated with occupation of the VIP-preferring receptors, the increase in cAMP caused by occupation of the secretin-preferring receptor does not cause an increase in enzyme secretion (Gardner et. al., 1979 a). The function altered (if any) by occupation of the secretin-preferring receptors is not known.

This indicates that an increase in the intracellular cAMP content does not always trigger enzyme secretion and suggests

that there may be an as yet, unknown mechanism activated upon occupation of the VIP-preferring receptor which allows the cell to differentiate between an increase in cAMP stimulated by each class of VIP-preferring or secretin-preferring receptor. One such mechanism may be compartmentalization of cellular cAMP within the acinar cell. Gardner et. al. (1982), suggested that occupation of VIP-preferring and secretin-preferring receptors causes accumulation of cAMP into different cellular compartments. The two compartments of cAMP appear to be acted on by phosphodiesterase enzymes having different sensitivities to various inhibitors (Gardner et. al., 1982).

The idea of compartmentalization implies that only one pool of cAMP is able to act to stimulate enzyme secretion. The mechanism by which the two different pools of cAMP can differentially activate secretion is not known. There are species differences in the ability of secretin and VIP to stimulate pancreatic enzyme secretion. In the pancreas from mouse, cat, dog, rat and guinea-pig, secretin and VIP caused an increase in cAMP (Robberecht et. al., 1977), although enzyme secretion was only increased in rat and guinea-pig (Robberecht et. al., 1977; Singh, 1982). This failure of VIP and secretin to increase enzyme secretion in mouse pancreas fragments was inconsistent with the earlier finding by Kulka and Sternlicht (1968) that dibutyryl cAMP caused an increase in enzyme secretion from whole mouse pancreas. Collen et. al. (1982) recently demonstrated that in dispersed acini from mouse pancreas VIP did cause an increase in amylase secretion;

the reason for the lack of effect of VIP in mouse pancreas in the study by Robberecht et. al. (1977) is not known. It has been suggested that the reason for such species differences in the ability of VIP and secretin to cause amylase secretion may be due to the lack of VIP-preferring receptors. One finding in support of this possibility is that studies of (^{125}I)-secretin binding to partially purified membranes from cat pancreas showed a single class of binding sites that had a high affinity for secretin and a low affinity for VIP (Multinovic et. al., 1976). The increase in cAMP that does occur in dog and cat could be due to occupation of the secretin-preferring receptors, causing compartmentalization of cAMP which does not lead to amylase secretion (Gardner et. al., 1982).

There are also differences in the classes of receptors for VIP and secretin in species where these agonists cause both increases in cAMP and amylase secretion. A recent study by Robberecht et. al. (1982) measured binding of (^{125}I)-VIP and the abilities of VIP and secretin to activate adenylate cyclase in plasma membranes from the whole pancreas of the rat. Unlike the receptors in guinea-pig, it was concluded that rat pancreas has three distinct classes of receptors - one class has a high affinity for secretin, the other a low affinity for secretin, each activates adenylate cyclase. Neither class of secretin receptor interacts with VIP. The third class of receptors interacts with VIP and secretin; however, activation of adenylate cyclase occurs only when these receptors are occupied by VIP.

Bissonette et. al. (1984) suggested that the study by Robberecht et. al. (1982) using whole pancreas would not enable determination of whether the receptors were present on acinar cells or duct cells. Using dispersed acini from rat pancreas four classes of receptors which interact with VIP and secretin were described (Bissonnette et. al., 1984). Three of these classes caused stimulation of amylase secretion - two by a cAMP-mediated mechanism and one by a non-cAMP-mediated mechanism. A fourth class of receptors interacted with VIP and secretin but did not increase cAMP or amylase secretion. Although the studies of rat pancreas by Robberecht et. al. (1982) and Bissonnette et. al. (1984) differ, it is clear that the rat pancreas is substantially different from the guinea-pig pancreas in the number of classes of receptors that interact with VIP and/or secretin.

Excitation by electrical field stimulation of non-cholinergic, non-adrenergic nerves in segments of guinea-pig pancreas resulted in enzyme secretion mediated by changes in cyclic nucleotide levels (Pearson et. al., 1981 a, b). The neurotransmitter released from the secretomotor nerves in the guinea-pig is proposed to be VIP, since VIP *in vitro* mimicked the effect of nerve stimulation (Pearson et. al., 1981 b) and VIP-like immunoreactivity has been found in nerve fibres surrounding acinar cells of guinea-pig pancreas (Buchan et. al., 1983).

A newly identified 27 amino-acid peptide, PHI (refers to the peptide (P) having N-terminal histidine (H) and C-terminal

isoleucine (I) was isolated by Tatemoto and Mutt (1980) from porcine intestinal mucosa and found to have actions similar to VIP on pancreatic secretion in rat and turkey (Dimaline and Dockray, 1980). PHI increased amylase secretion from dispersed acini of guinea-pig pancreas by binding to VIP-preferring receptors and stimulating an increase in cAMP (Jensen et. al., 1981). The apparent affinity of PHI for the VIP-preferring receptors on pancreatic acinar cells was approximately 25 times less than that of VIP while its affinity for the secretin-preferring receptors was approximately 300 times less than that for secretin but equal to that of VIP.

Helodermin is a recently purified peptide from the venom of the lizard *Heloderma suspectum*. On the basis of its biological properties it is related to VIP, secretin and PHI (Robberecht et. al., 1984 a). This peptide is responsible for the increase in cAMP in pancreatic acini caused by the venom of *Heloderma suspectum* and was found to bind to secretin receptors in acini and membranes from rat pancreas (Gillet et. al., 1983; Robberecht et. al., 1984 a). The ability of the venom on *Heloderma suspectum* to stimulate enzyme secretion is not due to helodermin but to another protein component of the venom, pancreatic secretory factor (PSF), which does not increase cAMP (Vandermeers et. al., 1984; Robberecht et. al., 1984 a; Dehaye et. al., 1984 a). In comparison with the binding of this peptide in pancreas, helodermin bound to a class of high-affinity VIP receptors capable of stimulating an increase in the activity of adenylate cyclase in rat liver membranes (Robberecht et. al., 1984 b).

Early studies with glucagon showed a depression of enzyme release in the stimulated pancreas (Dyck et. al., 1967; Wizemann et.al., 1974; Nakajima and Magee, 1970). Natural glucagon caused amylase secretion in mouse pancreas (Manabe and Steer, 1979; Singh, 1980) and in guinea-pig pancreas via an increase in intracellular cAMP content (Pandol et. al., 1983). However, unlike all other secretagogues that cause an increase in enzyme secretion by increasing cAMP, natural glucagon did not interact with the VIP-preferring receptors (Pandol et. al., 1983). Biologically-active synthetic glucagon did not stimulate enzyme secretion (Pandol et. al., 1980). The effect of natural glucagon was found to be due not to glucagon *per se* but to some previously undescribed peptide secretagogue which contaminates the natural glucagon. The peptide is structurally different from any known secretagogue and increases cAMP not by interacting with the VIP-preferring receptor but by acting on an as yet undefined receptor (Pandol et. al., 1983).

Studies which have examined the adrenergic control of pancreatic secretion have provided conflicting results. The early study by Greengard et. al. (1942) suggested that the inhibition of pancreatic secretion by adrenergic stimulation was due not to a direct action on the acinar cell but to vasoconstriction. An inhibition of secretion has been found more recently in rabbits (Hubel, 1970) and cats (Barlow et. al., 1974; Elisha et. al., 1984). However, there appear to be species differences in regard to adrenergic effects in pancreas, since

in the rat, stimulation by adrenergic agonists has been shown to increase (Furata et. al., 1978; Lingard and Young, 1983; Pearson and Singh, 1983), or to have no effect (Demol and Sarles, 1980) on pancreatic secretion. In dogs, the effect of adrenergic agonists was to decrease secretion (Kelly et. al., 1974; Rudick et. al., 1973) although contradictory results were obtained (Vaysse et. al., 1977). The possibility of a combined effect of adrenergic agonists *in vivo* on both vasoconstriction and secretion would result in difficulty in interpreting the agonist effects and could also lead to contradictory results.

Pearson et. al. (1984) further examined the mechanism whereby adrenergic stimulation may activate pancreatic secretion in the rat. Using atropine-treated rat pancreas, a non-cholinergic component of amylase secretion evoked by electrical field stimulation was identified. This secretion was reduced by β_1 - and β_2 -adrenergic antagonists although the β_1-antagonist caused a more pronounced reduction. Electrical field stimulation and β -adrenergic agonists stimulated amylase secretion and also an increase in cAMP with no effect on ^{45}Ca metabolism or acinar cell membrane potential and resistance. It was therefore suggested that stimulation of adrenergic nerves in rat pancreas causes an increase in cAMP which is responsible for amylase secretion. Although investigations of the adrenergic nerves in the pancreas have shown that they innervate mainly the islet cells (Ahren et. al., 1981), vessels (Alm et. al., 1967) and ganglia (Larsson and Rehfeld, 1979), a small number of adrenergic

fibres have been identified dispersed within the acinar portions of the gland (Ahren et. al., 1981). Pearson et. al. (1984) suggested that this small innervation together with overflow of noradrenaline released from the more richly supplied areas of the gland may be sufficient to account for the small adrenergic secretion in the rat. It has been suggested that β -receptors are present on both ductal cells and acinar cells since an increase in both fluid and protein secretion was stimulated by β -agonists (Lingard and Young, 1983). The increase in fluid secretion by β -agonists was identified to originate from the duct cells rather than acinar cells since β -adrenergic activation caused an increase in output of fluid having a high bicarbonate content (characteristic of duct fluid secretion)(Lingard and Young, 1983).

The finding that cholecystokinin stimulated adenylate cyclase activity in broken cell preparations of pancreas (Rutten et. al., 1972; Marois et. al., 1972; Svoboda et. al., 1976; Long and Gardner 1977) suggested a role for cAMP in mediating the action of CCK. However, the effects of CCK on pancreatic cAMP levels have been contradictory. An increase in cAMP was stimulated by CCK in rat pancreas (Deschodt-Lanckman et. al., 1975; Renckens et. al., 1980) and rat pancreatic acini (Kempen et. al., 1977), however, no increase was observed in studies on guinea-pig pancreas slices (Benz et. al., 1972; Albano et. al., 1976) or dissociated cells (Gardner et. al., 1976) or rat pancreatic acini (Pan et. al., 1982). The concentrations of CCK that increased calcium outflux, amylase release and cyclic guanosine

monophosphate (cGMP) did not increase cAMP (Deschodt-Lanckman et. al., 1975; Long and Gardner, 1977) indicating cAMP does not appear to mediate the action of CCK in stimulating enzyme secretion.

Recently it was reported that CCK, alone or in the presence of theophylline (a cyclic nucleotide phosphodiesterase inhibitor) did not cause an increase in cAMP in guinea-pig pancreas. However, concentrations of CCK which were supramaximal for stimulating amylase secretion caused a significant increase in cellular cAMP in the presence of other phosphodiesterase inhibitors, isobutylmethylxanthine and Ro 20-1724 (Gardner et. al., 1983). These results supported the earlier studies by Kempen et. al. (1977) and Renckens et. al. (1980) where CCK in the presence of isobutylmethylxanthine stimulated an increase in cellular cAMP. The augmentation of amylase secretion in guinea-pig pancreas by the phosphodiesterase inhibitors occurred with supramaximal concentrations of cholecystokinin but not with bombesin or carbachol (Gardner et. al., 1983). It would appear that this effect of CCK may not be physiologically important since it did not occur unless a cyclic nucleotide phosphodiesterase inhibitor, isobutylmethylxanthine was present suggesting that *in vivo* the cAMP is degraded too rapidly to cause stimulation of enzyme secretion.

An investigation of how occupation of the CCK receptor can activate increases in both calcium and cAMP may provide further information regarding the link between receptor activation and intracellular second messengers.

The studies described above have provided evidence to support a role for cAMP in mediating pancreatic secretion stimulated by a number of hormones, although species differences have been demonstrated. To further examine this role of cAMP, the effect of non-hormonal activators of adenylate cyclase : cholera toxin (Gill, 1977) and forskolin (Seamon et. al., 1981; Insel et. al., 1982) on pancreatic secretion have been examined.

In dispersed acini from guinea-pig pancreas, (^{125}I)-labelled cholera toxin was found to bind to sites that interact with cholera toxin but not with other secretagogues and to increase cAMP and stimulate enzyme secretion (Gardner and Rottman, 1979). In rat pancreas *in vivo* or in slices *in vitro* the cholera toxin induced increase in cAMP was not accompanied by an increase in enzyme secretion (Kempen et. al., 1975; Smith and Case, 1975). These results conflicted with the ability of VIP, secretin and derivatives of cAMP to increase enzyme secretion in rat pancreas *in vivo* or in fragments (Deschodt-Lanckman et. al., 1975; Robberecht et. al., 1977; Kempen et. al., 1977).

To clarify these results, Singh (1982) and Pan et. al. (1982) examined the effects of cholera toxin on dissociated rat pancreatic acini, since this preparation has been reported to be more responsive to secretagogues than pancreatic tissue slices (Peikin et. al., 1978; Williams et. al., 1978; Singh, 1982). Cholera toxin in the absence of a phosphodiesterase inhibitor, caused only a small stimulation of enzyme secretion (Pan et. al., 1982; Singh, 1982). This small effect of an

increase in cAMP on amylase secretion was in agreement with the small (two fold) increase in amylase secretion stimulated by VIP or secretin in rat acini (Collen et. al., 1982: Pan et. al., 1982; Singh et. al., 1982). In guinea-pig acini, however, VIP or secretin caused a four to six fold stimulation of secretion (Robberecht et. al., 1976; Gardner et. al., 1979).

Forskolin, a non-hormonal activator of adenylate cyclase in rat pancreatic acinar cells (Heisler, 1983) and in broken cell preparations and other intact tissues (Seamon et. al., 1981), caused a rapid and marked increase in cAMP but it was also a weak stimulant of amylase secretion in rat pancreatic acini (Heisler, 1983). The effect of forskolin on pancreatic acini from guinea-pig was not examined.

Early studies reported that the increase in amylase secretion stimulated by the combination of a secretagogue which acts via calcium with one which causes an increase in cAMP was shown to be greater than the sum of the increase caused by each agent acting alone (Gardner and Jackson, 1977; Peikin et. al., 1978; Uhlemann et. al., 1979).

This potentiation is a post-receptor effect which is not due to amplification of secretagogue-stimulated increases in cAMP or calcium since:

(1) Cholera toxin, VIP or secretin do not alter calcium transport in pancreatic acini or the changes in calcium transport caused by other secretagogues (Collen et. al., 1982; Pan et. al., 1982; Singh , 1982).

(2) Secretagogues which mobilize calcium do not alter cAMP or the increase in cAMP caused by cholera toxin, VIP or secretin (Collen et. al., 1982; Pan et. al., 1982; Singh, 1982).

(3) None of the secretagogues which mobilize cellular calcium alter the binding of(^{125}I)-VIP (Collen et. al., 1982).

(4) Potentiation of enzyme secretion occurs with two agents which bypass receptor activation: 8 -bromo cyclic AMP and ionophore A23187 (Collen et. al., 1982; Pan et. al., 1982).

This effect of potentiation by calcium of amylase secretion stimulated by secretagogues which act via cAMP is of minor importance in acini from guinea-pig, but causes a major increase in acini from rats and mice (Collen et. al., 1982; Pan et. al., 1982; Singh, 1982). The reason for this species variation is not known.

The question of how cAMP mediates pancreatic secretion has not been answered. It has been suggested that an alteration in the state of phosphorylation of specific proteins by cAMP may play a role in linking stimulus with response (Greengard, 1982). A number of studies have investigated protein phosphorylation and dephosphorylation in parotid glands (Kanamori and Hayakawa, 1980; Jahn et. al., 1980; Jahn and Söling, 1981 a), lacrimal gland (Jahn and Söling, 1981 b; Jahn et. al., 1982) and exocrine pancreas (Freedman and Jamieson, 1980, 1981; Burnham

and Williams, 1982 a, 1984; Jahn and Söling, 1983; Roberts and Butcher, 1983). The state of protein phosphorylation is controlled by specific protein kinases and phosphatases which can be regulated by calcium and cAMP (Cohen, 1982).

Studies examining the role of protein phosphorylation in rat exocrine pancreatic secretion demonstrated a secretagogue-stimulated phosphorylation of a protein M_r = 29,000 (Freedman and Jamieson, 1980) which was localized to a highly enriched ribosomal fraction and which showed a Ca^{2+} and cAMP-dependent phosphorylation in homogenates of pancreas (Freedman and Jamieson, 1981). In mouse exocrine pancreas derivates of cAMP stimulated phosphorylation of proteins M_r = 95,500, 32,000 and 20,000 (Roberts and Butcher, 1983), and a M_r = 92,000 proteins (Burnham and Williams, 1984). In guinea-pig pancreas a protein M_r = 29,000 - 35,000 which is phosphorylated by Ca^{2+} and cAMP was identified to be a ribosomal protein (Jahn and Söling, 1983). Although a number of different proteins have been identified in response to secretagogue stimulation their role (if any) in secretion is not known.

Several studies have identified cyclic nucleotide-activated protein kinases in rat pancreas (Lambert et. al., 1974; Jensen and Gardner, 1978; Lewis and Ronzio, 1979). Recently Burnham and Williams (1984) reported a cAMP-activated protein kinase activity in mouse pancreatic acini which was present in both the cytosolic and high-speed particulate fraction. Identification of protein substrates for this enzyme which are involved in stimulating exocytosis is required before the mechanism of action of cAMP in secretion is defined.

As described above, both Ca^{2+} and cAMP can stimulate the phosphorylation of the same protein(s). The potentiation by Ca^{2+} of cAMP-stimulated pancreatic secretion could be by an interaction between Ca^{2+} and cAMP on phosphorylation. This has yet to be investigated.

2.3 (c) Pancreatic Secretagogues which do not Appear to Act via Increasing the Intracellular Concentration of Calcium or cAMP

The venom from the lizard *Heloderma suspectum* or (Gila Monster venom) stimulated the release of amylase and the production of cAMP in pancreatic acini from guinea-pig (Raufman et. al., 1982) and rat (Dehaye et. al., 1984 a). This venom consists of two compounds: helodermin and pancreatic secretory factor (PSF). Helodermin as described in 2.3 (b), is a VIP-like component which increases cAMP production but the secretory effect is due to PSF which is a 17.5kDa protein that neither modifies cAMP concentration nor stimulates calcium fluxes.

Stimulation of amylase secretion from rat pancreatic acini by PSF required calcium in the extracellular fluid (Dehaye et. al., 1984 a), which suggested that perhaps PSF acted via an increase in cellular calcium. However, when PSF and a secretagogue which acts via calcium or cAMP (as described in 3.2 (a) and 3.2 (b)) were present simultaneously, an additive effect on secretion was seen (Dehaye et. al., 1984 a), which suggests that PSF may act independently of increases in calcium or cAMP.

The recent discovery that PSF has phospholipase A_2 activity (Dehaye et. al., 1984 b) has provided further insight into its possible action. This enzyme activity is calcium-dependent (Dehaye et. al., 1984 b), which would explain why the secretory action of PSF was dependent on extracellular calcium. The mechanism by which phospholipase A_2 activity stimulates pancreatic secretion is unknown at present. Possible modes of action could be via arachidonic acid or lysophospholipids, products released from phospholipids following phospholipase A_2 hydrolysis.

Arachidonic acid can be converted into prostaglandins and hydroxy-acids via the cyclo-oxygenase and lipoxygenase pathways, respectively (Van den Bosch, 1980). A role for prostaglandins in protein secretion from the exocrine pancreas was suggested by Marshall et. al. (1980, 1981), using whole mouse pancreas. However, experiments using either pancreatic fragments (Heisler, 1973; Baudin et. al., 1981), or acinar cell preparations (Chauvelot et. al., 1979; Stenson and Lobos, 1982; Putney et. al., 1981), failed to show any such role for prostaglandins in mediating pancreatic secretion. In a later study by Marshall et. al. (1982), it was suggested that prostaglandin E_2 derived from the breakdown of inositol phospholipid facilitated enzyme secretion by an action on the ampulla of Vater rather than on acini, as had been thought previously (Marshall et. al., 1980, 1981). Marshall et. al. (1982) proposed that prostaglandin E_2 caused relaxation of the smooth muscle of this sphincter and so allowed washout of

enzymes already present in the ducts, without increasing enzyme release fron acini. This idea was supported by their results showing that inhibition of agonist-stimulated secretion by indomethacin only occurred in intact mouse pancreas and not in acini. Also, smooth muscle relaxants increased amylase release and the presence of prostaglandin E_2 simultaneously with these agents did not cause an additive effect on secretion (Marshall et. al., 1982).

In another study, inhibitors of the cyclo-oxygenase pathway and lipoxygenase pathway did not alter amylase secretion stimulated by the calcium ionophore ionomycin, but did inhibit production of arachidonic acid derivatives (Rubin et. al., 1982). It would therefore appear that prostaglandins are not responsible for the effect of PSF in pancreatic acini. Although lysophospholipids do act as fusogens of cellular membranes (Poole et. al., 1970) there is no evidence for or against these lipids having a role in activating secretion from the exocrine pancreas. It is not clear how PSF acts to increase secretion.

Another agent which elicits enzyme secretion from the pancreas but whose mode of action is not clear is the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA). TPA stimulated enzyme secretion without elevating the intracellular content of cAMP or cGMP in guinea-pig pancreatic acinar cells (Gunther and Jamieson, 1979). Although TPA-induced secretion of protein does show a requirement for extracellular Ca^{2+}, it is less

susceptible to inhibition by removal of extracellular Ca^{2+} than secretion stimulated by caerulein in guinea-pig pancreatic acini (Gunther, 1981 a). It appears that TPA does not act in the same manner as other agonists which cause enzyme secretion in the pancreas via calcium-dependent mechanisms since TPA did not elicit any alteration in the efflux of Ca^{2+}, which occurs as a result of mobilization of calcium (Stolze and Schulz, 1980; Dormer et. al., 1981). The effect of secretagogues on Ca^{2+} fluxes will be discussed in detail in Section 2.5 of this thesis.

Although the mechanism by which TPA activates pancreatic secretion is not definitely known, recent evidence indicates that it may involve activation of the Ca^{2+}-and phospholipid-dependent protein kinase (protein kinase C). Tumour-promoting phorbol esters can substitute for diacylglycerol in the activation of protein kinase C *in vitro* (Castagna et. al., 1982) and can lower the Ca^{2+} requirement for activation of this enzyme by agents which act to increase cellular Ca^{2+} (Takai et. al., 1979 a; Castagna et. al., 982).

TPA binds to high affinity receptors in pancreatic acinar cells (Gunther, 1981 b) and it has been proposed that the TPA receptor may be identical to, or closely associated with this kinase (Castagna et. al., 1982; Niedel et. al., 1983). A recent study by Wooten and Wren (1984) showed that TPA caused a translocation of soluble protein kinase C from the cytosol to the particulate fraction, and that this redistribution was concurrent with stimulation of amylase secretion by TPA-treated acini. In

addition, polymix B, an inhibitor of protein kinase C, inhibited amylase release induced by TPA (Wooten and Wren, 1984), and carbachol (Wrenn and Wooten, 1984) in rat pancreatic acini. These results suggest a role for protein kinase C in activation of pancreatic enzyme secretion.

Recently, TPA has been shown to stimulate secretory responses in other tissues, for example, the release of serotonin from platelets (Knight and Scrutton, 1984) and the release of insulin from pancreatic islet cells (Hubinot et. al., 1984).

It has been suggested that activation of protein kinase C by agonists which increase intracellular calcium may play a role in stimulus-response coupling and that calcium and protein kinase C may act synergistically (Michell, 1983). The Ca^{2+} -dependency of the TPA-induced secretion from pancreas (Gunther, 1981) may be due to such a synergism between calcium and protein kinase C .

Even if it becomes clear that TPA acts via activation of protein kinase C, the mechanism by which this enzyme activates secretion remains unknown. The role of protein kinase C in mediating cell responses activated by agonists which mobilize Ca^{2+} is discussed in a separate section, 2.7.

2.4 : THE ROLE OF CALCIUM IN PANCREATIC ENZYME SECRETION

2.4 (a) Evidence for the Involvement of Calcium in Pancreatic Enzyme Secretion

Although the requirement for calcium of pancreatic secretion by a number of secretagogues (as described in Groups 1-4, 2.3 a) is now well recognised, it was only in 1966 when Hokin found that acetylcholine-stimulated secretion of amylase from mouse pancreas slices was dependent on extracellular calcium that the proposal that calcium may be an intracellular messenger in pancreatic secretion was initiated. That an increase in intracellular calcium content is the triggering event for enzyme secretion has been clearly demonstrated by mimicking the effects of secretagogues using procedures which artificially increase intracellular calcium and by-pass receptor activation. The divalent cation ionophore A23187 which allows calcium to enter into cells (Reed and Lardy, 1972) increased pancreatic secretion of amylase (Selinger et. al., 1974; Williams and Lee, 1974; Poulsen and Williams, 1977; Kanno and Saito, 1978; Gardner et. al., 1980; Stark and O'Doherty, 1982). Another cation ionophore, ionomycin, was also found to be effective in increasing amylase secretion from rat pancreatic acini (Halenda and Rubin, 1982).

The dependence of pancreatic enzyme secretion on intracellular and extracellular calcium has been examined by

studies of the effect of calcium depletion. Using incubated pancreas or perfused pancreas, omission of calcium from the extracellular medium reduced secretagogue-stimulated amylase secretion but did not abolish the release of amylase (Kanno, 1972; Williams and Chandler, 1975; Kanno and Nishimura, 1976). In another study, addition of the Ca^{2+}-chelating agent EGTA (ethylene glycol-bis-(β-amino ethyl ether) N, N'-tetra acetic acid) blocked amylase release from the isolated rat pancreas, but only after 60 min (Case and Clausen, 1973). In the superfused mouse pancreas incubated for 60 min in a Ca^{2+}-free solution containing EGTA, amylase secretion in response to short pulses of acetylcholine stimulation at 30 min intervals still occurred (Petersen and Ueda, 1976). In a Ca^{2+}-free solution containing EGTA, and in the presence of sustained stimulation, the initial effect of acetylcholine was relatively unaffected but the duration of secretion was reduced compared with that in a Ca^{2+} -containing medium (Petersen and Ueda, 1976). Re-admission of Ca^{2+} to the perfusion fluid during continued stimulation allowed full recovery of the acetylcholine effect and the amylase secretory response was sustained (Petersen and Ueda, 1976).

Other studies have used isolated acini rather than fragments of intact pancreas. In rat pancreatic acini incubated in either a normal Ca^{2+}-containing medium or a Ca^{2+}-free, EGTA containing medium caerulein-stimulated amylase secretion was similar for the first 10 min, after this time, however, the effect of caerulein in the Ca^{2+}-free medium was lost

(Williams, 1980). In isolated pancreatic acini from guinea-pig in the absence of extracellular Ca^{2+}, A23187 caused an initial stimulation of enzyme release, however, after 10 min of incubation, the acini were depleted of cellular Ca^{2+} and no stimulation by ionophore or any other secretagogue was possible (Gardner et. al., 1980).

These results showed that extracellular calcium is not required for the initial activation of enzyme secretion by secretagogues, but it is required for sustained secretion since cellular stores of calcium (Stolze and Schulz, 1980; Dormer et. al., 1981) become depleted and must be replenished.

A recent study by Dormer (1984) showed that an increase in intracellular Ca^{2+} was required to stimulate enzyme release. The Ca^{2+} -chelators EGTA and BAPTA (1,2-bis (2-aminophenoxy) ethane-NNN'N' -tetraacetic acid) were introduced into intact isolated rat pancreatic acini using a hypotonic swelling method. The carbachol-stimulated release of amylase was inhibited by these chelating agents and the results were consistent with this effect being due to chelation of intracellular Ca^{2+} rather than a non-specific effect, such as ATP depletion or cellular damage (Dormer, 1984).

2.4 (b) Evidence for the Involvement of Changes in Membrane Potential in Protein Secretion

Pancreatic acinar cells normally maintain a resting membrane potential of -40 to -50 mV. Secretagogues cause a

10 to 20 mV depolarization which is accompanied by a decrease in the input resistance of the plasma membrane (Nishiyama and Petersen, 1974; Iwatsuki and Petersen, 1977 b, c). The minimum latency of evoked depolarization after ionophonetic application of acetylcholine to the pancreatic acinar cell was reported to be 150 m sec (Nishiyama, 1984). Studies of the ionic dependence of acetylcholine-induced membrane potential and resistance changes indicated that the depolarization and change in membrane conductance is due to the opening of ion channels in the basolateral cell membrane which allows the passive influx of Na^+ and Cl^- and the efflux of K^+ down their electrochemical gradients (Nishiyama and Petersen, 1975; Iwatsuki and Petersen, 1977 a; Petersen and Maruyama, 1984).

Voltage-clamp studies have confirmed that acetylcholine opens conductance pathways in the mouse pancreatic acinar membrane which is made permeable to Na^+, K^+, and Cl^- (McCandless et. al., 1981). In addition, ion flux measurements have also shown that agonist-induced increases in ^{22}Na uptake (Putney et. al., 1980 a) ^{36}Cl uptake (Putney and Van de Walle, 1980 b) and ^{42}K efflux (Case and Clausen, 1973) occur.

Two possible mechanisms whereby a change in membrane potential may lead to secretion could be (1) by activation of voltage-dependent Ca^{2+} channels or (2) by Na^+ influx causing release of calcium from cellular stores, such as mitochondria. Studies showing that depolarization of the pancreatic cell membrane is

not important in the activation of pancreatic secretion indicated that voltage-dependent Ca^{2+} -channels do not play a role in stimulation of enzyme release. Evidence for this was provided by showing that a 10-fold elevation in extracellular K^+ concentration which depolarized the cells by 25 mV did not cause the release of amylase from superfused pancreas (Poulsen and Williams, 1977) or pancreatic acinar cells (Williams et. al., 1976). Also, in the presence of a high external K^+ concentration, CCK could still stimulate the normal release of enzyme from the pancreas (Argent et. al., 1973).

A number of studies have shown that an increase in intracellular Ca^{2+} precedes rather than occurs as a result of the change in membrane potential. Removal of extracellular Ca^{2+} and incubation with EGTA, caused a marked reduction in the amplitude of small depolarizations evoked by just suprathreshold doses of acetylcholine (Iwatsuki and Petersen, 1977 b) indicating an effect of Ca^{2+} on Na^+ permeability. This role of calcium in controlling membrane properties was further demonstrated by the intracellular microinjection of Ca^{2+} which caused a membrane depolarization and reduction in input resistance which mimicked that seen with acetylcholine (Iwatsuki and Petersen, 1977 c). In addition, injection into mouse pancreatic acinar cells of the calcium chelator EGTA abolished the action of acetylcholine on membrane potential and resistance changes (Laugier and Petersen, 1980).

The observation that ionophore A23187 caused a Ca^{2+} -dependent depolarization of pancreatic acinar cells (Poulsen and Williams, 1977) also supports the idea that depolarization in response to physiological stimulants may be mediated by Ca^{2+}. The depolarization by A23187 was abolished by omission of extracellular Na^{+} (Poulsen and Williams, 1977), confirming the results of Nishiyama and Petersen (1975) and Iwatsuki and Petersen (1977 b) that an influx of Na^{+} not Ca^{2+} is directly responsible for the depolarization caused by secretagogues. The study by Putney et. al. (1980 a) supported these results by demonstrating that the secretagogue-stimulated uptake of ^{22}Na into dispersed pancreatic acinar cells was abolished in the absence of extracellular Ca^{2+} and that the ionophore A23187 (albeit, at a high concentration of $2 \times 10^{-5}M$) stimulated ^{22}Na uptake.

Further evidence of calcium-activated increases in membrane permeability have been obtained recently with the use of the patch-clamp method which enables direct single-channel recording both in intact cells and excised micro-patches (Petersen and Maruyama, 1984). Two types of cation channels have been identified in pancreatic acinar cells (Maruyama and Petersen, 1982 a, b; Maruyama et. al., 1983 a; Petersen and Maruyama, 1984). One of these channels is a non-discriminatory cation channel with a conductance of approximately 30 pS and is voltage-insensitive. This channel is Ca^{2+} -dependent since it can be activated by submicromolar concentrations of Ca^{2+} and its opening and closure cannot be observed in the absence of

Ca^{2+} on the inside of the membrane. The channel is virtually impermeable to Cl^- but equally permeable to all the alkali metal ions (Yellen, 1982; Maruyama and Petersen, 1982 a, b). Although a permeability to Ca^{2+} has not been demonstrated it is thought that Ca^{2+} may also leak into the cells through these cation channels which then in turn maintains the cation channels in their open state (Petersen and Maruyama, 1984).

The second channel identified is highly selective to K^+, has a conductance of 200-250 pS and is also activated by Ca^{2+} but unlike the non-selective cation channel, it is voltage-activated. This latter channel has been found in pig pancreatic acinar cells (Maruyama et. al., 1983 a) but not in mouse and rat pancreatic acinar cells (Petersen and Maruyama, 1984) where only the non-specific cation channel has been found (Maruyama and Petersen, 1982 a, b).

The release of K^+ is a response to a secretagogue-stimulated increase in intracellular Ca^{2+} in salivary glands as well as in exocrine pancreas. In the parotid gland, the K^+ release response consists of two distinct phases (Putney, 1976). A transient phase occurs, lasting 2-4 min which does not require Ca^{2+} in the extracellular medium. In the absence of Ca^{2+} only one such transient response could be measured and it was necessary to incubate parotid gland in a Ca^{2+} -containing solution in order to enable re-activation of the transient phase (Putney, 1977). These observations led to the idea that agonists stimulate the release of a bound pool of Ca^{2+} which

activates the transient release of K^+, and that this pool is replenished by extracellular Ca^{2+}.

The second phase of increased K^+ permeability is more sustained, results in a net loss of tissue K^+ and requires extracellular Ca^{2+} (Selinger et. al., 1973; Putney, 1976 a). The biphasic nature of the K^+ release response in parotid gland reflects the dual mechanism of Ca^{2+} mobilization : intracellular release and influx. In mouse and rat parotid acini and mouse submandibular gland, Maruyama et. al. (1983 b) have identified the K^+ specific, voltage-Ca^{2+} -activated K^+ channel, the non-specific cation channel was not found in these cell types.

The second possible mechanism whereby a change in membrane potential might lead to enzyme secretion could be by an increase in Na^+ content. Omission of Na^+ from the extracellular medium caused an inhibition of agonist-stimulated amylase secretion in pancreas of cat (Argent et. al., 1973), rat (Case and Clausen, 1973) and mouse (Williams, 1975 a; Petersen and Ueda, 1976 a). However, it became clear that this effect of Na^+ was due to inhibition of fluid secretion in the preparations used since inhibition of amylase secretion by Na^+ removal did not occur in acini (Williams et. al., 1976). This indicated that amylase secretion was not dependent on Na^+ influx and so occurred independently of depolarization and that in the intact pancreas or in fragments (Argent et. al., 1973; Case and Clausen, 1973; Petersen and Ueda, 1976 a) amylase secretion

was stimulated but was unable to be released from the ducts into the extracellular fluid due to the absence of fluid secretion which requires Na^+. Secretagogues, although stimulating an increase in Na^+ uptake into acinar cells (Iwatsuki and Petersen, 1977 b; Putney et. al., 1980 b) caused only small increases in cytosolic Na^+ concentration (O'Doherty and Stark, 1982 b; Preissler and Williams, 1981), suggesting that a Na^+ extrusion mechanism is stimulated simultaneously with the influx of Na^+. Recently, Hootman et. al. (1983) provided the first evidence that the cellular Na^+-K^+ pump is stimulated by the secretagogues in guinea-pig pancreatic acini using the rate of (^3H)-ouabain binding as an index of Na^+-K^+-pump activity. Ouabain is a highly specific inhibitor of Na^+-K^+ pump activity which binds primarily to one conformational state of the pump enzyme - the phosphorylated intermediate (Jorgensen, 1980).

The secretagogue-stimulated Na^+-K^+ pump activity resulting in Na^+ extrusion and K^+ re-uptake evoked by secretagogues was Na^+ dependent but unaffected by the removal of Cl^- from the intracellular fluid (Hootman et. al., 1983).

Although there is no direct effect of a change in membrane potential as demonstrated by electrophysiology, on protein secretion, the ion fluxes which occur may have an indirect effect in the whole gland as they appear to be involved in fluid secretion. As discussed previously in 2.1 the mechanisms involved in acinar fluid secretion are not clearly defined.

The role of a $Na^+/K^+/Cl^-$ cotransporter has been suggested by Petersen (1984) and Singh (1984) while the studies by Seow et. al. (1984) and Seow and Young (1984) tend to put this idea into doubt and have led to the proposal of a Na^+/H^+, Cl^-/HCO^-_3 double antiport system being involved in fluid secretion. This area requires much further investigation.

2.4 (c) Cytoplasmic Calcium Concentrations in Pancreatic Acinar Cells

The intracellular free Ca^{2+} concentration of pancreatic acinar cells has been determined using four different methods. Ochs et. al. (1983) used the Ca^{2+} -selective fluorescent indicator, quin-2 which measures free, cytoplasmic but not membrane-bound Ca^{2+} (Pozzan et. al., 1981); O'Doherty and Stark (1982) used a Ca^{2+} -sensitive microelectrode inserted intracellularly. Maruyama et. al. (1983 a), used patch-clamp methods to record single-channel currents. The relationship between the open-state probability of the K^+ channel and membrane potential was determined in the excised patches at three different bath fluid concentrations of Ca^{2+} and also in an electrically isolated patch in an intact cell. By comparing the curve for open-state probability of K^+ channel vs membrane potential in the intact cell with that obtained for the excised patch, it was possible to determine the intracellular Ca^{2+} concentration (Maruyama et. al., 1983 a). Streb and Schulz (1983) used a method to allow them to measure the cytosolic free Ca^{2+} concentration which could be buffered by intracellular organelles

of rat pancreatic acinar cells. The cells were made permeable by addition of saponin which affects plasma membranes, and the free Ca^{2+} concentration of the surrounding medium was measured with a Ca^{2+} -sensitive macroelectrode. This medium was believed to represent an extended cytosol (Streb and Schulz, 1983), since free ion movement from within and outside the cell would be possible.

In the resting pancreatic acinar cell, the intracellular Ca^{2+} concentration was measured to be 1.8×10^{-7}M in mouse (Ochs et. al., 1983), 4.3×10^{-7}M (O'Doherty and Stark, 1982), between 10^{-8} and 10^{-7}M in pig (Maruyama et. al., 1983). The estimate made by O'Doherty and Stark (1982) was considerably greater than that by Maruyama et. al. (1983 a). The reason for this difference is likely to be due to microelectrode impalement causing the cells to become leaky and to allow an influx of Ca^{2+} to occur. It would appear that this was the case since the resting membrane potential was -26mV (O'Doherty and Stark, 1982) which is much lower than the normal resting membrane potentials of pancreatic acinar cells previously reported of 37mV (Nishoyama and Petersen, 1974) and 43mV (Poulsen and Williams, 1977). This lower resting membrane potential would be explained by a leaky membrane causing dissipation of electrochemical gradients, indicating the membranes of cells used by O'Doherty and Stark (1982) were probably damaged, and so their estimate for cytosolic Ca^{2+} concentration is likely to be incorrect.

The study by Streb and Schulz (1983) showed that as the free

Ca^{2+} concentration was decreased by addition of EGTA, Ca^{2+} was gradually released from the cells until a steady state was reached. If the medium concentration of Ca^{2+} was increased, Ca^{2+} was again taken up until the steady state level was reached. The "Ca^{2+} null point" was obtained and found to be equal to 4×10^{-7}M. This is the medium concentration at which no more Ca^{2+} uptake was observed and gives a measure of the cytosolic free Ca^{2+} concentration that can be buffered by the action of intracellular organelles. However, this value may not be the level which is obtained in the intact, non-permeabilized pancreatic acinar cell since it is higher than the estimates determined by Maruyama et. al. (1983 a), and Ochs et. al. (1983). The reason for this difference is not clear. One possibility is that Ca^{2+} pumps in the plasma membrane which contribute to maintaining a resting cytosolic Ca^{2+} concentration are disrupted by saponin and so the resting Ca^{2+} content is higher than in non-permeabilized cells.

In the presence of acetylcholine and Ca^{2+}, O'Doherty and Stark (1982) measured an increase in intracellular Ca^{2+}. However, they failed to see this effect when Ca^{2+} was absent from the extracellular medium, indicating that the microelectrode was not very sensitive to Ca^{2+} changes. When Ca^{2+} and secretagogues were present in the extracellular medium, a rapid transient increase in free Ca^{2+} content up to a maximum of 86×10^{-7}M was measured (Ochs et. al., 1983). In the absence of extracellular Ca^{2+}, an equivalent increase in free Ca^{2+} content could be elicited (although a higher concentration of agonist

was required), but this change decayed more rapidly than in acini in a Ca^{2+} -containing medium (Ochs et. al., 1983). In pig acini the free Ca^{2+} content identified by Maruyama et. al. (1983 a) was estimated to increase from 5 x 10^{-8}M to 10^{-6}M by acetylcholine or cholecystokinin-pentapeptide. It would therefore appear that secretagogue-stimulation of acinar cells causes at least a 10-fold change in the intracellular free Ca^{2+} concentration.

It is now well established that calcium is essential in triggering the electrophysiological and secretory responses of pancreatic acinar cells. It was not until the last few years that the sites of calcium storage and movements of calcium which occur upon receptor activation have been intensively studied. The question of how receptor activation stimulates a release of calcium from stores and an increase in the permeability of the cell membrane to calcium has for many years remained unanswered. Recent studies have provided evidence to support the role of inositol phospholipid hydrolysis as the crucial mechanism linking receptor activation to increased intracellular Ca^{2+}. Before a review of the processes involved in inositol phospholipid turnover and its role in calcium metabolism is presented it is necessary to discuss the movements of calcium and from what sites this occurs in order to correlate properties of agonist-stimulated inositol lipid hydrolysis with the cellular events occurring in calcium mobilization.

2.5 : CALCIUM FLUXES IN PANCREATIC ACINAR CELLS : THE EVENTS LEADING TO INCREASED INTRACELLULAR CALCIUM CONCENTRATION

The stimulation of the exocrine pancreas by secretagogues causes a biphasic movement of Ca^{2+}, an initial loss of Ca^{2+} followed by an uptake. The initial secretagogue-stimulated efflux of Ca^{2+} from whole-rat pancreas (Case and Clausen, 1973), fragments of mouse and rat pancreas (Williams and Chandler, 1975; Heisler and Grondin, 1973), mouse pancreatic acini (Dormer et. al., 1981) and isolated rat acinar cells (Stolze and Schulz, 1980; Schulz et. al., 1981) has been demonstrated by $^{45}Ca^{2+}$ flux measurement and also by atomic absorption spectrometry measurement of total Ca^{2+} content. Ca^{2+} efflux occurs as a result of the agonist-stimulated release of Ca^{2+} from cellular storage sites into the cell cytosol (Stolze and Schulz, 1980; Dormer et. al., 1981). The mechanism of this Ca^{2+} efflux, however, has not been defined.

The relationship between secretagogue-stimulated Ca^{2+} efflux and uptake is not clear, although Wakasugi et. al. (1981) suggested that these two processes are independent events. Lanthanum, at high concentrations (5mM) blocks both Ca^{2+} efflux and influx but at lower concentrations (1 and 2mM) blocks only Ca^{2+} efflux, indicating that an initial Ca^{2+} efflux is not a requirement for the activation of Ca^{2+} influx. However, these results do not preclude the possibility that a common mechanism activates the processes which lead to efflux and influx of Ca^{2+}. Lanthanum may have a higher affinity for the sites at which Ca^{2+} release from the cell occurs than for the sites at which the Ca^{2+} uptake mechanism acts.

The stimulated efflux is likely to be largely an active process since as the total intracellular Ca^{2+} concentration drops, this would lead to an increase in the gradient for Ca^{2+} to move into rather than out of the cell. There is some evidence to suggest that a component of the stimulated efflux requires energy since antimycin A (which is a redox inhibitor and inhibits the production of ATP) reduced secretagogue-stimulated efflux of Ca^{2+} in isolated mouse pancreatic acini (Dormer et. al., 1981) and dispersed rat pancreatic acinar cells (Stolze and Schulz, 1980). It was suggested that the Ca^{2+} efflux may be due to the active transport of Ca^{2+} by Ca^{2+} -pumps (Stolze and Schulz, 1980). Studies showing inhibition of carbachol-stimulated Ca^{2+} efflux by ouabain indicated that Ca^{2+} extrusion may also be via a Ca^{2+} - Na^{+} counter-transport driven by the primary action of the Na^{+} - K^{+} -pump (Stolze and Schulz, 1980).

The initial Ca^{2+} efflux is followed by the uptake of Ca^{2+}, the mechanism for this process, however, is not clear. There is at present no direct evidence to show that Ca^{2+} influx occurs by an increase in the permeability of the cell membrane due to the opening of "calcium-channels". The studies using the patch-clamp method have not identified channels in the pancreatic acinar cells which are specific for Ca^{2+}. It is possible, however, that Ca^{2+} may move through the non-specific cation channel (Maruyama and Petersen, 1982 a) although this has not been directly demonstrated and requires further investigation.

An indirect demonstration of an increase in the permeability of the pancreatic acinar cell membrane to Ca^{2+} was provided by Laugier and Petersen (1970). Sustained stimulation with agonist caused a sustained

depolarization and resistance reduction in the presence of extracellular Ca^{2+}; however, in a Ca^{2+} -free EGTA-containing medium only a transient depolarization and resistance change were seen. It was suggested that although the initial agonist-stimulated membrane changes do not require extracellular Ca^{2+}, the influx of Ca^{2+} into the cells is important in maintaining ion channels in their open state and so sustaining the evoked depolarization and membrane resistance changes, in the presence of sustained stimulation. The inability to detect any Ca^{2+} influx electrophysiologically may be due to a very small rate of ion flux which would not contribute significantly to change the electrical properties of the membrane.

An alternative mechanism for Ca^{2+} influx may be via a naturally occurring ionophore. Phosphatidic acid has been suggested to play a role in Ca^{2+} transport since it transfers Ca^{2+} across organic solvent layers and mimicked the responses of smooth muscle cells and parotid gland slices to a Ca^{2+} -mobilizing hormones (Salmon and Honeyman, 1980; Putney et. al., 1980 c).

A component of the stimulated Ca^{2+} uptake may be due to an active process since antimycin A inhibited secretagogue-stimulated Ca^{2+} uptake in pancreatic acinar cells and dispersed acinar preparations (Stolze and Schulz, 1980; Dormer et. al., 1981).

If uptake of Ca^{2+} was solely due to an increase in the plasma membrane permeability to this ion,this effect of ATP depletion on Ca^{2+} -influx would not be expected. Isolated subcellular organelles such as mitochondria, endoplasmic reticulum, Golgi membrane in addition

to plasma membranes possess energy-dependent Ca^{2+}-accumulation properties (Selinger et. al., 1970; Carafoli and Crompton, 1979; Bygrave, 1978; Ponnappa et. al., 1981), and may play an important role as Ca^{2+} storage sites in the pancreatic acinar cell. In unstimulated mouse pancreatic acini, antimycin A stimulated Ca^{2+} efflux and inhibited Ca^{2+} uptake (Dormer et. al., 1981).

What are the cellular sites from which Ca^{2+} is released and the Ca^{2+} sequestering sites where Ca^{2+} uptake occurs following secretagogue stimulation? Following stimulation with carbachol of pancreatic acinar cells which had been pre-loaded with $^{45}Ca^{2+}$, the cells released $^{45}Ca^{2+}$ and subsequently reuptake occurred to reach levels even above the control. When carbachol-stimulation was abolished with atropine, a rapid $^{45}Ca^{2+}$ uptake occurred. After addition of atropine, it was then possible to induce $^{45}Ca^{2+}$ release with another agonist, cholecystokinin, whereas without the interposed step of atropine this was not possible (Stolze and Sculz, 1980). These results indicated that Ca^{2+} is released from one store and is taken up into a second store. Refilling of this first store, involved in Ca^{2+} efflux, occurs only when receptor activation ceases. These results also show that the pool of Ca^{2+} released on receptor activation is common to both the CCK and acetylcholine receptors.

The fluorescent probe, chlorotetracycline (CTC) forms fluorescent complexes with membrane bound Ca^{2+} and was used to investigate the source of Ca^{2+} released by secretagogues. Chandler and Williams (1978 a, b) reported a decrease in CTC fluorescence induced by secretagogues in isolated pancreatic acini which was mimicked by mitochondrial inhibitors.

This suggested that Ca^{2+} was sequestered either in mitochondria or by an organelle (or membrane) requiring ATP produced by mitochondria, and that Ca^{2+} was released from these organelles in response to secretagogue-stimulation (Chandler and Williams, 1978 a, b).

Recent studies using subcellular fractionation of mouse pancreatic acinar cells and determination of Ca^{2+} content by atomic absorption spectroscopy and $^{45}Ca^{2+}$ have investigated the localization of sites of Ca^{2+} release and storage. One problem with measuring changes in Ca^{2+} levels in subcellular organelles following fractionation of stimulated cells is that re-distribution of Ca^{2+} may occur during the fractionation process. Dormer and Williams (1981) used a simple fractionation procedure and added ruthenium red (an inhibitor of mitochondrial Ca^{2+} transport) and EGTA, in an attempt to reduce this re-distribution. It was concluded that secretagogue-stimulation of mouse pancreatic acini causes the release of Ca^{2+} from a microsomal compartment since both $^{45}Ca^{2+}$ levels and net Ca^{2+} levels measured by atomic absorption spectrometry in this fraction decreased (Dormer and Williams, 1981). An increased exchange between zymogen granules and mitochondrial fractions was suggested since although $^{45}Ca^{2+}$ levels decreased, net Ca^{2+} levels in these organelles were not altered (Dormer and Williams, 1981).

The likely sites of Ca^{2+} release in pancreatic acini were suggested to be the endoplasmic reticulum and Golgi apparatus (Dormer and Williams, 1981). The plasma membrane was a contaminant of the microsomal fraction in the study by Dormer and Williams (1981) and so could not be excluded as a possible site of Ca^{2+} release. The validity of these

conclusions, however, is based on the assumption that little redistribution occurred, which is the subject of some controversy (Streb and Schulz, 1983).

To examine the sites of Ca^{2+} sequestration Wakasugi et. al. (1982) used saponin to permeabilize the cell membrane and so allow the cellular uptake of high molecular weight substances including those which inhibit mitochondrial or non-mitochondrial Ca^{2+} uptake. In saponin-treated cells $^{45}Ca^{2+}$ uptake induced with ATP was inhibited but not abolished by mitochondrial inhibitors. Non-mitochondrial inhibitors known to inhibit Ca^{2+} sequestration in sarcoplasmic reticulum (Martonosi and Feretos, 1964) inhibited ATP-induced $^{45}Ca^{2+}$ uptake partially or completely. The Ca^{2+} ionophore A23187 abolished $^{45}Ca^{2+}$ uptake completely and rapidly released previously accumulated $^{45}Ca^{2+}$ from the storage sites. These results suggested that both mitochondrial and non-mitochondrial storage sites are involved in an ATP-dependent Ca^{2+} uptake.

Electron microscopy of saponin-treated acinar cells incubated in the presence of ATP, Ca^{2+} and oxalate showed the presence of dense Ca^{2+} - oxalate precipitates in the rough endoplasmic reticulum and occasionally in mitochondria (Wakasugi et. al., 1982). These precipitates were absent without ATP and when A23187 was added to the ATP-containing incubation medium. It would appear that A23187 affects a Ca^{2+} storage pool located in the rough endoplasmic reticulum.

A further study to examine the site(s) of Ca^{2+} storage in exocrine pancreas was reported by Streb and Schulz (1983). As described in

2.4 (c) permeabilized rat pancreatic acinar cells were used and the measurement of the free Ca^{2+} content of the surrounding medium with a Ca^{2+} -electrode allowed determination of the cytosolic Ca^{2+} concentration ($4 \times 10^{-7}M$) buffered by intracellular organelles.

This steady state was found to be controlled by the nonmitochondrial Ca^{2+} pool since, when Ca^{2+} was added to the bathing medium, in the presence of three mitochondrial inhibitors at high concentrations, Ca^{2+} uptake occurred to maintain the same cytosolic Ca^{2+} steady state as controls. On the other hand, the mitochondrial uptake, which could be observed in the presence of vanadate to inhibit non-mitochondrial uptake, was unable to return free Ca^{2+} to steady state and could only buffer Ca^{2+} to $6.5 \times 10^{-7}M$, which was followed by Ca^{2+} release (Streb and Schulz, 1983).

An examination of nonmitochondrial Ca^{2+} uptake in the presence of vanadate showed two phases (Streb and Schulz, 1983). The first phase of rapid uptake occurred 3-5 min after addition of vanadate followed by a second phase which was a slower but continuous uptake. The non-mitochondrial pool of Ca^{2+} can be divided into two functionally different structures. One pool with a high uptake velocity but low capacity, and a second pool with considerably lower Ca^{2+} uptake velocity but high capacity that determines the final medium concentration of $4 \times 10^{-7}M$ at steady state.

In addition to examining the ability of cellular organelles to buffer changes in cytosolic Ca^{2+}, the role of non-mitochondrial Ca^{2+} stores in pancreatic acinar cells in response to secretagogue-stimulation was

also examined by Streb and Schulz (1983). When permeabilized cells were stimulated with CCK-8 or carbachol, Ca^{2+} release was followed by Ca^{2+} uptake to the prestimulation level. If non-mitochondrial uptake of Ca^{2+} was completely abolished by vanadate, some, though reduced, secretagogue-induced Ca^{2+} release was observed (Streb and Schulz, 1983). This suggested a role for the non-mitochondrial store of Ca^{2+}, the endoplasmic reticulum, in the initial Ca^{2+} efflux response. In addition, it has been suggested that since secretagogue-stimulation of Ca^{2+} efflux occurs in permeabilized cells, the Ca^{2+} may be released from the plasma membrane, otherwise one must envisage the transfer of a signal from plasma membrane internal organelles in an environment whereby the second messenger may leak out of the cell. Lanthanum which displaces Ca^{2+} from sites on the external surface of the cell membrane caused inhibition of agonist-stimulated Ca^{2+} efflux, again suggesting release of Ca^{2+} from a plasma membrane site (Schulz et. al., 1981). It is questionable, however, whether Ca^{2+} released from the plasma membrane would enter into the cytosol and then be pumped out by Ca^{2+} pumps. Since it is known that Ca^{2+} released from stores is sufficient to activate the secretory process, this indicates that Ca^{2+} efflux must occur as a result of release of Ca^{2+} into the cytosol from stores, and questions a plasma-membrane site of Ca^{2+} release. The site of the pool of Ca^{2+} responsible for the initial agonist-stimulated efflux is not yet known but is likely to be the rough endoplasmic reticulum.

In contrast to the results with pancreatic acini are those in parotid gland where the Ca^{2+} storage pools involved in secretagogue-stimulated release and uptake of Ca^{2+} do not appear to be intracellular. This

tissue resembles pancreas in that cholinergic receptor activation stimulated a Ca^{2+} efflux followed by an influx (Poggioli and Putney, 1982). When atropine is added during the influx stage there is an abrupt transient influx followed by a return of net influx to pre-stimulation levels (Poggioli and Putney, 1982). Following atropine addition, Ca^{2+} refilled the hormone sensitive pool since α-adrenoceptor activation caused a further Ca^{2+} efflux. However, during the period of refilling when a very large influx of Ca^{2+} occurred, the Ca^{2+} -dependent responses of K^{+} efflux and protein secretion were not stimulated. This last result suggests that this pool of Ca^{2+} refills rapidly without causing an increase in the cytosolic free Ca^{2+} content. Since this indicates Ca^{2+} does not enter the cytosol it appears that the hormone-sensitive Ca^{2+} pool is in some way sequestered in or in close association with the plasma membrane.

Therefore, it appears that in pancreatic acinar cells secretagogue-stimulation causes the efflux of Ca^{2+} which is associated with release of Ca^{2+} from cellular stores and is required for short term release of secretory product. Ca^{2+} uptake occurs to maintain an elevated cytostolic Ca^{2+} content to allow sustained secretion. Refilling of the secretagogue-sensitive Ca^{2+} pools requires removal of receptor activation.

In some tissues such as parotid gland (Poggioli and Putney, 1983) this pool may be to be membrane bound. Whereas in pancreatic acinar cells a non-mitochondrial pool which may be the endoplasmic reticulum appears to be involved in Ca^{2+} storage and release of Ca^{2+}.

What role does mitochondrial Ca^{2+} play in controlling pancreatic acinar cell Ca^{2+} content? The results of studies by Wakasugi et. al. (1982) and Streb and Schulz (1983) suggest that although an ATP-dependent accumulation of Ca^{2+} does occur into a mitochondrial pool this pool does not appear to play a role in maintaining cytosolic free Ca^{2+} concentration at a steady rate. It has been suggested that perhaps the mitochondrial pools act as an "emergency Ca^{2+} sink" which takes up Ca^{2+} when the internal free Ca^{2+} content increases to high levels (Streb and Schulz, 1983). However, there is no evidence to support this idea.

A recent study by Shears and Kirk (1984) has shown that in hepatocytes, mitochondrial Ca^{2+} is not mobilized in response to α-adrenergic activation which stimulates Ca^{2+} -dependent responses such as glycogen phosphorylase activity. Using a rapid cellular fractionation technique, the mitochondrial-rich fraction obtained from agonist-stimulated hepatocytes showed no loss of $^{45}Ca^{2+}$. Whereas a loss of total cell Ca^{2+} occurred. The study by Shears and Kirk (1984) conflicted with earlier studies using hepatocytes (Blackmore et. al., 1979 a, b); Murphy et. al., 1980; Dehaye et. al., 1980; Reinhart et. al., 1982). However, these studies either employed non-physiological Ca^{2+} -loading procedures; used hepatocytes which had not achieved a steady-state control level for mitochondrial Ca^{2+}; or long-term fractionation procedures; all of which could lead to a loss of mitochondrial Ca^{2+} which was not due to secretagogue activation.

The recent results of Shears and Kirk (1984) and Streb and Schulz (1983) have now cast doubt on any role of mitochondrial Ca^{2+} in the secretagogue-activated Ca^{2+} fluxes which occur.

How does receptor activation cause Ca^{2+} release? Na^{+} has been proposed as a possible mediator of Ca^{2+} release in some tissues such as sympathetic ganglia (Birks and Cohen, 1968) parasympathetic fibres (Poulsen, 1974), heart (Carafoli et. al., 1974). In the studies by Streb and Schulz (1983) using permeabilized cells, there would be no increase in intracellular Na^{+} concentration following receptor activation and yet Ca^{2+} release occurs. This indicates that in pancreatic acinar cells Ca^{2+} release occurs independently of any change in Na^{+}content.

Is inositol lipid hydrolysis associated with the control of the release of Ca^{2+} from stores? If the site of Ca^{2+} release is at the endoplasmic reticulum, how does the activation by secretagogue at the external surface of the cell membrane cause this Ca^{2+} release - is there an intracellular messenger released upon receptor activation? These questions will be discussed in the inositol phospholipid section to follow (2.8).

2.6 : THE RELATIONSHIP BETWEEN INHIBITION OF PROTEIN SECRETION, CYTOSOLIC CALCIUM CONCENTRATION AND RECEPTOR OCCUPATION IN THE EXOCRINE PANCREAS

The dose-response curve for amylase secretion stimulated by a number of secretagogues shows a characteristic biphasic shape where high concentrations of agonists cause a submaximal secretion of amylase (Williams, 1975 b; Savion and Selinger, 1978; Uhlemann et. al., 1979).

How does this inhibition of secretion occur? There is evidence to suggest that this is a post-receptor phenomenon and that Ca^{2+} is

responsible for the submaximal secretion of amylase. A comparison of the dose-response curves for amylase secretion and Ca^{2+} efflux in isolated mouse pancreatic acini showed that the secretagogue concentrations required to produce maximal amylase secretion was 2 to 3-fold less than that required to produce maximal Ca^{2+} efflux (Chandler and Williams, 1978; Korc et. al., 1979; Dormer et. al., 1981). These results indicated that the cytosolic Ca^{2+} concentration required for maximal amylase secretion is not the maximum level possible in the acinar cell. This was further supported in a study by Stark and O'Doherty (1982) showing, with the use of a Ca^{2+} selective microelectrode, that as acetycholine increased from 10^{-8}M to 10^{-5}M the intracellular concentration of Ca^{2+} continued to increase while amylase secretion progressively increased and then decreased. Studies showing that the inhibitory effect of high concentrations of secretagogues on amylase secretion can be reduced by decreasing the extracellular Ca^{2+} concentration in guinea-pig acini (Gardner et. al., 1979 b) and in mouse pancreatic fragments and acini (Roberts and Woodland, 1982; Burnham and Williams, 1982 b) support the idea that high levels of intracellular Ca^{2+} inhibit secretion.

The results of Gardner et. al. (1980) are also in agreement with this proposal since the addition of ionophore A23187 to secretagogue-stimulated guinea-pig pancreatic acini caused a reduction in the secretion of amylase due to the increased intracellular Ca^{2+} content The question of how Ca^{2+} causes this inhibition of secretion is however, not answered.

Supraoptimal concentrations of secretagogues cause morphological changes at the luminal area of the pancreatic acini. There is disruption

of the filamentous system surrounding the lumen, disappearance of microvilli and production of distended evaginations of the luminal membrane containing secretory material (Savion and Selinger, 1978). These changes lead to the eventual reduction in size of the lumen. It has been suggested that these changes in the microtubular and microfilamentous system could account for the inhibition of enzyme secretion caused by high concentrations of secretagogues (Savion and Selinger, 1978; Burnham and Williams, 1982 b). The morphological changes were found to occur only at the apical part of the cell membrane and both the inhibition of amylase secretion and the structural changes could be reversed by removal of the secretagogue (Savion and Selinger, 1978). It appears, however, that the collapse of the acinar cell lumen is not responsible for inhibition of secretion since cytochalasin B at a concentration which disrupted the structure of the microfilamentous network and microvilli inhibited enzyme secretion but the acinar lumen was still intact (Bauduin et. al., 1975).

The study by Burnham and Williams (1982 b) showed that the production of numerous cytoplasmic protrusions into the acinar lumen caused by high concentrations of cholecystokinin-octapeptide was reduced by exposure of acini to cytochalasin B, which disrupts microfilament networks (Brown and Spudich, 1981). Cytochalasin B (6.3×10^{-6}M) caused a decrease in the maximal secretion of amylase stimulated by CCK-8 and abolished the inhibition of secretion caused by high concentrations of this secretagogue (Burnham and Williams, 1982 b). This was taken as evidence that inhibition of secretion was due to alteration of the microfilament system by high concentrations of agonist.

In the presence of cytochalasin B (6.3×10^{-6}M) the maximal secretion of amylase stimulated by CCK-8 was reduced to a level of secretion which was not much greater than that stimulated by inhibitory concentrations of cholecystokinin-octapeptide (Burnham and Williams, 1982 b). However, from these results it is not clear whether a further reduction in amylase secretion would be expected with higher concentrations of CCK-8 and so the evidence to support the idea that the microfilament system is responsible for inhibition of secretion at high concentrations of agonist is not conclusive.

Cytochalasin B interaction with the pancreatic acinar cell not only results in disruption of the microfilament system but also alters monosaccharide transport (Bauduin et. al., 1975). The uptake of (^{3}H)-2-deoxyglucose into pancreatic gland *in vitro* was inhibited by pre-incubation for 2 hr with concentrations of cytochalasin B as low as 2.1×10^{-6}M, whereas ultrastructural changes were observed in the gland only when concentrations greater than 10^{-5}M were used (Bauduin et. al., 1975). The effect on glucose transport suggests the plasma membrane as a site of action of cytochalasin B and indicates that glucose transport is more sensitive to cytochalasin B than microfilament structure. Cytochalasin B (10^{-5}M) did not alter ATP levels of pancreatic gland - suggesting that cytochalasin B did not result in a depletion of energy for cellular processes.

Roberts and Woodland (1982) found no effect of a low concentration of cytochalasin B (1.1×10^{-6}M) on the inhibition caused by high concentrations of secretagogue, and concluded that microfilaments do not appear to be involved in this inhibitory effect. The concentration

of cytochalasin B used by Burnham and Williams (1982 b) was higher that that used by Roberts and Woodland (1982) and so could account for the differences in the results.

Microtubules do not appear to be involved since disruption of the microtubule system with vinblastine or colchicine did not alter the inhibition of amylase secretion (Williams and Lee, 1976; Roberts and Woodland, 1982).

How does Ca^{2+} inhibit secretion? If not by disruption of the microfilament system - by what other mechanisms? Another phenomenon activated by high concentrations of secretagogues in pancreatic acini cells is the activation of the lysosomal system whereby secretory granules are taken up by lysosomes to form autophagic vacuoles, which results in the formation of myocardial depressant factor - which is not normally released as part of the process of enzyme secretion (Savion and Selinger, 1978). It is possible that the resultant destruction of the secretory granules contributes to the decrease in enzyme secretion.

High concentrations of secretagogues activate other pancreatic functions such as stimulation of glucose uptake (Korc et. al., 1979), inhibition of amino acid uptake (Iwamoto and Williams, 1980) and inhibition of protein synthesis (Korc et. al., 1981) which also appear to be mediated by an increase in intracellular Ca^{2+}. Recent studies have investigated the relationship between secretagogue-receptor binding and biological functions in pancreatic acinar cells. Sankaran et. al. (1982) showed that there are two classes of receptors

for cholecystokinin-octapeptide and that occupation of the high-affinity receptors correlated with stimulation of secretion while occupation of the low-affinity receptors correlated with the inhibition of secretion. Regulation of the uptake of glucose and amino-isobutyric acid was the result of fractional occupancy of the low affinity cholecystokinin receptors (Sankaran et. al., 1982).

The study by Sankaran et. al. (1982) was supported by studies investigating occupancy of the muscarinic receptor using ^{3}(H)-quinuclidinyl benzilate in rat pancreatic acini (Larose et. al., 1981). This receptor also exists in two affinity states whereby occupation of the high affinity receptor (which was 40% of the total receptor population) corresponded with stimulation of secretion while occupation of the low affinity receptors corresponded with inhibition.

A close examination of the dose-response curves for Ca^{2+} efflux and amylase secretion (Chandler and Williams, 1978; Korc et. al., 1979; Dormer et. al., 1981) showed that increasing the concentration of secretagogues over levels at which Ca^{2+} efflux had plateaued, still caused a progressive decrease in maximal enzyme secretion. Since Ca^{2+} efflux occurs as a result of an increased cytosolic Ca^{2+} content, these results could be interpreted to show that the inhibition of secretion, although occurring at high concentrations of cytosolic Ca^{2+} is not linearly related to Ca^{2+} content. It could be suggested that some other as yet unknown, factor which continues to increase in concentration is also involved in inhibition of secretion. However, this conflicts with the study by O'Doherty and Stark (1982) in which acetylcholine 10^{-6} to 10^{-5}M continued to increase the Ca^{2+} content

(as measured with the Ca^{2+}-sensitive microelectrode) while amylase secretion progressively decreased.

The role of Ca^{2+} in inhibition of secretion still requires further investigation.

2.7 : CALCIUM-ACTIVATED STIMULUS-SECRETION COUPLING IN EXOCRINE PANCREAS

How might the agonist-stimulated increase in intracellular Ca^{2+} cause secretion? Regulation of protein phosphorylation has been shown to be influenced by Ca^{2+} in a number of secretory tissues and cells including the exocrine pancreas (Freedman and Jamieson, 1981; Burnham and Williams, 1982 a, 1984; Jahn and Söling, 1983; Roberts and Butcher, 1983), parotid gland (Kanamori and Hayakawa, 1980; Jahn and Söling, 1981 a), lacrimal gland (Jahn and Söling, 1981 b; Jahn et. al., 1982), adrenal medulla (Amy and Kirschner, 1982), mast cells (Sieghart et. al., 1978) and platelets (Lyons and Shaw, 1980).

There has been increasing evidence that Ca^{2+} may exert many of its actions through regulation of protein phosphorylation in numerous tissues (Cohen, 1982; Schulman, 1982) and so the effect of secretagogues on phosphorylation in exocrine pancreas has recently been examined.

In the mouse pancreatic acinar cells, stimulation with either carbachol or CCK-8 caused significant increases in the phosphorylation of a particulate protein, Mr =32,500 and soluble proteins with an Mr = 16,000

and 23,000 (Burnham and Williams, 1982 a). These alterations in phosphorylation correlated in a dose-dependent manner with the stimulation of enzyme secretion, and were mimicked by the Ca^{2+} ionophore A23187 (Burnham and Williams, 1982 a). Increased phosphorylation of a protein with an Mr = 32,000 by carbachol or A23187 only in the presence of Ca^{2+} was also observed in mouse pancreatic fragments (Roberts and Butcher, 1983). In guinea-pig exocrine pancreas a major protein Mr 29,000 - 35,000 was phosphorylated in response to carbachol; this protein was identified as being ribosomal (Jahn and Söling, 1983). Ca^{2+} -dependent phosphorylation of a protein with an Mr = 29,000 which was localized to a ribosomal fraction was also identified in rat pancreas in response to secretagogue stimulation (Freedman and Jamieson, 1981).

The regulatory role for Ca^{2+} in phosphorylation may involve alterations in the activity of Ca^{2+} -dependent protein kinases and/or phosphatases (Krebs and Beaven, 1979).

The initial studies which identified Ca^{2+} -dependent phosphorylation of protein stimulated by secretagogues did not identify the protein kinase(s) involved. Two possible modes of action whereby Ca^{2+} can alter protein phosphorylation are by activation of protein kinase C or by activation of calmodulin-dependent protein kinase.

2.7 (a) Protein Kinase C and Pancreatic Exocrine Secretion

Protein kinase C is a calcium-activated phospholipid-dependent protein kinase (Kishimoto et. al., 1980). This

protein kinase is normally active, but a quaternary complex of protein kinase C, diacylglycerol, calcium and phospholipid is enzymatically fully active for protein phosphorylation (Kikkawa et. al., 1983). Protein kinase C may be activated without a net increase in the intracellular calcium concentration since the addition of diacylglycerol results in a sharp increase in the affinity of the protein kinase for this ion if phosphatidylserine is present (Kishimoto et. al., 1980).

The protein kinase C has several characteristics including (a) an absolute dependency on phospholipid, mainly phosphatidylserine, but not calmodulin, for Ca^{2+} activation (Takai et. al., 1979 b); (b) preferential phosphorylation of type III histone (H1 histone) over other histones (Takai et. al., 1979 b); (c) inhibition of activity by phenothiazines (Mori et. al., 1980).

If protein kinase C has a role in controlling cell function then this implies that phosphorylation of a protein(s) is important in mediating this effect. Recent studies have investigated the presence of protein kinase C activity and the possible substrates for this enzyme which may be involved in mediating release of secretory product in exocrine pancreas.

Wrenn et. al. (1981) demonstrated calcium-activated kinase activity that was dependent on exogenous phospholipid in a soluble fraction of rat pancreas. More recently, a phospholipid -Ca^{2+}-dependent protein kinase activity and its endogenous

proteins have been localized to the pancreatic acinar cell population (Wrenn, 1983; Burnham and Williams, 1984; Wrenn, 1984; Wrenn and Wooten, 1984). An endogenous membrane protein (Mr = 18,000) from rat pancreatic zymogen granules was found to be specifically phosphorylated in the combined presence of Ca^{2+} and phosphatidylserine and it was suggested that this may be one mechanism whereby this enzyme may act in exocytosis (Wrenn, 1984).

Cytosolic and particulate preparations isolated from mouse pancreatic acinar cells showed Ca^{2+} -phospholipid dependent kinase activity which phosphorylated proteins of Mr = 40,000 and 62,000, with half maximal activation achieved at 1.2 x 10^{-5}M Ca^{2+} (Burnham and Williams, 1984). However, the role of these proteins in acinar function is not known. The study by Burnham and Williams (1982 a) identified a stimulated phosphorylation of proteins of M_r = 16,000, 23,000 and 32,500 by CCK-8 and carbachol. The molecular weight of the cytosolic and particulate proteins phosphorylated by Ca^{2+} -activated kinase activities (Burnham and Williams, 1984) were not the same as those proteins phosphorylated by secretagogue-stimulation (Burnham and Williams, 1982 a).

As suggested by Burnham and Williams (1984) the differences in proteins phosphorylated in intact vs cytosolic and particulate fractions may reflect the effect of homogenization (Bylund and Krebs, 1975) causing disruption of cellular compartments resulting in an increase in the susceptibility of certain

proteins to phosphorylation. Therefore, although identification of specific kinases is possible using fractionated cells, the proteins phosphorylated in the intact cell are more likely to be involved in regulation of cell function.

Protein kinase C activity may be directly involved in secretagogue-stimulation of pancreatic secretion since polymix B (which inhibits the action of this enzyme) completely inhibited carbachol-stimulated amylase release from intact rat pancreatic acini (Wrenn and Wooten, 1984). Polymix B was found to be over 100-fold more potent as an inhibitor of the Ca^{2+} -phospholipid-dependent protein kinase than of the calmodulin-dependent kinase (Wrenn and Wooten, 1984). The specificity of polymix B was examined and this agent was found not to alter muscarinic receptor binding or the structural integrity of the cell membrane (Wrenn and Wooten, 1984). However, before a definite role for protein kinase C action in pancreatic secretion is concluded the effect of polymix B in other parameters such as the final exocytotic seretory response should be examined.

An alternative approach to investigate the role of protein kinase C is by the use of phorbol esters. The finding that these compounds, which substitute for diacylglycerol in activating protein kinase C (Castagna et. al., 1982), stimulated the secretory response of pancreas (Gunther and Jamieson, 1979; Gunther, 1981 a; Wooten and Wrenn, 1984), platelets (Knight and Scrutton, 1984), adrenal medullary cells (Knight and Baker, 1983) and pancreatic islet cells (Hubinot et. al., 1984), has

led to the suggestion that diacylglycerol, by activating protein kinase C, may play a role in exocytosis.

The effect of the phorbol ester, TPA, on pancreatic exocrine function has been discussed in detail in 2.3 (c). The ability of TPA to stimulate pancreatic enzyme secretion (Gunther and Jamieson, 1979; Wooten and Wrenn, 1984) by binding to high affinity receptors (Gunther, 1979 b) has led to support for a role of protein kinase C in pancreatic secretion. Further studies showed that TPA caused translocation of protein kinase C from cytosol to particulate fraction which was concurrent with stimulation of amylase secretion (Wooten and Wrenn, 1984). In addition, inhibition of this enzyme also inhibited TPA-induced enzyme secretion from pancreatic acini (Wrenn and Wooten, 1984), providing further evidence to support protein kinase C as a possible mediator of secretion.

An increase in diacylglycerol occurs as a result of inositol phospholipid hydrolysis (described in section 2.8) by activation of receptors which mobilize calcium. What relationship does the activation of protein kinase C have with calcium in stimulating exocytosis? At least three possibilities exist: (1) that calcium and diacylglycerol stimulate secretion by two independent mechanisms; (2) that secretion is activated by calcium and protein kinase C can modify the sensitivity of this process to calcium; (3) that the actions of both calcium and diacylglycerol are mediated by protein kinase C.

It has been demonstrated in platelets that protein kinase C activation and Ca^{2+} mobilization are both essential to elicit full physiological responses such as release of serotonin (Kaibuchi et. al., 1982 a, 1983). When platelets are stimulated with the phorbol ester TPA an increase in phosphorylation of a 40kDa protein occurs with no increase in Ca^{2+}, whereas when stimulated with the Ca^{2+} ionophore A23187 an increase in phosphorylation of a 20kDa protein occurs. The addition of a physiological stimulus, such as thrombin, to platelets resulted in phosphorylation of both the 40kDa and 20kDa proteins. The combination of both TPA and A23187 mimicked the effect of thrombin, indicating protein kinase C and Ca^{2+} act synergistically to elicit the final response (Nishizuka, 1984).

Recent reports that certain phorbol esters can stimulate exocytosis with little or no apparent change in intracellular Ca^{2+} (Rink et. al., 1983; Di Virgilio et. al., 1984) has caused a questioning of the essential requirement for Ca^{2+} in control of secretion.

A direct measure of the dependence of secretion on intracellular free Ca^{2+} and protein kinase C activity has been possible with electrically permeabilized secretory cells. In adrenal medullary cells (Knight and Baker, 1983) and platelets (Knight and Scrutton, 1984) exposure to phorbol esters sensitizes secretion to Ca^{2+} by causing a leftward shift of the intracellular Ca^{2+} activation curve. This shift may be large enough to account for activation of secretion by phorbol esters in intact cells where intracellular

Ca^{2+} does not change. This result would support the idea that Ca^{2+} and protein kinase C do not simulate secretion by two independent pathways.

However, Di Virgilio et. al. (1984) found that phorbol esters can promote secretion from neutrophils at an ionized concentration of calcium of 10nM, which would require a bigger shift in calcium sensitivity than has so far been seen in electrically permeabilized cells (Baker, 1984). This effect at 10nM calcium suggests that protein phosphorylation by protein kinase C does not simply increase the calcium-sensitivity of the calcium-dependent processes but itself triggers responses which until now were considered to be strictly calcium dependent (Di Virgilio et. al., 1984). It is necessary that properties of the protein kinase C (such as its ability to phosphorylate substrates in the presence of a calcium-chelating agent) need to be known before a requirement for calcium in phorbol ester-stimulated secretion from neutrophils can be dismissed.

The studies with tumour promoters have provided considerable evidence to support a role for protein kinase C in secretion. The identification of the proteins phosphorylated which play a role in secretion have not been identified nor the possible mechanism(s) by which increased phosphorylation can stimulate secretion.

Cautionary consideration of these results with tumour promoters is necessary. Firstly, although phorbol esters appear to be

diacylglycerol analogues, it does not necessarily follow that the two classes of compounds should function identically. Diacylglycerols are rapidly metabolised in the cell by diacylglycerol kinase, tumour promoters are relatively stable (Blumberg et. al., 1984). The phorbol esters would therefore cause an abnormal, chronic stimulation of protein kinase C. It is possible that in the physiological situation, diacylglycerol released following agonist-activation of phosphoinositide hydrolysis may not cause such a prolonged stimulation of protein kinase C. The resulting effect is not known but should be investigated. Secondly, protein kinase C can be cleaved proteolytically to a 51KDa protein which is active and does not require calcium , diacylglycerol or phospholipids and which no longer binds to membrane (Takai et. al., 1977; Kishimoto et. al., 1983). When protein kinase C is in the active state it is more susceptible to proteolysis (Kishimoto et. al., 1983). Phorbol esters, by causing chronic stimulation of protein kinase C, may cause a greater conversion to the active fragment and lead to an altered pattern of phosphorylation (Blumberg et. al., 1984).

To further clarify the role of protein kinase C in secretion it is necessary to examine the ability of diacylglycerol released by agonist-stimulation to activate protein kinase C; to identify the substrates for this enzyme *in vivo* and to determine if any of these phosphorylated proteins play a role in secretion.

2.7 (b) Calcium-activated, Calmodulin-dependent Protein Kinase and Pancreatic Exocrine Secretion

Recent reviews by Cheung (1980) and Means and Dedman (1980) suggest that calmodulin may act as an intracellular Ca^{2+} receptor. Calmodulin forms a reversible complex with Ca^{2+}, this complex can activate several different enzymes which could be involved in mediating the cellular response. Enzyme activities modulated include phospholipase A_2, Ca^{2+} ATPases and cyclic nucleotide phosphodiesterases (Cheung, 1980; Means and Dedman, 1980). However, the role of calmodulin in pancreatic secretion has not been the subject of much research and it is not clear whether it does have a function in secretion.

Vandermeers et. al. (1977) purified and identified a "calcium-binding protein" in bovine and rat pancreas which showed physicochemical properties of calmodulin. Williams et. al. (1977) found that chlorpromazine (which shows anti-calmodulin effects; Norman et. al., 1980) inhibited the secretagogue-stimulated secretion of amylase. A possible role of calmodulin in pancreatic enzyme secretion was suggested by Heisler et. al. (1981) after showing inhibition of amylase secretion stimulated by ionophore A23187 or secretagogues by a number of agents believed to act as calmodulin antagonists. This was an indirect study and the agents used could exert their effects by other mechanisms, for example by acting like local anaesthetics to cause membrane stabilization, blockade of receptor binding (Seeman, 1972; Putney and Van de Walle, 1980 b). In addition to these effects,

psychotrophic drugs such as trifluoperazine, chlorpromazine, fluphenazine, which act as calmodulin antagonists are also equally potent as inhibitors of protein kinase C (Wrenn et. al., 1981; Schatzman et. al., 1981) which causes great difficulty in determining whether a Ca^{2+} -dependent increase in phosphorylation is due to calmodulin or protein kinase C by the use of calmodulin-inhibitors.

Membrane preparations from a variety of mammalian tissues contain a calcium-activated, calmodulin-independent protein kinase activity (Schulman, 1982). The first identification of a calmodulin-stimulated protein kinase activity in pancreas was reported by Gorelick et. al. (1983) where a protein with Mr = 51,000 was phosphorylated by this enzyme. Burnham and Williams (1984) identified a high speed particulate fraction and a cytosolic fraction of rat pancreatic acini which when depleted of endogenous calmodulin exhibited no Ca^{2+} -induced increase in protein phosphorylation. In the presence of calmodulin, a Ca^{2+} -induced phosphorylation of proteins with Mr = 92,000, 50,000 - 52,000 and 23,000 occurred. In the presence of phosphatidylserine, addition of Ca^{2+} did not cause any increase in phosphorylation indicating calmodulin-dependent protein kinase activity (Burnham and Williams, 1984).

An examination of the role of the calmodulin-dependent kinase found in rat pancreatic extracts was attempted with the use of fluphenazine (Wrenn and Wooten, 1984). Although this agent inhibited carbachol-stimulated amylase secretion it was not possible to determine whether this effect was due to inhibition

of protein kinase C activity or calmodulin-dependent kinase activity since this agent inhibited both kinases with equal potency (Wooten and Wrenn, 1984).

In conclusion, the question: How does Ca^{2+} activate pancreatic exocrine secretion? is still unanswered. The role of phosphorylation in secretion requires major investigation, along with investigations of other, as yet unknown mechanisms, whereby Ca^{2+} might mediate secretion.

2.8 : PHOSPHATIDYLINOSITOL : THE INITIAL DISCOVERY

Studies on inositol phospholipids and their role in cell function were initiated when Hokin and Hokin (1953) observed that acetylcholine and cholecystokinin stimulated an increase in the incorporation of the (^{32}P)-inorganic phosphate ((^{32}P)-P_i) into the total phospholipids of pigeon pancreas. The further identification that PtdIns was the major lipid into which (^{32}P)-P_i was incorporated in response to acetylcholine stimulation of pancreas (Hokin and Hokin, 1955, 1958 a) did not lead, however, to an immediate upsurge in research in this area.

During the twenty years following the Hokins' initial discovery, studies identified an agonist-stimulated increase in the incorporation of (^{32}P)-P_i or (^{3}H)-inositol into PtdIns of a number of tissues including pancreas (Hokin and Hokin, 1958 a; Hokin, 1968 a), brain Hokin and Hokin 1958 b), salivary gland (Eggman and Hokin, 1960) and adrenal medulla (Trifaró, 1969). However, it was still unresolved as to which reaction in PtdIns metabolism was controlled by the action of agonist.

The finding that agonist stimulation of mouse pancreas caused a loss of radioactivity from PtdIns pre-labelled with (^{32}P)-P_i or (^{3}H)-inositol and that this was accompanied by a decrease in the tissue PtdIns concentration (Hokin, 1974; Hokin-Neaverson, 1974 a; Hokin-Neaverson et. al., 1975) indicated that the breakdown of PtdIns occurred in response to agonist-stimulation. An increase in the levels of phosphatidic acid and diacylglycerol occurred concomittant

with the decrease in PtdIns in response to acetylcholine or cholecystokinin (Hokin, 1974; Hokin-Neaverson, 1974; Hokin-Neaverson et. al., 1975), suggesting that the breakdown of PtdIns and synthesis of phosphatidic acid were linked. When the acetylcholine-stimulated pancreas returned to its unstimulated state by addition of atropine, the net levels of PtdIns increased and those of phosphatidic acid decreased (Hokin, 1974). These results indicated that the breakdown of PtdIns, production of phosphatidic and synthesis of PtdIns were part of a cyclic reaction.

Michell and co-workers, after screening a variety of cell types in which there was an agonist-stimulated increase in PtdIns turnover, identified that the cells in which this lipid effect occurred had a major feature in common - they all utilized calcium as an intracellular second messenger. The finding that this PtdIns response was ubiquitous to so many cells suggested a functional role. Michell (1975) proposed that the breakdown of PtdIns plays a role in the coupling between the receptor-agonist interaction and the increase in intracellular calcium.

It was not until this time when Michell (1975) proposed that the breakdown of PtdIns may be a critical event in calcium-gating that the interest in the phosphoinositides largely increased. Since then there has been a proliferation of publications on this subject, with early studies measuring the synthesis of PtdIns by incorporation of (^{32}P)-P_i and (^{3}H)-inositol into PtdIns, and more recently by studies measuring the breakdown of PtdIns. Michell's initial hypothesis (1975) was modified recently due to the finding by Kirk et. al. (1981) of a

rapid agonist-stimulated breakdown of polyphosphoinositides in hepatocytes. Michell et. al. (1981) then proposed that the hydrolysis of PtdIns-4,5P_2 may be the initial agonist-activated event in the turnover of inositol phospholipids and that PtdIns is not hydrolysed but is phosphorylated to PtdIns-4P and PtdIns-4,5P_2 so as to refill this depleted pool of phospholipid. The development of research on inositol phospholipids in pancreatic function will be discussed in a chronological order so as to provide an understanding of the progress in this area from its initial discovery in 1953.

The structure and biosynthesis of PtdIns and the polyphosphoinositides; the enzymes involved in these reactions; the possible sites of location of the agonist-stimulated breakdown and the development of the methods of measuring the turnover of inositol phospholipid will be reviewed in the following section followed by a discussion on the calcium dependency of the agonist-stimulated hydrolysis of PtdIns, PtdIns-4,5P_2 and PtdIns-4P in pancreas and other tissues where relevant. Possible mechanisms whereby calcium mobilization may be controlled by phosphoinositide hydrolysis will be discussed in addition to the relationship between this lipid response and receptor occupation.

2.9 : "PTDINS EFFECT" 1953 - 1980

During the period 1953 - 1980, investigations of inositol phospholipid metabolism concentrated mainly on PtdIns and not the polyphosphoinositides. Apart from the early studies by the Hokins' in the 1950's and 1960's, interest in inositol phospholipid was minimal, until 1975 when Michell proposed a role for PtdIns in Ca^{2+}

mobilization. Following this proposal most studies examined PtdIns. The few studies of polyphosphoinositides reported a Ca^{2+}-dependent breakdown of PtdIns-4,5P_2 in iris smooth muscle (Akhtar and Abdel-Latif, 1978, 1980) human erythrocytes (Allan and Michell, 1978) and synaptosomes (Griffin and Hawthorne, 1978). It was thought that for inositol phospholipid hydrolysis to precede Ca^{2+} mobilization it should not be dependent on this ion and so any suggestion that the hydrolysis of the polyphosphoinositides might be involved in causing an increase in cytosolic Ca^{2+} was dismissed.

It has only been over the last three to four years that a role for PtdIns-4,5P_2 and PtdIns-4P in controlling cellular Ca^{2+} has been proposed (Michell et. al., 1981), and supported (Streb et. al., 1983; Burgess et. al., 1984; Joseph et. al., 1984; Prentki et. al., 1984; Suematsu et. al., 1984). For this reason it is relevant to discuss the polyphosphoinositides, the enzymes responsible for their metabolism and the possible mechanisms of activation, in the next section 2.10, entitled "PtdIns Effect" 1981 - 1984.

2.9 (a) Phosphatidylinositol : Metabolism

Phosphatidylinositol is the predominant inositol phospholipid of mammalian cells, representing between 2-12% of the total phospholipids of the cell (Michell, 1975), and consists of a glycerol backbone, two fatty acid chains and a phosphorylated alcohol. The structure of PtdIns in pancreas and in other mammalian tissues has been shown to be 1-stearoyl, 2-arachidonoyl-sn-glycero-3phosphorylinositol (Holub and Kuksis,

1971; Baker and Thompson, 1972) and is shown in Fig. 2.1. The fatty acids esterified to the glycerol backbone are n-octadecanoate having the formula : $CH_3(CH_2)_{16}(COOH)$ and cis-$\Delta^5,\Delta^8,\Delta^{11},\Delta^{14}$ -eicosatetraenoate having the formula $CH_3(CH_2)_4(CH{=}CHCH_2)_4(CH_2)_2(COOH)$.

The cycle of reactions shown in Fig. 2.2 was first suggested by Hokin and Hokin (1964) to be responsible for the agonist-stimulated turnover of PtdIns in the avian salt-gland and involves the hydrolysis of PtdIns by a phosphatidylinositol phosphodiesterase (phospholipase C) which had initially been found in the ox pancreas by Dawson (1959) and in liver by Kemp et. al. (1961).

The main characteristic of this cycle of PtdIns metabolism is that there is a turnover of the inositol phosphate headgroup whereas the diacylglycerol backbone of the molecule is conserved. The diacylglycerol released is phosphorylated to phosphatidic acid by diacylglycerol kinase. Phosphatidic acid interacts with CTP (cytidine triphosphate) to form cytidine diphosphodiacylglycerol (CDP-diacylglycerol) with which inositol finally interacts to form PtdIns.

The study by Dawson et. al. (1971) described a phosphatidyl-inositol-specific phosphodiesterase (phospholipase C) which catalysed the formation of inositol 1,2-cyclic-phosphate and inositol-1-phosphate from PtdIns. The enzyme has a partial cyclizing activity (Michell, 1975).

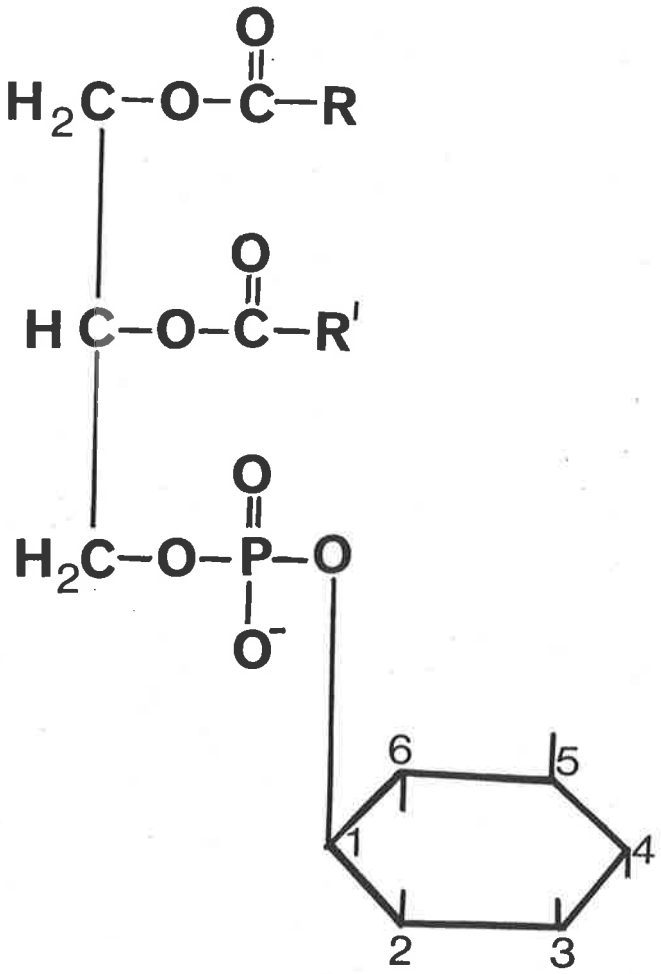
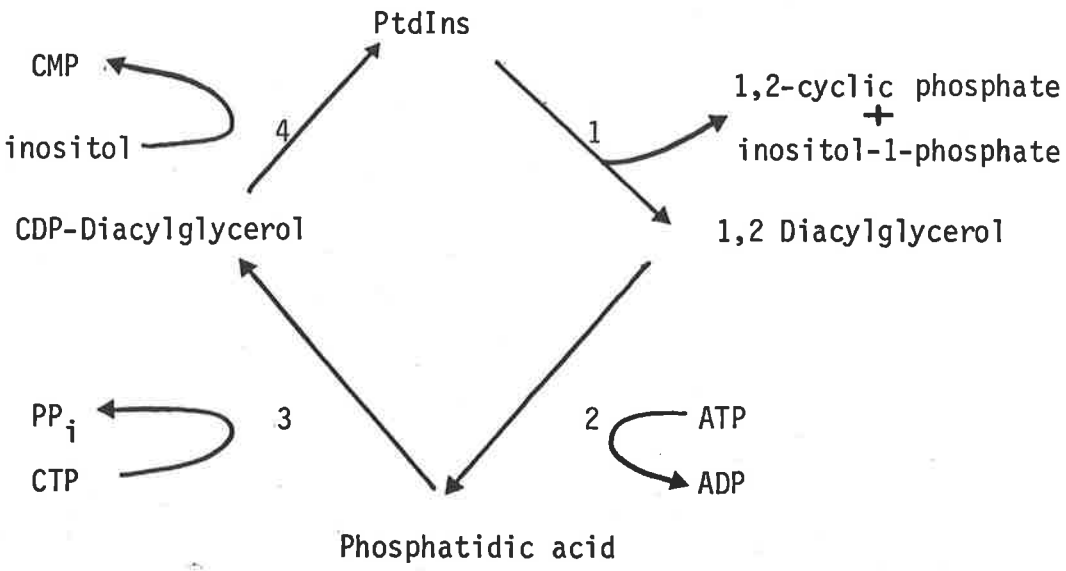


Fig. 2.1 : Phosphatidylinositol



Enzyme 1 : phospholipase C

2 : diacylglycerol kinase

3 : phosphatidic acid : CTP cytidyltransferase

4 : CDP-diacylglycerol inositol-3-phosphatidyl transferase

Fig. 2.2 : The PtdIns Cycle

One approach in studying phosphoinositide metabolism is to examine the properties of the enzyme(s) involved, in particular that of the enzyme(s) possibly responsible for hydrolysis. Since it is now generally believed that the initial phosphoinositide hydrolysed is PtdIns-4,5P_2 (Berridge, 1983; Downes and Wusterman, 1983), the enzyme to be investigated is polyphosphoinositide phosphodiesterase. However, in the period 1953 - 1980, it was thought that PtdIns was initially hydrolysed and so studies of the PtdIns-specific phospholipase C were carried out. Since a considerable amount of information was obtained on this phospholipase C and its regulatory factors these will be described even though this enzyme is not responsible for the initial agonist-stimulated alteration in PtdIns-4,5P_2. Nevertheless, it is important that the properties of PtdIns-phospholipase C be discussed since its possible activation can not be completely disregarded simply because PtdIns-4,5P_2 hydrolysis appears to be the crucial lipid event.

2.9 (b) The Subcellular Distribution of Phospholipase C

The subcellular location of the phospholipase C which could degrade PtdIns has been the subject of investigation since identification of this cellular site could provide information as to the possible mechanism of the activation of PtdIns hydrolysis. A soluble enzyme would have quite a different mechanism of control than one which is membrane bound. For

example, activation of a soluble enzyme may involve binding to substrate in the membrane whereas activation of a membrane-bound enzyme could involve a conformational change in the lipid environment allowing substrate-enzyme interaction. Phospholipase C has been found in two forms : cytosolic and lysosomal.

The cytosolic soluble form has been identified in the cytosolic supernatant of mammalian tissues including pancreas (Dawson, 1959), brain (Thompson, 1967), liver (Kemp et. al., 1961), platelets (Billah et. al., 1980) and the intestinal mucosa of guinea-pig (Atherton and Hawthorne, 1961). In platelets, all the phospholipase C activity was restricted to the soluble fraction (Billah et. al., 1980) while in homogenates of human foetus and uterus, over 90% of the enzyme activity was soluble (Direnzo et. al., 1981).

Irvine and Dawson (1978 a) investigated whether a particulate membrane-bound component of this enzyme was present in brain since earlier hypotheses suggested that it may exist (Friedel et. al., 1969; Lapetina and Michell, 1973). No membrane-bound form of the enzyme was identified (Irvine and Dawson, 1978). The contribution of a possible membrane-bound phospholipase C is not known, no studies have demonstrated the presence of this enzyme in the pure plasma membrane. The specificity of cytosolic phospholipase C for PtdIns has been examined in a number of tissues. Soluble phospholipase C purified from *B. cereus* bacterial culture supernatants hydrolysed

PtdIns specifically with no hydrolysis of other phospholipids (Ikezawa et. al., 1976; Low and Finean, 1977; Taguchi and Ikezawa, 1978). The action of this enzyme isolated from *S.aureus* on pure PtdIns was limited and was only effective when PtdIns was present with other lipids (Low and Finean, 1976). Soluble phospholipase C from higher plants (Irvine et. al., 1980) and guinea-pig intestinal mucosa (Atherton and Hawthorne, 1968) was also found to be specific for PtdIns.

The other from of PtdIns-specific phospholipase C is lysosomal in location. Fowler and de Duve (1969) showed that liver lysosomes could hydrolyse PtdIns into water-soluble products but did not investigate the nature of these products. Irvine et. al. (1977 a) identified the lysosomal location of phospholipase C by its close correlation with acid phosphatase in the subcellular fractionation of rat liver. This enzyme hydrolysed (^{32}P)-PtdIns to release inositol-1-phosphate (Irvine et. al., 1977 a) and was shown to be specific for PtdIns, both as a pure phospholipid (Irvine et. al., 1978) and as a membrane constituent (Irvine and Dawson, 1979).

2.9 (c) Activation and Modulation of PtdIns-specific Phospholipase C

Does receptor occupation activate PtdIns-phospholipase C? The evidence for a phospholipase C-type of hydrolysis was provided by identification of inositol phospholipid hydrolysis products released by agonist stimulation. Increases in 1,2 diacylglycerol (Banschbach et. al., 1974) phosphatidic acid

(Hokin-Neaverson, 1974 ; Lapetina et. al., 1981) and inositol (Hokin-Neaverson et. al., 1975; Fain and Berridge, 1979) were suggested to be due to the action of phospholipase C on PtdIns. However, the possibility that these products could also result from the hydrolysis of polyphosphoinositides was not suggested at the time. Therefore, various proposals based on the ability of agents or lipid changes to stimulate the enzyme were made on the assumption that receptor activation stimulated a phospholipase C specific for PtdIns.

The initial observation that PtdIns-specific phospholipase C enzyme was relatively inactive on a membrane substrate (Low and Finean, 1976; Irvine and Dawson, 1978), led to the suggestion that the physicochemical state of the membrane substrate may be the controlling factor in PtdIns hydrolysis (Irvine and Dawson, 1980). This suggestion was supported by the studies showing that positively charged proteins, KCl at physiological concentrations and choline-containing lipids inhibited the activity of the enzyme with pure PtdIns substrate (Allan and Michell, 1974 a; Irvine et. al., 1979 a). In addition, the introduction of phosphatidic acid or unsaturated fatty acids such as oleic or arachidonic acid into a membrane caused an increase in the activity of a soluble brain extract on PtdIns (Irvine et. al., 1979 b). The stimulation of phosphatidic acid, a product of PtdIns hydrolysis, on the PtdIns-phospholipase C has led to the suggestion of a mechanism whereby amplification of PtdIns hydrolysis could occur once the initial hydrolysis was stimulated (Irvine et. al., 1979 a).

In order to stimulate the activity of a PtdIns-specific phospholipase C present in a cytosolic soluble form, it is possible that receptor occupation would not activate the enzyme but rather alter the state of the substrate rendering it susceptible to enzyme action. For example, that the agonist-stimulation can cause a removal of the inhibitory effect of choline-containing phospholipids by causing a phase-shift in the membrane (Irvine et. al., 1982), or stimulation of a diacylglycerol kinase (an enzyme which may be soluble or in membranes (Kanoh and Akersson, 1978) to produce phosphatidate which could stimulate the enzyme and so the hydrolysis of PtdIns (Irvine et. al., 1979) have been suggested.

There is no direct evidence to show that the binding of hormones or neurotransmitter to receptors causes a conformational change in the membrane allowing enzyme-substrate interaction. No direct demonstration of a measured increase in PtdIns-specific phospholipase C activity following receptor activation has been provided. Although studies inferred that receptor activation stimulated this enzyme, these proposals were based on inositol phospholipid breakdown products, which could have been released from any of the phosphoinositides.

2.9 (d) The Calcium-Dependency of Phospholipase C Activity

The effect of Ca^{2+} on PtdIns-specific phospholipase C has been examined to investigate whether the activation of this enzyme may occur as a result of an increase in intracellular Ca^{2+}.

The initial studies of the cytosolic PtdIns phospholipase C in pancreas and liver described a Ca^{2+} -dependency (Dawson, 1959; Kemp et. al., 1961) which has been further investigated and confirmed in lymphocytes by Allan and Michell (1974 a, b).

The determination of the Ca^{2+} requirement of this enzyme has been complicated since the activity of the enzyme can be modified depending on the conditions in which the enzyme and substrate is incubated. The Ca^{2+} -dependency of the PtdIns phospholipase C isolated from lymphocytes was reported to depend on the pH at which it was assayed (Allan and Michell, 1974 b). The activity of this enzyme isolated from brain, increased as the Ca^{2+} concentration increased from 0.2mM to 1.0mM only in the absence of KCl (Irvine and Dawson, 1979 a). However, in the cell, the intracellular concentration of KCl could be as high as 140mM - a concentration which would inhibit any Ca^{2+} -activation of this phospholipase C, since 80mM KCl was found to be inhibitory in the study by Irvine and Dawson (1979 a). The addition of Ca^{2+} only slightly enhanced the hydrolysis of rat liver microsomal PtdIns by brain PtdIns-phospholipase C (Irvine and Dawson, 1979 a), and it was suggested that, as with brain membranes (Irvine and Dawson, 1978 a, b), sufficient Ca^{2+} is bound to the membrane substrate for action of the enzyme.

The lysosomal phospholipase C appears to be Ca^{2+} -independent since EDTA-insensitive hydrolysis of PtdIns was reported (Irvine et. al., 1977).

Although these studies show that Ca^{2+} can modulate the activity of PtdIns phospholipase C it is not possible to make a conclusion as to whether Ca^{2+} -activation of this enzyme occurs *in vivo*. The *in vitro* studies use conditions of varying ionic strength and pH, they require substantial cell manipulation and fractionation to isolate the enzymes and these conditions may not mimic those of enzyme and substrate *in vivo*.

2.10 : "PTDINS EFFECT" 1981 - 1984

The studies on PtdIns-specific phospholipase C provided information as to possible regulatory mechanisms which may be involved in activation of this enzyme. Albeit, no conclusion could be made due to the inability to assume that the effects *in vitro* mimic those *in vivo*. More recent studies of PtdIns phospholipase C have shown that the situation is even more complex than this.

A more detailed examination of the PtdIns phospholipase C has indicated that there are several forms of the enzyme which exist in rat brain (Hirasawa et. al., 1982 a) and other tissues (Hirasawa et. al., 1982 b; Hoffman and Majerus, 1982) and only one may be active at physiological pH (Hirasawa et. al., 1982 a). In addition, Hirasawa et. al. (1982 c) found that the PtdIns-phospholipase C of rat brain shows little activity under physiological conditions (neutral pH and micromolar Ca^{2+} concentration). However, when brain extract was treated with exogenous proteases, new forms of the PtdIns-phospholipase enzyme with an increase in Ca^{2+} -sensitivity over the original form were identified (Hirasawa et. al., 1982 c). This was the first demonstration of an

intracellular phospholipase that could be converted from an inactive to an active form by proteolytic cleavage. It is not known whether the Ca^{2+} -sensitive form exists in resting tissue nor whether proteolytic cleavage is part of the mechanism involved in activation of PtdIns-phospholipase C, if indeed it does occur.

Over the last four years considerable evidence has accumulated to support a role for the hydrolysis of PtdIns-4,5,P_2 in Ca^{2+} -mobilization (Streb et. al., 1983; Burgess et. al., 1984; Joseph et. al., 1984; Suematsu et. al., 1984).

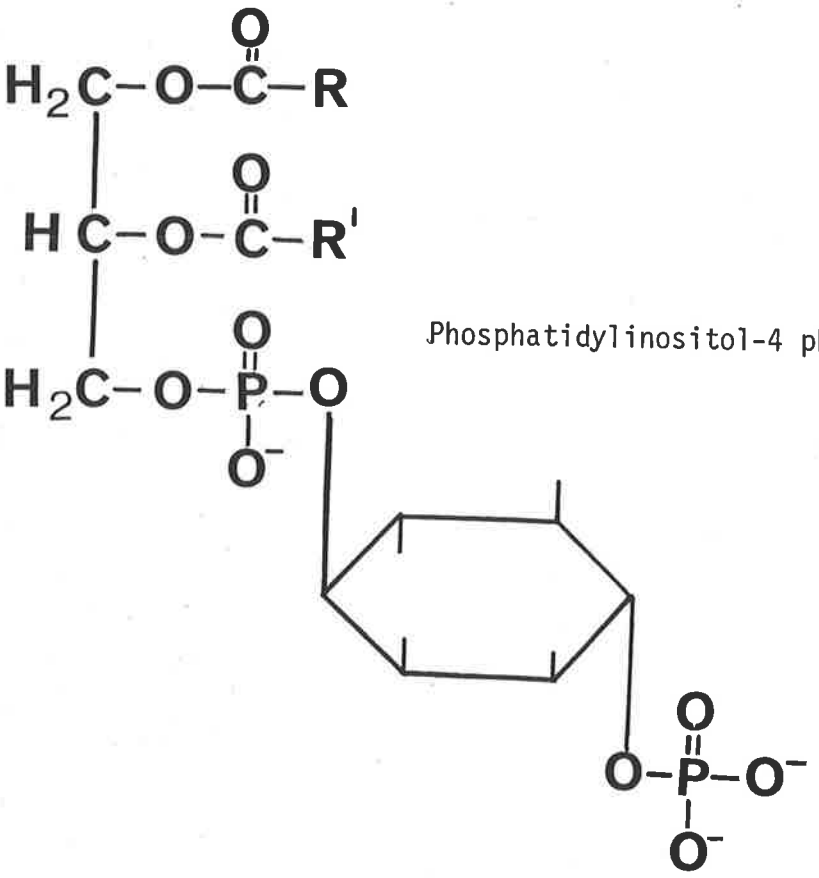
2.10 (a) Polyphosphoinositides : Metabolism

The polyphosphoinositides are the most polar of all phospholipids; they possess additional phosphate groups monoesterified to the inositol ring and so causing them to bind calcium strongly (Hendrickson, 1969). Phosphorylation of PtdIns at the hydroxyl group on position 4 of the inositol ring (by PtdIns kinase) produces PtdIns-4P and phosphorylation of this lipid at position 5 (by PtdIns-4P kinase) produces PtdIns-4,5P_2 (Fig. 2.3). These polyphosphoinositides comprise only about 1 - 2% of the total inositol lipid (Michell et. al., 1970). The pathways of conversion of inositol lipids are shown in Fig. 2.4.

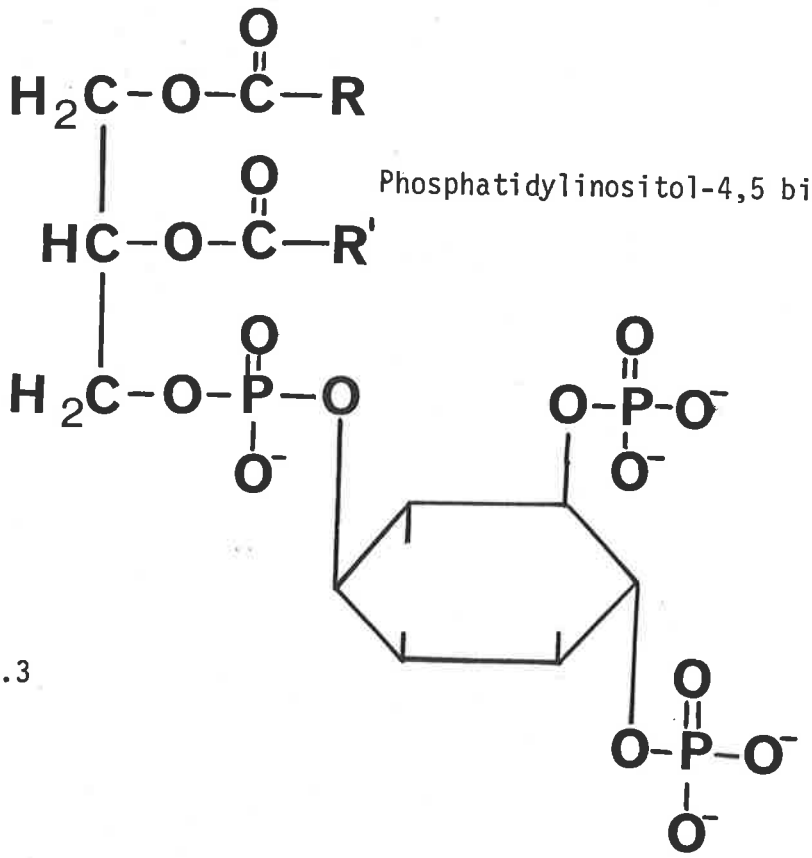
The polyphosphoinositides can be hydrolysed by a phosphodiesterase to remove the entire inositol-phosphate moiety (Ins-1,4,5P_3 or Ins-1,4P_2) or by phosphomonoesterases to remove specifically either the 4-P or 5-P and produce PtdIns-4-P or PtdIns. The

phosphomonoesterase activity has been found in both soluble and plasma membrane-bound enzymes (Keough and Thompson, 1970; Sheltaway et. al., 1972; Salway et. al., 1967). There appears to be two phosphomonoesterases, one specific for 4-phosphate and the other specific for 5-phosphate (Roach and Palmer, 1981).

The PtdIns-4,5P_2 phosphodiesterase activity in brain is found in both the soluble and plasma-membrane enriched fractions (Hawthorne, 1964; Hawthorne and White, 1975) and is soluble in kidney (Lapetina et. al., 1975) and iris-smooth muscle (Akhtar and Abdel-Latif, 1980). It is not known whether the soluble or membrane-bound form could be responsible for polyphosphoinositide hydrolysis *in vivo*. The work of Thompson and Dawson (1964 a, b) and Dawson and Thompson (1964) characterized many of the properties of the enzymes involved in the metabolism of the polyphosphoinositides. Studies in brain (Thompson and Dawson, 1964 a, b), erythrocytes (Downes and Michell, 1981) and hepatocytes (Seyfred and Wells, 1984 b) suggest that one polyphosphoinositide phosphodiesterase is responsible for the hydrolysis of both PtdIns-4,5P_2 and PtdIns-4P to release inositol trisphosphate (Ins-1,4,5P_3) and inositol bisphosphate (Ins-1,4,5P_2). However, whether one enzyme acts on both polyphosphoinositides has not been established.

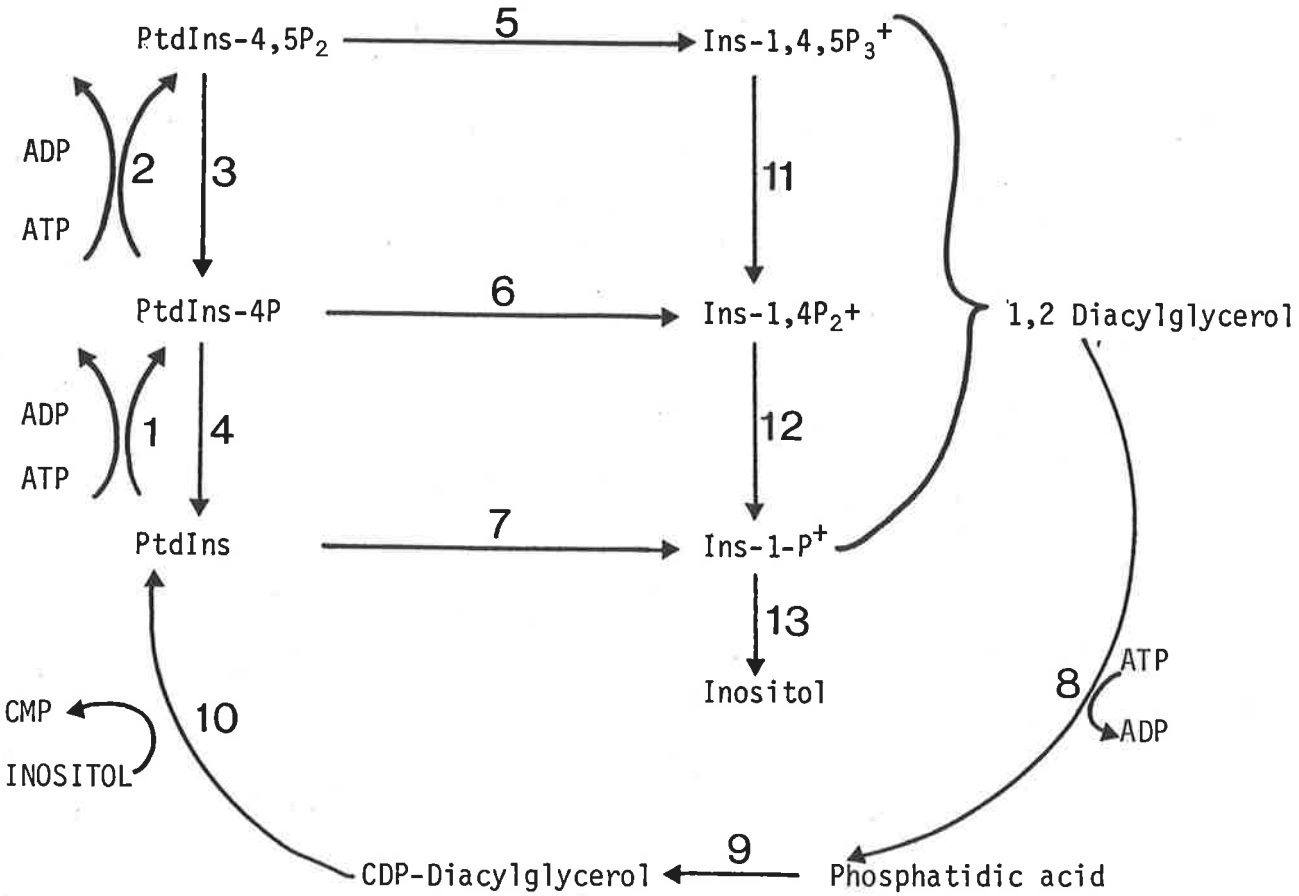


Phosphatidylinositol-4 phosphate



Phosphatidylinositol-4,5 bisphosphate

Fig. 2.3



Enzymes:
1. phosphatidylinositol kinase
2. phosphatidylinositol-4-phosphate kinase
3. phosphatidylinositol-4,5-bisphosphatase
4. phosphatidylinositol-4-phosphatase
5. polyphosphoinositide phosphodiesterase
6. polyphosphoinositide phosphodiesterase
7. phosphatidylinositol phosphodiesterase (phospholipase C)
8. diacylglycerol kinase
9. CTP-phosphatidate cytidyl transferase
10. CDP diacylglycerol inositol-3-phosphatidyl transferase
11. inositol-trisphosphatase
12. inositol-bisphosphatase
13. inositol-1-phosphatase

Fig. 2.4 : Pathways of Metabolism of Polyphosphoinositides

2.10 (b) Polyphosphoinositide Phosphodiesterase : Activation and Modulation

How might the polyphosphoinositide phosphodiesterase(s) be activated to hydrolyse PtdIns-4,5P_2 (and PtdIns-4P) on receptor occupation? The question of the Ca^{2+}-dependency of activation of this enzyme has received considerable attention.

The polyphosphoinositide phosphodiesterase was suggested to be a Ca^{2+} -dependent enzyme, since it was inactivated by the Ca^{2+} chelator EDTA (Ethylene-diamine-tetra-acetic acid) and activity restored by addition of Ca^{2+} (Thompson and Dawson, 1964 b). Early studies of polyphosphoinositide phosphodiesterase in erythrocytes by Allan and Michell (1978) showed that this enzyme was very sensitive to Ca^{2+} (in 100nM range - a concentration which is probably physiological) at low ionic strength. This suggested that perhaps hydrolysis of PtdIns-4,5P_2 occurred subsequent to an increase in cytosolic Ca^{2+} concentration. However, this was found not to be so. In a later study (Downes and Michell, 1982) when ionic strength was close to physiological, the polyphosphoinositide phosphodiesterase remained inactive up to 100 µM Ca^{2+} (about 1000-fold higher than occurs in healthy cells), indicating that this enzyme is probably not active in a normal erythrocyte. Although the erythrocyte is a convenient cell in which to investigate polyphosphoinositide phosphodiesterase there are no physiological stimuli which cause an increase in cytosol Ca^{2+}. It may be more appropriate therefore to examine this enzyme in other cells which possess receptors which on activation stimulate mobilization of Ca^{2+}.

More recently, the activity of PtdIns-4,5-bisphosphate phosphodiesterase in brain was examined. Although this enzyme hydrolysed PtdIns-4,5P_2 in any form at 1μM Ca^{2+}; when Mg^{2+} (1mM) and KCl 70mM were present (as an approximate parallel with the ionic environment *in vivo*) the enzyme could not act on PtdIns-4,5P_2 when this lipid was present in a lipid mixture similar to that in which it may exist *in vivo* (Irvine et. al., 1984). These results suggested that Ca^{2+} may not activate this enzyme when substrate is present in a physiological state.

A recent study by Cockcroft et. al. (1984) demonstrated the presence of a polyphosphoinositide phospholipase C which degraded endogenous PtdIns-4,5P_2 and PtdIns-4P in a plasma-membrane enriched fraction of human or rabbit neutrophils. Addition of Ca^{2+} (5×10^{-4}M) to these plasma-membrane fractions led to the specific hydrolysis of polyphosphoinositides. However, the incubation medium was a non-physiological one in that it was of low ionic strength (approx. 20mM) and the concentration of Ca^{2+} used was considerably higher than that expected in a normal cell. Therefore, this stimulation by Ca^{2+} cannot be ascribed a physiological effect and there is no evidence to show that it occurs in neutrophils following f-met-leu-phe activation.

A recent study of the dose-response of Ca^{2+} on the polyphosphoinositide phosphodiesterase in rat hepatocytes showed that the enzyme was activated by physiological concentrations of free

Ca^{2+} (200nM) when assayed at low ionic strength. However, the Ca^{2+}-requirement shifted to micromolar concentrations under isosmotic ionic conditions which were similar to *in vivo* conditions (Seyfred and Wells, 1984 b). The effect of ionic strength on this phosphodiesterase is in agreement with that observed in erythrocytes (Downes and Michell, 1982). It has been suggested that ionic strength alters the fluidity of the membrane (Seyfred and Wells, 1984 b) since a membranes' transition temperature is increased on increasing the ionic strength of the surrounding medium (Trauble, 1976). At lower ionic strength, the membrane could then be more fluid, and the phosphodiesterase having less physical constraints may have a lowered requirement for Ca^{2+}.

The activity of this enzyme to hydrolyse PtdIns-4,5P_2 present in a non-bilayer substrate was not affected by changes in Ca^{2+} within physiological limits ranging from 100nM to 10μM (Irvine et. al., 1984). It has therefore been suggested recently that the PtdIns-4,5P_2 phosphodiesterase has sufficient Ca^{2+} *in vivo* to hydrolyse its substrate and that the physicochemical form in which the substrate exists in a non-stimulated plasma membrane is unsuitable for hydrolysis (Irvine et. al., 1984). Perhaps then the activation of receptors in some way can alter the state of the substrate to make it more susceptible to enzyme action.

One possible way in which this could occur would be by changing the membrane lipid fluidity. Burgess et. al. (1983) showed

that noradrenaline and angiotensin II can increase the lipid fluidity, as measured by diphenylhexatriene-fluorescence polarization, of plasma membranes isolated from rat liver. This stimulated response did not occur when Ca^{2+} was removed from the membranes by EGTA (Burgess et. al., 1983). Divalent cations have been shown to reduce the mobility of phospholipids (Livingstone and Schachter, 1980) and since a small release of Ca^{2+} was observed from the isolated membranes following agonist-stimulation (Burgess et. al., 1983) it is possible that the agonist causes release of Ca^{2+} bound to membrane phospholipids, removes the mechanical constraints of this ion, and increases membrane fluidity (Burgess et. al., 1983). Perhaps this physical membrane change alters the substrate availability for the phosphodiesterase and inositol phospholipid hydrolysis could occur.

How well can the results of the activity of isolated enzymes with isolated substrates incubated in a variety of solutions which may or may not be physiological, but used to optimize activity, be related to effects *in vivo*? Although studies of the phosphodiesterases may provide information as to regulatory factors, it is difficult to relate these effects directly to the *in vivo* situation since this is not clearly known, in particular dependence on Ca^{2+}. If PtdIns hydrolysis, or PtdIns-4,5P_2 hydrolysis is to occur prior to an increase in intracellular Ca^{2+} concentration then this enzyme should not be dependent on an increase in cytosolic Ca^{2+} for its activation.

The Ca^{2+} -dependency of the phosphodiesterase enzyme is not clear and since the most recent studies showed an increased Ca^{2+} sensitivity of one form of the PtdIns-phospholipase C enzyme produced by proteolytic cleavage (Hirasawa et. al., 1982 c) it is possible that the Ca^{2+} normally present in the cell may be sufficient for the function of this enzyme.

The question of whether receptor occupation causes a conformational change in the membrane which makes the substrate available to the enzyme or alternatively stimulates the enzyme, or whether both these events occur, has not been answered.

It should be kept in mind that studies of the polyphosphoinositide phosphodiesterase may be meaningless and that the enzyme activated on receptor occupation may not yet have been described. For example, the possibility exists that receptor activation could stimulate the proteolytic cleavage of an inactive polyphosphoinositide phosphodiesterase to an active form (similar to the effect on PtdIns phospholipase C; Hirasawa et. al., 1982 c) which has not been discovered.

2.11 : SITE OF INOSITOL PHOSPHOLIPID METABOLISM

Although the basic details of the enzymic pathways for inositol phospholipid metabolism have been established there is uncertainty concerning the cellular location of the hydrolysis of inositol phospholipid. Since receptor activation occurs at the external surface of the cell then a site for inositol phospholipid breakdown in the

plasma membrane would be compatible with Michell's hypothesis (1981). If phosphoinositide hydrolysis occurred in intracellular membranes a "second messenger" would be required to transmit the signal from the plasma membrane to whatever internal membrane site the lipid breakdown occurred.

The synthesis of PtdIns in response to cholecystokinin or acetylcholine occurs in the endoplasmic reticulum in guinea-pig pancreas slices (Hokin and Huebner, 1967). The endoplasmic reticulum contains the highest level of phospholipid biosynthetic enzymes compared with other subcellular fractions (Wagner et. al., 1962). Phosphatidylinositol synthetase is entirely localized in the endoplasmic reticulum, is specific for CDP-diacylglycerol and *myo*-inositol and was purified to near homogeneity by Takenawa and Egawa (1977).

Although the synthesis of PtdIns occurs at the endoplasmic reticulum there must be a continuous interchange of lipids between the various membranes. A group of soluble phospholipid exchange proteins have been described (Helmkamp et. al., 1976; Demel et. al., 1977; Laffont et. al., 1981) and these could be responsible for refilling the pools of PtdIns in intracellular organelle membranes which do not synthesize PtdIns. A recent study by Seyfred and Wells (1984 a) showed that when rat hepatocytes were incubated with (^{32}P)-P_i and then fractionated, a similar rate of (^{32}P)-P_i incorporation into PtdIns of plasma membrane, mitochondria, nuclei, lysosomes and microsomes was found, suggesting rapid translocation of newly formed PtdIns to the sites where synthesis does not occur.

The enzyme CDP-digylceride synthetase (phosphatidic acid : CTP cytidyltransferase) converts phosphatidic acid directly to CDP-diacylglycerol. This conversion was first detected in liver microsomes *in vitro* (Carter and Kennedy, 1966), subsequently, the enzyme has been described in mitochondria and smooth and rough endoplasmic reticulum (Bishop and Strickland, 1970; Ter Schegget et. al., 1971).

The enzyme which converts 1,2 diacylglycerol to phosphatidic acid is 1,2 diacylglycerol kinase and is found both in the soluble and membrane fraction of brain, with a proportion of activity found in the plasma membrane (Lapetina and Hawthorne, 1971).. It has also been detected in plasma membranes of erythrocytes (Hokin and Hokin, 1963) and lymphocytes (Fisher and Mueller, 1971).

If inositol phospholipid hydrolysis does not occur at the endoplasmic reticulum then a mechanism to transport the diglyceride or phosphatidic acid to this site of PtdIns synthesis would be required. However, there is no evidence at present for a mechanism which transports the 1,2 diacylglycerol from one membrane to another. A study to investigate the presence of a phosphatidic acid-transfer protein showed clearly that platelet cytosol stimulated PtdIns transfer between liposomes and mitochondria whereas the transfer of phosphatidic acid between these two sites was not increased by platelet cytosol over a pH range between 4.5 to 10.5 (Laffont et. al., 1981). Other possible mechanisms for intermembranous exchange of phosphatidic acid, such as passive transfer (Stuhne-Sekalec and Stanacev, 1977, 1979) could not be excluded.

Synthesis of the polyphosphoinositides involves the enzymes phosphatidylinositol kinase (PtdIns-kinase) and phosphatidylinositol-4-phosphate kinase (PtdIns-4-P kinase) which phosphorylate PtdIns and

PtdIns-4-P, respectively. PtdIns kinase is known to be present in a number of subcellular sites including the plasma membrane (Harwood and Hawthorne, 1969; Smith and Wells, 1983), Golgi (Jergil and Sundler, 1983), lysosomal membrane (Collins and Wells, 1983), microsomes and nuclear envelopes (Smith and Wells, 1983). PtdIns-4-P kinase has been found associated with nuclear envelopes, plasma membranes and microsomes of rat liver (Smith and Wells, 1983), and is a soluble enzyme in brain (Kai et. al., 1968) and rat parotid gland (Oron et. al., 1978).

Although PtdIns-kinase and PtdIns-4-P kinase have been identified in a number of sites, the recent study by Seyfred and Wells (1984 a) showed that the plasma membrane contained the highest level of (^{32}P)-P_i -labelled polyphosphoinositides when compared with all the other subcellular fractions analysed. The general belief that these lipids are characteristic of the plasma membrane came mostly from the studies of erythrocyte membranes (Buckley and Hawthorne, 1972) and subcellular fractionation of brain (Eichberg and Hauser, 1973). The surface membranes of polymorphonuclear leukocytes (Wieneke and Woodin, 1967) and kidney (Hauser and Eichberg, 1973) also contain appreciable concentrations of polyphosphoinositides. However, very little is known about the polyphosphoinositides in intracellular membranes. These lipids have been found in adrenal chromaffin vesicle membranes (Buckley et. al., 1971; Phillips, 1973) and the secretory vesicles of the parotid (Oron et. al., 1978), membranes which do have a close functional relationship with the plasma membrane.

Despite the numerous studies of the enzymes involved in inositol phospholipid metabolism and their location, the site(s) of agonist-stimulated phosphoinositide breakdown has not been clearly identified.

The earliest studies of this question used avian salt gland (Hokin and Hokin, 1960). The disappearance of PtdIns occurred in the microsomal fraction which consisted largely of smooth membrane fragments derived from the extensive plasma membrane which is packed into numerous infoldings to give a very large secretory surface. There is only a very small amount of endoplasmic reticulum in this gland. In the mouse pancreas, the only fraction to show a significant decrease in (^{32}P)-PtdIns in response to acetylcholine was the rough endoplasmic reticulum (Harris and Hokin-Neaverson, 1977), whereas in rabbit neutrophils the site of PtdIns loss was the plasma membrane (Bennett et. al., 1982).

PtdIns hydrolysis has also been suggested to occur at the secretory vesicles in islets of Langerhans (Clements et. al., 1977) and synaptosomes (Pickard and Hawthorne, 1978). Using hepatocytes, Kirk and Michell (1981) reported that a constant proportion of the initial (^{32}P)-PtdIns complement was lost from all of the subcellular fractions derived from cells stimulated with vasopressin for 5 min. However, a study by Lin and Fain (1981) found that when hepatocytes were labelled with (^{3}H)-inositol, exposed to vasopressin or adrenaline, and then subject to cell fractionation a 16-19% loss of PtdIns was detected in the plasma-membrane enriched fraction.

The reasons for such variation in the localization of the disappearance of PtdIns are not clear. It is possible that a rapid movement of PtdIns by PtdIns exchange proteins (Helmkamp et. al., 1976) may contribute to this variation. For example, in hepatocytes, the PtdIns loss may occur at one site but there may be exchange of PtdIns between membranes

leading to re-establishment of an equilibrium distribution among all cellular compartments. In the pancreas, the localization of an agonist-stimulated decrease in PtdIns at the endoplasmic reticulum could be due to PtdIns breakdown at this site (Hokin-Neaverson, 1977) or alternatively could be due to the activity of a PtdIns-exchange protein. Since the endoplasmic reticulum is the site of synthesis of PtdIns (Hokin and Huebner, 1967) it is possible that during the time of incubation, the PtdIns-exchange protein could transfer the newly synthesized PtdIns to the site of degradation thereby causing a deficit at the endoplasmic retitulum. Therefore the site of inositol phospholipid breakdown in pancreas is not clear.

As for the results of PtdIns loss in synaptosomes (Pickard and Hawthorne, 1978) and secretory vesicles (Clements et. al., 1977) it has been suggested that this may not necessarily preclude the possibility that it occurs at the plasma membrane (Bennett et. al., 1982). The reason for this is that Bennett et. al., 1980) found that after secretion had occurred from neutrophils most of the plasma membrane fraction which in unstimulated cells is in the fraction containing the secretory granules. Since the studies by Pickard and Hawthorne (1978) and Clements et. al. (1977) did not show that their cell fractionation procedure (defined only for unstimulated cells) remains valid after secretion, it is possible that the plasma membrane may also be part of the fraction containing the secretory granule. In these tissues, cell stimulation results in exocytotic secretion, which involves fusion between the secretory vesicle membranes and plasma membranes and it is therefore likely that there is some plasma membrane associated with the secretory vesicle following stimulation.

To further examine whether inositol lipid hydrolysis occurs at the plasma membrane studies using isolated plasma membrane preparations were made, thereby avoiding any possible action of PtdIns exchange proteins.

Vasopressin and adrenaline induced a specific loss of PtdIns from isolated rat liver plasma membranes (Wallace et. al., 1982, 1983) and the vasopressin response was initially found to require deoxycholate (Wallace et. al., 1982). The use of this detergent to disrupt the lipid environment suggested that receptor occupation was unable to cause the appropriate changes in the membrane to allow lipid hydrolysis to occur. However, in a subsequent study the deoxycholate requirement was not found (Wallace et. al., 1983).

There are conflicting reports as to the requirement for addition of cytosol to the plasma membrane preparations. Harrington and Eichberg (1983) reported that noradrenaline caused a 50% loss of PtdIns from purified liver plasma membranes and that this effect did not occur if cytosol was absent from the incubation medium. In contrast to this study, Wallace et. al. (1983) demonstrated noradrenaline-stimulated PtdIns did not require added cytosol. The reason(s) for the different results in the two studies of isolated rat liver plasma membranes could be due to the contamination of the membrane fractions (Wallace et. al., 1983) with sufficient cytosol to allow the hydrolysis to occur or perhaps the preparation of membrane fractions (Harrington and Eichberg, 1983) caused disruption of structural links between receptor and effector.

It is not possible from these two studies to determine whether cytosol is required (and therefore that there are certain factors in cytosol, perhaps the phospholipase C, which are essential to link receptor occupation with enzyme activation), or whether the receptor-enzyme activation is a mechanism which can occur solely in the plasma membrane. This latter conclusion would require both substrate and enzyme to be membrane bound and that receptor activation would probably cause a conformational change allowing interaction.

All the above described studies measured decreases in PtdIns from various cellular sites. If, however, the hydrolysis of PtdIns-4,5P_2 is the initial agonist-stimulated event, then the studies identifying the site(s) of PtdIns loss may have been an indication of the site(s) of PtdIns phosphorylation rather than hydrolysis. As previously described, the PtdIns-kinase and PtdIns-4-P kinase enzymes have been found in a number of cellular sites. However, for phosphorylation to occur ATP is required. This might explain the requirement for added cytosol in membrane preparations (Harrington and Eichberg, 1983). Since the incubation media for the liver plasma membranes (Wallace et. al., 1983; Harrington and Eichberg, 1983) did not contain added ATP then cytosol would be required to provide the metabolic energy to allow phosphorylation to occur and so cause a loss of PtdIns in the membrane.

A recent study by Seyfred and Wells (1984 b) examined the site of vasopressin-induced phosphoinositide hydrolysis in rat hepatocytes. It was reported that after 45 sec of vasopressin treatment of (^{32}P)-P_i -prelabelled hepatocytes, the levels of (^{32}P)-PtdIns-4,5P_2 and (^{32}P)-PtdIns-4-P in the plasma membrane decreased by 40%, then gradually

returned to control levels after 10 min of treatment. This loss of polyphosphoinositides was specific for the plasma membrane since only small changes in PtdIns-4,5P_2 and PtdIns-4P were observed in the other subcellular fractions and these changes were attributed to contamination of these fractions by plasma membranes (Seyfred and Wells, 1984 b). The loss of plasma membrane (^{32}P)-PtdIns was 15% after a 45 sec incubation of prelabelled hepatocytes with vasopressin. This study by Seyfred and Wells (1984 b) of total (^{32}P)-phosphoinositide changes in hepatocytes are in good agreement with other studies (Litosch et. al., 1983; Creba et. al., 1983 and Thomas et. al., 1983) with respect to the extent and rapidity of response in hepatocytes.

A further analysis of polyphosphoinositide hydrolysis was made using (^{32}P)-P_i -labelled plasma membranes isolated from prelabelled hepatocytes. Using intracellular-like ionic conditions, and a Ca^{2+} concentration of 0.2μM, vasopressin stimulated a 20% decrease in PtdIns-4,5P_2 with a corresponding production of (^{32}P)-inositol trisphosphate and (^{32}P) -inositol bisphosphate. No change in (^{32}P)-P_i -labelled PtdIns or PtdIns-4P was observed and it was found that the increase in inositol bisphosphate resulted from an action of phosphatase enzyme on inositol trisphosphate (Seyfred and Wells, 1984 a). The requirement for Ca^{2+} of the polyphosphoinositide phosphodiesterase was demonstrated since no significant change in polyphosphoinositide hydrolysis occurred when the plasma membranes were incubated in the absence of Ca^{2+} (at physiological ionic strength); the half-maximal activity observed at 5μM Ca^{2+}. This study (Seyfred and Wells, 1984 a) suggests that there is a minimal amount of Ca^{2+} required for polyphosphoinositide breakdown in response to vasopressin which is supported by other studies

with hepatocytes showing a Ca^{2+}-requirement (Prpić et. al., 1982; Rhodes et. al., 1983; Thomas et. al., 1983).

If, as the above studies suggest, polyphosphoinositide hydrolysis occurs in the plasma membrane, rather than an intracellular membrane, then this site of hydrolysis would provide an efficient mechanism whereby binding of hormone or neurotransmitter to receptor and subsequent hydrolysis occur at the same or closely associated site - indicating that these two events are closely linked. It is still not known how receptor occupation could activate phosphoinositide hydrolysis.

2.12 : MEASUREMENT OF THE AGONIST-STIMULATED HYDROLYSIS OF INOSITOL PHOSPHOLIPID

The agonist-stimulated renewal of the inositol and phosphate moieties of the PtdIns molecule was not accompanied by an equivalent increase in the incorporation of glycerol into PtdIns (Hokin and Hokin, 1958 a, b). This led to the suggestion of a closed cycle of PtdIns breakdown and resynthesis (Hokin and Hokin, 1964) and so it was generally accepted in the early studies, that incorporation of radiactive precursor into PtdIns was an indirect measure of the hydrolysis step. There are a number of problems with this assumption which have made it necessary to develop methods to measure inositol phospholipid breakdown directly. Firstly, there are enzymic reactions which can occur to stimulate the synthesis of PtdIns (*de novo*) which do not occur as a result of phosphoinositide breakdown (see below). Secondly, the synthesis step of the inositol phospholipid cycle appears to be sensitive to a variety of factors which do not affect phosphoinositide breakdown (see below).

The earliest studies by the Hokins showed that in addition to the increase in the incorporation of (^{32}P)-P_i and (^{3}H)-inositol into PtdIns, (^{14}C)-glycerol incorporation into PtdIns and phosphatidic acid in pancreas slices was also stimulated by acetylcholine (Hokin and Hokin, 1958 a). This indicated that in addition to the turnover of the headgroup of PtdIns, enhanced *de novo* synthesis may contribute to the increase in incorporation of (^{32}P)-P_i into PtdIns in pancreas. Hokin and Hokin (1958 b) found, however, that enhanced *de novo* synthesis of PtdIns did not occur in slices of brain cortex from guinea-pig. More recently, studies of pancreas from rat (Calderon et. al., 1979; Chapman et. al., 1983) and human (Chapman et. al., 1983) have confirmed the original finding of Hokin and Hokin (1958 a), showing agonist-stimulated *de novo* synthesis.

It would therefore appear that the agonist-stimulated PtdIns -labelling responses in pancreas represent a combination of increased synthesis due to inositol phospholipid breakdown and also due to enhanced synthesis *de novo* since the newly synthesized phosphatidic acid must be able to enter the same pool as is generated from the diacylglycerol released during inositol phospholipid hydrolysis. Any reaction which increases the formation of diglyceride is capable of increasing the synthesis of PtdIns. Diacylglycerol may be derived from triacylglycerol or other non-inositide phospholipids and in the presence of (^{32}P)-P_i or (^{3}H)-inositol this may be converted to radio-labelled PtdIns. This has been observed in lymphocytes where calcium activated a triacylglycerol lipase to produce the diglyceride which then formed PtdIns (Allan and Michell, 1978). PtdIns would then be phosphorylated to form the polyphosphoinositides. A recent study (Ishizuka et. al., 1983) demonstrated that *de novo* synthesis of PtdIns, but not PtdIns breakdown, was stimulated by ionophore A23187 and compound 48/80 in rat mast cells.

The Ca^{2+}-dependent increase in *de novo* synthesis would provide incorrect evidence of a Ca^{2+}-dependent breakdown of inositol phospholipid in studies using resynthesis as a measure of hydrolysis. The effect of an increase in intracellular Ca^{2+} is often investigated using the Ca^{2+} ionophore A23187 which avoids the requirement for receptor occupation. In this case, it would not be possible to distinguish whether an increase in the PtdIns-labelling response is due to synthesis of PtdIns from diacylglycerol derived from PtdIns or from other lipid. Agonist-enhanced synthesis of PtdIns *de novo* has been reported in tissues other than pancreas (Hokin and Hokin, 1958 a) such as hepatocytes (Kirk and Michell, 1981), platelets (Prescott and Majerus, 1981), thyroid gland (Scott et. al., 1968), polymorphonuclear leukocytes (Sastry and Hokin, 1966) and adrenal cortex (Farese et. al., 1980).

In addition to agonist-stimulated *de novo* synthesis, cationic amphiphilic drugs, such as trifluoperazine, chlorpromazine and procaine, can cause an increase in labelling of PtdIns with (^{32}P)-P_i and (^{14}C)-glycerol in lymphocytes (Allan and Michell, 1975) by causing inhibition of phosphatidate phosphohydrolase (Brindley and Bowley, 1975), an enzyme which can convert phosphatidate to diglyceride. Therefore, an increase in incorporation of radioactive precursor does not occur as a result of breakdown of inositol lipid.

These studies show that it is necessary to be able to distinguish between inositol phospholipid turnover where the source of diacylglycerol is phosphoinositide and the increased labelling response due to production of diacylglycerol from a lipid other than inositol phospholipid.

Another problem with using the method of measuring the incorporation of radioactive precursor into phosphoinositide as an indication of the hydrolysis is the possibility that some agents which do not alter the breakdown step can alter the synthesis step. The incorporation of the (^{3}H)-inositol into PtdIns is catalysed by the CDP-diacylglycerol-inositol-3-phosphatidyl transferase which can be inhibited by Ca^{2+} (Egawa et. al., 1981 a). The inhibition by Ca^{2+} of incorporation of (^{3}H)-inositol into PtdIns has been demonstrated in blowfly salivary gland (Berridge and Fain, 1979), whereas the release of (^{3}H)-inositol due to agonist-stimulated phosphoinositide breakdown was not altered by removal of Ca^{2+} (Fain and Berridge, 1979). This reduction in PtdIns synthesis has been shown in other tissues such as vas deferens (Egawa et. al., 1981 b), platelets (Broeckman et. al., 1980), and iris-smooth muscle (Abdel-Latif and Luke, 1981). In addition, (^{32}P)-P_i incorporation into iris smooth muscle (Abdel-Latif and Luke, 1981) and rat parotid gland (Keryer et. al., 1979) has shown a dependence on the presence of sodium ion.

Since the incorporation of radioactive precursor into PtdIns can be altered by *de novo* synthesis and agents such as Ca^{2+}, it is not possible to use this as an indirect measure of inositol phospholipid breakdown. The measurement of the incorporation of radioactive precursors into inositol phospholipids has been used, however, in a number of studies on pancreas (Hokin and Hokin, 1955, 1958 a; Bauduin and Cantraine, 1972; Calderon et. al., 1979, 1980; Hokin, 1968 a, b, 1974; Farese et. al., 1981 a, 1982; Halenda and Rubin, 1982).

Another method used as a measure of inositol phospholipid hydrolysis is a decrease in its concentration measured either by radiochemical

or chemical assay. The use of (^{32}P)-P_i or (^{3}H)-inositol to pre-label inositol phospholipid prior to addition of agonist has enabled the measurement of an agonist-stimulated decrease in pancreatic PtdIns (Hokin, 1974; Hokin-Neaverson et. al., 1975; Hokin-Neaverson, 1977; Halenda and Rubin, 1982) and pancreatic polyphosphoinositide (Putney et. al., 1983; Orchard et. al., 1984). Other studies have measured a decrease in (^{14}C)-arachidonic acid-labelled PtdIns (Calderon et. al., 1979; Marshall et. al., 1980, 1981; Halenda and Rubin, 1982) in pancreatic tissue.

The concentration of phospholipids measured either by radiochemical or chemical means, measures the turnover of lipid and can be changed by alterations in breakdown, synthesis, or both. It is possible to measure the breakdown of inositol phospholipid as a decrease in the concentration of pre-labelled lipid providing the appropriate incubation time is used. For example, in platelets, an initial decrease in (^{32}P)-PtdIns-4,5P_2 occurs 15 sec after activation with platelet-activating-factor; this is subsequently followed by a rapid increase in incorporation of (^{32}P)-P_i into PtdIns-4,5P_2 such that at 30 sec the amount of (^{32}P)-PtdIns-4,5P_2 exceeds that of control (Lapetina, 1983). This effect is also seen with (^{32}P)-PtdIns and (^{32}P)-PtdIns-4P (Lapetina, 1983). Therefore, since concentration measurements can be the net result of breakdown and synthesis it is essential that the initial hydrolysis of lipid be measured very rapidly after agonist-activation to avoid the additional measurement of re-synthesis.

In experiments using (^{3}H)-inositol-labelled lipid, the same problem exists although an attempt to avoid measuring the re-synthesis steps

could be made by ensuring that the majority of the radioactive precursor has been incorporated into inositol lipid, the remaining free (^{3}H)-inositol removed by washing, and by incubating tissue in the presence of excess non-radioactive inositol to reduce the re-incorporation of (^{3}H)-inositol released from lipid following breakdown.

However, it is not possible to determine exactly to what extent any possible agonist-stimulated increase in the *de novo* synthesis contributes to the total inositol lipid concentration.

In some studies both a decrease in PtdIns concentration and an increase in phosphatidic acid concentration was measured (Hokin-Neaverson, 1974; Farese et. al.,1981 a, 1982). However, the phosphatidic acid concentration will be determined by inositol phospholipid breakdown and by the increase in formation of CDP-diacylglycerol and so too, will not provide a direct measure of phosphoinositide breakdown.

Any agent which may alter synthesis and not the breakdown of inositol phospholipid as described previously, could therefore cause a change in the total concentration of this lipid and so an incorrect estimation of the degree of agonist-stimulated breakdown.

Another problem in measuring inositol phospholipid concentration to assay hydrolysis is that a decrease in inositol phospholipid could occur due to activation of a phospholipase A_2. This enzyme characteristically requires Ca^{2+} and catalyses the deacylation of PtdIns so as to produce lysophosphatidylinositol and free fatty acid;

arachidonic acid (Van den Bosch, 1980). Since many studies use ionophore A23187 to increase the intracellular Ca^{2+} concentration as a test for the Ca^{2+} -dependency of the inositol lipid response, activation of phospholipase A_2 leading to a decrease in inositol phospholipid content would lead to erroneous conclusions.

Since phospholipase C activity releases 1,2 diacylglycerol and inositol-phosphates but phospholipase A_2 activity does not, then it would be more appropriate if investigating the agonist-activation of phospholipase C activity to measure the released products, which unlike measurement of total inositol lipid mass would avoid the detection of activation of phospholipase A_2 activity.

The most accurate method to measure breakdown of inositol phospholipids is to measure the release of the hydrolysis products. Hokin-Neaverson et. al. (1975) measured an acetylcholine-stimulated increase in (^{3}H)-inositol (which was believed at that time to be released from PtdIns in mouse pancreas), in addition to an increase in inositol levels measured chemically with gas-liquid-chromatography. Measurement of hydrolysis products were made in other gland tissue such as blowfly salivary gland (Fain and Berridge, 1979; Berridge and Fain, 1979), and rat parotid gland (Keryer et. al., 1979).

Hokin-Neaverson et. al. (1975) investigated whether a phosphodiesterase C type of cleavage was involved in the acetylcholine-stimulated loss of PtdIns in mouse pancreas. After examining the water-soluble products of PtdIns breakdown, no increase in the levels of inositol 1,2-cyclic phosphate or inositol-1-phosphate (products which would be released by

phospholipase C) were measured, while the levels of inositol increased. On the basis of this evidence it was suggested at the time, that other mechanisms of degradation which would release inositol as breakdown product were more likely than phospholipase C activity in mouse pancreas. These mechanisms could possibly be by action of a phospholipase D or reversal of the CDP-diacylglycerol-inositol-3-phosphatidyltransferase (Hokin-Neaverson et. al., 1975).

However, since the studies by Hokin-Neaverson et. al. (1975) on PtdIns breakdown in mouse pancreas no further investigation of the role of the hydrolysis response in pancreatic function, nor any examination of the calcium dependency of this response in pancreas were made until 1980.

A method was developed (Tennes and Roberts, 1981) to measure the agonist-stimulated breakdown of inositol phospholipid in mouse pancreas by assay of the release of (^{3}H)-inositol-labelled hydrolysis products released from inositol phospholipid labelled *in vivo* with *myo*-(2-^{3}H)-inositol. As described in Chapters 3 and 4 of this thesis, this technique provides a valid measure of inositol phospholipid hydrolysis (Tennes and Roberts, 1981, 1984) and eliminates the requirement for lengthy lipid extraction and chromatographic separation procedures used previously. This method facilitated the construction of dose-response curves and was used to investigate the role of calcium in the agonist-stimulated hydrolysis of inositol phospholipid in mouse pancreas (Tennes and Roberts, 1982, 1983). The validity of this technique is not dependent on whether PtdIns, PtdIns-4,5P_2 or PtdIns-4P is the initial lipid hydrolysed since the assay measures the release of (^{3}H)-inositol-labelled products which will accumulate independent of

which lipid is hydrolysed. Measurement of these breakdown products in total, without separating and identifying them, does not, however, allow determination of which phosphoinositide is hydrolysed nor would it detect if several pathways of hydrolysis of inositol phospholipid occurred.

The failure of Hokin-Neaverson et. al. (1975) to detect any increase in inositol 1,2-cyclic phosphate or inositol-1-phosphate could be explained by the action of two particular enzymes. Many tissues contain an active inositol 1,2-cyclic phosphate 2-phosphohydrolase (Dawson and Clarke, 1972) which could rapidly convert the cyclic derivative to inositol-1-phosphate. In addition, a *myo*-inositol-1-phosphatase has been found which would convert inositol-1-phosphate to inositol (Eisenberg, 1967). However, we now know that inositol 1,2-cyclic phosphate is not formed following agonist-activation of pancreas. Analysis of (^{3}H)-inositol-phosphates released from (^{3}H)-inositol-labelled phosphoinositides in rat pancreas showed increases in inositol-1-phosphate, inositol bisphosphate and inositol trisphosphate; no inositol 1,2-cyclic phosphate was identified (Rubin et. al., 1984).

The discovery that lithium inhibits *myo*-inositol-1-phosphatase (Naccarato et. al., 1974; Hallcher and Sherman, 1980) allowed the detection of an increase in the accumulation of inositol-1-phosphate due to agonist-stimulated breakdown of inositol phospholipid in mouse pancreatic fragments (Tennes and Roberts, 1984) and dispersed mouse pancreatic acinar cells (Hokin-Neaverson and Sadeghian, 1984). The results of these studies were supported by the identification of a carbachol- and caerulein-stimulated increase in inositol-1-phosphate in the

(^{3}H)-inositol-labelled water-soluble compounds extracted from rat pancreatic acini (Rubin et. al., 1984).

These independent studies provided the first evidence that, in the exocrine pancreas, a phospholipase C type of phosphodiesterase activity is involved in the hydrolysis of inositol phospholipid.

Subsequent to the development of the method described in this thesis, another method which measures the release of hydrolysis products was developed by Berridge et. al. (1982). This method utilized the ability of lithium to inhibit inositol-1-phosphatase and also the selective binding of inositol phosphates to an anion-exchange resin to measure agonist-stimulated increases in inositol-1-phosphate. This technique provides a good signal-to-noise ratio, particularly when using procedures for labelling tissues with radioactivity where a large amount of free *myo*-(2-^{3}H) inositol may be present.

However, the hydrolysis of polyphosphoinositide releases Ins-1,4,5P_3 and Ins-1,4P_2 which could rapidly be degraded by phosphatases to Ins-1-P. The increase in accumulation of Ins-1-P does not distinguish between whether the phospholipase C acts on PtdIns polyphosphoinositides or both. Therefore, any method which measures either increases in (^{3}H)-inositol-labelled products or more specifically in Ins-1-P, released from inositol lipid measures hydrolysis accurately but does not identify which lipid is initially hydrolysed by receptor activation. To investigate this question studies identifying increases in inositol phosphates released from lipid and their rates of release have recently been made (for example, Berridge, 1983; Downes and Wusterman, 1983; Rubin et. al., 1984).

The phosphosphoinositides of pancreas have been investigated by analysis of decreases in the levels of PtdIns-4,5P_2 and (^{32}P)-PtdIns-4P. Putney et. al. (1983) reported a loss of 15 - 20% of (^{32}P)-PtdIns-4,5P_2 in less than 1 min after stimulation of isolated rat pancreatic acini with carbachol of caerulein. A similar decrease in (^{32}P)-PtdIns-4,5P_2 was found in isolated cells, however, in neither of the tissue preparations did an agonist-stimlated decrease in PtdIns-4P occur (Putney et. al., 1983). A more recent study by Orchard et. al. (1984) showed a 30 - 50% decrease in (^{32}P)-PtdIns-4,5P_2 within 10-15 sec after stimulation of rat pancreatic acini with carbachol or CCK. These results of agonist-stimulated decreases in PtdIns-4,5P_2 have been reported in a number of tissues including rabbit iris smooth muscle (Akhtar and Abdel-Latif, 1978), rat hepatocytes (Kirk et. al., 1981; Thomas et. al., 1983; Creba et. al., 1983) blowfly salivary gland (Berridge, 1983), platelets (Billah and Lapetina, 1982) and rat parotid gland (Weiss et. al., 1982). Although these studies provide evidence for a rapid agonist-stimulated decrease in PtdIns-4,5P_2 they do not identify by which pathway the inositol lipids are hydrolysed.

As shown earlier in Fig. 2.4,PtdIns-4,5P_2 can be degraded by two pathways. The first pathway via the action of a phosphodiesterase would release diacylglycerol and Ins-1,4,5P_3 whereas the second pathway via the action of phosphomonoesterases which sequentially remove phosphate from the 5- and 4-position would yield PtdIns. The difference between these two pathways is the primary substrate which is hydrolysed by receptor activation. The first pathway has PtdIns-4,5P_2 as substrate whereas the second pathway has PtdIns as the initial lipid hydrolysed. The examination of the water-soluble

products released following the hydrolysis of inositol lipid can enable the identification of the lipid initially hydrolysed by the receptor activation mechanism.

Studies of phosphoinositide metabolism in insect salivary glands (Berridge, 1983) and rat parotid gland (Downes and Wusterman, 1983) have provided convincing evidence that agonists do hydrolyse polyphosphoinositides instead of PtdIns, at least in these secretory tissues. Firstly, following receptor activation, Ins-1,4,5P_3 and Ins-1,4P_2 increase with no delay (measured as early as 5 sec after agonist addition) and precede in time the increases in the levels of Ins-1-P and inositol (Berridge, 1983; Downes and Wusteman, 1983).

Secondly, the addition of agonist to tissue depleted of polyphosphoinositides caused little or no increase in free inositol or any inositol phosphates measured 10 min (Downes and Wusteman, 1983) or 5 min (Berridge, 1983) after receptor activation. (Since phosphorylation of PtdIns to PtdIns-4P and PtdIns-4,5P_2 is ATP-dependent, incubation of cells in dinitrophenol will inhibit the synthesis of polyphosphoinositides and so cause the depletion of these lipids from cell membranes).

A reduction in the agonist-stimulated hydrolysis of PtdIns by the metabolic inhibitor 2,4 dinitrophenol was reported by Hokin (1974) using mouse pancreas. At the time, it was not possible to explain why the hydrolysis of PtdIns would be an energy requiring mechanism (since the action of PtdIns-phospholipase C does not require ATP). This result was confirmed and extended using a number of metabolic

inhibitors in mouse pancreas (results described in Chapter 5 of this thesis). A metabolic requirement for phosphoinositide breakdown has since been reported in blood platelets (Holmsen et. al., 1982), rat parotid gland (Poggioli et. al., 1983; Downes and Wusterman, 1983), rat liver (Prpić et. al., 1982) and blowfly salivary gland (Berridge et. al., 1984). This result supports the proposal that PtdIns must first be converted to polyphosphoinositides before activation by agonists can stimulate hydrolysis.

It would therefore appear that hydrolysis of PtdIns is not associated with the initial events associated with receptor activation in blowfly salivary gland and rat parotid gland, however, the evidence at present does not eliminate the possibility that PtdIns may be involved at a later period following receptor activation. In addition, these results do not allow the identification of whether PtdIns-4,5P_2 and PtdIns-4P are both hydrolysed following receptor activation. That is, whether the increase in Ins-1,4P_2 is due to the action of inositol trisphosphatase on Ins-1,4,5P_3 or by the direct hydrolysis of PtdIns-4P by a phosphodiesterase.

The first direct measure of PtdIns-4,5P_2 hydrolysis in exocrine pancreas by measurement of agonist-induced increases in inositol-phosphates was described this year by Rubin et. al. (1984). At 1 min after carbachol-stimulation of rat pancreatic acini there was a 6.8-fold, 8.2-fold and 1.4-fold increase in (^{3}H)-Ins-1,4,5P_3 , (^{3}H)-Ins-1,4P_2 and (^{3}H)-Ins-1-P, respectively. These results indicate the action of a phosphodiesterase on PtdIns-4,5P_2. The Ins-1,4P_2 could arise by either the hydrolysis of PtdIns-4P or by the activity of an inositol bisphosphatase to dephosphorylate Ins-1,4,5P_3.

The rapid time course for the production of Ins-1,4,5P_3 and Ins-1,4P_2 compared with Ins-1-P (Rubin et. al., 1984) suggests that the hydrolysis of the polyphosphoinositides occurs prior to any possible hydrolysis of PtdIns. The lag period for production of Ins-1-P suggests that the increase in Ins-1-P is due to the removal of the phosphate from Ins-1,4P_2 by inositol bisphosphatase. In this case the proposal that PtdIns is not hydrolysed but phosphorylated to replace the polyphosphoinositide (Michell et. al., 1981) is supported. However, the results of the study by Rubin et. al. (1984) cannot eliminate the possibility that some Ins-1-P may be the product of the direct action of a phospholipase C on PtdIns.

As the above studies demonstrate, over the last few years there has been a great improvement in methods available to measure the hydrolysis of inositol phospholipid.

2.13 : THE CALCIUM DEPENDENCY OF INOSITOL PHOSPHOLIPID METABOLISM IN EXOCRINE PANCREAS

Hokin (1966) found that if Ca^{2+} was removed from the incubation medium, the acetylcholine-stimulated release of amylase from pigeon pancreas was abolished but a large stimulation of (^{32}P)-P_i incorporation into PtdIns and phosphatidic acid still occurred. This was the first indication that Ca^{2+} was not required for activation of the PtdIns response. Since this time the question of the Ca^{2+}-dependency of the inositol phospholipid response has been the subject of intense investigation and some controversy.

The initial proposal by Michell (1975) that PtdIns hydrolysis may precede the increase in intracellular Ca^{2+} led to the idea that the hydrolysis of PtdIns and (later of the polyphosphoinositides) should be independent of Ca^{2+}. A number of procedures have been used to examine the role of Ca^{2+} in phosphoinositide metabolism such as removal of this ion from the extracellular bathing medium, introduction of Ca^{2+} into the cell with the use of ionophores, and depletion of intracellular stores of Ca^{2+}.

In the early studies of PtdIns, it was confirmed that extracellular Ca^{2+} was not essential for the agonist-stimulated synthesis or loss of PtdIns and that A23187 did not stimulate PtdIns loss in a variety of tissues including rat parotid glands (Jones and Michell, 1975, 1976), hepatocytes (Billah and Michell, 1979), blowfly salivary gland (Fain and Berridge, 1979) and rat lacrimal gland (Jones et. al., 1979). However, the ability of the Ca^{2+} ionophore to cause the loss ot PtdIns in platelets (Bell and Majerus, 1980; Lapetina et. al., 1981) and neutrophils (Cockcroft et. al., 1981) led to the suggestion that the breakdown of PtdIns may not be involved in regulating Ca^{2+} fluxes but occur as a result of the increase in cytosolic Ca^{2+} (Cockcroft et. al., 1981; Hawthorne, 1982).

A stimulatory effect of ionophore on inositol lipid breakdown does show that there is a Ca^{2+}-dependent hydrolysis of phosphoinositide, however, it does not exclude the possibility that there may also be a receptor-activated Ca^{2+}-independent breakdown of this lipid.

Following the initial study by Hokin (1966) on the removal of extracellular Ca^{2+} on PtdIns synthesis, no study of the role of this ion

in agonist-stimulated inositol phospholipid metabolism in exocrine pancreas was reported until 1979.

Calderon et. al. (1979) reported that Ca^{2+} deprivation of rat pancreatic fragments by incubation in the calcium chelator EGTA did not prevent the carbachol-stimulated increase in incorporation of (^{3}H)-inositol, (^{32}P)-P_i, (^{3}H)-glycerol or (^{14}C)-arachidonic acid into inositol phospholipid. This study confirmed and extended the results of Hokin (1966). Farese et. al. (1982), in agreement with these earlier studies, showed (^{32}P)-P_i-labelling effects of PtdIns in rat pancreas to occur in the absence of Ca^{2+} but found that the agonist-stimulated decrease in PtdIns mass and increase in phosphatidic acid mass were dependent on Ca^{2+} (Farese et. al., 1980, 1982). In addition, the ionophore A23187 stimulated a decrease in PtdIns mass which led at the time to the suggestion that pancreatic inositol phospholipid changes follow rather than precede changes in intracellular Ca^{2+} (Farese et. al., 1980). However, as discussed earlier in Section 2.12 neither the measurement of incorporation of precursor into inositol phospholipid, nor the measure of total mass provides a measure of the initial agonist-stimulated event of hydrolysis. Any Ca^{2+}-dependency of these responses cannot be pre-supposed to be indicative of the hydrolysis response.

The results of Farese et. al. (1980, 1982) could be due to effects of Ca^{2+} other than to stimulate PtdIns hydrolysis. One alternative explanation would be that the increase in intracellular Ca^{2+} via the ionophore stimulates a phospholipase A_2 leading to a decrease in PtdIns mass. Halenda and Rubin (1982) demonstrated an agonist-stimulated

phospholipase A_2 activity in rat pancreatic acini, whereby the Ca^{2+} ionophore ionomycin stimulated the turnover of (^{14}C)-arachidonic acid-labelled PtdIns.

Another possible explanation for the apparent Ca^{2+}-dependent agonist-stimulate changes in PtdIns mass and phosphatidic acid mass might be that in the absence of Ca^{2+} the inhibitory effect of this ion on the conversion of phosphatidic acid to PtdIns (Egawa et. al., 1981) is removed. In this case PtdIns would be synthesized more rapidly and so the agonist-stimulated decrease in PtdIns would be compensated for and thus go undetected.

A close examination of the results of Farese et. al. (1982) revealed that in the presence of Ca^{2+} the carbachol-stimulated synthesis of PtdIns was increased 10.3-fold from control whereas in the absence of Ca^{2+} the increase was 25.1-fold above control. This increased synthesis was also apparent with CCK as agonist, with increases of 2.3-fold and 12-fold above control observed in the presence and absence of Ca^{2+}, respectively (Farese et. al., 1982).

These results show that in the absence of Ca^{2+}, a greater conversion of phosphatidic acid to PtdIns would occur. Therefore, it is possible that an agonist-stimulated loss of PtdIns did occur in the absence of Ca^{2+} in the study by Farese et. al. (1982) but that due to rapid re-synthesis, the decrease in PtdIns mass and increase in phosphatidic acid was small and so undetectable. In the presence of Ca^{2+} the loss of PtdIns could be detected due to a decrease in re-synthesis.

The study by Downes and Wusteman (1983) demonstrated that the polyphosphoinositides are hydrolysed and replaced from the PtdIns pool several times during even relatively brief periods (10 min) of agonist-stimulation of rat parotid gland. The agonist-stimulated decrease in PtdIns mass seen by Farese et. al. (1982) could be due to its conversion to polyphosphoinositides (Rubin et. al., 1984). It is possible that due to this rapid cycling of PtdIns, in the absence of Ca^{2+} an underestimation of PtdIns loss would be made.

The proposal by Farese et. al. (1982) of a Ca^{2+}-dependent loss of PtdIns is not the only explanation for the results of their study, the alternative explanations described above would also appear to be supported by their results and just as feasible.

The first study of the role of Ca^{2+} on the hydrolysis of inositol phospholipid in exocrine pancreas showed no such stimulatory effect of ionophore A23187 (at the concentration of 10^{-6}M, which stimulated amylase secretion); no requirement for intracellular stored Ca^{2+} and also that the agonist-stimulated hydrolysis still occurred when Ca^{2+} was removed from the extracellular medium (Tennes and Roberts, 1982). This study measured the release of (^{3}H)-inositol-labelled products from inositol phospholipid but did not identify which lipid was initially hydrolysed. These results are included in this thesis (Chapter 6).

Putney et. al. (1983), using rat pancreatic acini reported that ionomycin (2×10^{-6}M) for the first 2 min after addition, did not mimic the effects of carbachol or caerulein in inducing the loss of (^{32}P)-PtdIns-4,5P_2. However, ionomycin after 5-10 min caused a

parallel decrease in both (^{32}P)-PtdIns-4,5P_2 and (^{32}P)-PtdIns-4P. It was suggested (Putney et. al., 1983) that ionomycin may cause loss of polyphosphoinositides by a toxic mechanism which inhibits production of metabolic energy, since antimycin A caused a similar loss of polyphosphoinositide in parotid gland (Poggioli et. al., 1983). (Depletion of ATP would inhibit phosphorylation of PtdIns and so reduce polyphosphoinositide content).

An alternative explanation could be Ca^{2+}-stimulation of phospholipase C. Orchard et. al. (1984) using a high concentration of A23187 (2×10^{-5}M) did not observe any decrease in (^{32}P)-PtdIns-4,5P_2 of (^{32}P)-P_i pre-labelled rat pancreatic acini nor any increase in (^{32}P)-phosphatidic acid 1,2,5 or 10 min after A23187 addition. This high concentration of A23187 would cause a significant increase in the intracellular Ca^{2+} concentration and so be expected to stimulate any calcium-dependent hydrolysis of inositol phospholipid if it were to occur. The lack of effect of A23187 to stimulate inositol lipid hydrolysis both in this study by Orchard et.al. (1984) and also in that described by Tennes and Roberts (1982) suggests that the ability of ionomycin to induce a loss to phosphoinositide is not due to its effect to increase cytosolic Ca^{2+} and stimulate a phospholipase C, but perhaps due to some other toxic effect, in support of the suggestion by Putney et. al. (1983).

More recently, Rubin et. al. (1984) investigated the Ca^{2+}-dependency of polyphosphoinositide breakdown in rat pancreatic acini using the measurement of the release of Ins-1,4,5P_3 , Ins-1,4,P_2 , and Ins-1-P. These workers suggested that Ca^{2+} mobilization does not trigger the

breakdown of PtdIns-4,5P_2 since increases in Ins-1,4,5P_3 were not stimulated by ionomycin (10^{-6}M) after a 30 min incubation. This ionophore did, however, stimulate a small but significant increase in Ins-1,4P_2 and Ins-1-P over a 30 min incubation period (Rubin et. al., 1984). A toxic effect of ionomycin to deplete ATP (as suggested by Putney et. al., 1982) would not explain why ionomycin caused an increase in Ins-1,4P_2 and Ins-1-P. This result of Rubin et. al. (1984) could be interpreted to indicate a Ca^{2+}-stimulated hydrolysis of PtdIns-4P and perhaps also PtdIns, although it is not possible to determine if Ins-1-P was formed from Ins-1,4P_2, PtdIns or both. However, as previously described, studies using A23187 (10^{-6}M or 2 x 10^{-5}M) suggested that Ca^{2+} does not stimulate inositol phospholipid hydrolysis (Tennes and Roberts, 1982; Orchard et. al., 1984).

How then can the effect of ionomycin to stimulate increases in Ins-1,4P_2 and Ins-1-P (Rubin et. al., 1984) be explained? A non-specific toxic effect is one possibility. However, the mechanism that would allow an affect on PtdIns-4P_2 but not PtdIns-4,5P_2 is not known. Alternatively, because the study of Rubin et. al. (1984) isolated each of the (^{3}H)-inositol phosphates from free (^{3}H)-inositol, perhaps this allowed a much greater sensitivity in detecting a small degree of hydrolysis compared with measurement of increases in total (^{3}H)-inositol-labelled prodcuts (Tennes and Roberts, 1982) or (^{32}P)-phosphatidic acid (Orchard et. al., 1984). This would require that the phosphodiesterase enzyme stimulating the hydrolysis of PtdIns-4P has a requirement for Ca^{2+} whereas the enzyme hydrolysing PtdIns-4,5P_2 does not (since ionomycin did not cause the release of Ins-1,4,5P_3, Rubin et. al., 1984). There does not appear to be any direct evidence to show this.

It is not clear why A23187 and ionomycin appear to have different effects on phosphoinositides. It could be due to the different sensitivity of methods used to assay their effects on lipid hydrolysis. Perhaps these ionophores differ in their ability to increase cellular Ca^{2+}. If ionomycin causes a much greater increase in Ca^{2+} this might then activate the phospholipases responsible for release of Ins-1,4P_2 and Ins-1-P. These are only suggestions to explain the results and there is no evidence to support them at present.

The agonist-stimulated hydrolysis of inositol phospholipid in exocrine pancreas was not dependent on release of Ca^{2+} from intracellular stores (Tennes and Roberts, 1982) nor was the loss of (^{32}P)-PtdIns-4,5P_2 altered when a protocol was used which eliminated agonist-induced release of intracellular Ca^{2+} (Putney et. al., 1983). However, a role for extracellular Ca^{2+} in the agonist-stimulation of exocrine pancreas was suggested when it was found that although this event does not require Ca^{2+}, the addition of this ion to the incubation medium potentiated phosphoinositide hydrolysis stimulated by both cholinergic and peptide-receptor agonists (Tennes and Roberts, 1982). The effect of Ca^{2+} to potentiate agonist-stimulated inositol phospholipid hydrolysis and its possible site of action are described and discussed in Chapter 7 of this thesis. The more recent study of the specific inositol-phosphates released from pancreatic inositol phospholipid following receptor activation (Rubin et. al., 1984) did not examine a role for extracellular-Ca^{2+}. A role for extracellular Ca^{2+} in the agonist-stimulated hydrolysis of inositol phospholipid has recently been confirmed in hepatocytes (Creba et.al., 1983), lacrimal gland (Godfrey and Putney, 1984) and basophil 2H3 cells (Beaven et. al., 1984).

The hypothesis that inositol phospholipid hydrolysis mediated by phospholipase C is an early event in stimulus-secretion coupling occuring prior to an increase in intracellular Ca^{2+} is supported by the studies showing that an increase in intracellular Ca^{2+} does not mimic agonist-activated phosphoinositide hydrolysis (Tennes and Roberts, 1982; Putney et. al., 1983; Orchard et. al., 1984; Rubin et. al., 1984) and that intracellular Ca^{2+} is not required for agonist activation of this lipid response (Tennes and Roberts, 1982; Putney et. al., 1983).

Does inositol phospholipid hydrolysis control the mobilization of Ca^{2+}? One definitive way in which the proposal that the hydrolysis of inositol phospholipid controls cellular Ca^{2+} could be negated would be if receptor activation caused an increase in intracellular Ca^{2+} without an associated hydrolysis of phosphoinositide. This has not been demonstrated. Recent studies described in the following section (2.14) have provided supporting evidence for a role of inositol phospholipid hydrolysis (in particular, the breakdown products released) in mobilizing cellular Ca^{2+} and perhaps also in allowing Ca^{2+} to enter into cells.

2.14 : THE ROLE OF INOSITOL PHOSPHOLIPID HYDROLYSIS IN MEDIATING STIMULATION OF THE CELL RESPONSE

Inositol phospholipid hydrolysis may have two mechanisms whereby it mediates stimulation of the cell response agonists : one is by mobilizing Ca^{2+}, the other by activation of protein kinase C.

2.14 (a) The Role of Inositol Phospholipid Hydrolysis in Mobilizing Calcium

What evidence is there to distinguish between whether the agonist-activated inositol phospholipid hydrolysis does control cellular Ca^{2+} or whether the increase in intracellular Ca^{2+} is a parallel, independent event? A number of studies have investigated the ability of the breakdown products of inositol phospholipid hydrolysis to stimulate Ca^{2+} mobilization, and to mimic the effect of Ca^{2+}-mobilizing hormones to stimulate the cell response.

The first study to demonstrate a link between inositol phospholipids and cellular Ca^{2+} was reported by Berridge and Fain (1979). They suggested that if PtdIns is an essential component in activation of Ca^{2+} mobilization then a decrease in the level of this lipid should cause a corresponding decrease in the agonist-stimulated Ca^{2+} transport. This proposal was proven to be true when during stimulation of blowfly salivary gland with high concentrations of 5-hydroxytryptamine a gradual depletion of PtdIns (due to Ca^{2+} inhibition of resynthesis) and also a gradual decrease in $^{45}Ca^{2+}$ transport was measured (Berridge and Fain, 1979). If the glands were washed free of 5-hydroxytryptamine the increase in $^{45}Ca^{2+}$ transport was not restored. However, when the glands were incubated in the presence of 2mM-*myo*-inositol so as to allow resynthesis of PtdIns, the increase in $^{45}Ca^{2+}$ transport stimulated by 5-hydroxytryptamine was restored. These results showed a good correlation between PtdIns levels and $^{45}Ca^{2+}$ influx and led to the suggestion

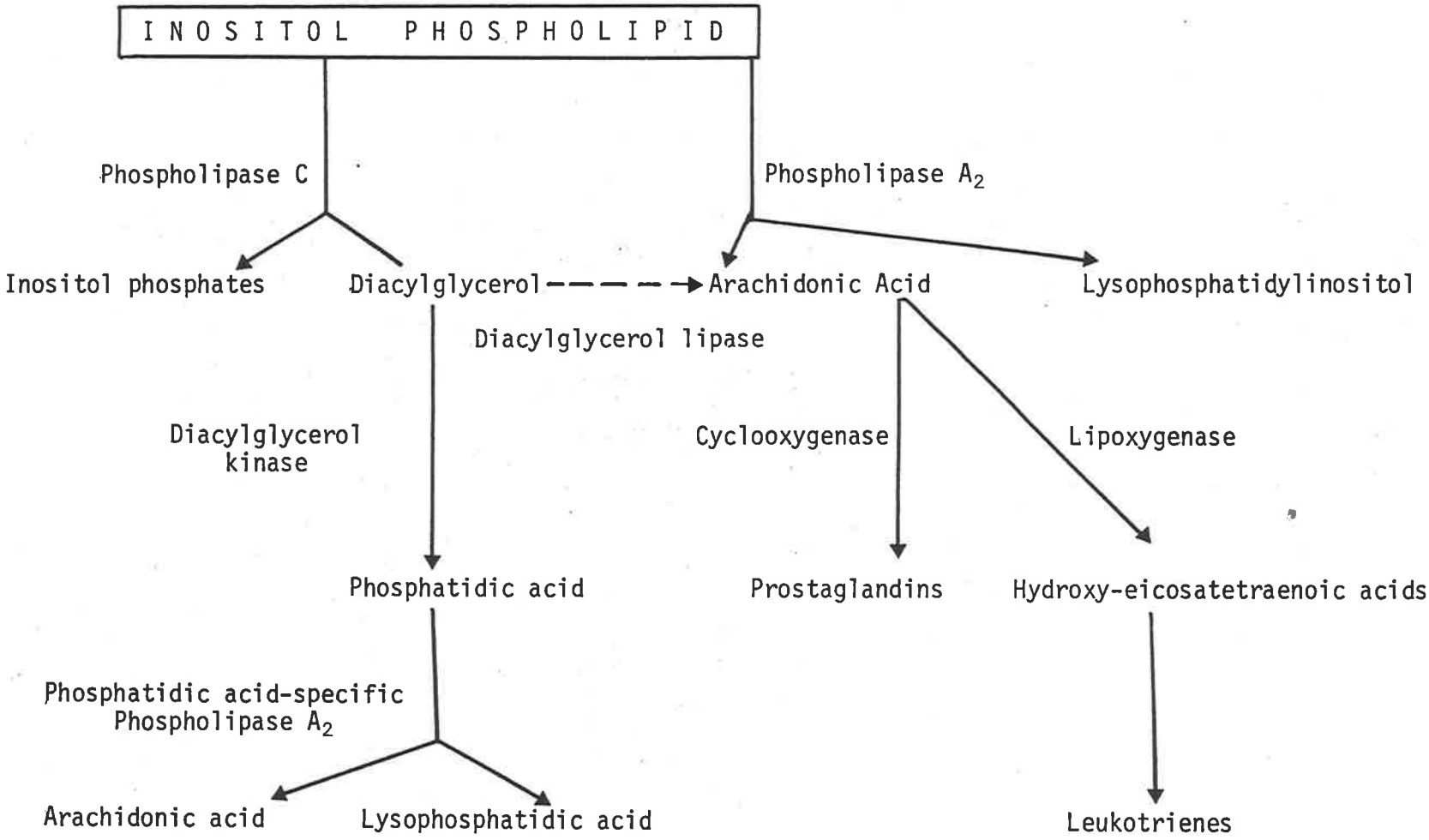


Fig. 2.5 : Inositol Phospholipid Hydrolysis Products : Possible Mediators of Mobilization of Cellular Calcium?

that PtdIns may play some role in opening calcium gates or channels in the plasma membrane by a conformational change due to PtdIns hydrolysis (Berridge and Fain, 1979). However, there was no evidence to support this idea.

The idea that the physicochemical change in the plasma membrane due to PtdIns loss may lead to an increase in the permeability of the plasma membrane to Ca^{2+} could account for the Ca^{2+} influx seen in a variety of cell types. However, in the pancreas the initial effect of agonist is to mobilize Ca^{2+} from cellular stores prior to causing an influx of Ca^{2+} (Stolze and Schulz, 1980; Dormer et. al., 1981).

Therefore, for inositol phospholipid hydrolysis to cause mobilization of cellular Ca^{2+} a mechanism must exist whereby the signal at the receptor on the plasma membrane would reach internal Ca^{2+} storage sites such as endoplasmic reticulum to stimulate release of Ca^{2+}.

To investigate this question workers examined the possibility that one or more of the breakdown products of inositol phospholipid hydrolysis may in some way release or transport Ca^{2+}. A schematic representation of the pathways involved in metabolism of products released by inositol phospholipid hydrolysis is shown in Fig. 2.5.

One of the first candidates for this role was phosphatidic acid which was found to translocate Ca^{2+} across both organic

solvent layers and liposomal membranes (Tyson et. al., 1976; Salmon and Honeyman, 1980; Putney et. al., 1980b; Serhan et. al., 1981). Phosphatidic acid stimulated Ca^{2+} uptake by nerve terminals (Harris et. al., 1981), and neuroblastoma cells (Ohsako and Deguchi, 1981) and the release of Ca^{2+} from the sacroplasmic reticulum of cardiac cells (Limas, 1980). Using a fluorescent phosphatidic acid analogue, Pagano et. al. (1983) demonstrated that when this analogue was transferred from phospholipid vesicles to cultured fibroblasts the endoplasmic reticulum was seen as highly fluorescent, indicating uptake and transfer to the site of PtdIns synthesis. It is possible that phosphatidic acid could transport Ca^{2+} across plasma membranes and also be transported to the internal cellular membrane to function as an ionophore at these sites before being converted back to PtdIns.

The addition of phosphatidic acid to smooth muscle cells (Salmon and Honeyman, 1980) and parotid slices (Putney et. al., 1980 b) mimicked the responses of these tissues to calcium-mobilizing hormones. In addition, Putney et. al. (1980 b) demonstrated that the ability of a variety of agents (including neomycin, lanthanum, cobalt and nickel) to inhibit the translocation of Ca^{2+} by phosphatidic acid from a water to chloroform phase correlated with their ability to inhibit agonist-activated ^{86}Rb release (which provides an indirect measure of changes in cellular Ca^{2+}) from parotid glands. It was therefore suggested that phosphatidic acid may act as a natural Ca^{2+} ionophore in

cells which show an agonist-stimulated hydrolysis of inositol phospholipid (Putney et. al., 1980 b).

If phosphatidic acid, acting as an ionophore, is to be involved in the agonist-stimulated Ca^{2+}-dependent cell response then its formation should be rapid enough to correlate with Ca^{2+} mobilization. In platelets, the time course of phosphatidic acid production correlated with the rapid release of serotonin (Lapetina, 1982), and Ca^{2+} influx (Imai et. al., 1982). However, the formation of phosphatidic acid following agonist-stimulation did not occur as quickly as the Ca^{2+}-dependent release of lysosomal enzymes in neutrophils (Cockcroft et. al., 1980), nor fast enough to stimulate Ca^{2+}-dependent phosphorylase an activity in isolated hepatocytes (Thomas et. al., 1983), indicating that phosphatidic acid may not mediate the initial changes in cellular Ca^{2+}.

Subsequent to the study by Putney et. al. (1980b), evidence was obtained which did not support a role for phosphatidic acid as an endogenous Ca^{2+} ionophore. Studies in our laboratory demonstrated that the local anaesthetic procaine inhibited the transfer of $^{45}Ca^{2+}$ by phosphatidic acid from an aqueous to organic phase but this agent did not inhibit the ability of the peptide agonist bradykinin to stimulate the Ca^{2+}-dependent response of smooth muscle contraction (Crouch et. al., 1981). The inability of local anaesthetics to inhibit the effect of peptide agonists to stimulate cellular response was also described for parotid gland (Marier et. al., 1978) since Substance P-stimulation of ^{86}Rb release was unaffected by

tetracaine and procaine. It would therefore appear that phosphatidic acid may not be acting as a Ca^{2+} ionophore *in vivo*.

There is evidence that phosphatidic acid can function to transport Ca^{2+}. However, whether it does act in this way to trigger activation of the cell response has not been confirmed, with some studies suggesting that it is not involved in agonist-stimulated mobilization of Ca^{2+} (Crouch et. al., 1981; Cockcroft et. al., 1980; Thomas et. al., 1983).

Another possible mediator of mobilization of Ca^{2+} could be arachidonic acid (or its metabolites). PtdIns is comprised of approximately 80% 1-stearoyl-2-arachidonyl-glycero-3-phosphoinositol (Geison et. al., 1976), and so is a rich source of arachidonic acid, which can be liberated by the action of phospholipase A_2 on phospholipids (Van den Bosch, 1980), by the action of a phosphatidic acid-specific phospholipase A_2 on phosphatidic acid (Billah, et. al., 1981) or by a digylceride lipase which cleaves arachidonic acid off diacylglycerol (Bell et. al., 1980). Both the phospholipase A_2 enzyme and the diglyceride lipase are stimulated by Ca^{2+} (Van den Bosch, 1980; Billah et. al., 1981; Bell et. al., 1980) and so the products of these reactions would not be involved in the initial increase in cytosolic Ca^{2+} following receptor occupation. Nevertheless the effect of arachidonic acid and its metabolites on Ca^{2+} mobilization has been investigated since these compounds could possibly act to modify the initial changes in cellular Ca^{2+}.

The agonist-stimulated release of arachidonic acid from inositol phospholipid occurs in a number of tissues including pancreas (Halenda and Rubin, 1982), salivary gland (Litosch et. al., 1982), neutrophils (Rubin et. al., 1981; Kramer et. al., 1984), and platelets, (Lapetina et. al., 1981) and requires extracellular Ca^{2+}.

The addition of arachidonate to neutrophils was found to cause a rapid and significant increase in the permeability of the plasma membrane to Ca^{2+} which was sensitive to inhibitors of the lipoxygenase-mediated metabolic pathway (Volpi et. al., 1980). Leukotriene B_4 stimulated the influx of Ca^{2+} in rabbit neutrophils (Naccache et. al., 1982) and caused the translocation of Ca^{2+} when added to liposomes (Serhan et. al., 1982). However, since leukotrienes are a product of arachidonic acid their production also requires Ca^{2+} and so can not be involved in the initial Ca^{2+} mobilization mechanism.

Despite the possible role for arachidonic acid metabolites in neutrophils, an examination of this role in other secretory tissue has demonstrated that there is no evidence to support a role for arachidonic acid, nor its products released by cyclooxygenase or lipoxygenase, in Ca^{2+} mobilization or other aspects of stimulus-secretion coupling in pancreas (Heisler, 1973; Chauvelot et. al., 1979; Bauduin et. al., 1981; Stenson and Lobos, 1982; Putney et. al., 1982), submandibular gland (Kurtzer and Roberts, 1982), parotid and lacrimal gland (Putney et. al., 1982), and blowfly salivary gland (Litosch

et. al., 1982). As described previously in Section 2.3 (c), the studies by Marshall et. al. (1980, 1981), using whole pancreas led to different conclusions. The reason for this was due to an effect of prostaglandins on the transport of enzyme in the ducts rather than due to a direct stimulation of pancreatic acinar cells to secrete (Marshall et. al., 1982).

Although prostaglandin production is stimulated by secretagogues in the exocrine pancreas (Banschbach and Hokin-Neaverson, 1980; Marshall et. al., 1980, 1981; Stenson and Lobos, 1982) its role (if any) in these cells is unknown. The metabolism of exogenous arachidonate by the exocrine pancreas is minimal in comparison to that converted to prostanoids by neutrophils and macrophages (Stenson and Parker, 1979; Stenson et. al., 1981). Perhaps in neutrophils where arachidonic acid and its metabolites appear to mobilize Ca^{2+} (Volpi et. al., 1980; Naccache et. al., 1982; Serhan et. al., 1982), these products may play a role in stimulus-secretion coupling to amplify the initial increase in Ca^{2+}.

In addition to releasing arachidonic acid, the phosphatidic acid-specific phospholipase A_2 (Billah et. al., 1980) causes the release of lysophosphatidic acid which acts as an effective Ca^{2+} ionophore in platelets (Gerrard et.al., 1979). The effect of lysophosphatidic acid on Ca^{2+} transport has not received much attention but since it also is formed as a result of the Ca^{2+}-dependent phospholipase A_2, it would not be responsible for the initial agonist-stimulated increase in Ca^{2+}.

The studies described above did not provide the answer to the question of how does inositol phospholipid hydrolysis control cellular calcium. Phospholipase C activity releases diacylclycerol which is converted to phosphatidic acid. Phosphatidic acid does not appear to act as a natural ionophore in cells, and the production of arachidonic acid and its metabolites from phosphatidic acid and inositol lipid is Ca^{2+}-dependent. Therefore another candidate for linking stimulus with response was required. The other products of phosphoinositide hydrolysis are the inositol-phosphates which have received considerable attention over the last two years.

The hydrolysis by phospholipase C of polyphosphoinositides causes the rapid increase in Ins-1,4,5P_3 and Ins-1,4P_2 in a number of tissues including pancreas (Rubin et. al., 1984), blowfly salivary gland (Berridge, 1983), hepatocytes (Thomas et. al., 1984), parotid gland (Downes and Wusteman, 1983; Berridge et. al., 1983), rat brain (Berridge et. al., 1983), and platelets (Agranoff et. al., 1983). The release of these products is so rapid that it was suggested they may function to mobilize cellular Ca^{2+} (Berridge, 1983), this idea was examined by Streb et. al. (1983).

Using pancreatic acinar cells whose plasma membranes were made permeable, the addition of Ins-1,4,5P_3 caused the release of intracellular Ca^{2+} from a non-mitochondrial store (Streb et. al., 1983). The site of Ins-1,4,5P_3 action to release Ca^{2+} was identified to be non-mitochondrial since incubation of cells in

the presence of mitochondrial inhibitors (which abolishes the uptake and storage of Ca^{2+} in mitochondria; Streb and Schulz, 1983) did not reduce the ability of Ins-1,4,5P_3 to release Ca^{2+}. Whereas when vanadate was used (to reduce the uptake and storage of non-mitochondrial Ca^{2+} (Streb and Schulz, 1983) an inhibition of Ins-1,4,5P_3 induced Ca^{2+} release was measured (Streb et. al., 1983). It is not yet clear which non-mitochondrial pools are involved in this effect of Ins-1,4,5P_3. The store of Ca^{2+} released by Ins-1,4,5P_3 appears to be the same store which is mobilized on receptor activation by carbachol since addition of Ins-1,4,5P_3 to cells inhibited the release of Ca^{2+} normally caused by carbachol (Streb et. al., 1984). Other inositol phosphates (Ins-1,4P_2 and Ins-1-P) were ineffective in causing this effect, as was *myo*-inositol, so the Ca^{2+} release appears to be specific for Ins-1,4,5P_3.

This study demonstrated that phosphoinositide hydrolysis may control cellular Ca^{2+} and so provided a possible answer to the question : what is the link between receptor occupation and Ca^{2+}-mobilization?

Since this initial discovery by Streb et. al. (1983), the ability of Ins-1,4,5P_3 to activate release of intracellular Ca^{2+} in hepatocytes (Burgess et. al., 1984; Joseph et. al., 1984), rat insulinoma microsomes (Prentki et. al., 1984) and skinned cells of porcine coronary artery (Suematsu et. al., 1984), has been demonstrated. These studies also confirmed that the site of Ca^{2+} release by Ins-1,4,5P_3 was non-mitochondrial, by

using experimental procedures which allowed selective accumulation of Ca^{2+} into mitochondrial or non-mitochondrial pools and testing the effect of Ins-1,4,5P_3 on Ca^{2+} release from these sites (Burgess et. al., 1984; Joseph et. al., 1984; Suematsu et. al., 1984) and also that Ins-1,4,5P_3 released Ca^{2+} from a microsomal fraftion of rat insulinoma but not from mitochondria (Prentki et. al., 1984).

The question of whether the intracellular level of Ins-1,4,5P_3 is increased sufficiently by agonist-activation for it to function to stimulate Ca^{2+} release has not been answered by direct measurements. At present there is no adequately sensitive and specific method for measuring the chemical levels of inositol phosphates, however, they can be estimated using (^3H)-inositol labelled tissue. The assumption is made that in all cells labelled with (^3H)-inositol the specific radioactivities of the three phosphoinositides and so their breakdown products, the inositol phosphates, will all be similar. This is considered to be a valid assumption (Burgess et. al., 1984; Rubin et. al., 1984), since the monoester phosphates of the polyphosphoinositides turn over much more rapidly than the other parts of the molecule (Michell, 1975; Downes and Michell, 1982). Therefore knowing the lipid ^3H (n mol per mg protein) and cell water per mg protein the net increase in cellular Ins-1,4,5P_3 can be calculated.

In pancreatic acinar cells, Ins-1,4,5P_3 at a concentration as low as 0.2μM caused a significant release of Ca^{2+} which was near maximal at 5μM (Streb et. al., 1983). Based on the finding by Weiss et. al. (1982) that the breakdown of PtdIns-4,5,P_2 in stimulated parotid cells is approximately 0.3 nmol per mg protein

per min, Streb et. al. (1983) calculated that this would result in an intracellular Ins-1,4,5P_3 concentration of approximately 30μM. This indicates that the hydrolysis of PtdIns-4,5,P_2 could release sufficient Ins-1,4,5P_3 to stimulate mobilization of Ca^{2+}. More recently, Rubin et. al. (1984) have calculated that in pancreatic acinar cells stimulated by a concentration of carbachol which causes a maximum rate of Ca^{2+} release, the concentration of Ins-1,4,5P_3 would be 3μM, still sufficient to cause maximum release of intracellular Ca^{2+}.

A study using non-permeabilized rat hepatocytes (Thomas et. al., 1984) reported that the dose-response curve for the initial rate of increase in cytosolic free Ca^{2+} (using fluorescent indicator Quin 2) was very similar to the initial rate of Ins-1,4,5P_3 production. The calculated increase in Ins-1,4,5P_3 which correlated with maximal cytosolic free Ca^{2+} concentration was about 0.6μM (Joseph et. al., 1984). In permeabilized rat hepatocytes half-maximal and maximal Ca^{2+} release were obtained when Ins-1,4,5P_3 at 0.1 and 0.5μM were added (Joseph et. al., 1984). The net increase in cellular Ins-1,4,5P_3 of permeabilized guinea-pig hepatocytes induced by a maximal concentration of adrenaline was calculated 53μM at 2 min when $^{45}Ca^{2+}$ release was maximal (Burgess et. al., 1984).

These studies which have examined and calculated the Ins-1,4,5P increase caused by agonist therefore suggest that a sufficient increase occurs to release Ca^{2+} from intracellular stores. However, many questions still remain to be answered before the

role of Ins-1,4,5P_3 in Ca^{2+} mobilization can be clearly understood. For example, by what mechanism does Ins-1,4,5P_3 cause the release of Ca^{2+} from intracellular stores? Does Ins-1,4,5P_3 allow Ca^{2+} to enter through plasma membranes? These questions and others require investigation.

2.14 (b) The Role of Inositol Phospholipid Hydrolysis in Activation of Protein Kinase C

The hydrolysis of inositol phospholipid releases diacylglycerol which activates protein kinase C (Takai et. al., 1979 a; Kishimoto et. al., 1980. The ability of phorbol esters to stimulate protein kinase C by substituting for diacylglycerol (Castagna et. al., 1982) and to stimulate pancreatic secretion (Gunther and Jamieson, 1979; Gunther, 1981 a; Wooten and Wrenn, 1984), the examination of protein kinase C-dependent protein phosphorylation in pancreatic exocrine secretion (Wrenn, 1983; Burnham and Williams, 1984; Wrenn and Wooten, 1984; Wrenn, 1984) and the relationship between Ca^{2+} mobilization and protein kinase C activation in the stimulation of the final cellular response such as secretion have been discussed in detail in 2.7 (a) of this thesis and therefore will not be further described here.

The direct evidence to support a role for diacylglycerol in activating protein kinase C has been obtained using isolated enzyme and *in vitro* incubations, since diacylglycerols having two long-chain fatty acyl residues cannot easily be dispersed in a form suitable for uptake into cells. The replacement of

one of the long-chain fatty acids of diacylglycerol with an acetyl group results in 1-oleoyl-2-acetyl-glycerol (phorbol ester) which can be dispersed effectively enough to be able to enter cells and activate protein kinase C (Kaibuchi et. al., 1982 a; Nishizuka, 1984).

The use of phorbol esters is therefore an indirect method used to examine the effect of diacylglycerol on the activation of protein kinase C.

The investigations of the role of protein kinase C activity would be aided greatly by the finding of a specific protein kinase C inhibitor. At present there are several non-specific agents which inhibit protein kinase C activity, for example, psychotrophic agents(such as trifluoperazine, fluphenazine, chlorpromazine), local anaesthetics, verapamil, polymix B (Mori et. al., 1980; Schatzman et. al., 1981; Wise et. al., 1982) but these agents also have other actions. The inhibition of protein kinase C by these drugs does not appear to be due to their interaction with the active centre of the enzyme since a catalytically active enzyme fragment obtained by limited proteolysis of protein kinase C is not susceptible to these drugs (Kishimoto et. al., 1983).

2.15 : THE RELATIONSHIP BETWEEN INOSITOL PHOSPHOLIPID HYDROLYSIS AND RECEPTOR OCCUPATION

The early study by Hokin (1968 b) compared the dose-response curve for agonist-stimulated amylase secretion with that for incorporation of

(^{32}P)-P_i into PtdIns and reported that higher concentrations of hormone than those necessary to elicit amylase secretion were required to stimulate turnover of PtdIns. It was suggested that this effect could be explained if the turnover of PtdIns is closely associated with receptor occupation (Michell et. al., 1976). This would mean that activation of a small proportion of the receptor population could be sufficient to increase the concentration of intracellular messenger, calcium and secretion. Higher concentrations of agonist would bring about an increased receptor occupation, increased turnover of PtdIns and increase in second messenger. Concentrations required to occupy receptors fully, would maximally stimulate the PtdIns response but be much higher than that required for the maximal cell response.

The first direct comparison between dose-response curves for receptor occupancy and inositol phospholipid hydrolysis was reported by Kirk et. al. (1981). The curve for vasopressin-stimulated loss of PtdIns and PtdIns-4,5P_2 in hepatocytes closely resembled that for the binding of (^{3}H-Lys^{8})-vasopressin to its receptor. The curve for activation of phosphorylase was shifted to much lower concentrations of vasopressin. This study demonstrated a close association between inositol phospholipid hydrolysis and receptor occupation.

In contrast to this conclusion, Weiss and Putney (1981) suggested that the PtdIns response may not be related to receptor occupation but may be more closely involved in the 'calcium-gating' mechanism itself. This idea was based on the finding that the concentration-dependence of methacholine-stimulated (^{32}P)-P_i-incorporation into PtdIns was similar to that for efflux of potassium (a measure of an increase in cytosolic

calcium) in rat parotid gland. The receptor-occupation curve for (^{3}H)-QNB binding to the muscarinic receptor was shifted to the right of these two curves. Weiss and Putney (1981) measured the incorporation of (^{32}P)-P_i into PtdIns, rather than its hydrolysis. Whether this is the reason for the difference in results compared with those of Kirk et. al. (1981) is not clear.

What does a close association between receptor occupation and inositol phospholipid hydrolysis mean? Kirk et. al. (1981) suggested that maximum hydrolysis of inositol phospholipid hydrolysis occurs with maximal receptor occupation. A consequence of this relationship would then be that increased occupation of one type of receptor population should produce an increase in inositol phospholipid hydrolysis (to a maximum) independent of occupation of another class of receptor. This idea was examined by Weiss and Putney (1981), however, again using (^{32}P)-P_i incorporation into PtdIns. Simultaneous stimulation of rat parotid gland with two agonists acting at different receptors (substance P and adrenergic) did not produce an additive effect. This did not appear to be due to saturation of a step involved in (^{32}P)-P_i-labelling since maximally effective concentrations of adrenaline and substance P each did not produce maximal possible PtdIns labelling, as seen with methacholine (Weiss and Putney, 1981).

In order to make a more direct comparison between the results of Kirk et. al. (1981) and so perhaps explain the conflicting results of Weiss and Putney (1981) it was necessary to measure the hydrolysis rather than synthesis of inositol lipid.

An examination of the effect of addition of a maximally effective concentration of one agonist to varying concentrations of another agonist on inositol phospholipid hydrolysis in exocrine pancreas is reported and discussed in Chapter 5 of this thesis. Briefly, the results of this study showed that the simultaneous stimulation of the muscarinic receptor (with a maximally effective concentration of carbachol or butyrylcholine) and the cholecystokinin receptor (with a high concentration of CCK-8) did not cause the degree of inositol phospholipid hydrolysis expected if each agonist acted independently of the other - there was no additive effect. It would appear that each receptor does not have its own individual mechanism for causing phosphoinositide hydrolysis but that the signals generated following occupation of different receptors converge on a common mechanism which limits the degree of hydrolysis of inositol lipid from a common pool. The possible explanations for this interactive effect are discussed in Chapter 5 of this thesis.

These results differ from those obtained in brain slices by Brown et. al. (1984). This recent study showed that cholinergic muscarinic, α-adrenergic and histamine H_1 receptors are linked to inositol phospholipid hydrolysis in rat cerebral cortical slices. Using a variety of agonists and antagonists this study reported a clear discrimination between the ability of full and partial agonists to cause phosphoinositide hydrolysis and also described the effect of competitive antagonists for each receptor.

The effects of agonists acting at different receptors on the hydrolysis of inositol phospholipid were additive, suggesting that the degree of

the phosphoinositide hydrolysis was proportional to receptor occupancy (Brown et. al., 1984). The reason for the difference in results using pancreas and brain slices is not known and is discussed in Chapter 5.

The mechanism stimulated by receptor occupation to cause phosphoinositide hydrolysis is unknown, but must be determined befofe a common rate-limiting step is understood. Does agonist-binding to receptor cause a conformational change in the membrane to allow access of phosphodiesterase to substrate, or does receptor occupation stimulate the enzyme? The answer to this question is not at present known and requires furhter investigation.

2.16 : INOSITOL PHOSPHOLIPID METABOLISM AND CYCLIC AMP

The review by Michell (1975) indicated that agonists whose actions are mediated by calcium cause an increase in the turnover of PtdIns while agonists that cause an increase in cAMP do not affect PtdIns turnover (mostly measured as synthesis of PtdIns).

However, an increase in intracellular Ca^{2+} may cause either a rise or fall in the cAMP content by activating or inhibiting adenylate cyclase in different cells (Malnoë et. al., 1982; Valverdes et. al., 1979). Similarly, an increase in cAMP content can lead to an increase or inhibition of Ca^{2+}-dependent responses, depending on cell type. These two messengers do interact in controlling cell function, although this interaction varies for different tissues (for a review see Rasmussen and Barrett, 1984). For example, in pancreatic acinar cells the presence of a Ca^{2+} mobilizing hormone with one that mobilizes cAMP

caused an increase in amylase secretion greater than the sum of the increase by each agent acting alone (Gardner and Jackson, 1977; Peikin et. al., 1978; Uhlemann et. al., 1979). On the other hand, in platelets, an increase in cAMP inhibited the thrombin-stimulated secretion of 5-hydroxytryptamine and platelet aggregation (Lapetina et. al., 1978), cAMP appears to act as an inhibitory second messenger in platelets to antagonize the mobilization of Ca^{2+} (Zavoico and Feinstein, 1984).

Does an increase in cyclic AMP alter cellular Ca^{2+} content by affecting phosphoinositide hydrolysis? The effect of cAMP could be examined by investigating whether an increase in cAMP (caused by activation of the appropriate receptor or by addition of cAMP, or dibutyryl cAMP (dbc AMP) results in (1) an alteration in the basal metabolism of phosphoinositide or (2) an alteration in phosphoinositide hydrolysis stimulated by the addition of a calcium mobilizing agonist.

The reports on the effect of cAMP on phosphoinositide metabolism are conflicting and differ for various tissues. This is not surprising because the effect of cAMP on Ca^{2+}-dependent responses varies for different tissues. Using the experimental approach in (1) studies of the effect of cAMP on basal phosphoinositide metabolism in adrenal cortex (Farese et. al., 1983), Leydig cells (Lowitt et. al., 1982) and kidney cortex (Bidot-Lopez et. al., 1981) reported that cAMP stimulated the *de novo* synthesis of inositol phospholipid by rapid stimulation of the synthesis of phosphatidic acid. The mechanism of this effect is not known but a protein synthesis inhibitor, cycloheximide, inhibited this effect of cAMP (Farese et. al., 1983) suggesting the

involvement of a labile protein. In rat hepatocytes and lymphocytes, however, dbcAMP had no effect on basal (^{32}P)-P_i incorporation into PtdIns (Kaibuchi et. al., 1982 b), whereas in blowfly salivary gland, the basal incorporation of (^{3}H)-inositol into PtdIns was reduced (Berrdige and Fain, 1979).

Few studies have been made of the effect of cAMP on inositol phospholipid metabolism stimulated by agonists. The studies measuring inositol phospholipid synthesis showed that the addition of dbcAMP to phenylephrine-stimulated hepatocytes had no effect on the agonist-activated increase in (^{32}P)-P_i incorporation into PtdIns whereas this latter response in phytohaemoglutinin-stimulated lymphocytes was inhibited (Kaibuchi et. al., 1982 b). In thrombin-stimulated platelets, dbcAMP substantially increased the rate of conversion of phosphatidate to PtdIns and so decreased the steady-state concentration of phosphatidate (Lapetina et. al., 1981) which had previously been thought to be due to inhibition of phospholipase C by cAMP (Billah et. al., 1979).

In the pancreas, secretin and vasoactive intestinal polypeptide (VIP) which act via cAMP to stimulate secretion, caused a decrease in PtdIns mass, with the effect of secretin being dependent on the extracellular Ca^{2+} concentration. From these results Farese et. al. (1981 a) proposed that agents which act via cAMP produce a Ca^{2+}-dependent hydrolysis of PtdIns. However, a recent study in our laboratory by M.F. Crouch (personal communication) showed that VIP did not stimulate the hydrolysis of inositol phospholipid (measured by the release of (^{3}H)-inositol-phosphates) in mouse pancreatic acini, indicating that cAMP does not affect the phospholipase C in pancreas as suggested by Farese et. al. (1981 b).

The mechanism of potentiation by cAMP of amylase secretion stimulated by Ca^{2+}-mobilizing hormones appears to occur subsequent to the increase in cellular Ca^{2+}. Forskolin (which activates adenylate cyclase, as described in 2.3 b) potentiated not only the effect of carbachol and CCK-8 but also the effect of A23187 to stimulate secretion of amylase from pancreatic acinar cells (Heisler, 1983). This result shows that cAMP acts at a site distal to the activation of an increase in cellular Ca^{2+} in exocrine pancreas.

2.17 : LITERATURE REVIEW - CONCLUSION

This review of the literature concerning both pancreatic exocrine function and inositol phospholipid hydrolysis demonstrates how rapidly knowledge in both these areas has increased over the last few years. Many questions still remain to be answered in regard to stimulus-secretion coupling. The results and discussions in the following chapters of this thesis describe studies which commenced in February 1980 examining inositol phospholipid hydrolysis in mouse exocrine pancreas and its possible role in secretion.

CHAPTER 3

MATERIALS AND METHODS

3.1 : EXPERIMENTAL ANIMALS

All experiments used male albino mice of the LaCa strain which were obtained from the Central Animal House, Waite Agricultural Institute, S. Australia. Mice were maintained in a room at constant temperature 22-25^{o}C and had free access to food in the form of M. & V. Mouse Cubes (Milling Industries, S. Australia) and water, unless otherwise stated. Lights were automatically switched on at 7.00 a.m. and off at 7.00 p.m. daily.

3.2 : MEASUREMENT OF THE HYDROLYSIS OF INOSITOL PHOSPHOLIPID

3.2(a) Assay Procedure

Male albino mice which had been starved for 16 hr (with water *ad libitum*) were injected intraperitoneally with 122kBq of *myo*-(2-^{3}H) inositol. The mice were then allowed food *ad libitum* for the next 8 hr and starved for the last 16 hr prior to the experiment. The mice were killed by cervical dislocation, the pancreata were then removed and cross-cut (1 mm) with a McIlwain tissue chopper. The tissue pieces were washed for 2 x 15 min in 10 ml of Krebs solution, containing 2 mM *myo*-inositol, at 37^{o}C. The tissue pieces were taken up into a glass pasteur pipette (tip diameter, 1.5 mm) and approximately equal aliquots were placed into plastic vials and incubated for 30 min at 37^{o}C in 1 ml of Krebs solution which contained agonists at appropriate concentrations and 2 mM *myo*-inositol. The tissue from one mouse (approximately 100 mg) was sufficient for about 6 vials and three mice were used for each experiment. The incubations were terminated by fast freezing in a dry ice/alcohol bath. In initial experiments, tissue pieces were

homogenised from the partially thawed state with an Ultraturrax (Janke and Kunkel) and the volumes of each sample adjusted to 2 ml with distilled water. From each homogenate, 0.2 ml samples were taken for determination of total radioactivity, and the remaining tissue homogenate was precipitated in the presence of 1.1mM carrier *myo*-inositol, with trichloroacetic acid (TCA) (final concentration 10% w/v) and centrifuged at 1020 xg for 15 min at 15°C. A 1 ml sample of the supernatant was taken for determination of acid-soluble radioactivity. The homogenate samples were placed in glass counting bottles, dissolved in 0.5 ml of tissue solubiliser (Soluene-350) and, after 1 hr, neutralised with 25 µl of glacial acetic acid. The solubilised homogenate and trichloroacetic acid supernatants were counted in a liquid scintillation spectrometer (Packard) using 15 ml of a commercial scintillant E299 (Amersham) per sample. For later experiments it was found necessary, for economic reasons, to reduce the volumes of Soluene-350 and scintillant used, therefore the assay was modified as follows. The tissue pieces were homogenised and a 0.1 ml sample from the homogenate was placed in a plastic miniature counting vial and 3.5 ml of commercial scintillant added. No Soluene-350 was necessary since 0.1 ml of homogenate dissolved readily in the scintillant. A 0.7 ml sample of the remaining homogenate was precipitated with TCA as above, and a 0.5 ml sample of the supernatant was placed in a miniature counting vial, neutralised with 50 µl of 6 M NaOH and 3.5 ml of commercial scintillant added. The counting efficiencies for the homogenate and supernatant samples were not affected by alteration of the assay method.

The agonist-stimulated hydrolysis of inositol phospholipid was measured as an increase in ^{3}H in the acid-soluble fraction above that measured in the control incubations, and is expressed as Bq/kBq ^{3}H incorporated into pancreatic tissue.

3.2(b) Radioactive Counting

All samples were counted on a Packard Tri-carb model 2002 liquid scintillation spectrometer. Samples were counted for 10 min each after a sufficient period of time had elapsed to avoid any effects of chemiluminescence. Quench corrections were obtained by the method of internal standardization. The internal standard consisted of *myo*-(2-^{3}H) inositol 37 kBq/ml or 370 kBq/ml, methiolate, 0.1 mg/ml and *myo*-inositol, 0.4 mM. To each sample, 20 µl of internal standard was added and the sample recounted for 1 min.

The counting efficiency of the Packard liquid scintillation spectrometer for the homogenate samples was 34-38% and for the TCA supernatant samples was 22-25%.

3.3 : EXTRACTION AND CHROMATOGRAPHIC SEPARATION OF PHOSPHOLIPIDS LABELLED *in vivo* WITH *myo*-(2-^{3}H) INOSITOL

Mouse pancreas, labelled *in vivo* with 1.85 MBq of *myo*-(2-^{3}H) inositol was chopped, washed for 30 min in Ca^{2+}-free Tris-Krebs solution and incubated in the presence and absence of carbachol (10^{-4}M) for 30 min as described in 3.2(a) (an increase in the amount of *myo*-(2-^{3}H) inositol injected into each mouse was necessary so that sufficient counts could be measured in the scrapings from thin layer chromatography sheets). The phospholipids were extracted from the tissue using the acidified solvent technique of Fain and Berridge (1979) in which the tissue was homogenised in chloroform/methanol (2:1 v/v) containing 0.25% HCl. The extract was dried under nitrogen and dissolved in chloroform/methanol (3:1 v/v). The extracts and the phospholipid standards, L-α-phosphatidylinositol (40 µg), L-α-phosphatidylinositol 4-monophosphate (40 µg) and L-α-phosphatidylinositol 4,5-bisphosphate (40 µg)

were separated by thin layer chromatography on silica gel-coated plastic sheets (Merck) which had been pre-soaked in 3% (w/v) magnesium acetate and dried. The sheets were equilibrated for 1 hr and developed with n/butanol/acetic acid/water (6:1:1 by volume) (Abdel-Latif et. al., 1977). The location of the phospholipid standards was determined by iodine-vapour staining, and the distribution of the radioactivity from the phospholipid extract was determined by scraping the silica gel from successive 0.5 cm strips of the chromatography sheet into 5 ml of commercial scintillation fluid and counted in the liquid scintillation spectrometer.

The residue from the lipid extract which was not soluble in chloroform/methanol (3:1 v/v) was dissolved in 1 ml of tissue solubiliser (Soluene-350), neutralised with 50 µl of glacial acetic acid, and 15 ml of scintillation fluid was added. Samples were taken at each step of the extraction procedure to calculate the recoveries for the extraction. The samples were counted in a liquid scintillation spectrometer and quench corrections made by internal standardization.

3.4 : EXTRACTION AND CHROMATOGRAPHIC SEPARATION OF ^{3}H-LABELLED COMPOUNDS RELEASED FROM *myo*-(2-^{3}H) INOSITOL-LABELLED INOSITOL PHOSPHOLIPID

The acid-soluble (^{3}H)-labelled compounds from both control and carbachol (10^{-4}M)-treated pancreas pieces were investigated. The trichloroacetic acid supernatants were mixed with ether (1:1 by volume) to remove the trichloracetic acid, for 1 hr at room temperature on a rotating turntable, then centrifuged at 1000 x g at room temperature for 5 min. The ether phase was removed and the ether wash repeated. Samples of the ether phase were applied to Whatman GF/F filter paper, dried, placed in counting bottles with 15 ml of scintillation fluid and counted in a liquid scintillation counter. Of the total ^{3}H present in the TCA-supernatant, 1% was

lost in the first ether-wash, and 0.3% was lost in the second ether-wash. The resulting lower phase was concentrated by evaporation in a Buchler Vortex Evaporator and chromatographed using the technique of Fain and Berridge (1979). Samples of the extracts of the TCA-supernatants and the standards, *myo*-inositol (150 µg) and phosphatidylinositol (40 µg) were applied to Whatman SG81 paper impregnated with 22% silicon dioxide. The paper was equilibrated for 1 hr and developed (descending) with chloroform/methanol/25% (w/v) ammonia solution (6:10:5, by volume). The chromatograms were stained with 1% $KMnO_4$ (w/v) containing 2% (w/v) Na_2CO_3 for location of inositol and iodine vapour for phosphatidylinositol. Strips (1 cm) of the chromatograms were cut, placed in counting bottles with 4 ml of scintillation fluid and counted each for 10 min in the liquid scintillation spectrometer.

3.5 : INDUCTION OF DIABETES AND MAINTENANCE OF DIABETIC MICE

Destruction of β-cells in pancreatic islets can be detected by the hyperglycaemia which results from the decreased ability of β-cells to release insulin. Diabetes is induced in a variety of animal species by streptozotocin (STZ) which destroys the β-cells of pancreatic islets and does not affect α-cells and others (Rerup, 1970). Recently, Nakadate et.al. (1981) found that the α_2- and β_2-adrenergic systems which modulate insulin release from the pancreatic β cells, alter the effect of STZ to cause diabetes in mice.

3.5(a) STZ-induced Diabetes

In the present study, a modification of the method by Nakadate et. al. (1981) in which isoprenaline is used to potentiate the diabetogenic action of STZ was used to induce diabetes in mice.

Male albino mice starved for 24 hr with water *ad libitum* and weighing 25-30 gm were injected intraperitoneally with 0.1 ml of isoprenaline hydrochloride (0.5 mg/kg) freshly dissolved in 0.8% NaCl. Streptozotocin (90 mg/kg) was freshly dissolved in cold citrate buffer (5mM, pH 4.5) and 0.1 ml given intravenously (I.V.) to the mice 10 min after the isoprenaline injection. Control mice were injected with isoprenaline hydrochloride (0.5 mg/kg) followed 10 min later by cold citrate buffer given I.V.

3.5(b) Blood Glucose Determination

Blood glucose concentration was measured using a colorometric method (Sigma Glucose Kit, No. 510-A) which determines glucose concentrations using the enzymatic procedure described by Raabo and Terkildsen (1960). The procedure is based on the following enzymatic reactions:

1. $\text{Glucose} + 2H_2O_2 + O_2 \xrightarrow[\text{Peroxidase}]{\text{Glucose oxidase}} \text{Gluconic Acid} + 2H_2O$
2. $H_2O_2 + \text{o-dianisidine} \longrightarrow \underset{\text{(Brown)}}{\text{Oxidized-o-dianisidine}}$
   (Colourless)

The intensity of the brown colour measured at 425-475 nm is proportional to the original glucose concentration (Keston, 1956).

Mice were anaesthetized with ether and a 50 µl sample of blood from the retro-orbital plexus of veins was taken up into heparinized blood capillary tubes. The 50 µl blood sample was immediately diluted with 0.45 ml of distilled water and mixed to haemolyze the blood. Glucose standards were made up in 0.5 ml of distilled water to enable a standard curve to be determined ranging from 50 mg glucose/100 ml blood to 800 mg glucose/100 ml blood. The samples to be used for reagent blanks consisted of 0.5 ml of water. To all samples, 0.25 ml

of 0.15 M $Ba(OH)_2$ was added and mixed, followed by 0.25 ml of 0.31 M $ZnSO_4$. The samples were centrifuged at 1000 x g at room temperature for 10 min and a 0.15 ml sample of the supernatant was taken and diluted to 0.3 ml with distilled water. After the addition of 3.0 ml of Combined Enzyme-Colour Reagent solution, consisting of glucose oxidase, peroxidase and o-dianisidine dihydrochloride, the samples were mixed thoroughly and incubated at room temperature for 45 min in the dark. At the end of the incubation period the optical density of the samples was read against the reagent blank at 450 nm on a Turner Spectrophotometer, Model 330. Blood glucose concentrations were determined from the standard curve obtained for each experiment. A representative glucose standard curve is shown in Fig. 3.1.

3.5(c) Insulin Maintenance of Diabetic Mice

Inspection of mice cages each day after streptozotocin-treatment indicated that the mice became polyuric 2-3 days after treatment. Blood glucose measurements made at this time showed that all streptozotocin-treated mice were already hyperglycaemic. Hyperglycaemia was defined as blood glucose concentrations over 300 mg/100 ml blood. Control mice had blood glucose concentrations of 154 ± 4 mg glucose/100 ml blood (n = 12). The hyperglycaemic mice were treated with insulin:

1. Lente (Insulin zinc suspension, Commonwealth Serum Laboratories, 100 units/ml) with an onset of approximately 3 hr, peak at 5-10 hr and duration of 4 hr.
2. Neuralin (Neutral insulin, Commonwealth Serum Laboratories, 100 units/ml) with an onset of approximately 30-60 min, a peak of 2-5 hr and a duration of 7 hr.

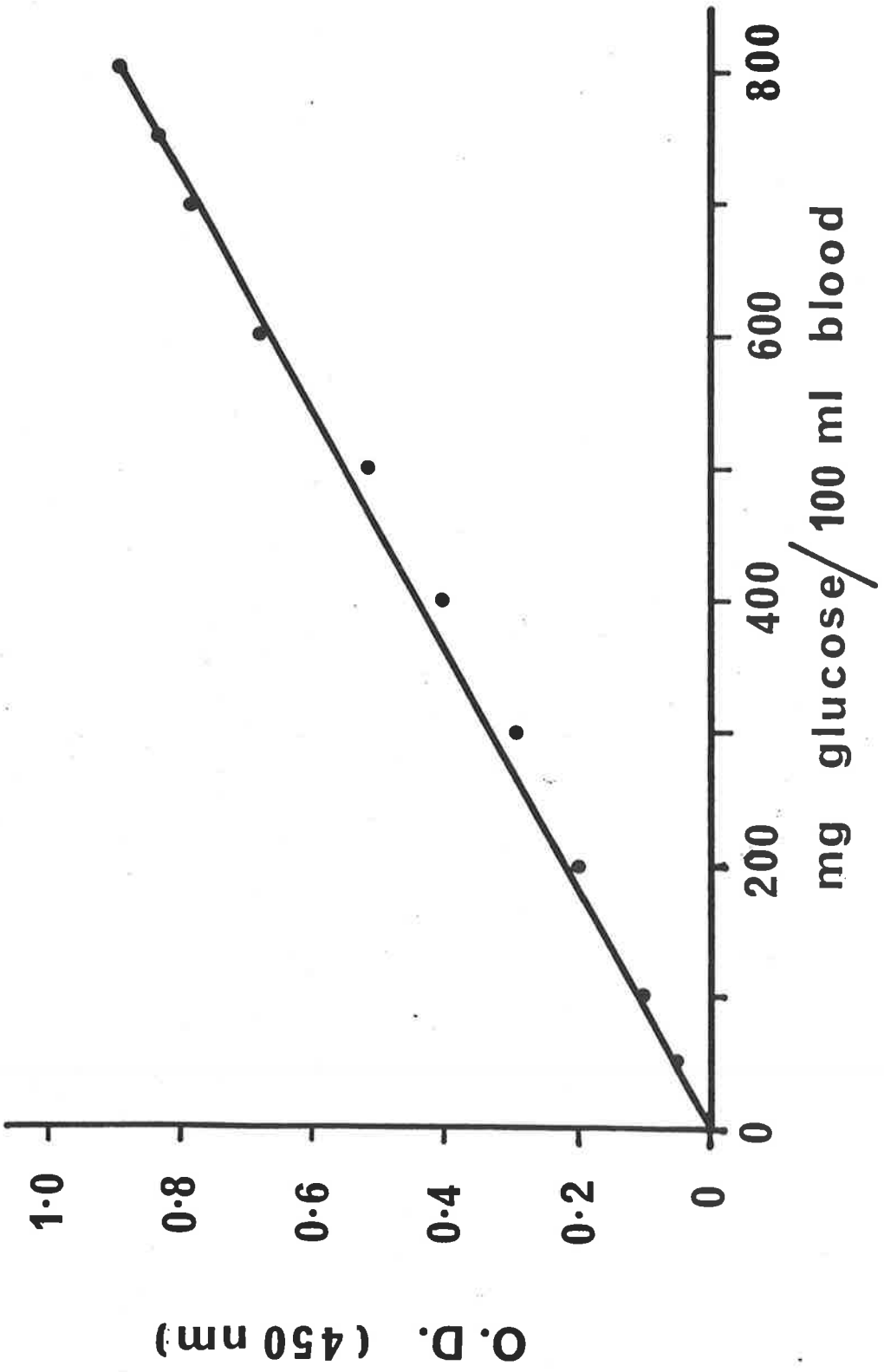


Fig. 3.1 : Glucose Standard Curve (Optical Density at 450nm)

Preliminary experiments using STZ-induced diabetic mice indicated that 1 unit of lente and 1 unit of neuralin insulin, diluted in normal saline and administered subcutaneously in the morning and evening, were effective in maintaining the diabetic mice for 5-7 days. Control mice were injected with equivalent volumes of 0.14 M NaCl twice daily. Although a number of insulin-treated mice died, blood glucose determination of the remaining mice 8 days after STZ treatment indicated that the mice were no longer hyperglycaemic and had blood glucose levels similar to control mice, as shown in Table 3.1.

3.5(d) Inositol Phospholipid Hydrolysis in Diabetic Mice

The control and diabetic mice maintained for 8 days, were used to investigate whether any of the agonist-stimulated hydrolysis of inositol phospholipid in pancreatic slices was occurring in the endocrine pancreas. The preparation of (^{3}H)-labelled mice as described in 3.2(a) involved starving overnight prior to injection with *myo*-(2-^{3}H) inositol. However it was found that diabetic mice could not tolerate fasting overnight, which agreed with findings of Korc et. al. (1981 b) using diabetic rats. An alternative *in vivo*-labelling method (Hokin-Neaverson et. al., 1975) was therefore used in which fed control and diabetic mice were injected intraperitoneally with 122kBq of *myo*-(2-^{3}H) inositol and 1 mg of pilocarpine hydrochloride. After 3½ hr the mice were killed and (^{3}H)-labelled pancreata used for measurement of agonist-stimulated inositol phospholipid breakdown as described in 3.2(a).

Table 3.1 : The effect of streptozotocin and insulin treatment on the blood glucose concentration of mice.

	Blood glucose mg/100 ml blood	
	A	B
Control	154 ± 4 (n = 12)	155 ± 5 (n = 12)
STZ-treated	406 ± 25 (n = 13)	108 ± 16 (n = 7)

The blood glucose concentration of the mice was determined at day 3 (A) and day 8 (B) after STZ treatment. Insulin treatment began on day 3 after the blood glucose assay was performed.

3.6 : DISSOCIATION OF PANCREATIC ACINAR CELLS

Mouse pancreatic acini were prepared using a modification of the method described by Williams et. al. (1978). Male albino mice were starved for 16 hr with water *ad libitum*, killed by cervical dislocation and pancreata removed and placed in 5 ml of dissociation medium consisting of Hepes buffered salt solution to which 260 units/ml collagenase (Worthington Biochemicals); 0.1 mg/ml α-chymotrypsin (Sigma); 0.1 mg/ml soybean trypsin inhibitor (crude soluble powder, type 11-S, Sigma) were added. The Hepes buffered salt solution consisted of (mM) : NaCl, 118; KCl 4.7; Glucose, 14; NaH_2PO_4, 1.2; N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (Hepes), 10; $CaCl_2$, 2.56; adjusted to pH 7.4 and gassed with 100% O_2. The pancreata were injected with the solution and incubated at 37°C with shaking at 120 cycles/min for 10 min. The solution was replaced with 5 ml of fresh dissociation medium, the pancreata reinjected and incubated for 30 min at 37°C with shaking at 120 cycles/min. Pancreatic tissue and buffer were pipetted up and down through polypropylene pipettes of decreasing tip diameter (3.5 mm, 2 mm, 1 mm) to dissociate the acini, and filtered through 75 μm mesh nylon cloth with 10 ml of Hepes buffered salt solution containing 1% bovine serum albumin (Fraction V. Sigma). 3 x 5 ml of this filtrate was layered on top of the centrifugation medium containing Hepes buffered salt solution and 4% bovine serum albumin and centrifuged at 50 x g for 4 min at 22°C. The pellet was resuspended in 4 ml of fresh centrifugation medium and centrifuged at 50 x g for 4 min. The pellet was resuspended in 15 ml of Hepes buffered incubation medium which was the same as the Hepes buffered salt solution but the 2.56 mM $CaCl_2$ was replaced with 1.28 mM $CaCl_2$, and incubated for 30 min at 37°C with shaking at 60 cycles/min. The lower calcium concentration was used to reduce clumping of acini (Williams et. al., 1978). Samples of dissociated acini were tested for cell damage using the trypan blue exclusion technique and viewed under a phase contrast light microscope (Olympus).

3.7 : AMYLASE SECRETION

3.7(a) Amylase Secretion in Pancreatic Slices

Male mice were starved for 16 hr (with water *ad libitum*) and then killed by cervical dislocation and pancreata were removed and cross-cut (1 mm) with a McIlwain tissue chopper. Tissue pieces were washed in 20 ml of Krebs solution at 37°C for 2 x 15 min and then taken up into a glass pasteur pipette (tip diameter 1.5 mm). Approximately equal aliquots of pancreatic pieces were placed in vials and incubated for 30 min at 37°C in 5 ml of Krebs solution in the absence or presence of agonist at the appropriate concentrations. At the end of the incubation a 50 µl sample of the incubating medium was taken and diluted 1/100 in Tris/Gelatine/NaCl buffer for determination of amylase released from the tissue. The tissue and the remaining incubation medium was homogenised and a 0.1 ml sample taken and diluted 1/1000 in Tris/Gelatine/NaCl buffer for determination of total amylase content of each tissue sample. Amylase standards were prepared from a stock solution of α-amylase (Type 11-A, bacterial) 10 µg/ml (6.95 units/ml) for determination of a standard curve for each experiment (a representative curve is shown in Fig. 3.2). All samples for amylase assay were diluted in Tris/Gelatine/NaCl buffer consisting of 0.2% (w/v) gelatine; 0.05 M Tris (hydroxymethyl) aminomethane (Tris), 0.15 M NaCl, adjusted to pH 7.0 with HCl. A sample of this buffer was used as the reagent blank.

Amylase secretion was determined using a colorometric assay (Roberts and Woodland, 1982) with Remazolbrilliant Blue-labelled starch synthesized according to Rinderknecht et. al. (1967). The amylase subtrate was prepared by dissolving Remazolbrilliant Blue-labelled starch in buffer consisting of 0.1 M Tris and 0.05 M NaCl, pH 7.0. 0.5 g of labelled starch/100 ml of buffer was stirred and heated to 90°C and left refrigerated overnight.

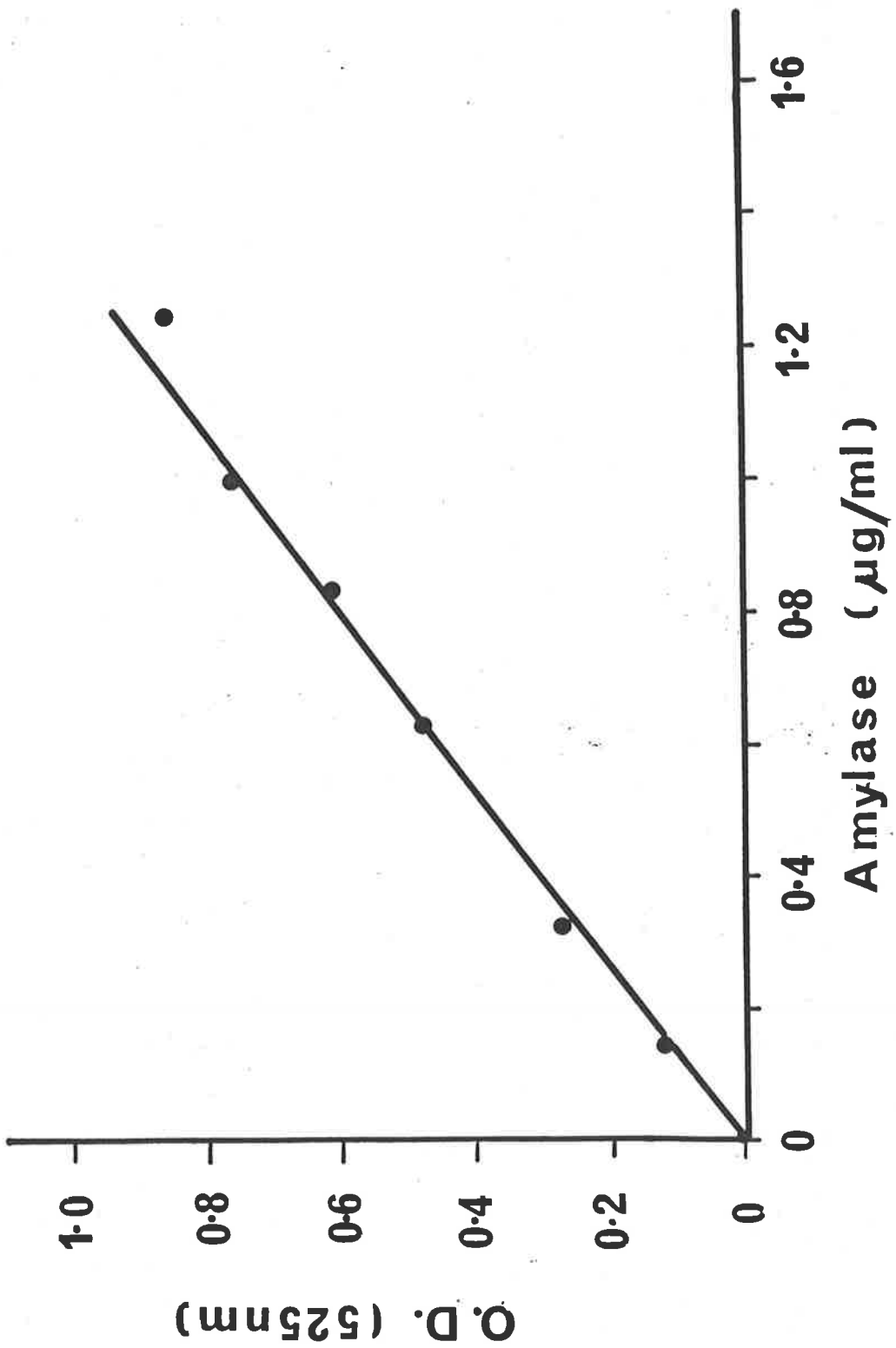


Fig. 3.2 : Amylase Standard Curve (Optical Density at 595nm)

Plastic tubes each containing 5 ml of amylase subtrate were placed in a heated water-bath and maintained at 37°C. 100 µl of each sample was added at 30 sec intervals to the heated subtrate, mixed and incubated at 37°C. The reaction was terminated after 30 min by the addition at 30 sec intervals of 2 ml of 0.5 M HCl containing 0.25 ml of Triton X-100. Samples were centrifuged at 1020 x g at 4°C for 10 min and the optical density read against a reagent blank at 595nm on a Turner Spectrophotometer, Model 330. Amylase secreted was calculated as a percentage of the total amylase present in each tissue sample.

3.7(b) Amylase Secretion in Pancreatic Acini

Pancreatic acini were prepared as described in 3.6. Following a 30 min pre-incubation in 15 ml of Hepes buffered incubation medium, the acini suspension was centrifuged at 50 x g at 22°C for 4 min. Acini were resuspended in 25 ml of incubation and 1.8 ml samples placed in vials containing 0.2 ml of incubation medium and agonist at appropriate concentrations. Acini were incubated with gentle shaking at 60 cycles/min and gassed with 100% O_2 for 30 min.

To measure the secretion of amylase from acini it was necessary to determine both the amount of amylase released and the total amylase present in each incubation sample. For determination of amylase released both at the start of the incubation, time = 0 min and at the end of the incubation, time = 30 min, 0.5 ml of acini suspension from each incubation vial was taken, placed in Eppendorf tubes and centrifuged at 8,400 x g at 22°C for 35 sec in a Select-a-Fuge 24 (Biodynamics) Eppendorf microcentrifuge. A 0.2 ml sample of the supernatant was immediately taken and placed in a tube. A 0.05 ml

sample of this supernatant was diluted 1/100 in Tris/Gelatine/NaCl buffer for determination of amylase released. To determine total amylase present, the acini pellet was suspended in the remaining incubation volume by vortexing and each sample was frozen in a dry ice/alcohol bath, and then thawed at 37^{o}C. The freezing and thawing procedure was repeated 5 times to ensure that all the acini were disrupted and all the amylase present in the acini was released. The samples were centrifuged at 50 x g for 5 sec and a 0.1 ml sample taken and diluted 1/1000 in Tris/Gelatine/NaCl buffer for determination of amylase present. The amylase content of all samples was determined using the colorometric assay as described in 3.7(a). The amylase released at the start of the incubation (the T=0 value), was subtracted from values for amylase released at the end of the incubation, to obtain amylase released during the 30 min incubation and expressed as a percentage of the total amylase present in the acini.

3.8 : DETERMINATION OF ADENOSINE TRIPHOSPHATE (ATP) CONTENT

To investigate whether agonist-stimulated breakdown of inositol phospholipid requires ATP, the effect of metabolic inhibitors on phosphoinositide breakdown was studied. To ensure that the concentrations of inhibitors used were effective in inhibiting ATP synthesis the ATP content of pancreatic tissue was measured.

The tissue for ATP assay was incubated under the same conditions as for the inositol phospholipid hydrolysis studies as described in 3.2. Tissue was pre-incubated for 10 min in the presence or absence of metabolic inhibitor prior to a 30 min incubation in Ca^{2+}-free Tris-Krebs solution. The incubation was terminated by the removal of the incubation solution, addition of 5 ml of cold perchloric acid (5% w/v) and fast-freezing in a

dry-ice/alcohol bath. Tissue was homogenised from the semi-thawed state, on ice, and centrifuged at 1000 x g for 10 min at $0^{o}C$. A 2.0 ml sample of the supernatant was added to 1.34 ml of neutralising solution consisting of 0.5 M KOH buffered with 0.1 M Hepes. ATP standards at concentrations ranging from $10^{-8}M$ to $10^{-5}M$ were prepared by dissolving ATP (Sigma) in cold perchloric acid (5% w/v). A 2.0 ml sample of these standards was neutralised with 1.34 ml of neutralising solution and stored on ice. Samples were assayed for ATP using the luciferase-luciferin assay described by Stanley and Williams (1969). The ATP content was determined from the standard curve obtained for each experiment. A representative curve is shown in Fig. 3.3. The tissue pellet obtained after centrifugation was washed several times in cold methanol to remove the perchloric acid and dried by heating in a water bath at $60^{o}C$ and then blowing with nitrogen. The pellet was dissolved in 2 ml of 1M NaOH and the protein content determined using the method of Lowry et. al. (1951) using bovine serum albumin as a standard to obtain a standard curve for each experiment. Results for ATP content of the tissue sample are expressed per mg protein.

3.9 : SOLUTIONS AND CHEMICALS

3.9(a) Solutions

Krebs solution consisted of (mM): NaCl, 106; KCl, 4.7; NaH_2PO_4, 1.15; $NaHCO_3$, 25; glucose, 2.8; Na fumarate, 2.7; Na glutamate, 4.9; Na pyruvate, 4.9; $MgCl_2$, 1.13; $CaCl_2$, 2.5.

Ca^{2+}-free Krebs-solution was similar to the standard Krebs solution but with the $CaCl_2$ and $MgCl_2$ replaced with 0.1mM ethyleneglycol-bis-(β-aminoethyl ether) N-N'-tetraacetic acid (EGTA). The EGTA was taken from a 0.1M stock solution the pH of which was adjusted to 7.4.

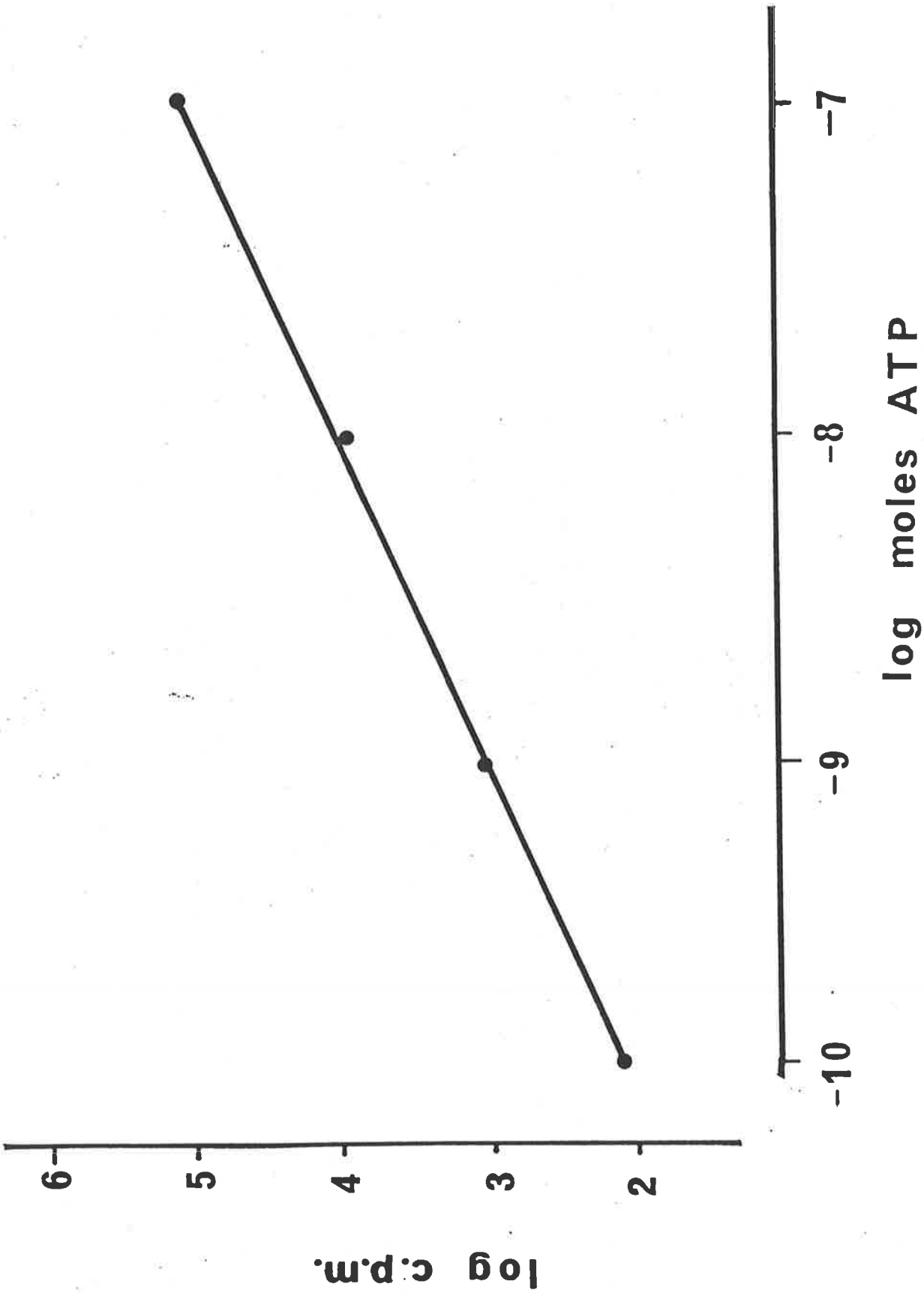


Fig. 3.3 : ATP Standard Curve

Tris-buffered Krebs solution (Tris-Krebs solution) consisted of (mM): NaCl, 126; KCl, 4.7; glucose, 2.8; sodium fumarate, 2.7; sodium glutamate, 4.9; sodium pyruvate, 4.9; tris (hydroxymethyl) aminomethane (Tris), 3; $MgCl_2$, 1.13; $CaCl_2$, 2.5; adjusted with HCl to pH 7.4 at 37^oC.

Ca^{2+}-free Tris-buffered Krebs solution (Ca^{2+}-free Tris-Krebs solution) was that of the Tris-Krebs solution but with the $CaCl_2$ replaced with 0.1mM EGTA, adjusted with HCl to pH 7.4 at 37^oC.

Na^+-free, Ca^{2+}-free Tris-buffered Krebs solution (Na^+-free, Ca^{2+}-free Tris-Krebs solution) consisted of (mM): KCl, 4.7; glucose, 2.8; fumaric acid, 2.7; glutamic acid, 4.9; pyruvic acid, 4.9; $MgCl_2$, 1.13; Tris, 161; EGTA, 0.1; adjusted with HCl to pH 7.4 at 37^oC. Solutions buffered with bicarbonate were bubbled with $95\%O_2/5\%CO_2$; Tris-buffered solutions were bubbled with 100% O_2.

Tris-buffered Krebs solutions were used for initial experiments investigating agonist-stimulated inositol phospholipid breakdown. However, pancreas slices incubated in this Krebs solution did not show a stimulation of amylase secretion in the presence of carbachol or cholecystokinin-octapeptide. When HCO_3-buffered Krebs was used, a significant stimulation of amylase secretion by secretagogues was measured. The reason for the inability of secretagogue to stimulate an increase in amylase secretion from pancreatic tissue incubated in Tris-Krebs solution is likely to be due to the bicarbonate requirement of fluid secretion by the pancreatic acinar cells.

Pancreatic juice flow from the isolated perfused pancreas is proportional to the bicarbonate concentration of the perfusing medium (Case et. al., 1970; Schulz, 1971). Although the ductular fluid secretion

is dependent on extracellular bicarbonate (Kanno and Yamamoto, 1977) the effect of bicarbonate removal on secretagogue-stimulated amylase release is not due to the inhibition of ductular fluid secretion since the agonists used, CCK-8 and carbachol, do not stimulate duct cells.

Secretagogues which stimulate enzyme secretion also stimulate the release of a chloride-rich fluid from acinar cells (Kanno and Yamamoto, 1977; Petersen and Ueda, 1977). The secretion of amylase from the perfused pancreas is unaffected by bicarbonate removal, however, the acinar fluid secretion is partially affected (Kanno and Yamamoto, 1977; Ueda and Petersen, 1977). Since the experiments in this study do not use a perfusion system it is likely that in Tris-Krebs solution the secretagogues do stimulate amylase release from the acinar cells into the duct, but due to the removal of bicarbonate and reduction in acinar fluid secretion, the amylase secretion is not transported along the duct to be released into the bathing medium. This would explain the difficulty in measuring an increase in amylase secretion from pancreatic slices incubated in Tris-Krebs solution.

3.9(b) Chemicals

(i) Radiochemicals:

myo-(2-^{3}H) inositol was obtained from:

Amersham Aust. Pty. Ltd.:

Specific Activity, Ci/mmol : 9.3, 16.4, 16.9, 25.0

Radioactive Concentration : 1 mCi/ml (37 MBq/ml).

New England Nuclear, Boston, Massachusets, U.S.A.
Specific Activity, Ci/mmol : 12.5, 15.8
Radioactive Concentration : 1 mCi/ml (37 MBq/ml).

The radiochemical purity of the *myo*-(2-^{3}H) inositol was checked each month in the laboratory by thin layer chromatography using the solvent system described in the specification sheet. 5 µl of 3.7 MBq/ml *myo*-(2-^{3}H) inositol and 5 µl of *myo*-inositol (150 µg) were applied to silica-gel coated plastic sheets (Merck), equilibrated for 1 hr and developed in n-propanol:ethyl acetate:water:ammonia (25% w/v) (5:1:3:1). The chromatogram was stained with 1% $KMnO_4$ (w/v) containing 2% (w/v) Na_2CO_3 for location of inositol and strips (1 cm) of chromatograms were scraped from the plate, placed in 7.5 ml of scintillation fluid and counted in a liquid scintillation spectrometer. One radioactive peak was obtained at an R_F = 0.33 which corresponds to the R_F for *myo*-inositol.

(ii) Chemicals:

Inositol-1-phosphate was a gift from Dr. R.M.C. Dawson, Department of Biochemistry, A.R.C. Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) was a gift from Dr. P. O'Connell, Upjohn Company, 301 Henrietta St., Kalamazoo, M.I. 49001, U.S.A.

Cholecystokinin octapeptide (CCK-8) was a gift from Dr. M.A. Ondetti, The Squibbe Institute for Medical

Research, P.O. Box 4000, Princeton, New Jersey, 68540, U.S.A.

The SIGMA Chemical Company, U.S.A. supplied:

carbamylcholine chloride (carbachol)

carbamyl-β-methylcholine chloride (bethanechol)

atropine sulphate

bombesin

myo-inositol

L-α-phosphatidylinositol Grade 1 from soybean

L-α-phosphatidylinositol 4-monophosphate from bovine brain

L-α-phosphatidylinositol 4,5-bisphosphate from bovine brain

adenosine 5'-triphosphate (ATP)

cycloheximide

cytochalasin B

colchicine

bovine serum albumin (Fraction V)

α-chymotrypsin Type 1-S bovine pancreas

α-amylase Type 11-A, bacterial

luciferase-luciferin

cholecystokinin-tetrapeptide

oligomycin

blood glucose kit No.510-A

neomycin sulphate

Remazol brilliant blue

isoprenaline hydrochloride

streptozotocin

soybean trypsin inhibitor (Type II-S)

ethylene glycol-bis-(β-aminoethyl ether) N,N'-tetrascetic acid (EGTA)

tris (hydroxymethyl) aminomethane (Tris)

(N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) (Hepes).

The Merck Company, Darmstadt, supplied:

Triton X-100.

The British Drug Houses Company, England, supplied:

2,4 dinitropherol and Amberlite Resin IRA-400.

Packard Instrument Pty. Ltd., Victoria, Australia, supplied the tissue solubiliser Soluene-350 and scintillation fluid E299.

The Worthington Biochemical Corporation, New Jersey, U.S.A. supplied the collagenase.

The Calbiochem-Behring Corporation, La Jolla, California, U.S.A. supplied ionophore A23187.

All other materials were reagent grade.

3.10 : STATISTICS

Results are presented as arithmetic means ± standard error. Students' t-test was used to determine the significance of differences in two group comparisons. For multiple group comparisons, results were analysed using two-way analysis of variance to determine the between groups significance, taking between experiment variation into account.

To test if there was an interaction between agonist and test-drug, factorial analysis (Snedecor and Cochran, 1980) was used. The factorial analysis of results was carried out in collaboration with Dr. W.N. Venables, Department of Statistics, University of Adelaide, using the statistics programs on the CYBER computer, Department of Mathematics, University of Adelaide.

CHAPTER 4

VALIDATION OF THE METHOD DEVELOPED TO MEASURE THE HYDROLYSIS OF INOSITOL PHOSPHOLIPID IN MOUSE EXOCRINE PANCREAS

4.1 : INTRODUCTION

In 1980 when this project began, the majority of published work on phosphoinositide metabolism measured the agonist-stimulated increase in the synthesis of PtdIns, rather than PtdIns breakdown. Michell (1975) proposed that PtdIns breakdown is the primary event in activation of inositol lipid metabolism. Resynthesis measurements provide an unsatisfactory indication of phosphoinositide breakdown (Michell, 1975). There is evidence that the breakdown and synthesis of PtdIns do not run parallel since some agents, including Ca^{2+} and Na^{+} which alter synthesis of PtdIns (Berridge and Fain, 1979; Egawa et. al., 1981 a; Abdel-Latif, 1981) do not alter agonist-stimulated PtdIns breakdown (Berridge and Fain, 1979; Jones and Michell, 1976) in a number of tissues.

To investigate the role of inositol phospholipids in cell control, it was clear that measurement of incorporation of radioactively-labelled compounds into inositol lipid (which measures synthesis), or chemical assays of total cell inositol lipid (which measures the net effect of synthesis and breakdown), would not be satisfactory.

The breakdown of inositol phospholipid can be measured by the use of radioactive-labelling methods to label all the phospholipids with, for example, inorganic phosphate (Jones and Michell, 1974; Fain and Berridge, 1979; Hokin-Neaverson, 1974) or (^{14}C)-labelled fatty acids (Halenda and Rubin, 1982; Lapetina, 1982) or to label the inositol phospholids with *myo*-(2-^{3}H)inositol (Hokin-Neaverson et. al., 1975; Fain and Berridge, 1979; Berridge et. al., 1983). To measure a decrease in radioactively-labelled inositol phospholipid, these studies have used methods that require the extraction and chromatographic separation of lipids. These methods are time consuming, often have a large variance and limit the number of samples

which can be handled in any one experiment. The method described in the present study permits the easy, reproducible measurement of inositol lipid breakdown which does not require extraction and separation of lipids and allows the processing of a large number of samples at a time.

In the last few years, a number of studies have shown a rapid breakdown of PtdIns-4,5P_2 following agonist-stimulation (Kirk et. al., 1981; Berridge et. al., 1983; Putney et. al., 1983). Recently, Michell et. al. (1981) suggested that the breakdown of PtdIns-4,5P_2 (and maybe simultaneously, PtdIns-4P) by the action of phosphodiesterases to remove the inositol phosphates, may be the initial agonist-stimulated event which initiates inositol lipid metabolism and that the loss of PtdIns occurs as it is phosphorylated to PtdIns-4P and then to PtdIns-4,5P_2 to replace the inositol lipid hydrolysed following receptor activation. The PtdIns decrease would, in this case, be due to the action of a kinase rather than a phospholipase.

Since the technique described in 3.2 measures the radioactively-labelled products released from *myo*-(2-^{3}H) inositol-labelled phosphoinositides it provides a measure of inositol phospholipid breakdown. Therefore if hydrolysis of PtdIns or PtdIns-4,5P_2 occurred, the method developed in this study would detect this.

One problem in using the release of *myo*-(2-^{3}H) inositol as an indicator of the hydrolysis of inositol phospholipid, is the possible involvement of the competing exchange reaction which shuttles inositol between the free and lipid-bound states (Eisenberg and Hasegawa, 1981). This reaction is catalysed by the phosphatidylinositol:inositol exchange enzyme described by Paulus and Kennedy (1960) and results in phosphatidyl-(2-^{3}H) inositol + *myo*-inositol $\overset{Mn^{2+}}{\rightleftharpoons}$ *myo*-(2-^{3}H) inositol + phosphatidylinositol. In the present study, it was investigated whether inositol-exchange contributes to the agonist-stimulated increase in *myo*-(2-^{3}H) inositol release which is

taken as a measure of inositol phospholipid breakdown in mouse pancreas (Tennes and Roberts, 1981).

Since inositol lipid hydrolysis is followed by its resynthesis, a possible complication with the use of radioactive-labelling methods to measure this hydrolysis is that both release and reincorporation of radioactive label may occur. The reutilization of the *myo*-(2-^{3}H) inositol released from inositol phospholipid was investigated so as to determine whether the increase in *myo*-(2-^{3}H) inositol content is actually measuring the combination of hydrolysis and resynthesis, rather than the hydrolysis of inositol phospholipid alone.

Recently, a method has been developed (Berridge et. al., 1982) which has allowed the investigation of the involvement of inositol exchange reactions and reutilization of *myo*-(2-^{3}H) inositol in phosphoinositide breakdown. The method uses lithium (Li^{+}) to inhibit the *myo*-inositol-1-phosphatase enzyme which converts the *myo*-inositol-1-phosphate released from inositol phospholipid to *myo*-inositol (Naccarato et. al., 1974). In the absence of Li^{+}, inositol phosphates released from inositol phospholipid by phosphodiesterases are rapidly converted to *myo*-inositol. In the presence of Li^{+}, the product which accumulates would be inositol-1-phosphate if the hydrolysis of inositol phospholipid is responsible for release of the labelled compound, and *myo*-inositol, if the exchange reaction was involved. This Li^{+} method allows differentiation between inositol exchange and inositol phospholipid hydrolysis. In addition, in the presence of Li^{+} there can be no reutilization of the (^{3}H)-labelled compound released from inositol phospholipid by phosphodiesterases as it will accumulate in the phosphate form, not as *myo*-inositol which is used in the resynthesis pathway. The enzyme reactions involved in inositol exchange and the effect of Li^{+} are summarized in Fig. 4.1, which includes the two possibilities of the receptor activated event being the hydrolysis of PtdIns or PtdIns-4,5P_2.

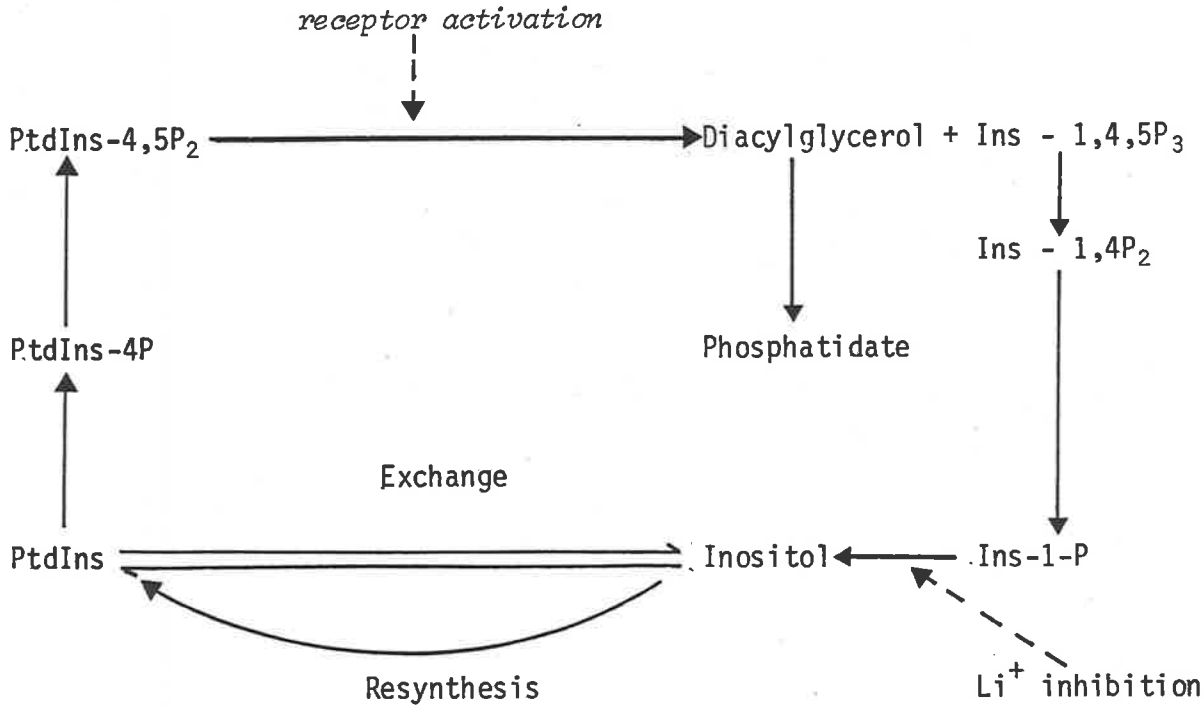


Fig. 4.1 : Inositol-Exchange Reaction and Lithium Inhibition of Inositol-1-Phosphatase

The tissue preparation used for the studies of phosphoinositide hydrolysis in this study is mouse pancreatic slices and although endocrine tissue only comprises 2-4% of the total pancreatic tissue (Bolender, 1974) it was necessary to determine if any of the inositol phospholipid breakdown measured in the tissue slices was occurring in the endocrine pancreas. Agonist-stimulated breakdown in pancreata from diabetic mice (whose β-cells of the endocrine tissue were destroyed with streptozotocin) and in pancreata from control mice were compared to investigate any contribution of endocrine pancreas to total inositol phospholipid hydrolysis. In addition, the sensitivity of the inositol phospholipid response to CCK-8 and CCK-4 in normal mice was examined. The two different molecular forms of CCK differ in their potency with respect to pancreatic secretion (Rehfeld, 1981). Since CCK-8 is a more potent stimulant of exocrine secretion while CCK-4 is more potent with respect to endocrine secretion, then a comparison of the stimulation of the lipid response by these two agonists allows examination of the possible contribution of the breakdown of inositol phospholipid in endocrine pancreas.

4.2 : MATERIALS AND METHODS

In those experiments to identify the *myo*-(2-^{3}H) inositol-labelled phospholipids and acid-soluble products, *myo*-(2-^{3}H) inositol-labelled pancreas pieces were washed and incubated in Ca^{2+}-free Tris-Krebs solution and inositol phospholipid breakdown was measured as described in 3.2. The *myo*-(2-^{3}H) inositol-labelled phospholipids and acid-soluble breakdown products were extracted, separated chromatographically and identified as described in 3.3 and 3.4.

For those studies investigating inositol-exchange and the reutilization of *myo*-(2-^{3}H) inositol, inositol phospholipid breakdown was measured in tissue

incubated in Tris-Krebs solution in the presence and absence of carbachol. In experiments using lithium, this Krebs solution was used but some or all of the NaCl was replaced with an equivalent concentration of LiCl. The acid-soluble (^{3}H)-labelled compounds released from inositol phospholipid were extracted, separated by paper chromatography and identified using the method of Fain and Berridge (1979) as described in 3.4. The *myo*-inositol-1-phosphate standard (40 μg) was included in the chromatographic separation.

Streptozotocin-induced diabetic mice were maintained for 5 days with insulin, (see 3.5(a) and 3.5(b)). Pancreas slices from control and diabetic mice were washed and incubated in Krebs solution and inositol phospholipid breakdown measured using the *myo*-(2-^{3}H) inositol-labelling technique described in 3.5(c) which avoided fasting the mice overnight.

To compare the effect of CCK-4 and CCK-8 on inositol phospholipid hydrolysis, *myo*-(2-^{3}H) inositol-labelled pancreas pieces were washed and incubated in Krebs solution in the presence and absence of agonist. Inositol phospholipid was measured as described in 3.2.

4.3 : RESULTS

4.3(a) Extraction and chromatographic separation of *myo*-(2-^{3}H) inositol-labelled phospholipids of mouse pancreas showed that nearly all (approximately 90%) of the *myo*-(2-^{3}H) inositol incorporated into pancreatic phospholipids was in the form of PtdIns, with only a small proportion of PtdIns-4P and PtdIns-4,5P_2 as shown in Table 4.1. The distribution of radioactivity in the different fractions from the extraction procedure is shown in Table 4.2. In both the control and carbachol-stimulated samples the majority of the label was found in the chloroform/methanol extract (which was used for the chromato-

graphic analysis of the phosphoinositides). The proportion of radioactivity in the water-soluble fraction significantly increased in the samples incubated with carbachol, when compared to the control tissues ($P < 0.05$).In both groups of samples there was a substantial amount of labelled material which was chloroform/methanol-insoluble and could not be chromatographed. The proportion of label in this form was not significantly different for control and stimulated samples ($P > 0.05$).

To validate the precipitation method as an assay for inositol phospholipid hydrolysis, it was necessary to show that all labelled inositol phospholipid was precipitated, and that the labelled *myo*-inositol released by hydrolysis was not carried down during the precipitation phase. Table 4.3 describes the chromatographic identification of (^{3}H)-labelled compounds in the acid-soluble fraction after *myo*-(2-^{3}H) inositol-labelled tissue was homogenised and precipitated with TCA. The labelled compounds of both control and carbachol-stimulated tissue had chromatographic mobilities corresponding to those of *myo*-inositol and inositol-1-phosphate. No radioactivity was present at the R_F value corresponding to PtdIns. When *myo*-(2-^{3}H) inositol was added to a homogenate made from unlabelled pancreatic pieces and the mixture immediately precipitated with TCA by the standard method, only a very small amount of the labelled *myo*-inositol (0.23 ± 0.02%, n = 4) was carried down with the precipitate.

The precision of the TCA precipitation method was tested by processing 7 samples of labelled pancreas, taken from the same pool of tissue pieces, through a 30 min incubation in Ca^{2+}-free Tris-Krebs, homogenisation and TCA precipitation as described in 3.2. The ^{3}H released

from pre-labelled tissue was 184 ± 8 Bq/kBq ^{3}H incorporated by the pancreas, the coefficient of variation being 11%. (The coefficient of variation is calculated as the standard deviation expressed as a percentage of the mean of the sample population).

The reproducibility of the method was tested by a comparison of the results of 6 experiments. Tissue samples were incubated for 30 min in Ca^{2+}-free Tris-Krebs solution in the presence of carbachol ($10^{-5}M$), homogenised and precipitated as described in 3.2. The ^{3}H released from inositol phospholipid above control was 94 ± 11 Bq/kBq ^{3}H incorporated by the pancreas (control tissue released 147 ± 6 Bq/kBq ^{3}H incorporated). A one-way analysis of variance indicated that there was no significant difference between the 6 experimental groups ($P < 0.05$), showing that the between-experiment variation was very small.

The amount of (^{3}H)-labelled acid-soluble compounds in control tissue, washed for 30 min and incubated for 30 min in Ca^{2+}-free Tris-Krebs solution was 147 ± 6 Bq/kBq ^{3}H incorporated by the pancreas ($n = 39$). This shows that approximately 15% of the total ^{3}H in the pancreas slices is not incorporated into phospholipids, indicating there is not a large pool of free *myo*-(2-^{3}H) inositol in unstimulated tissues.

Using this trichloroacetic acid precipitation method, carbachol and CCK-8 stimulated the breakdown of inositol phospholipid in a concentration-dependent manner in tissue incubated in Ca^{2+}-free Tris-Krebs solution as shown in Figs. 4.2 and 4.3.

Table 4.1 : Chromatography of *myo*-(2-^{3}H) inositol-labelled phospholipids of mouse pancreas.

	% Total c.p.m.	R_F	
Control	90.1 ± 2.2	0.38	(n = 7)
	8.2 ± 1.9	0.08	
Carbachol (10^{-4}M)	91.1 ± 1.3	0.38	(n = 5)
	6.6 ± 1.2	0.08	

The R_F of the inositol phospholipid standards were:

PtdIns, 0.38; PtdIns-4P and PtdIns-4,5P_2 , 0.08.

The isotope not accounted for in this table did not appear as a peak on the chromatogram. (n = the number of samples in the group).

Table 4.2 : Recovery of (^{3}H)-labelled material in the phospholipid extraction procedure.

	Control ^{3}H (d.p.m.)	Carbachol (10^{-4}M) ^{3}H (d.p.m.)
Lipid extract	72.6 ± 4 (n = 6)	66.6 ± 4.2 (n = 5)
Water-soluble fraction	5.6 ± 0.6 (n = 6)	11.9 ± 1.7 (n = 3)
Residue	17.2 ± 2.3 (n = 6)	20.5 ± 2.9 (n = 5)

The table shows the radioactivity in the final lipid extract, in the pooled upper phases of the extraction (water-soluble components) and in the chloroform/methanol-insoluble residue remaining in the extraction tube after the lipid extract was applied to the T.L.C. plate. Results are expressed as a percentage of the ^{3}H present in the tissue homogenate. (n = the number of samples in the group).

Table 4.3 : Chromatography of the (^{3}H)-labelled compounds in the acid-soluble fraction.

	% Total c.p.m.	R_F
Control	17.3 ± 2.8	0.17
	71.9 ± 0.6	0.44
	0	0.78
Carbachol	16.7 ± 7.1	0.19
(10^{-4}M)	75.8 ± 7.6	0.44
	0	0.78

The R_F values in this chromatographic system are *myo*-inositol, 0.44; PtdIns, 0.78; inositol-1-phosphate, 0.44. An inositol-1-phosphate standard was not available to be used for these experiments but a peak of radioactivity ran at R_F = 0.44 which corresponds to that found by Fain and Berridge (1979) for inositol-1-phosphate.

Fig. 4.2 : Carbachol-stimulated Hydrolysis of Inositol Phospholipid in Ca^{2+}-free Tris-Krebs Solution

Each point plotted represents inositol phospholipid hydrolysed above control expressed as Bq/kBq ^{3}H incorporated by pancreatic tissue, and is the mean ± S.E. of at least 16 samples.

The ^{3}H released from control tissue was 172 ± 9 Bq/kBq ^{3}H incorporated (n = 40).

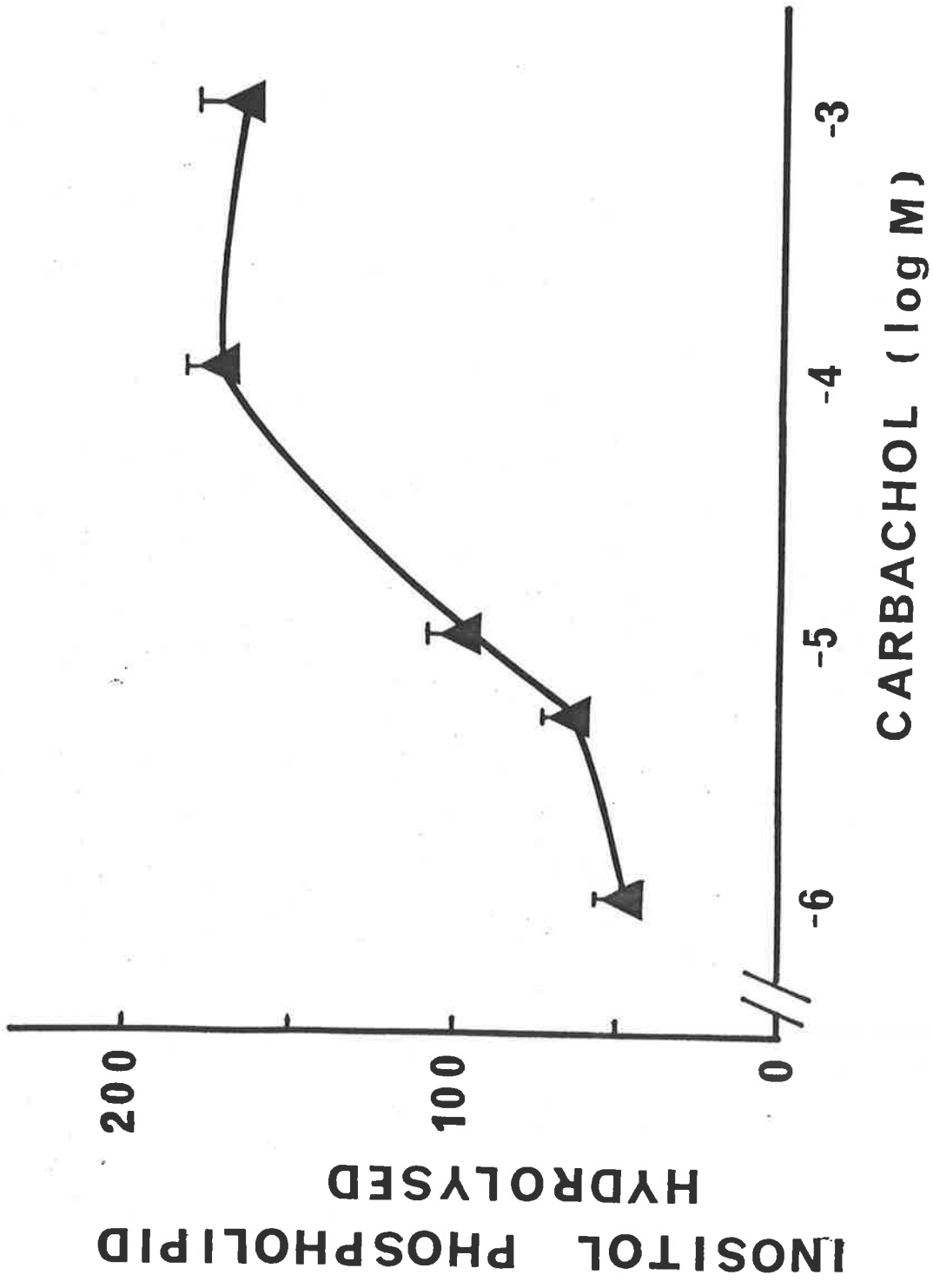


Fig. 4.2 : Carbachol-Stimulated Hydrolysis of Inositol Phospholipid in Ca^{2+}-free Tris-Krebs Solution

Fig. 4.3 : CCK-8-Stimulated Hydrolysis of Inositol Phospholipid in Ca^{2+}-free Tris-Krebs Solution

Each point plotted represents inositol phospholipid hydrolysed above control expressed as Bq/kBq ^{3}H incorporated by pancreatic tissue, and is the mean ± S.E. of at least 8 samples.

The ^{3}H released from control tissue was 153 ± 5 Bq/kBq ^{3}H incorporated (n = 40).

Where S.E. bars are not visible they did not extend beyond the limit of the symbol.

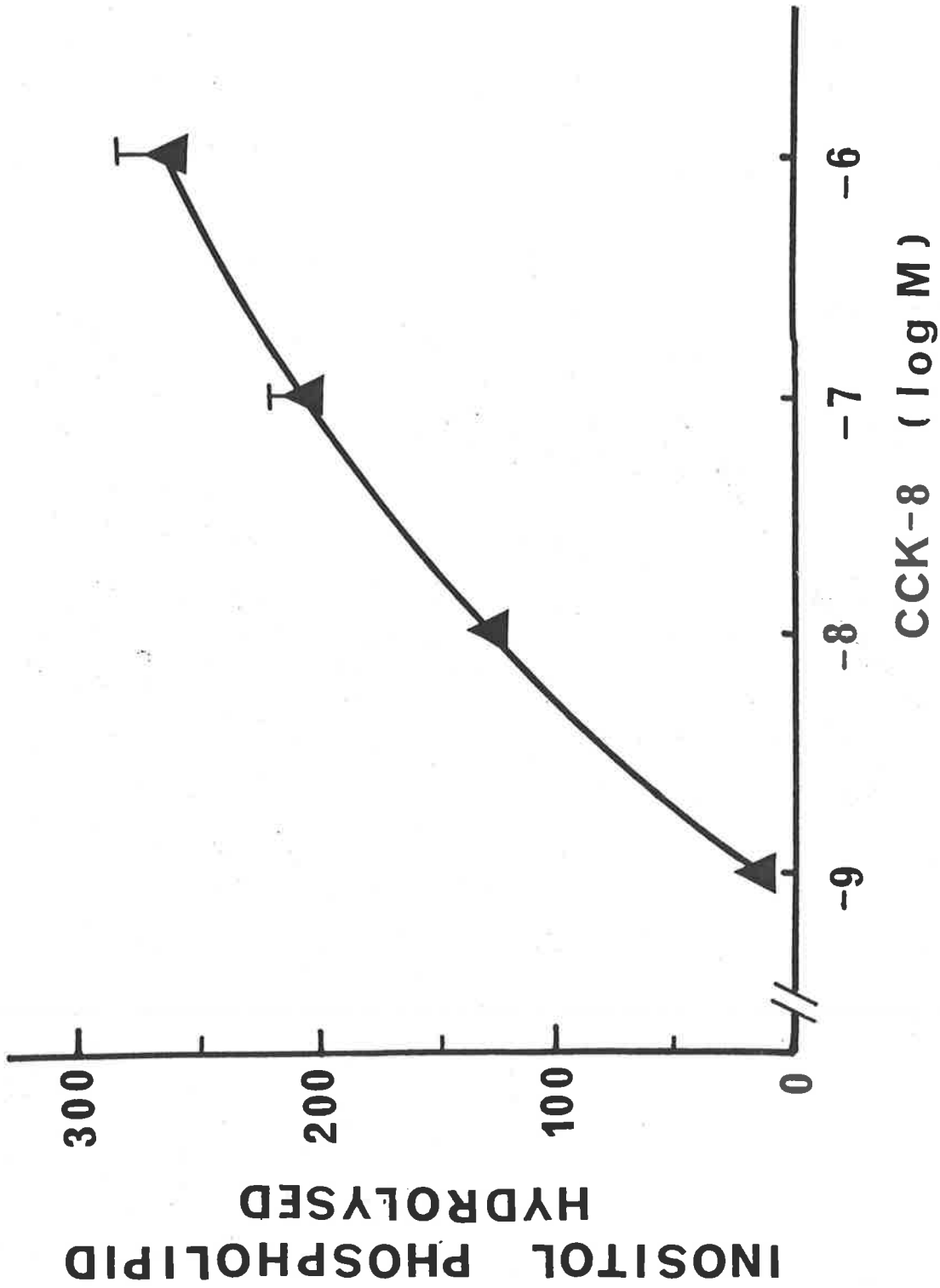


Fig. 4.3 : CCK-8-Stimulated Hydrolysis of Inositol Phospholipid in Ca^{2+}-free Tris-Krebs Solution

To investigate whether inositol-exchange was contributing to the increase in (^{3}H)-labelled compounds released following agonist-stimulation, tissue was incubated in Krebs solution containing 126mM LiCl in the presence and absence of carbachol (10^{-4}M). The acid-soluble ^{3}H in control samples was 168 ± 8 Bq/kBq ^{3}H incorporated by the pancreas (n = 12), while for carbachol-stimulated samples it increased to 378 ± 16 Bq/kBq ^{3}H incorporated by the pancreas (n = 12). The results of chromatography of the compounds in these acid-soluble fractions are shown in Table 4.4. In both control and carbachol-stimulated samples two radioactive peaks were obtained.

Carbachol-stimulation of the tissue did not cause any increase in the amount of radioactivity which ran at an R_F = 0.4 and chromatographed as inositol. The other peak of radioactivity which did not run as inositol did increase with carbachol-stimulation and ran at an R_F = 0.1 which correlates with the R_F for inositol-1-phosphate (Table 4.4). These R_F values are the same as those found by Fain and Berridge (1979) for inositol and inositol-1-phosphate.

A comparison of the effect of 0, 10 and 126mM LiCl on the release of (^{3}H)-inositol from pre-labelled pancreatic pieces, stimulated by carbachol (10^{-4}M) was made. As the concentration of LiCl increased, the percentage of radioactivity in the form of inositol-1-phosphate increased (Table 4.5). The requirement for such high concentrations of Li^+ to inhibit the inositol phosphatase probably reflects the preparation used, tissue slices. Using isolated acini from mouse pancreas, it was found that 10mM of LiCl was maximally effective in inhibiting this phosphatase (Crouch, M.F. and Roberts, M.L., unpublished).

Table 4.4 : Chromatography of *myo*-(2-^{3}H) inositol-labelled compounds in the acid-soluble fraction.

	CONTROL (LiCl) = 126mM	CARBACHOL (10^{-4}M) (LiCl) = 126mM
	c.p.m. (% of total ^{3}H incorporated by pancreas)	
R_F = 0.1	7.2 ± 0.8 (3)	27.3 ± 1.4 (3)
R_F = 0.4	8.4 ± 1.1 (3)	8.9 ± 1.0 (3)

Mean results ± S.E. are shown and represent the radioactivity present at each peak as a percentage of the total ^{3}H incorporated by the pancreas. *Myo*-inositol ran at R_F = 0.4; Inositol-1-phosphate ran at R_F = 0.1. The numbers in parentheses are the number of experiments. There were no other regions on the chromatograms which showed significant levels of ^{3}H and the 2 peaks accounted for approximately 90% of the TCA-soluble radioactivity (see Table 4.5).

Table 4.5 : The effect of lithium on (^{3}H)-inositol released from carbachol-stimulated pancreas pieces.

(LiCl)	0mM	10mM	126mM
	c.p.m. (% of total ^{3}H present on each chromatogram)		
R_F = 0.1	8.9	59.0	72.0
R_F = 0.4	76.0	31.0	23.7

The mean results of 2 (0mM, 10mM LiCl) or 3 (126mM LiCl) experiments are shown: Results show c.p.m. at two peaks corresponding to the R_F values = 0.1 and 0.4 expressed as a percentage of the total ^{3}H present on each chromatogram. The radioactivity not accounted for in this table did not occur as a peak on the chromatogram.

Fig. 4.4 : Inositol Phospholipid Hydrolysis in Pancreatic Tissue from Control and Diabetic Mice

Each point represents inositol phospholipid hydrolysed above control expressed as Bq/kBq ^{3}H incorporated by pancreas for control (●) and diabetic (■) mice; and is the mean ± S.E. of at least 8 samples.

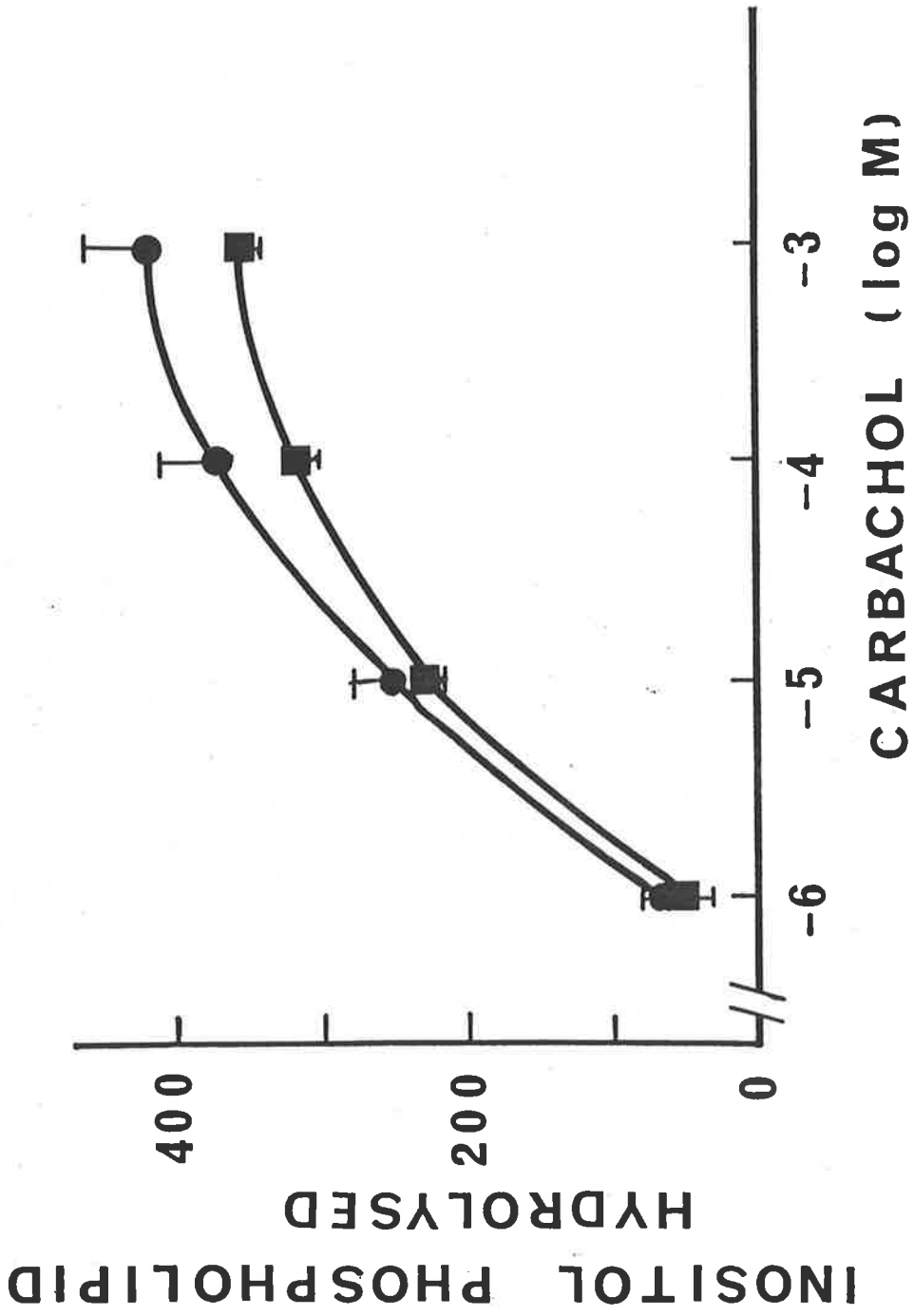


Fig. 4.4 : Inositol Phospholipid Hydrolysis in Pancreatic Tissue from Control and Diabetic Mice

To investigate whether some of the (^{3}H)-inositol released from inositol phospholipid by agonist-stimulation was reincorporated into inositol phospholipid, the carbachol stimulated breakdown of inositol lipid was compared in tissue incubated in Tris-Krebs solution and Tris-Krebs solution where NaCl was replaced with 126mM LiCl. In the absence of LiCl, carbachol (10^{-4}M) caused an increase in ^{3}H released above control of 218 ± 35 Bq/kBq ^{3}H incorporated by the pancreas (n = 8), and in the presence of LiCl carbachol (10^{-4}M) caused an increase above control of 210 ± 14 Bq/kBq ^{3}H incorporated by the pancreas (n = 12). There was no significant difference between these two groups ($P > 0.05$).

The breakdown of inositol lipid in diabetic and control mice was compared to investigate whether there was any significant breakdown of phosphoinositide occurring in the islet cells. A comparison of the concentration response curves in Fig. 4.4 showed that at each concentration of carbachol, there was no significant difference ($P > 0.05$) between inositol lipid breakdown in control and diabetic mice. In the unstimulated state there was no significant difference between ^{3}H released in control tissue, which was 252 ± 26 Bq/kBq ^{3}H incorporated by the pancreas, (n = 8), and in tissue from diabetic mice which was 277 ± 14 Bq/kBq ^{3}H incorporated by the pancreas (n = 8), ($P > 0.05$). The effect of CCK-8 and CCK-4 on inositol lipid hydrolysis is shown in Fig. 4.5. A comparison of the two concentration-response curves shows that the potency of CCK-8 to stimulate the lipid response is much greater than that of CCK-4. The difference between the dose-response curves for CCK-8 in Figs. 2.3 and 2.5 is due to the different Krebs solutions used. This effect is described in Chapter 6 of this thesis.

Fig. 4.5 : A Comparison of Inositol Phospholipid Hydrolysis Stimulated by CCK-8 and CCK-4 in Pancreatic Tissue Incubated in Krebs Solution

Each point plotted represents inositol phospholipid hydrolysed above control for CCK-8 stimulated (●) and CCK-4-stimulated (▲) pancreas; each is the mean ± S.E. of at least 8 samples and is expressed as Bq/kBq ^{3}H incorporated by pancreas.

The ^{3}H released from control tissue for the CCK-8 and CCK-4 experiments was (Bq/kBq ^{3}H incorporated by pancreas) : 271 ± 12 (n = 9) and 229 ± 12 (n = 10), respectively.

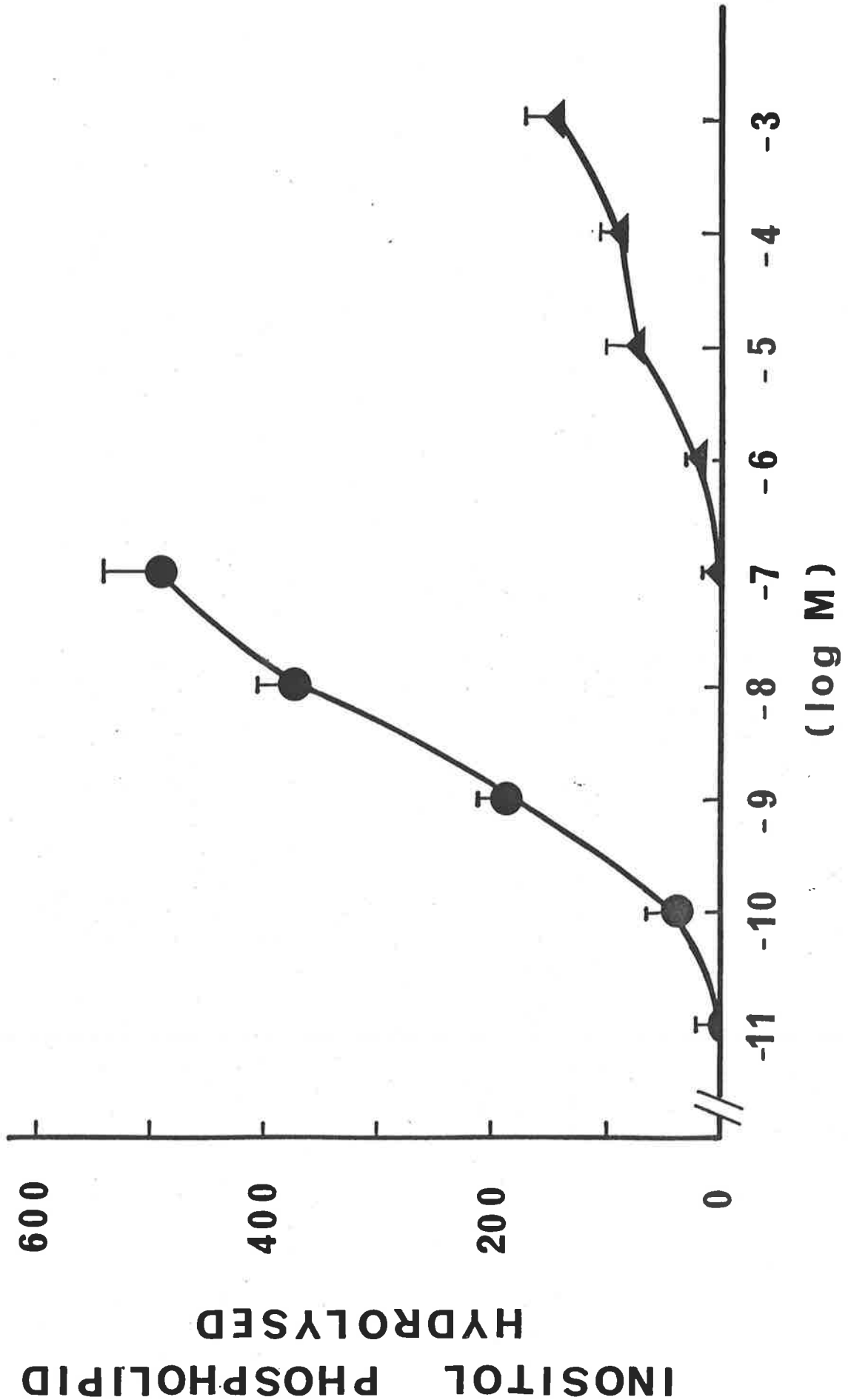


Fig. 4.5 : A Comparison of Inositol Phospholipid Hydrolysis Stimulated by CCK-8 and CCK-4 in Pancreatic Tissue Incubated in Krebs Solution

4.4 : DISCUSSION

When mouse pancreas, pre-labelled *in vivo* with *myo*-(2-^{3}H) inositol was incubated *in vitro* with carbachol, the proportion of (^{3}H)-labelled material in the TCA supernatant increased, which is taken as an increase in the hydrolysis of inositol phospholipid. It was necessary to extract and identify the *myo*-(2-^{3}H) inositol-labelled phospholipids and breakdown products and also to determine their presence in the TCA precipitate or supernatant, so as to establish whether the method could be used as an assay for phosphoinositide hydrolysis. The majority of the (^{3}H)-labelled lipid which could be extracted from the pre-labelled pancreas was PtdIns with only a small amount of polyphosphoinositide (Table 4.1). Hokin and Hokin 1958 b) found that PtdIns was the major phospholipid into which *myo*-(2-^{3}H) inositol was incorporated in brain. Michell et. al (1981), using *in vivo* labelling of hepatocytes with *myo*-(2-^{3}H) inositol, found that PtdIns-4P and PtdIns-4,5P_2 each represented 1-2% of the total inositol lipid. The results of the present study show that about 6-8% of total *myo*-(2-^{3}H) inositol-labelled phospholipid was in the form of PtdIns-4P and PtdIns-4,5P_2 (Table 4.1).

Although inositol and inositol-1-P were identified as (^{3}H)-labelled compounds in the acid-soluble fraction (Table 4.3) this result does not indicate whether the inositol lipid hydrolysed is PtdIns, PtdIns-4,5P_2 or PtdIns-4P. The action of a phospholipase C on PtdIns (Michell, 1975) would release inositol-1-P which is rapidly converted to inositol by the inositol-1-phosphatase enzyme, while the hydrolysis of PtdIns-4,5P_2 (Michell et. al.,1981) would release inositol-1,4,5P_3 which is rapidly converted successively by phosphatases to inositol-1,4P_2, inositol-1-P and inositol. A recent study by Berridge et. al. (1983) shows significant increases in the levels of inositol-1,4,5P_3, inositol-1,4P_2, and inositol-1-P after agonist stimulation, which supports the proposal that PtdIns-4,5P_2 hydrolysis may be the

initial agonist-stimulated event but does not exclude the possibility that PtdIns hydrolysis may occur also. Therefore, although the assay used in the present study does not identify which phosphoinositide(s) is hydrolysed it can be used to indicate inositol phospholipid breakdown since it measures (^{3}H)-inositol-labelled products released from *myo*-(2-^{3}H) inositol-labelled inositol phospholipid.

In all extraction methods for obtaining inositol lipid from tissues, an insoluble residue, presumably containing proteins, is obtained (Downes and Michell, 1982 b). This cannot be assayed for phosphoinositide content, whether one used a chemical method or a radio-chemical assay based on either (^{32}P)-P_i or *myo*-(2-^{3}H) inositol incorporation into phospholipids. About 20% of the *myo*-(2-^{3}H) inositol was incorporated into molecules which remained in the residue (Table 4.2). Although the nature of the labelled molecules in the residue has not been determined they did not contribute to the increase in acid-soluble ^{3}H following exposure to secretagogues because the proportion of counts in the residue did not decrease in carbachol-stimulated tissues (Table 4.2). On addition of carbachol, the decrease in the radioactivity of the lipid extract fraction (which is mostly PtdIns) is equal to the increase in the water-soluble fraction (Table 4.2). Since the TCA-precipitation confines inositol phospholipid to the precipitate and does not produce significant trapping of labelled hydrolysis products in this fraction, it is possible to use the change in acid-soluble radioactivity in the *myo*-(2-^{3}H) inositol-labelled pancreas as an assay for inositol lipid hydrolysis.

This assay has several advantages over alternative assays of inositol phospholipid hydrolysis which have been used to investigate the role of this phenomena in stimulus-response coupling. Many assays have used the agonist-stimulated incorporation of (^{32}P)-P_i into inositol lipid to

measure turnover, but this technique is actually measuring resynthesis of inositol phospholipid, rather than the primary event of breakdown (Michell, 1975; Michell et. al., 1981). Techniques of inositol phospholipid assay based on chemical estimation or radiochemical measurements of (^{32}P)-labelled phosphoinositides involve extraction of lipids from the tissues and separation of the inositol phospholipid from other phospholipids. These steps are time-consuming and lead to assays with large variance. The method described here is rapid, accurate and reproducible. *In vitro* labelling of PtdIns with *myo*-(2-^{3}H) inositol was used by Keryer and Rossignol (1978) to examine PtdIns turnover. This approach led to a large pool of free *myo*-(2-^{3}H) inositol in the tissue, so that addition of secretagogues stimulated breakdown and synthesis of (^{3}H)-PtdIns simultaneously. These workers found an initial increase, followed by a rapid decrease in levels of labelled PtdIns in parotid gland when incubated with secretagogues. *In vivo* labelling of inositol phospholipid with *myo*-(2-^{3}H) inositol results in a small pool of free, labelled *myo*-inositol since at the end of a 30 min wash and 30 min incubation only 15% of the total ^{3}H present in unstimulated tissue is acid-soluble.

The use of the lithium method to inhibit inositol-1-phosphatase (Berridge et. al., 1982) allowed investigation of whether the (^{3}H)-inositol released from inositol phospholipid by agonist-stimulation was reincorporated into inositol phospholipid and also whether inositol-exchange was stimulated. This study shows that lithium does inhibit inositol-1-phosphatase in mouse pancreas (Table 4.6) as has been found in brain and salivary glands (Berridge et. al., 1982). Naccarato et. al. (1974) found that 250mM Li^{+}, *in vitro*, completely inhibits inositol-1-phosphatase of rat mammary gland, while Hallcher and Sherman (1980) found that the hydrolysis of inositol-1-phosphate from bovine brain, *in vitro*, was half-maximally inhibited by 0.8mM Li^{+}.

If the (^{3}H)-inositol released from inositol phospholipid by agonist stimulation was reincorporated into inositol phospholipid then in the presence of 126mM LiCl it would be expected that the ^{3}H in the TCA supernatant would be greater than in the absence of LiCl since the inositol-1-phosphate would not be re-utilized. The results of this study show that (^{3}H)-inositol released is not re-incorporated and agree with previous results (Tennes and Roberts, 1981) (see Table 5.1) where CCK-8-stimulated inositol lipid hydrolysis was identical in tissue incubated in Ca^{2+}-free Tris-Krebs solution with either 126mM NaCl or 126mM LiCl. In addition, the results of this study show that inositol-exchange is not responsible for the increase in ^{3}H in the TCA supernatant following agonist stimulation since carbachol-stimulation of the tissue, in the presence of 126mM LiCl did not cause any increase in the amount of radioactivity which chromatographed as inositol (Table 4.4) (Tennes and Roberts, 1984).

In addition to hydrolysing inositol-1-phosphate, *myo*-inositol-1-phosphate shows minor activity when *myo*-inositol-1-phosphate (Naccarato et. al., 1974) and *myo*-inositol-3-phosphate (Hallcher and Sherman, 1980) act as substrates. If PtdIns-4,5P_2 is the initial inositol lipid to be hydrolysed then although Li^+ will inhibit the inositol-1-phosphatase conversion of inositol polyphosphates to inositol-1-phosphate, these polyphosphates will not accumulate since the inositol trisphosphatase and inositol bisphosphatase enzymes are active. Li^+ does not inhibit these bis- and trisphosphatases and so the inositol polyphosphates are converted to inositol-1-phosphate in the presence of Li^+(Berridge et.al.,1982).

Hokin (1974) using a double-labelling method to pre-label PtdIns *in vivo* with (^{32}P)-P_i and *myo*-(2-^{3}H) inositol, found that the decreases in (^{32}P)-PtdIns and (^{3}H)-PtdIns in pancreas pieces stimulated by acetylcholine were

proportionately the same. Since there was no differential effect on measurement of PtdIns breakdown using both radiochemical labels this suggests that there was no stimulation of inositol exchange, as has been shown in this study. Hokin-Neaverson et. al. (1975) measured an increase in free inositol released by acetylcholine stimulation but found no increase in inositol 1,2-cyclic phosphate, or other inositol phosphates and suggested that PtdIns hydrolysis may be due to a reversal of the cytidine nucleotide pathway of PtdIns synthesis which would result in PtdIns + cytidine monophosphate → inositol + cytidine diphosphate-diglyceride → cytidine triphosphate + phosphatidic acid (Hokin-Neaverson et. al., 1975). The results of the present study show that inositol phospholipid breakdown in mouse pancreas does not occur via this pathway since the accumulation of inositol-1-phosphate in the presence of LiCl indicated the initial agonist-stimulated event is a phospholipase C action. It is likely that Hokin-Neaverson et. al. (1975) found only inositol because the inositol phosphates formed were rapidly converted to *myo*-inositol by phosphatases. Tolbert et.al. (1980) found that the hormone-stimulated uptake of (^{3}H)-inositol into PtdIns in rat liver parenchymal cells did not correlate with (^{32}P)-P_i incorporation. The differential effect on labelling was not due to stimulation of the CDP-diacylglycerol : inositol-phosphatidyl transferase enzyme (Tolbert et.al., 1980), since both the phosphatidyl and inositol groups will both be turned over by this enzyme, but rather to the phosphatidylinositol : inositol exchange enzyme (Paulus and Kennedy, 1960; Eisenberg and Hasewaga, 1981). Another study using *myo*-(2-^{3}H) inositol in rat liver cells showed that the (^{3}H)-inositol labelling was mainly due to this exchange enzyme (Prpić et. al., 1982). Therefore, it would appear that although the released *myo*-(2-^{3}H) inositol from pre-labelled phospholipids is a good indicator of inositol phospholipid hydrolysis in the exocrine pancreas, this may not be so for all tissues.

Further confidence in the validity of this technique as an assay for inositol lipid hydrolysis can be derived from the fact that the concentration-dependent breakdown of inositol phospholipid stimulated by carbachol and CCK-8 (Figs. 4.2 and 4.3) confirms the results of Hokin (1974) and Hokin-Neaverson et. al. (1975) for mouse pancreas. Both carbachol and CCK-8 stimulate pancreatic secretion, apparently by binding to different receptors but activating the same stimulus-secretion pathway (Gardner and Jensen, 1980).

The use of pancreatic slices provides an accurate measure of inositol phospholipid breakdown in the exocrine pancreas since the destruction of islet cell function does not cause any significant effect on carbachol-stimulated inositol phospholipid breakdown in pancreatic slice preparations (Fig. 4.4). Carbachol and cholecystokinin-octapeptide regulate pancreatic islet function (Malaisse, 1972) and in a recent study, Best and Malaisse (1983) showed a carbachol-stimulated increase in the breakdown of inositol phospholipid in rat pancreatic islets. It is possible, that agonist-stimulated breakdown of inositol phospholipid does occur in the islet cells of the mouse pancreatic slice preparation, however, since endocrine tissue accounts for a very small percentage of total pancreatic tissue, this breakdown, if occurring, would contribute negligibly to the total phosphoinositide breakdown in slices (this is confirmed by the results in Fig. 4.4). The concentration response curves for inositol phospholipid breakdown stimulated by carbachol in control and diabetic mice are not identical. However, some difference could be expected. Korc et. al. (1981 a) have shown effects of insulin on the exocrine pancreas and since it is not possible to mimic the physiological release of insulin with 2 injections per day, it is likely that an effect of insulin could result in differences between control and diabetic mice.

That endocrine function contributes negligibly to the total inositol phospholipid breakdown measured in tissue slices was further confirmed by comparing the effect of CCK-8 and CCK-4 on the lipid response. CCK-4 is a more potent stimulator of endocrine function than CCK-8 (Rehfeld, 1981) and yet caused only a small degree of inositol lipid breakdown in comparison to CCK-8. Since CCK-4 does stimulate exocrine function, although to a lesser degree than endocrine secretion, it is likely that some of the inositol lipid hydrolysed by CCK-4 occurred in the exocrine pancreas. Although it is not possible from the present results to determine the amount of inositol lipid breakdown which can occur in the endocrine pancreas alone, it is clear that endocrine function does not contribute significantly to agonist-stimulated inositol lipid hydrolysis in pancreatic slices.

CHAPTER 5

INVESTIGATION OF INOSITOL PHOSPHOLIPID HYDROLYSIS AS AN EARLY EVENT IN ACTIVATION OF THE EXOCRINE PANCREAS

5.1 : INTRODUCTION

Following the validation of the method I developed which facilitated the measurement of inositol phospholipid hydrolysis, studies began to determine how closely this response is linked to receptor activation. A series of experiments were designed to identify at what stage in the chain of events leading to secretion the breakdown of this inositol lipid occurs. These experiments involved consideration of Na^+ as a second messenger in eliciting the lipid response, the possible requirement of protein synthesis and the possibility that microfilaments and microtubules (by providing an intact cytoskeletal network) may be involved.

One of the early events following activation of muscarinic and peptide receptors in exocrine pancreas is an increase in the influx of Na^+ which causes depolarization of the acinar cell membrane (Nishiyama and Petersen, 1975). Na^+ may act as a second messenger since Na^+ influx occurs very rapidly after receptor activation (Nishiyama and Petersen, 1975) and there is some evidence that Na^+ may stimulate the release of Ca^{2+} from mitochondria (Haworth et. al., 1980; Hughes et. al., 1980).

No investigation of the effect of Na^+ removal on inositol phospholipid breakdown has been made in any tissue. The only study of the role of Na^+ in phosphoinositide metabolism in the pancreas measured the *de novo* synthesis of inositol phospholipid (Calderon et. al., 1980) while in the parotid gland, the synthesis of PtdIns (Keryer et. al., 1979) and the net result of breakdown and synthesis, by assay of total PtdIns content (Jones and Michell, 1976) were measured. The technique developed in the present study allowed the first investigation of whether Na+ influx leads to the breakdown of inositol phospholipid by examining the effect of Na^+ removal on this lipid response directly.

Harris and Hokin-Neaverson (1977) found that the bulk of PtdIns hydrolysed in guinea-pig pancreas was located in the endoplasmic reticulum, which is the site of protein synthesis. This raised the possibility that inositol phospholipid breakdown may depend on the synthesis of a labile protein, indicating that this lipid response is not closely associated with receptor activation but may occur subsequent to a number of cellular events. The effect of the protein synthesis inhibitor cycloheximide, on inositol phospholipid hydrolysis was therefore investigated in this study.

In the exocrine pancreas, microtubule and microfilament disruption agents inhibit secretion by inhibiting the intracellular transport and release of exportable protein (Seybold et. al., 1975; Williams and Lee, 1976). A dependence of inositol lipid breakdown on an intact cytoskeleton may indicate the dependence of this response on release of secretory product. Alternatively, since a phosphatidylinositol phosphodiesterase has been shown to be associated with the microtubular subunit protein, tubulin (Daleo et. al., 1976; Quinn, 1973) perhaps the activation of interaction of enzyme with substrate may be altered by disruption of microtubules.

No study on the effect of microtubule and microfilament disruption agents on inositol lipid hydrolysis in pancreas has been made, although the breakdown of PtdIns in neutrophils appears to be independent of cytochalasin B (Cockcroft et. al., 1980). A dependence of PtdIns synthesis on an intact microtubular system in lymphocytes (Wassarman et. al., 1978) and on microfilament structure in cervical ganglia and pineal gland (Lakshmanan, 1978) has been suggested. The effect of a microtubule and microfilament disruption agent colchicine and cytochalasin B, respectively, on inositol lipid hydrolysis was investigated in this study to examine a possible dependence of this response on an intact cytoskeleton.

The conclusion from these studies was that the breakdown of inositol phospholipid is an early event in the activation of the exocrine pancreas, suggesting that it is closely linked to receptor activation.

Kirk et. al. (1981) suggested that the degree of inositol phospholipid hydrolysis was proportional to the degree of receptor occupancy. Their study showed a close correlation between the dose-response curves for (^{3}H-Lys^{8})-vasopressin binding to its receptors and the vasopressin-stimulated loss of PtdIns and PtdIns-4,5P_2 and so proposed that maximal inositol lipid hydrolysis occurred with maximal receptor occupation (Kirk et. al., 1981) in support of the earlier suggestion by Michell et. al. (1976). An implication of this proposal would be that the presence of one agonist at a concentration sufficient to occupy all receptors for that agonist should not affect the ability of another agonist acting at a different receptor in eliciting inositol phospholipid hydrolysis. The simultaneous presence of two different agonists acting independently should produce an additive effect on phosphoinositide hydrolysis.

However, Weiss and Putney (1981) suggested that the inositol phospholipid response may be more closely linked to the 'calcium-gating' mechanism than to receptor occupation. This idea was based on their study using rat parotid acinar cells where simultaneous stimulation with substance P and adrenergic agonists did not produce an additive effect on (^{32}P)-P_i incorporation into PtdIns (Weiss and Putney, 1981). Since this latter study did not measure the hydrolysis of phosphoinositide it was not possible to compare the results with those of Kirk et. al. (1981).

In view of the conflicting proposals of Weiss and Putney (1981) and Kirk et. al. (1981) the relationship between receptor occupation and inositol phospholipid hydrolysis in exocrine pancreas was examined by studying the effect of simultaneous activation of the cholecystokinin receptor and the muscarinic receptor.

Hokin (1974) showed that 2,4 dinitrophenol inhibited the agonist-stimulated decrease in PtdIns in mouse pancreas. However, Michell (1975) using cyanide and oligomycin was unable to conclusively confirm this requirement for metabolic energy. Since the initial proposed pathway for hydrolysis of PtdIns (Michell, 1975) by the action of phospholipase C is not an energy-requiring enzymic reaction, it was not clear how PtdIns breakdown would require ATP. In the present study, the dependency of agonist-stimulated hydrolysis of inositol phospholipid on cellular ATP was examined, since if ATP is necessary this may indicate that the decrease in PtdIns described by Hokin (1974) does not occur via a phospholipase C reaction on PtdIns but via a different pathway which requires ATP.

5.2 : MATERIALS AND METHODS

In experiments examining the role of Na^+, protein synthesis, microtubules, microfilaments and ATP, inositol phospholipid hydrolysis was measured as described in 3.2. Whereas, the studies examining the simultaneous addition of two agonists and the effect of duration of stimulation on phosphoinositide hydrolysis were carried out more recently and measured the release of (^{3}H)-inositol phosphates, as will be described.

In the experiments investigating the removal of extracellular Na^+ on inositol lipid hydrolysis, Na^+ was replaced with Tris (Na^+-free, Ca^{2+}-free Tris-Krebs solution) as described in 3.10 or with LiCl, where the NaCl of the Ca^{2+}-free Tris-Krebs solution (see 3.10) was replaced with 126mM LiCl. In these experiments, atropine (2.5×10^{-6}M) was included to block the action of any acetycholine which may have been released from nerve terminals in the tissue (Paton et. al., 1971). CCK-8 was used as an agonist in these experiments.

To study the effect of protein synthesis inhibition on inositol lipid hydrolysis, tissue was washed for 2 x 15 min and incubated for 30 min in Ca^{2+}-free Tris-Krebs solution. When cycloheximide (5×10^{-4}M) was to be used it was present in the second wash and also during the 30 min incubation. This concentration of cycloheximide was chosen since it was found in our Laboratory (M.L. Roberts, unpublished) that 5×10^{-4}M cycloheximide reduces protein synthesis in mouse pancreatic slices to 2.5% of control.

Williams and Lee (1976) using mouse pancreatic slices found that colchicine (3×10^{-5}M) reduced or abolished microtubules only after a 150 or 210 min incubation. In the present study, *myo*-(2-^{3}H)-inositol-labelled mouse pancreatic pieces were incubated in the absence or presence of colchicine (3×10^{-5}M) in a Ca^{2+}-free Tris-Krebs solution for 210 min prior to the addition of carbachol (5×10^{-6}M); the incubation was terminated after a further 30 min and inositol phospholipid hydrolysis measured.

Disruption of the microfilamentous network of acinar cells occurs when

pancreatic fragments are incubated for 90 min in the presence of cytochalasin B (2×10^{-5}M) (Stock et. al., 1978). The effect of microfilament disruption on phosphoinositide hydrolysis was measured using *myo*-(2-^{3}H) inositol-labelled mouse pancreatic slices, pre-incubated for 90 min with cytochalasin B (2×10^{-5}M) in Ca^{2+}-free Tris-Krebs solution and then incubated for 30 min in the presence of carbachol (5×10^{-6}M). Since cytochalasin B was dissolved in ethanol, an equivalent concentration (0.5% v/v, final concentration) of ethanol was added to control and carbachol-stimulated samples.

The effect of simultaneous stimulation of the muscarinic and cholecystokinin receptors was measured in *myo*-(2-^{3}H) inositol-labelled pancreatic slices incubated in Ca^{2+}-free Tris-Krebs solution in which the NaCl was replaced with 126mM LiCl. Pancreatic tissue was stimulated for 30 min with carbachol (10^{-3}M), CCK-8 (at a range of concentrations) and both agonists combined. The same experiments were performed with butyrylcholine chloride (10^{-3}M) instead of carbachol; when butyrylcholine was used, neostigmine (5×10^{-5}M) was included in the incubation solution to inhibit the degradation of the agonist by acetylcholine esterases. Both carbachol and butyrylcholine were present at maximally effective concentrations.

It was necessary to determine whether the degree of inositol phospholipid hydrolysis after a 30 min incubation in the presence of high concentrations of CCK-8 was limited due to depletion of pre-labelled phosphoinositide. The CCK-8-stimulated hydrolysis of inositol phospholipid was measured at 15, 30 and 60 min in tissue labelled *in vivo* with *myo*-(2-^{3}H) inositol and incubated in Ca^{2+}-free Tris-Krebs

solution in which the NaCl was replaced with 126mM LiCl. The two above studies measured inositol phospholipid hydrolysis as an increase in (^{3}H)-inositol phosphates.

It has been shown in the previous chapter that in the presence of Li^+ in the incubation medium, the (^{3}H)-labelled compounds released from the inositol phospholipid following stimulation are inositol-phosphates, and these were measured using the method of Berridge et. al. (1982).

Pancreatic tissue which had been labelled *in vivo* with 122 kBq of *myo*-(2-^{3}H) inositol, was incubated in Ca^{2+}-free Tris Krebs solution in the presence or absence of agonist(s). The incubation was terminated by freezing the samples in a dry ice/alcohol bath. Samples were homogenized from the frozen state and a 100µl sample was taken immediately from the homogenous solution for determination of total radioactivity. To the remaining homogenate 0.94 ml of chloroform/methanol (1:2, v/v) was added. Chloroform (0.31 ml) and water (0.31 ml) were then added to separate the phases. After centrifuging for 10 min at 4^{o}C at 500 x g a 1.2 ml sample of the upper phase was diluted to 3 ml with water and to each, 0.5 ml of Amberlite resin in the formate form (50%, 2/v in water) was added to bind (^{3}H)-inositol phosphates (Berridge et. al., 1982) and these were mixed on a rotating turntable. After washing the resin four times with 2.5 ml of 5mM *myo*-inositol, (^{3}H)-inositol phosphates were displaced from the resin by the addition of 0.5 ml of 1M ammonium formate/0.1M formic acid. These were mixed and after 10 min, 0.4 ml of the supernatant was counted in 3.5 ml of scintillation fluid in a liquid scintillation counter.

To investigate the role of ATP in the agonist-stimulated lipid response,

myo-(2-^{3}H) inositol-labelled pancreas pieces were incubated (as described in 3.2) in Ca^{2+}-free Tris-Krebs solution. Metabolic inhibitor was present for the final 10 min of the wash period prior to a 30 min incubation in the absence or presence of carbachol (10^{-5}M). Oligomycin was dissolved in ethanol; an equivalent concentration (1% v/v, final concentration) of ethanol was added to control and carbachol-stimulated samples. The pH of the 2,4DNP solution (10^{-3}M) and the KCN solution (10^{-3}M) was adjusted to pH 7.4 at 37^{o}C with NaOH and HCl respectively. The effect of these inhibitors on the ATP content of unstimulated tissue slices was measured using the luciferase-luciferin technique described in 3.9.

5.3 : RESULTS

The effect on inositol phospholipid hydrolysis of removal of Na^{+} from the extracellular medium is summarized in Table 5.1. Replacement of Na^{+} with either Li^{+} or Tris had no significant effect on the ^{3}H released from either the unstimulated or CCK-8-stimulated tissues ($P > 0.05$). In both experiments A and B, the CCK-8 stimulated groups were significantly different to the unstimulated groups ($P < 0.05$).

Table 5.1 : The effect of removal of extracellular Na^+ on CCK-8 stimulated inositol phospholipid breakdown

	INOSITOL PHOSPHOLIPID HYDROLYSED (Bq/kBq ^3H INCORPORATED BY PANCREAS)			
	EXPERIMENT A*		EXPERIMENT B*	
	CONTROL	CCK-8	CONTROL	CCK-8
Ca^{2+}-free Tris Krebs	227 ± 18 (8)	315 ± 20 (9)	183 ± 9 (5)	308 ± 7 (5)
Na^+-free, Ca^{2+}-free Tris Krebs	231 ± 14 (8)	306 ± 16 (8)	171 ± 14 (5)	309 ± 14 (5)

* The sodium was replaced in experiment A with Tris and in experiment B with lithium.
The numbers in brackets are the number of samples in each group.

Cycloheximide (5×10^{-4}M) did not cause any significant alteration in inositol phospholipid hydrolysis stimulated by carbachol (10^{-5}M), as shown in Table 5.2.

The results summarised in Table 5.3 show that after a 240 min incubation in Ca^{2+}-free Tris-Krebs solution it was not possible to measure a significant carbachol-stimulated breakdown of inositol phospholipid. The ^{3}H released from control tissue was 422 ± 34 Bq/kBq ^{3}H incorporated, which is more than twice that measured in the standard experiment with a total incubation time of 60 min. Analysis of variance indicated that there was no significant difference between any of the 4 groups ($P > 0.05$).

The effect of cytochalasin B (2×10^{-5}M) on carbachol-stimulated breakdown is shown in Table 5.4. Carbachol caused a significant breakdown of inositol phospholipid ($P < 0.05$) and cytochalasin B did not cause any significant alteration in the effect ($P > 0.05$).

Table 5.2 : The effect of cycloheximide on carbachol-stimulated inositol phospholipid hydrolysis

	INOSITOL PHOSPHOLIPID HYDROLYSED ABOVE CONTROL ($Bq/kBq^{3}H$ INCORPORATED BY PANCREAS)		
Cycloheximide ($5 \times 10^{-4}M$)	9 ± 13	(N = 10)	P > 0.05 (a)
Carbachol ($10^{-5}M$)	188 ± 22	(N = 10)	P < 0.05 (a)
Carbachol ($10^{-5}M$) + Cycloheximide ($5 \times 10^{-4}M$)	173 ± 11	(N = 10)	P > 0.05 (b)

^{3}H released from control tissue was 193 ± 13 $Bq/kBq^{3}H$ incorporated by pancreas (N = 10).

(a) = P (VS Control)

(b) = P (Interaction)

Table 5.3 : The effect of colchicine on carbachol-stimulated inositol phospholipid breakdown

		INOSITOL PHOSPHOLIPID HYDROLYSED (Bq/kBq ^{3}H INCORPORATED BY PANCREAS)		
Control		422 ± 34	(N = 5)	
Colchicine	(3×10^{-5}M)	380 ± 21	(N = 5)	P > 0.05 (a)
Carbachol	(5×10^{-6}M)	464 ± 32	(N = 5)	P > 0.05 (a)
Colchicine (3×10^{-5}M) + Carbachol (5×10^{-6}M)		369 ± 31	(N = 5)	P > 0.05 (a)

(a) =P (VS Control)

Table 5.4 : The effect of cytochalasin B on carbachol-stimulated inositol phospholipid breakdown

		INOSITOL PHOSPHOLIPID HYDROLYSED ABOVE CONTROL (Bq/kBq ^{3}H INCORPORATED BY PANCREAS)		
Cytochalasin B	(2×10^{-5}M)	22 ± 11	(N = 10)	P > 0.05 (a)
Carbachol	(5×10^{-6}M)	111 ± 22	(N = 10)	P > 0.05 (a)
Cytochalasin B (2×10^{-5}M) + Carbachol (5×10^{-6}M)		123 ± 16	(N = 10)	P > 0.05 (b)

^{3}H released from control tissue was 244 ± 17 Bq/kBq ^{3}H incorporated by the pancreas (N = 10).

(a) =P (VS Control)

(b) =P (Interaction)

The results in Fig. 5.1 show that CCK-8 and carbachol alone stimulated the hydrolysis of a significant proportion of the (^{3}H)-inositol phospholipid. However, simultaneous stimulation of both receptor classes produced an interaction since the amount of hydrolysis was less than the additive effect of each agonist acting individually. In fact, at 10^{-5}M and 10^{-6}M CCK-8 the presence of carbachol (10^{-3}M) produced little or no increase in the inositol phospholipid hydrolysed by CCK-8 alone. The results in Fig. 5.2 using another cholinergic agonist butyrylcholine (which acts as a partial agonist with respect to inositol phospholipid hydrolysis as shown in Chapter 7.3), showed a similar interaction effect.

The effect of duration of stimulation on inositol phospholipid hydrolysis is shown in Table 5.5. For each duration, agonist-induced hydrolysis of inositol phospholipid was measured in 12 samples and the amount of (^{3}H)-inositol phosphates present in unstimulated samples incubated for the same time was subtracted from that of the stimulated samples. Inositol phospholipid hydrolysis stimulated by CCK-8 increased significantly over the periods 15-30 min and 30-60 min ($P < 0.05$).

The effect of metabolic inhibitors on the carbachol-stimulated breakdown of inositol phospholipid and on the ATP content of tissue is shown in Tables 5.6 and 5.7 respectively. The relationship between the ATP content of tissue and inositol lipid hydrolysis in the presence of metabolic inhibitors is represented in Graph 5.3. None of the metabolic inhibitors used caused a significant alteration in inositol phospholipid hydrolysed in unstimulated tissue compared to control (in the absence of metabolic inhibitor) ($P > 0.05$). Although there is not a linear relationship between the concentration of ATP and inositol lipid hydrolysis, the

results show that 2,4 DNP and azide (10^{-2}M) which caused a significant decrease in lipid hydrolysis (Table 5.6) also produced a large significant decrease in ATP content (Table 5.7). Correspondingly, the inhibitors azide (10^{-3}M) and cyanide neither caused a significant decrease in ATP content nor a significant inhibition of carbachol-stimulated inositol lipid hydrolysis (Tables 5.7 and 5.6, respectively). The effects of oligomycin, however, did not agree with the above relationship between ATP content and inhibition of lipid hydrolysis, since oligomycin caused a very large inhibition of the breakdown of inositol lipid (similar to that for 2,4 DNP) but the ATP content was only reduced to 78% of control by this concentration of oligomycin.

Fig. 5.1 : The Effect of Simultaneous Addition of Carbachol and CCK-8 on the Hydrolysis of Inositol Phospholipid

Inositol phospholipid hydrolysis was measured as the release of (^{3}H)-inositol phosphates as described in materials and methods, 5.2. Pancreatic tissue was stimulated for 30 min with 10^{-3}M carbachol (●), CCK-8 (□) or both agonists combined (■). The hydrolysis of inositol phospholipid occurring in the unstimulated samples in each experiment was subtracted from that in stimulated samples and is expressed as Bq/kBq ^{3}H incorporated by pancreas. The values plotted are means ± S.E. of between 6 and 14 samples. The results for (^{3}H)-inositol phosphates present in control samples in each experiment were pooled and the mean ± S.E. calculated to be 23 ± 3 Bq/kBq^{3}H incorporated by pancreas (n = 12).

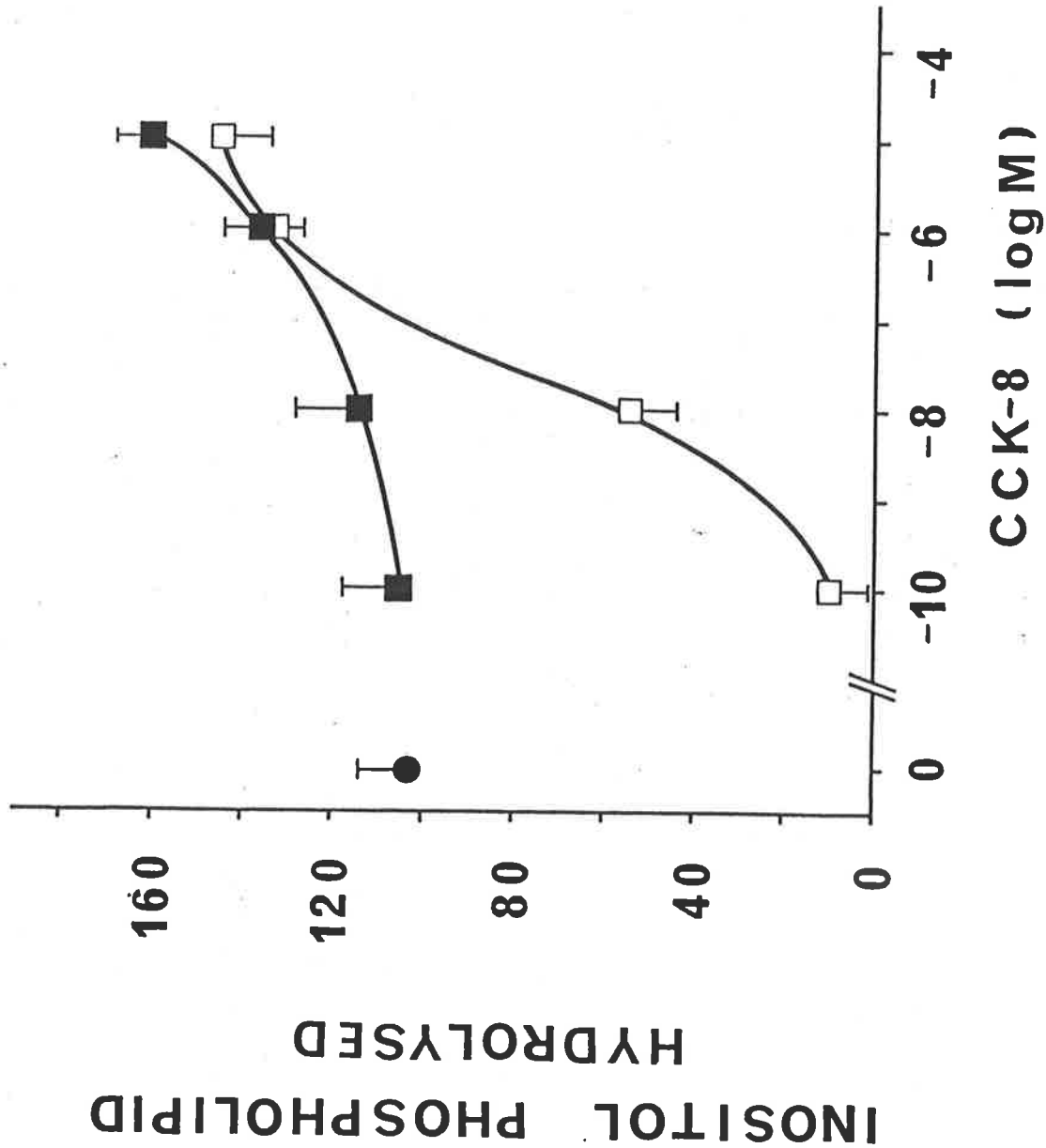


Fig. 5.1 : The Effect of Simultaneous Addition of Carbachol and CCK-8 on the Hydrolysis of Inositol Phospholipid

Fig 5.2 : The Effect of Simultaneous Addition of Butyrylcholine and CCK-8 on the Hydrolysis of Inositol Phospholipid

Inositol phospholipid hydrolysis was measured as the release of (^{3}H)-inositol phosphates as described in 5.2 Pancreatic tissue was stimulated for 30 min with 10^{-3}M butyrylcholine (△), CCK-8 (▲) or both agonists combined (□). Inositol phospholipid hydrolysis occurring above control is expressed as Bq/kBq ^{3}H incorporated by pancreas. The values plotted are means ± S.E. of between 6 and 11 samples. The results for (^{3}H)-inositol phosphates present in control samples in each experiment were pooled and the mean ± S.E. calculated to be 18 ± 2Bq/kBq ^{3}H incorporated by pancreas (n = 11).

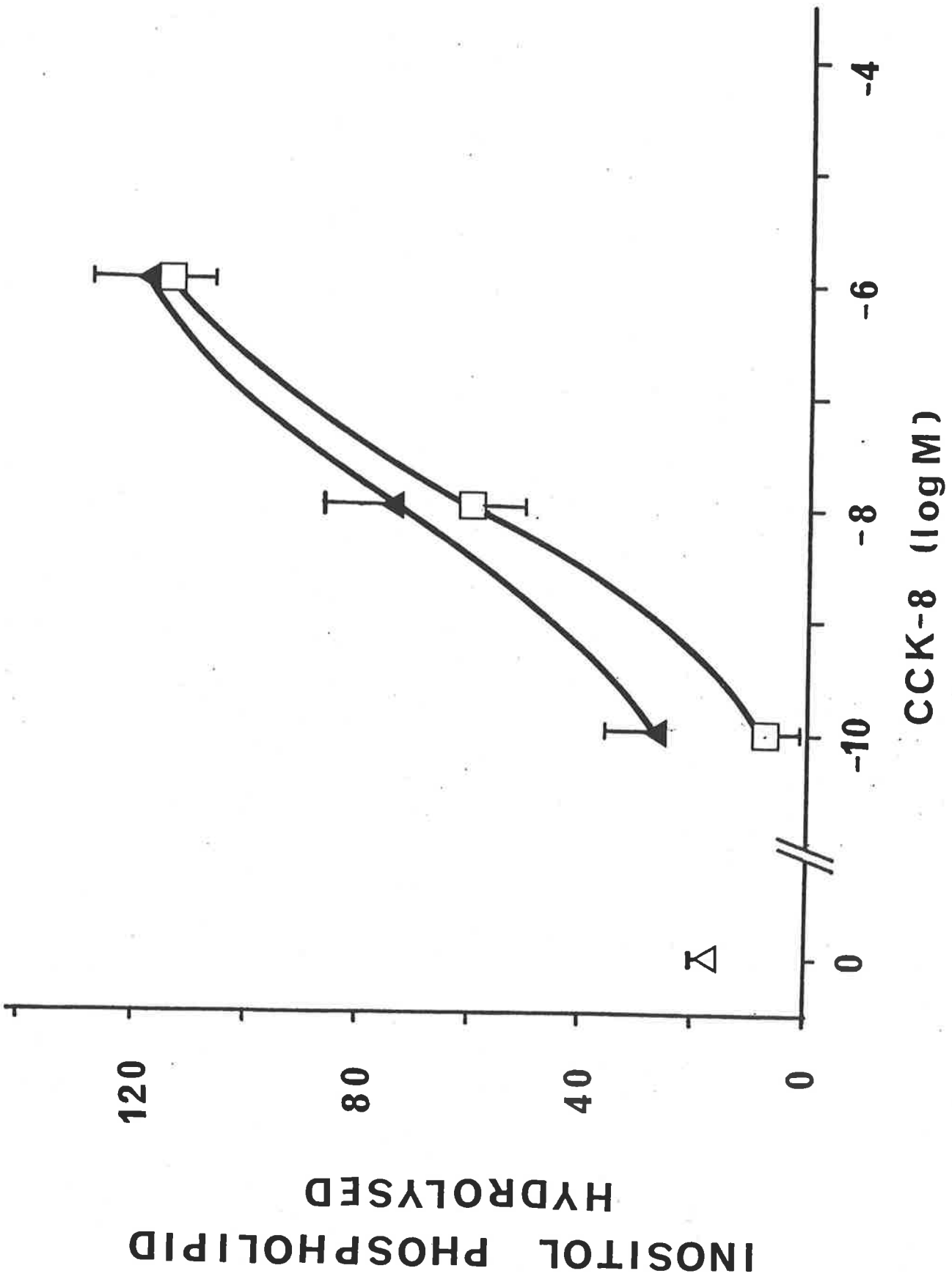


Fig. 5.2 : The Effect of Simultaneous Addition of Butyrylcholine and CCK-8 on the Hydrolysis of Inositol Phospholipid

Table 5.5 : The effect of duration of stimulation by CCK-8 (10^{-6}M) on inositol phospholipid hydrolysis

Duration of incubation (min)	Inositol phospholipid hydrolysed above control (Bq/kBq H incorporated by pancreas)	
15	90 ± 7	(N = 12)
30	144 ± 7	(N = 12)
60	236 ± 17	(N = 12)

The (^{3}H)-inositol present in control tissue incubated for 15, 30 and 60 min was : (Bq/kBq ^{3}H incorporated by pancreas) 25 ± 3 (n = 12), 20 ± 2 (n = 12), 15 ± 2 (n = 11), respectively.

Table 5.6 : The effect of metabolic inhibitors on the carbachol-stimulated hydrolysis of inositol phospholipid

			INOSITOL PHOSPHOLIPID HYDROLYSED ABOVE CONTROL (Bq/kBq ^{3}H INCORPORATED BY PANCREAS)		
Carbachol	(10^{-5}M)		119 ± 10	(N = 48)	(a)
Oligomycin Carbachol	(10^{-6}M) (10^{-5}M)	+	8 ± 18	(N = 12)	(b)
2,4 Dinitrophenol Carbachol	(10^{-3}M) (10^{-5}M)	+	3 ± 10	(N = 5)	(b)
Sodium azide Carbachol	(10^{-2}M) (10^{-5}M)	+	42 ± 8	(N = 13)	(b)
Sodium azide Carbachol	(10^{-3}M) (10^{-5}M)	+	98 ± 3	(N = 5)	(c)
Cyanide Carbachol	(10^{-3}M) (10^{-5}M)	+	93 ± 18	(N = 16)	(c)

(a) = P (VS Control) < 0.05

(b) = P (VS Carbachol) < 0.05

(c) = P (VS Carbachol) > 0.05

^{3}H released from control tissue = 226 ± 10 Bq/kBq ^{3}H incorporated by the pancreas (N = 42).

Table 5.7 : The effect of metabolic inhibitors on the ATP content of unstimulated pancreatic tissue slices

		(ATP) % CONTROL		
Oligomycin	(10^{-6}M)	78 ± 13	(N = 6)	(b)
2,4 Dinitrophenol	(10^{-3}M)	11 ± 5	(N = 10)	(a)
Sodium azide	(10^{-2}M)	15 ± 2	(N = 9)	(a)
Sodium azide	(10^{-3}M)	83 ± 15	(N = 9)	(b)
Cyanide	(10^{-3}M)	88 ± 15	(N = 6)	(b)

(a) = P (VS Control) < 0.05

(b) = P (VS Control) > 0.05

Fig. 5.3 : The ATP Content of Pancreatic Tissue in the Presence of Metabolic Inhibitor vs the Carbachol-Stimulated Hydrolysis of Inositol Phospholipid

(●) 2,4 DNP (10^{-3}M)

(□) Azide (10^{-2}M)

(■) Azide (10^{-3}M)

(○) Cyanide (10^{-3}M)

(▲) Oligomycin (10^{-6}M)

Each point plotted was obtained from values shown in Tables 5.6 and 5.7. Inositol phospholipid hydrolysed above control is expressed as Bq/kBq ^{3}H incorporated by the pancreas.

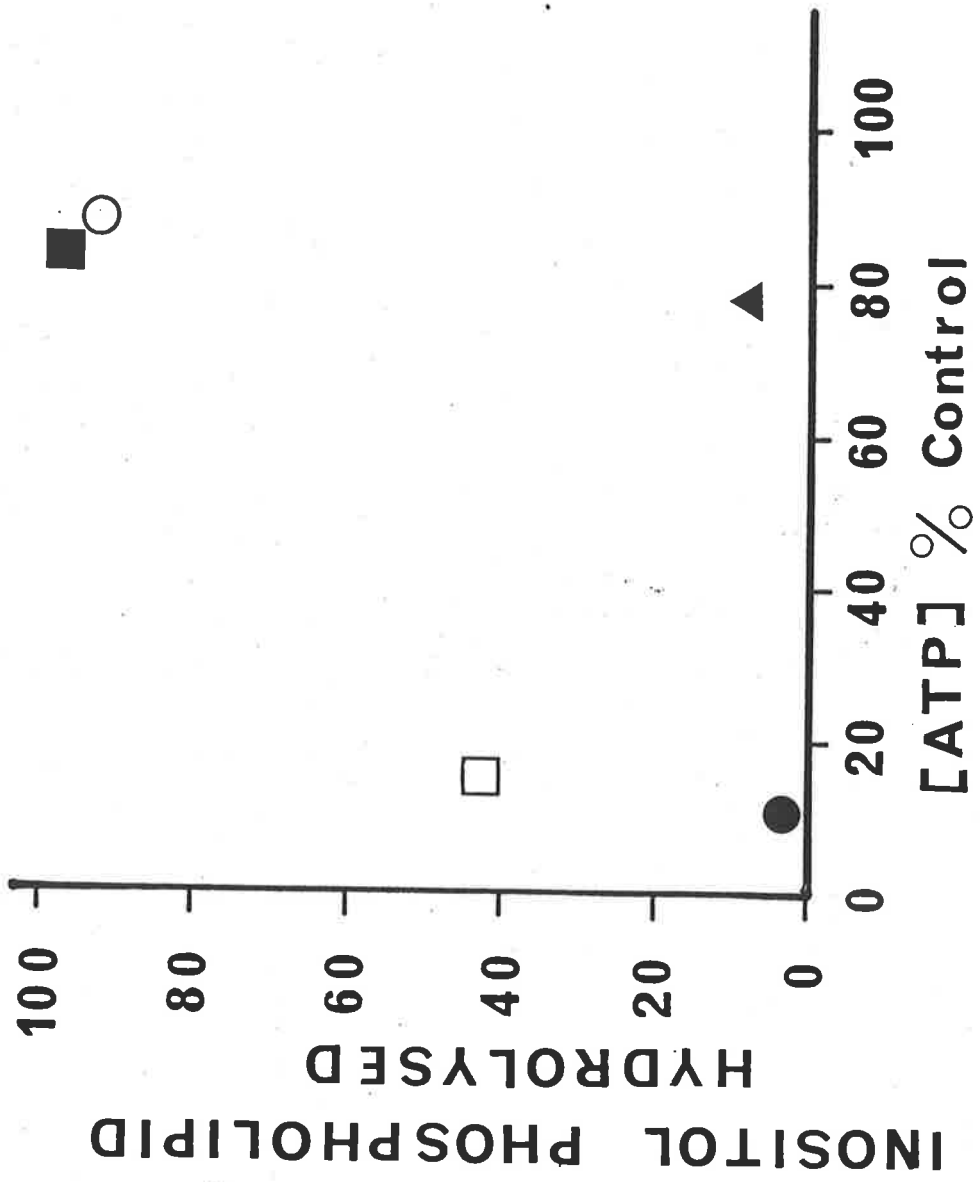


Fig. 5.3 : The ATP Content of Pancreatic Tissue in the Presence of Metabolic Inhibitor vs the Carbachol-Stimulated Hydrolysis of Inositol Phospholipid

5.4 : DISCUSSION

Activation of muscarinic or CCK receptors in the pancreas leads to an increase in the Na^+ permeability of the cell membrane. The alteration in Na^+ permeability can occur in the absence of extracellular Ca^{2+} (Nishiyama and Petersen, 1975; Iwatsuki and Petersen, 1977a). This raised the possibility that Na^+ influx may be the event which triggered inositol phospholipid breakdown in mouse pancreas. In the present study, the results obtained in the presence and absence of extracellular Na^+, demonstrate that the agonist-stimulated hydrolysis of inositol phospholipid is independent of receptor-stimulated Na^+ influx through the plasma membrane. The results of Calderon et. al. (1980) obtained using rat pancreatic fragments showed that Na^+ replacement with Li^+ stimulated the incorporation of (^{14}C)-acetate into inositol phospholipid. In the presence of Na^+, agonists caused an increase in inositol lipid synthesis whereas when Na^+ was removed, no increase in (^{14}C)-acetate incorporation above that caused by Na^+ removal alone occurred (Calderon et. al., 1980). The difference between the results of the present study, showing no effect of Na^+ removal on inositol lipid hydrolysed in control or stimulated tissue, and those of Calderon et. al. (1980) demonstrates that the synthesis pathway of the phosphoinositide cycle can be modified with no effect on inositol lipid breakdown and confirms that only results of the breakdown of this lipid can be used for a valid interpretation of the role of inositol lipid breakdown in cell function.

The agonist-stimulated breakdown of inositol lipid does not depend on the synthesis of a labile protein since cycloheximide, although inhibiting protein synthesis, had no effect on inositol phospholipid hydrolysis (Table 5.2). The only reported effects of cycloheximide on the phosphoinositide cycle in the pancreas are those by Farese et. al. (1981a,1982) in which the

secretagogue-stimulated increase in net phosphatidate content was inhibited by cycloheximide (10^{-4}M). Although the breakdown of PtdIns in these studies by Farese was not measured, it was suggested that the effect on phosphatidic acid content did not occur as a result of inhibition of PtdIns breakdown since the agonist-stimulated decrease in net PtdIns content was unaffected by cycloheximide. Since cycloheximide has been found to inhibit a membrane-bound diacylglycerolkinase from adrenal gland (Farese et. al., 1981 b), the site of action of cycloheximide may be at the conversion of diacylglycerol to phosphatidate in the pancreas (Farese et. al., 1982). Whatever its site of action, the results of the present study provide clear evidence that cycloheximide does not alter inositol lipid hydrolysis and further demonstrates the advantage of the assay used. The lack of dependency of inositol lipid hydrolysis on protein synthesis is likely to be common to a number of cell types since a recent study by Creba et. al. (1983) showed no effect of cycloheximide on PtdIns-4,5P_2 breakdown in rat hepatocytes.

The measurement of phosphoinositide turnover by either synthesis or total lipid content can be affected by agents, such as cycloheximide and Na^+, which may act at a step in the synthesis pathway. Measurement of the (^{3}H)-labelled products released from inositol lipid avoids the problem of the actions of agents such as these.

The lack of inhibitory effect of disruption of microfilaments on inositol lipid hydrolysis indicates that there is no requirement of lipid breakdown for an intact cytoskeletal network (which is necessary for transport and release of secretory products from the pancreas) and so indicates that this lipid hydrolysis is not dependent on these later events in pancreatic secretion. It was not possible to determine the effect of disruption of microtubules on inositol lipid hydrolysis using colchicine since the 210 min

pre-incubation with this agent allowed a large basal breakdown of lipid which resulted in a large pool of free (^{3}H)-inositol present. Under these conditions it is possible that both the breakdown and re-synthesis of inositol lipid occurred and that some of the released (^{3}H)-inositol was re-incorporated, causing difficulty in measuring the breakdown alone. This problem could have been overcome by using a different microtubule inhibitor, vinblastine, which does not require as long an incubation time as colchicine. Vinblastine disrupts microtubules and totally inhibits amylase secretion in mouse pancreatic fragments after only a 90 min pre-incubation, since the rate of vinblastine uptake is about 10 times greater than that of colchicine (Williams and Lee, 1976).

The results of these studies indicate that inositol lipid hydrolysis is closely associated with receptor activation since it occurs independently of a number of cellular events in stimulus-secretion coupling. To investigate the relationship between receptor occupation and inositol lipid the effect of simultaneous stimulation of different receptors was examined.

If as suggested by Kirk et. al. (1981) the degree of inositol phospholipid hydrolysis is proportional to the degree of receptor occupancy then simultaneous activation of two different classes of receptors should cause summation. The results shown in Figs. 5.1 and 5.2 demonstrate that the amount of inositol phospholipid hydrolysis produced by the combination of two agonists acting on different receptors was less than would be expected if they acted via independent mechanisms to stimulate hydrolysis. It is not possible to determine

how the interaction between occupation of the cholecystokinin and muscarinic receptors occurs. However, it is not due to depletion of (^{3}H)-labelled phosphoinositide since the amount of hydrolysis occurring at 30 min in the presence of CCK-8 and carbachol (or butyrylcholine) was not the maximal phosphoinositide hydrolysis possible. As shown in Table 5.5, the degree of hydrolysis following a 60 min incubation with CCK-8 (10^{-6}M) was considerably greater than that obtained at 30 min either in the presence of CCK-8 alone or in combination with carbachol or butyrylcholine (Figs. 5.1 and 5.2).

Since phosphoinositide depletion does not appear to cause the interaction effect it is possible that the phospholipase C responsible for hydrolysis of PtdIns-4,5P_2 is limited. At high concentrations of agonists the enzymes available may be maximally activated. Alternatively, there may be some, as yet, undefined interaction between receptors such that occupation of one class of receptors modulates the binding of agonist to another class of receptors. Therefore the reduced hydrolysis of inositol phospholipid may be due to reduced binding of agonists. These possibilities require investigation.

A recent study by Brown et. al. (1984) using rat cerebral cortical slices reported that the effects of agonists acting at different receptors on the hydrolysis of inositol phospholipid were additive. This result suggested that the degree of phosphoinositide hydrolysis is proportional to receptor occupancy. The reason for the difference in results using pancreas and brain slices is not known. One possible explanation could be that in brain slices the different classes of

agonists act on separate cell populations, and so have access to individual mechanisms for activation of hydrolysis.

At the time that these results were obtained, the metabolic-requiring step in the carbachol-stimulated hydrolysis of inositol phospholipid (Table 5.7) was difficult to identify since the proposed enzymic reaction responsible for hydrolysis was by the action of a phospholipase C (Michell, 1975) which does not require ATP. A possible explanation at that time was that ATP depletion caused a sufficient disruption of the intracellular environment to inhibit phospholipase C. It was not until Michell et. al. (1981) proposed that the breakdown of PtdIns-4,5P_2 may be the initial agonist-stimulated event, that another explanation for the ATP requirement of inositol lipid hydrolysis found in this study was available.

When pancreatic tissue is labelled *in vivo* with *myo*-(2-^{3}H)-inositol, PtdIns, PtdIns-4P and PtdIns-4,5P_2 are all radioactively labelled with the majority of the radioactivity being in PtdIns (Table 4.1). If PtdIns-4,5P_2 was the initial lipid hydrolysed on receptor stimulation, then the (^{3}H)-PtdIns-4P and (^{3}H)-PtdIns would be phosphorylated to replace PtdIns-4,5P_2 to enable a continued loss of (^{3}H)-PtdIns-4,5P_2 . This phosphorylation required ATP, therefore a reduction in ATP content will reduce the production of (^{3}H)-PtdIns-4,5P_2 and thereby inhibit the breakdown of this lipid and the increase in acid-soluble radioactivity, as measured in this assay.

The ATP content and carbachol-stimulated inositol lipid hydrolysis of pancreatic tissue were both reduced markedly by 2,4DNP and sodium azide (10^{-2}M) whereas sodium azide (10^{-3}M) and cyanide caused little reduction

in either ATP content or lipid hydrolysis. Cyanide and azide inhibit oxidative phosphcrylation by inhibiting the electron transfer between cytochrome ($a + a_3$) and O_2 whereas 2,4DNP and oligomycin act to uncouple electron transport and phosphorylation. However, unlike the other metabolic inhibitors used, the effect of oligomycin on inositol lipid hydrolysis was unrelated to its effect on ATP levels. The large inhibition of inositol lipid breakdown by oligomycin was not due to a correspondingly large reduction in ATP content. This suggested that perhaps oligomycin has other sites of action through which phosphoinositide breakdown is altered.

Oligomycin has at least two sites of action, one at the mitochondria to inhibit the formation of ATP (Lardy et. al., 1958) and another at the cell membrane to inhibit active transport and its associated ATPase activity. This effect on the $(Na^+ + K^+)$-ATPase has been demonstrated in red blood cells and kidney cortex slices (Whittam et. al., 1964), brain microsomes (Järnefelt,1962) and the electric organ of the electric eel (Glynn, 1963). A comparison of the effect of 2,4DNP (10^{-3}M) and oligomycin (10^{-6}M) showed that 2,4DNP had no effect on the $(Na^+ + K^+)$ ATPase whereas oligomycin inhibited almost 75% of the activity (Glynn, 1963).

Oligomycin exerts its inhibitory effect on $(Na^+ - K^+)$ by inhibiting the hydrolysis of ATP at the cell membrane and not by causing a decrease in ATP (Whittam et. al., 1964). Since the inhibition of inositol lipid hydrolysis by oligomycin is not accompanied by a decrease in ATP, perhaps the effect of oligomycin on this lipid response is a direct result of inhibiting the $(Na^+ + K^+)$ ATPase. The effect of ouabain on agonist-stimulated inositol lipid breakdown should be examined to determine if there is an association between this lipid response and the $(Na^+ + K^+)$ ATPase since ouabain inhibits this enzyme. A consequence of inhibition

of the ($Na^+ + K^+$) ATPase is an increase in the leakage of Na^+ into the cells and of K^+ out of the cells. Since the hydrolysis of inositol lipid is not dependent on Na^+ (Table 5.2) it is unlikely that the increase in intracellular Na^+ would explain the inhibition by oligomycin of inositol lipid breakdown, however, the effect of an alteration in K^+ concentration on inositol lipid hydrolysis is not known.

Other possible effects of oligomycin may be to inhibit the phospholipase responsible for the phosphoinositide breakdown or PtdIns kinase and PtdIns-4P kinase which act in the synthesis of PtdIns-4,5P_2. There is no evidence for the effect of oligomycin on these enzymes. If the formation of (^3H)-PtdIns-4,5,P_2 is inhibited in the presence of oligomycin this would indicate an effect on the kinase(s) whereas if the amount of (^3H)-PtdIns-4,5P_2 did not decrease in the presence of agonist and oligomycin then an effect on the phospholipase would be likely. These actions of oligomycin should be tested in order to clarify the question of how this agent which does not reduce ATP levels significantly, alters phosphoinositide breakdown.

The other studies which have not shown an inhibition of agonist-stimulated breakdown of inositol phospholipid by metabolic inhibitors did not report ATP levels (Michell, 1975; Miller and Kowel, 1981) and it is possible that the ATP content of the cells was not markedly reduced. The majority of studies of the effect of metabolic inhibitors on ATP production have used isolated mitochondria with only a few studies using mammalian tissue fragments or cells. It is important that ATP content of tissue is measured since the concentrations of metabolic inhibitors which effectively reduce ATP production in isolated mitochondria are not necessarily effective in tissue or isolated cells. The concentration and period of incubation with metabolic inhibitor will depend on the permeability of the particular cell for the agent.

The lack of effect of oligomycin (10^{-6}M), cyanide (10^{-3}M) and azide (10^{-3}M) on cellular ATP content was not expected. However, oligomycin takes a period of hours to penetrate cells (Michelangeli, 1979) and the 40 min incubation used in this study may not have been long enough. Cyanide (10^{-3}M) effective reduces ATP content in pancreatic fragments after a 90 min incubation (Williams and Lee, 1976) whereas in this study, the 40 min incubation may not have allowed effective penetration of the agent. Azide (2×10^{-3}M) reduced ATP content to 40% of control in isolated mouse lymphocytes after a 5 min incubation (Pozzan et. al., 1980), however, there are no reported effects of azide on the ATP content of pancreatic tissue and it is likely that 10^{-3}M azide does not penetrate pancreatic fragments as readily as isolated cells such as lymphocytes.

The results of the present study are in agreement with the inhibitory effect of 2,4DNP on the decrease in PtdIns in mouse pancreas (Hokin, 1974). A study by Poggioli et. al. (1983) showed that antimycin A, an inhibitor of mitochondrial respiration, inhibited both the agonist-stimulated decrease in (^{32}P)-PtdIns and the increase in (^{32}P)-phosphatidate synthesis in rat parotid cells, which is in agreement with the energy requirement for phosphoinositide breakdown in pancreas as found in the present study.

In conclusion, inositol phospholipid hydrolysis appears to be an early event in activation of the mouse exocrine pancreas since it is not linked to receptor occupation via an increase in Na^+, or synthesis of protein, nor via the processes involved in exocytosis since an intact cytoskeletal system was not required. The results suggest a close link

with receptor occupation and indicate that the binding of agonists to different receptors activates a common mechanism which limits the degree of inositol phospholipid. The interactive effect between different classes of receptors requires further investigation.

This study shows that agents reported to alter the synthesis of inositol phospholipid in pancreas have no effect on the breakdown of this lipid and so further demonstrates that synthesis results can not be used as an indirect measure of inositol lipid breakdown. The assay used in this study has the major advantage of measuring the (^{3}H)-inositol-labelled products released from inositol lipid and so is unaffected by agents which may alter synthesis reactions. The ATP requirement for agonist-stimulated breakdown of inositol lipid provides information regarding the possible pathway for lipid hydrolysis, supporting the proposal that PtdIns-4,5P_2 breakdown and not PtdIns may be the initial lipid hydrolysed. The close linking of the inositol phospholipid response to receptor occupation was in agreement with the idea that the lipid response may be involved in controlling Ca^{2+} entry to the cytoplasm and lead to the investigation of the relationship between Ca^{2+}-mobilization, inositol phospholipid hydrolysis and the Ca^{2+}-dependent response of secretion, which will be discussed in the following chapters.

CHAPTER 6

THE ROLE OF CALCIUM IN THE HYDROLYSIS OF INOSITOL PHOSPHOLIPID IN EXOCRINE PANCREAS

6.1 : INTRODUCTION

An essential argument in support of the proposal that inositol phospholipid hydrolysis precedes Ca^{2+} gating rather than occurs as a result of Ca^{2+} entering the cytosol would be inositol phospholipid breakdown is not dependent on an increase in the intracellular Ca^{2+} concentration. Despite the many studies that have failed to show an effect of the Ca^{2+} ionophore A23187 on PtdIns breakdown (Jones and Michell, 1975; Fain and Berridge, 1979; Billah and Michell, 1979) the question of the Ca^{2+}-dependency of inositol lipid breakdown has become confused by recent studies on neutrophils (Cockcroft et. al., 1981), platelets (Bell and Majerus, 1980; Lapetina et. al., 1981) and iris smooth muscle (Akhtar and Abdel-Latif, 1980) which have demonstrated stimulation of breakdown of PtdIns or PtdIns-4,5P_2 by Ca^{2+} ionophores.

Although Hokin-Neaverson (1977) stated that the breakdown of PtdIns in pancreas was not stimulated by increasing the intracellular Ca^{2+} concentration with ionophore A23187, Farese et. al. (1980) have shown a decrease in the total PtdIns mass of pancreatic fragments in response to the calcium ionophore. The results of Farese et. al. (1980) have been used to support the proposal that PtdIns hydrolysis in the pancreas follows, rather than precedes, Ca^{2+} entry (Hawthorne, 1982) even though inositol phospholipid breakdown was not measured. Because clarification of the relationship between inositol phospholipid hydrolysis and Ca^{2+}-mobilization is critical to an understanding of the role of inositol lipid breakdown in stimulus-secretion coupling, the involvement of intracellular and extracellular Ca^{2+} in secretagogue-induced inositol lipid breakdown was investigated in this study.

As described in the previous chapter, the breakdown of inositol phospholipid in the exocrine pancreas is stimulated by carbachol and CCK-8 in the absence of extracellular Ca^{2+}. Since these cells have intracellular stores from which Ca^{2+} can be released by agonists (Williams and Chandler, 1975; Petersen and Ueda, 1976; Schulz, 1980 b) this result does not eliminate a role for Ca^{2+} as the activator of inositol lipid breakdown in the pancreas.

In an attempt to determine whether the breakdown of inositol phospholipid in pancreas was activated by an increase in the intracellular Ca^{2+} concentration, the effect of A23187 on the breakdown of inositol phospholipid, rather than its effect on a decrease in inositol lipid mass (Farese et. al., 1980) (which measures the net result of breakdown and synthesis) was investigated.

In addition to investigating whether the hydrolysis of inositol phospholipid was activated by Ca^{2+}, the dependence of the agonist-stimulated breakdown of inositol lipid on Ca^{2+} released from stores was examined. When pancreatic fragments are incubated in Ca^{2+}-free solutions, the addition of secretagogues causes an increase in amylase secretion which decreases within 10 min, due to depletion of intracellular Ca^{2+} (Williams and Chandler, 1975; Kanno and Nishimura, 1976). If the agonist-stimulated hydrolysis of inositol phospholipid increases with time after depletion of Ca^{2+} stores, this would indicate that the lipid response was not dependent on Ca^{2+}. This was investigated in the present study.

Another method to investigate the dependence of the inositol lipid breakdown on stored Ca^{2+} would be to use an agent which inhibits the

release of Ca^{2+} from intracellular stores. 8-(N,N-Diethylamino)-octyl-3,4,5-trimethoxybenzoate-HCL(TMB-8) has been used as an intracellular Ca^{2+} antagonist (Charo et. al., 1976; Rittenhouse-Simmons and Deykin, 1978; Shaw, 1981). The mode and site of action of this agent have not been defined, although it has been proposed to immobilize Ca^{2+} at the intracellular membrane storage sites (Rittenhouse-Simmons and Deykin, 1978). TMB-8 was used in the present study in an attempt to study the role of intracellular Ca^{2+} stores in the activation of inositol lipid hydrolysis.

Studies of a number of tissues have shown different effects of removal of extracellular Ca^{2+} on agonist-stimulated inositol phospholipid breakdown. Although no alteration in PtdIns breakdown was found in blowfly salivary gland (Fain and Berridge, 1979) and parotid gland (Jones and Michell, 1975; Jones et. al., 1979), studies in lymphocytes (Hui and Harmony, 1979) and hepatocytes (Kirk et. al., 1981) showed a significant reduction in PtdIns breakdown on Ca^{2+} removal, while in polymorphonuclear leukocytes (Cockcroft et. al., 1981) this lipid response was abolished.

No study had been made of the effect of Ca^{2+} omission on inositol phospholipid breakdown in mouse pancreas although Farese et. al. (1980) showed that the carbachol-stimulated decrease in net PtdIns mass in rat pancreas was abolished by Ca^{2+} removal. The only other studies of Ca^{2+} omission in the pancreas measured the synthesis of PtdIns (Hokin, 1966; Calderon et. al., 1979, 1980). The breakdown of inositol phospholipid in mouse pancreas incubated in the presence and absence of extracellular Ca^{2+} was examined in the present study.

It was found that the hydrolysis of inositol phospholipid stimulated by both muscarinic and peptide receptor agonists was potentiated when Ca^{2+} was present in the extracellular fluid. Even though an increase in intracellular Ca^{2+} with ionophore may not stimulate inositol phospholipid breakdown a possible Ca^{2+}-requirement for the activation of this lipid response by agonist may exist. This raised the question: How does Ca^{2+} potentiate inositol phospholipid breakdown? An investigation of this question was important since it could provide a clearer understanding of the activation process of inositol lipid hydrolysis. This study examined whether the effect of Ca^{2+} was on the inside of the cell (for example, caused by the movement of Ca^{2+} through the plasma membrane) or on the outside of the cell (by binding to specific Ca^{2+} membrane-binding sites). To do this, the effects of various 'calcium antagonists' on agonist-stimulated inositol phospholipid breakdown were examined. Tetracaine, methoxyverapamil (D600), neomycin, and the metal cations lanthanum (La^{3+}) and manganese (Mn^{2+}) can act as Ca^{2+} antagonists since they inhibit transmembrane Ca^{2+} fluxes (Marier et. al., 1978; Kohlardt et. al., 1972; Goodman, et. al., 1974; Seeman, 1872; Putney et. al., 1978; Putney, 1981; Aub et. al., 1982) and/or interfere or compete with Ca^{2+} for the externally located membrane storage sites (Seeman, 1972; Weiss, 1974; Langer et. al., 1974).

In summary, the examination of the role of Ca^{2+} in inositol lipid breakdown resulted in the following findings:

1. The agonist-stimulated breakdown of inositol phospholipid occurs in the absence of extracellular Ca^{2+}.

2. The inositol lipid response was not activated by Ca^{2+} introduced into the cells with ionophore A23187.

3. The lipid breakdown stimulated by agonists was not dependent on the release of Ca^{2+} from intracellular stores since it occurred when this cellular Ca^{2+} had been depleted.

4. The putative intracellular calcium antagonist, TMB-8 could not be used as an inhibitor of the release of Ca^{2+} from stores since it acted to block the muscarinic receptor.

5. The presence of Ca^{2+} in the extracellular fluid potentiated both basal and agonist-stimulated lipid breakdown.

6. The potentiating effect of Ca^{2+} was not due to the movement of Ca^{2+} through the plasma membrane since neomycin had no effect on inositol lipid breakdown.

7. Two sites of potentiation by Ca^{2+} exist on the external surface of the cell membrane.

   One site is associated with potentiation of unstimulated inositol lipid hydrolysis and manganese can replace Ca^{2+} at this site which is lanthanum insensitive.

   The second site is associated with potentiation of agonist-stimulated hydrolysis of inositol lipid. Lanthanum and manganese compete with Ca^{2+} at this site to remove the potentiation effect.

6.2 : MATERIALS AND METHODS

In all experiments using pancreatic slices, tissue was labelled with *myo*-(2-^{3}H) inositol and inositol phospholipid hydrolysis measured

as described in 3.2. Amylase secretion was measured as described in 3.7 (a). In all experiments where tissue was incubated in the presence of agonist, A23187 or calcium antagonist, the appropriate controls were included so that the inositol lipid hydrolysed and amylase secreted in stimulated tissue could be calculated above basal values in unstimulated tissue.

6.2 (a) Inositol Phospholipid Hyrdolysis and Intracellular Ca^{2+}

These studies examined the role of intracellular Ca^{2+} in two ways. Firstly, intracellular Ca^{2+} was increased with the ionophore A23187 and secondly, intracellular Ca^{2+} was depleted by prolonged incubation of tissue in Ca^{2+}-free media.

To investigate the effect of increasing the intracellular Ca^{2+} concentration on amylase secretion and inositol lipid hydrolysis in pancreatic slices, the tissue was washed for 2 x 15 min in a solution similar to the Krebs solution but with the $MgCl_2$ and $CaCl_2$ omitted. A 5 min incubation in this Krebs solution containing ionophore A23187 (10^{-6}M) was followed by a 30 min incubation of tissue pieces in Krebs solution containing $CaCl_2$ but no $MgCl_2$ in the presence of A23187 (10^{-6}M). Ionophore A23187 was dissolved in ethanol, and ethanol (0.1% final concentration) was added to control samples for these experiments. Inositol phospholipid hydrolysis amylase secretion were measured.

To measure inositol lipid breakdown in pancreatic acini, mice were injected intraperitoneally with 1.22MBq of *myo*-(2-^{3}H) inositol using the procedure described in 3.2. Acini were

prepared and incubated for 30 min in the Hepes-buffered solution containing 2mM *myo*-inositol, as described in 3.6. The acini suspension was then centrifuged at 50 x $\bar{g}$ at 22°C for 4 min. Acini were resuspended in the Hepes-buffered solution and 0.8 ml aliquots placed in incubation vials containing 0.2 ml of Hepes-buffered solution and A23187 (10^{-6}M) or carbachol (10^{-4}M). Acini were incubated with gentle shaking at 60 cycles/min and gassed with 100% O_2 for 30 min. The incubation was terminated by fast freezing in a dry ice/alcohol bath and the samples assayed for inositol lipid breakdown using the technique described in 3.2.

Amylase secretion stimulated by A23187 (10^{-6}M) and carbachol (10^{-6}M) in acini incubated in Hepes-buffered solution, as above, was measured using the method described in 3.7 (b). (10^{-6}M carbachol was used instead of 10^{-4}M, since this lower concentration produced maximal amylase secretion).

To examine the effect of depletion of intracellular Ca^{2+} stores on inositol lipid hydrolysis, pancreatic fragments were washed for 2 x 15 min in Ca^{2+}-free Krebs solution and the incubated in Ca^{2+}-free Krebs solution with CCK-8 (10^{-8}M). The incubations were terminated at 5, 15 and 30 min and inositol lipid hydrolysis measured. The effect of this incubation procedure on cellular Ca^{2+}-stores was examined by incubating the tissue as above, and measuring CCK-8-stimulated amylase secretion at 5, 15 and 30 min.

In order to make a conclusion about the ability of CCK-8 to stimulate inositol phospholipid hydrolysis at a time when Ca^{2+} stores were depleted it was necessary to determine which of the two following events (causing an increase in phosphoinositide hydrolysis with time) occurred.

One explanation for a time-dependent increase in hydrolysis would be due to an increase in agonist-receptor interaction with time, such that the lipid response increased as an immediate result of receptor occupation at each time point. The second possible explanation would be that the hydrolysis of inositol phospholipid occurred as a secondary effect to activation of a process at the beginning of the incubation. For example, inositol phospholipid hydrolysis could be activated by the small amount of Ca^{2+} released from stores at the start of the incubation in Ca^{2+}-free Krebs solution. If this were the case, then the increase in lipid hydrolysis at 15 and 30 min could be due to a continued increase in enzyme-substrate interaction occurring as a result of the initial receptor activation when the incubation began. Therefore, a conclusion of lack of dependency of phosphoinositide hydrolysis on stored Ca^{2+} would be incorrect. To distinguish between these two alternatives atropine was used.

If inositol lipid hydrolysis occurs as an immediate result of receptor activation at each time point then addition of atropine at any time during the incubation will cause cessation of breakdown. If atropine addition does not cause cessation

of breakdown this would indicate that initial receptor activation stimulates inositol lipid hydrolysis via a mechanism which once activated does not require further receptor occupation. The latter possibility is unlikely since it would not provide the lipid response with a mechanism for 'switching off'. However, the two possibilities were examined using the following method.

Myo-(2-^{3}H) inositol-labelled pancreatic pieces were washed for 30 min in Ca^{2+}-free Krebs solution and then incubated in Ca^{2+}-free Krebs solution in the presence of carbachol (10^{-4}M). The incubation of 1/3 of the samples was terminated at 15 min while the remaining samples were incubated for a further 15 min. Atropine (10^{-4}M) was added to 1/2 of these remaining samples at 15 min. The appropriate controls were made, that is a control for 15 min and 30 min and a control for 30 min with atropine (10^{-4}M) added at 15 min.

To examine the effect of TMB-8 on inositol lipid hydrolysis pancreatic tissue slices were washed for 25 min in Ca^{2+}-free Tris-Krebs solution and then incubated with TMB-8 for 35 min. Inositol phospholipid hydrolysis was stimulated by adding agonists for the final 30 min of the incubation.

In the studies which investigated the effect of an agent or incubation procedure on both inositol phospholipid hydrolysis and amylase secretion it was necessary to use a Ca^{2+}-containing Krebs solution which was bicarbonate-buffered for both studies

since amylase secretion could not be stimulated in tissue incubated in Tris-Krebs solution, as described in 3.9 (a).

6.2 (b) Inositol Phospholipid Hydrolysis and Extracellular Ca^{2+}

The effect of Ca^{2+} on inositol phospholipid breakdown was investigated by measuring this response in pancreatic fragments incubated in Ca^{2+}-free Tris-Krebs solution and in Tris-Krebs solution, in the presence of a range of concentrations of CCK-8, carbachol or bethanechol.

To investigate the potentiation by Ca^{2+} of inositol phospholipid hydrolysis, the calcium antagonists tetracaine, methoxyverapamil (D600), neomycin, and metal cations La^{3+} and Mn^{2+} were used. *Myo*-(2-^{3}H) inositol-labelled pancreatic slices were incubated in Tris-Krebs solution or Ca^{2+}-free Tris-Krebs solution in the presence and absence of agonist and/or calcium antagonist for 30 min; inositol phospholipid hydrolysed was then measured.

Since the experiments using La^{3+} and Mn^{2+} required Tris-buffered Krebs solution (Ca^{2+} and Ca^{2+}-free) to avoid precipitation of the metal ion with HCO_3, the experiments using D600 and neomycin also used Tris-buffered Krebs solutions so that all results with Ca^{2+}-antagonists could be compared. In experiments using neomycin (10^{-2}M) or $MnCl_2$ (10^{-3}M) it was necessary to adjust the pH of the Tris-Krebs solution containing these agents to pH 7.4 at 37^{o}C; calcium antagonists used at other concentrations did not alter the pH of the Tris-Krebs in which

they were dissolved. D600 was dissolved in ethanol, an equivalent amount (0.1%,v/v) of ethanol was added to control samples.

To avoid the possibility of the metal cations causing release of acetycholine from nerve terminals, atropine (10^{-5}M) was present in experiments using CCK-8; in experiments using carbachol the addition of atropine (10^{-5}M) to tissue incubated with metal cation alone did not cause any significant difference in inositol lipid hydrolysed ($P > 0.05$).

To examine the effect of neomycin on amylase secretion, pancreatic pieces were incubated in Krebs solution in the presence and absence of neomycin and/or carbachol and amylase secreted was measured as described in 3.7 (a). The concentration of carbachol (10^{-6}M) was chosen for these experiments because it caused maximal amylase secretion.

6.3 : RESULTS

CCK-8, bethanechol and carbachol produced a concentration dependent increase in the breakdown of inositol lipid in pancreatic fragments incubated in Ca^{2+}-free Tris-Krebs solution, and the presence of extracellular Ca^{2+} potentiated the inositol phospholipid response (Figs. 6.1, 6.2, 6.3).

Since extracellular Ca^{2+} was not required for agonist-stimulated breakdown the role of intracellular Ca^{2+} was firstly examined, followed by an investigation of the potentiating effect of Ca^{2+}.

6.3 (a) Inositol Phospholipid Hydrolysis and Intracellular Ca^{2+}

The effect of increasing the intracellular Ca^{2+} concentration on the hydrolysis of inositol phospholipid was investigated with the ionophore A23187 and the results are shown in Table 6.1. Pancreatic slices incubated in the presence of A23187 (10^{-6}M) released 9 ± 9 Bq^{3}H/kBq ^{3}H incorporated by the pancreas from inositol lipid above control (n = 19); this was not a significant increase from control.. (For comparison, an EC_{50} of CCK-8 released approximately 230 Bq^{3}H/kBq ^{3}H incorporated by the pancreas above control.

To test whether the ionophore A23187 was effective in increasing the intracellular Ca^{2+} concentration under the experimental conditions used, the secretion of amylase from pancreatic fragments was measured. The results summarized in Table 6.1 show that A23187 caused a small but significant increase in amylase secretion above control. Since tissue slices were used, it is possible that the ionophore did not have access to all acinar cells, which may explain why secretion was low. It was therefore necessary to examine the effect of A23187 on inositol lipid hydrolysis and amylase secretion in pancreatic acini which would be more accessible to A23187, before the conclusion that this lipid response is not stimulated by an increase in intracellular Ca^{2+} could be made.

Fig. 6.1 : CCK-8-Stimulated Hydrolysis of Inositol Phospholipid

(●) Tris-Krebs solution

(▲) Ca^{2+}-free Tris-Krebs solution

^{3}H released (Bq/kBq ^{3}H incorporated by pancreas) in control tissue was 240 ± 14, n = 9 (●) and 153 ± 5, n = 40 (▲).

Each value plotted represents the inositol phospholipid hydrolysed above control expressed as Bq/kBq ^{3}H incorporated by pancreas and is the mean of at least 8 samples ± S.E.; where no error bar is visible it did not extend beyond the limits of the symbol.

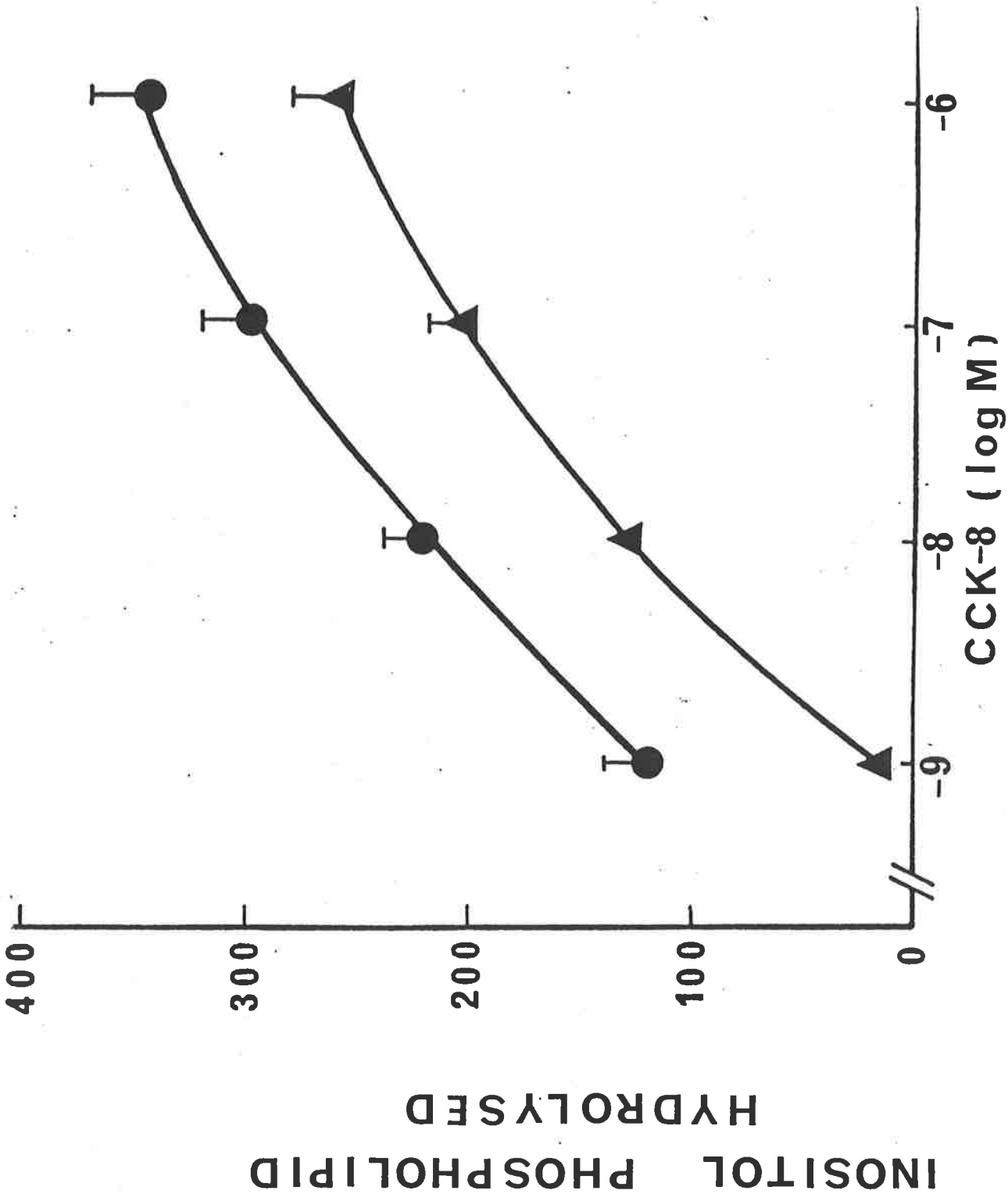


Fig. 6.1 : CCK-8-Stimulated Hydrolysis of Inositol Phospholipid

Fig. 6.2 : Bethanechol-Stimulated Hydrolysis of Inositol Phospholipid

(●) Tris-Krebs solution

(■) Ca^{2+}-free Tris-Krebs solution

^{3}H released (Bq/kBq ^{3}H incorporated by the pancreas) in control tissue was 271 ± 16, n = 22 (●) and 238 ± 23, n = 9 (■).

Each value plotted represents the inositol phospholipid hydrolysed above control expressed as Bq/kBq ^{3}H incorporated by pancreas and is the mean of at least 8 samples ± S.E.; where no error bar is visible it did not extend beyond the limits of the symbol.

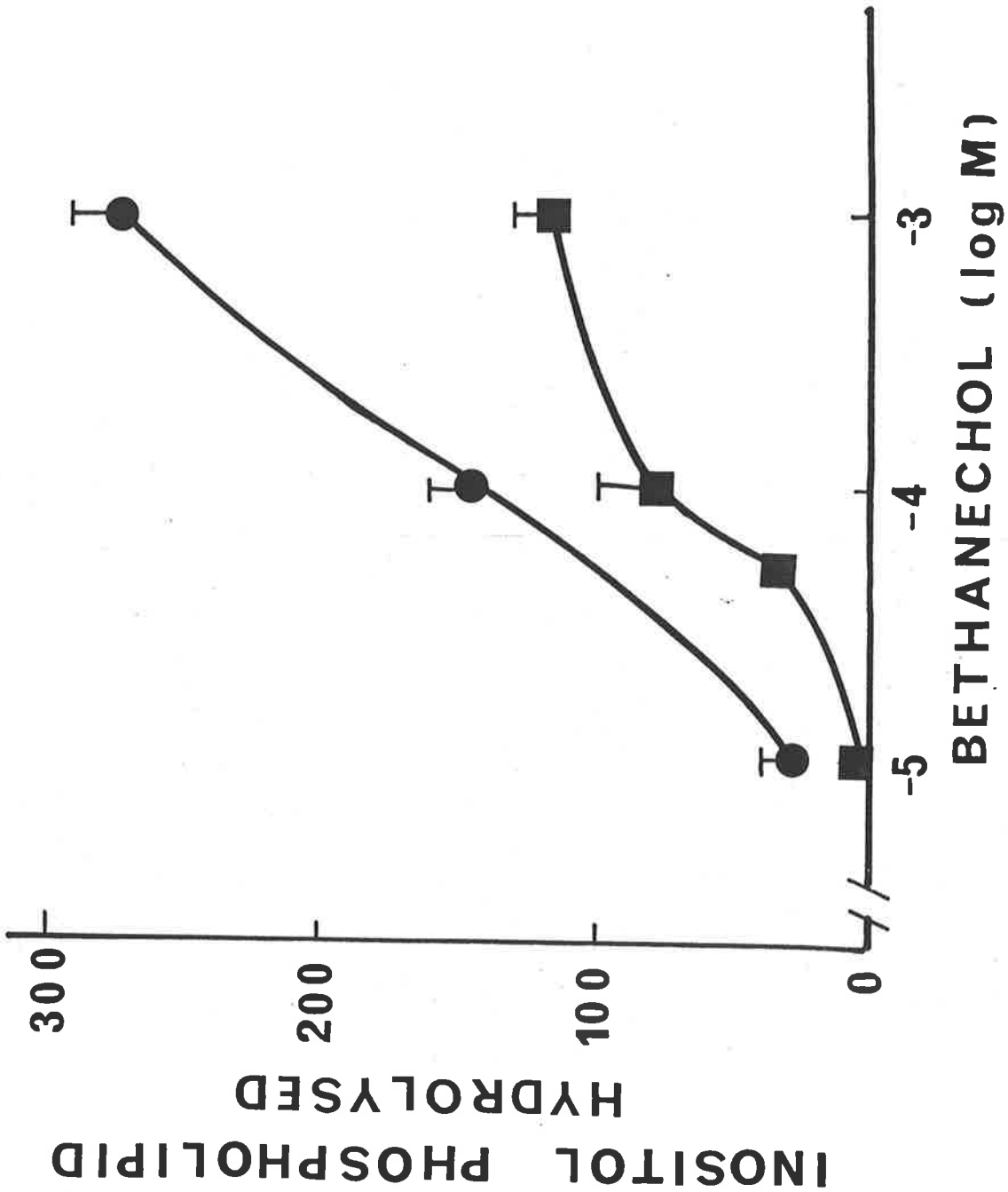


Fig. 6.2 : Bethanechol-Stimulated Hydrolysis of Inositol Phospholipid

Fig. 6.3 : Carbachol-Stimulated Hydrolysis of Inositol Phospholipid

(●) Tris-Krebs solution

(■) Ca^{2+}-free Tris-Krebs solution

^{3}H released (Bq/kBq ^{3}H incorporated by pancreas) in control tissue was 254 ± 10, n = 49 (●) and 172 ± 9, n = 44 (■).

Each value plotted represents the inositol phospholipid hydrolysed above control expressed as Bq/kBq ^{3}H incorporated by pancreas and is the mean of at least 8 samples ± S.E.; where no error bar is visible it did not extend beyond the limits of the symbol.

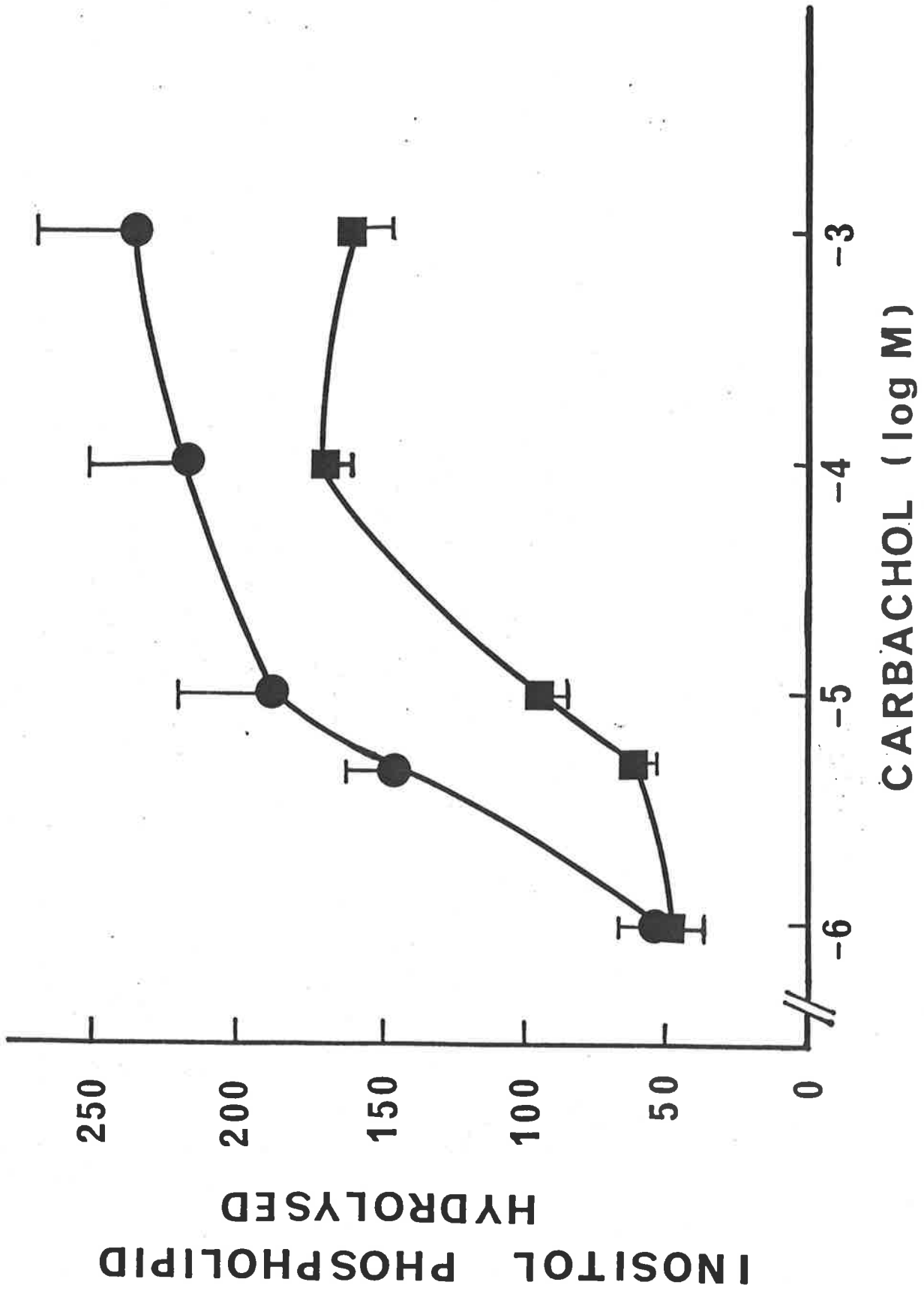


Fig. 6.3 : Carbachol-Stimulated Hydrolysis of Inositol Phospholipid

The results in Table 6.2 show that although A23187 caused an increase in amylase secretion from acini of almost twice control, there was no stimulation of inositol lipid hydrolysis, whereas activation of the muscarinic receptor stimulated both amylase secretion and lipid breakdown.

Since pancreatic cells possess cellular stores Ca^{2+}, it was investigated whether the agonist-induced hydrolysis of inositol phospholipid in the absence of extracellular Ca^{2+} ceased as intracellular stores of Ca^{2+} were depleted. Following a 30 min wash period in Ca^{2+}-free Krebs solution, the CCK-8-stimulated breakdown of inositol lipid measured after 5,15 and 30 min incubations of pancreas pieces in Ca^{2+}-free Krebs solution significantly increased ($P < 0.05$) at each increase in incubation time as shown in Fig. 6.4.

The 30 min wash period had a significant inhibitory effect on amylase secretion since the subsequent addition of CCK-8 (10^{-8}) for 5, 15 or 30 min failed to stimulate secretion above basal (Table 6.3) - indicating depletion of stored Ca^{2+}.

The results shown in Fig. 6.5 show that addition of atropine (10^{-4}M) 15 min after carbachol was added to pancreatic slices prevented any further breakdown of inositol phospholipid. In the absence of atropine, carbachol-stimulated inositol lipid hydrolysis at 30 min was significantly greater than that at 15 min ($P < 0.05$).

Table 6.1 : The effect of ionophore A23187 on the hydrolysis of inositol phospholipid and the secretion of amylase in pancreatic slices

	Inositol phospholipid hydrolysed (Bq/kBq ^{3}H incorporated by pancreas)	Amylase secreted (% total)
CONTROL	201 ± 11 (n = 19)	7.2 ± 0.25 (n = 20)
A23187 (10^{-6}M)	210 ± 10 (n = 19)[a]	10.1 ± 0.4 (n = 20)[b]

a : P(vs control) < 0.05

b : P(vs control) > 0.05

Table 6.2 : The effect of ionophore A23187 on the hydrolysis of inositol phospholipid and the secretion of amylase from pancreatic acini

	Inositol phospholipid hydrolysed (Bq/kBq ^{3}H incorporated by pancreas)	Amylase secreted (% total)
CONTROL	105 ± 5 (n = 5)	7.6 ± 0.9 (n = 11)
A23187 ($10^{-6}M$)	92 ± 7 (n = 8)[b]	13.7 ± 0.7 (n = 10)[a]
Carbachol*	300 ± 54(n = 7)[a]	15.4 ± 1.2 (n = 5)[a]

a : P(vs control) < 0.05

b : P(vs control) > 0.05

*Carbachol was present at a concentration of $10^{-4}M$ for the inositol phospholipid experiments and at $10^{-6}M$ for the amylase secretion experiments.

Table 6.3 : The effect of incubation of pancreatic slices in Ca^{2+}-free Krebs solution on CCK-8-stimulated amylase secretion

	Amylase secreted (% total)	
	CONTROL	CCK-8 (10^{-8}M)
5 min	6.5 ± 0.4 (n = 8)	6.9 ± 0.4 (n = 8)[a]
15 min	7.8 ± 0.4 (n = 8)	8.4 ± 0.4 (n = 8)[a]
30 min	9.1 ± 0.3 (n = 8)	9.9 ± 0.7 (n = 8)[a]

a : P(vs control) > 0.05

Fig. 6.4 : Time Course of CCK-8-Stimulated Inositol Phospholipid Hydrolysis in Ca^{2+}-free Tris-Krebs Solution

^{3}H released (Bq/kBq ^{3}H incorporated by the pancreas) in control tissue incubated for 5, 15 and 30 min was 121 ± 9 (n = 8), 134 ± 9 (n = 11), 148 ± 6 (n = 10), respectively.

Each value plotted represents inositol phospholipid hydrolysed above control expressed as Bq/kBq ^{3}H incorporated by pancreas and is the mean ± S.E.; for each time point, the number in parentheses is the number of samples.

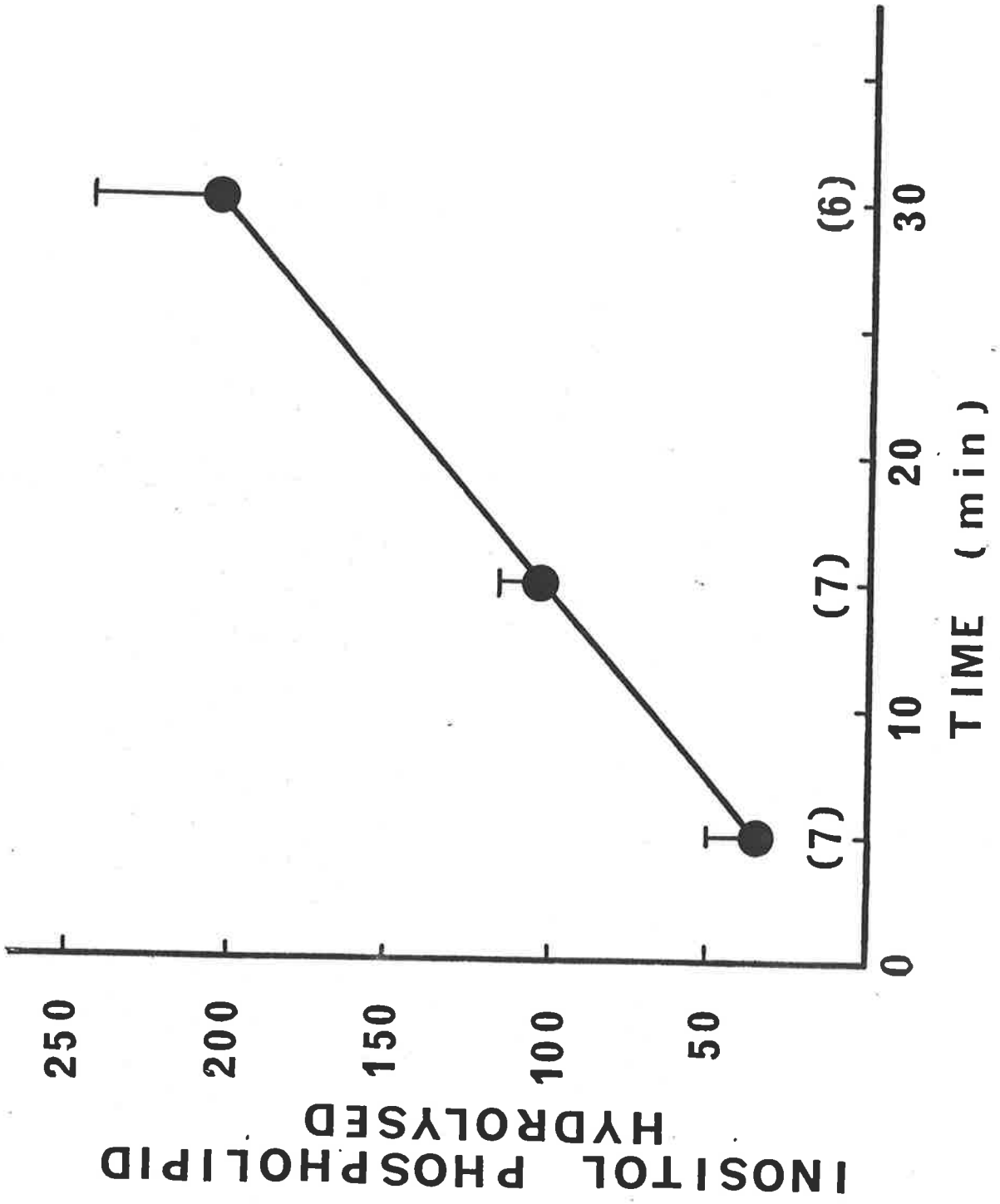


Fig. 6.4 : Time Course of CCK-8-Stimulated Inositol Phospholipid Hydrolysis in Ca^{2+}-free Tris-Krebs Solution

Fig. 6.5 : The Effect of Atropine on Carbachol-Stimulated Hydrolysis of Inositol Phospholipid in Ca^{2+}-free Krebs Solution

The ^{3}H released (Bq/kBq ^{3}H incorporated by the pancreas) from control tissue incubated for 15 min, 30 min and 30 min (with atropine added at 15 min) were: 174 ± 8 (n = 5), 162 ± 7 (n = 6), and 178 ± 8 (n = 6), respectively.

Each value plotted represents inositol phospholipid hydrolysed above control expressed as Bq/kBq ^{3}H incorporated by pancreas and is the mean ± S.E. of at least 6 samples.

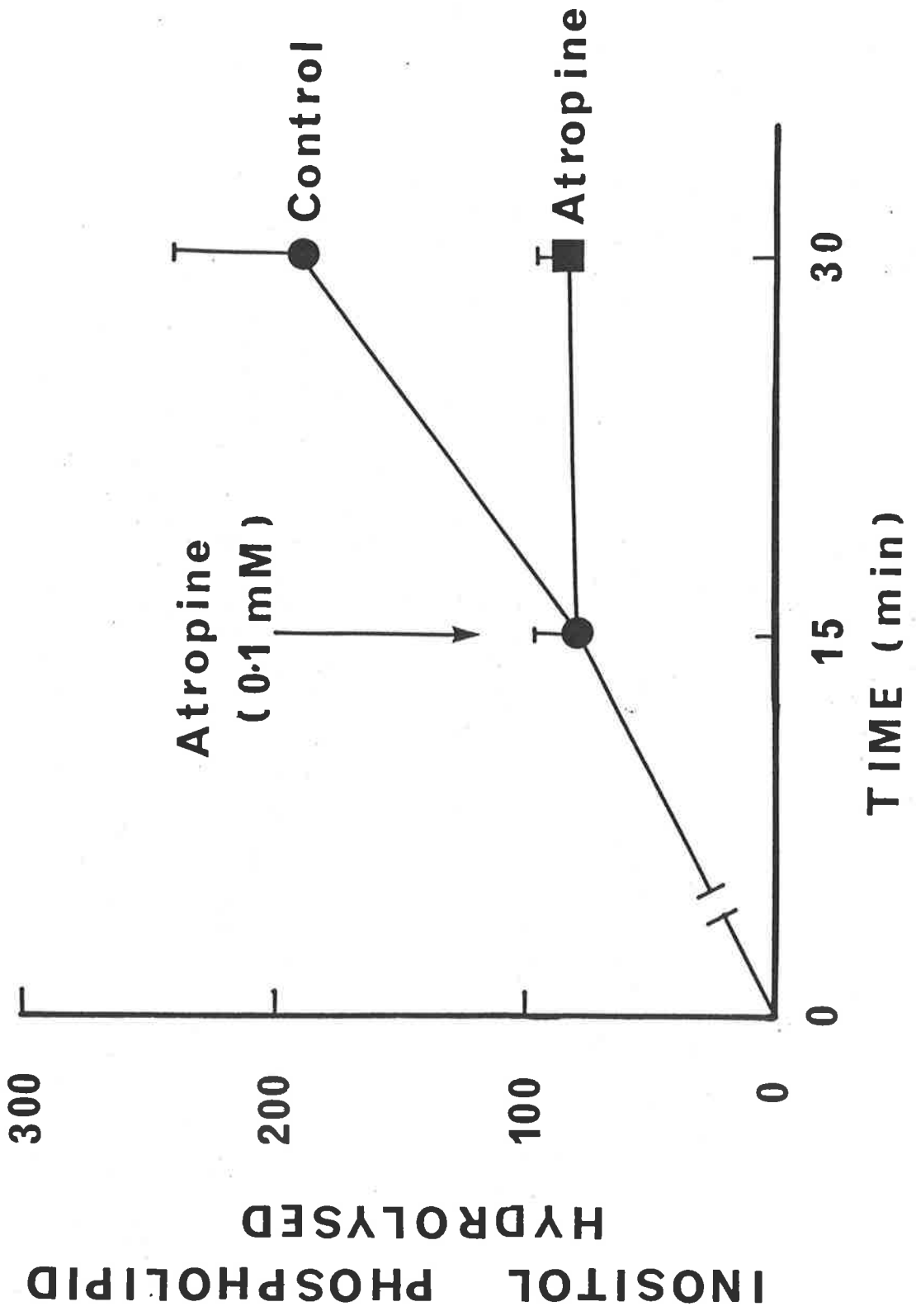


Fig. 6.5 : The Effect of Atropine on Carbachol-Stimulated Hydrolysis of Inositol Phospholipid in Ca^{2+}-free Krebs Solution

To further investigate whether the breakdown of inositol phospholipid occurred prior to the release of Ca^{2+} from intracellular stores, the putative intracellular calcium antagonist TMB-8 was used. TMB-8 (2×10^{-4}M) inhibited completely the hydrolysis of inositol phospholipid induced by carbachol (Table 6.4), whereas the breakdown of phospholipid produced by an equipotent concentration of CCK-8 (10^{-8}M) was reduced by 28 ± 19% (n = 5), which was not a significant decrease ($P > 0.05$). A higher concentration of TMB-8 (10^{-3}M) did not inhibit completely but did significantly reduce the effect of CCK-8 (10^{-8}M) on lipid hydrolysis by 44 ± 9% (n = 10), (Table 6.4). TMB-8 alone had no significant effect on inositol phospholipid hydrolysis since TMB-8, 2×10^{-4}M and 10^{-3}M, caused the release of ^{3}H (Bq/kBq ^{3}H incorporated by the pancreas) above control of 24 ± 8 (n = 4) and 20 ± 21 (n = 4), which were not significantly increased above control ($P > 0.05$).

The specificity of TMB-8 to act as a calcium antagonist was investigated in our laboratory by examining the effect of TMB-8 on (^{3}H)-QNB binding to pancreatic muscarinic receptors. The results of this study are discussed in 6.4 of this chapter. Briefly, it was found that the effect of TMB-8 could not be attributed to inhibition of a Ca^{2+}-dependent hydrolysis of inositol phospholipid.

These studies investigating intracellular Ca^{2+} indicate that the hydrolysis of inositol phospholipid is not activated by an increase in intracellular Ca^{2+}, nor is the agonist-stimulated lipid response dependent on Ca^{2+} released from stores.

Table 6.4 : The effect of TMB-8 on carbachol and CCK-8-stimulated hydrolysis of inositol phospholipid

	Inositol phospholipid hydrolysed above control (Bq/kBq ^3H incorporated by the pancreas)	
	TMB-8*	
	(-)	(+)
CCK-8 ($10^{-8}M$)	133 ± 14 (n = 13)[a]	76 ± 13 (n = 10)[b]
Carbachol ($10^{-5}M$)	130 ± 32 (n = 4)[a]	5 ± 10 (n = 5)[c]

*TMB-8 concentrations used were $10^{-3}M$ for incubations with CCK-8 and $2 \times 10^{-4}M$ for incubations with carbachol. The ^3H released in control samples was 198 ± 8 Bq/kBq ^3H incoroprated by the pancreas (n = 33).

a : P(vs control) < 0.05

b : P(vs CCK-8) < 0.05

c : P(vs carbachol)< 0.05

6.3 (b) Inositol Phospholipid Hydrolysis and Potentiation by Ca^{2+}

As shown in Figs. 6.1, 6.2 and 6.3, Ca^{2+} potentiated the hydrolysis of inositol phospholipid stimulated by CCK-8, carbachol and bethanechol. A significant increase in the release of ^{3}H from *myo*-(2-^{3}H) inositol-labelled inositol phospholipid also occurred in unstimulated tissue. The results for acid-soluble ^{3}H present in control tissue incubated in Tris-Krebs solution or Ca^{2+}-free Krebs solution were pooled from data of Figs. 6.1, 6.2 and 6.3 and calculated to be 257 ± 8 Bq/kBq ^{3}H incorporated into pancreas (n = 80) and 170 ± 6 Bq/kBq ^{3}H incorporated into pancreas (n = 93), respectively; these two groups were significantly different ($P < 0.05$).

The dose-response curve for carbachol-stimulated inositol lipid hydrolysis in a Ca^{2+}-containing HCO_3 -buffered Krebs solution showed an upward shift compared with carbachol-stimulated hydrolysis in a Ca^{2+}-containing Tris-buffered Krebs solution Fig. 6.6). The lipid hydrolysis in tissue incubated in a Ca^{2+}-containing solution buffered with Hepes is also shown.

The reason for the difference in inositol lipid hydrolysis stimulated by carbachol in a Tris-buffered or HCO_3-buffered solution is not clear. The Hepes-buffered solution which is gassed with O_2 as is the Tris-solution appeared to resemble more closely the response in HCO_3-buffered solution at high concentrations of carbachol indicating that the difference is not due to the gas used. The Tris may be entering the cells and causing a change in intracellular pH which affects lipid hydrolysis.

Fig. 6.6 : Carbachol-Stimulated Inositol Lipid Hydrolysis in a Ca^{2+}-containing Krebs solution. Solutions were buffered with HCO_3 (●), Hepes (▲) and Tris (■)

Results plotted represent inositol lipid hydrolysed above control expressed as Bq/kBq ^3H incorporated by pancreas and are the mean ± S.E. of at least 4 samples.

^3H released in control tissue was: (Bq/kBq ^3H incorporated by pancreas) 237 ± 10 (n = 12) for HCO_3-Krebs, 254 ± 10 (n = 49) for Tris-Krebs, and 226 ± 7 (n = 4) for Hepes-Krebs.

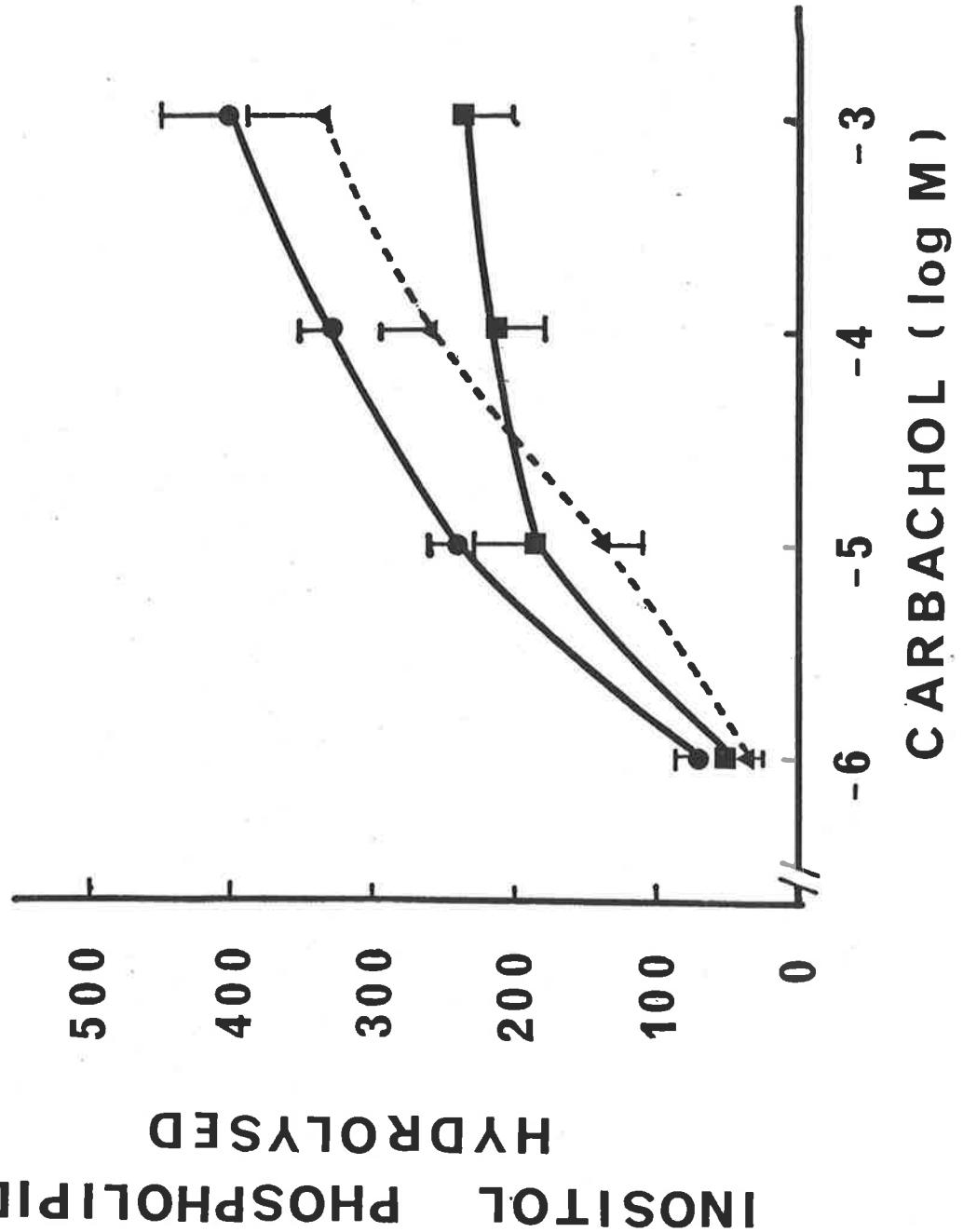


Fig. 6.6 : Carbachol-Stimulated Inositol Lipid Hydrolysis in a Ca^{2+}-containing Krebs Solution. Solutions were buffered with HCO_3, Hepes and Tris

To examine the mechanism of potentiation by Ca^{2+} of agonist-stimulated inositol phospholipid hydrolysis a number of Ca^{2+}-antagonists were used. These agents (tetracaine, D600, neomycin, La^{3+} and Mn^{2+}) inhibit transmembrane Ca^{2+} fluxes and in addition tetracaine, La^{3+} and Mn^{2+} also compete with Ca^{2+} for binding sites on the external surface of the cell.

The Ca^{2+} potentiation effect was firstly examined using tetracaine (5×10^{-5}M) which did not alter the effect of carbachol on inositol phospholipid hydrolysis in Tris-Krebs solution (Table 6.5) but did significantly reduce the effect of this agonist in Ca^{2+}-free Tris-Krebs solution (Table 6.6). To examine whether this inhibitory effect of tetracaine was specifically associated with muscarinic receptor activation, the ability of tetracaine to reduce CCK-8-stimulated lipid hydrolysis was also examined. The results in Table 6.6 show that tetracaine did not alter inositol lipid hydrolysis stimulated by the peptide CCK-8 in Ca^{2+}-free Tris-Krebs solution. A direct comparison between the effect of tetracaine on carbachol and CCK-8-stimulated lipid hydrolysis could not be made, however, since these two agonists were not present at equiactive concentrations.

D600 (10^{-4}M) did not cause a significant decrease in CCK-8-stimulated inositol lipid hydrolysis and although a decrease in the effect of carbachol on the lipid response was observed, this was not significant ($P > 0.05$) (Table 6.7).

The effects of neomycin (3×10^{-3}M and 10^{-2}M) on carbachol-stimulated inositol phospholipid hydrolysis are shown in Table 6.8. Neomycin did not cause a significant reduction in inositol lipid hydrolysed even at the high concentration of 10^{-2}M which significantly reduced carbachol-stimulated amylase secretion (Table 6.9).

The reduction in amylase secretion was used as an indirect indication that Ca^{2+} influx had been inhibited by neomycin. $^{45}Ca^{2+}$ flux measurements were not carried out in this study, however, there is clear evidence to show that neomycin (3×10^{-3}M and 10^{-2}M) effectively inhibits the entry of Ca^{2+} from the extracellular medium into the receptor regulated pool of Ca^{2+} in rat parotid gland (Marier et. al., 1978; Putney, 1981; Aub et. al., 1982). Therefore it seemed reasonable to assume that the effect of neomycin to inhibit amylase secretion was by a reduction in Ca^{2+} influx and so the results indicated that agonist-stimulated inositol phospholipid hydrolysis was not potentiated by Ca^{2+} influx.

To examine the possible role of Ca^{2+} bound to the negatively charged sites on the cell surface, La^{3+} and Mn^{2+} were used. These ions also block the entry of Ca^{2+} into cells but since the results using neomycin indicated that Ca^{2+} entry was not responsible for the potentiation of the lipid response then any alteration of lipid breakdown by these metal ions could be attributed to an effect on membrane-bound Ca^{2+}.

La^{3+} (10^{-3}M) significantly reduced bethanechol and CCK-8-stimulated

inositol phospholipid hydrolysis in Tris-Krebs solution but did not alter the effect of these agonists on inositol lipid hydrolysed in Ca^{2+}-free Tris-Krebs solution (Fig. 6.7). The hydrolysis of inositol phospholipid induced by bethanechol ($5 \times 10^{-5}M$) and CCK-8 ($10^{-8}M$) was reduced by 47.8% and 47.3% respectively, in Tris-Krebs solution. La^{3+} ($10^{-3}M$) did not have any effect on the inositol phospholipid hydrolysed in unstimulated tissue incubated in either the presence or absence of Ca^{2+}.

Table 6.5 : The effect of tetracaine on the agonist-stimulated hydrolysis of inositol phospholipid in Tris-Krebs solution

	Inositol phospholipid hydrolysed above control (Bq/kBq ^{3}H incorporated by the pancreas)		
Tetracaine ($5 \times 10^{-5}M$)	-20 ± 9	(n = 14)	a
Carbachol ($5 \times 10^{-6}M$)	120 ± 17	(n = 16)	b
Tetracaine ($5 \times 10^{-5}M$) + Carbachol ($5 \times 10^{-6}M$)	90 ± 11	(n = 14)	c

a : P(vs control) > 0.05

b : P(vs control) < 0.05

c : P(interaction)> 0.05

^{3}H released in coltrol tissue was 264 ± 14 Bq/kBq ^{3}H incorporated by the pancreas (n = 13)

Table 6.6 : The effect of tetracaine on the agonist-stimulated hydrolysis of inositol phospholipid in Ca^{2+}-free Tris-Krebs solution

	Inositol phospholipid hydrolysed above control (Bq/kBq ^3H incorporated by the pancreas)		
Tetracaine ($5 \times 10^{-5}M$)	-4 ± 8	(n = 12)	a
Carbachol ($5 \times 10^{-6}M$)	73 ± 7	(n = 18)	b
Tetracaine ($5 \times 10^{-5}M$) + Carbachol ($5 \times 10^{-6}M$)	38 ± 8	(n = 17)	c
CCK-8 ($10^{-8}M$)	109 ± 13	(n = 10)	b
Tetracaine ($5 \times 10^{-5}M$) + CCK-8 ($10^{-8}M$)	100 ± 13	(n = 9)	d

P(vs control) = a > 0.05; b < 0.05

P(interaction) = c < 0.05; d > 0.05

^3H released in control tissue was 148 ± 6 Bq/kBq ^3H incorporated by the pancreas (n = 27)

Table 6.7 : The effect of D600 on the agonist-stimulated hydrolysis of inositol phospholipid in Tris-Krebs solution

	Inositol phospholipid hydrolysed above control (Bq/kBq ^3H incorporated by the pancreas)		
D600 ($10^{-4}M$)	-14 ± 13	(n = 15)	a
Carbachol ($5 \times 10^{-6}M$)	117 ± 16	(n = 11)	b
D600 ($10^{-4}M$) + Carbachol ($5 \times 10^{-6}M$)	67 ± 16	(n = 10)	c
CCK-8 ($10^{-8}M$)	118 ± 15	(n = 10)	b
D600 ($10^{-4}M$) + CCK-8 ($10^{-8}M$)	163 ± 14	(n = 10)	c

a : P(vs control) > 0.05

b : P(vs control) < 0.05

c : P(interaction)> 0.05

^3H released in control tissue was 282 ± 10 Bq/kBq ^3H incorporated by the pancreas (n = 20)

Table 6.8 : The effect of neomycin on the carbachol-stimulated hydrolysis of inositol phospholipid in Tris-Krebs solution

	Inositol phospholipid hydrolysed above control (Bq/kBq ^3H incorporated by the pancreas)		
Neomycin ($3 \times 10^{-3}M$)	29 ± 13	(n = 19)	a
Neomycin ($10^{-2}M$)	11 ± 12	(n = 10)	a
Carbachol ($5 \times 10^{-6}M$)	175 ± 12	(n = 25)	b
Neomycin ($3 \times 10^{-3}M$) + Carbachol ($5 \times 10^{-6}M$)	172 ± 11	(n = 15)	c
Neomycin ($10^{-2}M$) + Carbachol ($5 \times 10^{-6}M$)	184 ± 17	(n = 10)	c

a : P(vs control) > 0.05

b : P(vs control) < 0.05

c : P(interaction)> 0.05

^3H released in control tissue was 222 ± 8 Bq/kBq ^3H incorporated by the pancreas (n = 25)

Table 6.9 : The effect of neomycin on carbachol-stimulated amylase secretion

	Amylase secreted (% total)		
Control	7.4 ± 0.6	(n = 5)	
Neomycin (10^{-2}M)	17.7 ± 0.5	(n = 5)	a
Carbachol (10^{-6}M)	11.2 ± 0.9	(n = 5)	b
Neomycin (10^{-2}M) + Carbachol (10^{-6}M	8.5 ± 0.3	(n = 5)	c

a : P (vs control) > 0.05

b : P (vs control) < 0.05

c : P (interaction) < 0.05

To further examine the ability of an ion, such as La^{3+} to reduce the potentiation by Ca^{2+} of inositol phospholipid hydrolysis another cation, with differing ability to displace Ca^{2+} from membrane sites was examined. The effect of Mn^{2+} on carbachol-stimulated and CCK-8-stimulated inositol lipid hydrolysis is shown in Fig. 6.8. In Tris-Krebs solution, Mn^{2+} (10^{-3}M) significantly reduced inositol lipid hydrolysis stimulated by carbachol to a level similar to that in Ca^{2+}-free Tris-Krebs solution, but did not alter the effect of CCK-8. In unstimulated tissue incubated in the absence of Ca^{2+}, Mn^{2+} caused a significant increase in inositol lipid hydrolysed, however, this effect did not occur when Ca^{2+} was present in the incubation medium (Fig. 6.8).

In Ca^{2+}-free Tris-Krebs solution, inositol phospholipid hydrolysis in the presence of carbachol and Mn^{2+} was equivalent to the additive effects of these two agents alone. In contrast, Mn^{2+} potentiated the effect of CCK-8 on inositol lipid hydrolysis; that is, in the presence of CCK-8 and Mn^{2+}, inositol lipid hydrolysed was significantly greater than the addition of these two agents alone.

Fig. 6.7 : The Effect of Lanthanum on Bethanechol-Stiumlated and CCK-8-Stimulated Hydrolysis of Inositol Phospholipid in Ca^{2+} and Ca^{2+}-free Tris-Krebs Solution

Each histogram plotted represents the mean ± S.E. of inositol phospholipid hydrolysed (Bq/kBq ^{3}H incorporated by the pancreas) of at least 9 samples.

(□) = Ca^{2+}-free Tris-Krebs solution

(▨) = Tris-Krebs solution

(*) = P(interaction) < 0.05

Inositol lipid hydrolysis stimulated by bethanechol and CCK-8 was significantly greater than control ($P < 0.05$) in both Krebs solutions.

Concentrations of agents used:

La^{3+} : $10^{-3}M$

Bethanechol : $5 \times 10^{-5}M$

CCK-8 : $10^{-8}M$

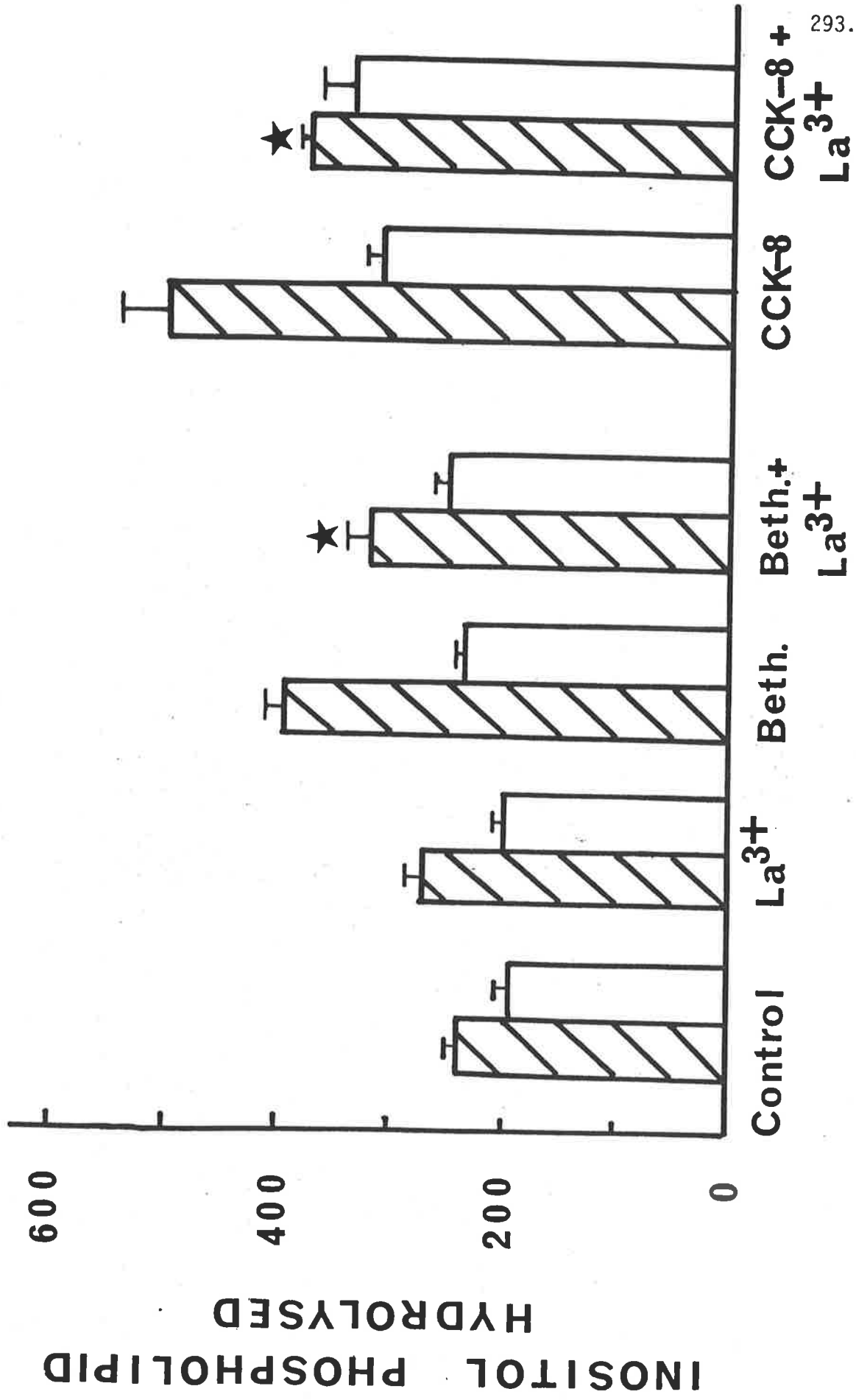


Fig. 6.7 : The Effect of Lanthanum on Bethanechol-Stimulated and CCK-8-Stimulated Hydrolysis of Inositol Phospholipid in Ca^{2+} and Ca^{2+}-free Tris-Krebs Solution

Fig. 6.8 : The Effect of Manganese on Carbachol-Stimulated and CCK-8-Stimulated Hydrolysis of Inositol Phospholipid in Ca^{2+} and Ca^{2+}-free Tris-Krebs Solution

Each histogram plotted represents the mean ± S.E. of inositol phospholipid hydrolysed (Bq/kBq ^{3}H incorporated by the pancreas) of at least 10 samples.

(□) = Ca^{2+}-free Tris-Krebs solution

(▨) = Tris-Krebs solution

(*) = P vs (interaction) < 0.05

(**) = P vs (control) < 0.05

Inositol lipid hydrolysis stimulated by carbachol and CCK-8 was significantly greater than control ($P < 0.05$) in both Krebs solutions.

Concentrations of agents used:

Mn^{2+} : $10^{-2}M$

Carbachol : $10^{-6}M$ in Ca^{2+}-free Tris-Krebs solution

$5 \times 10^{-6}M$ in Tris-Krebs solution

CCK-8 : $10^{-8}M$

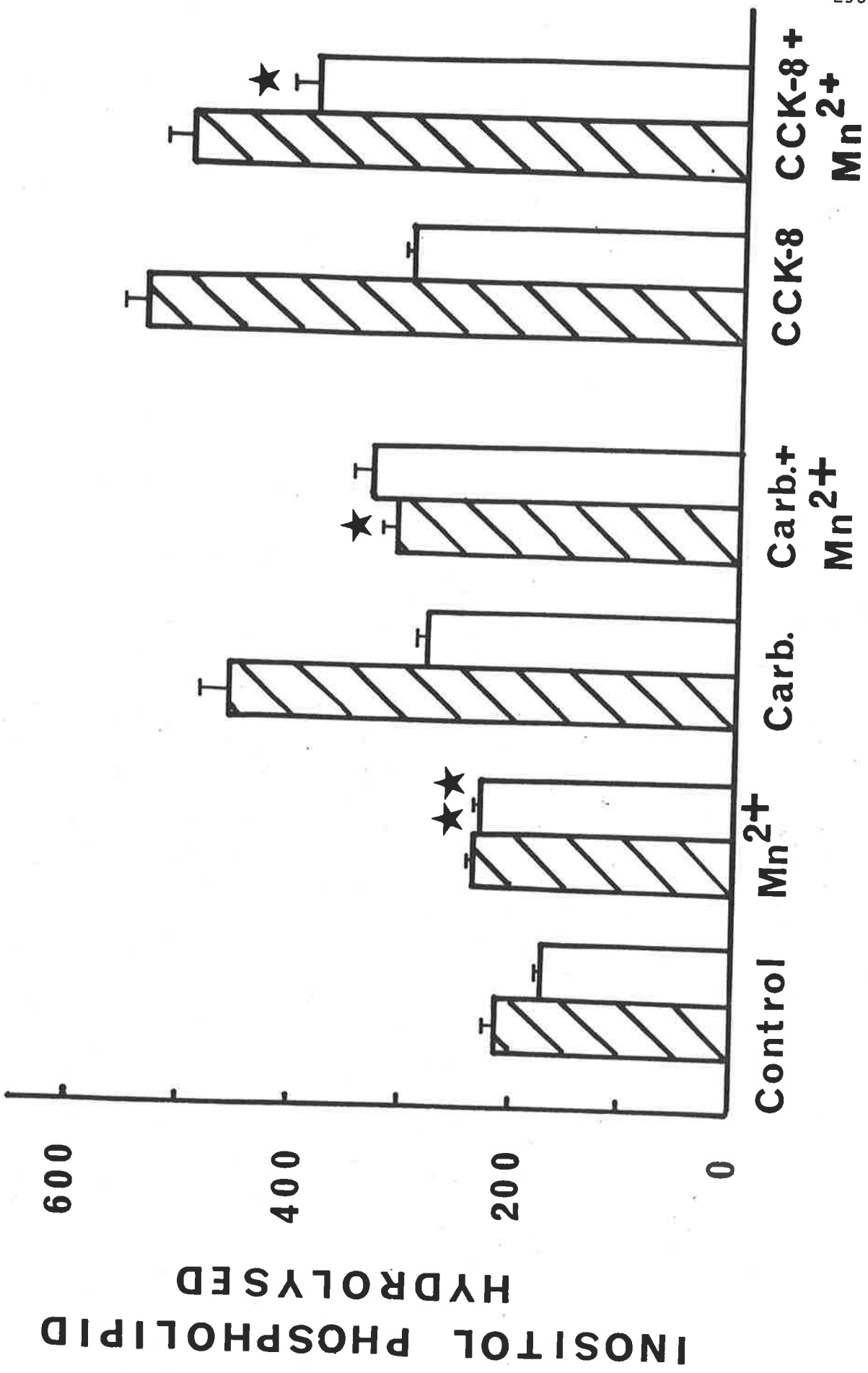


Fig. 6.8 : The Effect of Manganese on Carbachol-Stimulated and CCK-8-Stimulated Hydrolysis of Inositol Phospholipid in Ca^{2+} and Ca^{2+}-free Tris-Krebs Solution

6.4 : DISCUSSION

Secretagogue-activation of the exocrine pancreas releases Ca^{2+} from a membrane-store into the cytosol, increasing the free Ca^{2+} concentration. Ca^{2+} is pumped out of the cell resulting in Ca^{2+} efflux, which is followed by an associated influx of Ca^{2+} from the extracellular fluid into the cell (Stolze and Schulz, 1980; Dormer et. al., 1981). The cellular location of the membrane-bound store(s) of Ca^{2+} have been discussed in detail in Chapter 2.5 of this thesis. There is considerable evidence that the endoplasmic reticulum plays a major role in release of Ca^{2+} following receptor activation (Dormer and Williams, 1981; Streb and Schulz, 1983).

If the hydrolysis of inositol phospholipid is involved in controlling the intracellular Ca^{2+} concentration in the exocrine pancreas, it may act to release Ca^{2+} from these Ca^{2+} storage sites and/or be associated with the mechanism which allows an increase in the plasma-membrane permeability to Ca^{2+}, causing Ca^{2+} influx. For inositol lipid hydrolysis to be the initial event in secretagogue-stimulated Ca^{2+} mobilization it would occur prior to the increase in the cytosolic Ca^{2+} concentration. Also, it must be possible to activate the lipid response in a Ca^{2+}-free medium and independently of the release of Ca^{2+} from cellular stores. The Ca^{2+}-dependency of inositol phospholipid breakdown was investigated in the present study by examining the possible requirements for intracellular and extracellular Ca^{2+}.

It was shown that in the absence of extracellular Ca^{2+}, secretagogues stimulated inositol phospholipid hydrolysis. However, it was possible that activation of this lipid response was dependent on Ca^{2+} released

from intracellular stores.. The intracellular Ca^{2+} concentration can be increased artificially with the ionophore A23187 and receptor activation by-passed.

In the presence of the ionophore A23187, Ca^{2+} stimulated the secretion of amylase but not the breakdown of inositol lipid in either pancreatic slices or acini. Since ionophore A23187 incorporates into both the plasma membrane and intracellular membranes jn pancreatic acinar cells (Chandler and Williams, 1977), the increase in intracellular Ca^{2+} may be due to both release from Ca^{2+}-stores and influx of Ca^{2+} from the intracellular medium. The results of the present study indicate that the breakdown of inositol phospholipid in mouse exocrine pancreas does not occur as a result of an increase in the intracellular concentration of Ca^{2+}.

Farese et. al. (1980) studying rat pancreas, found that ionophore A23187 in the presence of Ca^{2+} caused a decrease in total PtdIns content, leading to the proposal that PtdIns breakdown in pancreas was Ca^{2+}-activated. Instead of assuming that Ca^{2+} was activating the phospholipase C involved in the agonist-stimulated hydrolysis of inositol lipid, the decrease in PtdIns mass with A23187 could be explained by an effect of Ca^{2+} on two other processes.

Firstly, inhibition of PtdIns synthesis by Ca^{2+} has been demonstrated in many tissues (Berridge and Fain, 1979; Tolbert et. al., 1980; Egawa et. al., 1981 a). The basal synthesis of PtdIns which occurs in unstimulated tissue would be inhibited by the high intracellular concentration of Ca^{2+} caused by A23187 and so the total PtdIns content would be less than in tissue incubated without ionophore.

Secondly, a decrease in total PtdIns mass would occur if a phospholipase A_2 was activated. An increase in the activity of this enzyme in pancreas was demonstrated by Marshall et. al. (1980) by measuring the agonist-stimulated release of (^{14}C)-arachidonic acid from pre-labelled PtdIns. More recently, Halenda and Rubin (1982) showed that the Ca^{2+} ionophore, ionomycin, stimulated the turnover of the arachidonoyl group of PtdIns; indicating activation by Ca^{2+} of a phospholipase A_2 which acts on PtdIns in pancreas.

A less specific effect of the ionophore A23187 on the pancreatic acinar cell membrane may also alter PtdIns content. In the study by Farese et. al. (1980), ionophore A23187 was used at a high concentration, ($2 \times 10^{-5}M$). Chandler and Williams (1977) showed that A23187 ($10^{-5}M$) increased amylase release from acinar cells but also produced marked disruption of cell morphology, increased release of lactate dehydrogenase (LDH) and increased the permeability to trypan blue. This effect of A23187 is found not only in isolated cells, since Selinger et. al. (1974) showed increased LDH release with A23187 ($6 \times 10^{-6}M$) from pancreatic fragments. Perhaps the high concentration of ionophore used by Farese et. al. (1980) in pancreatic fragments was able to disrupt cellular membranes causing a conformational change possibly allowing an interaction between PtdIns and cause PtdIns breakdown. The concentration of ionophore used in the present study ($10^{-6}M$) does not cause alteration in the cellular morphology of acinar cells (Chandler and Williams, 1977).

Therefore, from the study by Farese et. al. (1980) measuring PtdIns mass, it can not be concluded that the agonist-stimulated breakdown of PtdIns follows rather than precedes the mobilization of Ca^{2+}.

In the study by Halenda and Rubin (1982) an increase in intracellular Ca^{2+} with ionomycin, stimulated a decrease in PtdIns, labelled *in vivo* with *myo*-(2-^{3}H) inositol. It is not possible to determine from this result whether the decrease is due to activation of phospholipase C or phospholipase A_2 (or both). Since phospholipase A_2 is activated by Ca^{2+} then agonist-activation of this event is not involved in mobilization of Ca^{2+}. The breakdown of inositol phospholipid with phospholipase C is the event to be examined. The lack of effect of Ca^{2+} with ionophore on phospholipase C activity was demonstrated in the present study by measuring the release of (^{3}H)-inositol-labelled products from inositol lipid which eliminates any complicating effect of activation of phospholipase A_2.

Subsequent to the study of the effect of A23187 on inositol phospholipid hydrolysis (Tennes and Roberts, 1982) described in this thesis, other studies on the ability of A23187 to stimulate a decrease in levels of PtdIns-4,5P_2 and PtdIns-4P (Putney et. al., 1983; Orchard et. al., 1984), an increase in phosphatidic acid (Orchard et. al., 1984) or an increase in release of Ins-1,4,5P_3, Ins-1,4P_2 and Ins-1-P (Rubin et. al., 1984) were reported. These results have been compared and discussed in detail in Section 2.13 of the literature review. Briefly, an increase in intracellular Ca^{2+} with A23187 or ionomycin did not mimic agonist-activated phosphoinositide hydrolysis (Putney et. al., 1983; Orchard et. al., 1984; Rubin et. al., 1981).

One important point regarding the use of ionophores to examine the Ca^{2+}-dependency of phosphoinositide hydrolysis is whether the increase in intracellular Ca^{2+} produced by ionophore mimics that caused by agonists.

In the following chapter of this thesis, it is shown that the dose-response curves of amylase secretion are biphasic. High concentrations of agonists which cause a submaximal secretion of amylase stimulate inositol phospholipid hydrolysis whereas low concentrations which stimulate amylase secretion produce very little lipid hydrolysis. There is evidence to suggest that the high agonist concentrations cause very large increases in intracellular Ca^{2+} (Gardner et. al., 1980; Roberts and Woodland, 1982; Burnham and Williams, 1982 b); Stark and O'Doherty, 1982).

Other studies have also shown that dose-response curves for agonist-stimulated inositol phospholipid breakdown are shifted to the right of those for the physiological Ca^{2+}-dependent cell response (Fain and Berridge, 1979; Kirk et. al., 1981; Creba et. al., 1983).

Therefore, although A23187 or ionomycin are used in concentrations which stimulate the physiological response, the increase in Ca^{2+} could be minimal in comparison to that caused by agonists at concentrations required to stimulate inositol phospholipid hydrolysis.

A study by Stark and O'Doherty (1982) demonstrated that A23187 (10^{-5}M) caused a greater increase in cytosolic Ca^{2+} concentration than A23187 (10^{-6}M) in pancreatic acinar cells. Maximal amylase secretion was achieved with A23187 (10^{-6}M) while A23187 (10^{-5}M) caused a marked reduction in secretion. These results suggest that to cause an increase in Ca^{2+} content similar to that obtained by a concentration of agonist which stimulates a significant hydrolysis of inositol phospholipid and a reduction in amylase secretion, A23187 at a concentration of 10^{-5}M

should be used. A23187 is usually used at 10^{-6}M; a hesitation in using a high concentration (10^{-5}M) of ionophore is that it has been shown to have cell damaging effects (Selinger et. al., 1974).

However, in a very recent study, Orchard et. al. (1984) using rat pancreatic acini, reported that a high concentration of A23187 (2×10^{-5}M) did not cause any decrease in (^{32}P)-PtdIns-4,5P_2 nor any increase in (^{32}P)-phosphatidic acid at 1,2,5 or 10 min after ionophore addition. Therefore, if inositol phospholipid hydrolysis was activated by a high intracellular concentration of Ca^{2+} then this study would have detected it. Since no effect was seen, this result supports the suggestion of the present study that an increase in intracellular Ca^{2+} does not activate the mechanism which is stimulated by agonists to cause inositol phospholipid hydrolysis.

Although the breakdown of inositol phospholipid was not stimulated by an increase in intracellular Ca^{2+} with ionophore it was possible that the receptor-activated event required intracellular stored Ca^{2+}. In the present study, CCK-8 stimulated inositol phospholipid hydrolysis in pancreatic slices incubated in Ca^{2+}-free Krebs solution, and this continued for at least 30 min under conditions in which intracellular Ca^{2+} stores were depleted. The procedures that were used, namely washing the tissue for 30 min in Ca^{2+}-free Krebs solution which contained EGTA, appeared to deplete the stores of Ca^{2+} even prior to incubation with secretagogue, since using this procedure, CCK-8 did not stimulate amylase secretion at any of the time intervals of 5, 15 or 30 min incubation.

The lack of stimulation of amylase secretion 5 min after the addition of CCK-8 disagrees with the results of Williams and Chandler (1975) using mouse pancreas fragments. These workers showed that after a 30 min incubation in Ca^{2+}-free Krebs solution, an increase in amylase secretion was stimulated by bethanechol in the first 10 min. In the present study, some stored Ca^{2+} could have been released in the first 5 min to stimulate a small secretion of amylase which was not detected with the assay used. By 15 min and 30 min, however, the Ca^{2+} stores would have been depleted since Chandler and Williams (1975) showed that the agonist-stimulated amylase secretion initially increases but rapidly declines by 15 and 30 min due to insufficient intracellular Ca^{2+}. The lack of stimulated amylase secretion at these time intervals was supported by the results of the present study.

The assumption that inositol lipid hydrolysis occurring at 15 and 30 min was activated by agonist at these times, when Ca^{2+} stores were depleted, was validated by the action of atropine to inhibit any further breakdown of inositol phospholipid in the presence of carbachol during the 15 min - 30 min period of incubation.

To confirm these results which indicate that intracellular Ca^{2+} stores are not required for agonist-activation of inositol phospholipid hydrolysis the effect of the putative intracellular Ca^{2+} antagonist TMB-8 was examined. TMB-8 was proposed to inhibit the release of stored Ca^{2+} on the basis of the studies showing that TMB-8 inhibits contraction of rabbit aortic strip (which is dependent on intracellular Ca^{2+}), Ca^{2+} exchange in resting guinea-pig ileum and caffeine-induced Ca^{2+} efflux from skeletal muscle sarcoplasmic reticulum (Malagodi and Chiou, 1974;

Chiou and Malagodi, 1975). TMB-8 had not been used previously on pancreatic tissue. In the present study, TMB-8 (2×10^{-4}M) inhibited completely the inositol phospholipid hydrolysis stimulated by carbachol but did not significantly reduce CCK-8-stimulated lipid breakdown at this concentration. Only when TMB-8 (10^{-3}M) was used was the effect of CCK-8 reduced significantly.

If inositol phospholipid hydrolysis was activated by release of Ca^{2+} from intracellular stores, and if TMB-8 acted as an intracellular Ca^{2+} antagonist, one might expect TMB-8 to be equally effective in inhibiting the effects of equiactive concentrations of carbachol and CCK-8. The difference in the inhibitory potencies of TMB-8 toward the two agonists suggested that the antagonism was at a site that was not shared by carbachol and CCK-8. One possibility could be that TMB-8 acts on one pool of Ca^{2+} associated with the carbachol receptor but has less affinity for the pool associated with the CCK-8 receptor. This explanation is unlikely since Stolze and Schulz (1980) showed that the pool of Ca^{2+} mobilized on receptor activation which is responsible for the initial Ca^{2+} efflux from pancreatic cells is common to both carbachol and CCK-8 receptors.

A more likely explanation for the differential effect of TMB-8 is that this agent blocked the muscarinic receptor. Recent studies have shown that a wide range of local anaesthetics and other substances with hydrophobic regions inhibit muscarinic receptor binding (Fairhurst et. al., 1980; Aguilar et. al., 1980; Putney and Van De Walle, 1980 b). A study in our laboratory by J.A. Kennedy showed that (^{3}H)-QNB binding to muscarinic receptors was inhibited in mouse pancreatic acini and

mouse submandibular gland by TMB-8 (2×10^{-4}M) (Tennes et. al., 1983). Since the effect of TMB-8 on (^{3}H)-QNB binding is not unique to the pancreas it is likely that muscarinic receptors in all tissues would be blocked by this agent.

It is not known whether the inhibition by TMB-8 of CCK-8-stimulated inositol lipid breakdown can be explained by a less potent action of TMB-8 on CCK receptors since CCK receptor binding in the presence of TMB-8 has not been measured. However, a non-specific inhibitory action of TMB-8 on both types of receptors is a possibility since the recent study by Mürer et. al. (1981) suggested that TMB-8 may be a general membrane-active compound in platelets, where it caused considerable leakage of cytoplasmic constituents. If TMB-8 does alter the plasma membrane, as the results of Mürer et. al. (1981) suggest then it is possible that TMB-8 may act to interfere with the interaction of both muscarinic agonists and peptides on their respective receptors.

The present study suggests that the inhibition of carbachol-stimulated inositol phospholipid hydrolysis by TMB-8 was due to inhibition of the binding of the agonist to receptors rather than to any effect on the release of Ca^{2+} from stores. TMB-8 should be used with caution as an intracellular Ca^{2+} antagonist since it acts with reasonable potency to block at least one class of membrane receptors. Recently, the agent Quin 2-tetraacetoxymethyl ester (Tsien et. al., 1982) has been used as an indicator of intracellular free Ca^{2+} content and can be used as an intracellular Ca^{2+} buffer. This agent which binds to Ca^{2+} effectively would provide a means whereby the Ca^{2+} released from stores

would be inactivated. Unfortunately there was not time to do the Quin-2 experiments but nevertheless it seems reasonable to conclude from the experiments with A23187 and with depletion of intracellular Ca^{2+} that the agonist-stimulated hydrolysis of inositol phospholipid is not dependent on the release of cellular stored Ca^{2+}.

Although the activation of agonist-stimulated inositol phospholipid hydrolysis does not require Ca^{2+}, this lipid response can be modified by extracellular Ca^{2+}, as shown by its potentiation effect on inositol lipid hydrolysis in both unstimulated and agonist-stimulated tissue.

In the unstimulated tissue, the *myo*-(2-^{3}H)-inositol-labelled inositol phospholipid content of tissue incubated in Ca^{2+}-free solution was appreciably higher than that in a Ca^{2+}-containing solution. The reason for this effect in control tissue is not clear. A possible explanation may be that this effect is due to an inhibition by Ca^{2+} of incorporation of *myo*-(2-^{3}H) inositol into inositol phospholipid. However, this is unlikely since using the *in vivo* labelling method there is only a small pool of free *myo*-(2-^{3}H) inositol and the results in Chapter 4 indicate that (^{3}H)-inositol released from inositol phospholipid is not re-incorporated within 30 min. Alternatively, removal of Ca^{2+} bound to inositol phospholipid on the external surface of the cell membrane, by EGTA may alter the properties of the lipid and decrease basal turnover.

Previous studies, using *in vitro* labelling of PtdIns with (^{32}P)-P_i showed that the PtdIns content of unstimulated hepatocytes (Billah and Michell, 1979) and rat parotid gland fragments (Jones and Michell, 1975)

was greater in Ca^{2+}-free media than in the presence of Ca^{2+}. Since these studies relied on the incorporation of (^{32}P)-P_i into PtdIns it is likely that this effect of Ca^{2+} on PtdIns content was due to inhibition by Ca^{2+} of PtdIns synthesis (Berridge and Fain, 1979; Tolbert et. al., 1980; Egawa et. al., 1981 a).

The potentiation by extracellular Ca^{2+} of agonist-stimulated PtdIns loss had been shown to occur in hepatocytes (Kirk et. al., 1981) and lymphocytes (Hui and Harmony, 1979), however, no such effect had previously been reported for the pancreas. The following discussion of the results of the present study describes identification of two sites on the external surface of the plasma membrane which are associated with potentiation of the hydrolysis of inositol phospholipid in stimulated and unstimulated pancreatic cells.

A role for extracellular Ca^{2+} in phosphoinositide hydrolysis (Tennes and Roberts, 1982) has subsequently been confirmed in hepatocytes (Creba et. al., 1983), lacrimal gland (Godfrey and Putney, 1984), and leukaemic basophil 2H3 cells (Beaven et. al., 1984). Evidence for an extracellular site for the effect of Ca^{2+} in these cells is described in detail later in this discussion.

Although inositol lipid breakdown is not stimulated by an increase in intracellular Ca^{2+}, the potentiation by Ca^{2+} occurring in agonist-stimulated cells could be due to a stimulated influx of Ca^{2+} from the extracellular medium to the cell cytosol or by an effect of Ca^{2+} binding to the external surface of the cell membrane. An attempt was made to investigate this using agents which might inhibit either or both of these effects of Ca^{2+}.

Tetracaine, a local anaesthetic, is a lipophilic compound which incorporates well into biological membranes (Seeman, 1972). This agent antagonises the contraction of smooth muscle (Feinstein, 1966; Feinstein and Paimre, 1969) and inhibits catecholamine secretion and Ca^{2+} flux in adrenal glands (Jaanus et. al., 1967; Rubin et. al., 1967). In addition, local anaesthetics can compete with and displace the membrane bound Ca^{2+} from the fixed negative sites on the membrane (Bondani and Karler, 1970; Feinstein and Paimre, 1969). At the time that these studies were commenced it was thought that local anaesthetics were Ca^{2+}-antagonists in glands (Marier et. al., 1978). No studies of the action of tetracaine on the pancreas had been made previously so its action as a Ca^{2+}-antagonist was examined.

Since tetracaine had no effect on carbachol-stimulated inositol phospholipid breakdown in the presence of extracellular Ca^{2+}, this suggested that perhaps Ca^{2+} influx or Ca^{2+} bound to the plasma membrane may not be responsible for potentiating this response. Surprisingly, however, tetracaine inhibited the effect of carbachol in the absence of extracellular Ca^{2+}. A possible explanation for this is that tetracaine may have a non-specific membrane-disruption effect or alternatively be acting more specifically to block the muscarinic receptor. The lack of effect of tetracaine on peptide-receptors had been demonstrated previously in parotid gland by Marier et. al. (1978) since the ^{86}Rb efflux response stimulated by carbachol was inhibited by tetracaine while substance P was unaffected. A direct comparison between the effect of tetracaine on carbachol and CCK-8-stimulated inositol lipid breakdown cannot be made since equiactive concentrations of agonists were not used. It is possible, however, that the inability

of tetracaine to alter the effect of CCK-8 in Ca^{2+}-free Tris-Krebs solution was due to the lack of effect of the local anaesthetic on the peptide receptor.

Although local anaesthetics had been found to inhibit cholinergic muscarinic receptors in excitable tissues (Fields et. al., 1978; Burgermeister et. al., 1978) it was not until Putney and Van de Walle (1980 b) showed procaine (10^{-3}M) inhibited (^{3}H)-QNB binding in parotid gland that the first direct evidence of this effect in secretory, non-excitable tissue was shown. The studies with tetracaine described in the present study were just completed when the study by Putney and Van de Walle (1980 b) offered an explanation for the effect of tetracaine in the absence of extracellular Ca^{2+}. Aguilar et. al. (1980) provided evidence to suggest that local anaesthetics inhibit (^{3}H)-QNB binding to the muscarinic receptor by acting at some accessory site but not on the true receptor site and so by an allosteric-like interaction with receptors inhibit specific binding. The absence of inhibition by tetracaine of carbachol-stimulated inositol phospholipid hydrolysis in Tris-Krebs solution suggests that perhaps Ca^{2+} binds to this accessory site and so prevents the local anaesthetic binding. It is well known that local anaesthetics and Ca^{2+} compete for the negatively charged surface binding sites on the plasma membrane(Seeman, 1972). In Ca^{2+}-free Tris-Krebs solution, EGTA would remove the Ca^{2+} from these sites, allowing tetracaine free access and thereby inhibiting muscarinic receptor binding and the inositol lipid response. Direct effects of Ca^{2+} on (^{3}H)-QNB binding have been described (Aronstam et. al., 1977) but this finding has been disputed by Fairhurst et. al. (1980) and Aguilar et. al. (1980).

These experiments with tetracaine did not eliminate the possible role of Ca^{2+} bound to the cell surface nor Ca^{2+} influx in potentiation of inositol phospholipid hydrolysis.

The next step was to use an agent which would inhibit Ca^{2+} influx and so allow an examination of whether this event, in association with activation of the receptor could cause potentiation of phosphoinositide hydrolysis. The Ca^{2+}-antagonist D600 was one such candidate. It was at first questionable whether D600 would block Ca^{2+} influx in pancreas since its well known action was to block voltage-dependent Ca^{2+} channels such as those in cardiac fibres (Kohlardt et. al., 1972) and smooth muscle (Golenhofen and Hermstein, 1975), whereas Ca^{2+} influx occurs via a potential-independent mechanism in pancreas (Poulsen and Williams, 1977).

However, D600 (10^{-4}M) was shown to inhibit secretagogue-stimulated enzyme secretion from pancreas (Schulz, 1975) and cause a small reduction in CCK-8-stimulated Ca^{2+} influx into pancreas cells (Kondo and Schulz, 1976), suggesting that this agent may be a possible Ca^{2+}-antagonist in pancreas. Nishiyama and Petersen (1975) found that D600 (10^{-4}M) had no effect on acetylcholine-induced depolarization and resistance changes in mouse pancreas, however, this result does not necessarily mean that D600 does not alter Ca^{2+} influx since the electrical changes in the acinar cell membrane could be the result of release of Ca^{2+} from intracellular stores.

It was therefore decided to examine D600 as a Ca^{2+}-antagonist in the pancreas. The results of the present study showing no significant reduction in agonist-stimulated inositol phospholipid hydrolysis by D600, suggested that agonist-stimulated Ca^{2+} influx was not responsible

for the potentiation effect caused by Ca^{2+}. However, this conclusion was modified by the results of a more recent study by Dormer et. al. (1981), which showed that D600 (10^{-4}M) did not alter bethanechol-stimulated Ca^{2+} uptake in pancreatic acini, in contrast to the conclusion by Kondo and Schulz (1976).

The action of D600 as a Ca^{2+}-antagonist in pancreas then became questionable. In addition, another study reported at that time (Putney, 1981) suggested that the inhibition by D600 (10^{-3}M) of agonist-stimulated ^{86}Rb efflux in parotid gland may be due to blockade of the muscarinic receptor rather than inhibition of Ca^{2+} influx. Fairhurst et. al. (1980) had found D600 blocked this receptor in brain.

However, the lack of effect of D600 (at a lower concentration than that used by Putney (1981) to inhibit receptor binding) on the acetylcholine-stimulated depolarization of acinar cell membranes (Nishiyama and Petersen, 1975) suggested that D600 (at 10^{-4}M) did not act as a receptor-blocking agent in the pancreas. Due to the controversy as to the specificity of D600 it then became clear that a more specific antagonist of Ca^{2+} was required.

Neomycin, a cationic aminoglycoside antibiotic inhibited the influx of Ca^{2+} in rat parotid gland (Marier et. al., 1978; Putney, 1981; Aub et. al., 1982). The efflux of ^{86}Rb from parotid gland fragments occurs in two phases. The initial transient phase occurs as a result of release of Ca^{2+} from cellular stores whereas the subsequent sustained phase is dependent on the influx of Ca^{2+} from the external medium

(Putney, 1978). Neomycin (3×10^{-3}M) significantly reduced the carbachol-stimulated sustained phase of ^{86}Rb efflux but had no effect on the transient phase (Marier et. al., 1978). These results indicate inhibition of Ca^{2+} influx and also that neomycin (3×10^{-3}M) does not act to block binding of agonist to the muscarinic receptor since both the transient and sustained phases would be inhibited. A subsequent study by Aub et. al. (1982) examined the refilling of the cellular pool of Ca^{2+} mobilized by receptor activation and found that neomycin 3×10^{-3}M and 10^{-2}M, reduced and inhibited respectively, the influx into this pool.

In the present study neomycin (10^{-2}M) caused inhibition of agonist-stimulated amylase secretion indicating a possible Ca^{2+}-antagonist action but had no effect on inositol phospholipid hydrolysis. These results suggest that the potentiation by Ca^{2+} of lipid hydrolysis is not due to the passage of Ca^{2+} through the acinar cell plasma membrane. A more definite conclusion could be made if the effect of neomycin on $^{45}Ca^{+}$ flux were made in association with inositol lipid hydrolysis studies, but it was not possible to do $^{45}Ca^{2+}$ flux measurements at that time. The results in parotid gland provided good evidence that neomycin does inhibit Ca^{2+} influx in exocrine gland (Marier et. al., 1978; Putney et. al., 1981; Aub et. al., 1982) so it seems reasonable that the inhibition of amylase secretion by neomycin was due to inhibition of Ca^{2+} influx.

Neomycin binds strongly to PtdIns-4,5P_2 (Llodhi et. al., 1976) and this effect appears to be responsible for the inability of the phosphoinositide phosphodiesterase to hydrolyse PtdIns-4,5P_2 and PtdIns-4P in

erythrocyte membranes in the presence of neomycin (5×10^{-3} and 10^{-2}M), (Downes and Michell, 1981). Although neomycin may bind to phosphoinositides, the results of the present study suggest that if this does occur in exocrine pancreas, it does not significantly affect receptor-activated inositol lipid breakdown.

Since the potentiation by Ca^{2+} of inositol lipid hydrolysis did not appear to be due to Ca^{2+} influx the effect of Ca^{2+} bound to the external surface of the plasma membrane was examined.

The aim of the use of the cations, La^{3+} and Mn^{2+} was to compare the ability of these ions which have different affinities for the Ca^{2+}-binding site on the plasma membrane with their ability to reduce the potentiation of agonist-stimulated inositol phospholipid hydrolysis by Ca^{2+}. An ion's potency as a Ca^{2+}-displacer has been found to be dependent on its non-hydrated ionic radius (Langer et. al., 1974). The closer this radius is to that of Ca^{2+} (0.99Å) the more effective it is in competing with Ca^{2+} for its binding site. Therefore, the effects of La^{3+} (1.016Å) and Mn^{2+} (0.80Å) on inositol phospholipid hydrolysis were compared. These ions can also inhibit Ca^{2+} influx but the results with neomycin eliminated this effect in contributing to the potentiation so the role of externally bound Ca^{2+} could be more specifically examined.

La^{3+}, which does not enter pancreatic acinar cells (Wakasugi et. al., 1981) has a similar ionic radius to Ca^{2+} and is able to displace competitively the divalent cation from superficial sites on the surface of the cell membrane. Since La^{3+} has a higher valency than Ca^{2+} it binds to these sites with greater affinity than

Ca^{2+} and can inhibit the binding of Ca^{2+} and block its movement across the membrane (Seeman, 1972).

An early study by Chandler and Williams (1974) showed that not only did La^{3+} ($10^{-2}M$) decrease $^{45}Ca^{2+}$ influx in pancreatic fragments but also caused a reduction in total $^{45}Ca^{2+}$ content, suggesting externally bound Ca^{2+} was displaced. This reduction in total $^{45}Ca^{2+}$ content would not be due to an undetected $^{45}Ca^{2+}$ efflux since La^{3+} (1,2 and 5mM) was found to inhibit Ca^{2+} efflux from pancreatic acinar cells (Wakasugi et. al., 1981).

A study by Keryer and Rossignol (1978) used La^{3+} to examine the Ca^{2+}-dependency of PtdIns metabolism in rat parotid glands. The method of *in vitro*-labelling used, resulted in a large pool of free *myo*-(2-^{3}H) inositol since on addition of carbachol a sharp rise in (^{3}H)-PtdIns content occurred followed by a decrease in (^{3}H)-PtdIns. When La^{3+} ($5 \times 10^{-4}M$) was also present with carbachol, the synthesis of (^{3}H)-PtdIns was reduced to near control levels (Keryer and Rossignol, 1978). It was not possible from their study to determine whether carbachol-stimulated loss of PtdIns was inhibited since their technique measured the net result of synthesis and breakdown, and synthesis was reduced with La^{3+}.

6.4 (a) The Effect of Lanthanum in Agonist-stimulated Tissue

The effect of La^{3+} on CCK-8 and bethanechol-stimulated inositol lipid hydrolysis in Tris-Krebs solution was similar to the effect of removal of Ca^{2+} from the extracellular medium,

indicating that La^{3+} antagonizes the potentiation effect of Ca^{2+}. This effect of La^{3+} could be due to displacement of Ca^{2+} from binding sites on the external surface of the plasma membrane.

An alternative explanation for the effect of this trivalent ion may be to inhibit receptor binding. In a study by El-Fakahany and Richelson (1981), lanthanides caused an increase in the maximal binding capacity but an apparent decrease in the binding affinity of (^3H)-QNB for intact mouse neuroblastoma cells. This effect of lanthanides on (^3H)-QNB binding was reduced by Ca^{2+}. The results presented here show that in the absence of Ca^{2+}, when any possible sites for altering agonist binding would be freely accessible to La^{3+}, agonist-stimulated inositol lipid breakdown was unchanged, suggesting that La^{3+} does not reduce phosphoinositide hydrolysis by an action on agonist binding to receptors.

The results of the present study suggest that Ca^{2+} acts to potentiate the agonist-stimulated hydrolysis of inositol phospholipid at a site on the outside of the pancreatic acinar cell which is La^{3+} sensitive.

How might Ca^{2+} bound to this site potentiate the agonist-stimulated hydrolysis of inositol phospholipid? One possibility may be through an action on receptor binding. There is conflicting evidence as to the effect of Ca^{2+} on (^3H)-QNB binding to muscarinic receptors, it may increase (Hedlund and Bartfai, 1979), decrease (Birdsall et. al., 1979) or have no

effect (Aronstam et. al., 1977; El-Fakahany and Richelson, 1981) on antagonist binding. However, the effect of Ca^{2+} on (^3H)-QNB binding may not reflect the actual effect on agonist-binding since the binding of agonists appears to be more sensitive to the effects of sodium ions and guanine nucleotides than the binding of classical antagonists (Birdsall et. al., 1980; Ehlert et. al., 1980 a, b). No studies of the effect of Ca^{2+} on agonist binding to muscarinic or peptide receptors in pancreas have been made so the possibility that Ca^{2+} may enhance agonist receptor binding cannot be excluded. However, if this were to occur the action must be common to the interaction of both muscarinic and peptide agonists to their receptors since both are potentiated.

The mechanism by which Ca^{2+} causes potentiation is not known. One possibility could be that although the interaction between enzyme and phosphoinositide may not have an absolute requirement for Ca^{2+} (since it occurs in EGTA containing solutions), this ion may be able to increase the efficacy of the interaction and thereby cause potentiation. Because so little is known about the mechanism which links receptor-occupation with inositol phospholipid hydrolysis it is difficult to determine how Ca^{2+} might have its effect.

A recent report by Beaven et. al. (1984) confirmed the ability of La^{3+} to alter agonist-stimulated hydrolysis of inositol phospholipid. La^{3+} (10^{-4}M and 5×10^{-4}M) inhibited the antigen-stimulated hydrolysis of inositol phospholipid and Ca^{2+} influx

in rat basophil 2H3 cells. The inhibition by La^{3+} of Ca^{2+} influx in these cells was not responsible for the effect on phosphoinositide since La^{3+} ($10^{-5}M$) substantially inhibited Ca^{2+} influx and histamine release but had a negligible effect on lipid breakdown. In addition, A23187 and Ca^{2+} caused only a small increase in (^{3}H)-inositol-phosphates. Therefore, the effect of Ca^{2+} was identified to be extracellular. The study by Beaven et. al. (1984) suggested that a common Ca^{2+} binding site(s) for both the activation of breakdown and the mechanism which stimulates the influx of Ca^{2+}. The mode of action of Ca^{2+}, however, is not known.

6.4 (b) The Effect of Lanthanum in Unstimulated Tissue

The potentiation by Ca^{2+} of inositol phospholipid hydrolysis in unstimulated tissue was not reduced by La^{3+}. The differential effect of this trivalent ion on Ca^{2+} potentiation in unstimulated and agonist-stimulated tissue suggests that 2 sites of potentiation of lipid hydrolysis may exist; one involved in stimulated-hydrolysis, the other in basal turnover.

To further examine the potentiation of agonist-stimulated inositol lipid hydrolysis by Ca^{2+}, Mn^{2+} which has an atomic radius of within 0.19$\mathring{A}$ of Ca^{2+} (as compared to that for La^{3+} of within 0.026$\mathring{A}$ of Ca^{2+}) was examined.

6.4 (c) The Effect of Manganese in Agonist-stimulated Tissue

Mn^{2+} significantly reduced carbachol-stimulated inositol phospholipid hydrolysis in Tris-Krebs solution; the effect of CCK-8 was reduced but this was not significant. Since equiactive concentrations of carbachol and CCK-8 were not used the conclusion that the two receptor types have differential sensitivities to Mn^{2+} can not be made. It is possible that the breakdown of lipid stimulated by a lower concentration of CCK-8 would be inhibited by Mn^{2+} (10^{-2}M).

There appears to be a similarity between the effect of La^{3+} and Mn^{2+} to remove the potentiation by Ca^{2+} of agonist-stimulated breakdown of inositol phospholipid. In the absence of extracellular Ca^{2+}, Mn^{2+} replaced Ca^{2+} to potentiate CCK-8-stimulated inositol lipid breakdown. Although carbachol-stimulated breakdown of lipid was increased with Mn^{2+} this was not significantly greater than the additive effect of each agent alone. Mn^{2+} appears to be partially active at the Ca^{2+} potentiation site.

Mn^{2+} (10^{-3}M and 10^{-2}M) was shown to inhibit amylase secretion from pancreas stimulated by either peptidergic or by cholinergic stimuli (Heisler et. al., 1972; Kanno and Nishimura, 1976; Argent et. al., 1982), although Petersen and Ueda (1976) did not detect any effect of Mn^{2+} (5×10^{-3}M) on acetylcholine-stimulated amylase secretion. The study by Argent et. al. (1982) concluded that not only does Mn^{2+} inhibit Ca^{2+} influx but also displaces Ca^{2+} from cellular membranes of pancreatic acinar cells.

The ability of Mn^{2+} to remove the Ca^{2+} potentiation confirms the results with La^{3+} that there is a Ca^{2+}-binding site on the external surface of the pancreatic acinar cell which is involved in potentiating phosphoinositide hydrolysis.

6.4 (d) The Effect of Manganese in Unstimulated Tissue

Mn^{2+} mimicked the effect of Ca^{2+} in the absence of secretagogues to cause an increase in inositol phospholipid hydrolysis in Ca^{2+}-free Tris-Krebs solution.

This stimulatory action on lipid hydrolysis was not due to the action of Mn^{2+} to cause release of acetylcholine from nerve terminals since it occurred in the presence of atropine.

Stimulation of the inositol-exchange enzyme by Mn^{2+} (Paulus and Kennedy, 1960) would cause an apparent increase in the breakdown of inositol phospholipid due to an increase in free (^{3}H)-inositol. However, this effect does not explain the results obtained in the present study. If inositol-exchange was responsible for the increase in lipid breakdown it would occur both in the presence and absence of Ca^{2+}, since this enzyme is only inhibited by high concentrations of Ca^{2+} (5mM) (Egawa et. al.,1981 a)which are not attained intracellularly. Because Mn^{2+} only has a stimulatory effect in Ca^{2+}-free Tris-Krebs solution, then activation of inositol-exchange was not involved.

Unlike La^{3+}, however, Mn^{2+} can enter into pancreatic acinar cells and displace Ca^{2+} from the binding sites of intracellular organelles (Argent et. al., 1982). It is unlikely that this effect of Mn^{2+} was responsible for an increased lipid hydrolysis in the absence of secretagogues since the results of the present study show that an increase in intracellular Ca^{2+} concentration does not stimulate inositol phospholipid hydrolysis in exocrine pancreas.

6.4 (e) Concluding Summary

These studies using calcium antagonists led to the conclusion that there are two sites at which Ca^{2+} acts to potentiate inositol phospholipid breakdown:

(i) The site associated with potentiation of basal breakdown

This site is not La^{3+}-sensitive and could be intracellular since Mn^{2+}, which enters cells, can mimic the effect of Ca^{2+}. However, since neomycin which would block Ca^{2+} entry, did not alter this potentiation by Ca^{2+} it appears that the site is extracellular and Mn^{2+} can replace Ca^{2+} at this site.

(ii) The site associated with potentiation of agonist-stimulated breakdown

This site is external since neomycin did not reduce potentiation. The removal of potentiation by La^{3+} and Mn^{2+} indicates that this site is accessible to both ions,

acting to remove Ca^{2+} from the external surface of the cell membrane. There is a suggestion, from the results with CCK-8+Mn^{2+} and CCK-8+La^{3+} in the absence of extracellular Ca^{2+}, that these ions are partially active at this site to replace Ca^{2+}. To examine this more fully would require dose-response curves for CCK-8-induced lipid hydrolysis in the presence of La^{3+} or Mn^{2+} at a range of concentrations.

An extracellular site of potentiation by Ca^{2+} has also been demonstrated recently in rat hepatocytes (Creba et. al., 1983) and lacrimal gland (Godfrey and Putney, 1984).

In rat hepatocytes, the hormone-stimulated loss of (^{32}P)-PtdIns-4,5P_2 was reduced by 50% in the presence of EGTA, but not abolished since a significant decrease in this lipid was still seen after 15 min of Ca^{2+}-deprivation (Creba et. al., 1983). The potentiation by Ca^{2+} was not due to Ca^{2+} influx since an increase in intracellular Ca^{+} with ionophore did not stimulate hydrolysis (Creba et. al., 1983).

Godfrey and Putney (1984) using rat lacrimal glands measured inositol phospholipid hydrolysis as release of (^{3}H)-inositol-phosphates. Lowering the external Ca^{2+} concentration to less than 1μM caused a 50% decrease in agonist-stimulated inositol-phosphate formation compared with samples containing 1mM Ca^{2+}.

Using the protocol of 'cross-receptor inactivation' the

potentiation effect was identified to be extracellular (Godfrey and Putney, 1984). 'Cross-receptor inactivation' is a method in which Ca^{2+} stores are released by addition of agonist and the subsequent refilling of these stores is blocked by antagonist (Putney, 1977). This protocol then allows the binding of an agonist to a different receptor without the concomittant release of Ca^{2+} from stores. Godfrey and Putney (1984) showed that incubation of lacrimal gland in EGTA, and elimination of internal Ca^{2+} release (using the above protocol) did not result in an additional inhibition of agonist-stimulated inositol lipid hydrolysis beyond that seen when external Ca^{2+} was removed. This suggests that there may be a role for extracellular Ca^{2+} or membrane-bound Ca^{2+} in coupling receptors to subsequent enzyme reactions. In lacrimal gland phosphoinositide breakdown depends partially on extracellular Ca^{2+}, but does not appear to result from the receptor-induced increase in intracellular Ca^{2+}.

The mechanism by which Ca^{2+} potentiates inositol lipid hydrolysis is not known. A recent study by Burgess et. al. (1983) showed that the ability of noradrenaline to alter the order of membrane lipids and so increase the fluidity of isolated rat liver plasma membranes was dependent on the presence of Ca^{2+}. Addition of EGTA increased membrane fluidity and removed the effect of noradrenaline. It was suggested (Burgess et. al., 1983) that hormones act to alter membrane fluidity by displacing Ca^{2+} bound to the plasma membrane and thereby remove the mechanical constraints of Ca^{2+} on phospholipids. To examine this question,

an examination of the ability of agonists to release Ca^{2+} from isolated plasma membranes labelled with $^{45}Ca^{2+}$, could be made.

Burgess et. al. (1983) suggested that Ca^{2+} ions may be directly involved in the step linking receptor-activation and changes in membrane lipids. The problem with using isolated plasma membranes to examine the potentiation effect of Ca^{2+} is that the cell fractionation process involved in preparing plasma membranes is one which could cause loss of Ca^{2+} from some essential site. In the study by Burgess et. al. (1983) it is possible that EGTA may have access to Ca^{2+} binding sites which are not normally accessible in intact cells. For example, EGTA may remove Ca^{2+} from both the extracellular and intracellular sides of the plasma membrane, rather than examine extracellular Ca^{2+}.

A more specific identification of the location of these Ca^{2+}-binding sites on the external surface of the cell may provide a clearer understanding of the modulation by Ca^{2+} of this lipid response.

In conclusion, it appears that the agonist-stimulated hydrolysis of inositol phospholipid occurs prior to an increase in intracellular Ca^{2+} since it is independent of the release of Ca^{2+} from membrane stores. Also, the lipid response is not stimulated by an increased cytosolic concentration of Ca^{2+}. The potentiation

by Ca^{2+} of inositol lipid hydrolysis in stimulated and unstimulated tissue appears to occur at two distinct sites on the external surface of the cell. The mechanism of potentiation requires further investigation.

CHAPTER 7

THE RELATIONSHIP BETWEEN THE HYDROLYSIS OF INOSITOL PHOSPHOLIPID AND THE SECRETION OF AMYLASE IN MOUSE EXOCRINE PANCREAS

7.1 : INTRODUCTION

Although the hydrolysis of PtdIns, PdtIns-4,5P_2 and PtdIns-4P has been studied in a variety of tissues there has been little attempt to correlate the inositol phospholipid hydrolysis with the physiological response of the cell.

The extent of platelet activation and inositol phospholipid breakdown stimulated by platelet-activating factor show similar time-related changes (Lapetina, 1982). A comparison of the dose-dependent relationship between inositol phospholipid breakdown and the final cell response has been made in blowfly salivary salivary gland (Fain and Berridge, 1979), hepatocytes (Kirk et. al., 1981; Creba et. al., 1983) and guinea-pig ileum (Watson and Downes, 1983). These dose-response curves for agonist-stimulated inositol phospholipid hydrolysis are displaced to the right in relation to the dose-response curves for the final physiological responses of fluid secretion and Ca^{2+} transport (Fain and Berridge, 1979), glycogen phosphorlyase activation and Ca^{2+} release (Kirk et. al., 1981) and muscle contraction (Watson and Downes, 1983). These studies showed that inositol phospholipid hydrolysis is produced by concentrations of agonists which are supramaximal for the physiological response. This discrepancy in the dose-response curves for the events can be explained by the presence of spare receptors (Stephenson, 1956)whereby the Ca^{2+}-dependent final cell response can be maximal when only a proportion of the total receptor population is occupied. Kirk et. al. (1981) showed that the vasopressin-stimulated breakdown of PtdIns-4,5P_2 correlated well with the occupation of receptors by (Lys^8)-vasopressin in hepatocytes. However, there has not been a convincing correlation of the degree of inositol phospholipid hydrolysis and the extent of the Ca^{2+}-dependent response of secretion in the exocrine pancreas.

Secretion of protein from the exocrine pancreas is stimulated by a number of agonists which cause an increase in the intracellular Ca^{2+} concentration. A biphasic dose-response relationship is characteristic of pancreatic amylase secretion stimulated by cholinergic agonists and cholecystokinin peptides (Williams, 1975 b; Jensen et. al., 1980; Williams et. al., 1981), where increasing the concentration of agonist produces an increase in enzyme secretion to a maximum, and further increases in agonist concentration cause submaximal secretion. Recent studies (Savion and Selinger, 1978; Roberts and Woodland, 1982; Burnham and Williams, 1982 b) have shown that the decrease in pancreatic amylase secretion with high agonist concentrations is a Ca^{2+}-dependent phenomenon.

The aim of this study was to compare the ability of a number of secretagogues acting through different classes of receptors to stimulate both the breakdown of inositol phospholipid and the secretion of amylase and thus determine if the relationship between these two responses supported the proposal that inositol phospholipid breakdown may control the agonist-stimulated entry of Ca^{2+} into these cells.

7.2 : MATERIALS AND METHODS

Amylase secretion and inositol lipid hydrolysis in response to carbachol, bethanechol (carbamyl-β-methylcholine chloride), butyryl-choline chloride, CCK-8 and bombesin were measured in mouse pancreatic slices. The dose-response curve for bombesin-induced amylase secretion obtained with this preparation differed from that previously described for guinea-pig acini and rat acini (Deschodt-Lanckman et. al., 1976; Jensen et. al., 1978; Ehlemann et. al., 1979) and so the effect of bombesin on acini isolated from mouse pancreas was examined. Amylase secretion in

pancreatic slices and acini was measured as described in 3.7(a) and 3.7 (b). Inositol phospholipid hydrolysis was measured as described in 3.2. Tissue was incubated in the presence of a range of concentrations of secretagogues.

To investigate the extent of inositol lipid hydrolysis at the low agonist concentrations, the recently developed method of Berridge et. al. (1982) was used. This method has been shown to be a sensitive assay for the measurement of inositol lipid hydrolysis (Berridge et. al., 1982; Watson and Downes, 1983). These studies used *in vitro* labelling of tissue with *myo*-(2-^{3}H) inositol which would result in a significant pool of free (^{3}H)-inositol in the tissue. Since the method of Berridge et. al. (1982) allows the separation of accumulated (^{3}H)-inositol-1-phosphate from this free pool of (^{3}H)-inositol it can provide a good signal-to-noise ratio for assay of increases of inositol lipid hydrolysis. Lithium is used to inhibit inositol-1-phosphatase.

The present experiments aimed to use the method of Berridge et. al. (1982) on *in vivo* labelled pancreatic tissue to examine the breakdown of inositol lipid at low concentrations of carbachol.

Pancreatic tissue which had been labelled *in vivo* with 122kBq of *myo*-(2-^{3}H) inositol, was washed and then incubated in Krebs solution (in which the NaCl was replaced with 126mM LiCl) for 30 min in the presence of carbachol at a range of concentrations. The incubation was terminated by freezing the samples in a dry-ice/alcohol bath. Samples were assayed for the release of (^{3}H)-inositol phosphates as described in 5.2.

7.3 : RESULTS

Secretion of amylase in mouse pancreatic slices was stimulated by agonists acting on cholinergic, cholecystokinin or bombesin receptors. The relative potencies of agonists to stimulate a maximal secretion were in the order CCK-8 > bombesin > carbachol > bethanechol = butyrylcholine (Fig. 7.1). The secretion of amylase for all agonists was biphasic, high concentrations of CCK-8, bombesin, carbachol and bethanechol being able to reduce amylase secretion by at least 45% of that produced by their optimal concentrations. Butyrylcholine was less efficacious than the other agonists in causing inhibition of secretion (Fig. 7.1).

The relative potencies of agonists to stimulate the breakdown of inositol phospholipid (Fig. 7.1) were in the same order as that for stimulation of a peak amylase secretion. A comparison of amylase secretion and breakdown of inositol phospholipid in pancreatic slices (Fig. 7.1) shows that the maximal secretion of amylase occurred at concentrations of agonists which produced only a small degree of breakdown of inositol lipid. For the different secretagogues, the degree of breakdown corresponding to peak secretion of amylase was remarkably constant and ranged between 68-87 Bq/kBq ^{3}H incorporated by the pancreas, above control (Table 7.1). Concentrations of agonists which increased the breakdown of inositol phospholipid above this level inhibited amylase secretion. With butyrylcholine, increasing its concentration from $10^{-5}M$ to $10^{-4}M$ stimulated an increase in the breakdown of inositol phospholipid and a reduction in secretion. However, increases in concentrations from $10^{-4}M$ to $10^{-2}M$ did not cause any further increase in inositol phospholipid breakdown and similarly, no further decrease in secretion occurred.

The inhibition of amylase secretion by high concentrations of bombesin was unexpected since previous studies had shown little inhibition when bombesin was used on rat or guinea-pig pancreatic acini (Deschodt-Lanckman et. al., 1976; Jensen et. al., 1978; Uhlemann et. al., 1979). In order to confirm the results using bombesin in mouse pancreas, amylase secretion by bombesin in mouse pancreatic acini was measured. High concentrations of bombesin produced submaximal secretion (Fig. 7.2) similar to that measured in mouse pancreatic slices. The potency of bombesin to stimulate maximal amylase secretion was greater in mouse pancreatic acini (Fig. 7.2) than in pancreatic slices (Fig. 7.1).

Using the method described by Berridge et. al. (1982) the hydrolysis of inositol phospholipid in the presence of carbachol 10^{-8}M, 10^{-7}M, 10^{-6}M and 10^{-4}M was measured and the (^{3}H)-inositol-1-phosphate present above control (Bq/kBq ^{3}H incorporated by the pancreas) was 28 ± 6, 27 ± 4, 84 ± 24 and 283 ± 17, respectively (n = 8). The (^{3}H)-inositol-1-phosphate in control tissue was 30 ± 1 Bq/kBq ^{3}H incorporated by the pancreas. The method of Berridge et. al. (1982) appears to be no more sensitive than the method developed in the present study since inositol phospholipid breakdown at low concentrations of carbachol (10^{-8}M and 10^{-7}M) was not significantly increased above control and the hydrolysis stimulated by 10^{-6}M and 10^{-4}M carbachol was similar to that measured with the present assay (see Fig. 7.1, carbachol dose-response curve).

Fig. 7.1 : The Effect of Secretagogues on Amylase Secretion and the Hydrolysis of Inositol Phospholipids

Amylase secretion was measured as the increase in amylase released by the stimulated tissue above the control value which was obtained for each experiment, and is expressed as a percentage of the total amylase in the tissue (●). The results for basal secretion of amylase from experiments with each secretagogue were pooled and the mean ± S.E. calculated to be 8.4 ± 0.2% of the total amylase present (n = 85).

Inositol phospholipid hydrolysis was measured as the release of water-soluble (^{3}H)-labelled products from the inositol phospholipids above the control value obtained for each experiment and is expressed as Bq/kBq ^{3}H incorporated by the pancreas (■). The results for ^{3}H released from control samples in each experiment were pooled and the mean ± S.E. calculated to be 259 ± 11 Bq/kBq ^{3}H incorporated by the pancreas (n = 48) during the 30 min incubation.

All values plotted are the mean ± S.E. of at least 6 samples. Where no error bar is visible it did not extend beyond the limits of the symbol.

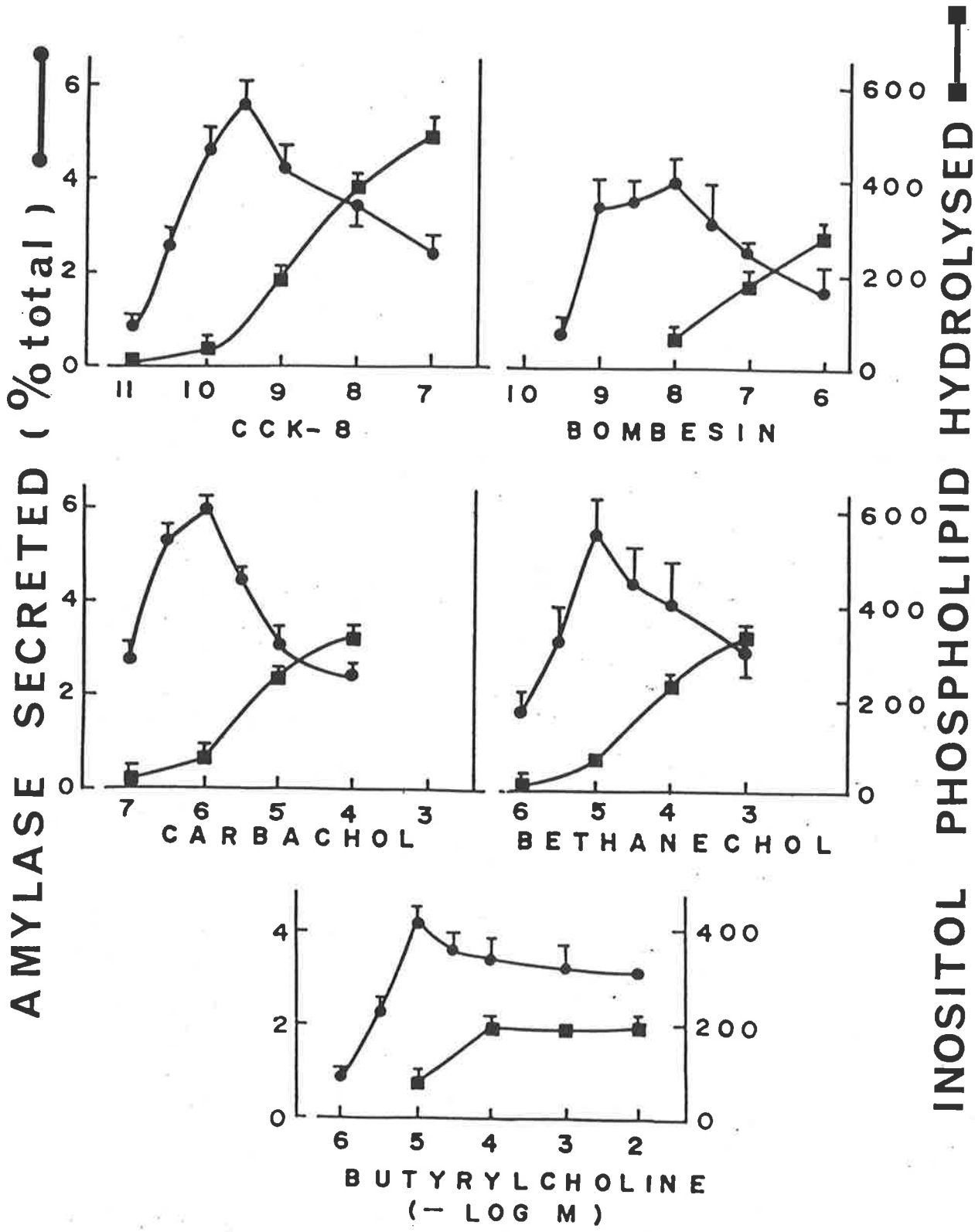


Fig. 7.1 : The Effect of Secretagogues on Amylase Secretion and the Hydrolysis of Inositol Phospholipids

Table 7.1 : The breakdown of inositol phospholipid corresponding to maximal amylase secretion.

AGONIST	CONCENTRATION OF AGONIST PRODUCING MAXIMAL AMYLASE SECRETION	AMYLASE SECRETED ABOVE CONTROL (% TOTAL)	INOSITOL PHOSPHOLIPID HYDROLYSED ABOVE CONTROL (Bq/kBq ^{3}H INCORPORATED BY PANCREAS)
Bombesin	10^{-8}M	3.98 ± 0.55 (12)	73 ± 8 (8)
Carbachol	10^{-6}M	5.94 ± 0.4 (19)	73 ± 11 (11)
Bethanechol	10^{-5}M	5.54 ± 0.77 (13)	68 ± 9 (8)
Butyrylcholine	10^{-5}M	4.2 ± 0.3 (13)	87 ± 9 (8)
CCK-8	3.2×10^{-10}M	5.57 ± 0.47 (15)	80*

*This value for CCK-8 was determined from the curve in Fig. 7.1 for inositol phospholipid hydrolysis; all other values are the means of experimentally determined data.

All values represent the mean ± S.E. of the number of samples in brackets.

Fig. 7.2 : Bombesin-Stimulated Amylase Secretion in Mouse Pancreatic Acini

Amylase secretion was measured as the increase in amylase released by the stimulated tissue above the control value which was obtained for each experiment as is expressed as a percentage of the total amylase present in the tissue. Results represent the mean ± S.E. of at least 4 samples. The results for amylase released from control tissue in each experiment were pooled and calculated to be 7.7 ± 1.4% of the total amylase present in acini, (n = 4).

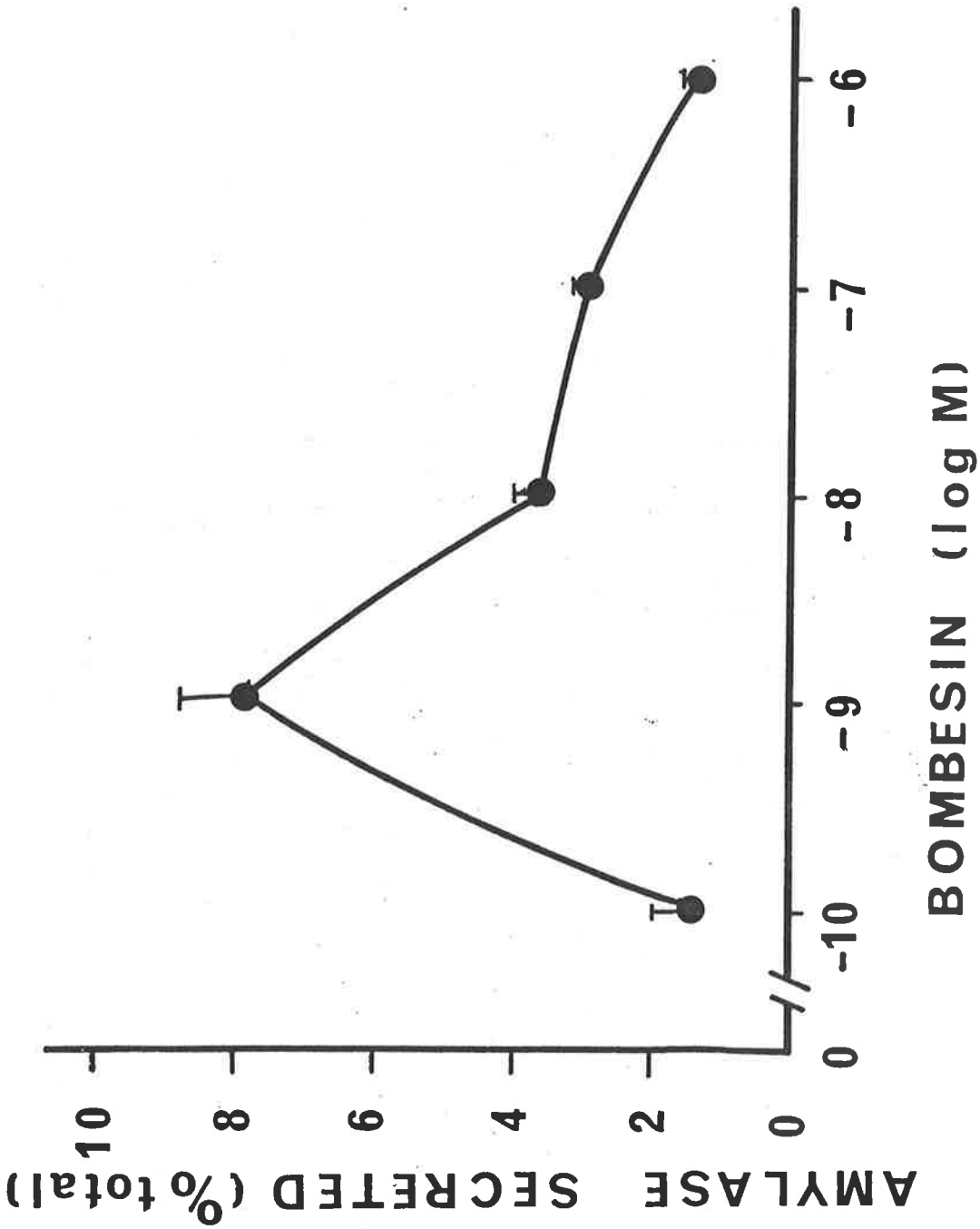


Fig. 7.2 : Bombesin-Stimulated Amylase Secretion in Mouse Pancreatic Acini

Fig. 7.3 : Comparison of Receptor Occupancy Curves (----), Amylase Secretion (□) and Inositol Phospholipid Hydrolysis (○) in the Mouse Exocrine Pancreas (Roberts et. al., 1984). Results for Amylase Secretion and Lipid Hydrolysis were Obtained from Fig. 7.1 and Expressed as a percentage of the Maximal Response

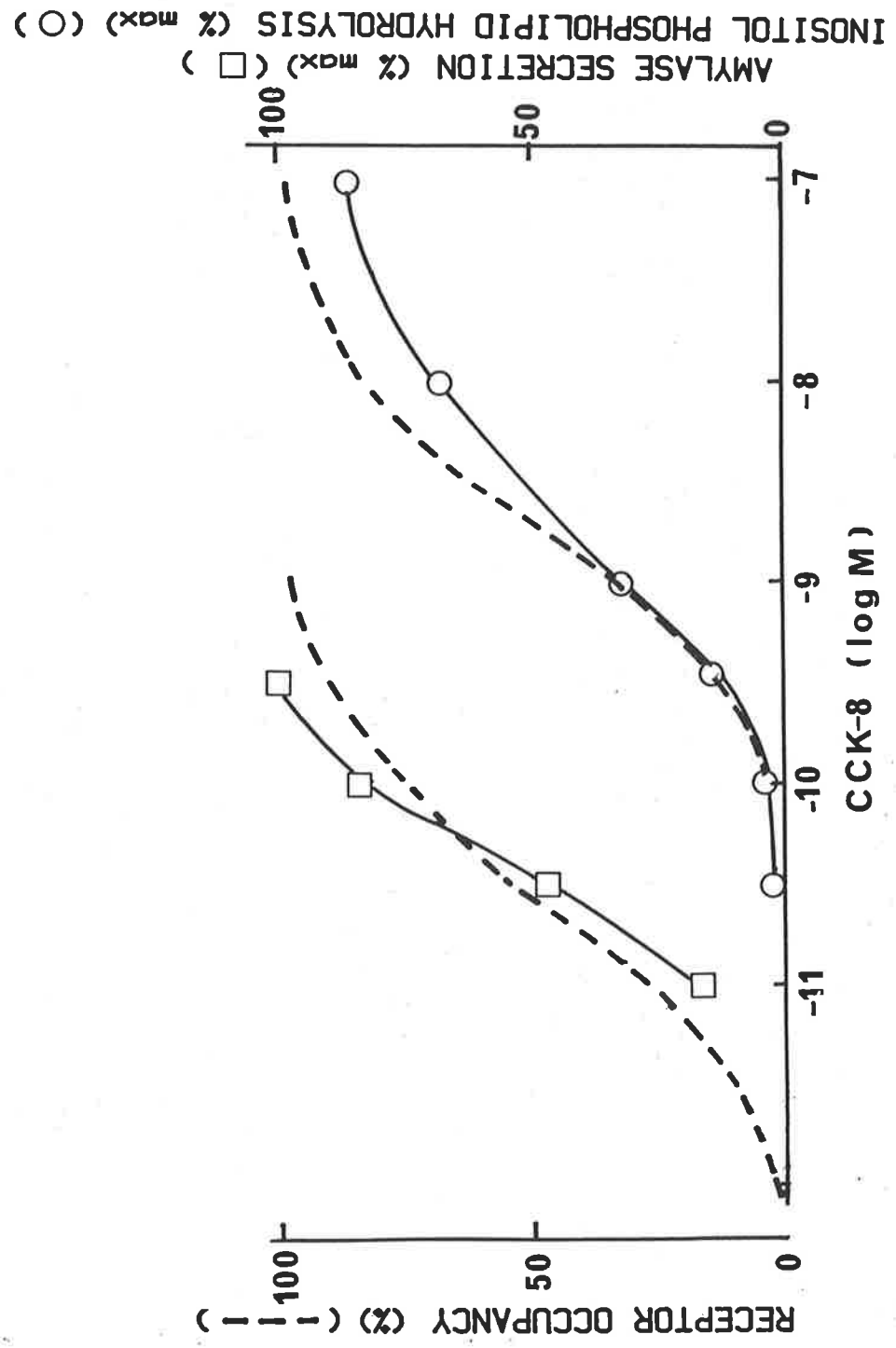


Fig. 7.3 : Comparison of Receptor Occupancy Curves, Amylase Secretion and Inositol Phospholipid Hydrolysis in the Mouse Exocrine Pancreas

7.4 : DISCUSSION

Both the stimulation and inhibition of amylase secretion from the pancreas are due to an increase in the intracellular Ca^{2+} concentration (Petersen and Ueda, 1976a; Williams et. al., 1978; Roberts and Woodland, 1982; Burnham and Williams, 1982b). The inhibition is due to a high Ca^{2+} concentration since this effect can be decreased by reducing the extracellular concentration of Ca^{2+} (Roberts and Woodland, 1982; Burnham and Williams, 1982b) and mimicked by the addition of Ca^{2+} with ionophore A23187 (Gardner et. al., 1980; Stark and O'Doherty, 1982). This inhibitory effect of Ca^{2+} on the secretory response may be a result of disruption of the microfilament system (Savion and Selinger, 1978; Burnham and Williams, 1982 b).

In the present study a variety of agonists were used to stimulate the exocrine pancreas from mice. The results show that there appears to be a correlation between the agonist-stimulated breakdown of inositol phospholipid and the inhibition of amylase secretion. Concentrations of secretagogues which produce a breakdown of more than about 9% of the inositol phospholipid (above control) of the pancreas inhibit amylase secretion. Butyrylcholine, which is only a partial agonist with respect to stimulation of the breakdown of inositol phospholipid, did not produce a large inhibition of amylase secretion.

Bombesin was chosen as an agonist since earlier studies with pancreatic acini from rat (Deschodt-Lanckman et. al., 1976) and guinea-pig (Jensen et. al., 1978) showed that little inhibition of secretion occurred. If inositol lipid hydrolysis is involved in the inhibition of secretion, as proposed by this study, then bombesin should not stimulate inositol lipid hydrolysis to a large degree. The results using mouse pancreatic

slices showed bombesin did cause inhibition of secretion and a corresponding increase in inositol phospholipid breakdown. The inhibition of amylase secretion by bombesin in mouse pancreas was supported by the results showing the same effect of this secretagogue on amylase secretion in mouse pancreatic acini. Experiments carried out in this laboratory by M.F. Crouch using guinea-pig pancreatic acini showed that high concentrations of bombesin did not cause a submaximal secretion of amylase in this species and correspondingly little hydrolysis of inositol phospholipid was stimulated.

The reason for the difference in potency of bombesin to stimulate inositol lipid hydrolysis in acini and slices is not clear. One possibility may be that there is greater access of bombesin for its receptor in acini than in slices. Williams et. al. (1978) reported a similar difference in potency of caerulein to stimulate amylase secretion in rats, where this secretagogue had greater potency in acini than in slices.

Since the agonist-stimulated breakdown of inositol phospholipid in the pancreas does not result from an increase in the intracellular concentration of Ca^{2+} (Tennes and Roberts, 1982) the present findings suggest that the converse may apply, that is, the agonist-stimulated breakdown of inositol phospholipid may control the increase in intracellular Ca^{2+} concentration provided by high concentrations of agonists and thus produce inhibition of amylase secretion.

In the pancreas, the cholecystokinin receptor exists in both high and low affinity states in rat (Sankaran et. al., 1980), guinea-pig (Jensen et. al., 1980) and mouse (Sankaran et. al., 1982). The muscarinic receptor in the rat pancreas also exists in these two states (Larose et.al., 1981).

It has been proposed that occupation of high affinity CCK receptors in mouse pancreas stimulates secretion while occupation of the low affinity receptors causes inhibition of secretion (Sankaran et. al., 1982). Using the values for the affinity of these two classes of CCK receptor in mouse pancreas, given by Sankaran et. al. (1982), the theoretical occupancy curves for the high and low affinity binding sites were calculated by Roberts et. al. (1984). The concentration-response curves for stimulation of amylase secretion and inositol phospholipid hydrolysis by CCK-8 obtained in the present study were plotted with these receptor-occupancy curves for the CCK receptor. As can be seen in Fig. 7.3, the increase in amylase secretion correlates with occupation of the high affinity receptors while hydrolysis of inositol phospholipid appears to correlate with occupation of the low affinity receptors.

The ability of high concentrations of bombesin to cause a decrease in maximal secretion and a corresponding increase in inositol phospholipid hydrolysis was in agreement with the results using the other secretagogues in mouse pancreas. It therefore appears that the mouse pancreas is different to the rat (Deschodt-Lanckman et. al., 1976) and guinea-pig (Jensen et. al., 1978) in respect to its response to high concentrations of bombesin.

Bombesin receptors have been demonstrated in the guinea-pig pancreas and appear to exist as a single class of high affinity binding sites (Jensen et. al., 1978). The results of the present study using mice suggest that unlike the guinea-pig, there may be high and low affinity states of this receptor in mouse pancreas. Studies of the binding of bombesin to its receptors in mouse pancreas is required to clarify this point. An alternative explanation is that there is only a single class

of bombesin receptor in the mouse pancreas and that in this species the coupling of receptor activation to the inositol phospholipid response is different, resulting in a greater efficiency for stimulating inositol phospholipid hydrolysis than in the guinea-pig. It is not known how this occurs but it is obviously an immediate area of concern.

The high concentrations of agonists which are required to produce breakdown of inositol phospholipids suggest that the breakdown of the lipid may be associated with activation of low affinity receptors, at least for the CCK and muscarinic receptor. Agonist-modulated glucose uptake (Sankaran et. al., 1982; Korc et. al., 1979) and amino acid uptake (Iwamoto and Williams, 1980) by pancreatic acinar cells are Ca^{2+}-dependent processes influenced by concentrations of agonists in the same range as those producing inhibition of secretion. The function of inositol phospholipid hydrolysis may also be to control the entry of Ca^{2+} for these and similar processes.

Low concentrations of five agonists which stimulated secretion of amylase in mouse pancreatic slices did not stimulate a significant breakdown of inositol phospholipid, measured using either the method developed in this study, or with that described by Berridge et. al. (1982). The method of Berridge et. al. (1982) did not cause an amplification of inositol lipid hydrolysis compared with the method used throughout the study. Since *in vivo* labelling of inositol phospholipid results in a small pool of free (^3H)-inositol, the method developed in the present study has a favourable signal-to-noise ratio and so is quite a sensitive assay for inositol lipid breakdown in mouse pancreas.

Recently Streb et. al. (1983) have shown that inositol-1,4,5-trisphosphate causes the release of Ca^{2+} from a non-mitochondrial intracellular store in rat pancreatic acini with permeabilized plasma membranes. The threshold concentration of inositol-1,4,5-trisphosphate which causes release of Ca^{2+} is somewhat less than 100nM. It is known from electrophysiological studies, that the release of Ca^{2+} takes place within 500 msec of the agonist binding to the receptors (Nishiyama and Petersen, 1975). If inositol-1,4,5-trisphosphate is the second messenger then it should reach a concentration of about 100nM within the first 500 msec of stimulation. It may be that the very small amount of inositol phospholipid hydrolysed by low concentrations of agonist is sufficient to generate the amount of inositol-1,4,5-trisphosphate which is required to release Ca^{2+} from cellular stores to stimulate secretion.

There have been other studies which have attempted to correlate the inositol phospholipid effect with the secretory response in the exocrine pancreas. An apparent correlation of increased PtdIns turnover and inhibition of amylase secretion has been reported previously for the pigeon pancreas (Hokin, 1968 b) and rat pancreas (Calderon et. al., 1980). These studies used the incorporation of (^{32}P)-P_i or (^{14}C)-acetate into PtdIns as a measure of PtdIns hydrolysis and it is now accepted that this indirect assay can lead to erroneous conclusions (Michell and Kirk, 1981). Recent studies (Crouch and Roberts, 1984) in this laboratory have shown a correlation between stimulation of amylase secretion at low concentrations of agonist and an increase in the synthesis of inositol phospholipid in mouse pancreatic acini. This further demonstrates that one can not use synthesis results to make conclusions about inositol lipid breakdown.

In addition, a recent study by Chapman et. al. (1983) showed that the stimulation of synthesis of PtdIns may not always occur as a direct result of inositol lipid breakdown since an agonist-stimulated increase in the synthesis *de novo* of PtdIns was measured.

A comparison of the study by Hokin (1974) investigating muscarinic stimulation of PtdIns breakdown with that by Williams (1975 b) on acetylcholine-stimulated amylase secretion in mouse pancreatic slices suggested a correlation between PtdIns breakdown and inhibition of amylase secretion. The present study provides the first direct comparison between inositol phospholipid breakdown and secretion in the pancreas by examining the effects of agonists which varied in their ability to inhibit amylase secretion. Farese et. al. (1982) using rat pancreatic fragments, found that decreases in PtdIns concentration correlated with increases in amylase secretion. The reason for the difference between our results and those of Farese is not clear, although Farese et. al. (1982) measured total PtdIns content rather than breakdown. Total PtdIns content is a balance between breakdown and synthesis and is affected by factors which do not necessarily affect breakdown. Amongst these are agonist-stimulated synthesis *de novo* of PtdIns (Calderon et. al., 1980; Chapman et. al., 1983), inhibition of PtdIns synthesis by Ca^{2+} (Berridge and Fain, 1979; Egawa et. al., 1981 a) and any other factor which will alter the basal or stimulated rate of PtdIns synthesis. A further difficulty in comparing our results with those of Farese et. al. (1982) arises from their failure to find the decrease in amylase secretion with high concentrations of CCK-8 and carbachol which was seen in the present study and in other studies using rats and mice (Savion and Selinger, 1978; Roberts and Woodland, 1982; Burnham and Williams, 1982 b; Sankaran et. al., 1982).

The results of this study suggest that at high concentrations of agonists the degree of inositol phospholipid hydrolysis and the increase in intracellular Ca^{2+} concentration are correlated. This supports the proposal that the lipid hydrolysis may control the 'calcium-gating' response. At low concentrations of agonist the degree of hydrolysis of inositol phospholipid is very small, but may be sufficient to generate the amount of inositol-1,4,5-trisphosphate which is required to release Ca^{2+} from intracellular stores.

CHAPTER 8

CONCLUSION

8.1 : CONCLUDING DISCUSSION

At the time of commencing this study, most of the studies examining phosphoinositide metabolism measured synthesis not breakdown of this lipid. The few studies which did measure a decrease in inositol phospholipid or an increase in the release of breakdown products required lengthy extraction and chromatographic separation of lipids and metabolites (for example, Hokin-Neaverson, 1974; Jones and Michell, 1976; Fain and Berridge, 1979). The method described in Chapters 3 and 4 of this thesis provided a rapid and reproducible direct assay of inositol phospholipid hydrolysis by measuring the release of breakdown products. *In vivo* labelling with *myo*-(2-^{3}H) inositol allowed a large incorporation of ^{3}H into inositol phospholipid with a small amount of free (^{3}H)-inositol thereby providing a sensitive assay. The release of (^{3}H)-inositol-labelled products is a valid measure of hydrolysis in the mouse exocrine pancreas since agonist-activation does not stimulate the inositol-exchange enzyme nor is there any significant re-incorporation into phosphoinositide of (^{3}H)-inositol released from this labelled lipid over the period of incubation used for the assays (as described in Chapter 4).

This method did not enable identification of which lipid is initially hydrolysed by receptor activation. However, information regarding this was provided by the studies examining the effect of ATP depletion on inositol phospholipid hydrolysis (as described in Chapter 5). Since the agonist-stimulated release of (^{3}H)-inositol-labelled products required ATP and no ATP-dependent hydrolysis of PtdIns is known, this indicated that phosphorylation of PtdIns and perhaps also PtdIns-4P was necessary for activation of inositol phospholipid hydrolysis. The hydrolysis of

PtdIns alone could not account for the agonist-stimulated increase in (^{3}H)-inositol labelled products, therefore it could be concluded that PtdIns-4,5P_2 and/or PtdIns-4P are the major inositol phospholipids hydrolysed in exocrine pancreas.

In studies validating this method (see Chapter 4), the acid-soluble products of inositol phospholipid hydrolysis were extracted and identified to be mostly inositol, with a small proportion of Ins-1-P. Ins-1,4,5P_3 and Ins-1,4P_2 were not examined but it is likely that over the 30 min incubation period much of the inositol-polyphosphate would have been converted to Ins-1-P or inositol.

The assay developed by Berridge et. al. (1982) which uses lithium to inhibit the conversion of Ins-1-P to inositol by inositol-1-phosphatase provides a rapid measure of inositol phospholipid hydrolysis which gives a good signal-to-noise ratio and is now being used by a number of laboratories. However, this method (Berridge et. al., 1982) also does not allow identification of which phosphoinositide(s) is hydrolysed since it measure total (^{3}H)-inositol-phosphates.

The first study to separate and identify the (^{3}H)-inositol-phosphates released from *myo*-(2-^{3}H) inositol-labelled phosphoinositide in exocrine pancreas was reported this year by Rubin et. al. (1984). The time course of release of inositol-phosphates was examined in an effort to determine which inositol lipid(s) was hydrolysed following receptor activation. At 1 min after carbachol stimulation of rat pancreatic acini (^{3}H)-Ins-1,4,5P_3 and (^{3}H)-Ins1,4P_2 were increased 8.2-fold and 6.8-fold above control), respectively; whereas only a small increase in Ins-1-P (1.4-fold above control) occurred. Rubin et. al.(1984) suggested that the greater latency in Ins-1-P production compared to Ins-1,4,5P_3 and Ins-1,4P_2

indicated that it was formed from the breakdown of Ins-1,4,5P_3 and Ins-1,4P_2 and not PtdIns. The results of Rubin et. al. (1984) showed that PtdIns-4,5P_2 is rapidly hydrolysed following receptor activation and supported earlier studies showing a more rapid time course of release of Ins-1,4,5P_3 and Ins-1,4P_2 compared with the release of Ins-1-P and inositol in blowfly salivary gland (Berridge, 1983) and rat parotid gland (Downes and Wusteman, 1983). These studies provide evidence that agonists hydrolyse polyphosphoinositide(s) rather than PtdIns. However, it is not possible to determine from these studies (Berridge, 1983; Downes and Wusteman, 1983; Rubin et. al., 1984) whether PtdIns-4P is also hydrolysed since the Ins-1,4P_2 could result either from Ins-1,4,5P_3 or PtdIns-4P.

Therefore one of the major questions yet to be answered is whether agonist-stimulated hydrolysis of PtdIns-4P occurs in addition to the breakdown of PtdIns-4,5P_2. Although the development of the method described in this thesis (Tennes and Roberts, 1981), allowed considerable progress in the study of inositol phospholipid in exocrine pancreas this assay would not be chosen to investigate the contribution of PtdIns-4P hydrolysis to total phosphoinositide hydrolysis; neither would the method of Berridge et. al. (1982) nor simply the isolation and identification of released inositol-phosphates, be used.

The difficulty in examining PtdIns-4P hydrolysis is that the product released can also increase as a result of PtdIns-4,5P_2 hydrolysis since Ins-4,5,P_3 can be converted by a phosphomonoesterase. If an inhibitor of the Ins-1,4,5P_3 phosphomonoesterase was available (such as lithium, which inhibits Ins-1-P phosphomonoesterase), then increases in Ins-1,4,P_2

could be attributed to the hydrolysis of PtdIns-4P not Ins-1,4,5P_3. Unfortunately, no such inhibitor is at present known and so future research must use an alternative method to examine this question.

One such method has been described recently (Aub and Putney, 1984), which analyses the kinetics of formation of inositol phosphates by using a computer simulation program. Estimates were made of the rate constants for breakdown of Ins-1,4P_2 and Ins-1,4,5P_3 in rat parotid cells. When these rate constants were applied to the measured steady-state levels of Ins-1,4P_2 and Ins-1,4,5P_3, rates of flux through each of these species could be calculated. The value for the rate of formation of Ins-1,4,5P_3 was less than that for Ins-1,4P_2 suggesting that this latter compound must also be formed by a pathway other than the hydrolysis of Ins-1,4,5P_3; which could be PtdIns-4P hydrolysis. The rate of formation of Ins-1-P could be accounted for by the flux through Ins-1,4P_2,suggesting that a significant PtdIns breakdown does not occur and that all of the Ins-1-P could be formed by hydrolysis of Ins-1,4P_2.

This method (Aub and Putney, 1984) allowed a clear identification of PtdIns-4P hydrolysis. However, the functional role, if any, of this response is unknown. It would therefore appear that future studies of inositol phospholipid using this kinetic analysis will allow more conclusive interpretations that those studies which simply assay increases in (^{3}H)-inositol-phosphates.

With the aid of this type of kinetic analysis, it may then be possible to examine the Ca^{2+}-dependency of the agonist-stimulated hydrolysis of

PtdIns-4P; a question which has not yet been investigated. There is some suggestion that the hydrolysis of PtdIns-4P may be Ca^{2+}-activated since ionomycin caused a small but significant increase in Ins-1,4P_2 in rat pancreatic acini (Rubin et. al., 1984). Since ionomycin did not cause an increase in Ins-1,4,5P_3 it was possible to attribute the Ins-1,4P_2 rise to PtdIns-4P hydrolysis. However, if the Ca^{2+}-dependency of the agonist-stimulated hydrolysis of PtdIns-4P was to be examined, it would be necessary to use a kinetic analysis of increases in inositol-phosphates since receptor activation would increase Ins-1,4,5P_3 leading to production of Ins-1,4P_2 which for example, may occur in the absence of Ca^{2+} whereas hydrolysis of PtdIns-4P may not. Without kinetic analysis it would be impossible to accurately determine from which compound Ins-1,4P_2 was formed. It is likely that this form of kinetic analysis of inositol phospholipid hydrolysis will be most useful in future research in this area.

One of the most important questions still unanswered is how does receptor occupation cause an increase in inositol phospholipid hydrolysis? The binding of agonist to receptor could cause a conformational change in the membrane resulting in activation of the enzyme responsible for hydrolysis or alternatively, allow substrate to be made available to enzyme. At present it is not known which of these events (or whether both) occur.

The results presented in this thesis (Chapter 5) show that there is a close association between occupation of receptor and inositol phospho-lipid hydrolysis, since this lipid response was found to be an early event in activation of the exocrine pancreas. To examine the mechanism

whereby receptor occupation stimulates the hydrolysis of lipid, studies investigating the effect of simultaneous addition of two different agonists were made.

The results in Chapter 5 suggest that the muscarinic and cholecystokinin receptors in exocrine pancreas do not have their own individual mechanism for causing phosphoinositide hydrolysis but that the signals generated following occupation of different receptors converge on a common mechanism which limits the degree of hydrolysis of inositol phospholipid. The reason for this interaction effect is not known, but it does not appear to be due to depletion of (^{3}H)-labelled phosphoinositide. Further examination is required to determine whether the lack of additive effect of simultaneous addition of muscarinic and cholecystokinin receptor agonists is due to maximal stimulation of the enzyme responsible for hydrolysis, that is, if receptor activation does stimulate the enzyme. It is possible that the enzyme is active at all times but that receptor occupation is required to cause phosphoinositide to be accessible to the enzyme; in this case the interaction effect would not be due to maximal activation of enzyme. An alternative explanation could be that occupation of one class of receptor causes an alteration in the ability of a different agonist to bind to its receptor. This could be examined using receptor-binding techniques. If no alteration in binding properties was observed the interaction effect could be due to a physicochemical change in the membrane whereby binding to one receptor population alters the conformation of the membrane such that the ability of the other receptor population to activate inositol phospholipid hydrolysis is reduced. It may be possible to test this by examining whether the interaction effect is observed in

cells with membranes disrupted (with for example, saponin, filipin or electrically permeabilized). This may provide information as to whether transmission of conformational changes in membrane from one receptor to another alters the link between receptor occupation and inositol lipid hydrolysis.

The mechanism which is responsible for activating inositol lipid hydrolysis must be examined. The activation of adenylate cyclase by occupation of the β-receptor has been clearly identified. The protein components involved and the conformational changes causing interaction of these components to activate and deactivate the adenylate cyclase are clearly understood (for a review see Schramm and Selinger, 1984). Is it possible that receptor and inositol phospholipid could exist in a comparable structured complex? Studies using cell free systems in which isolated plasma membrane fractions are activated by agonist and inositol lipid hydrolysis measured could provide a means to examine this question. Various compounds may be found (for example, analagous to GTP in the adenylate cyclase system) which are required for activation of the lipid response - this would allow one to build up some kind of model for the activation mechanism. Another possible way to examine this may be to isolate the receptor and see if it is associated with a lipid-enzyme complex which is still able to be activated. There are always problems with these kinds of experiments, however, since it is possible that the isolation procedures may disrupt some essential link. If this did occur, it would still provide information as the structural proximity of the lipid and receptor.

A further examination of the ability of Ca^{2+} to potentiate inositol

phospholipid hydrolysis may allow identification of a Ca^{2+}-sensitive step in the mechanism linking receptor occupation with the lipid response.

As studies in Chapter 6 have shown, neither intracellular nor extracellular Ca^{2+} is required for the agonist-stimulated hydrolysis of inositol phospholipid, but the presence of Ca^{2+} in the extracellular medium potentiates this hydrolysis of lipid. This potentiation is not due to the movement of Ca^{2+} through the plasma membrane since neomycin, an agent which blocks Ca^{2+} influx in exocrine glands (Marier et. al., 1978; Putney, 1981; Aub et. al., 1982) does not alter the potentiation effect and an external site of potentiation was suggested (Tennes and Roberts, 1982). A role for extracellular Ca^{2+} in phosphoinositide hydrolysis has subsequently been confirmed in hepatocytes (Creba et. al., 1983), lacrimal gland (Godfrey and Putney, 1984) and leukaemic basophil 2H3 cells (Beaven et. al., 1984).

The site of Ca^{2+}-potentiation was examined with La^{3+} and Mn^{2+}-agents which can displace Ca^{2+} from membrane binding sites. Two sites of potentiation by Ca^{2+} have been identified on the external surface of the pancreatic cell membrane. One site is associated with potentiation of inositol phospholipid hydrolysis in unstimulated cells. This site is La^{3+}-sensitive but Mn^{2+} can replace Ca^{2+} at this site. The mechanism by which the basal hydrolysis of inositol phospholipid can be potentiated by extracellular Ca^{2+} is not clear. It could indicate that the enzyme responsible for hydrolysis is active at all times and that Ca^{2+} bound to specific sites on the membrane allows a minimal access of enzyme to substrate. The second site is associated with potentiation of agonist-

stimulated inositol lipid hydrolysis. La^{3+} and Mn^{2+} both compete with Ca^{2+} at this site to remove the potentiation effect. A recent study by Beaven et. al. (1984) showed that a La^{3+}-sensitive site exists on the external surface of the basophil 2H3 cell and that occupation of this site inhibited antigen-stimulated inositol phospholipid hydrolysis supporting the results obtained in the present study in the exocrine pancreas (Tennes and Roberts, 1982).

The mechanism of potentiation requires further investigation. One suggestion is that the occupation of receptor causes a conformational change in the lipid membrane allowing interaction between phosphoinositide and enzyme, Ca^{2+} in some as yet unknown manner could increase the efficacy of this interaction. A more specific effect of Ca^{2+} to potentiate agonist-stimulated inositol phospholipid hydrolysis could be due to a Ca^{2+}-dependent hydrolysis of PtdIns-4P. Since La^{3+} removed the potentiation effect of Ca^{2+} (Chapter 6) it would be interesting to examine Ins-1,4P_2 and Ins-1,4,5P_3 formation in the presence of La^{3+}, with the kinetic analysis described by Aub and Putney (1984). If removal of Ca^{2+} from the external binding sites with La^{3+} reduced the contribution by (^{3}H)-PtdIns-4P of (^{3}H)-Ins-1,4P_2 to the total (^{3}H)-inositol-labelled products such that only (^{3}H)-PtdIns,4,5P_2 hydrolysis occurred, this would identify the site of Ca^{2+} potentiation at the step linking receptor occupation with hydrolysis of PtdIns-4P.

The site of Ca^{2+}-binding was proposed to be external since Ca^{2+} potentiation was unaffected by neomycin and removed by La^{3+} which does not appear to enter pancreatic cells (Wakasugi et. al., 1981). Ca^{2+} could be bound to phospholipids and to the negatively charged groups

(sialic acid residues) on cell surface glycoproteins. If neuraminidase was used to remove the sialic acid residues and the ability of Ca^{2+} to potentiate phosphoinositide hydrolysis was also removed, this would identify the site of Ca^{2+} binding to be on glycoproteins and so provide additional information for the development of a model of how receptor occupation activates inositol lipid hydrolysis and how this mechanism could be modulated by Ca^{2+}.

Studies over the last few years have demonstrated that the receptor-transducing mechanism uses PtdIns-4,5P_2 as substrate (whether PtdIns-4P is also used is not known). An important consequence of this is that the ATP requirement to refill the small pool of PtdIns-4,5P_2 provides a mechanism for controlling the responsiveness of the cell by altering phosphorylation reactions which control the availability of substrate. For example, if a cell is subject to a large, long-term stimulation, and is metabolically very active it is possible that ATP could be used for a number of cellular processes, and ATP levels could drop. If so, this could provide a protective mechanism for the cell and a means of "switching-off" since the formation of PtdIns-4,5P_2 would be reduced, Ca^{2+} mobilization would decrease and so the Ca^{2+}-activated responses would eventually inactivate.

Depletion of PtdIns-4,5P_2 from the cell could provide a means of examining which cellular responses are controlled by this lipid hydrolysis. ATP depletion to reduce PtdIns-4,5P_2 content could not be used since this would also interfere directly with the numerous ATP-dependent cellular responses. An inhibition of PtdIns-4P kinase would deplete PtdIns-4,5P_2, however, no such inhibitor is known. It is possible

that incubation of cells with lithium in the presence of agonist would eventually reduce the formation of PtdIns by inhibiting the conversion of Ins-1-P to inositol. However, PtdIns can also be formed *de novo*. To eliminate this effect perhaps cationic amphiphilic drugs which inhibit phosphatidate phosphohydrolase and so reduce *de novo* synthesis of PtdIns (Allan and Michell, 1974) could be used. One problem with using these drugs is that they could also have non-specific effects, such as acting in a local-anaesthetic manner and so may not prove useful for this aim. Alternatively, if cells could be depleted of inositol, then this would lead to eventual depletion of PtdIns and so also PtdIns-4,5,P_2.

Recent studies have provided one answer for the question of how inositol phospholipid hydrolysis causes an increase in intracellular Ca^{2+} by the identification of release of Ca^{2+} by Ins-1,4,5P_3 from non-mitochondrial stores in pancreas (Streb et. al., 1983) and other cells (Burgess et. al., 1984; Joseph et. al., 1984; Prentki et. al., 1984; Suematsu et. al., 1984).

However, the mode of action of Ins-1,4,5P_3 to act on this non-mitochondrial pool is not known, nor has the identity of this pool been clearly demonstrated. Is the pool intracellular or is some Ca^{2+} released from the plasma membrane? The study by Poggioli and Putney (1982) identified the hormone-sensitive Ca^{2+} pool in parotid gland cells to be in close proximity to (or perhaps in) the plasma membrane. There is at present no evidence to show that Ins-1,4,5P_3 releases Ca^{2+} from the plasma membrane, this requires investigation. One experiment to examine this question would be to observe if addition of Ins-1,4,5P_3 to isolated

plasma membrane fractions, isolated from parotid glands and pre-labelled with $^{45}Ca^{2+}$, resulted in the release of Ca^{2+}. If so, then this would identify a role for Ins-1,4,5P_3 at the plasma membrane. Ins-1,4,5P_3 can also release Ca^{2+} from an intracellular, non-mitochondrial pool in other cells, such as pancreas (Streb and Schulz, 1983), the identity of the site(s) is yet to be determined and requires investigation.

Although Ins-1,4,5P_3 mobilizes Ca^{2+} from a cellular site(s), a number of cells, including pancreas require Ca^{2+} influx to sustain secretion and also to refill Ca^{2+} stores. It is not known whether Ins-1,4,5P_3 plays a role in this. The question of how does receptor occupation cause Ca^{2+} influx has not been answered. Ins-1,4,5P_3 may play a role, or phosphatidic may act as an ionophore or perhaps the conformational change in the membrane may open up pathways in the membrane through which Ca^{2+} can pass. One method to examine if Ins-1,4,5P_3 can increase the permeability of the membrane to Ca^{2+} would be to study the effect of injection of this compound into an intact cell. It may be necessary to use a large cell such as an oocyte which shows large increases in cytoplasmic free Ca^{2+} concentration during fertilization (Cuthbertson et. al., 1981) and during steroid-induced meiotic division (Baulieu et. al., 1978) to exmaine this effect since injection of Ins-1,4,5P_3 could be damaging to a small cell such as pancreatic acinar cell.

The physiological role of inositol phospholipid hydrolysis in pancreas was examined by comparing the dose-response curves for amylase secretion with those for phosphoinositide hydrolysis (Chapter 7). Using a number of secretagogues acting through different classes of receptors it was demonstrated that at high concentrations of agonists the degree of

inositol phospholipid hydrolysis correlates with the degree of inhibition of amylase secretion. Since this effect on secretion appears to be due to an increase in intracellular Ca^{2+} (Gardner et. al., 1980; Roberts and Woodland, 1982; Burnham and Williams, 1982 b) these results suggest that inositol lipid breakdown and the increase in intracellular Ca^{2+} are correlated. In the studies described in Chapter 7, at low agonist concentrations when amylase secretion is stimulated, the degree of inositol phospholipid hydrolysis is small but may be sufficient to cause release of a significant amount of Ins-1,4,5P_3 to stimulate release of Ca^{2+} from stores and so activate secretion. To maintain secretion, Ca^{2+} influx would be required, and it still remains to be examined by what mechanism this occurs.

The state of knowledge of the "PtdIns Effect" has increased enormously over the last few years. From PtdIns being a candidate for Ca^{2+}-mobilization (Michell, 1975) we now know a considerable amount about the pathways involved, the initial phosphoinositide to be hydrolysed and the requirement for phosphorylation to maintain this pool of PtdIns-4,5P_2. One possible mechanism by which inositol phospholipid mobilizes Ca^{2+} has been discovered - Ins-1,4,5P_3, for which there is substantial evidence. Another important intracellular second messenger has also been identified - diacylglycerol. Ins-1,4,5P_3 and diacylglycerol may play separate roles or a synergistic role in cell activation via Ca^{2+} and protein kinase C.

BIBLIOGRAPHY

Abdel-Latif, A.A., Akhtar, R.A., and Hawthorne, J.N. (1977). Acetylcholine increases the breakdown of triphosphoinositide of rabbit iris muscle pre-labelled with (^{32}P)-phosphate. Biochem. J. 162, 61-73.

Abdel-Latif, A.A. (1981). Sodium ion and the effect of acetylcholine on phospholipid and phosphoprotein phosphate turnover in the rabbit iris smooth muscle. Biochem. Pharmacol. 30, 1371-1374.

Abdel-Latif, A.A.,and Luke, B. (1981). Sodium ion and the neuro-transmitter-stimulated ^{32}P-labelling of phosphoinositides and other phospholipids in the iris muscle. Biochim. Biophys. Acta. 673, 64-74.

Adrian, T.E., Besterman, H.S., Mallison, C.N., Greenberg, G.R., and Bloom, S.R. (1979). Inhibition of secretin-stimulated pancreatic secretion by pancreatic polypeptide. Gut. 20, 37-40.

Aquilar, J.S., Criado, M., and DeRobertis, E. (1980). Inhibition by local anaesthetics, phentolamine and propranolol of (^{3}H)-quinuclydinyl benzylate binding to central muscarinic receptors. Eur. J. Pharmacol. 68, 317-326.

Ahren, B., Ericson, L.E., Lundquist, I., and Sundler, F. (1981). Adrenergic innervation of pancreatic islets and modulation of insulin secretion by the sympatho-adrenal system. Cell Tissue Res. 216, 15-30.

Akhtar, R.A., and Abdel-Latif, A.A. (1978). Calcium-ion requirement for acetylcholine-stimulated breakdown of triphosphoinositide in rabbit iris smooth muscle. J. Pharmacol. Exp. Ther. 204, 655-668.

Akhtar, R.A., and Abdel-Latif, A.A. (1980). Requirement for calcium ions in acetylcholine-stimulated phosphodiesterase cleavage of PtdIns-4,5P_2 in rabbit iris smooth muscle. Biochem. J. 192, 783-791.

Albano, J., Bhoola, K.D., and Harvey, R.F. (1976). Intracellular messenger role of cyclic GMP in exocrine pancreas. Nature 262, 404-406.

Agranoff, B.W., Murthy, P., and Seguin, E.B. (1983). Thrombin-induced phosphodiesteratic cleavage of phosphatidylinositol bisphosphate in human platelets. J. Biol. Chem. 258, 2076-2078.

Albinus, M., Case, R.M., Reed, J.D., Shaw, B., Smith, P.A., Gomez-Pan, A., Hall, R., Besser, G.M., and Schally, A.U. (1976). Effect of growth hormone-releasing inhibiting hormone (GH-RIH or somatostatin) on secretory processes in stomach and pancreas. In: "Stimulus-Secretion Coupling in the Gastrointestinal Tract", pp. 407-409, Eds: R.M. Case and H. Goebell. MTP Press, Lancaster.

Allan, D., and Michell, R.H. (1974 a). Phosphatidylinositol cleavage in lymphocytes - requirement for calcium ions at a low concentration and effects of other cations. Biochem. J. 142, 599-604.

Allan, D., and Michell, R.H. (1974 b). Phosphatidylinositol cleavage catalysed by the soluble fraction from lymphocytes-activity at pH 5.5 and pH 7.0. Biochem. J. 142, 591-597.

Allan, D., and Michell, R.H. (1975). Enhanced synthesis *de novo* of phosphatidylinositol in lymphocytes treated with cationic amphiphilic drugs. Biochem. J. 148, 471-478.

Allan, D., and Michell, R.H. (1978). The influence of intracellular Ca^{2+} on the metabolism of inositol phospholipids in lymphocytes and erythrocytes. In: "Cyclitols and Phosphoinositides", pp. 325-348. Eds: W.W. Wells and F. Eisenberg. Academic Press, N.Y.

Alm, P., Cegrell, L., Ehinger, B., and Falck (1967). Remarkable adrenergic nerves in the exocrine pancreas. Z. Zellforsch. Mikrosk. Anat. 83, 178-186.

Amy, C.M., and Kirschner, N. (1981). Phosphorylation of adrenal-medulla cell-proteins in conjunction with stimulation of catecholamine secretion. J. Neurochem. 36, 847-854.

Argent, B.E., Case, R.M., and Scratcherd, T. (1973). Amylase secretion by the perfused cat pancreas in relation to the secretion of calcium and other electrolytes and as influenced by the external ionic environment. J. Physiol. (Lond.) 230, 575-593.

Argent, B.E., Case, R.M., and Hirst, F.C. (1982). The effects of manganese, cobalt and calcium on amylase secretion and calcium homeostasis in rat pancreas. J. Physiol. (Lond.) 323, 353-375.

Aronstam, R.S., Abood, L.G., and Baumgold, J. (1977). Role of phospholipids in muscarinic binding by neural membranes. Biochem. Pharmacol. 26, 1689-1695.

Atherton, R.S., and Hawthorne, J.N. (1968). The phosphoinositide inositolphosphohydrolase of guinea-pig intestinal mucosa. Eur. J. Biochem. 4, 68-75.

Aub, D.L., McKinney, J.S., and Putney, J.W. Jr. (1982). Nature of the receptor-regulated calcium pool in the rat parotid gland. J. Physiol. (Lond.) 331, 557-565.

Aub, D.L., and Putney, J.W. Jr. (1984). Metabolism of inositol phosphates in parotid cells : implications for the pathway of the phosphoinositide effect and for the possible messenger role of inositol trisphosphate. Life Sci. 34, 1347-1355.

Baker, P.F. (1984). Multiple controls for secretion? Nature 310, 629-630.

Baker, R.R., and Thompson, W. (1972). Positional distribution and turnover of fatty acids in phosphatidic acid, phosphoinositides, phosphatidylcholine and phosphatidylethanolamine in rat brain, *in vivo*. Biochim. Biophys. Acta. 270, 489-503.

Banschbach, M.W., Geison, R.L., and Hokin-Neaverson, M. (1974). Acetylcholine increases the level of diglyceride in mouse pancreas. Biochem. Biophys. Res. Commun. 58, 714-718.

Banschbach, M.W., and Hokin-Neaverson, M. (1980). Acetylcholine promotes the synthesis of prostaglandin E in mouse pancreas. FEBS Lett. 117, 131-133.

Banwell, J.G., Northam, B.E., and Cooke, W.T. (1967). Secretory response of the human pancreas to continuous intravenous infusion of pancreozymin-cholecystokinin (Cecekin). Gut. 8, 380-387.

Barlow, T.E., Greenwell, J.R., Harper, A.A., and Scratcherd, T. (1974). The influence of the splanchnic nerves on the external secretion, blood flow and electrical conductance of the cat pancreas. J. Physiol. (Lond.) 236, 421-433.

Bauduin, H., and Cantraine, F. (1972). 'Phospholipid effect' and secretion in the rat pancreas. Biochim. Biophys. Acta. 270, 248-253.

Bauduin, H., Galand, N., and Boeynaems, J.M. (1981). *In vitro* stimulation of prostaglandin synthesis in the rat pancreas by carbamylcholine, caerulein and secretin. Prostaglandins 22, 35-52.

Bauduin, H., Stock, C., Vincent, D., and Grenier, J.F. (1975). Microfilamentous system and secretion of enzyme in the exocrine pancreas. Effect of cytochalasin B. J. Cell Biol. 66, 165-181.

Baulieu, F.E., Godeau, F., Schorderet, M., and Schorderet-Slatkine, S.S. (1978). Steroid-induced meiotic division in *Xenopus Laevis* oocytes : surface and calcium. Nature 275, 593-598.

Beaven, M.A., Moore, J.P., Smith, G.A., Hesketh, T.R., and Metcalfe, J.C. (1984). The calcium signal and phosphatidylinositol breakdown in 2H3 cells. J. Biol. Chem. 259, 7137-7142.

Beglinger, C., Taylor, I.L., Grossman, M.I., and Solomon, T.E. (1984). Pancreatic polypeptide inhibits exocrine pancreatic responses to six stimulants. Am. J. Physiol. 246, G288-G291.

Bell, R.L., and Majerus, P.W. (1980). Thrombin-induced hydrolysis of phosphatidylinositol in human platelets. J. Biol. Chem. 255, 1790-1792.

Bell, R.L., Stanford, N., Kennerly, D.A., and Majerus, P.W. (1980). Diglyceride lipase : A pathway for arachidonate release from human platelets. Adv. Prostagl. Thromb. Res. 6, 219-224.

Bennett, J.P., Cockcroft, S., and Gomperts, B.D. (1980). Use of cytochalasin B to distinguish between early and late events in neutrophil activation. Biochim. Biophys. Acta. 601, 584-591.

Bennett, J.P., Cockcroft, S., Caswell, A.H., and Gomperts, B.D. (1982). Plasma-membrane location of phosphatidylinositol hydrolysis in rabbit neutrophils stimulated with formylmethionyl-leucylphenylalanine. Biochem. J. 208, 801-808.

Benz, L., Eckstein, B., Matthews, E.K., and Williams, J.A. (1972). Control of pancreatic amylase release *in vitro* : effects of ions, cyclic AMP and colchicine. Br. J. Pharmacol. 46, 66-67.

Berridge, M.J., and Fain, J.N. (1979). Inhibition of phosphatidyl-inositol synthesis and the inactivation of calcium entry after prolonged exposure of the blowfly salivary gland to 5-Hydroxytryptamine. Biochem. J. 178, 59-69.

Berridge, M.J., Downes, C.P., and Hanley, M.R. (1982). Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. Biochem. J. 206, 587-595.

Berridge, M.J. (1983). Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. Biochem. J. 212, 849-858.

Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P., and Irvine, R.F. (1983). Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem. J. 212, 473-482.

Berridge, M.J., Buchanan, P.B., and Heslop, J.P. (1984). Relationship of polyphosphoinositide metabolism to the hormonal activation of the insect salivary gland by 5-Hydroxytryptamine. Mol. Cell. Endocrinol. 36, 37-42.

Best, L., Malaisse, W.J. (1983). Phosphatidylinositol and phosphatidic acid metabolism in rat pancreatic islets in response to neuro-transmitter and hormonal stimuli. Biochim. Biophys. Acta. 750, 157-163.

Bidot-Lopez, P., Farese, R.V., and Sabir, M.A. (1981). Parathyroid hormone and adenosine-3',5'-monophosphate acutely increase phospholipids of the phosphatidate-polyphosphoinositide pathway in rabbit kidney cortex tubules *in vitro* by a cycloheximide-sensitive process. Endocrinology, 108, 2078-2081.

Bieger, W., Martin-Achard, A., Bassler, M., and Kern. H.F. (1976). Studies on intracellular transport of secretory proteins in the rat exocrine pancreas. IV : Stimulation by *in vivo* infusion of caerulein. Cell Tissue Res. 165, 435-453.

Billah, M.M., and Michell, R.H. (1979). Phosphatidylinositol metabolism in rat hepatocytes stimulated by glycogenolytic hormones. Effects of angiotensin, vasopressin, adrenaline, ionophore A23187 and calcium-ion deprivation. Biochem. J. 182, 661-668.

Billah, M.M., Lapetina, E.G., and Cuatrecasas, P. (1979). Phosphatidyl-inositol-specific phospholipase C of platelets : Association with 1,2 diacylglycerol-kinase and inhibition by cyclic-AMP. Biochem. Biophys. Res. Commun. 90, 92-98.

Billah, M.M., Lapetina, E.G., and Cuatrecasas, P. (1981). Phospholipase A_2 activity specific for phosphatidic acid - a possible mechanism for the production of arachidonic acid in platelets. J. Biol. Chem. 256, 5399-5403.

Billah, M., Lapetina, E.G., and Cuatrecasas, P. (1980). Phospholipase A_2 and phospholipase C activities of platelets : Differential substrate specificity, Ca^{2+}-requirement, pH dependence and cellular localization. J. Biol. Chem. 255, 10227-10231.

Billah, M.M., and Lapetina, E.G. (1982). Rapid decrease of phosphatidylinositol 4,5-bisphosphate in thrombin-stimulated platelets. J. Biol. Chem. 257, 12705-12708.

Birdsall, N.J.M., Burgen, A.S.V., Hulme, E.C., and Wells, J.W. (1979). The effects of ions on the binding of agonists and antagonists to muscarinic receptors. Br. J. Pharmacol. 67, 371-377.

Birdsall, N.J.M., Berrie, C.P., Burgen, A.S.V., and Hulme, E.C. (1980). Modulation of the binding properties of muscarinic receptors : Evidence for receptor-effector coupling. In: "Receptors for Neurotransmitters and Peptide Hormones", pp. 107-116. Eds: G. Pepeu, M.J. Kuhar, and S.J. Enna, Raven Press, New York.

Birks, R.I., and Cohen, M.W. (1968). The action of sodium pump inhibitors on neuromuscular transmission. Proc. Royal Soc. Lond. B. 170, 381-399.

Bishop, H.H., and Strickland,K.P.(1970). On the specificity of cytidine diphosphate diglycerides in monophosphoinositol biosynthesis by rat brain preparations. Can. J. Biochem. 48, 270-277.

Bishop, A.E., Polak, J.M., Green, I.C., Bryant, M.G., and Bloom, S.R. (1980). The location of VIP in the pancreas of man and rat. Diabetologia 18, 73-80.

Bissonnette, B.M., Collen, M.J., Adachi, H., Jensen, R.T., and Gardner, J.D. (1984). Receptors for vasoactive intestinal peptide and secretin on rat pancreatic acini. Am. J. Physiol. 246, G710-G717.

Blackburn, W.R., and Vinijchaikul, K. (1969). The pancreas in Kwashiorkor : An Electron microscopic study. Lab. Invest. 20, 305-318.

Blackmore, P.F., Dehaye, J.P., Strickland, W.G., and Exton, J.H. (1979 a). Alpha-adrenergic mobilization of hepatic mitochondrial calcium. FEBS Lett. 100, 117-120.

Blackmore, P.F., Dehaye, J.P., and Exton, J.H. (1979 b). Studies on alpha-adrenergic activation of hepatic glucose output - role of mitochondrial calcium release in alpha-adrenergic activation of phosphorylase in perfused rat liver. J. Biol. Chem. 254, 6945-6950.

Blumberg, P.M., Jaken, S., König, B., Sharkey, N.A., Leach, K.A., Geng, A.Y., and Yeh, E. (1984). Mechanism of action of the phorbol ester tumour promoters : Specific receptors for lipophilic ligands. Biochem. Pharmacol. 33, 933-940.

Bolender, R.P. (1974). Stereological analysis of guinea-pig pancreas, I. Analytical model and quantitative description of non-stimulated pancreatic exocrine cells. J. Cell Biol. 61, 269-287.

Bondani, A., and Karler, R. (1970). Interaction of calcium and local anaesthetics with skeletal muscle microsomes. J. Cell. Physiol. 75, 199-211.

Bonner-Weir, S., and Orci, L. (1982). New perspectives on the microvasculature of the islets of Langerhans in the rat. Diabetes 31, 883-889.

Brindley, D.N., and Bowley, M. (1975). Drugs affecting synthesis of glycerides and phospholipids in rat liver : Effects of clofibrate, halofenate, fenfluramine, amphetamine, cinchocaine, chlorpromazine, demethylimipramine, mepyramine and some of their derivatives. Biochem. J. 148, 461-469.

Brindley, D.N., Allan, D., and Michell, R.H. (1975). Redirection of glyceride and phospholipid synthesis by drugs including chlorpromazine, fenfluramine, imipramine, mepyramine and local anaesthetics. J. Pharm. Pharmacol. 27, 462-464.

Broekman, M.J., Ward, J.W., and Marcus, A.J. (1980). Phospholipid metabolism in stimulated human platelets : Changes in phosphatidylinositol, phosphatidic acid and lysophospholipids. J. Clin. Invest. 66, 275-283.

Brown, E., Kendall, D.A., and Nahorski, S.R. (1984). Inositol phospholipid hydrolysis in rat cerebral cortical slices : I. Receptor characterisation. J. Neurochem. 42, 1379-1387.

Brown, S.S., and Spudich, J.A. (1981). Mechanism of action of cytocholasin : Evidence that it binds to actin filament ends. J. Cell Biol. 88, 487-491.

Buchan, A.M.J., Davison, J.S., and Dickson, V. (1983). Vagal non-adrenergic, non-cholinergic (NANC) control of pancreatic electrolyte and enzyme secretion in the guinea-pig. J. Physiol. (Lond.) 346, 117P.

Buckely, J.T., LeFebvre, Y.A., and Hawthorne, J.N. (1971). Identification of an actively phosphorylated component of adrenal medulla chromaffin granules. Biochim. Biophys. Acta. 239, 517-519.

Buckley, J.T., and Hawthorne, J.N. (1972). Erythrocyte membrane polyphosphoinositide metabolism and regulation of calcium binding. J. Biol. Chem. 247, 7218-7223.

Burgermeister, W., Klein, W.L., Nirenberg, M., and Witkop, B. (1978). Comparative binding studies with cholinergic ligands and histrionicotoxin at muscarinic receptors of neural cell lines. Mol. Pharmacol. 14, 751-767.

Burgess, G.M., Giraud, F., Poggiolo, J. and Claret, M. (1983). α-adrenergically mediated changes in membrane lipid fluidity and Ca^{2+} binding in isolated rat liver plasma membranes. Biochim. Biophys. Acta. 731, 387-396.

Burgess, G.M., Godfrey, P.P., McKinney, J.S., Berridge, M.J., Irvine, R.F., and Putney, J.W. Jr. (1984). The second messneger linking receptor activation to internal Ca release in liver. Nature 309, 63-66.

Burnham, D.B., and Williams J.A. (1982 a). Effects of carbachol, cholecystokinin and insulin on protein phosphorylation in isolated pancreatic acini. J. Biol. Chem. 257, 10523-10528.

Burnham, D.B., and Williams, J.A. (1982 b). Effects of high concentrations of secretagogues on the morphology and secretory activity of the pancreas : A role for microfilaments. Cell Tissue Res. 222, 201-212.

Burnham, D.B., and Williams, J.A. (1984). Activation of protein kinase activity in pancreatic acini by calcium and cAMP. Am. J. Physiol. 246, G500-G508.

Bygrave, F.L. (1978). Mitochondria and the control of intracellular calcium. Biol. Rev. 53, 43-79.

Bylund, D.B., and Krebs, E.G. (1975). Effects of denaturation on the susceptibility of proteins to enzymic phosphorylation. J. Biol. Chem. 250, 6355-6361.

Calderon, P., Furnelle, J., and Christophe, J. (1979). *In vitro* lipid metabolism in the rat pancreas. III. Effects of carbamylcholine and pancreozymin on the turnover of phosphatidylinositols, 1,2-diacylglycerols and phosphatidylcholines. Biochim. Biophys. Acta. 574, 404-413.

Calderon, P., Furnelle, J., and Christophe, J. (1980). Phosphatidylinositol turnover and calcium movement in the rat pancreas. Am. J. Physiol. 238, G247-G254.

Carafoli, E., Tiozzo, R., Lugli, G., Crovetti, F., and Kratzing, G. (1974). The release of calcium ions from heart mitochondria by sodium. J. Mol. Cell. Cardiol. 6, 361-371.

Carafoli, E., and Crompton, M. (1979). The regulation of intracellular calcium. In: "Current Topics in Membranes and Transport", Vol.10, pp. 151-216. Eds: F. Bronner and A. Kleinzeller. Academic Press, New York.

Caro, L.G., and Palade, G.E. (1964). Protein synthesis, storage and discharge in the pancreatic exocrine cell. An autoradiographic study. J. Cell Biol. 20, 473-495.

Carter, J.R., and Kennedy, E.P. (1966). Enzymatic synthesis of cytidine diphosphate diglyceride. J. Lipid Res. 7, 678-683.

Case, R.M., Harper, A.A., and Scratcherd, T. (1968). Water and electrolyte secretion by the perfused pancreas of the cat. J. Physiol. (Lond.)196, 133-149.

Case, R.M., Harper, A.A., and Scratcherd, T. (1969). The secretion of electrolytes and enzymes by the pancreas of the anaesthetized cat. J. Physiol. (Lond.) 201, 335-348.

Case, R.M., Scratcherd, T., and Wynne, R.D.A. (1970). The origin and secretion of pancreatic juice bicarbonate. J. Physiol. (Lond.) 210, 1-15.

Case, R.M., and Scratcherd, T. (1972). Actions of dibutyryl cyclic adenosine 3',5'-monophosphate and methyl xanthines on pancreatic exocrine secretion. J. Physiol. (Lond.) 223, 649-667.

Case, R.M., and Clausen, T. (1973). The relationship between calcium exchange and enzyme secretion in the isolated rat pancreas. J. Physiol. (Lond.) 235, 75-102.

Case, R.M. (1978). Synthesis, intracellular transport and discharge of exportable proteins in the pancreatic acinar cell and other cells. Biol. Rev. 53, 211-354.

Case, R.M., Hunter, M., Novak, I., and Young, J.A. (1984). The anionic basis of fluid secretion by the rabbit mandibular salivary gland. J. Physiol. (Lond.) 349, 619-630.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257, 7847-7851.

Chance, R.E., Ciezkowski, M., Jaworek, J., Konturek, S.J., Swierczek, J., and Tasler, J. (1981). Effect of pancreatic polypeptide and its C-terminal hexapeptide on meal- and secretin-induced pancreatic secretion in dogs. J. Physiol. (Lond.) 314, 1-9.

Chandler, D.E., and Williams, J.A. (1974). Pancreatic acinar cells : Effect of lanthanum ions on amylase release and calcium ion fluxes. J. Physiol. (Lond.) 243, 831-846.

Chandler, D.E., and Williams, J.A. (1977). Intracellular uptake and α-amylase and lactate dehydrogenasae releasing actions of the divalent cation ionophore A23187 in dissociated pancreatic acinar cells. J. Membr. Biol. 32, 201-230.

Chandler, D.E., and Williams, J.A. (1978 a). Intracellular divalent cation release in pancreatic acinar cells during stimulus-secretion coupling I. Use of chlorotetracycline as a fluorescent probe. J. Cell Biol. 76, 371-385.

Chandler, D.E., and Williams, J.A. (1978 b). Intracellular divalent cation release in pancreatic acinar cells during stimulus-secretion coupling II. Subcellular localization of the fluorescent probe chlorotetracycline. J. Cell Biol. 76, 386-399.

Chapman, B.A., Wilson, J.A., Colley, P.W., Pirola, R.C., and Somer, J.B. (1983). Increased phospholipid synthesis in the stimulated rat and human pancreas. Biochem. Biophys. Res. Commun. 115, 771-776.

Charo, I.F., Feinman, R.D., and Detwiler, T.C. (1976). Inhibition of platelet secretion by an antagonist of intracellular calcium. Biochem. Biophys. Res. Commun. 72, 1462-1467.

Chauvelot, L., Heisler, S., Huot, J., and Cagnon, D. (1979). Prostaglandins and enzyme secretion from dispersed rat pancreatic acinar cells. Life Sci. 25, 913-920.

Cheung, W.Y. (1980). Calmodulin plays a pivotal role in cellular regulation. Science 207, 19-27.

Chey, W.Y., Shay, H., and Shuman, C.R. (1963). External pancreatic secretion in diabetes mellitus. Ann. Intern. Med. 59, 812-821.

Chiou, C.Y., and Malagodi, M.H. (1975). Studies on the mechanism of action of a new Ca^{2+} antagonist, 8(N,N-Diethylamino) octyl 3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscles. Br. J. Pharmacol. 53, 279-285.

Chipperfield, A.R. (1984). Chloride-dependent, diuretic-sensitive K efflux from mouse pancreas and its stimulation by hypertonicity. J. Physiol. (Lond.) 346, 114P.

Christophe, J.P., Conlon, T.P., and Gardner, J.P. (1976). Interaction of porcine vasoactive intestinal peptide with dispersed pancreatic acinar cells from the guinea-pig : Binding a radioiodonated peptide. J. Biol. Chem. 251, 4629-4634.

Clemente, F., de Caro, A., and Figarella, C. (1972). Composition of human pancreatic juice - immunoenzymological study. Eur. J. Biochem. 31, 186-193.

Clements, R.S., Rhoten, W.B., and Starnes, W.R. (1977). Subcellular localization of alterations in phosphatidylinositol metabolism following glucose-induced insulin release from rat pancreatic islets. Diabetes, 26, 1109-1116.

Cockcroft, S., Bennett, A.P., and Gomperts, B.D. (1980). f-MetLeuPhe-induced phosphatidylinositol turnover in rabbit neutrophils is dependent on extracellular calcium. FEBS Lett. 110, 115-118.

Cockcroft, S., Bennett, J.P., and Gomperts, B.D. (1981). The dependence on Ca^{2+} of phosphatidyyinositol breakdown and enzyme secretion in rabbit neutrophils stimulated by formylmethionyl-leucylphenylalanine or ionomycin. Biochem. J. 200, 501-508.

Cockcroft, S., Baldwin, J.M., and Allan, D. (1984). The Ca^{2+}-activated polyphosphoinositide phosphodiesterase of human and rabbit neutrophil membranes. Biochem. J. 221, 477-482.

Cohen, P. (1982). The role of protein phosphorylation in the neural and hormonal control of cellular activity. Nature 296, 613-620.

Collen, M.J., Sutcliff, V.E., Pan, G-Z, and Gardner, J.D. (1982). Post-receptor modulation of action of VIP and secretin on pancreatic enzyme secretion by secretagogues that mobilize cellular calcium. Am. J. Physiol. 242, G423-G428.

Collins, C.A., and Wells, W.W. (1983). Identification of phosphatidyl-inositol kinase in rat liver lysosomal membranes. J. Biol. Chem. 258, 2130-2134.

Creba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H., and Kirk, C.J. (1983). Rapid breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in rat hepatocytes stimulated by vasopressin and other Ca^{2+}-mobilizing hormones. Biochem. J. 212, 733-747.

Crouch, M.F., Roberts, M.L., and Tennes, K.A. (1981). Mepacrine inhibition of bradykinin-induced contractions of the rabbit ear vein. Agents and Actions 11, 330-334.

Crouch, M.F., and Roberts, M.L. (1984). The effects of gamma-hexachlorocyclohexane on amylase secretion and inositol phospholipid metabolism in mouse pancreatic acini. Biochim. Biophys. Acta. (in press).

Cuthbertson, K.S.R., Whittingham, D.G., and Cobbold, P.H. (1981). Free Ca^{2+} increases in exponential phases during mouse oocyte activation. Nature 294, 754-757.

Dagorn, J-C. (1978). Non-parallel enzyme secretion from rat pancreas : *in vivo* studies. J. Physiol. (Lond.) 280, 435-448.

Daleo, R.G., Piras, M.M., and Piras, R. (1976). Digylceride kinase activity of microtubules. Characterization and comparison with the protein kinase and ATPase activities associated with vinblastine-isolated tubulin of chick embryonic muscles. Eur. J. Biochem. 68, 339-346.

Dawson, R.M.C. (1959). Studies on the enzymic hydrolysis of monophosphoinositides by phospholipase preparations from *P. notatum* and ox pancreas. Biochim. Biophys. Acta. 33, 68-77.

Dawson, R.M.C., and Thompson, W. (1964). The triphosphomonoesterases of brain tissue. Biochem. J. 91, 244-250.

Dawson, R.M.C., Freinkel, N., Jungalwala, F.B., and Clarke, N. (1971). The enzymic formation of *myo*-inositol 1,2-cyclic phosphate from phosphatidylinositol. Biochem. J. 122, 605-607.

Dawson, R.M.C., and Clarke, N.G. (1972). D-*myo*-inositol, 1,2-cyclic phosphate 2-phosphohydrolase. Biochem. J. 127, 113-118.

Debas, H.T., and Grossman, M.I. (1973). Pure cholecystokinin : pancreatic protein and bicarbonate response. Digestion 9, 469-481.

Debas, H.T., Konturek, S.J., and Grossman, M.I. (1975). Effect of extragastric and truncal vagotomy on pancreatic secretion in the dog. Am. J. Physiol. 228, 1172-1177.

Dehaye, J-P., Blackmore, P.F., Venter, J.C., and Exton, J.H. (1980). Studies on the α-adrenergic activation of hepatic glucose output. α-adrenergic activation of phosphorylase by immobilized epinephrine. J. Biol. Chem. 255, 3905-3910.

Dehaye, J-P., Winand, J., Michel, P., Robberecht, P., Waelbroek, M., Vandermeers, A., Vandermeers-Piret, M-C., and Christophe, J. (1984 a). Pancreatic secretory factor (PSF), a protein from Gila monster venom stimulating enzyme secretion from rat pancreatic acini. FEBS Lett. 166, 283-287.

Dehaye, J-P., Winand, J., Michel, P., Poloczek, P., Damien, C., Vandermeers-Piret, M-C., Vandermeers, A., and Christophe, J. (1984 b). Phospholipase A_2 activity of pancreatic secretory factor, a new secretagogue isolated from the venom of *Heloderma suspectum*. FEBS Lett. 172, 284-288.

Demel, R.A., Kalsbeek, R., Wirtz, K.W.A., and van Deenen, L.M.M. (1977). Protein mediated net transfer of phosphatidylinositol in model systems. Biochim. Biophys. Acta. 466, 10-22.

Demol, P., and Sarles, H. (1980). Action of catecholamines on exocrine pancreatic secretion of conscious rats. Arch. Int. Pharmacodyn. 243, 149-163.

Deschodt-Lanckman, M., Robberecht, P., De Neef, P., Labrie, F., Christophe, J. (1975). *In vitro* interactions of gastrointestinal hormones on cyclic adenosine 3',5'-monophosphate levels and amylase output in the rat pancreas. Gastroenterology 68, 381-325.

Deschodt-Lanckman, M., Robberecht, P., De Neef, P., Lammens, M., and Christophe, J. (1976). *In vitro* action of bombesin and bombesin-like peptides on amylase secretion, calcium efflux and adenylate cyclase activity in the rat pancreas. J. Clin. Invest. 58, 891-898.

Dimaline, R., and Dockray, G.J. (1980). Actions of a new peptide from porcine intestine (PHI) on pancreatic secretion in the rat and turkey. Life Sci. 27, 1947-1951.

Direnzo, G.C., Johnston, J.M., Okazaki, T., Okita, J.R., MacDonald, P.C., and Bleasdale, J.E. (1981). Phosphatidylinositol-specific phospholipase C in fetal membranes and uterine decidua. J. Clin. Invest. 67, 847-856.

Di Virgilio, F., Lew, D.P., and Pozzan, T. (1984). Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic Ca^{2+} levels. Nature 310, 691-693.

Dockray, G.J. (1972). The action of secretin, cholecystokinin-pancreozymin and caerulein on pancreatic secretion in the rat. J. Physiol. (Lond.) 255, 679-692.

Dockray, G.J. (1973). Vasoactive intestinal peptide : Secretin-like action on the avian pancreas. Experientia 29, 1510-1511.

Dollinger, H.C., Raptis, S., and Pfeiffer, E.F. (1976). Effects of somatostatin on exocrine and endocrine pancreatic function stimulated by intestinal hormones in man. Hormone Metabolic Res. 8, 74-78.

Domschke, W., Tympner, F., Domschke, S., and Demling, L. (1975). Exocrine pancreatic function in juvenile diabetes. Digest. Dis. 20, 309-315.

Dormer, R.L., and Williams, J.A. (1981). Secretagogue-induced changes in subcellular Ca^{2+} distribution in isolated pancreatic acini. Am. J. Physiol. 240, G130-G140.

Dormer, R.L., Poulsen, J.H., Licko, V., and Williams, J.A. (1981). Calcium-fluxes in isolated pancreatic acini : effects of secretagogues. Am. J. Physiol. 240, G38-G49.

Dormer, R.L. (1984). Introduction of calcium chelators into isolated rat pancreatic acini inhibits amylase release in response to carbamylcholine. Biochem. Biophys. Res. Commun. 119, 876-883.

Downes, C.P., and Michell, R.H. (1981). The polyphosphoinositide phosphodiesterase of erythrocyte membranes. Biochem. J. 198, 133-140.

Downes, C.P., and Michell, R.H. (1982 a). The control by Ca^{2+} of the polyphosphoinositide phosphodiesterase and the Ca^{2+}-pump ATPase in human erythrocytes. Biochem. J. 202, 53-58.

Downes, C.P. and Michell, R.H. (1982 b). Phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate : lipids in search of a function. Cell Calcium 3, 467-502.

Downes, C.P., and Wusteman, M.M. (1983). Breakdown of polyphosphoinositides and not phosphatidylinositol accounts for muscarinic agonist-stimulated inositol phospholipid metabolism in rat parotid glands. Biochem. J. 216, 633-640.

Dyck, W.P., Rudick, J., Hoexter, B., and Janowitz, H.D. (1969). Influence of glucagon on pancreatic exocrine secretion. Gastroenterology 56, 531-537.

Eagle, H. (1959). Amino acid metabolism in mammalian cell cultures. Science 130, 432-437.

Egawa, K., Takenawa, T., Sacktor, B. (1981 a). Inhibition by Ca^{2+} of the incorporation of *myo*-inositol into phosphatidylinositol. Mol. Cell. Endocrinol. 21, 29-35.

Egawa, K., Sacktor, B., and Takenawa, T. (1981 b). Ca^{2+}-dependent and Ca^{2+}-independent degradation of phosphatidylinositol in rabbit *vas deferens*. Biochem. J. 194, 129-136.

Eggman, L.O., and Hokin, L.E. (1960). The relationship between secretory activity and the incorporation of ^{32}P into phosphoinositide and phosphatidic acid in salivary glands and pigeon oesophageol mucosa *in vitro*. J. Biol. Chem. 235, 2569-2571.

Ehlert, F.J., Roeske, W.R., Rosenberger, L.B., and Yamamura, H.I. (1980 a). The influence of guanyl-5'-yl imidodiphosphate and sodium on muscarinic receptor binding in the rat brain and longitudinal muscle of the rat ileum. Life Sci. 26, 245-252.

Ehlert, F.J., Yamamura, H.I., Triggle, D.J., and Roeske, W.R. (1980 b). The influence of guanyl-5'-yl imidodiphosphate and sodium chloride on the binding of the muscarinic agonist, (^{3}H)cis methyldioxolane Eur. J. Pharmacol. 61, 317-318.

Eichberg, J., and Hauser, G. (1973). Sub-cellular distribution of polyphosphoinositides in myelinated and unmyelinated rat brain. Biochim. Biophys. Acta. 326, 210-223.

Eisenberg, F. Jr. (1967). D-Myoinositol 1-phosphate as product of cyclization of glucose 6-phosphate : a substrate for a specific phosphatase in rat testis. J. Biol. Chem. 242, 1375-1382.

Eisenberg, F. Jr., and Hasegawa, R. (1981). Clarification of the roles of competing exchange reactions in phosphatidylinositol metabolism. Trends Biochem. Soc. 6, 9-10.

Ekholm, R., and Edlund, Y. (1959). Ultrastructure of the human exocrine pancreas. J. Ultrastructural Res. 2, 453-481.

El-Fakahany, E., and Richelson, E. (1981). Effects of lanthanides on muscarinic acetylcholine receptor function. Mol. Pharmacol. 19, 282-290.

Elisha, E.E., Hutson, D., and Scratcherd, T. (1984). The direct inhibition of pancreatic electrolyte secretion by noradrenaline in the isolated perfused cat pancreas. J. Physiol. (Lond.) 351, 77-85.

Fain, J.N., and Berridge, M.J. (1979). Relationship between hormonal activation of phosphatidylinositol hydrolysis, fluid secretion and calcium flux in the blowfly salivary gland. Biochem. J. 178, 45-58.

Fairhurst, A.S., Whittaker, M.L., and Ehlert, F.J. (1980). Interactions of D600 (Methoxyverapamil) and local anaesthetics with rat brain α-adrenergic and muscarinic receptors. Biochem. Pharmacol. 29, 155-162.

Farese, R.V., Larson, R.E., and Sabir, A.M. (1980). Effects of Ca^{2+} ionophore A23187 and Ca^{2+} deficiency on pancreatic phospholipids and amylase release *in vitro*. Biochim. Biophys. Acta, 633, 479-484.

Farese, R.V., Sabir, M.A., and Larson, R.E. (1981 a). Effects of dibutyryl cyclic AMP and theophylline on rat pancreatic phospholipids *in vitro*. Ca^{2+}-sensitive decrease in phosphatidylinositol and cycloheximide-sensitive increase in phosphatidic acid. Biochim. Biophys. Acta. 665, 463-470.

Farese, R.V., Sabir, M.A., and Larson, R.E. (1981 b). Effects of adrenocorticotrophin and cycloheximide on adrenal diglyceride kinase. Biochemistry 20, 6047-6051.

Farese, R.V., Larson, R.E., and Sabir, M.A. (1982). Ca^{2+}-dependent and Ca^{2+}-independent effects of pancreatic secretagogues on phosphatidylinositol metabolism. Biochim. Biophys. Acta. 710, 391-399.

Farese, R.V., Sabir, M.A., Larson, R.E., and Trudea III, W. (1983). Further observations on the increases in inositide phospholipids after stimulation by ACTH, cAMP and insulin, and on discrepancies in phosphatidylinositol mass and $^{32}PO_4$-labelling during inhibition of hormonal effects by cycloheximide. Cell Calcium 4, 195-218.

Feinstein, M.B. (1966). Inhibition of contraction and calcium exchangeability in rat uterus by local anaesthetics. J. Pharmacol. Exp. Ther. 152, 516-524.

Feinstein, M.B., and Paimre, M. (1969). Pharmacological action of local anaesthetics on excitation-contraction coupling in striated and smooth muscle. Fed. Proc. 28, 1643-1648.

Fields, J.Z., Roeske, W.R., Morkin, E., and Yamamura, H.I. (1978). Cardiac muscarinic cholinergic receptors : Biochemical identification and characterization. J. Biol. Chem. 253, 3251-3258.

Fisher, D.B., and Mueller, G.C. (1971). Studies on the mechanism by which phytohaemagglutinin rapidly stimulates phospholipid metabolism of human lymphocytes. Biochim. Biophys. Acta. 248, 434-448.

Fölsch, U.R., and Creutzfeldt, W. (1975). Electrolyte secretion by a pancreatic duct model in the rat *in vivo* and accumulation of cyclic adenosine 3',5'-monophosphate *in vitro* in response to gastrointestinal hormones. In: "Stimulus-secretion Coupling in the Gastrointestinal Tract", pp.381-389. Eds: R.M. Case and H. Goebell. MTP Press, Lancaster.

Fölsch, U.R., Winckler, K., and Wormsley, K.G. (1978 a). Influence of repeated administration of cholecystokinin and secretin on the pancreas of the rat. Scand. J. Gastroenterol. 13, 663-671.

Fölsch, U.R., Lankisch, P.G., and Creutzfeldt, W. (1978 b). Effect of somatostatin on basal and stimulated pancreatic secretion in the rat. Digestion 19, 194-203.

Fowler, S., and de Duve, C. (1969). Digestive activity of lysosomes. III. The digestion of lipids by extracts of rat liver lysosomes. J. Biol. Chem. 244, 471-481.

Freedman, S.D., and Jamieson, J.D. (1980). Correlation between protein phosphorylation and secretagogue action in the exocrine pancreas. J. Cell Biol. 87, 171 a.

Freedman, S.D., and Jamieson, J.D. (1981). Ribosomal localization of a 29,000 Dalton protein whose phosphorylation is stimulated *in situ* by secretagogues in the rat exocrine pancreas and parotid. J. Cell Biol. 91, 12029.

Friedel, R.O., Brown, J.D., and Durrel, J. (1969). The enzymic hydrolysis of phosphatidylinositol by guinea-pig brain : Subcellular distribution and hydrolysis products. J. Neurochem. 16, 371-378.

Furuta, Y., Hashimoto, K., and Washizaki, M. (1978). Beta-adrenoceptor stimulation of exocrine secretion from the rat pancreas. Br. J. Pharmacol. 62, 25-29.

Gardner, J.D., Conlon, T.P., and Adams, T.D. (1976). Cyclic AMP in pancreatic acinar cells : Effects of gastrointestinal hormones. Gastroenterology 70, 29-35.

Gardner, J.D., and Jackson, M.J. (1977). Regulation of amylase release from dispersed pancreatic acinar cells. J. Physiol. (Lond.) 270, 439-454.

Gardner, J.D. (1979). Regulation of pancreatic exocrine function *in vitro* : initial steps in the actions of secretagogues. Ann. Rev. Physiol. 41, 55-66.

Gardner, J.D., and Rottman, A.J. (1979). Action of cholera toxin on dispersed acini from guinea-pig pancreas. Biochim. Biophys. Acta. 585, 250-265.

Gardner, J.D., Rottman, A.J., Natarajan, S., and Bodansky, M. (1979). Interaction of secretin$_{5-27}$ and its analogues with hormone receptors on pancreatic acini. Biochim. Biophys. Acta. 583, 491-503.

Gardner, J.D., and Jensen, R.T. (1980). Receptor for secretagogues on pancreatic acinar cells. Am. J. Physiol. 238, G63-G66.

Gardner, J.D., Walker, M., Rottman, A.J. (1980). Effect of A23187 on amylase release from dispersed acini prepared from guinea-pig pancreas. Am. J. Physiol. 238, G458-G466.

Gardner, J.D., Korman, L.Y., Walker, M.D., and Sutliff, V.E. (1982). Effects of inhibitors of cyclic nucleotide phosphodiesterase on the actions of vasoactive intestinal peptide and secretin on pancreatic acini. Am. J. Physiol. 242, G547-G551.

Gardner, J.D., Sutliff, V.E., Walker, M.D., and Jensen, R.T. (1983). Effects of inhibitors of cyclic nucleotide phosphodiesterase on actions of cholecystokinin, bombesin and carbachol on pancreatic acini. Am. J. Physiol. 245, G676-G680.

Geison, R.L., Banschbach, M.W., Sadeghian, K., and Hokin-Neaverson, M. (1976). Acetylcholine-stimulation of selective increases in stearic and arachidonic acids in phosphatidic acid in mouse pancreas. Biochem. Biophys. Res. Commun. 68, 343-349.

Gerrard, J.M., Kindom, S.E., Petersen, D.A., Peller, J., Krantz, K.E., and White, J.G. (1979). Lysophosphatidic acids : influence on platelet aggregation and intracellular calcium flux. Am. J. Pathol. 96, 423-438.

Gill, D.M. (1977). Mechanism of action of cholera toxin. In: "Advances in Cyclic Nucleotide Research", Vol. 8, pp.85-118. Eds: P. Greengard and G.A. Robinson, Raven Press, New York.

Gillet, L., Robberecht, P., König, W., amd Christophe, J.A. (1983). Interaction of Gila Monster Venom with secretin receptors in rat pancreatic membranes. Reg. Peptides 6, 305.

Glynn, I.M. (1963). 'Transport Adenosinetriphosphatase' in electric organ. The relation between ion transport and oxidative phosphorylation. J. Physiol. (Lond.) 169, 452-465.

Godfrey, P.P., and Putney, J.W. Jr. (1984). Receptor-mediated metabolism of the phosphoinositides and phosphatidic acid in rat lacrimal acinar cells. Biochem. J. 218, 187-195.

Goldfine, I.D., and Williams, J.A. (1983). Receptors for insulin and CCK in the acinar pancreas : Relationship to hormone action. Int. Rev. Cytol. 85, 1-37.

Golenhofen, K., and Hermstein, N. (1975). Differentiation of calcium activation mechanisms in vascular smooth muscle by selective suppression with verapamil and D600. Blood Vessels 12, 21-37.

Goodman, F.R., Weiss, G.B., and Adams, H.R. (1974). Alterations by neomycin of ^{45}Ca movements and contractile responses in vascular smooth muscle. J. Pharmacol. Exp. Ther. 188, 472-480.

Gorelick, F.S., Cohn, J.A., Freedman, S.D., Delahunt, N.G., Gershoni, J.M., and Jamieson, J.D. (1983). Calmodulin-stimulated protein kinase activity from rat pancreas. J. Cell. Biol. 97, 1294-1295.

Greenberg, G.R., McCloy, R.F., Adrian, T.E., Chadwick, V.S., Baron, J.H., and Bloom, S.R. (1978). Inhibition of pancreas and gallbladder by pancreatic polypeptide. Lancet 2, 1280-1281.

Greengard, H., Roback, R.A., and Ivy, A.C. (1942). The effect of sympathomimetic amines on pancreatic secretion. J. Pharmacol. Exp. Ther. 74, 309-318.

Greengard, P. (1982). Protein phosphorylation : an overview. In : "Handbook of Pharmacology", Vol.58, pp.357-361. Eds: J.A. Nathanson and J.W. Kebabian, Springer-Verlag, Berlin.

Griffin, H.D., and Hawthorne, J.N. (1978). Calcium-activated hydrolysis of phosphatidylinositol-*myo*-inositol 4-phosphate and phosphatidylinositol-*myo*-inositol 4,5-bisphosphate in guinea-pig synaptosomes. Biochem. J. 176, 541-552.

Grossman, M.I., Greengard, H., and Ivy, A.C. (1943). The effect of dietary composition on pancreatic enzymes. Am. J. Physiol. 138, 676-682.

Gunther, G.R., and Jamieson, J.D. (1979). Increased intracellular cyclic GMP does not correlate with protein discharge from pancreatic acinar cells. Nature 280, 318-320.

Gunther, G.R. (1981 a). Effect of 12-O-Tetradecanoyl-phorbol-13-acetate on Ca^{2+} efflux and protein discharge in pancreatic acini. J. Biol. Chem. 256, 12040-12045.

Gunther, G.R. (1981 b). Binding of phorbol ester derivatives by pancreatic acinar cells. J. Cell Biol. 91, A210.

Halenda, S.P., and Rubin, R.P. (1982). Phospholipid turnover in isolated rat pancreatic acini. Biochem. J. 208, 713-721.

Hallcher, L.M., and Sherman, W.R. (1980). The effects of lithium ion and other agents on the activity of *myo*-inositol-1-phosphatase from bovine brain. J. Biol. Chem. 255, 10896-10901.

Harrington, C.A., and Eichberg, J. (1983). Norepinephrine causes α_1-adrenergic receptor-mediated decrease of phosphatidylinositol in isolated rat liver plasma membranes supplemented with cytosol. J. Biol. Chem. 258, 2087-2090.

Harris, D.W., and Hokin-Neaverson, M. (1977). Subcellular location of acetylcholine-stimulated breakdown of phosphatidylinositol in mouse pancreas. Fed. Proc. 36, 639.

Harris, R.A., Schmidt, J., Hitzemann, B.A., and Hitzemann, R.J. (1981). Phosphatidate as a molecular link between depolarization and neurotransmitter release in the brain. Science, 212, 1290-1291.

Harwood, J.L., and Hawthorne, J.N. (1969). The properties and subcellular distribution of phosphatidylinositol kinase in mammalian tissues. Biochim. Biophys. Acta. 171, 75-88.

Hauser, G., and Eichberg, J. (1973). Improved conditions for preservation and extraction of polyphosphoinositides. Biochim. Biophys. Acta. 326, 201-209.

Haworth, R.A., Hunter, D.R., and Berkoff, H.A. (1980). Na^+ releases Ca^{2+} from liver, kidney and lung mitochondria. FEBS Lett. 110, 216-218.

Hawthorne, J.N. (1964). The biochemistry of the inositol lipids. Vitamins and Hormones 22, 57-79.

Hawthorne, J.N., and White, P.A. (1975). *Myo*-inositol lipids. Vitamins and Hormones 33, 529-573.

Hawthorne, J.N. (1982). Is phosphatidylinositol now out of the calcium gate? Nature 295, 281-282.

Hedlund, B., and Bartfai, T. (1979). The importance of thiol- and disulfide groups in agonist and antagonist binding to the muscarinic receptor. Mol. Pharmacol. 15, 531-544.

Heisler, S., Fast, D., and Tenenhouse, A. (1972). Role of Ca^{2+} and cyclic 3',5'-AMP in protein secretion from rat exocrine pancreas. Biochim. Biophys. Acta. 279, 561-572.

Heisler, S. (1973). Effects of various prostaglandins and serotonin on protein secretion from rat pancreas. Experientia 29, 1234-1235.

Heisler, S., and Grondin, G. (1973). Effect of lanthanum on ^{45}Ca flux and secretion of protein from rat exocrine pancreas. Life Sci. 13, 783-794.

Heisler, S., Chauvelot, L., Desjardins, D., Noel, C., Lambert, H., and Desy-Audet, L. (1981). Stimulus-secretion coupling in exocrine pancreas : Possible role of calmodulin. Can. J. Physiol. Pharmacol. 59, 994-1001.

Heisler, S. (1983). Forskolin potentiates calcium-dependent amylase secretion from rat pancreatic acinar cells. Can. J. Physiol. Pharmacol. 61, 1168-1176.

Helmkamp, G.M. Jr., Wirtz, K.W.A., and van Deenen, L.L.M. (1976). Phosphatidylinositol exchange protein. Effects of membrane structure on activity and evidence for a ping-pong mechanism. Archiv. Biochem. Biophys. 174, 592-602.

Hendrickson, H.S. (1969). Physical properties and interactions of phosphoinositides. Ann. N.Y. Acad. Sci. 165, 668-676.

Hirasawa, K., Irvine, R.F., and Dawson, R.M.C. (1982 a). Heterogeneity of the calcium-dependent phosphatidylinositol phosphodiesterase in rat brain. Biochem. J. 205, 437-442.

Hirasawa, K., Irvine, R.F., and Dawson, R.M.C. (1982 b). Heterogeneity of the calcium-dependent phosphatidylinositol phosphodiesterase of rat liver and kidney as revealed by column chromatofocusing. Biochem. Biophys. Res. Commun. 107, 533-537.

Hirasawa, K., Irvine, R.F., and Dawson, R.M.C. (1982 c). Proteolytic activation can produce a phosphatidylinositol phosphodiesterase highly sensitive to Ca^{2+}. Biochem. J. 206, 675-678.

Hoffman, S.L., and Majerus, P.W. (1982). Identification and properties of two distinct phosphatidylinositol-specific phospholipase C enzymes from sheep seminal vesicular glands. J. Biol. Chem. 257, 6461-6469.

Hokin, L.E., and Hokin, M.R. (1955). Effects of acetylcholine on the turnover of phosphoryl units in individual phospholipids of pancreas slices and brain cortex slices. Biochim. Biophys. Acta. 18, 102-110.

Hokin, L.E., and Hokin, M.R. (1958 a). Phosphoinositides and protein secretion in pancreas slices. J. Biol. Chem. 233, 805-810.

Hokin, L.E., and Hokin, M.R. (1958 b). Acetylcholine and the exchange of inositol and phosphate in brain phosphoinositide. J. Biol. Chem. 233, 818-821.

Hokin, L.E., and Hokin, M.R. (1960). Studies on the carrier function of phosphatidic acid in sodium transport I. The turnover of phosphatidic acid and phosphoinositide in the avian salt gland on stimulation of secretion. J. Gen. Physiol. 44, 61-85.

Hokin, L.E., and Hokin, M.R. (1963). Diglyceride kinase and other pathways for phosphatidic acid synthesis in the erythrocyte membrane. Biochim. Biophys. Acta. 67, 470-484.

Hokin, L.E., and Huebner, D. (1967). Radioautographic localization of the increased synthesis of phosphatidylinositol in response to pancreozymin or acetylcholine in guinea-pig pancreas slices. J. Cell Biol. 33, 521-530.

Hokin, L.E. (1966). Effects of calcium omission on acetylcholine-stimulated amylase secretion and phospholipid synthesis in pigeon pancreas slices. Biochim. Biophys. Acta. 115, 219-221.

Hokin, M.R., and Hokin, L.E. (1953). Enzyme secretion and the incorporation of ^{32}P into phospholipids of pancreas slices. J. Biol. Chem. 203, 967-977.

Hokin, M.R., and Hokin, L.E. (1964). Interconversions of phosphatidyl-inositol and phosphatidic acid involved in the response to acetylcholine in the salt gland. In: "Metabolism and Physiological Significance of Lipids",pp.423-434. Eds: R.M.C. Dawson amd D.N. Rhodes. John Wiley and Sons Ltd., London.

Hokin, M.R. (1968 a). Studies on chemical mechanisms of the action of neurotransmitters and hormones. I. Relationship between hormone-stimulated ^{32}P incorporation into phosphatidic acid and into phosphatidylinositol in pigeon pancreas slices. Archiv. Biochem. Biophys. 124, 271-279.

Hokin, M.R. (1968 b). Studies on chemical mechanisms of the action of neurotransmitters and hormones. II. Increased incorporation of ^{32}P into phosphatides as a second, adaptive response to pancreozymin or acetylcholine in pigeon pancreas slices. Archiv. Biochim. Biophys. 124, 280-284.

Hokin, M.R. (1974). Breakdown of phosphatidylinositol in the pancreas in response to pancreozymin and acetylcholine. In: "Secretory Mechanisms of Exocrine Glands", pp.101-113. Eds: N.A. Thorn and O.H. Petersen. Munskgaard, Copenhagen.

Hokin-Neaverson, M. (1974). Acetylcholine causes a net decrease in phosphatidylinositol and a net increase in phosphatidic acid in mouse pancreas. Biochem. Biophys. Res. Commun. 58, 763-768.

Hokin-Neaverson, M., Sadeghian, K., Majumder, A.L., and Eisenberg, F. Jr. (1975). Inositol is the water-soluble product of acetylcholine-stimulated breakdown of phosphatidylinositol in mouse pancreas. Biochem. Biophys. Res. Commun. 67, 1537-1544.

Hokin-Neaverson, M. (1977). Metabolism and role of phosphatidylinositol in acetylcholine-stimulated membrane function. Adv. Exp. Med. Biol. 83, 429-446.

Hokin-Neaverson, M., and Sadeghian, K. (1984). Lithium-induced accumulation of inositol-1-phosphate during cholecystokinin octapeptide- and acetylcholine-stimulated phosphatidylinositol breakdown in dispersed mouse pancreas acinar cells. J. Biol. Chem. 259, 4346-4352.

Holmsen, H., Kaplan, K.L., and Dangelmaier, C.A. (1982). Differential energy requirements for platelet responses : A simultaneous study of aggregation, 3 secretory processes, arachidonate liberation, phosphatidylinositol breakdown and phosphatidic formation. Biochem. J. 208, 9-18.

Holub, B.J., and Kuksis, A. (1971). Differential distribution of orthophosphate-^{32}P and ^{14}C-glycerol among molecular species of phosphatidylinositols of rat liver *in vivo*. J. Lipid Res. 12, 699-705.

Hootman, S.R., Ernst, S.A., and Williams, J.A. (1983). Secretagogue regulation of Na^+-K^+ pump activity in pancreatic acinar cells. Am. J. Physiol. 245, G339-G346.

Howard, F., and Yudkin, J. (1963). Effect of dietary change upon the amylase and trypsin activities of the rat pancreas. Br. J. Nutrition 17, 281-294.

Hubel, K.A. (1970). Response of rabbit pancreas *in vitro* to adrenergic agonists and antagonists. Am. J. Physiol. 219, 1590-1594.

Hubinot, C.J., Best, L., Sener, A., and Malaisse, W.J. (1984). Activation of a protein kinase C by a tumour-promoting phorbol ester in pancreatic islets. FEBS Lett. 170, 247-253.

Hughes, B.P., Blackmore, P.F., and Exton, J.H. (1980). Exploration of the role of sodium in the α-adrenergic regulation of hepatic glycogenolysis. FEBS Lett. 121, 260-264.

Hui, D.J., and Harmony, J.A.K. (1980). Phosphatidylinositol turnover in mitogen-activated lymphocytes. Suppression by low density lipoproteins. Biochem. J. 192, 91-98.

Ikezawa, H., Yamanegi, M., Taguchi, R., Miyashita, T., and Ohyabu, T. (1976). Studies on phosphatidylinositol phosphodiesterase (phospholipase C type) of *bacillus cereus* I. Purification, properties and phosphatase-releasing activity. Biochim. Biophys. Acta. 450, 154-164.

Imai, A., Ishizuka, Y., Kawai, K., and Nozawa, Y. (1982). Evidence for coupling of phosphatidic acid formation and calcium influx in thrombin-activated human platelets. Biochem. Biophys. Res. Commun. 108, 752-759.

Insel, P.A., Stengel, D., Terry, N., and Hanoune, J. (1982). Regulation of adenylate cyclase of human platelet membranes by forskolin. J. Biol. Chem. 257, 7485-7490.

Irvine, R.F., Hemington, N., and Dawson, R.M.C. (1977). Phosphatidyl-inositol-degrading enzymes in liver lysosomes. Biochem. J. 164, 277-280.

Irvine, R.F., and Dawson, R.M.C. (1978 a). Distribution of calcium-dependent phosphatidylinositol-specific phosphodiesterase in rat brain. J. Neurochem. 31, 1427-1434.

Irvine, R.F., Hemington, N., and Dawson, R.M.C. (1978 b). Hydrolysis of phosphatidylinositol by lysosomal enzymes of rat liver and brain. Biochem. J. 176, 475-484.

Irvine, R.F., and Dawson, R.M.C. (1979). Neural phospholipases hydrolysing phosphatidylinositol and their possible role in stimulated turnover of this phospholipid. Biochem. Soc. Trans. 7, 353-357.

Irvine, R.F., Hemington, N., and Dawson, R.M.C. (1979 a). The calcium-dependent phosphatidylinositol-phosphodiesterase of rat brain, mechanisms of suppression and stimulation. Eur. J. Biochem. 99, 525-530.

Irvine, R.F., Letcher, A.J., and Dawson, R.M.C. (1979 b). Fatty-acid stimulation of membrane phosphatidylinositol hydrolysis by brain phosphatidylinositol phosphodiesterase. Biochem. J. 178, 497-500.

Irvine, R.F., and Dawson, R.M.C. (1980). The control of phosphatidyl-inositol in cell membranes. Biochem. Biophys. Res. Commun. 8, 27-30.

Irvine, R.F., Letcher, A.J., and Dawson, R.M.C. (1980). Phosphatidyl-inositol in higher plants. Biochem. J. 192, 279-283.

Irvine, R.F. (1982). The enzymology of stimulated inositol lipid turnover. Cell Calcium 3, 295-309.

Irvine, R.F., Letcher, A.J., and Dawson, R.M.C. (1984). Phosphatidyl-inositol-4,5-bisphosphate phosphodiesterase and phosphomonoesterase activities of rat brain. Biochem. J. 218, 177-185.

Ishizuka, Y., Imai, A., Kakashima, S., and Nozawa, Y. (1983). Evidence for *de novo* synthesis of phosphatidylinositol coupled with histamine release in activated rat mast cells. Biochem. Biophys. Res. Commun. 111, 581-587.

Iwatsuki, N., and Petersen, O.H. (1977 a). Pancreatic acinar cell : localization of acetylcholine receptors and the importance of chloride and calcium for acetylcholine-evoked depolarization. J. Physiol. (Lond.) 269, 723-733.

Iwatsuki, N., and Petersen, O.H. (1977 b). Pancreatic acinar cells : the acetylcholine equilibrium potential and its ionic dependency. J. Physiol. (Lond.) 269, 735-751.

Iwatsuki, N., and Petersen, O.H. (1977 c). Acetylcholine-like effects of intracellular calcium application in pancreatic acinar cells. Nature 268, 147-149.

Iwamoto, Y., and Williams, J.A. (1980). Inhibition of pancreatic α-aminoisobutyric acid uptake by cholecystokinin and other secretagogues. Am. J. Physiol. 238, G440-G444.

Jaanus, S.D., Miele, E., and Rubin, R.P. (1967). The analysis of the inhibitory effect of local anaesthetics and propranolol on adreno-medullary secretion evoked by calcium or acetylcholine. Br. J. Pharmacol. Chemother. 31, 319-330.

Jahn, R., Unger, C., and Söling, H.D. (1980). Specific protein phosphorylation during stimulation of amylase secretion by β-agonists or dibutyryl adenosine 3',5'-monophosphate in the rat parotid gland. Eur. J. Biochem. 112, 345-352.

Jahn, R., and Söling, H.D. (1981 a). Phosphorylation of the same specific protein during amylase release evoked by β-adrenergic or cholinergic agonists in rat and mouse parotid glands. Proc. Natl. Acad. Sci. USA 78, 6903-6906.

Jahn, R., and Söling, H.D. (1981 b). Protein phosphorylation during secretion in the rat lacrimal gland. A general role of EC-protein in stimulus-secretion coupling in exocrine organs? FEBS Lett. 131, 28-30.

Jahn, R., Padel, U., Porsch, P.H., and Söling, H.D. (1982). Adrenocorticotrophic hormone and alpha-melanocyte-stimulating hormone induce secretion and protein phosphorylation in the rat lacrimal gland by activation of a cAMP-dependent pathway. Eur. J. Biochem. 126, 623-629.

Jahn, R., and Söling, H.D. (1983). Phosphorylation of the ribosomal protein S6 in response to secretagogues in the guinea-pig exocrine pancreas, parotid and lacrimal gland. FEBS Lett. 153, 71-76.

Jamieson, J.D., and Palade, G.E. (1967 a). Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. J. Cell Biol. 34, 577-615.

Jamieson, J.D., and Palade, G.E. (1967 b). Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. Transport to condensing vacuoles and zymogen granules. J. Cell Biol. 34, 597-615.

Jamieson, J.D., and Palade, G.E. (1968). Intracellular transport of secretory proteins in the pancreatic exocrine cell. III. Dissociation of intracellular transport from protein synthesis. J. Cell Biol. 39, 580-588.

Jamieson, J.D., and Palade, G.E. (1971 a). Condensing vacuole conversion and zymogen granule discharge in pancreatic exocrine cells : metabolic studies. J. Cell Biol. 48, 503-522.

Jamieson, J.D., and Palade, G.E. (1971 b). Synthesis, intracellular transport and discharge of secretory proteins in stimulated pancreatic exocrine cells. J. Cell Biol. 50, 135-158.

Jamieson, J.D. (1972). Transport and discharge of exportable proteins in pancreatic exocrine cells : *in vitro* studies. In: "Current Topics in Membranes and Transport", Vol.3, pp.273-338. Eds : F. Bronner and A. Kleinzeller. Academic Press, New York-London.

Jansen, J.B.W., and Lamers, C.B.H.W. (1983). Radioimmunassay of cholecystokinin in human tissue and plasma. Clinica Chimica Acta. 131, 305-316.

Järnefelt, J. (1962). Properties and possible mechanism of the Na^+ and K^+-stimulated microsomal adenosine triphosphate. Biochim. Biophys. Acta. 59, 643-654.

Jensen, R.T., and Gardner, J.D. (1978). Cyclic nucleotide-dependent protein kinase activity in acinar cells from guinea-pig pancreas. Gastroenterology 75, 806-817.

Jensen, R.T., Moody, T., Pert, C., Rivier, J.E., and Gardner, J.D. (1978). Interaction of bombesin and litorin with specific membrane receptors on pancreatic acinar cells. Proc. Natl. Acad. Sci. USA 75, 6139-6143.

Jensen, R.T., and Gardner, J.D. (1979). Interaction of physalaemin, substance P and eledoisin with specific membrane receptors on pancreatic acinar cells. Proc. Natl. Acad. Sci. USA 76, 5679-5683.

Jensen, R.T., Lemp, G.F., and Gardner, J.D. (1980). Interaction of cholecystokinin with specific membrane receptors on pancreatic acinar cells. Proc. Natl. Acad. Sci. USA 77, 2078-2083.

Jensen, R.T., Tatemoto, K., Mutt, V., Lemp, G.F., and Gardner, J.D. (1981). Actions of a newly isolated intestinal peptide PHI on pancreatic acini. Am. J. Physiol. 241, G498-G502.

Jergil, B., and Sundler, R. (1983). Phosphorylation of phosphatidylinositol in rat liver Golgi. J. Biol. Chem. 258, 7968-7973.

Jones, L.M., and Michell, R.H. (1974). Breakdown of phosphatidylinositol provoked by muscarinic cholinergic stimulation of rat parotid-gland fragments. Biochem. J. 142, 583-590.

Jones, L.M., and Michell, R.H. (1975). The relationship of calcium to receptor-controlled stimulation of phosphatidylinositol turnover. Effects of acetylcholine, adrenaline, calcium ions, cinchocaine, and a bivalent cation ionophore on rat-parotid fragments. Biochem. J. 148, 479-485.

Jones, L.M., and Michell, R.H. (1976). Cholinergically stimulated phosphatidylinositol breakdown in parotid-gland fragments is independent of the ionic movement. Biochem. J. 158, 505-507.

Jones, L.M., Cockcroft, S., and Michell, R.H. (1979). Stimulation of phosphatidylinositol turnover in various tissues by cholinergic and adrenergic agonists, by histamine and by caerulein. Biochem. J. 182, 669-672.

Jorgensen, P.L. (1980). Sodium and potassium ion pump in kidney tubules. Physiol. Rev. 60, 864-917.

Joseph, S.K., Thomas, A.P., Williams, R.J., Irvine, R.F., and Williamson, J.R. (1984). *Myo*-Inositol 1,4,5-trisphosphate : A second messenger for the hormonal mobilization of intracellular Ca^{2+} in liver. J. Biol. Chem. 259, 3077-3081.

Kai, M., Salway, J.G., and Hawthorne, J.N. (1968). The diphosphoinositide kinase of rat brain. Biochem. J. 106, 791-801.

Kaibuchi, K., Kimihiko, S., Hoshijima, M., Takai, Y., and Nishizuka, Y. (1982 a). Phosphatidylinositol turnover in platelet activation, calcium mobilization and protein phosphorylation. Cell Calcium 3, 323-335.

Kaibuchi, K., Takai, Y., Ogawa, Y., Kimura, S., Nishizuka, Y., Nakamura,T., Tomomura, A., and Ichihara, A. (1982 b). Inhibitory action of adenosine 3',5'-monophosphate on phosphatidylinositol turnover : difference in tissue response. Biochem. Biophys. Res. Commun. 104, 105-112.

Kaibuchi, K., Takai, M., Sawamura, M., Hoshijima, M., Fujikura, T., and Nishizuka (1983). Synergistic functions of protein phosphorylation and calcium mobilization in platelet activation. J. Biol. Chem. 258, 6701-6704.

Kanamori, T., and Hayakawa, T. (1980). Cyclic AMP-dependent ^{32}P-incorporation into a protein in rat parotid slices. Biochem. Int. 1, 395-402.

Kanno, T. (1972). Calcium-dependent amylase release and electrophysiological measurements in cells of pancreas. J. Physiol. (Lond.) 226, 353-371.

Kanno, T., and Saito, A. (1976). The potentiating influences of insulin on pancreozymin-induced hyperpolarization and amylase release in the pancreatic acinar cell. J. Physiol. (Lond.) 261, 505-521.

Kanno, T., and Nishimura, O. (1976). Stimulus-secretion coupling in pancreatic acinar cells : inhibitory effects of calcium removal and manganese addition on pancreozymin-induced amylase release. J. Physiol. (Lond.) 257, 309-324.

Kanno, T., and Yamamoto, M. (1977). Differentiation between the calcium-dependent effects of cholecystokinin-pancreozymin and the bicarbonate-dependent effects of secretin in exocrine secretion of the rat pancreas. J. Physiol. (Lond.) 264, 787-799.

Kanno, T., and Saito, A. (1978). Influence of external potassium concentration on secretory responses to cholecystokinin-pancreozymin and ionophore A23187 in the pancreatic acinar cell. J. Physiol. (Lond.) 278, 251-263.

Kanoh, H., and Akersson, B. (1978). Properties of microsomal and soluble diacylglycerol kinase in rat liver. Eur. J. Biochem. 85, 225-232.

Kelly, G.A., Rose, R.C., and Nahrwold, D.L. (1974). Effect of isoproterenol on pancreatic responses to exogenous and endogenous cholecystokinin. Gastroenterology 66, 722.

Kemp, P., Hübscher, G., and Hawthorne, J.N. (1961). Phosphoinositides 3. Enzymic hydrolysis of inositol-containing phospholipids. Biochem. J. 79, 193-200.

Kemper, H.J.M., De Pont, J.J.H.H.M., and Bonting, S.L. (1975). Rat pancreas adenylate cyclase. III. Its role in pancreatic secretion assessed by means of cholera toxin. Biochim. Biophys. Acta. 392, 276-287.

Kempen, H.J.M., De Pont, J.J.H.H.M., and Bonting, S.L. (1977). Rat pancreatic adenylate cyclase. IV. Effect of hormones and other agents on cyclic AMP levels and enzyme release. Biochim. Biophys. Acta. 496, 65-76.

Keough, K.M.W., and Thompson, W. (1970). Triphosphoinositide phospho-diesterase in developing brain of rat and in subcellular fractions of brain. J. Neurochem. 17, 1-11.

Keryer, G., Herman, G., and Rossignol, B. (1979). Sodium requirement in secretory processes regulated through muscarinic receptors in rat parotid glands : Its effect on amylase secretion and phosphatidyl-inositol labelling. FEBS Lett. 102, 4-8.

Keryer, G., and Rossignol, B. (1978). Lanthanum as a tool to study the role of phosphatidylinositol in the calcium transport in rat-parotid glands upon cholinergic stimulation. Eur. J. Biochem. 85, 77-83.

Keston, A.S. (1956). Specific colorimetric enzymatic analytical reagents for glucose. Abstract of Papers, 129th Meeting, ACS, Dallas (TX), April 1956, p.310.

Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R., Nishizuka, Y. (1983). Protein kinase C as a possible receptor protein of tumour-promoting phorbol esters. J. Biol. Chem. 258, 11442-11445.

Kirk, C.J., and Michell, R.H. (1981). Phosphatidylinositol metabolism in rat hepatocytes stimulated by vasopressin. Biochem. J. 194, 155-165.

Kirk, C.J., Creba, J.A., Downes, C.P., and Michell, R.H. (1981). Hormone-stimulated metabolism of inositol lipids and its relationship to hepatic receptor function. Biochem. Soc. Trans. 9, 377-379.

Kishimoto, A., Takai, Y., Mari, T., Kikkawa, U., and Nishizuka, Y. (1980). Activation of calcium and phospholipid-dependent protein kinase by diacylclycerol : Its possible relation to phosphatidylinositol turnover. J. Biol. Chem. 255, 2273-2276.

Kishimoto, A., Kajikawa, N., Shiota, M., and Nishizuka, Y. (1983). Proteolytic activation of a calcium-activated, phospholipid-dependent protein kinase by calcium-dependent neutral protease. J. Biol. Chem. 258, 1156-1164.

Knight, D.E., and Baker, P.F. (1983). The phorbol ester TPA increases the affinity of exocytosis for calcium in 'leaky' adrenal medullary cells. FEBS Lett. 160, 98-100.

Knight, D.E., and Scrutton, M.C. (1984). Cyclic nucleotides control a system which regulates Ca^{2+} sensitivity of platelet secretion. Nature 309, 66-68.

Kohlardt, M., Bauer, P., Krause, H., and Fleckenstein, A. (1972). Differentiation of the transmembrane Na and Ca channels in mammalian cardiac fibres by the use of specific inhibitors. Pflügers Arch. 335, 309-321.

Kondo, S., and Schulz, I. (1976). Calcium ion uptake in isolated pancreas cells induced by secretagogues. Biochim. Biophys. Acta. 419, 76-92.

Korc, M., Williams, J.A., and Goldfine, I.D. (1979). Stimulation of the glucose transport system in isolated mouse pancreatic acini by cholecystokinin and analogues. J. Biol. Chem. 254, 7624-7629.

Korc, M., Iwamoto, Y., Sankaran, H., Williams, J.A., Goldfine, I.D. (1981 a). Insulin action in pancreatic acini from streptozotocin-treated rats. I. Stimulation of protein synthesis. Am. J. Physiol. 240, G56-G62.

Korc, M., Bailey, A.C., and Williams, J.A. (1981 b). Regulation of protein synthesis in normal and diabetic rat pancreas by cholecystokinin. Am. J. Physiol. 241, G116-G121.

Kramer, C.M., Franson, R.C., and Rubin, R.P. (1984). Regulation of phosphatidylinositol, calcium metabolism and enzyme secretion by phorbol dibutyrate in neutrophils. Lipids 19, 315-323.

Kulka, L.G., and Sternlicht, E. (1968). Enzyme secretion in mouse pancreas mediated by adenosine-3',5'-cyclic phosphate and inhibited by adenosine-3'-phosphate. Proc. Natl. Acad. Sci. USA 61, 1123-1128.

Kurtzer, R.J., and Roberts, M.L. (1982). Calcium-dependent K^+ efflux from rat submandibular gland. The effects of trifluoperazine and quinidine Biochim. Biophys. Acta. 693, 479-484.

Laffont, F., Chap, H., Soula, G., and Douste-Blazy, L. (1981). Phospholipid exchange proteins from platelet cytosol possibly involved in phospholipid effect. Biochem. Biophys. Res. Commun. 102, 1366-1371.

Lakshmanan, J. (1978). Involvement of post-synaptic cytoskeleton in nerve growth factor-induced 'PI-Effect'. FEBS Lett. 95, 161-164.

Lambert, M., Camurs, J., and Christophe, J. (1974). Phosphorylation of protein components of isolated zymogen granule membranes from the rat pancreas. FEBS Lett. 49, 228-232.

Langer, G.A., Serena, S.D., and Nudd, L.M. (1974). Cation exchange in heart cell culture : Correlation with effects on contractile force. J. Mol. Cell Cardiol. 6, 149-161.

Lapetina, E.G., and Hawthorne, J.N. (1971). Diglyceride kinase of rat cerebral cortex. Biochem. J. 122, 171-179.

Lapetina, E.G., and Michell, R.H. (1973). A membrane-bound activity catalysing phosphatidylinositol breakdown to 1,2 diacylglycerol, D-*myo*-inositol 1 : 2 cyclic phosphate, and D-*myo*-inositol 1-phosphate. Biochem. J. 131, 433-442.

Lapetina, E.G., Sequin, E.B., and Agranoff, B.W. (1975). Preparation of ^{32}P-labelled inositides and their degradation by soluble kidney enzymes. Biochim. Biophys. Acta. 398, 118-124.

Lapetina, E.G., Chandrabose, K., and Cuatracasas, P. (1978). Ionophore A23187- and thrombin-induced platelet aggregation : Independence from cycloxygenase products. Proc. Natl. Acad. Sci. USA 75, 818-822.

Lapetina, E.G., Billah, M.M., and Cuatrecasas (1981). The phosphatidyl-inositol cycle and the regulation of arachidonic acid production. Nature 292, 367-369.

Lapetina, E.G. (1982). Platelet-activating factor stimulates the phosphatidylinositol cycle. J. Biol. Chem. 257, 7314-7317.

Lapetina, E.G. (1983). Metabolism of inositides and the activation of platelets. Life Sci. 32, 2069-2082.

Lardy, H.A., Johnson, D., and McMurray, W.C. (1958). Antibiotics as tools for metabolic studies. I. A survey of toxic antibiotics in respiratory, phosphorylative and glycolytic systems. Archiv. Biochim. Biophys. 78, 587-597.

Larose, L., Dumont, Y., Asselin, J., Morisset, F., and Poirier, G.G. (1981). Muscarinic receptor of rat pancreatic acini : (^{3}H)-QNB binding and amylase secretion. Eur. J. Pharmacol. 76, 247-254.

Larsson, L-I. (1979). Innervation of the pancreas by substance P, enkephalin, vasoactive intestinal polypeptide and gastrin/CCK immunoreactive nerves. J. Histochem. Cytochem. 27. 1283-1284.

Larsson, L-I., and Rehfeld, J.F. (1979). Peptidergic and adrenergic innervation of pancreatic ganglia. Scand. J. Gastroenterol. 14, 433-437.

Laugier, R., and Petersen, O.H. (1970). Pancreatic acinar cells : Electrophysiological evidence for stimulant-evoked increase in membrane calcium permeability in the mouse. J. Physiol. (Lond.) 303, 61-72.

Laugier, R., and Petersen, O.H. (1980). Effects of intracellular EGTA injection on stimulant-evoked membrane potential and resistance changes in pancreatic acinar cells. Pflügers Arch. 386, 147-152.

Lee, P.C., Jensen, R.T., and Gardner, J.D. (1980). Bombesin-induced desensitization of enzyme secretion in dispersed acini from guinea-pig pancreas. Am. J. Physiol. 238, G213-G218.

Lewis, D.S., and Ronzio, R.A. (1979). An assessment of the role of protein kinase and zymogen granule phosphorylation during secretion by the rat exocrine pancreas. Biochim. Biophys. Acta. 583, 422-433.

Lifson, N., Kramlinger, K.G., Mayrand, R.R., and Lender, E.J. (1980). Blood flow to the rabbit pancreas with specific reference to the islets of Langerhans. Gastroenterology, 79, 466-473.

Limas, C.J. (1980). Phosphatidate releases calcium from cardiac sarcoplasmic reticulum. Biochem. Biophys. Res. Commun. 95, 541-546.

Lin, T.M., Evans, D.C., Chance, R.E., and Spray, G.F. (1977). Bovine pancreatic polypeptide action on gastric and pancreatic secretion in dogs. Am. J. Physiol. 232, E311-E315.

Lin, S.H., and Fain, J.N. (1981). Vasopressin and epinephrine stimulation of phosphatidylinositol breakdown in the plasma membrane of rat hepatocytes. Life Sci. 29, 1905-1912.

Lingard, J.M., and Young, J.A. (1983). β-adrenergic control of exocrine secretion by perfused rat pancreas *in vitro*. Am. J. Physiol. 245, G690-G696.

Litosch, I., Saito, Y., and Fain, J.N. (1982). 5-HT-stimulated arachidonic acid release from labelled phosphatidylinositol in blowfly salivary glands. Am. J. Physiol. 243, C222-C226.

Litosch, I., Lin, S-H., and Fain, J.N. (1983). Rapid changes in hepatocyte phosphoinositides induced by vasopressin. J. Biol. Chem. 258, 13727-13732.

Livingstone, C.J., and Schachter, D. (1980). Calcium modulates the lipid dynamics of rat hepatocyte plasma membranes by direct and indirect mechanisms. Biochemistry 19, 4823-4827.

Llodhi, S., Weiner, N.D., and Schacht, J. (1976). Interactions of neomycin and calcium in synaptosomal membranes and polyphosphoinositide monolayers. Biochim. Biophys. Acta. 426, 781-785.

Long, B.W., and Gardner, J.D. (1977). Effects of cholecystokinin on adenylate cyclase activity in dispersed pancreatic acinar cells. Gastroenterology 73, 1008-1014.

Low, M.G., and Finean, J.B. (1976). Action of phosphatidylinositol-specific phospholipases C on membranes. Biochem. J. 154, 203-208.

Lowitt, S., Farese, R.V., Sabir, M.A., and Root, A.W. (1982). Rat Leydig cell phospholipid content is increased by luteinizing hormone and 8-bromo-cyclic AMP. Endocrinology 111, 1415-1417.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, A.J. (1951). Protein measurement with the Folin Phenol Reagent. J. Biol. Chem. 193, 265-275.

Luft, R., Efendic, S., Hökfelt, T., Johansson, O., and Arimura, A. (1974). Immunohistochemical evicence for the localization of somatostatin-like immunoreactivity in a cell population of the pancreatic islets. Med. Biol. 52, 428-430.

Lyons, R.M., and Shaw, J.O. (1980). Interaction of Ca^{2+} and protein-phosphorylation in the rabbit platelet-release action. J. Clin. Invest. 65, 242-255.

Malagodi, M.H., and Chiou, C.Y. (1974). Pharmacological evaluation of a new Ca^{2+}-antagonist, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxy-benzoate hydrochloride (TMB-8) : Studies in smooth muscles. Eur. J. Pharmacol. 27, 25-33.

Malaisse, W.J. (1972). Insulin secretion and food intake. Proc. Nutr. Soc. 31, 213-217.

Malnoë, A., Cox, J.A., and Stein,E.A.(1982). Ca^{2}-dependent regulation of calmodulin-binding and adenylate cyclase activation in bovine cerebellar membranes. Biochim. Biophys. Acta. 714, 84-92.

Manabe, T., and Steer, M.L. (1979). Effects of glucagon on pancreatic content and secretion of amylase in mice. Proc. Soc. Exp. Biol. Med. 161, 538-542.

Margulis, L. (1973). Colchicine-sensitive microtubules. Int. Rev. Cytol. 34, 333-361.

Marier, S.H., Putney, J.W. Jr., and Van De Walle, C.M. (1978). Control of calcium channels by membrane receptors in the rat-parotid gland. J. Physiol. (Lond.) 279, 141-151.

Marois, C., Morisset, J., and Dunnigan, J. (1972). Presence and stimulation of adenyl cyclase in pancreatic homogenate. Rev. Can. Biol. 31, 253-257.

Marshall, P.J., Dixon, J.F., and Hokin, L.E. (1980). Evidence for a role in stimulus-secretion coupling of prostaglandins derived from release of arachidonoyl residues as a result of phosphatidyl-inositol breakdown. Proc. Natl. Acad. Sci. USA 77, 3292-3296.

Marshall, P.J., Boatman, D.E., and Hokin, L.E. (1981). Direct demonstration of the formation of prostaglandin E_2 due to phosphatidylinositol breakdown associated with stimulation of enzyme secretion in the pancreas. J. Biol. Chem. 256, 844-847.

Marshall, P.J., Dixon, J.F., and Hokin, L.E. (1982). Prostaglandin E_2 derived from phosphatidylinositol breakdown in the exocrine pancreas facilitates secretion by an action on the ducts. J. Pharmacol. Exp. Therap. 221, 645-649.

Martonosi, A., and Feretos, R. (1964). Sarcoplasmic reticulum I. The uptake of Ca^{2+} by sarcoplasmic reticulum fragments. J. Biol. Chem. 239, 648-658.

Maruyama, Y., and Petersen, O.H. (1982 a). Single channel currents in isolated patches of plasma membrane from basal surface of pancreatic acini. Nature 299, 159-161.

Maruyama, Y., and Petersen, O.H. (1982 b). Cholecystokinin activation of single-channel currents is mediated by internal messenger in pancreatic acinar cells. Nature 300, 61-63.

Maruyama, Y., Petersen, O.H., Flanagan, P., and Pearson, G.T. (1983 a). Quantification of Ca^{2+}-activated K^+ channels under hormonal control in pig pancreas acinar cells. Nature 305, 228-232.

Maruyama, Y., Gallacher, D.V., and Petersen, O.H. (1983 b). Voltage and Ca^{2+}-activated K^+ channel in baso-lateral acinar cell membranes of mammalian salivary glands. Nature 302, 827-829.

Means, A.R., and Dedman, J.R. (1980). Calmodulin : an intracellular calcium receptor. Nature 285, 73-77.

Meldolesi, J. (1970). Effect of caerulein on protein synthesis and secretion in the guinea-pig pancreas. Br. J. Pharmacol. 40, 721-731.

Meldolesi, J. (1976). Discussion. In: "Stimulus-Secretion Coupling in the Gastrointestinal Tract", pp.329-330. Eds: R.M. Case and H. Goebell. MTP Press, England.

Michelangeli, F. (1979). Effects of oligomycin on energy metabolism in isolated oxyntic cells. Biochem. Pharmacol. 28, 941-943.

Michell, R.H., Hawthorne, J.N., Coleman, R., and Karnovsky, M.L. (1970). Extraction of polyphosphoinositides with neutral and acidified solvents : A comparison of guinea-pig brain and liver, and measurements of rat liver inositol compounds which are resistant to extraction. Biochim. Biophys. Acta. 210, 86-91.

Michell, R.H. (1975). Inositol phospholipids and cell surface receptor function. Biochim. Biophys. Acta. 415, 81-147.

Michell, R.H., Jafferji, S.S., and Jones, L.M. (1976). Receptor-occupancy dose-response curve suggests that phosphatidylinositol breakdown may be intrinsic to the mechanism of the muscarinic cholinergic receptor. FEBS Lett. 69, 1-5.

Michell, R.H., and Kirk, C.J. (1981). Studies of receptor-stimulated inositol lipid metabolism should focus upon measurements of inositol lipid breakdown. Biochem. J. 198, 247-248.

Michell, R.H., Kirk, C.J., Jones, L.M., Downes, C.P., and Greba, J.A. (1981). The stimulation of inositol lipid metabolism that accompanies calcium mobilization in stimulated cells : Defined characteristics and unanswered questions. Phil. Trans. R. Soc. Lond. B. 296, 123-137.

Michell, R.H. (1983). Ca^{2+} and protein kinase C : Two synergistic cellular signals. Trends Biochem. Sci. 8, 264-266.

Miller, J.C., and Kowal, C.N. (1981). The relationship between the incorporation of ^{32}P into phosphatidic acid and phosphatidylinositol in rat parotid acinar cells. Biochem. Biophys. Res. Commun. 102, 999-1007.

Mori, T., Takai, Y., Minakuchi, B.Y., and Nishizuka, Y. ((1980). Inhibitory action of chlorpromazine, dibucaine, and other phospholipid-interacting drugs on calcium activated, phospholipid-dependent protein kinase. J. Biol. Chem. 255, 8378-8380.

Morisset, J.A., and Webster, P.D. (1971). *In vitro* and *in vivo* effects of pancreozymin, urecholine and cyclic AMP on rat pancreas. Am. J. Physiol. 230, 202-208.

Multinovic, S., Schulz, I., and Rosselin, G. (1976). The interaction of secretin with pancreatic membranes. Biochim. Biophys. Acta. 436, 113-127.

Mürer, E.H., Stewart, G.J., Davenport, K., and Siojo, E. (1981). Effect of three calcium antagonists on platelet secretion and metabolism. Biochem. Pharmacol. 30, 523-530.

Murphy, E., Coll, K., Rich, T.L., and Williamson, J.R. (1980). Hormonal effects on calcium homeostasis in isolated hepatocytes. J. Biol. Chem. 255, 6600-6608.

Naccache, P.H., Molski, T.F.P., Becker, E.L., Borgeat, P., Picard, S., Vallerand, P., and Sha'afi R.I. (1982). Specificity of the effect of lipoxygenase metabolites of arachidonic acid on calcium homeostasis in neutrophils. J. Biol. Chem. 257, 8608-8611.

Naccarato, W.F., Ray, R.E., and Wells, W.W. (1974). Biosynthesis of *myo*-inositol in rat mammary gland : Isolation and properties of enzymes. Archiv. Biochem. Biophys. 164, 194-201.

Nakadate, T., Nakaki, T., Muraki, T., and Kato, R. (1981). Adrenergic receptors and the onset of streptozotocin-induced diabetes in mice. Eur. J. Pharmacol. 75, 45-51.

Ng, K.H., Morisset, J., and Poirier, G.G. (1979). Muscarinic receptors of the pancreas : Correlation between displacement of quinuclidinyl-^{3}H-benzilate binding and amylase secretion. Pharmacology, 18,263-270.

Niedel, J.E., Kuhn, L.J., and Vandenbark, G.R. (1983). Phorbol diester receptor co-purifies with protein kinase C. Proc. Natl. Acad. Sci. USA 80, 36-40.

Nishiyama, A., and Petersen, O.H. (1974). Pancreatic acinar cells : Membrane potential and resistance change evoked by acetylcholine. J. Physiol. (Lond.) 238, 145-158.

Nishiyama, A., and Petersen, O.H. (1975). Pancreatic acinar cells : Ionic dependence of acetylcholine-induced membrane potential and resistance change. J. Physiol. (Lond.) 244, 431-465.

Nishiyama, A. (1984). The role of Ca^{2+} stores in secretion. Cell Calcium 5, 89-110.

Nishizuka, Y. (1984). Turnover of inositol phospholipids and signal transduction. Science 225, 1365-1370.

Norman, J.A., Drummond, A.H., and Moser, P. (1980). Inhibition of calcium-dependent regulator-stimulated phosphodiesterase activity by neuroleptic drugs is unrelated to their clinical efficacy. Mol. Pharmacol. 16, 1089-1094.

Ochs, D.L., Korenbrot, J.I., and Williams, J.A. (1983). Intracellular free calcium concentrations in isolated pancreatic acini : Effects of secretagogues. Biochem. Biophys. Res. Commun. 117, 122-128.

O'Doherty, J., and Stark, R.J. (1982). Stimulation of pancreatic acinar secretion : Increases in cytosolic calcium and sodium. Am. J. Physiol. 242, G513-G521.

Ohsako, S., and Deguchi, T. (1981). Stimulation by phosphatidic acid of calcium influx and cyclic GMP synthesis in neuroblastoma cells. J. Biol. Chem. 256, 10945-10948.

Olinger, E.J., and Gardner, J.D. (1979). Action of VIP and secretin on adenylate cyclase activity in acinar cells from guinea-pig pancreas. Gastroenterology 77, 704-713.

Oron, Y., Sharani, Y., Lefkovitz, H., and Selinger, A. (1978). Phosphatidylinositol kinase and diphosphoinositide kinase in the rat-parotid gland. In: "Cyclitols and Phosphoinositides", pp.383-398. Eds: W.W. Wells and F. Eisenberg. Academic Press, New York.

Orchard, J.L., Davis, J.S., Larson, R.E., and Farese, R.V. (1984). Effects of carbachol and pancreozymin (cholecystokinin-octapeptide) on polyphosphoinositide metabolism in rat pancreas *in vitro*. Biochem. J. 217, 281-287.

Otsuki, M., and Williams, J.A. (1982). Effect of diabetes mellitus on the regulation of enzyme secretion by isolated rat pancreatic acini. J. Clin. Invest. 70, 148-156.

Pan, G-Z., Collen, M.J., and Gardner, J.D. (1982). Action of cholera toxin on dispersed acini from rat pancreas : Post receptor modulation involving cyclic AMP and calcium. Biochim. Biophys. Acta. 720, 338-345.

Pagano, R.E., Longmuir, K.J., and Martin, O.C. (1983). Intracellular translocation and metabolism of a fluorescent phosphatidic acid analogue in cultured fibroblasts. J. Biol. Chem. 258, 2034-2040.

Palade, G.E., and Siekevitz, P. (1956). Pancreatic microsomes : An integrated morphological and biochemical study. J. Biophys. Biochem. Cytol. 2, 671-690.

Palade, G.E., Siekevitz, P., and Caro, L.G. (1962). Structure, chemistry and function of the pancreatic exocrine cell. In: Ciba Foundation Symposium, "The Exocrine Pancreas", pp.23-55. Eds: A.V.S. De Reuck and M.P. Cameron. Churchill, London.

Pandol, S.J., Sutliff, V.E., Jones, S.W., Charlton, C.G., O'Donohue, T.L., Gardner, J.D., and Jensen, R.T. (1983). Action of natural glucagon on pancreatic acini : Due to contamination by previously undescribed secretagogues. Am. J. Physiol. 245, G703-G710.

Paton, W.D.M., Vizi, E.S., and Aboo Zar, M. (1971). The mechanism of acetylcholine release from parasympathetic nerves. J. Physiol. (Lond.) 215, 819-848.

Paulus, H., and Kennedy, E.P. (1960). The enzymatic synthesis of inositol monophosphatide. J. Biol. Chem. 235, 1303-1311.

Pearson, G.T., Singh, J., Daoud, M.S., Davison, J.S., and Petersen, O.H. (1981 a). Control of pancreatic cyclic nucleotide levels and amylase secretion by noncholinergic, nonadrenergic nerves : A study employing electrical field stimulation of guinea-pig segments. J. Biol. Chem. 256, 11025-11031.

Pearson, G.T., Davison, J.S., Collins, R.C., Petersen, O.H. (1981 b). Control of enzyme secretion by noncholinergic, nonadrenergic nerves in guinea-pig pancreas. Nature 290, 259-261.

Pearson, G.T., and Singh, J. (1983). An adrenergic nervous pathway for control of enzyme secretion in the rat pancreas. J. Physiol. (Lond.) 334, 134P.

Pearson, G.T., Singh, J., and Petersen, O.H. (1984). Adrenergic nervous control of cAMP-mediated amylase secretion in the rat pancreas. Am. J. Physiol. 246, G563-G573.

Peikin, S.R., Rottman, A.J., Batzri, S., and Gardner, J.D. (1978). Kinetics of amylase release by dispersed acini prepared from guinea-pig pancreas. Am. J. Physiol. 235, E743-E749.

Petersen, H., Solomon, T., and Grossman, M.I. (1978). Effect of chronic pentagastrin, cholecystokinin and secretin on pancreas of rats. Am. J. Physiol. 234, E286-E293.

Petersen, O.H. (1970). Some factors influencing stimulation-induced release of potassium from the cat submandibular gland to fluid perfused through the gland. J. Physiol. (Lond.) 208, 431-447.

Petersen, O.H., and Ueda, N. (1976 a). Pancreatic acinar cells : The role of calcium in stimulus-secretion coupling. J. Physiol. (Lond.) 254, 583-606.

Petersen, O.H., and Ueda, N. (1976 b). The importance of calcium for caerulein- and secretin-evoked fluid and enzyme secretion in the perfused rat pancreas. J. Physiol. (Lond.) 263, 223-224P.

Petersen, O.H., and Ueda, N. (1977). Secretion of fluid and amylase in the perfused rat pancreas. J. Physiol. (Lond.) 264, 819-835.

Petersen, O.H., and Iwatsuki, N. (1978). The role of calcium in pancreatic acinar cell stimulus-secretion coupling : An electrophysiological approach. Ann. N.Y. Acad. Sci. 307, 599-617.

Petersen, O.H., Maruyama, Y., Graf, J., Laugier, R., Nishiyama, A., and Pearson, G.T. (1981). Ionic currents across pancreatic acinar cell membranes and their role in fluid secretion. Phil. Trans. R. Soc. Lond. B. 296, 151-166.

Petersen, O.H. (1984). The role of Ca^{2+} stores in secretion. In: "Secretion : Mechanisms and Control", pp.149-168. Eds: R.M. Case, J.M. Lingard and J.A. Young. Manchester University Press.

Petersen, O.H., and Maruyama, Y. (1984). Calcium-activated potassium channels and their role in secretion. Nature 307, 693-696.

Phillips, J.H. (1973). PtdIns kinase, a component of the chromaffin granule membrane. Biochem. J. 136, 579-587.

Pickard, M.R., and Hawthorne, J.N. (1978). Labelling of nerve ending phospholipids in guinea-pig brain *in vivo* and effect of electrical stimulation on phosphatidylinositol metabolism in prelabelled synaptosomes. J. Neurochem. 30, 145-155.

Ponnapa, B.C., Dormer, R.L., and Williams, J.A. (1981). Characterization of an ATP-dependent Ca^{2+} uptake system in mouse pancreatic microsomes. Am. J. Physiol. 240, G122-G129.

Poggioli, J., and Putney, J.W. Jr. (1982). Net calcium fluxes in rat-parotid acinar cells : Evidence for a hormone-sensitive calcium pool in or near the plasma membrane. Pflügers Archiv. 392, 239-243.

Poggioli, J., Weiss, S.J., McKinney, J.S., and Putney, J.W. Jr. (1983). Effects of antimycin A on receptor-activated calcium mobilization and phosphoinositide metabolism in rat-parotid gland. Mol. Pharmacol. 23, 71-77.

Poulsen, J.H. (1974). Liberation of acetylcholine by ouabain in the cat submandibular gland. Pflügers Archiv. 380, 281-286.

Poulsen, J.H., and Williams, J.A. (1977). Effects of the calcium ionophore A23187 on pancreatic acinar cell membrane potentials and amylase release. J. Physiol. (Lond.) 264, 323-339.

Pozzan, T., Corps, A.N., Montecucco, C., Hesketh, T.R., and Metcalfe, J.C. (1980). Cap formation by various ligands on lymphocytes shows the same dependence on high cellular ATP levels. Biochim. Biophys. Acta. 602, 558-566.

Pozzan, T., Rink, T.J., and Tsien, R.Y. (1981). Intracellular free Ca^{2+} in intact lymphocytes. J. Physiol. (Lond.) 318, 12P-13P.

Preissler, M., and Williams, J.A. (1981). Pancreatic acinar cell function : Measurement of intracellular ions and pH and their relation to secretion. J. Physiol. (Lond.) 321, 437-448.

Prescott, S.M., and Majerus, P.W. (1981). The fatty acid composition of phosphatidylinositol from thrombin-stimulated human platelets. J. Biol. Chem. 256, 579-582.

Prentki, M., Biden, T.J., Janjic, D., Irvine, R.F., Berridge, M.J., and Wollheim, C.B. (1984). Rapid mobilization of Ca^{2+} from rat insulinoma microsomes by inositol-1,4,5-trisphosphate. Nature 309, 562-564.

Prpić, V., Blackmore, P.F., and Exton, J.H. (1982). Phosphatidylinositol breakdown induced by vasopressin and epinephrine in hepatocytes is calcium-dependent. J. Biol. Chem. 257, 11323-11331.

Putney, J.W. Jr. (1976). Biphasic modulation of potassium release in rat-parotid gland by carbachol and phenylephrine. J. Pharmacol. Exp. Ther. 198, 375-384.

Putney, J.W. Jr. (1977). Muscarinic, α-adrenergic and peptide receptors regulate the same calcium influx sites in the parotid gland. J. Physiol. (Lond.) 268, 139-149.

Putney, J.W. Jr. (1978). Stimulus-permeability coupling : Role of calcium in the receptor regulation of membrane permeability. Pharmacol. Rev. 30, 209-245.

Putney, J.W. Jr., Van De Walle, C.M., and Leslie, B.A. (1978). Receptor control of calcium influx in parotid acinar cells. Mol. Pharmacol. 14, 1046-1053.

Putney, J.W. Jr., and Van De Walle, C.M. (1980 a). Role of calcium in stimulation of ^{36}Cl uptake by dispersed pancreatic acinar cells. Biochem. Biophys. Res. Commun. 95, 1461-1466.

Putney, J.W. Jr., and Van De Walle, C.M. (1980 b). The relationship between muscarinic receptor binding and ion movements in rat-parotid cells. J. Physiol. (Lond.) 299, 521-531.

Putney, J.W. Jr., Landin, C.A., and Van De Walle, C.M. (1980 a). Effect of carbachol on radiosodium uptake by dispersed pancreatic acinar cells. Pflügers Arch. 385, 131-136.

Putney, J.W. Jr., Weiss, S.J., Van De Walle, C.M., and Haddas, R.A. (1980 b). Is phosphatidic acid a calcium ionophore under neurohumoral control? Nature 284, 345-347.

Putney, J.W. Jr. (1981). Calcium antagonists and calcium-gating mechanisms in the exocrine glands. In: "New Perspectives on Calcium Antagonists", pp.169-175. Ed: G.B. Weiss. American Physiological Society : Bethesda.

Putney, J.W. Jr., De Witt, L.M., Hoyle, P.C., and McKinney, J.S. (1982). Calcium, prostaglandins and the phosphatidylinositol effect in exocrine gland cells. Cell Calcium 2, 561-571.

Putney, J.W. Jr., Burgess, G.M., Halenda, S.P., McKinney, J.S. and Rubin, R.P. (1983). Effects of secretagogues on (^{32}P)-phosphatidylinositol 4,5-bisphosphate metabolism in the exocrine pancreas. Biochem. J. 212, 483-488.

Quinn, P.J. (1973). The association between phosphatidylinositol phosphodiesterase activity and a specific subunit of microtubular protein in rat brain. Biochem. J. 133, 273-281.

Raabo, E., and Terkildsen, T.C. (1960). On the enzymatic determination of blood glucose. Scand. J. Clin. Lab. Invest. 12, 402.

Rasmussen, H., and Barrett, P.Q. (1984). Calcium messenger system : An integrated view. Physiol. Rev. 64, 938-984.

Raufman, J-P., Jensen, R.T., Sutliff, V.E., Pisano, J.J., and Gardner, J.D. (1982). Actions of Gila monster venom on dispersed acini from guinea-pig pancreas. Am. J. Physiol. 242, G470-G474.

Redman, C.M., Siekevitz, P., and Palade, G.E. (1966). Synthesis and transfer of amylase in pigeon pancreatic microsomes. J. Biol. Chem. 241, 1150-1158.

Reed, P.W., and Lardy, H.A. (1972). A23187 : A divalent cation ionophore. J. Biol. Chem. 247, 6970-6977.

Rehfeld, J.F. (1981). Four basic characteristics of the gastrin-cholecystokinin system. Am. J. Physiol. 240, G255-G266.

Reinhart, P.H., Taylor, W.M., and Bygrave, F.L. (1982). A procedure for the rapid preparation of mitochondria from rat liver. Biochem. J. 204, 731-735.

Renckens, B.A.M., Van Emst-De Vries, S.E., Depont, J.J.H.H.M., and Bonting, S.L. (1980). Rat pancreas adenylase cyclase. VII. Effect of extracellular calcium on pancreozymin-induced cyclic AMP formation. Biochim. Biophys. Acta. 630, 511-518.

Rerup, C.C. (1970). Drugs producing diabetes through damage of the insulin secreting cells. Pharmacol. Rev. 22, 485-518.

Rhodes, D., Prpić, V., Exton, J.H., and Blackmore, P.F. (1983). Stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis in hepatocytes by vasopressin. J. Biol. Chem. 258, 2770-2773.

Rinderknecht, H., Wilding, P., and Haverback, B.J. (1967). A new method for the determination of α-amylase. Experientia 23, 805.

Rinderknecht, H., Renner, I.G., Douglas, A.P., and Adham, N.F. (1978). Profiles of pure pancreatic secretions obtained by direct pancreatic duct cannulation in normal healthy human subjects. Gastroenterology 75, 1083-1089.

Rink, T.J., Sanchez, A., and Hallam, T.J. (1983). Diacylclycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. Nature 305, 317-319.

Rittenhouse-Simmons, S., and Deykin, D. (1978). The activation by Ca^{2+} of platelet phospholipase A_2 : Effects of dibutyryl cyclic adenosine monophosphate and 8-(N,N-Diethylamino)-octyl-3,4,5-trimethoxy-benzoate. Biochim. Biophys. Acta. 543, 409-422.

Roach, P.D., and Palmer, F.B.St. C. (1981). Human erythrocyte cytosol PtdIns-4,5P_2 phosphatase. Biochim. Biophys. Acta. 661, 323-333.

Robberecht, P., Deschodt-Lanckman, M., Camus, J., Bruylands, J., and Christophe, J. (1971). Rat pancreatic hydrolases from birth to weaning and dietary adaptation after weaning. Am. J. Physiol. 221, 376-381.

Robberecht, P., Deschodt-Lanckman, M., De Neef, P., Borgeat, P., and Christophe, J. (1974). *In vivo* effects of pancreozymin, secretin, vasoactive intestinal polypeptide and pilocarpine on the levels of cyclic AMP and cyclic GMP in the rat pancreas. FEBS Lett. 43, 139-143.

Robberceht, P., Conlon, T.P., and Gardner, J.D. (1976). Interaction of porcine vasoactive intestinal peptide with dispersed acinar cells from guinea-pig : Structural requirements for effects of VIP and secretin on cellular cAMP. J. Biol. Chem. 251, 4635-4639.

Robberecht, P., Deschodt-Lanckman, M., Lammers, M., De Neef, P., Christophe, J. (1977). *In vitro* effects of secretin and vasoactive intestinal polypeptide on hydrolase secretion and cyclic AMP levels in the pancreas of five animal species : A comparison with caerulein. Gastroenterol. Clin. Biol. 1, 519-525.

Robberecht, P.W., Waelbroeck, M., Noyer, M., Chatelain, P., De Neef, P., and Christophe, J. (1982). Characterization of secretin and vasoactive intestinal peptide receptors in rat pancreatic plasma membranes using the native peptides secretin-(7-27) and five secretin analagues. Digestion 23, 201-210.

Robberecht, P., Waelbroeck, M., Dehaye, J.P., Winand, J., Vandermeers, A., Vandermeers-Piret, M-C., and Christophe, J. (1984 a). Evidence that helodermin, a newly extracted peptide from Gila monster venom, is a member of the secretin, VIP, PHI family of peptides with an original pattern of biological properties. FEBS Lett. 166, 277-282.

Robberecht, P., Waelbroeck, M., De Neef, P., Camus, J-C., Vandermeers, A., Vandermeers-Piret, M-C., and Christophe, J. (1984 b). Specific labelling by (^{125}I) helodermin of high affinity VIP receptors in rat liver membranes. FEBS Lett. 172, 55-58.

Roberts, M.L., and Woodland, M.J. (1982). Calcium causes the biphasic dose-response curve for pancreatic amylase secretion. Experientia 38 , 256-257.

Roberts, M.L., and Butcher, F.R. (1983). The involvement of protein phosphorylation in stimulus-secretion coupling in the mouse exocrine pancreas. Biochem. J. 210, 353-359.

Roberts, M.L., Tennes, K.A., and Crouch, M.F. (1984). The role of phosphatidylinositol in pancreatic exocrine secretion. In: "Secretion : Mechanisms and Control", pp.169-172. Eds: R.M. Case, J.M. Lingard and J.A. Young. Manchester University Press.

Rose, B., and Loewenstein, W.R. (1975). Permeability of cell junction depends on local cytoplasmic calcium activity. Nature 254, 250-252.

Rothman, S.S., and Brooks, F.P. (1965). Pancreatic secretion *in vitro* in 'Cl-free', 'CO_2-free' and low Na^+ environment. Am. J. Physiol. 209, 790-796.

Rothman, S.S., and Brooks, F.P. (1965). Pancreatic secretion *in vitro* in 'Cl-free', 'CO_2-free' and low Na^+ environment. Am. J. Physiol. 209, 790-796.

Rothman, S.S. (1975). Protein transport by the pancreas. Science 190, 747-753.

Rothman, S.S., and Wilking, H. (1978). Differential rates of digestive enzyme transport in the presence of cholecystokinin-pancreozymin. J. Biol. Chem. 253, 3543-3549.

Rubin, R.P., Feinstein, M.B., Jaanus, S.D., and Paimre, M. (1967). Inhibition of catecholamine secretion and calcium exchange in perfused cat adrenal glands by tetracaine and magnesium. J. Pharmacol. Exp. Ther. 155, 463-471.

Rubin, R.P., Sink, L.E., and Freer, R.J. (1981). On the relationship between formylmethionyl-leucyl-phenylalanine stimulation of arachidonyl phosphatidylinositol turnover and lysosomal enzyme secretion by rabbit neutrophils. Mol. Pharmacol. 19, 31-37.

Rubin, R.P., Kelly, K.L., Halenda, S.P., and Laychock, S.G. (1982). Arachidonic-acid metabolism in rat pancreatic acinar cells - calcium mediated stimulation of the lipoxygenase system. Prostaglandins 24, 179-193.

Rubin, R.P., Godfrey, P.P., Chapman, A., and Putney, J.W. Jr. (1984). Secretagogue-induced formation of inositol phosphates in rat exocrine pancreas : Implications for a messenger role for inositol trisphosphate. Biochem. J. 219, 655-659.

Rudick, J., Gonda, M., Rosenberg, I.R., Chapman, M.L., Dreiling, D.A,, and Janowitz, H.D. (1973). Effects of a beta-adrenergic receptor stimulant (isoproterenol) on pancreatic exocrine secretion. Surgery 74, 338-343.

Rutten, W.J., De Pont, J.J.H.H.M., and Bonting, S.L. (1972). Adenylate cyclase in the rat pancreas : Properties and stimulation by hormones. Biochim. Biophys. Acta. 274, 201-213.

Saito, A., Williams, J.A., and Kanno, T. (1980). Potentiation of cholecystokinin-induced exocrine secretion by both exogenous and endogenous insulin in isolated and perfused rat pancreata. J. Clin. Invest. 65, 777-782.

Sakamoto, C., Goldfine, I.D., and Williams, J.A. (1984). The somatostatin receptor on isolated pancreatic acinar cell plasma membranes : Identification of subunit structure and direct regulation by cholecystokinin. J. Biol. Chem. 259, 9623-9627.

Salmon, D.M., and Honeyman, T.W. (1980). Proposed mechanism of cholinergic action in smooth muscle. Nature 284, 344-345.

Salway, J.G., Kai, M., and Hawthorne, J.N. (1967). Triphosphoinositide phosphomonoesterase activity in nerve cell bodies, neuroglia and subcellular fractions from whole rat brain. J. Neurochem 14, 1013-1024.

Sankaran, H., Goldfine, I.D., Deveney, C.W., Wong, K-Y., and Williams, J.A. (1980). Binding of cholecystokinin to high affinity receptors on isolated rat pancreatic acini. J. Biol. Chem. 255, 1849-1853.

Sankaran, H., Goldfine, I.D., Bailey, A., Licko, V., and Williams, J.A. (1982). Relationship of cholecystokinin receptor binding to regulation of biological functions in pancreatic acini. Am. J. Physiol. 242, G250-G257.

Sastry, P.S., and Hokin, L.E. (1966). Studies on the role of phospholipids in phagocytosis. J. Biol. Chem. 241, 3354-3361.

Savion, N., and Selinger, Z. (1978). Morphological changes in rat pancreatic slices associated with inhibition of enzyme secretion by high concentrations of secretagogues. J. Cell Biol. 76, 467-482.

Schatzman, R.C., Wise, B.C., and Kuo, J.F. (1981). Phospholipid-sensitive calcium-dependent portein kinase : Inhibition by antipsychotic drugs. Biochem. Biophys. Res. Commun. 98, 669-677.

Scheele, G.A., and Palade, G.E. (1975). Studies on the guinea-pig pancreas : Parallel discharge of exocrine enzyme activities. J. Biol. Chem. 250, 2660-2670.

Scheele, G.A. (1980). Biosynthesis, segregation and secretion of exportable proteins by the exocrine pancreas. Am. J. Physiol. 238, G467-G477.

Schramm, M., and Selinger, Z. (1984). Message transmission : Receptor controlled adenylate cyclase system. Science 225, 1350-1356.

Schulman, (1982). Calcium-dependent protein phosphorylation. In: "Handbook of Pharmacology", Vol.58, pp.425-470. Eds: J.A. Nathanson and J.W. Kebabian. Springer-Verlag, Berlin.

Schulz, I. (1971). Influence of bicarbonate-CO_2 and glycodiazine buffer on the secretion of the isolated cat's pancreas. Pflügers Arch. 329, 283-306.

Schulz, I. (1975). The role of extracellular Ca^{2+} and cyclic nucleotides in the mechanism of enzyme secretion from the cat pancreas. Pflügers Arch. 360, 165-181.

Schulz, I., and Ullrich, K.J. (1978). Transport processes in the exocrine pancreas. In: "Membrane Transport in Biology", Vol.4A, pp.811-852, Eds: G. Giebisch, D.C. Tosteson and H.H. Ussing. Springer-Verlag, Berlin.

Schulz, I. (1980 a). Bicarbonate transport in the exocrine pancreas. Ann. N.Y. Acad. Sci. 341, 191-209.

Schulz, I. (1980 b). Messenger role of calcium in function of pancreatic acinar cells. Am. J. Physiol. 239, G335-G347.

Schulz, I., and Stolze, H.H. (1980). The exocrine pancreas : The role of secretagogues, cyclic nucleotides, and calcium in enzyme secretion. Ann. Rev. Physiol. 42, 127-156.

Schulz, I., Kimura, T., Wakasugi, H., Haase, W., and Kribben, A. (1981). Analysis of Ca^{2+} fluxes and Ca^{2+} pools in pancreatic acini. Phil. Trans. R. Soc. Lond. B. 296, 105-113.

Scott, T.W., Mills, S.C., and Freinkel, N. (1968). The mechanism of thyrotrophin action in relation to lipid metabolism in thyroid tissue. Biochem. J. 109, 325-332.

Scratcherd, T., Case, R.M., and Smith, P.A. (1975). A sensitive method for the biological assay of secretin and substances with 'secretin-like' activity in tissues and biological fluids. Scand. J. Gastroenterol. 10, 821-828.

Scratcherd, T., Hutson, D., and Case, R.M. (1981). Ionic transport mechanisms underlying fluid secretion by the pancreas. Phil. Trans. R. Soc. Lond. B. 296, 167-178.

Scratcherd, T., and Hutson, D. (1982). The role of chloride in pancreatic secretion. In: "Electrolyte and Water Transport Across Gastrointestinal Epithelia", pp.61-69. Eds: R.M. Case, A. Garner, L.A. Turnberg and J.A. Young. Raven Press, New York.

Seamon, K.B., Padgett, W., and Daly, J.W. (1981). Forskolin : Unique diterpene activator of adenylate cyclase in membranes and intact cells. Proc. Natl. Acad. Sci. USA 78, 3363-3367.

Seeman, P.S. (1972). The membrane actions of anaesthetics and tranquilizers. Pharmacol. Rev. 24, 583-655.

Selinger, Z., Naim, E., and Lasser, M. (1970). ATP-dependent calcium uptake by microsomal preparations from rat parotid and submaxillary glands. Biochim. Biophys. Acta. 203, 326-334.

Selinger, Z., Batzri, S., Eimerl, S., and Schramm, M. (1973). Calcium and energy requirements for K^+ release mediated by the epinephrine α-receptor in rat parotid slices. J.Biol. Chem. 248, 369-372.

Selinger, Z., Eimerl, S., Savion, N., and Schramm, M. (1974). A Ca^{2+} ionophore (A23187) stimulating hormone and neurotransmitter action in the rat parotid and pancreas glands. In: "Secretory Mechanisms in Exocrine Glands", pp.68-78. Eds: N.A. Thorn and O.H. Petersen. Munskgaard, Copenhagen.

Seow, F., and Young, J.A. (1984). The secretory mechanisms of rat pancreatic acini. Proc. Aust. Physiol. Pharmacol. Soc. 15, 159P.

Seow, F., Christie, J.A., and Young, J.A. (1984). Effect of 'loop' diuretics on rat pancreatic acinar secretion. Proc. Aust. Physiol. Pharmacol. Soc. 15, 158P.

Serhan, C.N., Fridovich, J., Goetzl, E.J., Dunham, P.B., and Weissman, G. (1982). Leukotriene B_4 and phosphatidic acid are calcium ionophores. J. Biol. Chem. 257, 4746-4752.

Seybold, J., Bieger, W., and Kern, H.F. (1975). Studies on intracellular transport of secretory proteins in the rat exocrine pancreas. II. Inhibition by antimicrobubular agents. Virchows Archiv. A. Pathol. Anat. Histol. 368, 309-327.

Sewell, W.A., and Young, J.A. (1975). Secretion of electrolytes by the pancreas of the anaesthetized rat. J. Physiol. (Lond.) 252, 379-376.

Seyfred, M.A., and Wells, W.W. (1984 a). Subcellular incorporation of ^{32}P into phosphoinositides and other phospholipids in isolated hepatocytes. J. Biol. Chem. 259, 7659-7665.

Seyfred, M.A., and Wells, W.W. (1984 b). Subcellular site and mechanism of vasopressin-stimulated hydrolysis of phosphoinositides in rat hepatocytes. J. Biol. Chem. 259, 7666-7672.

Shaw, J.O. (1981). Effects of extracellular Ca^{2+} and the intracellular Ca^{2+} antagonist 8-(N,N-Diethylamino) octyl-3,4,5-trimethoxy-benzoate on rabbit platelet conversion of arachidonic acid into thromboxane. Prostaglandins 21, 571-579.

Shears, S.B., and Kirk, C.J. (1984). Determination of mitochondrial calcium content in hepatocytes by a rapid cellular-fractionation technique : α-adrenergic agonists do not mobilize mitochondrial Ca^{2+}. Biochem. J. 219, 383-389.

Sheltawy, A., Brammer, M., and Borrill, D. (1972). Subcellular distribution of triphosphoinositide phosphomonoesterase in guinea-pig brain. Biochem. J. 128, 579-586.

Sieghart, W., Theoharides, T.C., Alper, S.L., Douglas, W.W., and Greengard, P. (1978). Calcium-dependent protein phosphorylation during secretion by exocytosis in mast cells. Nature 275, 329-331.

Siekevitz, P., and Palade, G.E. (1960). A cytochemical study on the pancreas of the guinea-pig. V. *In vivo* incorporation of leucine-I-^{14}C into the chymotrypsinogen of various cell fractions. J. Biophys. Biochem. Cytol. 7, 619-630.

Singer, M.V., Solomon, T.E., and Grossman, M.I. (1980). Effect of atropine on secretion from intact and transplanted pancreas in dog. Am. J. Physiol. 238, G18-G22.

Singer, M.V., Solomon, T.E., Rammert, H., Caspary, F., Niebel, W., Goebell, H., and Grossman, M.I. (1981). Effect of atropine on pancreatic responses to HCl and secretin. Am. J. Physiol. 240, G376-G380.

Singh, M. (1980). Effect of glucagon on digestive enzyme synthesis, transport and secretin in mouse pancreatic acinar cells. J. Physiol. (Lond.) 306, 307-322.

Singh, M. (1982). Role of cyclic adenosine monophosphate in amylase release from dissociated rat pancreatic acini. J. Physiol. (Lond.) 331, 547-555.

Singh, J. (1984). Effects of acetylcholine and caerulein on $^{86}Rb^{+}$ efflux in the mouse pancreas : Evidence for a sodium-potassium-chloride cotransport system. Biochim. Biophys. Acta. 775, 77-85.

Sjöstrand, F.S., and Hanzon, U. (1954 a). Membrane structures of cytoplasm and mitochondria in exocrine cells of mouse pancreas as revealed by high resolution electron microscopy. Exp. Cell Res. 7, 393-414.

Sjöstrand, F.S., and Hanzon, U.(1954 b). Ultrastructure of Golgi apparatus of exocrine cells of mouse pancreas. Exp. Cell Res. 7, 415-429.

Smith, C.O., and Wells, W.W. (1983). Phosphorylation of rat liver nuclear envelopes. II. Chracterization of *in vitro* lipid phosphorylation. J. Biol. Chem. 258, 9368-9373.

Smith, P.A., and Case, R.M. (1975). Effects of cholera toxin on cyclic adenosine-3', 5'-monophosphate concentration and secretory processes in exocrine pancreas. Biochim. Biophys. Acta. 399, 277-290.

Snedecor, G.W., and Cochran, W.G. (1980). Factorial Experiments. In: "Statistical Methods", pp.298-330, Seventh edition. Iowa State University Press, Iowa, USA.

Snook, J.T. (1971). Dietary regulation of pancreatic enzymes in the rat with emphasis on carbohydrate. Am. J. Physiol. 221, 1383-1387.

Söling, H.D., and Unger, K.O. (1972). The role of insulin in regulation of α-amylase synthesis in the rat pancreas. Eur. J. Clin. Invest. 2, 199-212.

Stark, R.J., and O'Doherty, J. (1982). Effect of ionophore A23187 on cytosolic Ca^{2+} and enzyme secretion. Am. J. Physiol. 243, C196-C199.

Steer, M.L., and Glazer, G. (1976). Parallel secretion of digestive enzymes by the *in vitro* rabbit pancreas. Am. J. Physiol. 231, 1860-1865.

Steer, M.L., and Manabe, T. (1979). Cholecystokinin-pancreozymin induces the parallel discharge of digestive enzymes from the *in vitro* rabbit pancreas. J. Biol. Chem. 254, 7228-7229.

Stening, G.F., and Grossman, M.I. (1969). Gastrin-related peptides as stimulants of pancreatic and gastric secretion. Am. J. Physiol. 217, 262-266.

Stenson, W.F., and Parker, C.W. (1979). Metabolism of arachidonic acid in ionophore-stimulated neutrophils. J. Clin. Invest. 64, 1457-1465.

Stenson, W.F., Nickells, M.W., and Atkinson, J.P. (1981). Metabolism of exogenous arachidonic acid in ionophore-stimulated neutrophils. Prostaglandins 21, 675-689.

Stenson, W.F., and Lobos, E. (1982). Metabolism of arachidonic acid by pancreatic acini : Relation to amylase secretion. Am. J. Physiol. 242, G493-G497.

Stephenson, R.P. (1956). A modification of receptor theory. Br. J. Pharmacol. 11, 379-393.

Stock, C., Launay, J.F., Grenier, J.F., and Bauduin, H. (1978). Pancreatic acinar cell changes induced by caerulein, vinblastine, deuterium oxide, and cytochalasin B *in vitro*. Lab. Invest. 38, 157-164.

Stolze, H., and Schulz, I. (1980). Effect of atropine, ouabain, antimycin A, and A23187 on 'trigger Ca^{2+} pool' in exocrine pancreas. Am. J. Physiol. 238, G338-G348.

Stanley, P.E., and Williams, S.G. (1969). Use of the Liquid Scintillation Spectrometer for determining adenosine triphosphate by the Luciferase enzyme. Anal. Biochem. 29, 381-392.

Streb, H., and Schulz, I. (1983). Regulation of cytosolic free Ca^{2+} concentration in acinar cells of rat pancreas. Am. J. Physiol. 245, G347-G357.

Streb, H., Irvine, R.F., Berridge, M.J., and Schulz, I. (1983). Release of Ca^{2+} from a non-mitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature 306, 67-69.

Stuhne-Sekalec, L., and Stanacev, N.Z. (1977). Translocation of spin-labelled radioactive lipids from isolated guinea-pig liver microsomal to mitochondrial membranes. Can. J. Biochem. 55, 1159-1165.

Stuhne-Sekalec, L., and Stanacev, N.Z. (1979). Biosynthesis, utilization and translocation of endogenous isomeric spin-labelled radioactive cytidinediphosphodiglycerides from isolated guinea-pig liver microsomal to mitochondrial membranes. Can. J. Biochem. 57, 1019-1025.

Suematsu, E., Hirata, M., Hashimoto, T., and Kuriyama, H. (1984). Inositol 1,4,5-trisphosphate releases Ca^{2+} from intracellular store sites in skinned single cells of porcine coronary artery. Biochem. Biophys. Res. Commun. 120, 481-485.

Susini, C., Esteve, J.P., Bommelaer, G., Vaysse, N., Ribet, A. (1978). Inhibition of exocrine pancreatic secretion by somatostatin in dogs. Digestion 18, 384-399.

Svoboda, M., Robberecht, P., Camus, J., Deschodt-Lanckman, M., Christophe, J. (1976). Subcellular distribution and response to gastro-intestinal hormones of adenylate cyclase in rat pancreas. Eur. J. Biochem. 69, 185-193.

Swanson, C.H., and Solomon, A.K. (1975). Micropuncture analysis of the cellular mechanisms of electrolyte secretion by the *in vitro* rabbit pancreas. J. Gen. Physiol. 65, 22-45.

Taguchi, R., and Ikezawa, H. (1978). Phosphatidylinositol-specific phospholipase C from *Clostridium novyi* Type A. Arch. Biochim. Biophys. 186, 196-201.

Takai, Y., Kishimoto, A., Inoue, M., and Nishizuka, Y. (1977). Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characterization of an active enzyme from bovine cerebellum. J. Biol. Chem. 252, 7603-7609.

Takai, Y., Kishimoto, A., Kikkawa, V., Mori, T., and Nishizuka, Y. (1979 a). Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated phospholipid dependent protein kinase system. Biochem. Biophys. Res. Commun. 91, 1218-1224.

Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., and Nishizuka, Y. (1979 b). Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. J. Biol. Chem. 254, 3692-3695.

Takenawa, T., and Egawa, K. (1977). CDP-diglyceride : Inositol transferase from rat liver. Purification and properties. J. Biol. Chem. 252, 5419-5423.

Tartakoff, A.M., Jamieson, J.D., Scheele, G.A., and Palade, G.E. (1975). Studies on the pancreas of the guinea-pig : Parallel processing and discharge of exocrine proteins. J. Biol. Chem. 250, 2671-2677.

Tatemoto, K., and Mutt, U. (1980). Isolation of two novel candidate hormones using a chemical method for finding naturally occurring polypeptides. Nature 285, 417-418.

Tennes, K.A., and Roberts, M.L. (1981). Agonist-stimulated breakdown of *myo*(2-^{3}H)inositol-labelled phosphatidylinositol in mouse pancreas. Aust. J. Exp. Biol. Med. Sci. 59, 791-801.

Tennes, K.A., and Roberts, M.L. (1982). The role of calcium in agonist-stimulated hydrolysis of phosphatidylinositol in mouse pancreas. Biochim. Biophys. Acta. 719, 238-243.

Tennes, K.A., Kennedy, J.A., and Roberts, M.L. (1983). Inhibition of agonist-induced hydrolysis of phosphatidylinositol and muscarinic receptor binding by the calcium antagonist 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate-HCl. (TMB-8). Biochem. Pharmacol. 32, 2116-2118.

Tennes, K.A., and Roberts, M.L. (1984). The contribution of inositol exchange to agonist-stimulated breakdown of *myo*(2-^{3}H)inositol-labelled phosphatidylinositol in mouse exocrine pancreas. Aust. J. Exp. Biol. Med. Sci. 62, 303-308.

Ter Schegget, J., Van den Bosch, H., Van Baak, M.A., Hostetler, K.Y., and Vorst, P. (1971). The synthesis and utilization of dCDP-digylceride by a mitochondrial fraction from rat liver. Biochim. Biophys. Acta. 239, 234-242.

Thomas, A.P., Marks, J.S., Coll, K.E., and Williamson, J.R. (1983). Quantitation and early kinetics of inositol lipid changes induced by vasopressin in isolated and cultured hepatocytes. J. Biol. Chem. 258, 5716-5725.

Thomas, A.P., Alexander, J., and Williamson, J.R. (1984). Relationship between inositol polyphosphate production and the increase of cytosolic free Ca^{2+} induced by vasopressin in isolated hepatocytes. J. Biol. Chem. 259, 5574-5584.

Thompson, W., and Dawson, R.M.C. (1964 a). The hydrolysis of triphosphoinositide by extracts of rat brain. Biochem. J. 91, 233-236.

Thompson, W., and Dawson, R.M.C. (1964 b). The triphosphoinositide phosphodiesterase of brain tissue. Biochem. J. 91, 237-245.

Tolbert, M.E.M., White, A.C., Aspry, K., Cutts, J., and Fain, J.N. (1980). Stimulation by vasopressin and α-catecholamines of phosphatidylinositol formation in isolated rat liver parenchymal cells. J. Biol. Chem. 255, 1938-1944.

Träuble, H. (1976). Membrane Electrostatics. In: "Structure of Biological Membranes", pp.509-550. Eds: S. Abrahamsson and I. Pascher. Plenum Press, New York and London.

Trifaró, J.M. (1969). Phospholipid metabolism and adrenal medullary activity. The effect of acetylcholine on tissue uptake and incorporation of orthophosphate-^{32}P into nucleotides and phospholipids of bovine adrenal medulla. Mol. Pharmacol. 5, 382-393.

Tsien, R.Y., Pozzan, T., and Rink, T.J. (1982). T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocytes. Nature 295, 68-71.

Tyson, C.A., Van de Zande, H., and Green, D.E. (1976). Phospholipids as ionophores. J. Biol. Chem. 251, 1326-1332.

Ueda, N., and Petersen, O.H. (1977). The dependence of caerulein-evoked pancreatic fluid secretion on the extracellular calcium concentration. Pflügers Arch. 370, 179-183.

Ueda, N., Suzuki, Y., Okamura, K., and Namiki, M. (1980). Dibutyryl cyclic GMP inhibits caerulein - but not Ach- , bombesin- or secretin-evoked fluid secretion in the perfused rat pancreas. In: "Biology of Normal and Cancerous Exocrine Pancreatic Cells", pp.55-60. Eds: R.A. Ribet, L. Pradayrol and C. Susini. Elsevier/North Holland : Amsterdam.

Uhlemann, E.R., Rottman, A.J., and Gardner, J.D. (1979). Actions of peptides isolated from amphibian skin on amylase release from dispersed pancreatic acini. Am. J. Physiol. 236, E571-E576.

Valverdes, I., Vandermeers, A., Anjaneyula, R., and Malaisse, W.J. (1979). Calmodulin activation of adenylate cyclase in pancreatic islets. Science 206, 225-227.

Van den Bosch, H. (1980). Intracellular phospholipases A. Biochim. Biophys. Acta. 604, 191-246.

Vandermeers, A., Vandermeers-Piret, M.C., Rathe, J., Kutzner, R., Delforge, A., and Christophe, J. (1977). A calcium dependent protein activator of guanosine 3',4'-monophosphate phosphodiesterase in bovine and rat pancreas. Eur. J. Biochem. 81, 379-386.

Vandermeers, A., Vandermeers-Piret, M-C., Robberecht, P., Waelbroeck, M., Dehaye, J.P., Winand, J., and Christophe, J. (1984). Purification of a novel pancreatic secretory factor (PSF) and a novel peptide with VIP- and secretin-like properties (helodermin) from Gila monster venom. FEBS Lett. 166, 273-276.

Vanysse, N., Bastic, M.J., Pascal, J.P., Martinel, C., Fourtanier, G., and Ribet, A. (1977). Effects of catecholamines and their inhibitors on the isolated canine pancreas. Gastroenterology 72, 711-718.

Volpi, M., Naccache, P.H., and Sha'afi, R.I. (1980). Arachidonate metabolite(s) increase the permeability of the plasma membrane of the neutrophils to calcium. Biochem. Biohpys. Res. Commun. 92, 1231-1237.

Wagner, H., Lissau, A., Holzl, J., and Horhammer, L. (1962). The incorporation of ^{32}P into the inositol phosphatides of rat brain. J. Lipid Res. 3, 177-180.

Wakasugi, H., Stolze, H., Haase, W., and Schulz, I. (1981). Effect of La^{2+} on secretagogue-induced Ca^{2+} fluxes in rat isolated pancreatic acinar cells. Am. J. Physiol. 240, G281-G289.

Wakasugi, H., Kimura, T., Haase, W., Kribben, A., Kaufman, R., and Schulz, I. (1982). Calcium uptake into acini from rat pancreas : Evidence for intracellular ATP-dependent calcium sequestration. J. Membr. Biol. 65, 205-220.

Wallace, M.A., Randazzo, P., Li, S-Y., and Fain, J. (1982). Direct stimulation of phosphatidylinositol degradation by addition of vasopressin to purified rat liver plasma membranes. Endocrinology 111, 341-343.

Wallace, M.A., Poggioli, J., Giraud, F., and Claret, M. (1983). Nonpinephrine-induced loss of phosphatidylinositol from isolated rat liver plasma membrane. FEBS Lett. 156, 239-243.

Walsh, J.H., Lamers, C.B., and Valenzuela, J.E. (1982). Cholecystokinin-octapeptide-like immunoreactivity in human plasma. Gastroenterology 82, 438-444.

Wassarman, P.M., Ukena, T.E., Josefowicz, W.J., and Karnovsky, M.J. (1977). Colchicine inhibits phosphatidylinositol turnover induced by lymphocytes by concanavolin. Nature 265, 741-742.

Watson, S.P., and Downes, C.P. (1983). Substance P induced hydrolysis of inositol phospholipids in guinea-pig ileum and rat hypothalamus. Eur. J. Pharmacol. 93, 245-253.

Weiss, G.B. (1974). Cellular pharmacology of lanthanum. Ann. Rev. Pharmacol. 14, 343-354.

Weiss, S.J., and Putney, J.W. Jr. (1981). The relationship of phosphatidyl-inositol turnover to receptors and calcium ion channels in rat parotid acinar cells. Biochem. J. 194, 463-468.

Weiss, S.J., McKinney, J.S., and Putney, J.W. Jr. (1982). Receptor-mediated net breakdown of phosphatidylinositol 4,5-bisphosphate in parotid acinar cells. Biochem. J. 206, 555-560.

Whittam, R., Wheeler, K.P., Blake, A. (1964). Oligomycin and active transport reactions in cell membranes. Nature 203, 720-724.

Wieneke, A.A., and Woodin, A.M. (1967). The polyphosphoinositide content of the leukocyte, erythrocyte and macrophage. Biochem. J. 105, 1039-1045.

Williams, J.A., and Lee, M. (1974). Pancreatic acinar cells : Use of a Ca^{2+} ionophore to separate enzyme release from the earlier steps in stimulus secretion coupling. Biochem. Biophys. Res. Commun. 60, 542-548.

Williams, J.A. (1975 a). Na^{+} dependence of *in vitro* pancreatic amylase release. Am. J. Physiol. 229, 1023-1026.

Williams, J.A. (1975 b). An *in vitro* evaluation of possible cholinergic and adrenergic receptors affecting pancreatic amylase secretion. Proc. Soc. Exp. Biol. Med. 150, 513-516.

Williams, J.A., and Chandler, D.E. (1975). Ca^{2+} and pancreatic amylase release. Am. J. Physiol. 229, 1729-1732.

Williams, J.A., and Lee, M. (1986). Microtubules and pancreatic amylase release by mouse pancreas *in vitro*. J. Cell Biol. 71, 795-806.

Williams, J.A., Cary, P., and Moffatt, B. (1976). Effects of ions on amylase release by dissociated pancreatic acinar cells. Am. J. Physiol. 231, 1562-1567.

Williams, J.A., Poulsen, J.A., and Lee, M. (1977). Effects of membrane stabilizers on pancreatic amylase release. J. Membr. Biol. 33, 185-196.

Williams, J.A., Korc, M., and Dormer, R.L. (1978). Action of secretagogues on a new preparation of functionally intact, isolated pancreatic acini. Am. J. Physiol. 235, E517-E524.

Williams, J.A. (1980). Regulation of pancreatic acinar cell function by intracellular calcium. Am. J. Physiol. 238, G269-G279.

Williams, J.A., Sankaran, H., Korc, M., and Goldfine, I.D. (1981). Receptors for cholecystokinin and insulin in isolated pancreatic acini : Hormonal control of secretion and metabolism. Fed. Proc. 40, 2497-2502.

Williams, J.A., Sankaran, H., Roach, E., and Goldfine, I.D. (1982 a). Quantitative electron microscope audoradiographs of ^{125}I-cholecystokinin in pancreatic acini. Am. J. Physiol. 243, G291-G296.

Williams, J.A., Bailey, A., Preissler, M., and Goldfine, I.D. (1982 b). Insulin regulation of sugar-transport in isolated pancreatic acini from diabetic mice. Diabetes 31, 674-682.

Wise, B.C., Glass, D.B., Jen Chou, C-H., Raynor, R.L., Katoh, N., Schatzman, R.C., Turner, R.S., Kibber, R.F., and Kuo, J.F. (1982). Phospholipid-sensitive Ca^{2+}-dependent protein kinase from heart. II. Substrate specificity and inhibition by various agents. J. Biol. Chem. 257, 8489-8495.

Wizemann, V., Schulz, I., and Simon, B. (1973). SH-groups on the surface of pancreas cells involved in secretin stimulation and glucose-mediated secretion. Biochim. Biophys. Acta. 307, 366-371.

Wizemann, V., Weppler, P., and Mahrt, R. (1974). Effect of glucagon and insulin on the isolated exocrine pancreas. Digestion 11, 432-435.

Wooten, M.W., and Wrenn, R.W. (1984). Phorbol ester induces intracellular translocation of phospholipid/Ca^{2+}-dependent protein kinase and stimulates amylase secretion in isolated pancreatic acini. FEBS Lett. 171, 183-186.

Wrenn, R.W., Katoh, N., Schatzman, R.C., and Kuo, J.F. (1981). Inhibition by phenothiazine antipsychotic drugs of calcium-dependent phosphorylation of cerebral cortex proteins regulated by phospholipid or calmodulin. Life Sci. 29, 725-733.

Wrenn, R.W. (1983). Phospholipid-sensitive calcium-dependent protein kinase and its endogenous substrate proteins in rat pancreatic acinar cells. Life Sci. 32, 2385-2392.

Wrenn, R.W. (1984). Phosphorylation of a pancreatic zymogen granule membrane protein by endogenous calcium/phospholipid-dependent protein kinase. Biochim. Biophys. Acta. 775, 1-6.

Wrenn, R.W., and Wooten, M.W. (1984). Dual calcium-dependent protein phosphorylation systems in pancreas and their differential regulation by polymix B. Life Sci. 35, 267-276.

Yellen, G. (1982). Single Ca^{2+}-activated non-selective cation channels in neuroblastoma. Nature 296, 357-359.

Zavoico, G.B., and Feinstein, M.B. (1984). Cytoplasmic Ca^{2+} in platelets is controlled by cyclic AMP : Antagonism between stimulators and inhibitors of adenylate cyclase. Biochem. Biophys. Res. Commun. 120, 579-585.