# THE EFFECTS OF TACHYKININS AND THEIR METABOLITES ON ARTICULAR CARTILAGE CHONDROCYTE AND SYNOVIOCYTE FUNCTION

by

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# DECLARATION

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

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The work described herein has been the subject of the following publications and presentations:-

## PUBLICATIONS

HALLIDAY, D.A., McNEIL, J.D. AND SCICCHITANO, R. (1992)
Failure of tachykinins including substance P and its fragments to influence proteoglycan and protein synthesis in bovine chondrocytes *in vitro*.
Biochimica et Biophysica Acta 1137, 29-33.

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The substance P fragment, SP-(7-11) increases prostaglandin $E_2$, intracellular $Ca^{2+}$ and collagenase production in bovine articular chondrocytes.
Biochemical Journal 292 (1), 57-62.

HALLIDAY, D.A., McNEIL, J.D. AND SCICCHITANO, R. (1993)
A metabolite of substance P, SP-(7-11) is involved in the pathogenesis of inflammatory joint disease.
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HALLIDAY, D.A., McNEIL, J.D., BETTS, W.H. AND SCICCHITANO, R. (1993)
A role for the C-terminal fragment SP-(7-11) in the pathogenesis of arthritis.
Regulatory Peptides (in press).

## PUBLISHED ABSTRACTS

HALLIDAY, D.A., McNEIL, J.D., BETTS, W.H. AND SCICCHITANO, R. (1992)
Intracellular calcium increases following exposure to substance P-(7-11) in isolated bovine articular chondrocytes.
Arthritis and Rheumatism 35 (9), S174, C44.

HALLIDAY, D.A., McNEIL, J.D. AND SCICCHITANO, R. (1992)
A C-terminal metabolite of substance P is involved in chondrocyte mediated cartilage destruction.
Arthritis and Rheumatism 35 (9), S174, C47.

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Australian and New Zealand Journal of Medicine 22, (6), 746.

HALLIDAY, D.A., McNEIL, J.D., BETTS, W.H. AND SCICCHITANO, R.(1992)
Effects of neuropeptides on articular cartilage chondrocyte function.
Australian and New Zealand Journal of Medicine 22, (2), 179.

## PRESENTATIONS

HALLIDAY, D.A. Substance P and connective tissues of the joint. (Oral presentation at the State Scientific Meeting of the Australian Rheumatology Association, May 1990).

HALLIDAY, D.A., McNEIL, J.D., WIEBKIN, O.W. and SCICCHITANO, R. Effect of substance P on proteoglycan production by cultured bovine articular chondrocytes. (Oral Presentation at the International Union of Pure and Applied Biophysics Satellite Congress, Palmerston North New Zealand, 7-10th August 1990).

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HALLIDAY, D.A., McNEIL, J.D. and SCICCHITANO, R. Mammalian tachykinins and their fragments have no direct effect on bovine chondrocyte proteoglycan production. (Poster presentation at the International Meeting on the Biology and Pathology of the Extracellular Matrix, Lorne Victoria, November 24-29, 1991).

HALLIDAY, D.A., McNEIL, J.D., BETTS, W.H. and SCICCHITANO, R. Differential effects of substance P, neurokinins and substance P fragment, SP-(7-11) on prostaglandin $E_2$ and collagenase production in bovine articular chondrocytes. (Poster presentation at the International Meeting on the Biology and Pathology of the Extracellular Matrix, Lorne, Victoria, November 24-29, 1991).

HALLIDAY, D.A., McNEIL J.D., BETTS, W.H. and SCICCHITANO, R. Effects of neuropeptides on articular cartilage chondrocyte function. (Poster presentation at the 35th Annual Scientific Meeting of the Australian Rheumatology Association, Perth ,WA, December 12-14, 1991).

HALLIDAY, D.A., McNEIL J.D. and SCICCHITANO, R. A role for the C-terminal fragment SP-(7-11) in the pathogenesis of arthritis. (Poster presentation at the International Symposium on Substance P and related peptides, Shizuoka, Japan, November 3-6, 1992)

HALLIDAY, D.A., McNEIL J.D. and SCICCHITANO, R. The substance P fragment SP-(7-11) increases intracellular calcium and interacts with a guanine nucleotide-binding protein. (Poster presentation at the Third Hanson Symposium, "Cellular Signalling", Adelaide, Australia, November 9-11, 1992).

HALLIDAY, D.A., McNEIL J.D. and SCICCHITANO, R. A role for the C-terminal fragment SP-(7-11) in the pathogenesis of arthritis. (Oral presentation at the 31st

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HALLIDAY, D.A., McNEIL J.D. and SCICCHITANO, R. Evidence for substance P (NK-1) receptors on bovine articular chondrocytes. (Oral presentation at the 37th Annual Scientific Meeting of the Australian Rheumatology Association, Adelaide, Australia, May 26-28,1993).

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# THESIS SUMMARY

The effect of mammalian tachykinins and the metabolites of substance P (SP) have been studied on bovine articular chondrocyte and synoviocyte function.

Chondrocytes were isolated from articular cartilage and grown in monolayer culture *in vitro*. Cartilage slices were also used *in vitro* in some experiments. Both human and bovine synovial cells were cultured from explant cultures *in vitro*.

Bovine articular chondrocytes in monolayer culture failed to alter proteoglycan synthesis as measured by CPC precipitation of $^{35}SO_4^=$ labelled glycosaminoglycans in response to SP, neurokinin A (NKA) or neurokinin B (NKB). Furthermore the N-terminal, SP-(1-4), SP-(1-6) and the C-terminal SP-(7-11) fragment of SP also failed to alter proteoglycan synthesis. The molecular size of proteoglycan present in the cell layer was assessed by column chromatography following exposure to SP. No alteration of proteoglycan monomer was observed following exposure to SP. Similar profiles were obtained from proteoglycans released into the cell supernatants. Total protein production was also unaffected by SP.

However the C-terminal fragment of SP, SP-(7-11) significantly increased prostaglandin $E_2$ ($PGE_2$) production. Intact tachykinins and the N-terminal fragments of SP did not alter $PGE_2$ production. SP-(7-11) was found to increase $PGE_2$ production by approximately 60% at $10^{-4}$ M. This effect was completely inhibited by the cycloxygenase inhibitor, indomethacin. Furthermore spontaneous $PGE_2$ production was unaffected by extracellular EGTA. However the stimulatory effect of SP-(7-11) could be completely inhibited when chondrocytes were cultured in the presence of extracellular EGTA. The non-peptide NK-1 receptor antagonist, CP-96,345 was found to inhibit the stimulatory effect of SP-(7-11) on $PGE_2$ production. $PGE_2$ production was inhibited by 60-70% when SP-(7-11) was incubated in the presence of $10^{-4}$ M CP-96, 345.

Bovine articular chondrocytes which had been previously exposed to hr IL-1$\alpha$ had an enhanced response to SP-(7-11), suggesting that hr IL-1$\alpha$ augments the effect of SP-(7-11).

Bovine articular chondrocytes also increase the production of matrix metalloproteinase I (MMP-I) in response to SP-(7-11). This response was maximal at $10^{-4}$ M SP-(7-11) where MMP-I synthesis was approximately five times that of control and could be blocked by cycloheximide. SP did not alter MMP-I synthesis. In response to SP-(7-11) chondrocytes were found to secrete latent MMP-I. Tissue inhibitor of metalloproteinase (TIMP) production was not induced by SP-(7-11).

Intracellular calcium concentration $[Ca^{2+}]_i$ was measured using the fluorescent dye FURA-2AM. Chondrocytes loaded with FURA-2AM also respond to SP-(7-11). This response was maximal at 10 μM and the change in $[Ca^{2+}]_i$ was approximately 140 nM. This effect could be completely blocked by extracellular EGTA. Following the initial stimulation with SP-(7-11), subsequent stimulation with SP-(7-11) had no further effect suggesting desensitization of this mechanism.

Both bovine and human rheumatoid synoviocytes cultured from explants failed to alter $PGE_2$ production in response to tachykinins or the fragments of SP. These studies demonstrated that SP metabolism by the metalloendopeptidase, neutral endopeptidase (NEP), CALLA or CD10 antigen into SP-(7-11) is of biological significance.

The major finding from this work has been that the metabolism of SP and the subsequent production of SP-(7-11) is important in regulating the biological activity of SP on chondrocytes in the synovial joint.

# GLOSSARY

| | |
|---|---|
| APMA | 4-aminophenylmercuricacetate |
| Brij 35 | Polyoxyethylene lauryl ether |
| $[Ca^{2+}]_i$ | Intracellular free calcium concentration |
| CALLA | Common acute lymphoblastic leukaemia antigen |
| CGRP | Calcitonin gene related peptide |
| CP-96,345 | Dihyrochloride salt of (2S,3S)-*cis*--2-(diphenylmethyl)-N- ((2-methoxyphenyl)methyl)-1-azabicyclo[2.2.2]octan-3-amine |
| CPC | Cetyl pyridinium chloride |
| DMEM | Dulbecco's Modification of Eagle's Medium |
| EDTA | Ethylenediamine tetraacetic acid |
| EGTA | Ethylene glycol-bis(β-aminoethyl Ether) |
| ELISA | Enzyme Linked Immunosorbant Assay |
| FCS | Foetal calf serum |
| FURA-2 AM | 108964-32-5 5-oxazolecarboxylic acid, 2-(6-(bis(2-acetyl)methoxy)-2-oxoethyl)-5(2-(2-(bis(2- (acetyloxy) methoxy)-2-oxoethyl)amino-5-methylphenoxy)-2-benzofuranyl)- (acetoxy)methylester. |
| GAG | Glycosaminoglycan |
| GuHCl | Guanidine hydrochloride |
| HBSS | Hank's balanced salt solution |
| HEPES | (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) |
| IL-1α | Interleukin 1 alpha |
| Kav | Partition coefficient described by the formula:- $Kav = \frac{Ve-Vo}{Vt-Vo}$ |
| MMP-I | Matrix metalloproteinase I, vertebrate collagenase |
| MMP-III | Matrix metalloproteinase III, stromelysin |
| NEP | Neutral endopeptidase 24.11 |
| NKA | Neurokinin A, substance K |
| NKB | Neurokinin B, neuromedin B |
| OA | Osteoarthritis |
| PBS | Phosphate buffered saline |
| $PGE_2$ | Prostaglandin $E_2$ |
| PMSF | Phenylmethylsulfonyl fluoride |
| RA | Rheumatoid arthritis |
| SBTI | Soy bean trypsin inhibitor |
| SDS PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SP | Substance P |

| | |
|---|---|
| SP-(1-4) | **H-Arg-Pro-Lys-Pro-OH.** |
| SP-(1-6) | **H-Arg-Pro-Lys-Pro-Gln-Gln-OH.** |
| SP-(7-11) | **H-Phe-Phe-Gly-Leu-Met-$NH_2$.** |
| SP-(8-11) | **H-Phe-Gly-Leu-Met-$NH_2$.** |
| SP-(9-11) | **H-Gly-Leu-Met-$NH_2$** |
| TCA | Trichloroacetic acid |
| TIMP | Tissue inhibitor of metalloproteinase |
| TNF-α | Tumor necrosis factor alpha |
| Ve | Elution volume |
| Vo | Void volume |
| vt | Total volume |

# CHAPTER I

# INTRODUCTION AND REVIEW

# A. GENERAL INTRODUCTION

There is considerable evidence to suggest that the peripheral sensory nervous system influences the pathogenesis of joint diseases including rheumatoid arthritis (RA). In particular a neuropeptide called substance P (SP) which is released from sensory nerves by antidromic stimulation, may directly influence the function of connective tissue cells in the joint, including the articular chondrocytes and synoviocytes. This thesis presents evidence that a metabolite of SP may be involved in chondrocyte-mediated joint destruction.

In humans, many joints of the extremities are diarthrodial or synovial joints. These joints possess a synovial cavity and therefore are freely moveable. The weight-bearing surface of movement in the joint is the articular cartilage which in normal joints is bathed in a small volume of highly viscous fluid known as synovial fluid. Synovial fluid (McCarty 1985) is essentially a filtrate of blood plasma to which hyaluronan is added by cells, known collectively as synoviocytes, in the synovial lining. Articular cartilage and synovial fluid are enveloped in a capsule and held to the neighbouring bone by ligaments and muscle. The blood supply to the joint arises from blood vessels which enter the subchondral bone and form an arterial circle around the joint. These vessels then subdivide into a capillary network which is dense adjacent to the joint cavity. The innervation of synovial joints by peripheral nerves is predominantly sympathetic and sensory. The sensory fibres form proprioceptive endings in the ligaments and synovium and are sensitive to the position and movement of the joint, particularly to stretching and twisting.

There are several cell types which are important in normal joint biology, particularly the articular cartilage chondrocyte and several subclasses of synoviocytes. The synovial cavity is lined with a thin layer (only 2-3 cells thick) of synoviocytes which are fibroblastic connective tissue cells possessing long cytoplasmic processes which intertwine with each other. Electron microscopy of the normal synovial surface has revealed three different types of synoviocyte (Woodward et al 1969), designated

types A, B and C. Type A cells are generally characterized by distinctive intracellular organelles such as numerous lysosomes, smooth-walled vacuoles and poorly developed endoplasmic reticulum. Type A cells are macrophage-like, since they serve phagocytic functions but they also synthesize an array of macromolecules and form the majority of synoviocytes present. The second most abundant synoviocytes, type B, have few vacuoles but abundant endoplasmic reticulum. More importantly, type B synoviocytes synthesize hyaluronan which is responsible for synovial fluid viscosity. The intermediate or type C cell, combines features of type A and B synoviocytes, suggesting that type A and B cells represent different functional stages of the same basic cell type. The fourth cell type in the synovium is the resident phagocytic cell of monocytic origin (Edwards et al 1982).

There are striking morphological and biochemical changes in the joint in the diseased state. Although dramatic changes occur in the synovial tissues in joint diseases such as RA, the major focus in this discussion will be the articular cartilage in which the chondrocyte is the only cell type present.

Chondrocytes are the only cells which maintain and manufacture all the extracellular matrix macromolecules of articular cartilage. During development chondrocytes synthesize and secrete the complex macromolecules seen in adult tissue (see Glowacki et al 1983; Solursh 1982). The differentiation of cartilage is essentially the differentiation of chondrocytes. Mature adult articular chondrocytes stem from precursor mesenchymal cells which produce types I and II collagen. As these cells differentiate into more mature chondrocytes there is a switch in gene expression to synthesis of cartilage-specific proteoglycans and type II collagen (Castagnola et al 1988).

Chondrocytes in human adult articular cartilage vary in size, shape and distribution in the horizontal layers or zones of articular cartilage. In fixed tissue sections, chondrocytes appear to increase in size and become more widely scattered with increasing distance from the surface. Chondrocytes on the articular surface appear to be single cells and comparatively elongated (Zone 1). Moving towards the

subchondral bone, Zone 2 contains both single cells which become grouped when zone 2 merges with zone 3 chondrocytes. These chondrocytes tend to be organised into a vertical orientation similar to that observed in growth plate cartilage (Roy and Meachim 1968). Beside histological differences there are also biochemical differences between chondrocytes in the various layers of the articular cartilage. These differences are in the orientation and diameter of collagen fibres and the content and type of proteoglycans (Serafini-Fracassini and Smith 1974). Therefore, beside morphological specialization, there is also metabolic specialization. Chondrocytes isolated from deep in the articular zone but not the superficial zone (Zone 1) synthesize more keratan sulphate and have a well-organised matrix rich in proteoglycan (Aydelotte et al 1986; Aydelotte et al 1988; Aydelotte and Kuettner 1988). The regulation of cartilage macromolecules by chondrocytes is a highly dynamic process which involves factors produced by chondrocytes acting in an autocrine fashion and has been studied in chondrocytes by Hardingham and Muir (1972). Matrix macromolecule synthesis can also be regulated by extracellular factors such as growth factors and hormones during development (Vetter et al 1986 ; Schlechter 1986).

Inflammation of the joint results in dramatic changes to all of these structures leading to loss of joint function. Joint inflammation is characterised by the selective infiltration of inflammatory cells into the synovium. These cells release a number of mediators which have pathological effects on both synoviocyte and chondrocyte function. In particular, the effects of cytokines released from these inflammatory cells on chondrocyte and synoviocyte function have been studied. In general these inflammatory mediators push the balance of connective tissue turnover in the joint towards degradative processes.

The possibility that the peripheral nervous system could participate in the early stages of joint destruction has received comparatively little attention. Pain signals which originate in the joint are transmitted to higher cortical centres, in part by sensory nerve fibres for which the neuropeptide SP is a neurotransmitter. Furthermore several studies have indicated that SP can be released into the joint by antidromic stimulation

of small, unmyelinated C-afferent nerves. Substance P has several biochemical features which enable it to act as an intercellular communicator-peptide between cells of the central and peripheral nervous systems. However less studied is the observation that SP can signal inflammatory cells to initiate a number of pro-inflammatory events in a similar fashion to cytokines.

The role of SP and the related members of the tachykinin family (see below) has been studied in some detail with regard to joint inflammation in animal models. However the effects of these peptides on the function of the connective tissues of the joint in particular chondrocytes have not been determined. For these reasons I have chosen to examine in detail the effect of these neuropeptides on chondrocyte, and to a lesser extent synoviocyte function, and determine whether they may contribute significantly to joint pathology. The results of these studies form the basis of this thesis.

## B. EVIDENCE FOR A ROLE OF THE PERIPHERAL NERVOUS SYSTEM IN THE PATHOGENESIS OF ARTHRITIS

Rheumatoid arthritis is an inflammatory synovitis which has several characteristics that suggest involvement of the nervous system in its pathogenesis (for reviews of this hypothesis see Levine et al 1985; Levine et al 1987; Iversen 1985; Fitzgerald 1989; Matucci-Cerinic and Partsch 1992). The first is the relative symmetry of joint involvement and the finding that specific joints are more frequently and often more severely involved. If the neurologic hypothesis is valid it would also be expected that differences in joint pathology between high and low innervation-density joints (Mitchell and Fries 1982) would be discernible. Usually the pathogenesis of RA is explained by invoking self-perpetuating immune responses (Klareskog et al 1982). These theories fail to explain two of the most prominent clinical features of RA, namely that distal joints are more likely to develop RA and that RA is characteristically an asymmetric disease.

There is now substantial evidence that neuropeptides influence inflammatory reactions, through direct stimulation of inflammatory cells by a variety of neuropeptides and their fragments, the most prominent being the tachykinin SP.

## B.1. Innervation of the joint

Direct examination of nerve fibres supplying the joints shows that several classes are present. Electron microscopy (Langford and Schmidt 1983) has revealed that in the medial and posterior articular nerves of the knee, 20% of the nerve fibres are finely myelinated with the remainder being unmyelinated. Studies utilizing sympathectomy have revealed that none of the myelinated and 50% of the unmyelinated axons, arise from the dorsal root ganglia.

The presence of nerve bundles supplying the synovium had not been clearly demonstrated until recently. A number of studies have been previously performed (Kellgren and Samuel 1950; Harvey 1987), however the results have been conflicting. Some authors suggest the synovium is poorly innervated (Harvey 1987) whilst others suggest the opposite (Kennedy et al 1982). The earlier studies used techniques which involved the impregnation of silver which is not specific for nerve fibres and may account for the contradictory results.

More recently antisera directed to neural cytoskeletal proteins and neuroactive peptides have been used to identify neuronal constituents in a variety of tissues (Hökfelt et al 1980; Holzer 1988). More conclusive studies have been performed using the marker, protein gene product 9.5 (PGP 9.5) (Lundberg 1988). This protein was first identified in extracts of human brain and is a major component of the neuronal cytoplasm and has proved to be the most sensitive marker for neuronal innervation. Using PGP 9.5 antiserum, Mapp et al (1990) have demonstrated that high numbers of nerve fibres are present in the normal synovium.

Immunostaining for SP and calcitonin gene-related peptide (CGRP) in sensory and sympathetic nerves was found predominantly in the perivascular networks in normal synovium. In RA synovium a similar innervation was seen as in normal

synovium in the deep tissue. Of importance is the observation that fibres immunoreactive for PGP 9.5, neuropeptide Y (NPY), SP and CGRP were not visible in the superficial tissues and the superficial cell layer in synovium from patients with RA. This observation is possibly explained by an increased release of these neuropeptides thereby reducing the neuronal store below that detectable by immunocytochemistry or alternately by formation of synovial pannus as seen in RA.

The observation that C-fibres which innervate the synovium contain SP, neurokinin A (NKA) and CGRP suggests that these neuropeptides should also be located in synovial fluid. The levels of these peptides, in particular SP, in both normal and inflamed synovial fluids have been analysed by a number of groups using both radioimmunoassay and high pressure liquid chromatography. In phlogistic arthropathies, Devillier et al (1983) found increases in SP levels in the synovial fluid. This was later confirmed by Agro and Stanisz (1992), Menkes et al (1990) and Matucci-Cerinic et al (1991). However Marshall et al (1990) reported that SP levels in RA patients were not significantly different from those found in osteoarthritis patients, while Larsson et al (1989) found no SP-like immunoreactivity in either control or rheumatoid groups. Neurokinin A was found in all control groups but not in RA patients, while CGRP was found in both groups. The discrepancies in the quantification of neuropeptides in synovial fluid can be explained in light of patient selection and the rapid metabolism of neuropeptides in synovial fluid. An immunohistochemical study by Mapp et al (1990) found elevated quantities of SP in afferent fibres in the normal synovium but a lack of SP in the same fibres in RA.

These data suggest that in the early phases of joint inflammation large amounts of neuropeptides may be released form sensory nerve terminals, resulting in a permanent depletion. Therefore it can be hypothesized that SP and other neuropeptides may have a predominant role in the early phase of joint pathology, when neuropeptides are not depleted.

*a. Clinical Evidence*

Implicit in the hypothesis that the nervous system contributes to the pathophysiology of RA is the postulate that neurologic alterations should influence the expression of the disease. Possibly, the development of RA may be associated with a predisposing neurological abnormality, although the neurologic abnormalities before the onset of RA have not been systemically studied. However patients with RA are more likely to have autonomic dysfunction (Bennett and Scott 1965; Leden et al 1983). Furthermore neurologic lesions can alter the bilateral symmetry of RA in humans and in experimentally-induced arthritis in the rat. Patients, who acquire either central or peripheral lesions to the nervous system and later develop RA are spared joint inflammation, erosions of bone and cartilage and formation of nodules in the paretic limbs (Thompson and Bywaters 1962; Glick 1967). Similarly, in the adjuvant arthritis model, an experimentally-induced bilateral symmetric arthritis is delayed in onset and diminished in severity on the side which has been previously damaged neurologically (Courtright and Kuzell 1965). This sparing effect has been previously explained by the decreased use of these joints, however the correlation between joint use and severity of arthritis is poor (Glick 1967). This phenomenon is not restricted to RA. Patients who develop Heberden's nodes, a form of osteoarthritis, only develop osteoarthritis on the side unaffected by a previous stroke (Winter 1952). Gout is also less common on the paretic side (Glynn and Clayton 1976).

A weaker, although relevant, argument is the observation that stress, defined as hyperactivity of the autonomic nervous system, seems to trigger exacerbations in RA (Baker 1982). Surgical intervention of the nervous system has not been explored systematically, however it has been reported that sympathectomy is beneficial in advanced RA (Herfort 1956). Hyperactivity of the autonomic nervous system has however been studied in the spontaneously hypertensive rat. This strain has a tonically increased sympathetic tone (Nilsson and Folkow 1982); its control strain is the Wistar Kyoto rat or normotensive rat. When adjuvant arthritis is induced in each strain, the

spontaneously hypertensive rat shows significantly higher scores for bone and cartilage erosions than the normotensive control.

***b. Experimental evidence***

Both afferent (sensory) and efferent (sympathetic) arms of the peripheral nervous system appear to be involved in the inflammatory processes of RA. The unmyelinated, afferent neurons which innervate the joint contain SP (Leeman and Gamse 1981). Up to 90% of SP in primary afferent fibres is transported peripherally from cell bodies in the dorsal root ganglion. This 'peripheral pool' of SP can be released by antidromic (that is action potentials in the direction of peripheral terminals) activation. SP is contained in a subset of unmyelinated afferents that transmit pain signals. Studies in humans indicate that half these unmyelinated afferents in the synovium elicit pain sensation (Kellgren 1950). Therefore nocioceptors are largely present in the synovial afferent innervation.

Evidence that SP is directly involved in joint inflammation comes from experiments in the adjuvant arthritis model, measurements of levels of SP in synovial fluid and studies on the direct effects of SP on RA synovial cells *in vitro*. Adjuvant arthritis is induced by intradermal injection of *Mycobacterium butyrium* (Pearson and Wood 1959). After inoculation, a bilateral inflammation and destruction of joints is seen in 20-30 days in this experimental model of arthritis. Earlier studies established that the joints more severely affected were those in which innervation density and therefore SP concentration was highest (Lembeck et al 1981; Mitchell and Fries 1982). Furthermore, the development of adjuvant arthritis can be specifically blocked by the administration of capsaicin, a neurotoxin which is relatively selective for unmyelinated sensory neurons (Gamse et al 1982). Direct infusion of SP into the rat knee increased the severity of adjuvant arthritis, whereas injection of the SP receptor antagonist [$Pro^2$, $Trp^{7,9}$]-SP had no effect (Engberg et al 1981). These data indicate that release of intraneuronal SP in joints directly contributes to the severity of adjuvant arthritis (Levine et al 1984).

The question of which neural components contribute to joint injury in adjuvant arthritis has been addressed. Levine et al (1984) found that neonatal administration of capsaicin, which causes peripheral sympathectomy and eliminates small-diameter afferents, attenuated joint injury. To determine whether the central or peripheral nervous systems contributed to this process, rats underwent dorsal rhizotomy. This surgical procedure selectively eliminates central connections in joint afferents while leaving peripheral connections intact. Rats which underwent dorsal rhizotomy developed a more severe arthritis which could be reduced by prior sympathectomy or capsaicin treatment. This observation suggests that large diameter afferents which are cut during dorsal rhizotomy also influence inflammation (Levine et al 1986).

Taken together, these results indicate that no single class of nerve fibre is responsible for the neurologic component of inflammation in adjuvant arthritis. Rather, large diameter afferents, sympathetic efferents and central nervous system circuits that modulate these fibre systems, all influence the severity of joint disease.

### *c. Effect of SP on synoviocytes in vitro*

Two studies have addressed the possibility that SP may have direct effects on human synoviocytes (Lotz et al 1987; Partsch et al 1991). In cultures of human rheumatoid synoviocytes, SP was found to stimulate prostaglandin $E_2$ and collagenase secretion in a dose-dependent manner (Lotz et al 1987). A similar result was found with the SP-(4-11) fragment, however the magnitude of the response was not as great as for the full length peptide. The specificity of this effect was demonstrated through the use of the antagonist [D-Pro$^2$, D-Phe$^7$, D-Trp$^9$]-SP or D-SP (Folkers et al 1981) . When the antagonist was titrated against SP at $10^{-8}$ M and $10^{-9}$ M, the levels of $PGE_2$ were reduced. Collagenase activity is released spontaneously *in vitro,* however this was shown to increase 5-8 fold after the addition of $10^{-8}$ M SP. The effect was again blocked by the antagonist, D-SP. To determine whether SP was able to stimulate synoviocyte proliferation, $^3$H-thymidine incorporation was measured and this demonstrated the ability of SP to stimulate proliferation.

The results from this study suggest that SP may directly contribute to the pathogenesis of RA. Stimulation of synoviocyte proliferation leads to an enhancement of pannus formation and collagenase production which in turn leads to the destruction of articular cartilage.

## C. TACHYKININS

Tachykinins are peptide neurotransmitters which are defined by their ability to rapidly contract a variety of smooth muscles and a common carboxyl-terminal amino acid sequence , phe-**X**-gly-leu-met-$NH_2$, where **X** is an aromatic or branched aliphatic amino acid and the C-terminal methionine is amidated. Three mammalian tachykinins have been isolated so far; SP, NKA and neurokinin B (NKB). In addition there are amino-terminal extended (neuropeptide γ and neuropeptide K) and a truncated form of NKA called NKA (3-10) (Helke et al 1990; Leeman and Mroz 1974; Pernow 1983), (see table 1).

Mammalian tachykinins are widely distributed in both the central and peripheral nervous systems. The biological responses evoked by this group of peptides are diverse. Members of this peptide family excite neurons, evoke behavioural responses, are potent vasodilators and contract, in concert with other classical neurotransmitters, many smooth muscles. Tachykinins are of special interest in the striatonigral system and in the afferent transmission of nocioception. More recently tachykinins have found more divergent roles as growth factors and messengers between the nervous and immune systems.

### C.1. Molecular biology of tachykinins

Two distinct genes called the SP/NKA or (preprotachykinin PPT gene I or A) encode the mammalian tachykinins (Nakanishi 1987; Nakanishi 1991). The NKB gene is called the preprotachykinin II or B gene. SP, NKA and the NKA derivatives, neuropeptide K, neuropeptide γ and NKA (3-10) are encoded by mRNAs from SP/NKA gene transcription (Nawa et al 1983; Nawa et al 1984; Krause et al 1987).

**Table 1.** The known mammalian tachykinin neuropeptides and their amino acid sequences. The C-terminal sequence homology characteristic of this family of neuropeptides is shown in shading and bold. Figure adapted from Helke et al (1990).

| Gene | PPT mRNA | Tachykinin | Amino Acid Sequence |
|---|---|---|---|
| SP/NKA (PPT I) gene | α, β, γ | Substance P | Arg-Pro-Lys-Pro-Gln-Gln-**Phe**-Phe-**Gly-Leu-Met-$NH_2$** |
| | β, γ | Neurokinin A | His-Lys-Thr-Asp-Ser-**Phe**-Val-**Gly-Leu-Met-$NH_2$** |
| | β | Neuropeptide K | Asp-Ala-Asp-Ser-Ser-Ile-Glu-Lys-Gln-Val-Ala-Leu-Leu-Lys-Ala-Leu-Tyr-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-**Phe**-Val-**Gly-Leu-Met-$NH_2$** |
| | γ | Neuropeptide γ | Asp-Ala-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-**Phe**-Val-**Gly-Leu-Met-$NH_2$** |
| | β, γ | Neurokinin A(3–10) | Thr-Asp-Ser-**Phe**-Val-**Gly-Leu-Met-$NH_2$** |
| NKB (PPT II) gene | | Neurokinin B | Asp-Met-His-Asp-Phe-**Phe**-Val-**Gly-Leu-Met-$NH_2$** |

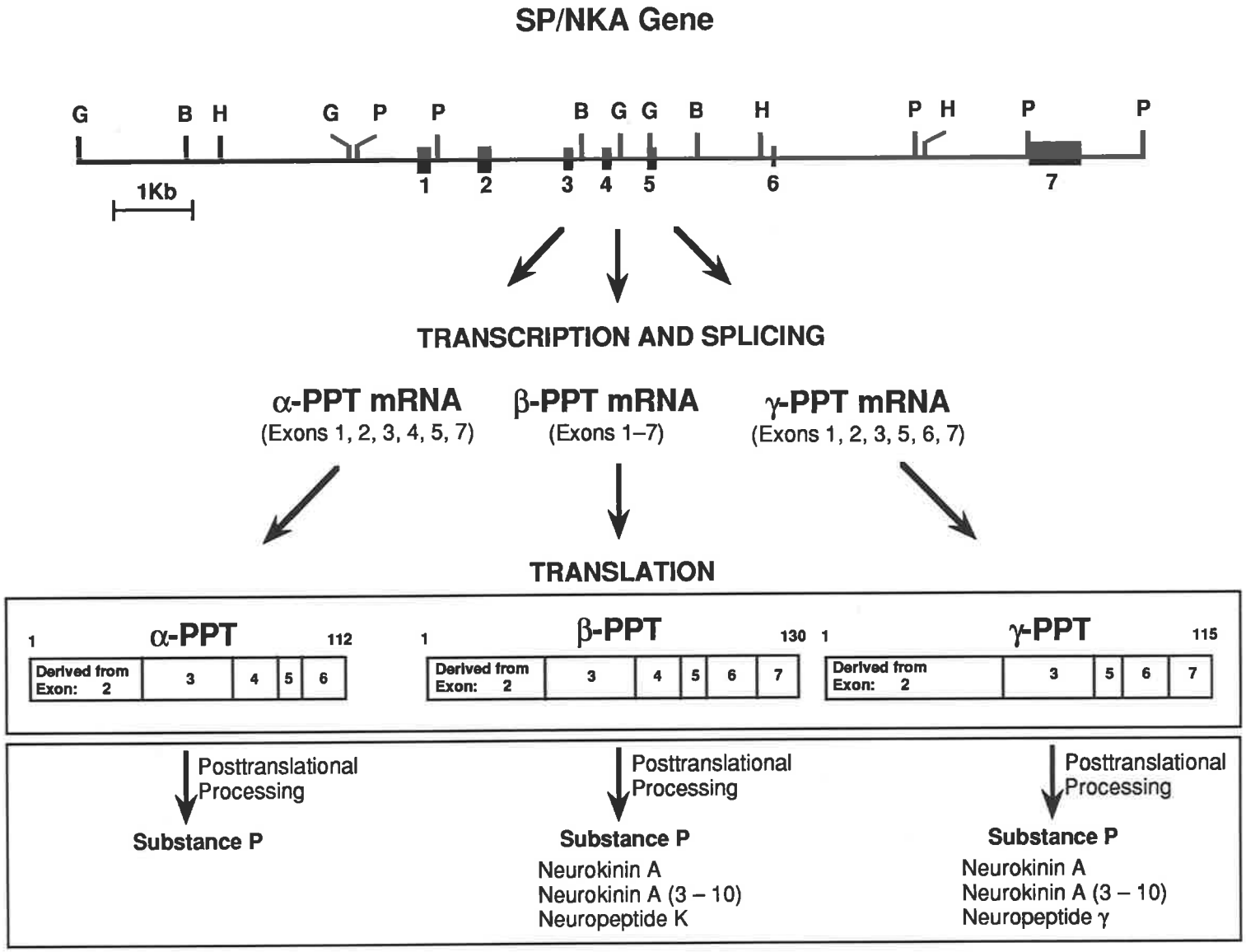
NKB is the only peptide derived from the NKB mRNA (Kotani et al 1986; Bonner et al 1987). Alternate splicing of the primary transcripts of the SP/NKA gene results in three mRNA products called α, β and γ-PPT (Nawa et al 1984). The β-PPT mRNA contains sequences from all seven exons of this gene, whereas the α-PPT mRNA lacks the sixth exon and the γ-PPT mRNA lacks the fourth exon. SP is encoded in part of exon 3, whereas NKA is encoded by exon 6. Differential posttranslational processing of the NKA portions of β- and γ-PPT precursors results in different $NH_2$ terminally extended forms of NKA. Consequently, SP is the only tachykinin peptide derived from α-PPT mRNA, whereas SP, NKA, neuropeptide K and NKA(3-10) can potentially be derived from β-PPT mRNA and SP, NKA, neuropeptide γ and NKA(3-10) can be potentially derived from γ-PPT mRNA (see figure 1.1).

Tachykinin gene expression can therefore be regulated at a number of levels, including SP/NKA gene transcription, primary transcript splicing, PPT mRNA transport, translation efficiency, stability and specific posttranslational processing mechanisms. Transcription activation generally occurs at promoter/enhancer regions of genes with *trans*-acting factors binding to *cis* regulatory sequences. On the basis of the homology of the promoter/enhancer regions of the SP/NKA gene to established consensus sequences for other regulated genes, it is possible that cAMP, estrogen, nerve growth factor and other serum factors and phorbol esters may regulate PPT gene transcription.

The peripheral biosynthesis of SP (molecular weight 1,348) (Tregear et al 1971) has been studied in the rat dorsal root ganglion (Harmar et al 1981). Substance P appears to be synthesized in the cell bodies of ganglia since $^{35}$S-methionine and $^{3}$H-proline are both incorporated into SP after a lag period of one hour. Biosynthetic blockade by cycloheximide suggests SP is translated on ribosomes like other cellular proteins.

Ligation experiments with peripheral nerves have shown that SP migrates to peripheral nerve terminals after synthesis which occurs in the dorsal root ganglion (Holton 1958). This observation has been extended by biochemical and

**Figure 1.1.** Diagram showing the transcription and splicing of the SP/NKA gene primary transcript and the translation and posttranslational processing of the α, β and γ-preprotachykinin (PPT) precursors. Transcription of the gene and splicing yields α, β and γ-PPT mRNA. The mRNAs are translated and the signal peptide cleaved. The abbrevitions above the SP/NKA gene are sites for restriction endonuclease action. G, (Bg III) B, (Bam H1) H, (Hind III) P, (Pst I). Figure adapted from Helke et al (1990).


SP/NKA Gene
G B H G P P B G G B H P H P P
1Kb
1 2 3 4 5 6 7
TRANSCRIPTION AND SPLICING
α-PPT mRNA
(Exons 1, 2, 3, 4, 5, 7)
β-PPT mRNA
(Exons 1–7)
γ-PPT mRNA
(Exons 1, 2, 3, 5, 6, 7)
TRANSLATION
1 α-PPT 112
Derived from Exon: 2
3
4
5
6
1 β-PPT 130
Derived from Exon: 2
3
4
5
6
7
1 γ-PPT 115
Derived from Exon: 2
3
5
6
7
Posttranslational Processing
Substance P
Posttranslational Processing
Substance P
Neurokinin A
Neurokinin A (3 – 10)
Neuropeptide K
Posttranslational Processing
Substance P
Neurokinin A
Neurokinin A (3 – 10)
Neuropeptide γ

immunohistochemical studies (Brimijoin et al 1980; Gamse et al 1982). Calculations of the rate of accumulation of SP following ligation of the sciatic nerve demonstrated that about 90% of SP synthesized in the sensory ganglion is transported peripherally. The average velocity of this transport is 1 mm/hr in the sciatic nerve, however there also appears to be rapid and stationary populations of SP (Brimijoin et al 1980). The fast moving population is approximately five times faster than the average velocity and is bound to intra-axonal particles, whereas the slower group is soluble in the axoplasma. This transport is blocked by capsaicin, a neurotoxin (Gamse et al 1980) or colchicine (Hokfelt et al 1975). To date the function of these two SP populations is unknown.

## C.2. Mammalian tachykinin receptors

### *a. Pharmacology of tachykinin receptors*

Three main classes of mammalian tachykinin receptor have been described although there are receptor subtypes within these groups (Buck et al 1991). Tachykinin receptors are designated NK-1, NK-2 or NK-3, with the preferred endogenous ligands being SP, NKA and NKB respectively, although individual tachykinins bind to a greater or lesser extent to all three receptor types.

Early studies used pharmacologically constructed agonists since antagonists were unable to discriminate between receptors. For these reasons chemical modifications (see for reviews Wormser et al 1986; Drapeau et al 1987; Regoli et al 1988b) have been made to improve the selectivity of neurokinin agonists for one or the other receptor type. Usually the modified neurokinin constructs consisted of the shortest peptide sequence that maintained full activity and selectivity. Often the N-terminal sequence contributed to some of these non-discriminating effects particularly for SP (Drapeau et al 1987). Receptor selectivity was usually determined in biological not molecular assay systems, using mono-receptor tissues. For example, the presence of NK-1 receptors only was determined in the dog carotid artery with intact endothelium (D'Orleans-Juste 1985), in which addition of SP leads to vasodilation.

Similar biological assay systems have been developed using the rabbit pulmonary artery (D'Orleans-Juste 1986) where NKA causes contraction. The rat portal vein is sensitive only to NKB (Mastrangelo et al 1987). Degradation of the native agonist in these biological systems highlighted the need for selective agonists resistant to proteolytic degradation. Usually the minimal amino acid constructs are resistant to degradation (Hruby 1983). A number of chemical modifications have been made to generate receptor-specific agonists. These include modifications to the SP-(4-11) octapeptide [B-Ala$^4$, Sar$^9$, Met ($O_2$)] which have produced highly specific NK-1 receptor agonists. Improvement of NK-3 receptor agonist selectivity has been produced by replacement of Val$^7$ by an N-methyl residue (MePhe$^7$) which has high affinity for NK-3 receptors. Selective NK-2 receptor agonists have been made by replacement of Met$^{10}$ by norleucine (Nle) and also by the reduction of the decapeptide chain by three N-terminal amino acids (Drapeau et al 1987; Wormser et al 1986; Ploux et al 1987).

### *b. Biochemistry of tachykinin receptors*

The biochemical characterisation of tachykinin receptors initially began with attempts to solubilize and purify the SP receptor protein. NK-1 receptors were purified from bovine brainstem (Nakata et al 1988), chick brain membranes (Too and Hanley 1988) and from the human IM-9 lymphoblast cell line (Payan et al 1984a; McGillis et al 1987b). These IM-9 receptors were shown to retain their high affinity for SP in the solubilized state, which was comparable with intact cells. The binding of SP with the solubilized protein was inhibited by GTP which demonstrates that the receptors are G-protein coupled (Nakata et al 1988). Cross-linking studies of receptors isolated from the IM-9 cells (McGillis et al 1987b) revealed a 58 kDa and a 33 kDa protein by sodium dodecyl sulphate polyacrylamine gel electrophoresis (SDS-PAGE) Using high performance liquid chromatography (HPLC) the 33 kDa protein was purified to homogeneity and found in high quantities when compared to the density of SP

receptors on IM-9 cells. The function of the 33 kDa protein from this cell line has not been determined.

*c. Molecular structure of tachykinin receptors*

Although a number of studies have been performed using improved agonists to determine the presence or absence of tachykinin receptors in particular tissues, until recently little was known of the molecular structure of tachykinin receptors.

The first receptor sequenced was the bovine NK-2 receptor (Masu et al 1987). This was followed by the isolation of the rat and human NK-1 (Hershey and Krause 1990; Yokota 1989; Gerard et al 1991), NK-2 (Graham et al 1991) and NK-3 (Tsuchida et al 1990) receptor cDNAs. The gene for the NK-1 receptor is located on chromosome 2 and that for the NK-2 receptor on chromosome 10 (Tsuchida et al 1990). The NK-1 receptor consists of 407 (Yokota et al 1989), NK-2, 390 (Sasai and Nakanishi 1989) and NK-3, 452 (Shigemoto et al 1990) amino acid residues. There is between 86-95% homology between bovine, rat and human tachykinin receptors and 54-66% homology between tachykinin receptors in a given species.

Structurally the receptors consist of an extracellular amino terminus and seven transmembrane helices with alternating extracellular and cytoplasmic loops, and a cytoplasmic carboxy terminus. The basic structure of the tachykinin receptors places them in the superfamily of GTP-binding protein-linked receptors which also includes the adrenergic (Dohlman et al 1987; Libert et al 1989), rhodopsin (Nathans and Hogness 1983) and muscarinic cholinergic receptors (Bonner et al 1987; Kubo et al 1986).

The three tachykinin receptors have seven hydrophobic segments, each consisting of 20-25 uncharged amino acid residues. The amino-terminal and carboxyl-terminal regions of the three receptors also show features similar to other G protein-coupled receptors. All three receptors possess N-glycosylation sites at the amino-terminus and contain many serine and threonine residues. Possible phosphorylation sites are present at the carboxyl-terminus. The core sequences covering the seven

putative transmembrane domains and their extending cytoplasmic portions are highly conserved (Shigemoto 1990; Nakanishi et al 1991) (see figure 1.2 ).

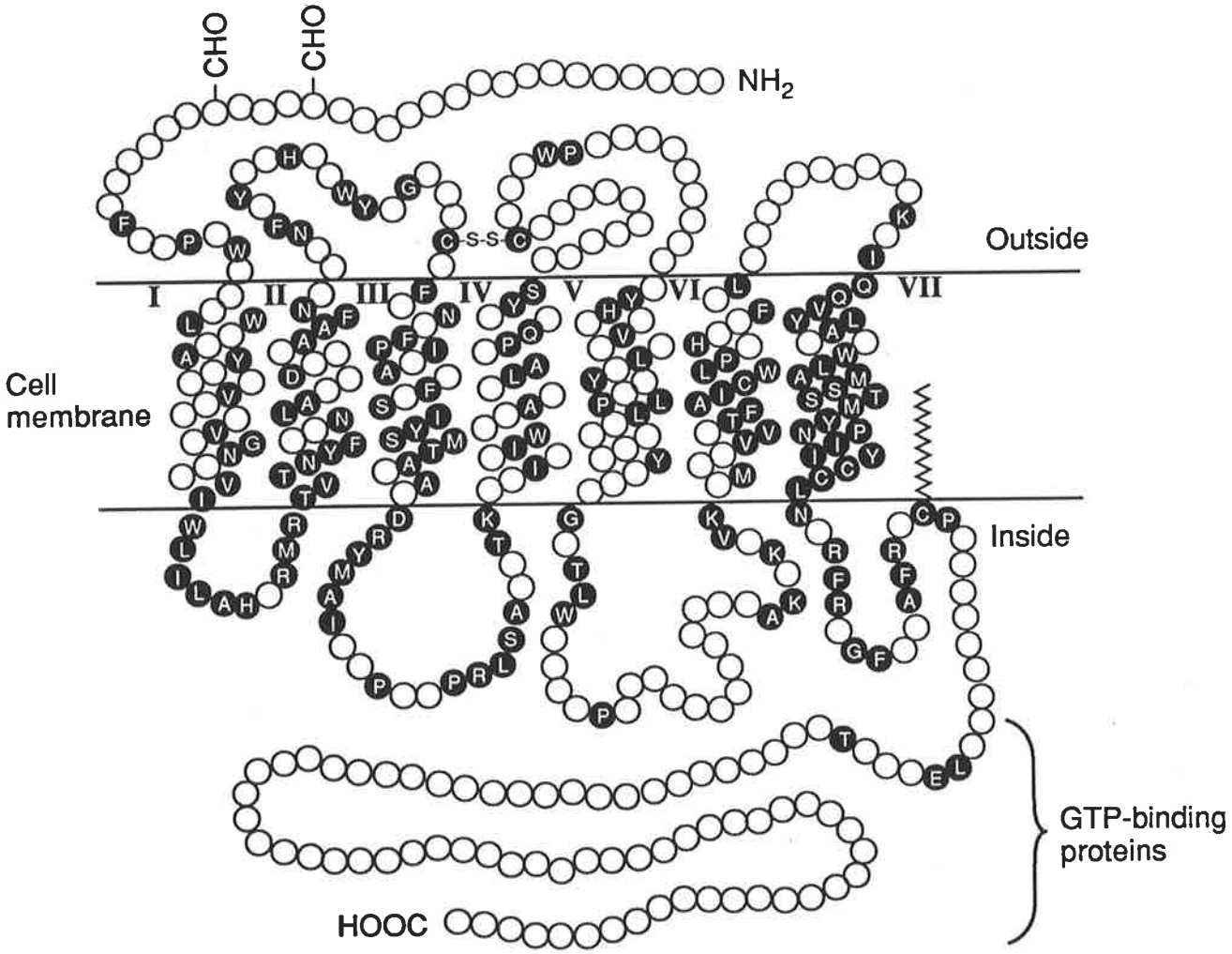
Structural analysis of the β2-adrenergic receptor, revealed two cysteine residues that are found in the third and fourth extracellular loops and are involved in the formation of a disulfide linkage between these two loops (Dixon et al 1987). Another feature of the β2-adrenergic receptor is a second cysteine which immediately follows transmembrane segment VII and is responsible for anchoring the receptor to the plasma membrane (O'Dowd et al 1989). These three cysteines are also conserved in all three tachykinin receptors and may play a crucial role in the fundamental structure for G protein-coupled receptors.

Beside these sequence characteristics of tachykinin and other G protein-coupled receptors, several other features are also common to tachykinin receptors;

1. Both NK-2 and NK-3 receptors have an aspartic acid **(D)** in transmembrane segment II which is common to other G protein-coupled receptors. NK-1 receptors have a glutamic acid **(E)** in place of this aspartic acid.
2. NK-1 and NK-2 receptors share one histidine **(H)** residue each in the transmembrane segments V and VI, which is characteristic of tachykinin receptors.
3. NK-1 and NK-3 receptors are highly conserved throughout the third cytoplasmic loops as well as in portions of the carboxyl-terminal. The NK-2 receptor diverges from the other two in these regions. The conservation of the intracellular regions, particularly on the short homologous sequences near transmembrane segments V and VI has lead to the hypothesis that these areas may be important for coupling of the receptors to G proteins.
4. The number of serine **(S)** and threonine **(T)** residues in the third cytoplasmic loop and in the carboxyl-terminal cytoplasmic regions differs among the three tachykinin receptors.

**Figure 1.2.** A model of the primary structures of the rat NK-1, NK-2 and NK-3 receptors. The three receptors have seven hydrophobic transmembrane spanning segments, each consisting of 20-25 uncharged amino acid residues and share significant homology with the G protein-coupled receptors. Black circles are amino acids which are common to all three receptors. The extracellular region of the NK-1 receptor is important for SP binding and the C-terminal region is coupled to GTP-binding proteins for intracellular signalling. Figure adapted from Nakanishi (1991).

## Model for NK-(1–3) Receptors



Studies by Harada et al (1987) and Shigemoto et al (1990) have demonstrated that all three receptors are desensitised in response to repeated applications of agonist application and that they differ in the amplitude of that desensitization. As a consequence it may be possible that the sequence divergence and the different distribution of threonine and serine residues in these cytoplasmic regions may participate in evoking differing desensitization behaviours between the tachykinin receptors.

### *d. Signal transduction systems triggered by NK-1*

Since the NK-1 receptor was isolated, sequenced and cloned in several species including human, it has been possible to determine the intracellular outcome of receptor / agonist binding. Studies by Krause et al (1992) on human and rat NK-1 receptor function in transfected Chinese Hampster Ovary (CHO) cells have demonstrated that receptor stimulation results in a rapid ($t_{1/2}$ =15 seconds) and transient increase in inositol 1,4,5-trisphosphate ($IP_3$). The flux through this pathway lasted for at least 30 minutes. Levels of cAMP also increased with a $t_{1/2}$ = 1-2 minutes.

Similar pathways were stimulated by NKA and NKB in transfected mammalian cells. All three receptors were reported to rapidly stimulate phosphatidylinositol hydrolysis and cAMP formation in these cells. Both phospholipase C and adenylate cyclase were activated by tachykinin peptide plus GTP in membrane preparations of the cells (Nakanishi et al 1992). These results demonstrate that tachykinin receptors have the potential to couple both phospholipase C and adenylate cyclase to initiate phosphatidylinositol hydrolysis and cAMP formation.

The effect of SP on intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) levels has been investigated in stably transformed CHO cells (Mochizuki-oda et al 1992). SP was shown to induce a rapid release of $Ca^{2+}$ from intracellular stores followed by a sustained rise in $[Ca^{2+}]_i$ which was dependent on extracellular $Ca^{2+}$. The time course of $Ca^{2+}$ mobilization from intracellular stores correlated well with the formation of $IP_3$. Patch clamp recordings suggested that SP triggered the activation of calcium channels by a second

messenger which was identified as $IP_3$. Taken together these results suggest that $IP_3$ may regulate both SP-induced $Ca^{2+}$ mobilization from intracellular stores and $Ca^{2+}$ entry through cation channels in CHO-NK1 cells.

To determine which region of the NK-1 receptor is necessary for mediating SP-induced cAMP production, wild type and chimeras of NK-1 and NK-2 receptors were transfected into CHO cells (Blount and Krause 1992). The response to SP of clonal cell lines which possessed the wild type NK-1 receptor was compared with cell lines which had either their putative third cytoplasmic loop or carboxyl tail replaced with the analogous domains of the NK-2 receptor. The outcome of these experiments suggested that the third cytoplasmic loop domain of the NK-1 receptor plays an important role in G protein coupling and/or activation which determines the cAMP response.

## C.3. TACHYKININ RECEPTOR ANTAGONISTS

### *a. NK-1 receptor antagonists*

To further understand the physiological role of tachykinin neuropeptides *in vitro* and *in vivo* a number of receptor antagonists have been developed. These antagonists can be divided into two broad categories, peptide and non-peptide. Early attempts to create specific (NK-1) SP receptor antagonists pioneered by the groups of Regoli and Folkers were based around the replacement of $Phe^7$ and $Gly^9$ in SP with D-Trp or another D-amino acid. These peptide-based NK-1 antagonists including [D-$Pro^4$, D-$Trp^{7,9,10}$, $Phe^{11}$] SP-(4-11) and [D-$Trp^4$, D- $Trp^{7,9}$, $Norleucine^{11}$] displayed both low affinity and poor selectivity for NK-1 receptors (Regoli et al 1987).

Improvements in both affinity and selectivity were developed in the compound L-668,169 (cyclo[Gln, D-Trp, N-MePhe(R), Gly(ANC-2), Leu, Met]$_2$), (McKnight et al 1988). Similar modifications were made to the non-mammalian tachykinin physalaemin resulting in the compound D-$Pro^9$, (spiro-Gamma-lactam )$leu^{10}$, $Trp^{11}$] physalaemin (1-11), referred to as GR82332 which was a selective NK-1 receptor antagonist *in vitro* as well as *in vivo* , blocking the increase in tracheal insufflation

pressure induced by intravenous SP in the guinea pig (Hagan et al 1991) and inhibiting chemically induced-nocioception in the mouse (Birch et al 1992).

The macrocyclic peptide FK224 was originally isolated from the cultivation broth of the bacterial strain *Streptomyces violaceoniger*. This tachykinin antagonist is novel in that it can block both NK-1 and NK-2 receptors. In guinea pigs FK224 can block contractions of ileum induced by SP and contractions of the rat vas deferens induced by NKA. The NKB-induced NK-3 contraction of rat portal vein remained intact in the presence of FK224 (Morimoto et al 1992). *In vivo* FK224 blocks guinea pig airway constriction and oedema induced by intravenous SP and NKA, suggesting FK224 way be useful in treating asthma (Murai et al 1992).

The first non-peptide NK-1 antagonist designated CP-96,345 was produced by Snider et al (1991) and its binding site in the NK-1 receptor has been determined by Gether et al (1993) and Fong et al (1993). In bovine caudate membranes, CP-96,345 was found to be equipotent with SP at displacing the binding of [$^3$H]-SP to NK-1 sites, possessing a $K_i$ value of 0.6 nM, whilst displaying insignificant affinity for NK-2 or NK-3 binding sites. Substance P induces salivation in the rat which acts specifically via NK-1 receptors. CP-96,345 was shown to completely inhibit this response *in vivo* (Snider et al 1991). In the central nervous system, CP-96,345 was shown to antagonise SP- induced increases in the firing rate of locus coeruleus neurons (brain slice preparation ) in the guinea pig (McLean et al 1991). Since finding that CP-96,345 is a selective, high affinity NK-1 receptor antagonist of both central and peripheral NK-1 binding sites, attention has been given to the role of this compound in blocking pain and neurogenic inflammation. In the rat intra-arterial administration of SP causes a fall in blood pressure and increased plasma extravasation. Both of these effects were blocked by administration of CP-96,345 (Lembeck et al 1992).

A second non-peptide NK-1 antagonist was developed by Garrett et al (1991). RP67,580 was found to inhibit the binding of [$^3$H]-SP in rat brain membranes and like CP-96,345 was shown not to bind to either NK-2 or NK-3 receptors. The discovery of these two non-peptide antagonists led to the discovery of marked differences in the

pharmacological profile of NK-1 receptors in different species. It was shown for instance that CP-96,345 was approximately 100 times more potent in antagonising NK-1 sites in bovine, guinea pig and human brain tissue as compared with rat and mouse brain (Gitter et al 1991, Beresford et al 1991; Watling et al 1991). This contrasts with RP 67580 which was shown to have greater affinity for rat and mouse brain slices compared to NK-1 binding sites on U373MG human astrocytoma cells (Fardin et al 1992). These studies suggest the presence of pharmacologically distinct rat/mouse and guinea pig/human type NK-1 receptors although the presence of both receptor subtypes within the same species has not been demonstrated.

Possibly the most selective NK-1 antagonist developed so far is CP-99,994 (McLean et al 1992) which was able to block the SP-induced excitation of neurons from the locus coeruleus in tissue slices from guinea pig brain *in vitro* with an $IC_{50}$ of 25 nM. *In vivo* CP-99,994 was able to block the hyperlocomotor activity induced by intraventricular infusion of [$Sar^9$, $Met(O_2)^{11}$]-SP with an $IC_{50}$ of 0.59 mg/kg. In receptor binding assays using the IM-9 cell line, CP-99,994 exhibited greater than 10, 000 fold selectivity for the NK-1 relative to the NK-2 and NK-3 receptors ($K_i$ of 0.17 nM). Furthermore CP-99,994 lacks affinity for the $Ca^{2+}$ channel binding site which both CP-96,345 and racemic RP 67,580 demonstrate (Schmidt et al 1992 ).

***b. NK-2 and NK-3 receptor antagonists***

The approach used to develop selective antagonists for the NK-2 and NK-3 receptors was similar to that for NK-1 receptor antagonists. The incorporation of D-amino acids into SP yielded the first peptide NK-2 antagonists. The resultant antagonists developed were weak and displayed poor affinity and selectivity for the NK-2 receptor (Buck and Shatzer 1988). Better antagonists have been developed using NKA as the template for modification. The first selective NK-2 antagonists MEN10, 207 and MEN 10, 208 were developed by substituting D-Trp at positions 6, 8 and 9 into NKA(4-10) and NKA(3-10) (Maggi et al 1990). These were further improved by substitution of Tyr in position 5 and Arg at position 10 and the replacement of Arg10

with Lys resulted in the compound MEN 10,376 which had greatly improved NK-2 selectivity and affinity (Maggi et al 1991).

The first non-peptide NK-2 antagonist SR48968 (Advenier et al 1992a; Emonds-Alt et al 1992; Advenier et al 1992b) was shown to inhibit the binding of [$^{125}$I]-iodohistidyl NKA to NK-2 binding sites present in the rat duodenum with a Ki of 0.5 nM. In contrast SR48968 displayed only weak affinity for NK-1 binding sites in rat cerebral cortex membranes or human IM-9 cells. When SR48968 was examined *in vivo* it was found to produce a dose-dependent inhibition of [Norleucine$^{10}$] NKA(4-10)-induced bronchoconstriction in the anaesthetised guinea pig, following both intravenous and intraduodenal administration ( Advenier et al 1992a).

Unlike the considerable improvements seen in the development of selective NK-1 and NK-2 antagonists, to date the development of NK-3 antagonists has been limited. No non-peptide NK-3 antagonists are available. Initially [D-Pro$^2$, D-Trp$^{6,8}$, Norleucine$^{10}$]-NKB was found to block NKB-induced contractions of the guinea pig ileum (Jacoby et al 1986). Improved affinity has been reported with the NKA-derived analog [Trp$^7$, B-Ala$^8$]-NKA(4-10) which blocked NKB-induced contractions of the rat portal vein. However this compound also has significant antagonist effects for NK-1 and NK-2 receptors and therefore its effectiveness as a selective NK-3 antagonist is limited (Drapeau et al 1990). Until non-peptide, high affinity NK-3 antagonists are produced, the biological action of NKB will not be fully appreciated.

The relative contribution of each tachykinin and tachykinin receptors to a particular physiological and pathophysiological response can now be determined through the use of these antagonists.

### C.4. Tachykinins modulate the immune response

Beside the neurotransmitter role of neuropeptides in the central and peripheral nervous systems, there is now substantial evidence that they can regulate immunological responses (see for reviews: Mc Gillis et al 1987a ; Kimball 1989; Payan et al 1986b). Lymphoid organs are innervated by nerves (Fink and Weihe 1988;

Popper et al 1988) which come into close association with lymphocytes and thereby provide a means for the nervous system to communicate with the immune system, a process termed **neuroimmunomodulation** (Solomon 1987).

The first evidence that SP was involved in inflammation was its potential to act as a mediator of neurogenic inflammation (see Foreman and Jordan 1983). Substance P released antidromically from sensory nerve endings in the skin, causes vasodilation and plasma exudation. The direct effects of tachykinins and their fragments have now been studied in some detail on a variety of cells in the immune system. Lymphocyte proliferation and immunoglobulin synthesis are modulated by SP ( Payan et al 1983; Stanisz et al 1986; Scicchitano et al 1987). Functional receptors for SP have been localised on lymphocytes (Payan et al 1983), IM-9 lymphoblasts (Payan et al 1984a; McGillis 1987b), circulating human T lymphocytes (Payan et al 1984) and guinea pig macrophages (Hartung et al 1986). Substance P also has a number of effects on neutrophil functions including lysosomal enzyme release and phagocytosis ( Bar-Shavit et al 1980; Serra et al 1988; Hafstrom et al 1989), chemotaxis (Wiedermann et al 1989; Iwamoto et al 1990; Perianin et al 1989), leukotriene synthesis and antibody-dependent cell-mediated cytotoxicity (Wozniak et al 1989; Wozniak et al 1993; Hafstrom et al 1989). Substance P also modulates macrophage and monocyte chemotaxis and oxy radical, thromboxane and cytokine production (Hartung and Toyka 1983; Ruff et al 1985; Hartung et al 1986; Wagner et al 1987; Lotz et al 1988; Kimball et al 1988). Substance P can also stimulate or enhance release of cytokines such as interleukin 1 (IL-1) and interleukin-6 (IL-6) (Lotz et al 1988; Kimball et al 1988; Laurenzi et al 1990) and interferon gamma (Wagner et al 1987). Production of other inflammatory mediators including histamine (Fewtrell et al 1982; Foreman and Jordan 1983; Shibata et al 1985; Shanahan et al 1985) and prostaglandin (Hartung et al 1986) are also stimulated by SP. These are mediators which are intimately involved in the pathophysiology of arthritis.

Other investigations have suggested SP is involved in repair mechanisms of the connective tissues (Nilsson et al 1985; Tanaka et al 1988). Substance P was shown

to stimulate proliferation of smooth muscle cells and skin fibroblasts (Nilsson et al 1985) suggesting that SP may be implicated in wound healing responses during tissue damage and inflammation. This activity may be due to the sequence homology between SP and brain-derived acidic fibroblast growth factor (Gimenez-Gallego et al 1985).

A number of studies in rabbits have shown that SP levels in the joint can be directly modulated by the cytokines, IL-1α and tumour necrosis factor alpha (TNF-α). Intra-articular injection of either human recombinant IL-1α or TNF-α into the rabbit knee resulted in increased SP levels in joint lavages 24 hours after injection (O'Byrne et al 1990). It was also found that IL-1α injection induced increased amounts of prostaglandin $E_2$ and SP in joint synovial fluid. This effect on $PGE_2$ production and SP release could be inhibited by pretreating the joints with indomethacin, triamicinolone or dexamethasone. The mechanism of SP release in response to IL-1α or TNF-α was not determined. Possibilities may include cytokine stimulation of $PGE_2$ production in both connective and inflammatory cells and the subsequent stimulation of afferent sensory neurons by $PGE_2$.

## C.5. Neutral endopeptidase and its relevance to SP-mediated joint destruction

### *a. Metabolism of tachykinins by neutral endopeptidases*

The literature concerning the inactivation and degradation of neuropeptides is voluminous and mainly centres around degradation at the synaptic cleft. The metabolism of neuropeptides represents a major mechanism by which biological activity of small peptides is regulated (Lynch and Snyder 1986). Classically, the amount of peptide activity in a tissue will be regulated by enzymatic degradation of that peptide.

In the synaptic cleft several cytosolic proteases have been reported to degrade SP (Nyberg et al 1984; Orlowski et al 1983). However, a membrane-bound protease seems to be predominantly involved in the inactivation of SP, analogous with acetylcholine degradation by acetylcholinesterase in the synapse. Several membrane-bound proteases found in the brain have the ability to degrade SP. A metal chelator-

sensitive endopeptidase from human brain [EC 3.4.24] was shown to cleave SP at the $Gln^6$-$Phe^7$, $Phe^7$-$Phe^8$ and $Phe^8$-$Gly^9$ bonds (Lee et al 1981), while a phosphoramidon-sensitive neutral endopeptidase [EC 3.4.24.11] cleaved SP at the $Gln^6$-$Phe^7$, $Phe^7$-$Phe^8$ and the $Gly^9$-$Leu^{10}$ bonds (Matsas et al 1983). Neutral endopeptidase, also known as endopeptidase 24.11, common acute lymphoblastic leukaemia antigen (CALLA) or CD10 antigen, was first found in the brush border of the rabbit kidney (Kerr and Kenny 1974) and later in many other non-neuronal tissues (Gafford et al 1983; Hersh 1982; Schwartz et al 1981). This enzyme appears to be identical to enkephalinase A found in the brain (Hersh 1982). The 742 amino acid sequence has been recently determined (Malfroy et al 1988) in humans and displays high homology with rat (94%) and rabbit (93%) endopeptidases. The significance of the small differences are not clear, however it has been shown that human and rat NEPs are identical in terms of their optimal pH activity, sensitivity to inhibitors and kinetic parameters for the hydrolysis of a number of neurokinins (Connelly et al 1985; Johnson et al 1984). Several important domains are fully conserved between rat, rabbit and human NEP, namely the hydrophobic region (residues 21-43) which is likely to be a transmembrane spanning region and two fragments (residues 568-580 and 627-635). These fragments have been proposed to serve as binding sites for the zinc atoms of this metallopeptidase (De Vault et al 1987).

Neutral endopeptidase 24.11 may be important for the modulation of tachykinin activity in the joint. Neutrophils, amongst other inflammatory cells infiltrate the synovium in rheumatoid arthritis. Neutrophils possess a membrane-bound endopeptidase able to deactivate a wide range of neuropeptides (Connelly et al 1985). The neutrophil cell membrane enzyme has similar characteristics to the NEP purified from kidney. This was shown by inhibition of its activity by phosphoramidon and thiorphan which are specific inhibitors of kidney endopeptidase (Connelly et al 1985). Antisera to human renal NEP inhibited hydrolysis by the neutral endopeptidase found in neutrophils. Immunohistochemistry using light and electron microscopy localized neutral endopeptidase on the membrane of human neutrophils. Furthermore neutral

endopeptidase activity is higher in neutrophil membrane preparations in patients with adult respiratory disease syndrome and septic pneumonia (Johnson et al 1985).

Since neutrophil endopeptidase has all the characteristics of the enzyme which degrades SP it may be important in the control of SP activity in the rheumatoid synovial joint.

Although the presence of SP in the joint has been demonstrated the physiological consequences of its metabolism have been largely ignored. As discussed previously, NEP is primarily a membrane-bound protein, however a soluble form has been identified in serum and shown to increase in patients with adult respiratory distress syndrome (Johnson et al 1985), sarcoidosis ( Almenoff et al 1984 ) and end-stage renal failure ( Deschodt-Lanckman 1989). Neutral endopeptidase has been detected in the synovial fluids of patients with rheumatic diseases. In a study by Applebloom et al (1991) patients with RA had greater NEP levels in their synovial fluid than patients with osteoarthritis (OA), microcrystalline or seronegative spondylarthropathy. No significant differences were found however, in serum NEP levels. The elevated levels of NEP in RA were subsequently shown to be correlated with polymorphonuclear leucocyte and lymphocyte numbers but not monocyte/macrophage numbers.

Quantitative studies of peptidases in articular tissues are few and have been based predominantly on immunoreactivity rather than specific functional assays and have failed to determine cellular sources or regulation of peptidase activity (Vainio 1970; Sreedharan et al 1990; Kamori et a 1991). One possible site of peptide metabolism may be cells within the synovial lining which have the ability to rapidly degrade bradykinin (Bathon et al 1989). Neutral endopeptidase has been identified in sonicates of human synovial tissue (Sreedharan et al 1990) and expression of the same enzyme was found on rabbit synovial fibroblasts (Werb and Clark 1989). The synovium is in an important position for delivery of peptides to the joint, derived from the bloodstream and SP-containing sensory nerves, which are found in close proximity to the synovial lining (Gronblad et al 1988; Pereira da Silva 1990; Konttinen et al

1990). Furthermore the thin normal lining of the synovium is greatly hypertrophied in RA (Harris 1989) and may have a greater ability to degrade peptides than in normal synovium. The possibility that human synoviocytes are capable of degrading SP and other kinins including bradykinin has been studied by Bathon et al (1992). Cultured synoviocytes derived from both OA and RA synovium were found to rapidly cleave SP with 50% hydrolysis occurring within 30 minutes. However contrary to the results obtained with bradykinin, the NEP-24.11 inhibitor, phosphoramidon (Hudgin et al 1981) only partially inhibited the metabolism of SP by synoviocytes, suggesting the possibility of involvement of other enzymes. The dipeptidyl (amino) peptidase IV (DAP IV; EC 3.4.14.5) inhibitor diprotin A (Umezawa et al 1984) also partially inhibited hydrolysis, while a combination of phosphoramidon and diprotin A almost completely inhibited hydrolysis. In particular the membrane-bound metalloendopeptidases NEP-24.11 (EC 3.4.24.11) and DAP IV (EC 3.4.14.5), which are known to hydrolyse SP (Wang et al 1991), were found to be present and biologically active on cell membranes of cultured human synoviocytes. Furthermore the membrane antigens CD10/CALLA (NEP-24.11) and CD26 (DAP IV) were analysed on synoviocytes using flow cytometry (Knapp 1989). Using monoclonal antibodies directed against CD10 and CD26, Bathon et al (1992) found cultured synoviocytes from both OA and RA patients expressed these antigens. The percentage of positive cells and the intensity of fluorescence for each antigen were similar in RA and OA and remained consistent even after several cell passages *in vitro*.

These studies reveal two important considerations which should be considered when studying the effect of tachykinins on connective tissues. The first is the ability of the synovium to hydrolyse a large number of pro-inflammatory peptides including SP. This suggests the synovium has a unique anti-inflammatory function which is enhanced as the synovial lining hypertrophies. Secondly the rapid metabolism of tachykinins by either NEP or DAP IV would result in the generation of a number of neuropeptide metabolites which have not been quantified.

The metabolites of SP are also biologically active. The N-terminal tetrapeptide fragment (metabolite) of SP, Arg-Pro-Lys-Pro, resembles in its structure, the phagocytosis-stimulating peptide tuftsin (Thr-Lys-Pro-Arg) (Nishioka et al 1972). Indeed SP was shown to enhance phagocytosis and this activity rested in the N-terminal fragment SP-(1-4) of SP (Bar-Shavit et al 1980). Human neutrophils also respond to SP fragments (Wozniak et al 1993). Hafstrom et al (1989) found that the SP-(7-11) fragment induced chemiluminescence and aggregation of neutrophils at high concentrations (>10 μM), whereas the entire SP molecule was less potent and the SP-(1-4) fragment was inactive.

Since the inflamed synovial joint has been identified as a site for rapid degradation of many regulatory peptides and the observation that fragments may have greater biological activity than the native peptide (Hafstrom et al 1989; Wozniak et al 1993), new possibilities arise for the mode of SP-mediated joint destruction which to date have not been studied in detail.

## D. MECHANICAL PROPERTIES OF HYALINE ARTICULAR CARTILAGE

Cartilage is a fundamental biological material which during embryogenesis forms the framework for bone formation and later in life forms a protective covering for bones to withstand compressive loads, yet move freely against each other. Yet adult cartilage is devoid of blood vessels, nerves and lymphatics. Cartilage composition is maintained by chondrocytes which synthesise and deposit a complex extracellular matrix (ECM) composed mainly of collagens and proteoglycans. It is noteworthy to consider that chondrocytes have the capacity to synthesise the largest proteins secreted by any cell.

The ECM of articular cartilage is hyperhydrated. Water content in articular cartilage varies from 65-80% of total weight and is critical in normal joint function and resistance to wear. Under load, this extracellular water is forced out of the cartilage

matrix. When the load is released the extruded water is rapidly taken back into the cartilage ECM.

The non-cellular component of articular cartilage is composed mainly of type II collagen and cartilage-specific proteoglycan found as aggregates with hyaluronan. Collagen fibres provide structural integrity through their three dimensional knit with proteoglycans. Cartilage proteoglycans are highly viscous and strongly hydrophobic, properties important in the compressive load-bearing function of articular cartilage.

Early considerations of cartilage characteristically used terms such as' inert', 'low metabolism' and 'low, inactive cell content'. The functions were described as providing mechanical and structural support to the underlying bone. It is now clear that these observations lacked an understanding of the complexity and regenerative capacity of articular cartilage. In fact articular cartilage is metabolically active. In particular chondrocytes synthesise collagen and large amounts of proteoglycan. In normal cartilage the turnover of proteoglycan may exceed 800 days (Maroudas 1973). However proteoglycan synthesis increases dramatically in damaged cartilage (Cheung et al 1978) although the newly synthesised fibrocartilage does not have the weight bearing properties of undamaged cartilage (Akeson et al 1972). The control of both proteoglycan and collagen biosynthesis is clearly fundamental in the homeostasis of articular cartilage. Synthetic and degenerative processes are part of normal chondrocyte function. Any perturbation of these balances as seen in joint disease will lead to changes in either proteoglycan or collagen content, resulting in changes in the mechanical properties in the tissue.

## D.1. Extracellular matrix of the articular cartilage

### *a. Cartilage-specific proteoglycan (aggrecan)*

Proteoglycans were first identified in articular cartilage which is the richest source of proteoglycans in the body. The abundance of proteoglycan in cartilage has meant that extraction, separation and purification methods have now been developed to investigate proteoglycan structure and function in detail (Sajdera and Hascall 1969).

However it is now known that proteoglycans are found in all tissues and have a variety of structures, functions and sizes. Sequence data are now available and this has led to the identification of several other classes of proteoglycan designated by the suffix "an". For example versican (Zimmerman et al 1989), biglycan (Fisher et al 1989), syndecan (Saunders et al 1989) and betaglycan (Andres et al 1989). Cartilage-specific proteoglycans are known as 'aggrecans' due to their ability to form aggregates when bound to hyaluronan.

Proteoglycans in articular cartilage are very large polyanionic molecules possessing many glycosaminoglycan (GAG) side chains consisting mainly of chondroitin sulphate which is covalently bound to serine residues on the core protein via a specific trisaccharide carbohydrate sequence, xylose-galactose-galactose ( Roden and Smith 1966; Matthews and Lozaiyte 1958; Partridge et al 1961; Muir 1958) (Figure 1.3). The core protein and GAG structure are very diverse and the amount present in different tissues and the functions of the GAGs are still largely unknown. The large aggregating proteoglycans which are found in articular cartilage contain a high molecular mass core protein (230 kDa) to which are attached during post translational processing mainly in the golgi, a large number of chondroitin sulphate and keratan sulphate chains which make up 90% of the total mass of the secreted protein (Hardingham et al 1986). Interestingly these side-chains are not distributed evenly along the length of the core protein. Approximately 60% of these GAG side chains are concentrated, that is they are clustered, towards the C-terminal end of the core protein (Oldberg et al 1987; Doege et al 1987). The N-terminal portion is made up of repeats which are considerably shorter than the more C-terminal clusters (Doege et al 1987). These repeats are made up of 25 amino acids in bovine cartilage, however the implications of such clustering are not clear. The other major glycosaminoglycan-rich region, the keratan sulphate rich region is located near the hyaluronan-binding region. The N-terminal end consists of two globular domains separated by a short peptide sequence (Wiedemann et al 1984). These two domains (G1 and G2) have homologous structures (Doege et al 1987) but it is only the N-terminal G1 domain (link protein)

which appears to bind hyaluronan in a very specific fashion (Morgelin et al 1988; Fosang and Hardingham 1989). Hyaluronan to which large proteoglycans bind to form aggregates, does not share the same synthetic pathway as the proteoglycan, but rather is synthesised in the plasma membrane of chondrocytes and is only available for aggregation after secretion (Ratcliffe et al 1987). The tight binding of proteoglycan to hyaluronan is stabilized by link protein which provides a mechanism for immobilizing proteoglycans in the articular cartilage matrix.

### *b. The domains of aggrecan*

The complete primary structures for the large aggregating proteoglycan has been deduced from rat chondrosarcoma (Doege et al 1986; Doege et al 1987), as well as the partial sequences from bovine (Oldberg et al 1987; Antonsson et al 1989) and human sources (Baldwin et al 1989). The complete coding sequence of the large aggregating chondroitin sulphate proteoglycan from human sources has recently been recorded using a combination of cDNA and genomic exon sequencing. The composite sequence is 7,137 nucleotides long encoding 2,316 amino acids. Human and rat aggrecan amino acid sequences are 75% identical, with the conserved amino acids between domains ranging from 100% to 60%. The human sequence contains two regions of highly conserved repeats not found in rat aggrecan. The first is composed of 11 repeats in the keratan sulphate domain and the second consists of a 19 amino acid sequence repeated 19 times in the serine-glycine-containing region immediately following the keratan sulphate rich region.

These sequence data have been used to compile a detailed structural model for aggrecan. As mentioned, the two globular domains G1 and G2 form the amino-terminus of aggrecan core protein while another globular region G3 makes up the carboxyl-terminus. The primary function of the G1 sequence is to bind hyaluronan and consists of three disulfide-bonded loop regions designated A, B and B' (Doege et al 1990). B and B' have approximately 100 amino acids which are common to both whereas the A domain does not share this homology. The G2 domain has sequences

homologous to the G1 B and B' regions but lacks the A sequence. The C-terminal G3 domain contains a disulfide-bonded globular structure which contains regions with homology to hepatic cell surface lectin (Sai et al 1981; Rhodes et al 1988; Deak et al 1986). G3 also has additional disulfide containing domains, one has homology with the epidermal growth factor (EGF) repeat (Baldwin et al 1989) and one is related to complement B proteins (Patthy 1987). The Mel-14 antigen is similar to the G3 domain in that it contains a lectin-like region and an EGF-like sequence (Lasky et al 1989) but has a transmembrane and cytoplasmic domain which G3 lacks. The G3 domain has a highly conserved amino acid sequence across species, however it remains to be established what properties G3 confer on aggrecan organization within the matrix (see figure 1.3).

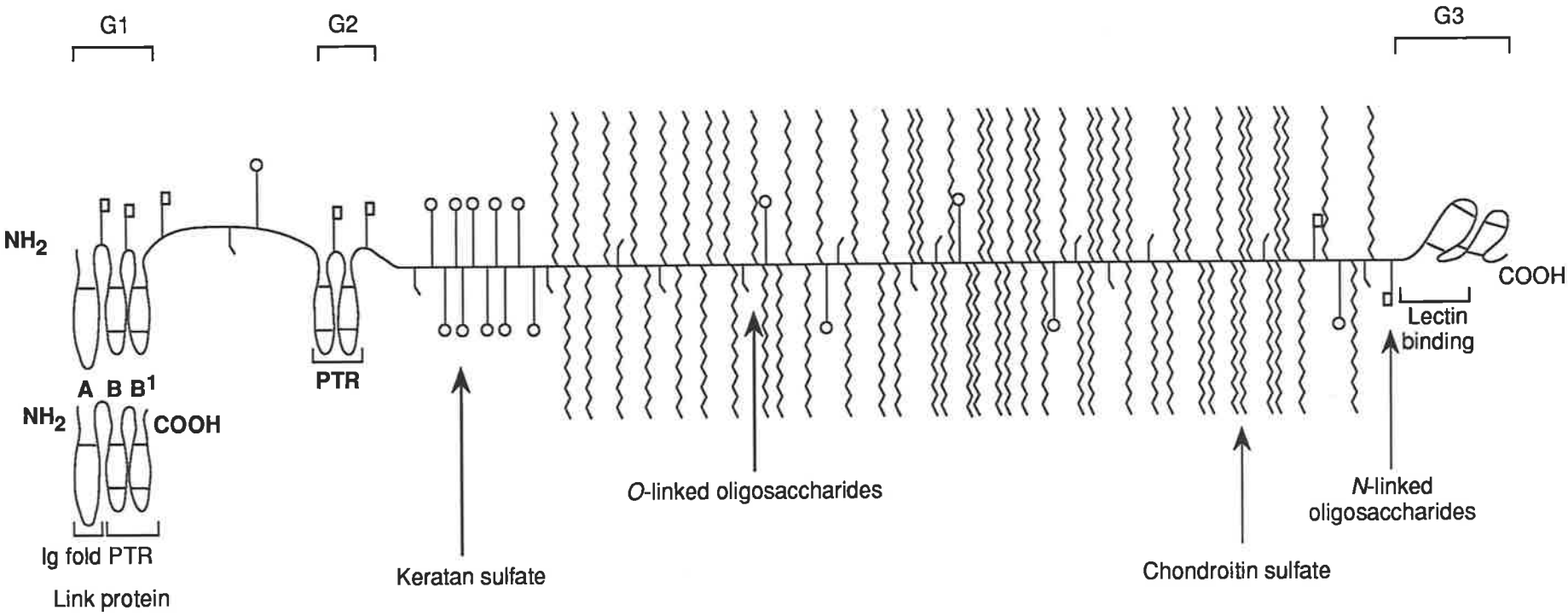
Aggrecan is therefore a complex molecule with multiple domain structures in which a number of structural elements are related to proteins with other functions. These proteins include members of the Ig superfamily and cell surface receptors for cell-cell interactions and cell-matrix interactions. A comprehensive understanding of these interrelationships will provide the basis for an understanding of the physiological functions of aggrecan's components.

### *c. Alteration to aggrecan structure and metabolism during normal ageing*

The molecular changes seen in human aggrecan are a consequence of synthetic and catabolic events regulated by cellular and extracellular factors. The age-related changes are characterized by a decrease in the size and number of chondroitin sulphate chains, an increase in 6-sulfation relative to 4-sulfation and an increase in the size and number of keratan sulphate chains. Changes to the amino acid composition of core protein are also present, however the hyaluronan-binding region appears to be constant at all ages (Bayliss and Ali 1978; Roughley et al 1980). The changes seen between newborn and mature adult cartilage can be explained by essentially three mechanisms;

**Figure 1.3** A schematic representation of the carbohydrate and core protein structures of aggregating proteoglycan found in articular cartilage. The G1 region forms the link protein region which binds to hyaluronan. Figure adapted from Hardingham et al (1990).

## Articular Cartilage Proteoglycan



1. The variation of core protein amino acid composition could be explained in terms of altered gene expression. However recent recombinant DNA evidence suggests that cartilage proteoglycan is encoded by one gene which undergoes alternate splicing to produce two RNA transcripts (Baldwin et al 1989). It is unlikely therefore that multiple genes code for these changes to core protein.
2. Changes in the glycosaminoglycan chain length and sulfation position are obvious examples of post-translational enzyme activity which probably contributes to the polydispersity and heterogeneity of proteoglycans seen during aging.
3. Proteolytic modification of the core protein is characterized by the appearance of a poorly glycosylated hyaluronic acid-binding region in adult cartilage (Roughley et al 1984; Bayliss et al 1985).

Hyaluronan and link protein undergo extensive changes during aging. The concentration of hyaluronan increases 3-4 fold from birth to 90 years of age to accommodate the increasing number of proteoglycan monomers and binding region fragments (Holmes et al 1988). However there is an age-related decrease in hyaluronan chain size extracted from matrix which indicates that there is a slow accumulation of degraded hyaluronan that together with the smaller proteoglycan monomers results in a lower mass aggregate in aged adult cartilage.

Comparatively little is known about the age-related changes to proteoglycan extracellular assembly and subsequent immobilization within the matrix. The processes which are involved in the normal aging process of articular cartilage in many ways reflect some of the processes which occur in disease. However the inflammatory component alters the balance between synthesis and degradation in favour of degradation and results in much greater aggrecan loss from the matrix.

## D.2. Cartilage matrix degradation

### *a. Matrix metalloproteinases*

Extracellular matrix integrity results from the dynamic balance between synthesis and degeneration of its different protein components. Remodelling of the ECM occurs during development, growth and wound healing, however excessive matrix degradation contributes to the pathogenesis of many diseases besides RA (Tryggvason et al 1987). The remodelling and specific degradation of the articular cartilage ECM is controlled by a family of enzymes coupled with specific inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). The number of recognised metalloproteinases has risen rapidly in the past few years and is expected to increase as protein and cDNA information becomes available. Only those metalloproteinases relevant to the joint will be reviewed.

Matrix metalloproteinases are normally active at neutral pH and characteristically have a $Zn^{2+}$ at their active site and require $Ca^{2+}$ for structural stability. Inhibition of activity can be achieved chemically by the use of specific cation chelators or by naturally occurring metalloproteinases inhibitors (Cawston et al 1981). The cells which synthesise and secrete TIMPs are usually the same cells which produce the metalloproteinase proenzymes (Kuettner et al 1977). Matrix metalloproteinases are usually secreted from connective tissue cells such as fibroblasts in an inactive proenzyme or latent form. Activation of the latent enzyme can be achieved with proteases such as trypsin or plasmin or by organomercurial compounds such as 4-amino-phenylmercuric acetate (APMA). Other methods for activation include alteration of pH and temperature increases. Any of these treatments leads to the subsequent loss of a 10-12 kDa portion of the proenzyme and conversion to the active enzyme (Harris et al 1984).

The primary structures of the metalloproteinases have revealed that these proteins contain several distinct domains which are common amongst all family members. The first similarity is a leader sequence which targets the molecule for

secretion and is present on the preproenzyme but is absent in the proenzyme. The catalytic domains contain conserved histidine residues that are postulated to be the zinc binding domain (Sanchez-Lopez 1988). The proenzyme contains a highly conserved sequence PRCGVPDV which is cleaved to form the latent enzyme. Another conserved domain is the hemopexin sequence found in domain 3 which derives its name from the heme-binding protein and is found in all metalloproteinases except PUMP-1 (Putative metalloproteinase-1) (Muller et al 1988) now known at matrilysin (Busiek et al 1992) . The function of the hemopexin domain is unknown, however it may be involved in substrate specificity or may be a region which recognises a cell-surface receptor.

Interstitial collagenase or matrix metalloproteinase I (MMP-I) which is a 52 kDa protein, degrades types I, II and III collagen (Goldberg et al 1986). Neutrophil secreted collagenase (MMP-8), a 75 kDa protein, degrades types I, II and III collagen (MacCartney and Tschesche 1983) and stromelysin (MMP-3) or stromelysin , a 53 kDa protein which degrades articular cartilage proteoglycan (Chin et al 1985). These metalloproteinases share both sequence and activation properties which will be discussed in greater detail.

### *b. Collagen degradation*

Interstitial collagenase, 3/4ase or MMP-I is synthesized as a preproenzyme and secreted in two forms, a major 52 kDa proenzyme and a minor 55 kDa glycosylated form (Wilhelm et al 1986). The activation of the latent enzyme occurs through several mechanisms as previously mentioned but also *in vitro* through incubation with the chaotropic agents, NaI and NaSCN (Abe and Nagai 1972), activation by nonenzymatic tissue activators (Nagase et al 1991) and a process of autoactivation (Stricklin et al 1977). After each treatment the latent enzyme is converted to a stable 42 kDa active enzyme through removal of an 81 amino acid peptide from the amino terminal end of the molecule (Grant et al 1987).

The regulation of MMP-I synthesis by biological agents such as cytokines and growth factors is important to the pathogenesis of RA. Usually the stimulation of

MMP-I secretion is correlated with a similar stimulation of MMP-I mRNA synthesis suggesting regulation occurs at the transcriptional level (Brenner et al 1989; Brinckerhoff 1987). Rheumatoid arthritis is characterised by an infiltration into the synovial joint of lymphocytes, macrophages and neutrophils which secrete a number of cytokines resulting in cartilage degradation. Neutrophils are of particular interest since collagenase is synthesized during their maturation and stored in specific granules until it is secreted upon cell activation under the influence of several agents including phorbol esters (Hasty et al 1986). Neutrophil collagenase (MMP-8) differs from MMP-I in both molecular weight (MacCartney and Tschesche 1983) and its ability to degrade type I collagen more rapidly than type III (Hasty et al 1987a). The similarities of neutrophil collagenase with other interstitial collagenases have been investigated using monoclonal antibodies raised against human neutrophil collagenase (Hasty et al 1984). This monoclonal failed to react against human skin or human synovial collagenase (Hasty et al 1987b) whereas a polyclonal antibody raised against neutrophil collagenase reacted with both interstitial and neutrophil collagenases, suggesting conservation of some epitopes between collagenases of different cell origin.

Stromelysin (MMP-3), is particularly important in articular cartilage degradation since it can also cleave type II collagen in the procollagen form by removal of both N-and C-terminal peptides (Galloway et al 1983; Okada et al 1986). The biosynthesis of MMP-3 is similar to MMP-I resulting in the secretion of 60 kDa and 57 kDa proenzymes (Wilhelm et al 1987). Treatment of pro-MMP-3 with either trypsin or organomercurials results in the loss of 84 residues from the amino-terminus, resulting in a 45 kDa stable active enzyme (Wilhelm et al 1987). It is interesting to note that the same compounds which stimulate MMP-I production *in vitro* also stimulate MMP-3. Tight co-ordination of MMP-I and MMP-3 gene expression has been observed in rabbit synovial cells (Frisch et al 1987; Vance et al 1989).

MMP- 6 was first detected in human articular cartilage extracts (Sapolsky et al 1976) and further characterised in the same tissue by Woessner and Seltzer (1984). Polymorphonuclear leukocytes also contain MMP-6 (Azzo and Woessner 1986). This

enzyme exhibits the classical characteristics of other matrix metalloproteinases, however the maximal activity is at pH 5.3. MMP-7 is the most recent addition to the family of proteoglycan degradative enzymes (Woessner and Taplin 1988). The molecular weight of the pro-MMP-7 is 28 kDa which can be activated by organomercurials resulting in an active 19 kDa enzyme. The phenotype of the substrate specificity of MMP-7 is distinct from the other metalloproteinases making this a distinct member of the family.

This set of matrix metalloproteinases are capable of degrading articular cartilage macromolecules. The regulation of metalloproteinase synthesis, metalloproteinase activation and metalloproteinase inhibitor production is critical to the pathology of RA. The overproduction of metalloproteinases or reduction in production of TIMP may contribute significantly to articular cartilage degradation and the subsequent loss of joint function.

### c. *Aggrecan degradation in articular cartilage*

Articular cartilage proteoglycans are large on a molecular scale and so provide ample sites for attack by many different classes of proteinases, primarily by the enzyme MMP-3 (Sellers et al 1978; Fosang et al 1991; Murphy et al 1992). Elevated levels of metalloproteoglycanase activity have been extracted from osteoarthritic cartilage compared with normal tissue ( Pelletier et al 1987). The analysis of proteoglycan degradation products can be made *ex vivo* from cartilage explants (Ratcliffe et al 1986) or by determining the degradation products *in vitro* following incubation of purified aggrecan with purified stromelysin (Galloway et al 1983). The normal metabolic turnover of aggrecan from adult bovine articular cartilage has been studied in some detail. Eight major peptides derived from the core protein were released into the culture medium (Campbell et al 1989; Ilic et al 1992). The two largest peptides (Mr 300 kDa and 250 kDa) have a functional G1 as well as G2 and keratan and chondroitin sulfate attachment regions (Ilic et al 1990) . These peptides are passively lost from the proteoglycan aggregates (Bolis et al 1989). The other six

peptides result from the proteolytic cleavage of core protein at three distinct sites. Three peptides (Mr 230, 200 and 170 kDa) contain the G2 domain as well as the keratan and chondroitin sulphate attachment regions and the same N-terminal sequence ARGSVILXAX which corresponds to the interglobular domain of the human aggrecan (Doege et al 1991). Two smaller peptides (130 and 100 kDa) are derived from the proteolytic cleavage at two distinct sites in the chondroitin sulfate attachment domain 2 of aggrecan.

Studies of cartilage proteoglycan cleavage by stromelysin and PUMP have been carried out in G1-G2 fragments prepared from pig laryngeal cartilage. This fragment was cleaved by stromelysin to generate two fragments (110 kDa and 56 kDa). Immunoblotting showed the 110 kDa fragment to be the G2 domain and the 56 kDa to be the G1 domain. Amino acid analysis indicated a single cleavage occurred between an asparagine and a pair of phenylalanine residues in both human and rat core protein sequence which is located within a region with high sequence homology to the interglobular domain (Fosang et al 1991). The action of PUMP was similar to the action of stromelysin. However the 110 kDa fragment was further cleaved into two similarly sized 55-60 kDa components. Therefore PUMP was able to cleave at an additional site in the proteoglycan core protein which was unavailable to stromelysin.

No significant degradation of proteoglycan aggregate could be found following incubation with human gingival fibroblast gelatinases (72 kDa) and pig neutrophil enzymes (97 kDa). Human fibroblast collagenase was able to digest both monomers and aggregates and yield fragments which were different from stromelysin degradation.

Recent investigations of articular cartilage structure have revealed that cartilage integrity is also due to a number of binding proteins which function as cross-links between type II collagen fibrils and mediate cell-matrix interactions ( Heinegard and Oldberg 1989). These proteins include type IX collagen and the glycoproteins anchorin and decorin. These hold proteoglycan aggregates *in situ* under considerable pressure. Proteinases which degrade these proteins would markedly modify

proteoglycan stability and therefore cartilage turnover. It is known that type IX collagen is degraded by stromelysin but its activity on other non-collagenous-binding proteins is not known ( Okada et al 1989).

*d. Cartilage specific tissue inhibitors of metalloproteinases*

Following secretion, the biological activity of latent metalloproteinases is regulated by activators of proenzymes and also by several inhibitors of metalloproteinases, TIMPs.

The first demonstration of naturally occurring inhibitors of metalloproteinases was from 1M guanidine-HCl extracts of bovine nasal cartilage (Kuettner et al 1977; Kuettner et al 1976). Later a neutral metalloproteinase released from primary cultures of bovine articular chondrocytes was characterized ( Morales and Kuettner 1982) which was able to degrade proteoglycan and acted in concert with other metalloproteinases to degrade the collagen-proteoglycan framework. An inhibitor of these metalloproteinases isolated from cultures of bovine articular chondrocytes was subsequently characterised (Morales et al 1983; McGuire-Golding et al 1983). The metalloproteinase inhibitor had a molecular weight of 35 kDa, was relatively heat stable and resistant to inactivation by trypsin. This particular inhibitor was active against rat uterine collagenase and gelatinase but not bacterial collagenases such as thermolysin or collagenase purified from *Clostridium histolyticum.*

More recently two forms of TIMPs have been delineated. TIMP-1 has now been cloned, sequenced and expressed in a recombinant form (Docherty et al 1985; Carmichael et al 1986) and has a molecular mass of 28.5 kDa. TIMP-1 is a glycoprotein which is synthesized by most connective-tissue cells *in vitro* as well as endothelial cells ( Herron et al 1986), astrocytes and gliomas (Apodaca et al 1990). TIMP-1 is the predominant inhibitor of metalloproteinases and is found in a variety of body fluids including synovial fluid (Cawston 1986). TIMP-1 also contains 12 cysteine residues which are all thought to be involved in six disulfide bond-pairs making this a very stable protein resistant to many forms of denaturation (Carmichael et al 1986).

TIMP-1 specifically acts against matrix metalloproteinases and forms essentially an irreversible complex with metalloproteinases but not metalloendopeptidases. TIMP-1 binds to the latent proenzyme form of gelatinase (type IV collagenase) (Wilhelm et al 1989) and many other members of the matrix metalloproteinase family (Ward et al 1991).

TIMP-2 has also been isolated from articular cartilage extracts (Bunning et al 1984; Murray et al 1986) and more recently from human melanoma cells (Stetler-Stevenson et al 1989; Goldberg et al 1989), as a 21 kDa protein bound to the proform of 72 kDa gelatinase. Although the sequence homology between TIMP-1 and TIMP-2 is only 40%, the six disulfide bond-pairs and other key residues are conserved suggesting these domains are important for inhibitor activity (Williamson et al 1990). This family of metalloproteinase inhibitors has been expanded to include the so-called large inhibitor of metalloproteinase (LIMP; 76 kDa) which has been isolated from human lung fibroblast culture medium (Cawston et al 1990). LIMP can inhibit the activity of collagenase, gelatinase and stromelysin via formation of tight binding complexes with these metalloproteinases. The inhibitory activity of LIMP was later shown to consist of a 72 kDa-progelatinase bound to TIMP-2 (Curry et al 1992).

The mechanism of metalloproteinase inhibition by TIMPs is not fully appreciated, however it appears that the N-terminal domain of TIMP-1 retains the inhibitory activity. Murphy et al (1991) created recombinant TIMP-1 and a truncated version containing only the three N-terminal loops. The truncated forms were found to exist as two glycosylated variants (24 kDa and 19.5 kDa). All truncated forms were found to inhibit and form complexes with active forms of metalloproteinases, indicating that the major inhibitory region of TIMP-1 resides in these three N-terminal loops. TIMP-2 has a similar structural-functional spectrum of metalloproteinase inhibition both at the enzyme and proenzyme levels.

Understanding the roles of metalloproteinases which degrade both collagen and proteoglycan and their interaction with TIMPs, will be fundamental to a fuller appreciation of articular cartilage physiology during development and disease.

*e. Articular chondrocyte function during inflammation*

Cartilage destruction is a common feature of RA. During inflammation the balance between cartilage degradation and synthesis is disturbed. Degradative processes involving the proteoglycan and collagen content in the articular cartilage are predominant. It is unlikely that enzymes released into the synovial fluid by inflammatory cells are the major contributor to cartilage degradation since numerous protease inhibitors are present in synovial fluid (Dingle 1984; Hadler et al 1981). A second mechanism is through the direct stimulation of chondrocytes and/or synoviocytes to release proteolytic enzymes which degrade the cartilage matrix, while TIMP production is inhibited. In the past decade, cytokines released by inflammatory cells have featured strongly in many papers concerning the degradation of articular cartilage by chondrocytes (see Dayer and Demczuk 1984). A number of cytokine molecules, predominantly IL-1α and TNF-α have been shown to regulate chondrocyte metalloproteinase biosynthesis. Rheumatoid synovial fluid contains a milieu of cytokines including an IL-1α -like activity which arises from mononuclear leukocytes, synovium and chondrocytes (Saklatvala and Dingle 1980; Dinarello 1989; Wood, Ihrie and Hamerman 1985).

Interleukin-1α is a 17 kDa cytokine that is primarily a product of macrophages and monocytes (Dinarello 1989). Two forms of IL-1 have been described, alpha and beta. Interleukin-1α and IL-1β bind to the same receptor but with different affinities and these receptors are present on bovine articular chondrocytes (Harvey et al 1991).

Interleukin-1α has both systemic and local effects such as augmentation of T and B lymphocyte function, chemotaxis of neutrophils, proliferation of synovial fibroblasts and production of prostaglandin $E_2$ and collagenase (Dayer et al 1986). The direct implication of IL-1 in RA comes from clinical and biochemical evidence. Clinical evidence comes from studies of IL-1β levels in plasma from patients with RA (Eastgate et al 1988). These patients had strikingly higher levels of IL-1β than age-matched controls and the level correlated positively with diagnostic scores for RA.

Most studies of the effect(s) of IL-1 and other cytokines on articular cartilage degradation have been performed *in vitro*, with either cartilage slice, agar or monolayer culture. This method largely ignored the effects of naturally occurring IL-1 inhibitors, which act at a number of different levels ( Dayer and Secklinger 1989; Larrick 1989).

Early studies in this area showed that conditioned media of activated rabbit peritoneal macrophages contain a factor capable of inducing *de novo* synthesis of latent collagenase and neutral proteinase (Deshmukh-Phadke et al 1978; Ridge et al 1980; Desmukh-Phadke et al 1980) in chondrocyte monoculture. Similar results have been observed in homologous and heterologous chondrocyte suspension (Herman et al 1981) and cartilage-organ culture systems (Herman et al 1982; Herman et al 1984). Explants of porcine or bovine synovium produce factors capable of degrading cartilage proteoglycan (Fell and Jubb 1977; Saklatvala and Dingle 1980). This effect also occurs in rheumatoid (Steinberg et al 1979) and osteoarthritic synovium (Appel et al 1982). The problem with the earlier studies was their inability to distinguish between the many cytokines present in the conditioned medium of activated macrophages or synovial fluids, many of which share biological properties with IL-1. Recombinant cytokines are now available and the effects of recombinant IL-1α have been tested on bovine, porcine, rat, mouse and human cartilage *ex vivo* (Smith et al 1989), *in vitro* (Mitchell and Cheung 1991; Schnyder et al 1987; Gilman 1987) and *in vivo* (van der Loo and van der Berg 1990).

Whether IL-1 is the predominant pathologic messenger molecule in these systems or is one of many effector cytokines in arthritis is still largely unknown. Despite much experimentation on both IL-1 and TNF-α and their effects on cartilage integrity, there are essentially only two pieces of evidence that they are major contributors. Firstly they can degrade cartilage *in vitro* which is due to *de novo* chondrocyte metalloproteinase production. Secondly IL-1 and TNF-α are spontaneously produced by RA synovial tissue cells and chondrocytes and have been detected in RA synovial fluid. It has been proposed by Hollander et al (1991a & b) that

if IL-1 and TNF-α are major mediators of cartilage degradation in joint diseases then three predictions should be satisfied;

1. IL-1 and TNF-α should only be raised in conditions during periods of cartilage degradation.
2. Inhibitors of IL-1 and TNF-α should inhibit cartilage degradation.
3. Recombinant human IL-1 and TNF-α should cause degradation of human cartilage.

The first of these predictions is not satisfied due to the observation that the concentrations of cartilage glycosaminoglycan in RA synovial fluid do not correlate with IL-1 or TNF concentrations (Bensouyard et al 1990). Furthermore very high TNF levels have been detected in normal synovial fluid (Westacott et al 1990). The second and third predictions was discounted by a study in which normal and diseased human cartilage were incubated with RA synovial fluid. Glycosaminoglycan loss was increased following incubation with RA synovial fluid but not normal human serum. However recombinant IL-1α, IL-1β and TNF-α had no degradative effect in this system. The authors conclude that RA synovial fluid degrades cartilage by synergistic interaction between cytokines and some other component of synovial fluid. Studies with chondrocytes and cytokines have demonstrated elegantly that cartilage degradation in disease is a highly complex event and its elucidation is limited by the systems which are currently used to investigate this process.

The possibility that the peripheral nervous system may also contribute to the pathogenesis of arthritis has received far less attention. The effects of tachykinins on cells of the nervous and immune systems have been investigated however to date no thorough investigation on the effects of tachykinins on the connective tissue cells of the joint have been done.

## E. SUMMARY

Tachykinins are clearly involved in the pathogenesis of inflammatory joint disease by virtue of their ability to effect microvasculature, inflammatory cells and connective tissue cells of the joint. In all of these studies the rapid metabolism of neuropeptides by NEP has been largely ignored. In view of this the effect of SP metabolites on connective tissue cell function should also be examined. To date the effect of tachykinins or their metabolites on articular chondrocyte function has not been investigated.

In this thesis I have therefore posed the following questions:-

1. What effect does SP, NKA, NKB and the N-and C-terminal metabolites of SP have on aggrecan production in bovine articular chondrocytes *in vitro* ?

2. Is prostaglandin $E_2$ production influenced by tachykinins and their metabolites? Can exposure to hr IL-1$\alpha$ alter the effect of tachykinins on prostaglandin $E_2$ production?

3. Do SP or SP metabolites influence chondrocyte-mediated collagenase (MMP-I) or tissue inhibitor of metalloproteinase (TIMP) synthesis?

4. Do tachykinins or their fragments alter $[Ca^{2+}]_i$ in bovine articular chondrocytes?

# CHAPTER II

# THE EFFECTS OF TACHYKININS AND THEIR METABOLITES ON BOVINE ARTICULAR CARTILAGE CHONDROCYTE PROTEOGLYCAN PRODUCTION *IN VITRO*

# A. INTRODUCTION

The contribution of tachykinins to the exacerbation of inflammatory joint disease has been investigated in some detail at the cellular level. Studies have concentrated on the effect of SP on inflammatory cells including, lymphocytes, mast cells, neutrophils and macrophages.

The effect of tachykinins on connective tissue cells of the joint has been less thoroughly investigated. In particular the effect of SP has been investigated on human rheumatoid synoviocytes *in vitro* (Lotz et al 1987; Matucci-Cerinic et al 1991). The synovium is richly innervated by unmyelinated C-afferent fibres which contain SP, NKA and CGRP (Mapp et al 1990). These neuropeptides are released into the joint by antidromic stimulation where they may effect the function of synoviocytes and/or chondrocytes. Extensive destruction of the articular cartilage is a prominent feature of RA, a process which may be augmented by SP due to its ability to stimulate synoviocyte proliferation and $PGE_2$ and collagenase production.

However the molecular weight of SP (1348 Da) is well below the exclusion limit of cartilage so that the increased SP levels in synovial fluid seen in traumatic and RA will be paralleled by increased levels within cartilage extracellular fluid (Marshall et al 1990; Matucci-Cerininc et al 1991; Agro and Stanisz 1992).

Chondrocytes can both detect and repair damaged articular cartilage predominantly through the production of aggrecan and type II collagen. During development chondrocytes can remodel the articular cartilage via the production of MMP-I and MMP-3 as discussed in the literature review.

The effect of tachykinins on proteoglycan production in articular cartilage has not been investigated. The hypothesis that SP contributes to the accelerated cartilage destruction seen in arthritis by modulating chondrocyte synthetic function, particularly proteoglycan (aggrecan ) production was tested.

The aims of this study were to determine the effects of tachykinins and SP fragments on bovine articular chondrocyte:-

1. Proteoglycan production, both proteoglycan released into the culture medium and incorporated into the cell layer.
2. Proteoglycan size, both secreted into the medium and incorporated into the cell layer.
3. Protein production.

Since it is likely that a number of SP metabolites are generated in synovial fluid due to the hydrolysis of SP by NEP and other enzymes, the effect of N- and C-terminal metabolites of SP on chondrocyte function was also investigated. These studies were performed in monolayer cultures of normal, bovine articular chondrocytes isolated from the metacarpophalangeal joint.

## B. MATERIALS AND METHODS

### *B.1. Materials*

Bovine hocks were supplied by SAMCOR, South Australia. Chemicals and biologicals were purchased from the following sources: collagenase purified from *Clostridium histolyticum*, trypsin, bovine testicular hyaluronidase, phenylmethylsulfonyl fluoride (PMSF), soy bean trypsin inhibitor (SBTI), Sigma Co. St. Louis, MO, USA. Dulbecco's modification of Eagle's medium (DMEM) and foetal calf serum (FCS), Cytosystems, Castle Hill, New South Wales, Australia. Penicillin/streptomycin and tissue culture plates, Flow Laboratories, Virginia, USA. $Na_2{}^{35}SO_4$ [65 MBq/ml in aqueous solution] and L-[4,5-$^3$H] Leucine (9.25 MBq in aqueous solution], Amersham International, Amersham UK. Tachykinins (SP, NKA, NKB) and SP fragments, AUSPEP, Melbourne, Australia. Lyophilised SP was diluted in a solution of phosphate buffered saline (PBS)/acetic acid ($10^{-3}$ M final concentration), 1:1 (v:v). Peptides were stored under nitrogen at -70$^0$ C to prevent

oxidation. Human recombinant (hr IL-1α) was a gift from Dr. P.T. Lomedico (Hoffmann-LaRoche, Nutley, NJ, USA.; specific activity $10^8$ units/mg. Cetylpyridinium chloride (CPC), AJAX Chemicals, Sydney, Australia. Sepharose CL-4B chromatography gel, Pharmacia Fine Chemicals, Uppsala, Sweden.

### *B.2. Methods*

#### a. *Cell culture*

Bovine chondrocytes were isolated as described by Kuettner et al (1981). Briefly, after enzymatic digestion for 18 hours in the presence of bacterial collagenase (2 mg/ml) and 3% penicillin/streptomycin, chondrocytes were washed in DMEM. Cell viability was determined using trypam blue exclusion. Chondrocytes were seeded at high density (5 x $10^5$ cells/ml), into 24 well tissue culture plates (1 ml/well) and cultured in DMEM containing 10% heat-inactivated FCS, 1% L-glutamine and 1% penicillin/streptomycin. Chondrocytes which are cultured under these conditions maintain their differentiated phenotype, produce type II collagen (as assessed by SDS PAGE) and continue to produce keratan sulphate as measured by ELISA (data not shown).

#### b. *Labelling of proteoglycans in chondrocyte culture*

Chondrocytes were found to be confluent 5-7 days after inoculation. The medium containing DMEM supplemented with FCS was removed and the cell layers were washed twice with PBS to remove any traces of FCS. Chondrocytes were then exposed to 5 μCi/ml of $Na_2{}^{35}SO_4$ for 18 hours in the presence of ($10^{-8}$—$10^{-4}$ M) SP or serum-free medium control which contained the neuropeptide diluent. In this assay 10 % FCS yielded optimal stimulation of proteoglycan production, as measured by CPC precipitation and was therefore used as a 'positive control'. At the end of the experiments, culture supernatants were collected, rendered cell-free by centrifugation and stored at -$20^0$ C until assayed.

c. ***Cetylpyridinium chloride assay of glycosaminoglycan in cell supernatants and cell layers***

Glycosaminoglycan production (including release into the culture supernatant and incorporation into cell layers) was measured using a modification of the method of Marsh et al (1979). After incubation with medium or SP, culture supernatants were recovered and the cell layer was washed three times with 1 ml PBS. Hyaluronan was added to the supernatants (as a carrier molecule) and also to the PBS washings of the cell layer. An equivalent volume of 1% CPC, 0.1M NaCl was added and allowed to precipitate for 3 hours at $37^0$ C. The resultant precipitate was spun for 10 minutes at 1, 300 g at room temperature. Following centrifugation, the pellet was washed twice with 1% CPC. Pellets were then suspended in scintillation fluid (Readysafe, Beckman Instruments) and counted on a Beckman LS6000LL scintillation counter. The cell layer was solubilized with 1 ml 0.2 M NaOH for 2 hours at $37^0$ C prior to CPC assay.

d. ***Measurement of total protein production***

Total protein production was measured by incubation of cultured chondrocytes with 2 μCi/ml $^3$H-L Leucine for 24 hours in the presence or absence of SP. Protein present in the cell supernatants and cell layers was determined by trichloroacetic acid (TCA) precipitation. The cell layer was recovered by trypsinization and cells were disrupted by sonication in the presence of 0.2% Triton X-100. 0.2 volumes of 50% TCA were added to the supernatants or cell sonicates and incubated for 30 minutes at $4^0$ C. Following centrifugation at 1, 300 g for 5 minutes the resultant pellet was washed twice in 10% TCA before resuspension in a small volume of 0.1M NaOH before scintillation counting.

**e.** ***Determination of proteoglycan monomer size by Sepharose CL-4B chromatography***

Confluent cultures of bovine chondrocytes were radiolabelled for 18 hours with

20 μCi/ml $Na_2{}^{35}SO_4$ in the presence or absence of $10^{-6}$ M SP. After radiolabelling, supernatants were pooled and kept at $-20^0$ C until chromatographic analysis was performed. Samples (1 ml) were eluted under dissociative conditions with 4 M GuHCl, 0.5 M Na acetate, pH 5.8 on a Sepharose CL-4B column (1.6 X 27 cm; 58.3 $cm^3$), and 1.5 ml fractions were collected.

Cell layer-associated proteoglycans were extracted for 24 hours at $4^0$ C with 4 M GuHCl in the presence of 0.05 M $Na_2EDTA$, 5 mM benzamidine HCl, 0.5 mM PMSF,

0.1 M 6-amino hexanoic acid and SBTI to inhibit endogenous proteases. The resultant extract was pooled and fractionated as per culture supernatants.

**f.** ***Statistics***

Data are expressed as means ± S.E.M., n = number of experiments. All experiments were performed in triplicates. Student's t-test was used to test for significant differences between means.

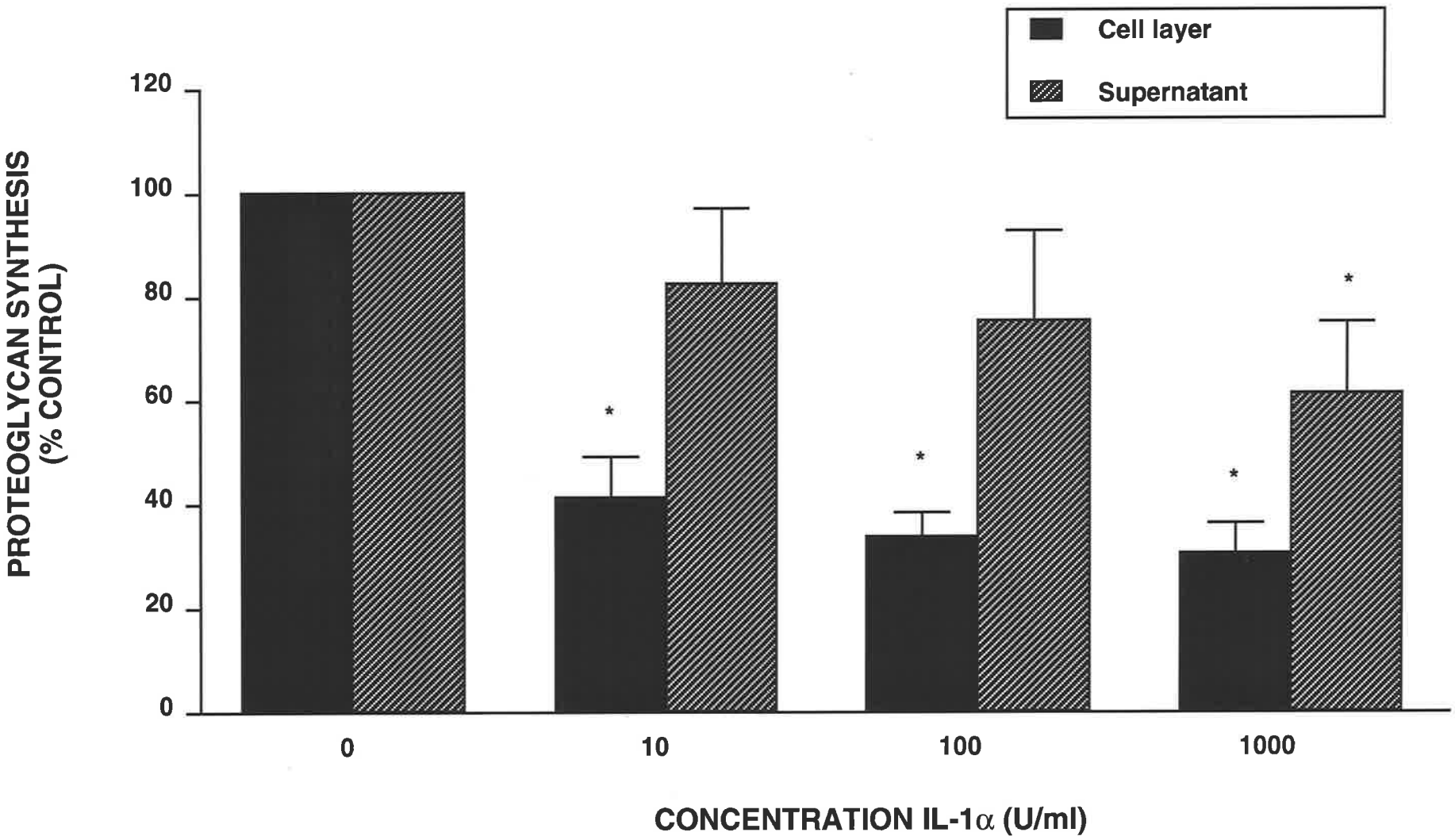
## C. RESULTS

**c.1.** ***Effect of hr IL-1α on chondrocyte proteoglycan production***

The inhibitory effect of IL-1α on chondrocyte proteoglycan production was confirmed. Bovine chondrocytes were incubated for 18 hours in serum-free medium with hr IL-1α or its diluent control. Figure 2.1 shows that hr IL-1α significantly suppressed the synthesis of sulphated proteoglycans, measured as amounts released into

**Figure 2.1. The effect of hr IL-1α on proteoglycan production by bovine articular chondrocytes *in vitro*.**

Monolayers of confluent chondrocytes in serum-free DMEM were exposed to hr IL-1α for 18 hours in the presence of 5 μCi/ml $Na_2\,^{35}SO_4$. Proteoglycans released into supernatants (hatched bars) or cell layers (solid bars) were extracted with CPC, as outlined in the methods section. Data are means ± S.E.M. expressed as a percent of medium control of four separate experiments. (*) indicates significant ($p < 0.05$) suppression of proteoglycan production. Control counts were: supernatant, 12, 005 ± 1, 983 dpm; cell layer, 202 ± 21 dpm.


Cell layer
Supernatant
PROTEOGLYCAN SYNTHESIS
(% CONTROL)
120
100
80
60
40
20
0
*
*
*
*
0
10
100
1000
CONCENTRATION IL-1α (U/ml)

culture supernatants or incorporated in the cell layer. This inhibition was most evident for the cell layer. With 10 units of IL-1α, cell-layer absolute counts decreased from control values of 201 ± 21 dpm to 106 ± 12 dpm (n = 4, $p < 0.05$).

### c.2. *Effect of tachykinins on chondrocyte proteoglycan production*

Table 2.1 shows the effect of SP, NKA and NKB on proteoglycan production in both supernatants and cell layers. Tachykinins failed to influence proteoglycan synthesis significantly in either the cell layer or supernatants. Table 2.2 shows the lack of effect of any of the SP fragments, SP-(1-4), SP-(1-6) and SP-(7-11), on proteoglycan production. In these experiments 10% FCS was used as a positive control to stimulate proteoglycan production (data not shown in table).

### *c.3. Effect of Substance P on total protein production*

SP did not stimulate total protein production, as measured both in the supernatants or the cell layers (Fig. 2.2). This is in contrast to the effect of 10 % FCS where total protein production increased to 250 % of control cells. N- and C-terminal fragments were not tested for total protein production in view of the results from the previous experiments on proteoglycan production.

### *c.4. Effect of Substance P on proteoglycan molecular weight profiles*

Molecular sieve chromatography of $^{35}$S-sulphate - labelled material, extracted from the cell layers of bovine chondrocytes is shown in figure 2.3. Virtually all of the $^{35}$S-containing material present in the cell layer is extracted under these conditions. Elution profiles of proteoglycans produced by cells exposed to SP or medium were identical and all high molecular weight material in control and SP-exposed chondrocytes, elutes in the void volume. This indicates that there is no significant production of smaller molecular weight proteoglycan monomer by the cell layer. Fractionation profiles for proteoglycans in supernatants are also identical for medium and SP-exposed chondrocytes (figure 2.4) indicating that a smaller molecular weight

**Table 2.1. The effect of tachykinins on proteoglycan production by bovine chondrocytes.**

Chondrocyte monolayers were exposed to SP, NKA and NKB for 18 hours in serum-free medium. Data are means ± S.E.M. from 3-6 experiments performed in triplicate, expressed as percent of control. Control values were: Supernatant 11, 850 ± 670 dpm, Cell layer 1, 370 ± 110.6 dpm.

| TACHYKININ (M) | PROTEOGLYCAN PRODUCTION | | | |
|---|---|---|---|---|
| | SUPERNATANT | | CELL LAYER | |
| Substance P | Mean ± | SEM (% control) | Mean ± | SEM (% control) |
| $10^{-8}$ | 101.7 | 11.7 | 89.1 | 7.9 |
| $10^{-6}$ | 104.9 | 9.3 | 101.5 | 9.5 |
| $10^{-4}$ | 86.3 | 7.4 | 85.6 | 7.1 |
| Neurokinin A | | | | |
| $10^{-8}$ | 98.4 | 9.4 | 112.8 | 7.6 |
| $10^{-6}$ | 112.4 | 11.3 | 88.0 | 11.2 |
| $10^{-4}$ | 98.9 | 7.1 | 95.2 | 9.3 |
| Neurokinin B | | | | |
| $10^{-8}$ | 80.8 | 9.6 | 117 | 32.5 |
| $10^{-6}$ | 84 | 12 | 121.3 | 11.2 |
| $10^{-4}$ | 84.1 | 7.6 | 121.4 | 11.2 |

* None of the values in this table were significantly different from medium control.

**Table 2.2. The effect of SP fragments on proteoglycan production by bovine chondrocytes.**

Conditions as in table I. Data are means ± S.E.M. expressed as percent of control values from 3-6 experiments performed in triplicate. Control values were: Supernatant, 12, 260 ± 2170 dpm, Cell layer 1, 417 ± 110 dpm.

| FRAGMENT (M) | PROTEOGLYCAN PRODUCTION | | | |
|---|---|---|---|---|
| | SUPERNATANT | | CELL LAYER | |
| SP-(1-4) | Mean | ± SEM (% control) | Mean | ± SEM (% control) |
| $10^{-8}$ | 121.2 | 19.3 | 112.5 | 15.3 |
| $10^{-6}$ | 108.3 | 15.9 | 86.2 | 10.6 |
| $10^{-4}$ | 86.7 | 14.7 | 82.5 | 12.4 |
| SP-(1-6) | | | | |
| $10^{-8}$ | 120.4 | 19.3 | 110.3 | 13 |
| $10^{-6}$ | 114.5 | 16.9 | 91.4 | 10.2 |
| $10^{-4}$ | 89.7 | 14.7 | 84.5 | 2.4 |
| SP-(7-11) | | | | |
| $10^{-8}$ | 100.3 | 8.9 | 98.6 | 8.1 |
| $10^{-6}$ | 104.3 | 8.5 | 99.7 | 9.5 |
| $10^{-4}$ | 89.1 | 10.1 | 117.0 | 18.2 |

* None of the values in this table were significantly different from medium control.

**Figure 2.2. The effect of SP on protein production by bovine chondrocytes *in vitro*.**

Monolayers of confluent chondrocytes were cultured for 18 hours in serum-free DMEM containing 5μ Ci/ml L-[$^3$H] leucine and SP, 10 % FCS or medium control. Cell layer and supernatant protein synthesis was measured by TCA precipitation, as outlined in the methods section. Data are means ± S.E.M. of four experiments performed in triplicate and are expressed as a percent of control values (medium in absence of SP or FCS). Control counts were: supernatant 1, 021 ± 305 dpm; cell layer 1, 709 ± 133 dpm.

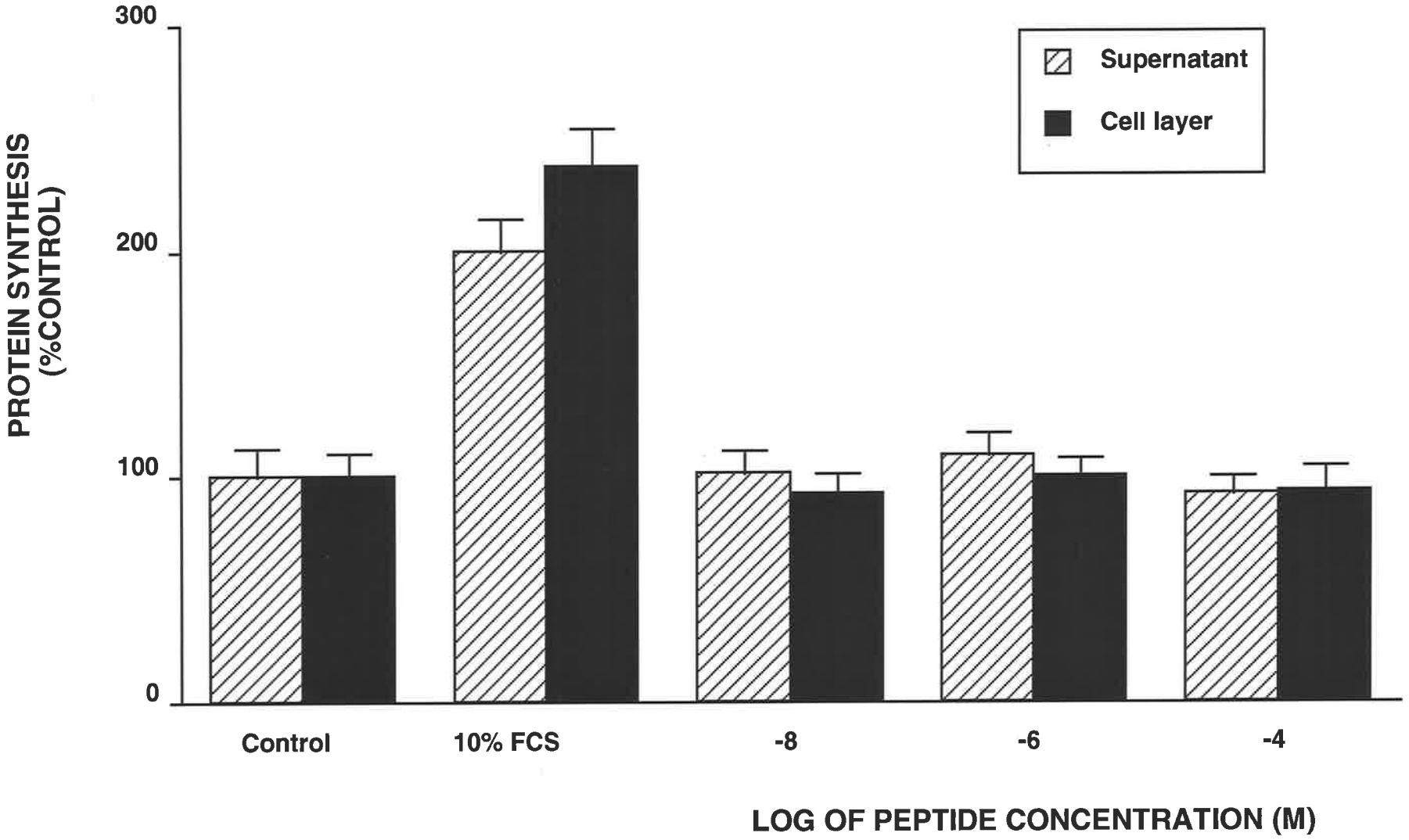

300
200
100
0
PROTEIN SYNTHESIS
(%CONTROL)
Supernatant
Cell layer
Control
10% FCS
-8
-6
-4
LOG OF PEPTIDE CONCENTRATION (M)

**Figure 2.3. Sepharose CL-4B elution profile of proteoglycans extracted from the cell layer of bovine chondrocytes following exposure to SP.**

$Na_2\ ^{35}SO_4$ (20 μCi/ml) labelled proteoglycans present in the cell layer of bovine chondrocytes which were exposed to medium (squares) or $10^{-6}$ M SP (circles) for 18 hours. Labelled proteoglycans were extracted with 4 M GuHCl, as outlined in the methods section. 1 ml of the pooled extract was loaded and fractionation was carried out over 24 hours at $4^0$ C. The elution buffer was 4 M GuHCl, 0.5 M sodium acetate, pH 5.8 containing protease inhibitors. column dimensions: 1.6 x 27 cm; 58.3 $cm^3$; 1.0 ml fractions were collected and radioactivity counted. $V_o$ = fraction 17, $V_t$ = fraction 35. Plots are representative of three separate fractionations.

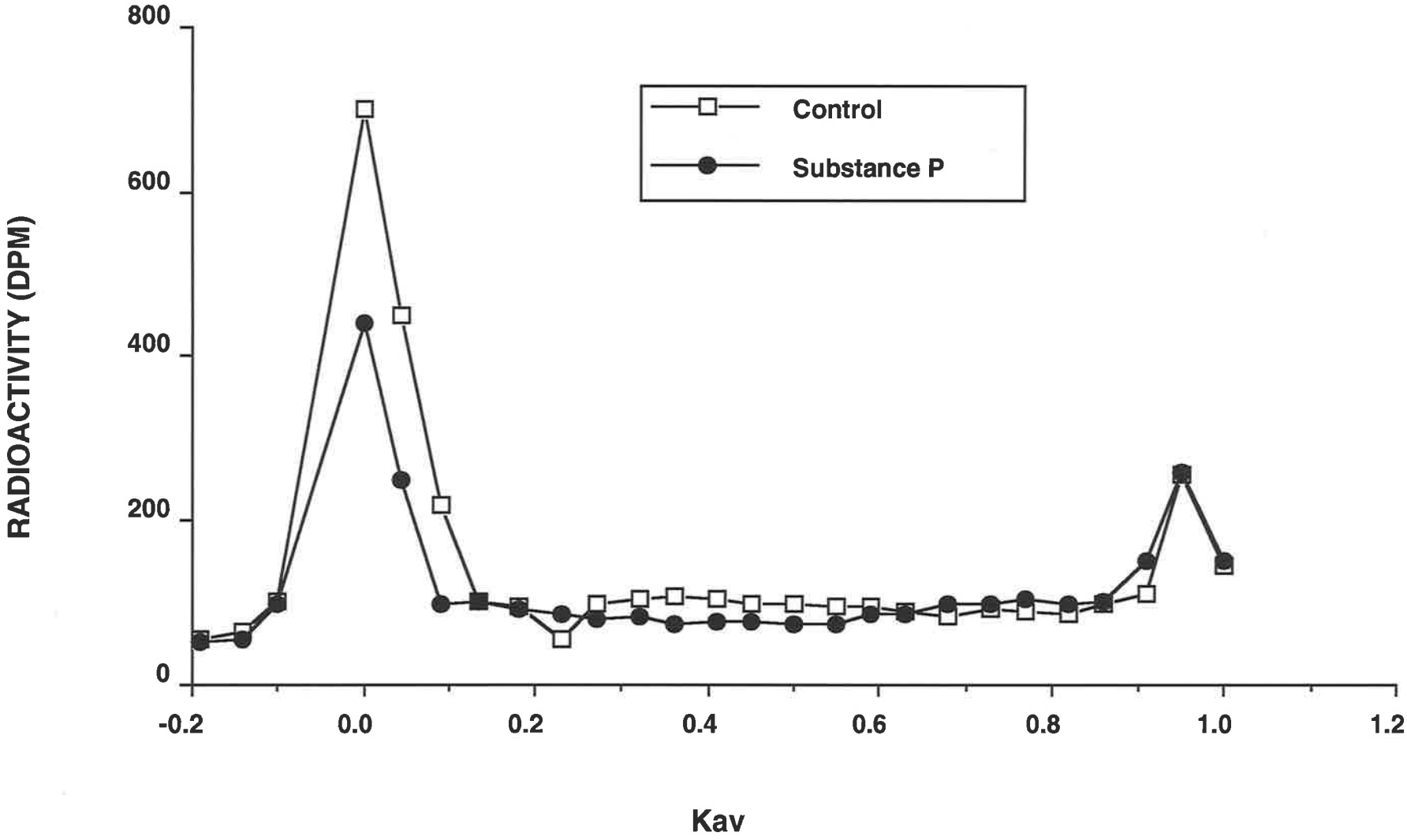
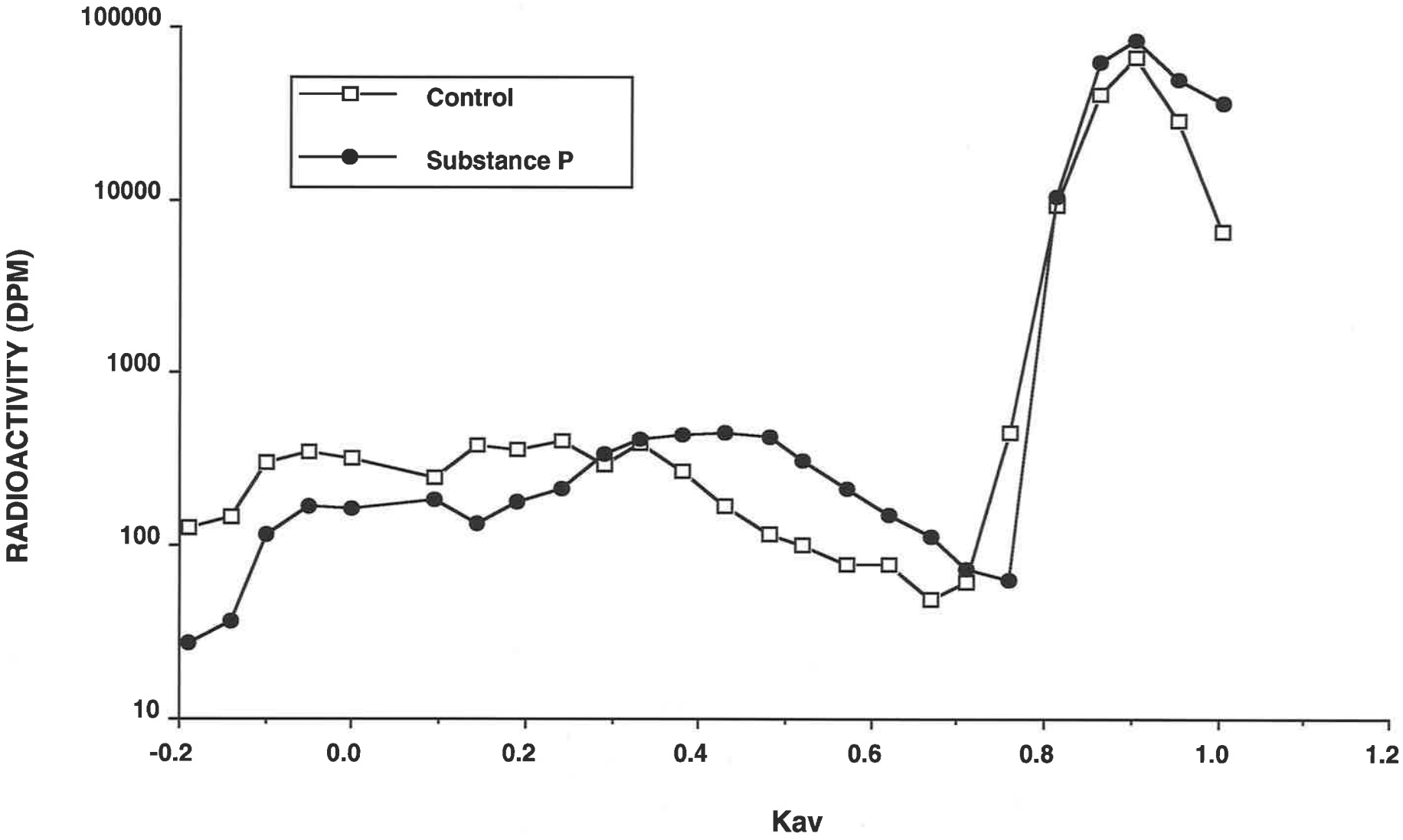

800
600
400
200
0
RADIOACTIVITY (DPM)
Control
Substance P
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Kav

**Figure 2.4. Sepharose CL-4B elution profile of proteoglycans present in bovine chondrocyte supernatants following exposure to SP.**

Supernatants were taken from chondrocyte cultures which had been exposed to $10^{-6}$ M SP for 24 hours. Supernatants were pooled and eluted using 4 M GuHCl, 0.5 M sodium acetate, pH 5.8 at $4^0$ C containing the same protease inhibitors as cell layer extracted proteoglycans. The column dimensions for these experiments were the same as for fractionation of cell layer-extracted proteoglycans. This profile is representative of several different experiments.


100000
10000
1000
100
10
RADIOACTIVITY (DPM)
Control
Substance P
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Kav

monomer was not secreted into the supernatant following exposure of chondrocytes to SP.

## D. DISCUSSION

The initial experiments in this study were directed towards confirming an already characterised response of chondrocytes to a biologically active molecule. Proteoglycan synthesis is known to be suppressed by catabolin, now known as IL-1$\alpha$ (Tyler 1985). This was reconfirmed using ($^{35}SO_4$) labelled glycosaminoglycan and subsequent CPC precipitation assay. Following this initial study the effects of tachykinins on chondrocyte function were assessed. Mammalian tachykinins and the C- and N-terminal fragments of SP were found not to influence proteoglycan production, either secreted proteoglycan or proteoglycan incorporated into the cell layer. Furthermore total protein production was not altered in response to SP. Foetal calf serum was found to be a potent inducer of both total protein and proteoglycan production, confirming previous reports (Mc Quillan et al 1986a; Mc Quillan et al 1986b). This applied to proteoglycans secreted into the medium as well as that fraction of the proteoglycans retained within the cell layer. In addition there was no alteration in the size of the proteoglycan species retained within the cell layer insofar as it remained excluded by Sepharose 4B-CL.

These findings in themselves do not preclude an alteration in the glycosaminoglycan content of the proteoglycans synthesized., However further studies indicated that this was unlikely. If, for example, SP induced a significantly greater keratan sulphate content of the proteoglycan monomer, at the expense of chondroitin sulphate, then an overall decrease in proteoglycan monomer molecular size would have been expected and this was not found.

These studies do not necessarily preclude a role for SP in modulating any chondrocyte function. In these experiments the ability of SP to influence proteoglycan

production directly was examined, but not to prime chondrocytes and modulate synthesis in response to a second stimulus. For example Wozniak et al (1989 and 1993) have shown that while SP has little effect on peripheral blood neutrophil superoxide anion production, it dramatically enhanced the response to a second stimulus such as *formyl* -methionyl-leucyl-phenylalanine and platelet activating factor. It is possible that tachykinins and SP fragments may directly stimulate the release of metalloproteinases, e.g. stromelysin, from synoviocytes and/or chondrocytes. No published evidence for direct stimulation by SP of chondrocyte metalloproteinase synthesis exists.

## E. SUMMARY

These experiments demonstrated that SP, NKA, NKB and the fragments of SP, SP-(1-4), SP-(1-6) and SP-(7-11) had no direct effect on bovine articular chondrocyte proteoglycan production. Furthermore SP had no effect on total protein production.

# CHAPTER III

# MODULATION OF PROSTAGLANDIN $E_2$ PRODUCTION BY THE TACHYKININ FRAGMENT SP-(7-11) IN BOVINE ARTICULAR CHONDROCYTES BUT NOT SYNOVIOCYTES.

# A. INTRODUCTION

Prostaglandins are synthesized in response to cell injury, whether the nature of the injury is mechanical, chemical, immunological or microbial. The appearance of prostaglandins in tissues is an index of cell damage and prostaglandins play an important role in the pathogenesis of the inflammation seen in inflammatory joint diseases. The prostaglandins are 20 carbon fatty acid derivatives characterized by a five membered ring and they are generated by the oxidation of polyunsaturated fatty acids, in particular arachidonic acid. The release of arachidonic acid from cell membranes can be stimulated by a number of factors including immunological stimuli (Dayer et al 1985), calcium ionophores (Lapetina and Cuatrecasas 1979) and tumour promoting agents ( Ohuchi and Levine 1978). Following this initial release, arachidonic acid is oxygenated by cycloxygenase (the first enzyme in the prostaglandin cascade) to produce two unstable endoperoxide intermediates, $PGG_2$ and $PGH_2$. The structural requirements for the conversion of fatty acids into prostaglandin precursors have been determined by Nugteren et al (1967). Cis-double bonds are required at positions 8,11 and 14 and prostaglandins must possess a chain length of 20 carbon atoms. 5,8,11,14 eicosatetraenoic acid is the most plentiful of these precursor molecules and leads to the synthesis of prostaglandins of the dienoic series of which prostaglandin $E_2$ ($PGE_2$) is a member (Morrison and Needleman 1979). $PGH_2$ is further isomerized to form two main products, $PGE_2$ and $PGF_{2\alpha}$. $PGF_{2\alpha}$ is formed by the action of 9-ketoreductase on $PGE_2$. It appears that *de novo* synthesis of prostaglandins must precede their release and biological action since there is little evidence to suggest that prostaglandins are stored intracellularly (Kunze and Vogt 1971). Prostaglandins are generally labile in most tissues, and $PGE_2$ is rapidly (<30 seconds) hydrolysed to inactive metabolites in the circulation (Samuelsson et al 1978).

Several lines of evidence suggest that the prostaglandins, primarily $PGE_1$ and $PGE_2$ may act as mediators of inflammation (Vane 1976). Firstly , prostaglandins cause inflammation when injected subacutely, they are vasodilators and they potentiate the

formation of edema induced by other agents, for example they also sensitize tissue to pain stimulation. Secondly $PGE_1$ and $PGE_2$ have been found in inflammatory exudates ( Higgs and Salmon 1979) and thirdly, their biosynthesis is strongly inhibited by drugs which have anti-inflammatory action .

The first non-steroidal anti-inflammatory drug found to inhibit prostaglandin synthesis was aspirin. Aspirin inhibits cyclo-oxygenase, the first enzyme in the synthetic pathway for $PGE_2$ (Roth et al 1975; Roth and Majerus 1975). Other non-steroidal anti-inflammatory drugs have been shown to act via this pathway, in particular indomethacin (Rome and Lands 1975).

Prostaglandins are important inflammatory mediators in arthritis and there is ample evidence for the stimulation of their synthesis in chondrocytes and synoviocytes by cytokines (Chin and Lin 1988; Gilman 1987; Meyer et al 1990; Dayer et al 1986; Kumkumian et al 1989).

Tachykinins represent a novel class of peptides which potentially can modulate prostaglandin production in connective tissue cells of the joint and this possibility has not been fully studied. Studies in human rheumatoid synoviocytes by Lotz et al (1987) demonstrated that SP stimulated the production of $PGE_2$ *in vitro*. These experiments only investigated the effect of SP but not SP fragments which are likely to be immediately generated when SP comes into contact with synoviocyte membranes (Bathon et al 1992).

For these reasons tachykinins and SP metabolites have been studied for their ability to induce synthesis of a major prostaglandin pro-inflammatory mediator in joint disease, namely $PGE_2$.

The possibility that both chondrocytes and synoviocytes respond to tachykinins and their metabolites by synthesis of $PGE_2$ was studied.

The aims of these experiments were to:-

1. Determine whether tachykinins and their metabolites influence $PGE_2$ production in bovine chondrocytes.

2. Investigate the possibility that pre-exposure to IL-1α modulates the effect of SP or SP-(7-11) on $PGE_2$ synthesis.
3. Determine whether synoviocytes alter their $PGE_2$ secretion in response to tachykinins and their metabolites.
4. Determine whether the NK-1 receptor antagonist CP-96,345 can block the effect of SP-(7-11) on $PGE_2$ production by bovine chondrocytes.

# B. MATERIALS AND METHODS

## *B.1. Materials*

Rabbit anti-$PGE_2$ polyclonal antibody was purchased from Sigma (St. Louis, Mo, USA). Radiolabelled [5,6,8,11,12,14,15 (n)-$^3$H]-$PGE_2$ (5.2-6.3 TBq/mmol) was purchased from Amersham International (Amersham UK). Prostaglandin $E_2$ standard was purchased from Caymen Chemicals ( Ann Arbor, Michigan, USA). Dextran T70 was from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals used in the $PGE_2$ assay were of analytical grade and purchased from either Sigma or Ajax Chemicals (Sydney, Australia). Human recombinant IL-1α was from the same source as previously mentioned. Tissue culture plates (Falcon tissue culture ware) , media and FCS were purchased from Cytosystems (Castle Hill, New South Wales, Australia). CP-96,345 was a gift from Dr. Saul B. Kadin, Central Research Division, Pfizer Inc. Groton, CT, USA.

## B.2. Methods

### *a. Cell culture*

Chondrocytes were isolated as described previously and cultured until confluent in DMEM containing 10% FCS. When chondrocytes were confluent, medium was removed and the cell layer was washed twice with PBS. Chondrocytes were then incubated with the neuropeptide under study in serum-free medium (500 µl) for 18

hours. Human recombinant IL-1α (100 U/ml) was used as a 'positive' control for $PGE_2$ production. Supernatants were collected and stored at $-20^0$ C until assayed for $PGE_2$.

Human and bovine synoviocytes were grown from explants obtained from synovial joints of patients undergoing joint replacements or from beef hocks, at the time of cartilage collection. Synovium was collected and diced into 1 $mm^2$ pieces and maintained in a small volume of tissue culture medium in 25 $cm^3$ tissue culture flasks. The medium contained DMEM, 20 mM HEPES buffer, 10% heat inactivated FCS, 1% penicillin/streptomycin, 1% L-glutamine and 1% fungizone. Confluent synoviocytes were established more rapidly from bovine than human synovium explants. Synoviocytes were passaged into larger 50 $cm^3$ tissue culture flasks following removal of FCS by washing with PBS and recovery of cells from the plastic substrate with trypsin. Synoviocytes were seeded into 24 well tissue culture plates at a density of ($10^5$ cells/well). Cells were usually confluent within 48 hours. Experimental cultures were exposed to neuropeptides for 24 hours in serum-free medium 250 µl. At the completion of each experiment, supernatants were collected and stored at - $20^0$ C until assayed for $PGE_2$.

### *b. Prostaglandin $E_2$ assay*

Standard curves were established for each assay between $PGE_2$ concentrations of 10 pg-10 ng. Standard $PGE_2$ was diluted in 1 mM $Na_2CO_3$, pH 10. Prostaglandins present in either chondrocyte or synoviocyte supernatants were assayed using a modification of the radioimmunoassay (RIA) described by Jarre and Behrman (1974). Aliquots (100 µl) of sample were added to the RIA assay buffer which consisted of 0.1% gelatin, 0.9% NaCl, 0.01 M tris base and 0.05% $NaN_3$, pH 7.3. To this mixture 100 µl of $^3$H-$PGE_2$ suspended in 1mM $Na_2CO_3$ was added (stock solution contained 20µl $^3$H-$PGE_2$ (7.07 TBq/mmol) in 10 ml of 1 mM $Na_2CO_3$). Anti-$PGE_2$ antibody (100 µl) was added to the reaction mixture (stock solution contained 1 vial anti-$PGE_2$ reconstituted with 10 ml RIA buffer). This mixture was then incubated at $37^0$ C for 2 hours. After incubation samples were cooled to $4^0$ C for 1 hour. To separate bound

from unbound $PGE_2$, 500 µl of a solution containing 1% activated charcoal, 1% Dextran T70, 0.05% $NaN_3$ was added to each sample and mixed. Samples were then centrifuged at $4^0$ C in a pre-cooled centrifuge for 20 minutes at 2, 000 g. Aliquots (500 µl) of supernatant were carefully removed and radioactivity was determined with a Beckman LS-6000 LL scintilation counter. Standard $PGE_2$ was assayed under the same conditions. Standard curves were plotted on a logarithmic scale and quantities of $PGE_2$ in samples were determined from the standard curve.

### *c. Statistics*

Differences between groups were determined by using Student's *t*-test. Data represent means ± S.E.M. of between 3-5 separate experiments.

## C. RESULTS

### *c.1. Prostaglandin $E_2$ production in bovine chondrocytes is increased by SP-(7-11)*

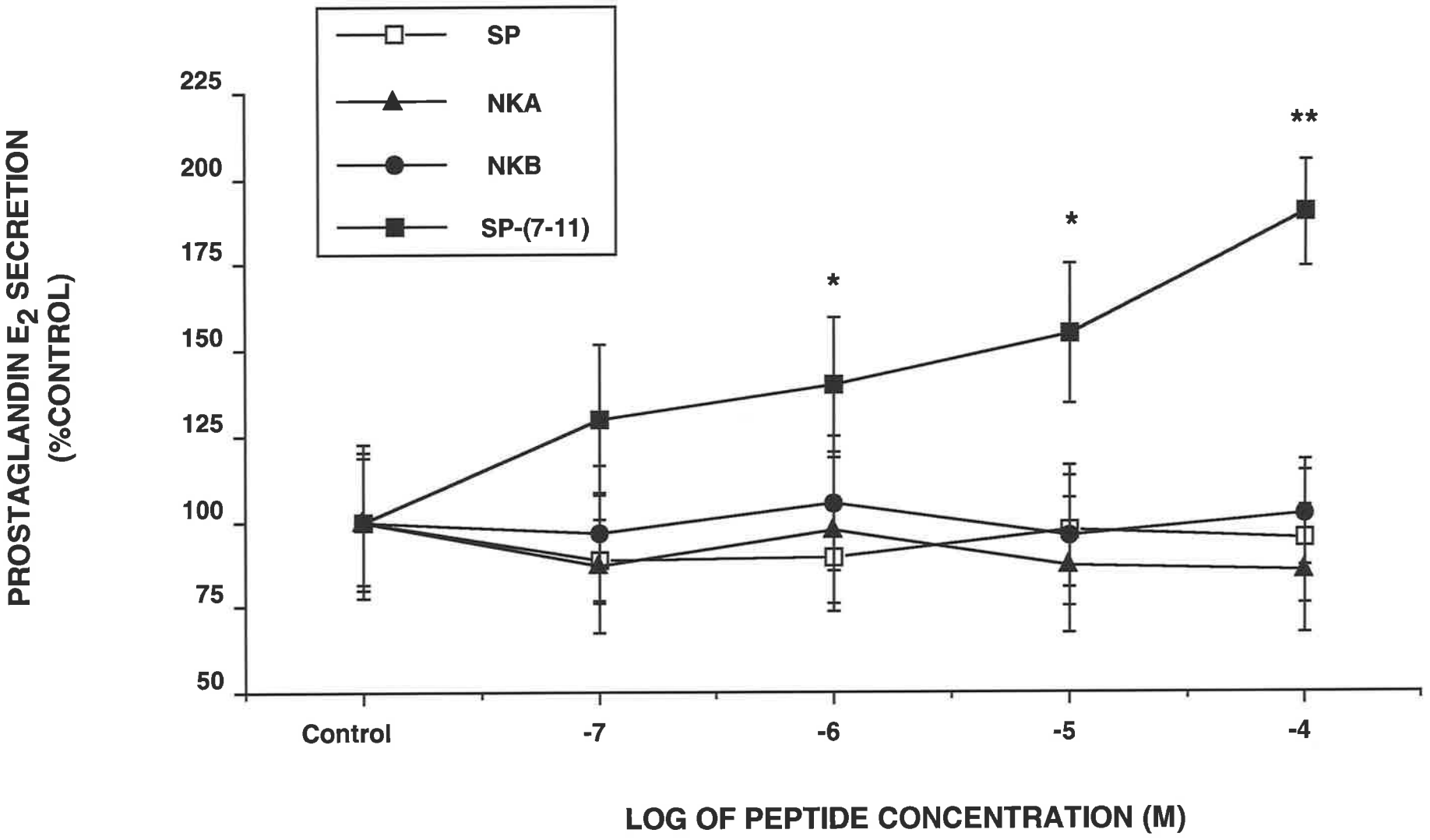
Confluent chondrocytes were incubated in serum-free medium containing $10^{-7}$—$10^{-4}$ M neuropeptide for 24 hours. Incubation with hr IL-1α (100 U/ml) was used as a positive control since is has been established previously that hr IL-1α stimulates $PGE_2$ synthesis in chondrocytes. Typically hr IL-1α at this concentration stimulated $PGE_2$ production 2-3 fold higher than control levels.

The C-terminal fragment of SP, SP-(7-11) significantly increased $PGE_2$ secreted into the medium, at concentrations greater than $10^{-6}$ M (Figure 3.1; $p < 0.05$ compared to control). The maximum effect was noted at $10^{-4}$ M SP-(7-11) when $PGE_2$ secretion increased from control levels of 16.9 ± 3.2 to 34.8 ± 4.2 ng/ml ($p < 0.01$; n = 4). In contrast SP, NKA and NKB had no effect on $PGE_2$ secretion.

Spontaneous $PGE_2$ secretion in bovine chondrocytes was blocked by 93% in the presence of the cycloxygenase inhibitor, indomethacin (15 µM and 30 µM) and the effect of ($5 \times 10^{-5}$ M) SP-(7-11) was inhibited completely in the presence of 15 µM

**Figure 3.1. Effect of SP, NKA, NKB and the SP fragment SP-(7-11) on bovine chondrocyte $PGE_2$ synthesis.**

Confluent chondrocytes were incubated with neuropeptide ($10^{-7}$—$10^{-4}$ M) for 18 hours. $PGE_2$ secreted into the medium was determined by radioimmunoassay. Values represent mean ± S.E.M. for four separate experiments, each performed in quadruplicate. *$p < 0.05$ compared with control; **$p < 0.01$ compared with control. Data are expressed as percentage of control value which was 16.9 ± 3.2 ng/ml.


SP
NKA
NKB
SP-(7-11)
225
200
175
150
125
100
75
50
*
*
**
Control
-7
-6
-5
-4
PROSTAGLANDIN E2 SECRETION (%CONTROL)
LOG OF PEPTIDE CONCENTRATION (M)

indomethacin (5 X $10^{-5}$ M, SP-(7-11) alone, 89.7 ± 15 ng/ml ; 15 μM indomethacin alone, 6.2 ± 0.8 ng/ml ; 15 μM indomethacin + 5 X $10^{-5}$ M SP-(7-11), 5.86 ± 0.73 ng/ml (figure 3.2).

To determine whether extracellular calcium was important in mediating the effect of SP-(7-11), chondrocytes were incubated with EGTA and $PGE_2$ secretion was assessed. Spontaneous $PGE_2$ secretion by chondrocytes was uneffected by incubating chondrocytes with either 100 μM or 2 mM EGTA. However when chondrocytes were exposed to SP-(7-11) in the presence of 100 μM EGTA for 24 hours, $PGE_2$ production was reduced, (medium alone 17.5 ± 2.08 ng/ml ; 100 μM EGTA + SP-(7-11), 16.3 ± 4.08 ng/ml, $p < 0.05$; n = 4) (figure 3.3).

To determine the length of the carboxyl-terminal fragment necessary to stimulate $PGE_2$, the effect of the C-terminal fragments, SP-(8-11) and SP-(9-11) was determined. Neither of these fragments stimulated $PGE_2$ secretion. In a separate series of experiments the effects of the N-terminal fragments SP-(1-4) and SP-(1-6) were compared to SP-(7-11). Only SP-(7-11) increased $PGE_2$ synthesis whereas the N-terminal fragments had no effect (figure 3.4).

### *c.2. The effect of SP-(7-11) is suppressed by pertussis toxin*

Chondrocytes were incubated for 0.5, 1 and 2 hours in serum-free medium in the presence of 1-100 ng/ml pertussis toxin. Following preincubation, medium containing pertussis toxin was removed and monolayers were washed with PBS. Chondrocytes were then cultured in the presence of $10^{-5}$ M SP-(7-11) for a further 24 hours. Figure 3.5 shows that peincubation with pertussis toxin significantly suppressed the stimulation of $PGE_2$ secretion by SP-(7-11) at all concentrations of pertussis toxin tested ($p < 0.01$ n = 4). However the degree of the suppression of $PGE_2$ secretion induced by SP-(7-11) was reduced following longer exposure time to pertussis toxin.

**Figure 3.2. Indomethacin blocks the effect of SP-(7-11) on chondrocyte $PGE_2$ synthesis.**

Chondrocytes were incubated with either 15 μM or 30 μM indomethacin alone or with $5 \times 10^{-5}$ M SP-(7-11). SP-(7-11), ($5 \times 10^{-5}$ M) alone which was used as a positive control. Values represent mean ± S.E.M. of four separate experiments expressed as a percentage of control (spontaneous secretion) value which was 14.3 ± 2.4 ng/ml.

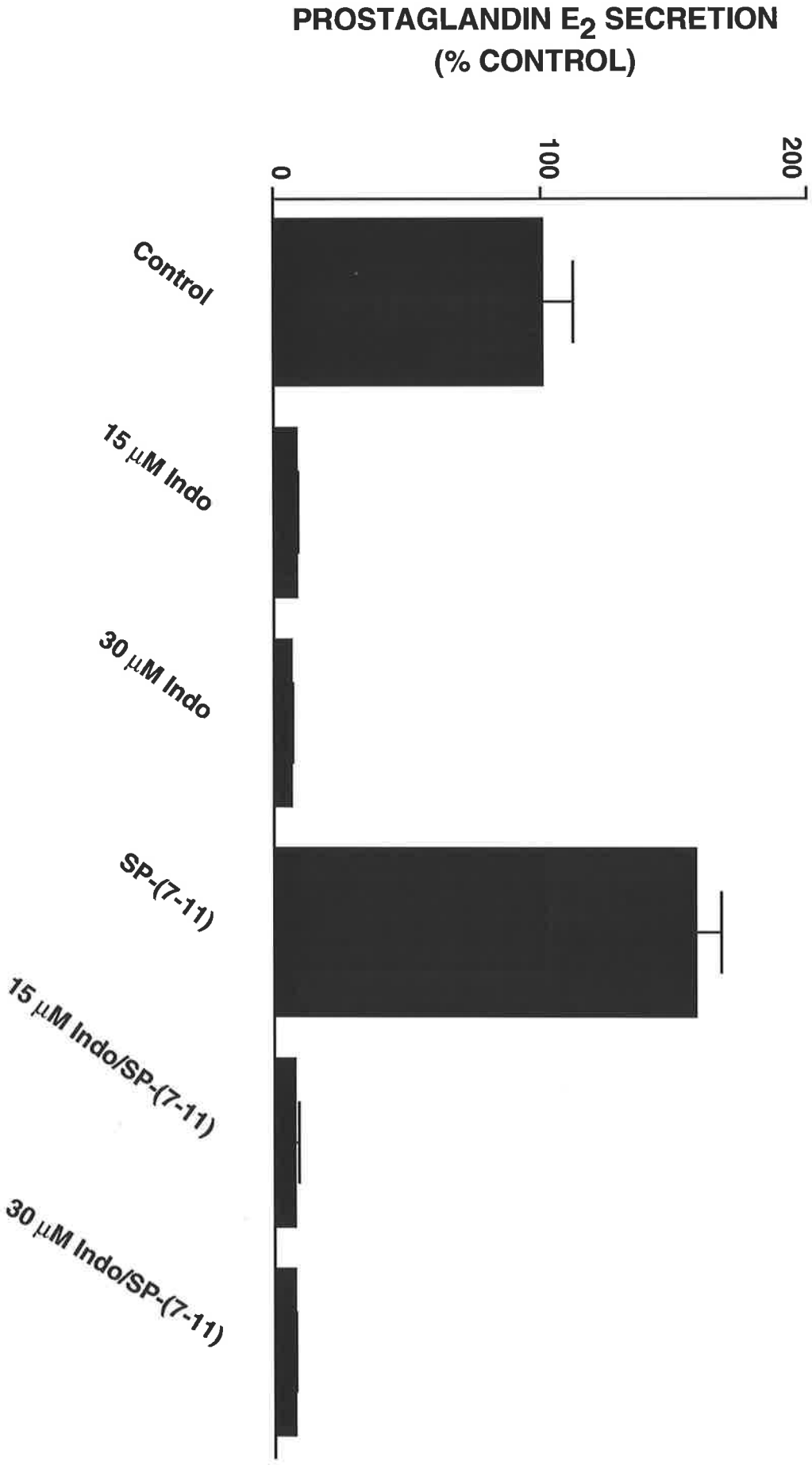

PROSTAGLANDIN $E_2$ SECRETION
(% CONTROL)
0
100
200
Control
15 μM Indo
30 μM Indo
SP-(7-11)
15 μM Indo/SP-(7-11)
30 μM Indo/SP-(7-11)

**Figure 3.3. Extracellular EGTA blocks the effect of SP-(7-11) on chondrocyte $PGE_2$ production.**

Chondrocytes were exposed to EGTA alone (100 μM or 2 mM ), SP-(7-11) alone ($10^{-5}$ M), (positive control) or EGTA (100 μM or 2 mM) + SP-(7-11) ($10^{-5}$ M) for 18 hours. Values represent mean ± S.E.M. of three separate experiments performed in quadruplicate. Data are expressed as percentage of control value (spontaneous secretion) which was 18.2 ± 4.3 ng/ml.

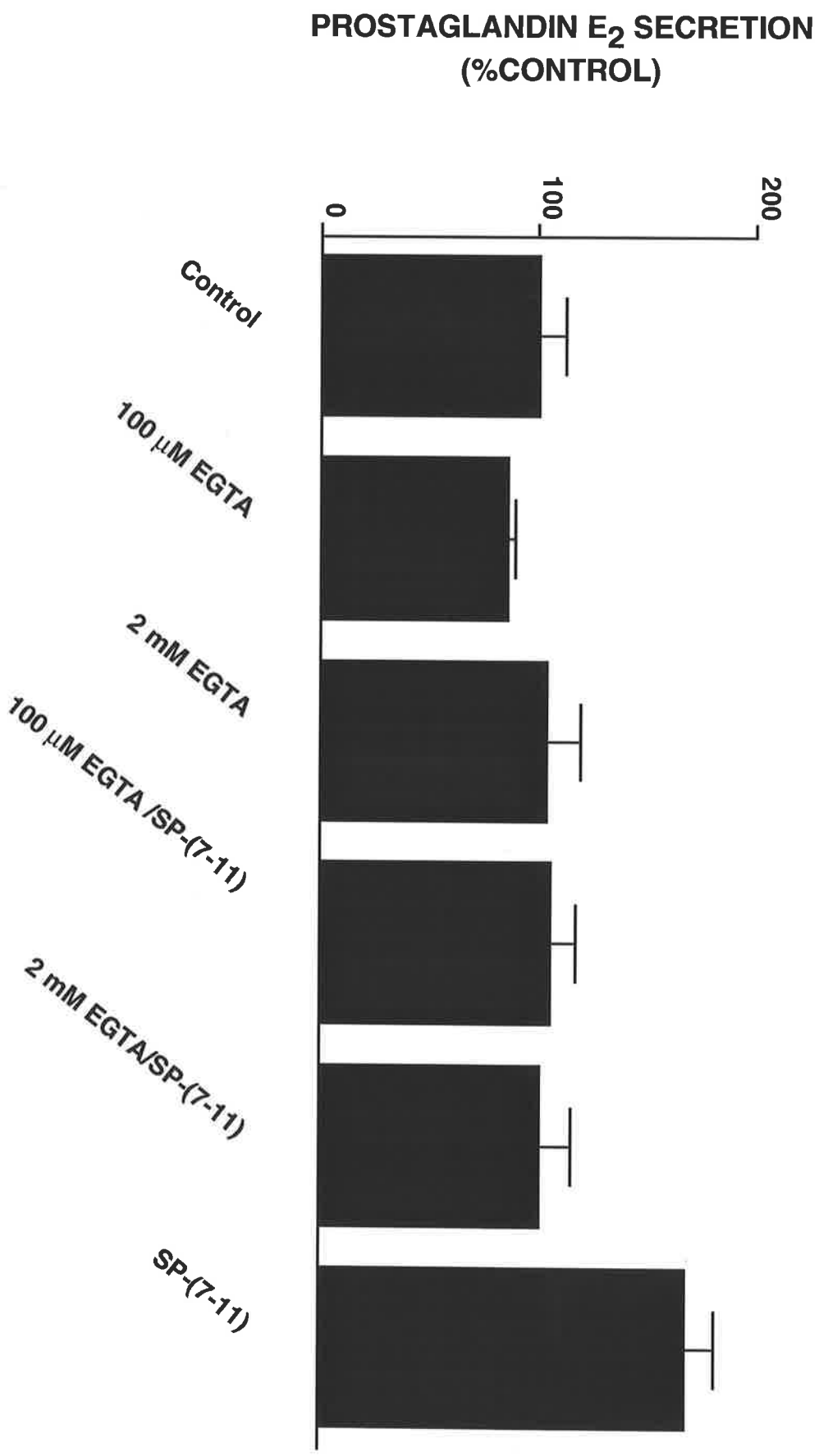

PROSTAGLANDIN $E_2$ SECRETION
(%CONTROL)
0
100
200
Control
100 μM EGTA
2 mM EGTA
100 μM EGTA /SP-(7-11)
2 mM EGTA/SP-(7-11)
SP-(7-11)

**Figure 3.4. Effect of N-terminal and C-terminal SP fragments on $PGE_2$ secretion in bovine chondrocytes.**

Experimental protocol is the same as in figure 3.1. Values represent mean ± S.E.M. for four separate experiments each performed in quadruplicate expressed as % control. Control values were 15.8 ± 3.6 ng/ml (*$p < 0.05$, **$p < 0.01$, compared with control).

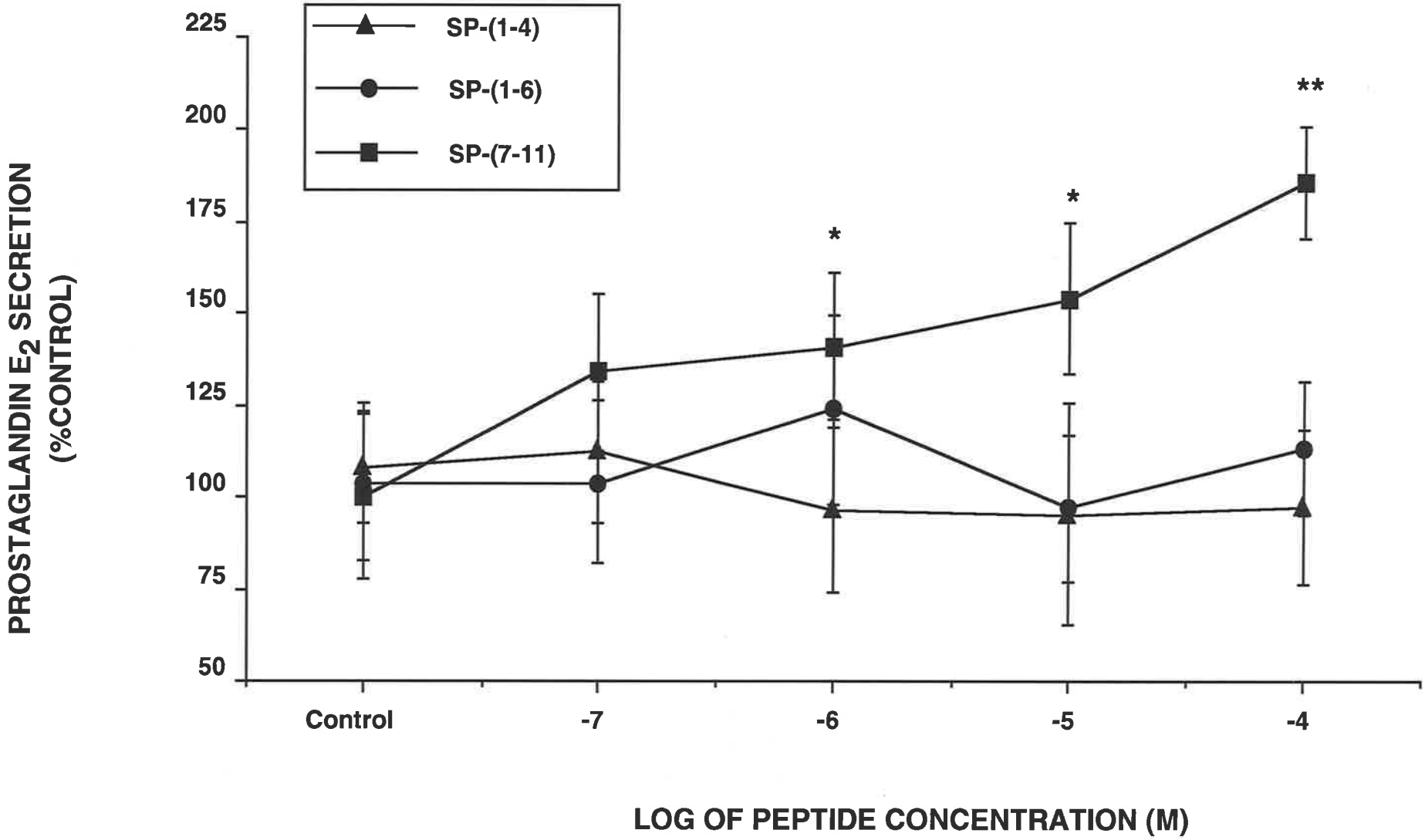
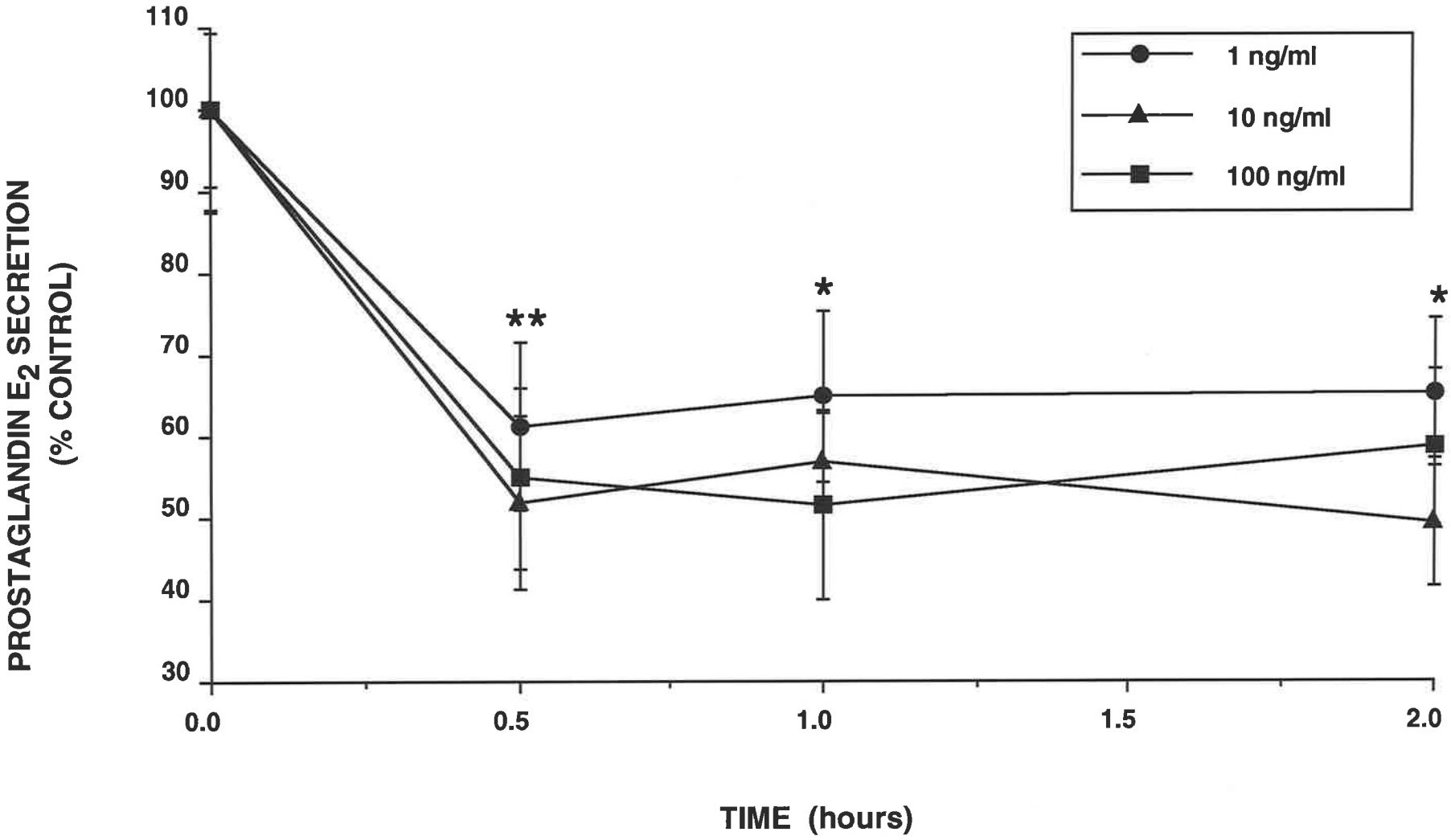

SP-(1-4)
SP-(1-6)
SP-(7-11)
225
200
175
150
125
100
75
50
PROSTAGLANDIN $E_2$ SECRETION
(%CONTROL)
*
*
**
Control
-7
-6
-5
-4
LOG OF PEPTIDE CONCENTRATION (M)

**Figure 3.5. Preincubation with pertussis toxin inhibits the stimulatory effect of SP-(7-11).**

Chondrocytes were incubated with pertussis toxin for (0, 30, 60 and 120 minutes), at concentrations of 1, 10 and 100 ng/ml. Following this incubation with pertussis toxin cell layers were washed with PBS. SP-(7-11) ($10^{-5}$ M) was then added and incubated with chondrocytes for a further 24 hours. Values are expressed as percent control. The control was SP-(7-11) alone without any preincubation with pertussis toxin. Values represent mean ± S.E.M. of four separate experiments (*$p < 0.05$, ** $p < 0.01$).


PROSTAGLANDIN $E_2$ SECRETION
(% CONTROL)
110
100
90
80
70
60
50
40
30
0.0
0.5
1.0
1.5
2.0
TIME (hours)
1 ng/ml
10 ng/ml
100 ng/ml
**
*
*

### *c.3 . The non-peptide NK-1 antagonist CP-96, 3 45 inhibits the production of $PGE_2$ in response to SP-(7-11).*

To determine whether SP-(7-11) mediated its stimulatory effect on $PGE_2$ production via interaction with NK-1 receptors, CP-96,345 was incubated in the presence of SP-(7-11). When SP-(7-11), ($5 \times 10^{-5}$ M) was co-incubated with CP-96,345 at concentrations greater than $10^{-6}$ M, $PGE_2$ synthesis was significantly inhibited ($p < 0.05$). This inhibition was maximal when the CP-96,345 concentration was $10^{-4}$ M. Further experiments demonstrated that CP-96,345 had no effect on spontaneous $PGE_2$ secretion. The receptor specificity was demonstrated by co-incubating 100 U/ml hr IL-1α in the presence of CP-96,345. Prostaglandin production was not inhibited by CP-96,345 in the presence of 100 U/ml hr IL-1α (see figure 3.6)

### *c.4. Does preincubation with hr IL-1α modulate the effect of either SP or SP-(7-11)?*

The failure to detect changes in $PGE_2$ production by chondrocytes following incubation with tachykinins may have been due to the experimental design. For these reasons time-course experiments were performed and the response to tachykinins following preincubation with IL-1α was assessed. Figure 3.7 shows the release of $PGE_2$ by chondrocytes in response to SP ($5 \times 10^{-5}$ M) or medium alone. There was no significant difference in the rate of secretion in SP-treated chondrocytes, again suggesting that SP has no effect on chondrocyte $PGE_2$ production. Chondrocytes which had been pre-exposed to hr IL-1α for 24 hours, washed with PBS and then exposed to SP did not alter their $PGE_2$ production in response to SP (figure 4.8).

Chondrocytes which had been exposed to $10^{-5}$ M SP-(7-11) produced significantly more $PGE_2$ ($p < 0.05$) in the first 30 minutes of incubation (data not shown) and thereafter than control chondrocytes. SP-(7-11) therefore appears to rapidly induce the synthesis of $PGE_2$ in bovine chondrocytes (figure 3.9).

**Figure 3.6. The non-peptide NK-1 antagonist CP-96,345 inhibits the stimulatory effect of SP-(7-11) on bovine chondrocyte $PGE_2$ secretion but does not inhibit the effect of hr IL-1α.**

Chondrocytes were incubated with SP-(7-11) (5 X $10^{-5}$ M), 100 U/ml hr IL-1α or medium alone (diluent control) and titrated against CP-96,345 ($10^{-8}$—$10^{-4}$ M) for 24 hours.

Spontaneous $PGE_2$ secretion was, 32.6 ± 0.12 ng/ml. SP-(7-11), 5 X $10^{-5}$ M alone $PGE_2$ secretion was 80.35 ± 0.28 ng/ml, and 100 U/ml hr IL-1α $PGE_2$ secretion was 130 ± 2.7 ng/ml. CP-96, 345 had no effect on spontaneous $PGE_2$ secretion at any concentration tested. Date are mean of four separate experiments ± S.E.M expressed as a percentage of the appropriate control (* $p< 0.05$, ** $p< 0.01$)

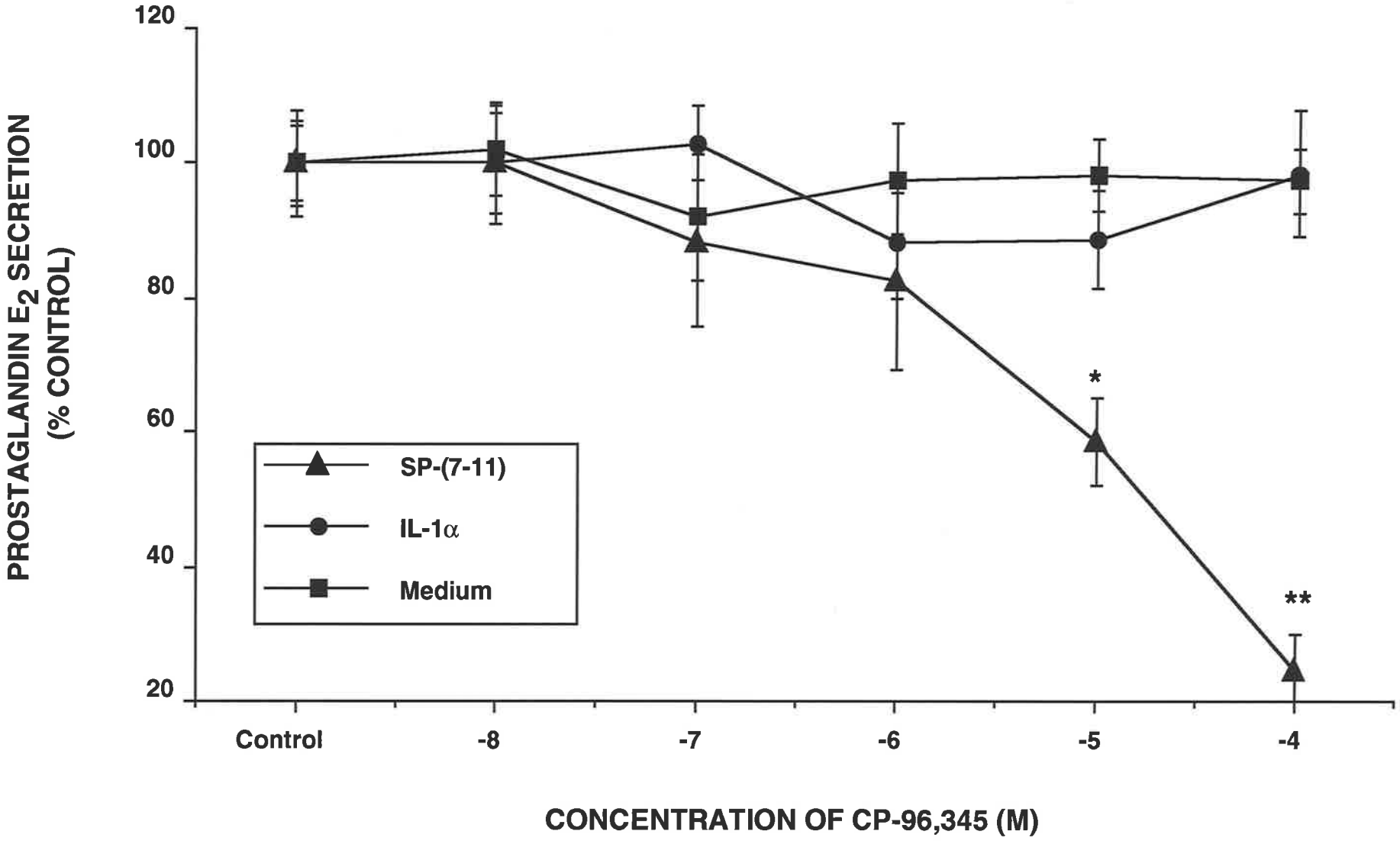

PROSTAGLANDIN $E_2$ SECRETION
(% CONTROL)
120
100
80
60
40
20
SP-(7-11)
IL-1α
Medium
*
**
Control
-8
-7
-6
-5
-4
CONCENTRATION OF CP-96,345 (M)

**Figure 3.7. Time course release of $PGE_2$ into chondrocyte supernatants in the presence or absence of SP.**

Bovine chondrocytes were incubated with SP ($5 \times 10^{-5}$ M) or with vehicle alone (ie 0.01 M acetic acid). Supernatants were collected at the indicated time points and assayed for $PGE_2$. Data are representative of four experiments.

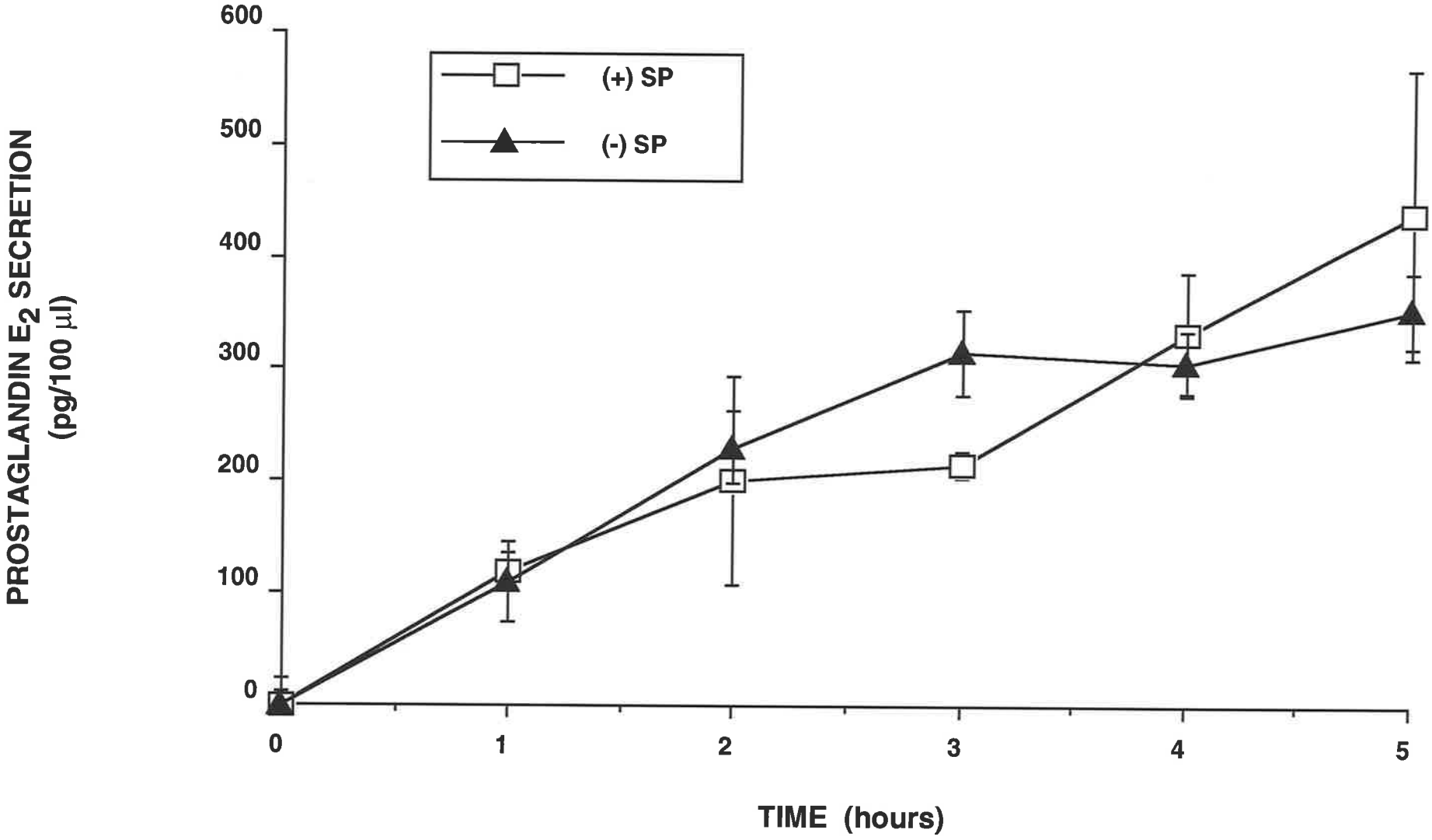

PROSTAGLANDIN $E_2$ SECRETION
(pg/100 µl)
600
500
400
300
200
100
0
(+) SP
(-) SP
0
1
2
3
4
5
TIME (hours)

**Figure 3.8. Preincubation with hr IL-1α has no effect on the time course of chondrocyte $PGE_2$ production in the presence of SP.**

Chondrocytes were incubated with hr IL-1α (50 U/ml) for 24 hours in serum-free medium. Following incubation medium was removed and the cell layer was washed with PBS. SP ($10^{-5}$ M) or vehicle alone was added, and supernatants were removed at the times indicated. This experiment was performed four times and data are representative of these results.

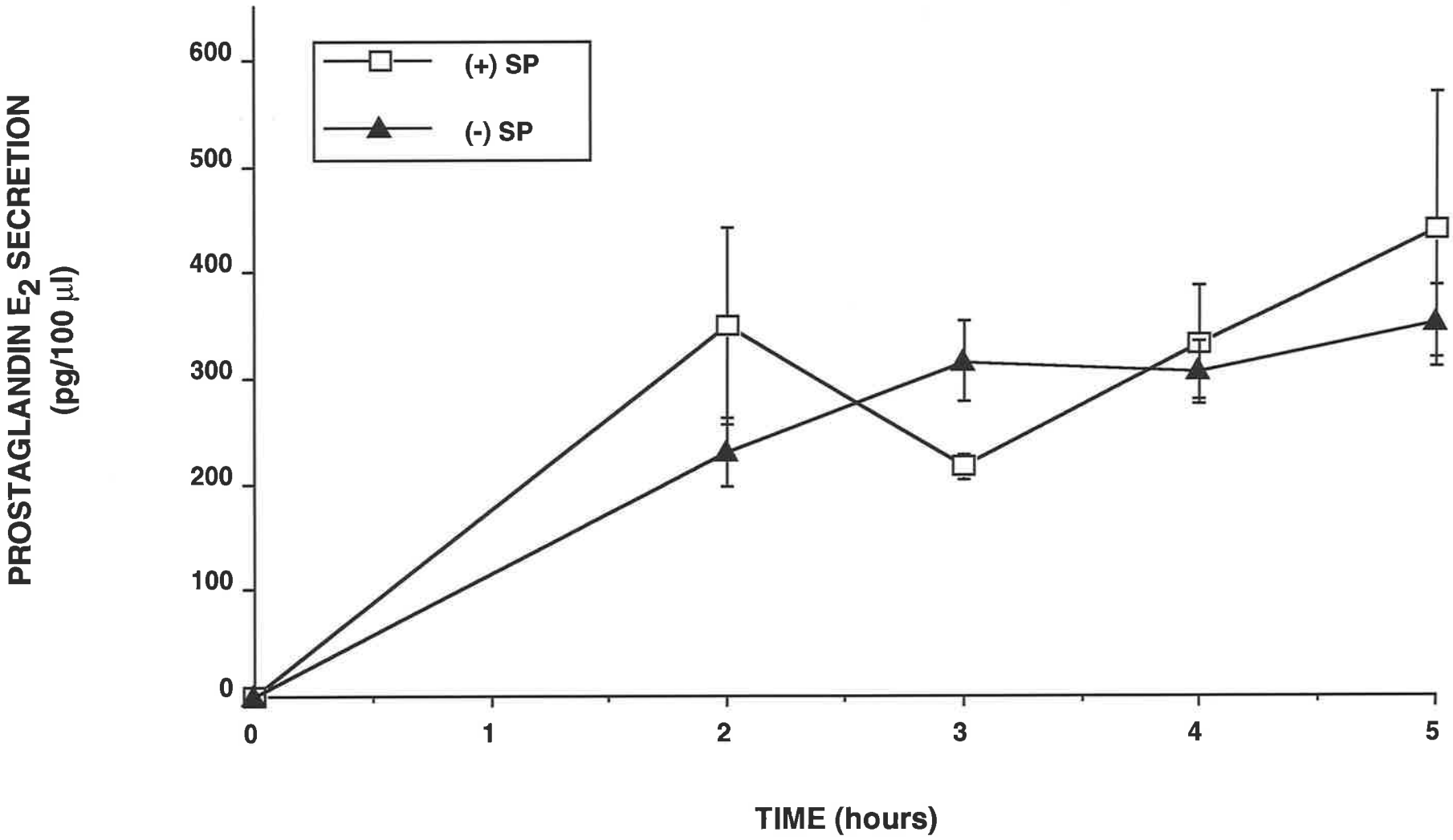
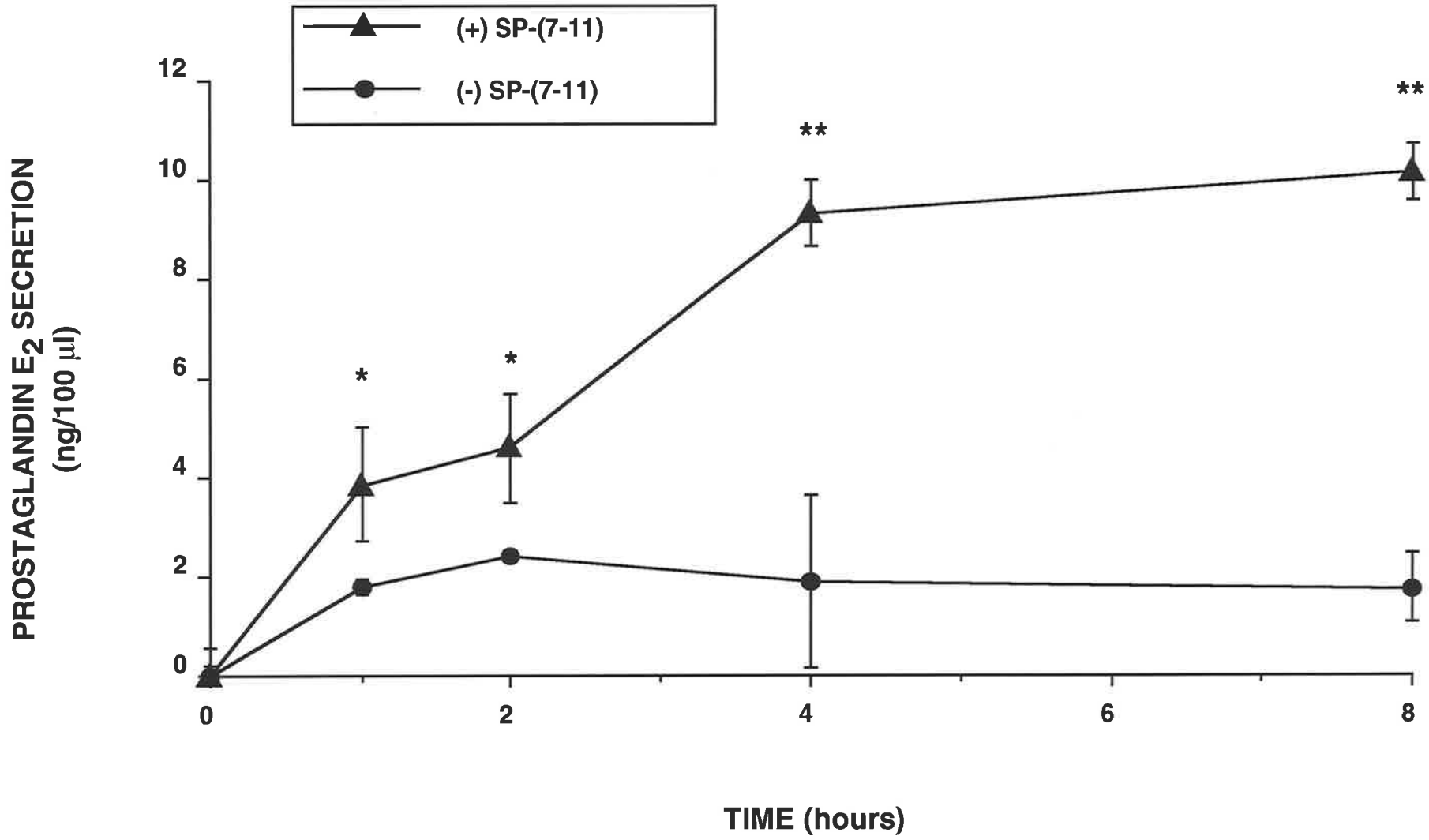

PROSTAGLANDIN $E_2$ SECRETION
(pg/100 µl)
600
500
400
300
200
100
0
(+) SP
(-) SP
0
1
2
3
4
5
TIME (hours)

**Figure 3.9. $PGE_2$ secretion from bovine chondrocytes in the presence or absence of SP-(7-11).**

Chondrocytes were incubated with $10^{-5}$ M SP-(7-11) or vehicle control (0.01 M acetic acid) for the times indicated. Supernatants were collected at the times indicated and assayed for $PGE_2$. Data are expressed as mean ± S.E.M. for a representative experiment (n = 4) performed in quadruplicate (*$p< 0.05$, ** $p<0.01$.


(+) SP-(7-11)
(-) SP-(7-11)
PROSTAGLANDIN $E_2$ SECRETION (ng/100 µl)
12
10
8
6
4
2
0
0
2
4
6
8
*
*
**
**
TIME (hours)

The dose-response to SP following preincubation with IL-1α was also determined. Chondrocytes were incubated for 24 hours with 50 U/ml hr IL-1α. Monolayers were washed, SP was added at concentrations between $10^{-7}$— $10^{-5}$ M and cells were incubated for a further 24 hours. Chondrocytes which had been exposed previously to hr IL-1α consistently produced more $PGE_2$ than chondrocytes which had not. However the response of chondrocytes to SP was not altered following exposure to hr IL-1α (figure 3.10).

In contrast preincubation of chondrocytes with 50 U/ml hr IL-1α for 24 hours followed by washing and exposure to SP-(7-11) for a further 24 hours, resulted in a significant enhancement in the stimulatory effect of SP-(7-11). Furthermore the concentration at which chondrocytes responded to SP-(7-11) was decreased ten fold (figure 3.11). These results indicate that neuropeptides and cytokines may be involved and interact in stimulating synthesis of $PGE_2$ in bovine chondrocytes.

### *c.5. Effects of tachykinins and their fragments on human and bovine synoviocyte $PGE_2$ production.*

The amount of $PGE_2$ produced by both human and bovine synoviocytes *in vitro* was significantly less than the amount produced by bovine chondrocytes after equivalent incubation time. In order to concentrate the amount of $PGE_2$ produced by synoviocytes, the amount of medium that the cells were bathed in, was halved. Confluent human and bovine synoviocytes were found to respond to hr IL-1α. Figure 3.12 shows that human synoviocytes isolated from patients with RA demonstrated increased production of $PGE_2$ in response to hr IL-1α *in vitro*.. Generally no increase in $PGE_2$ secretion was observed until the hr IL-1α concentration was above 100 U/ml.

However when human synoviocytes were incubated with SP or SP-(7-11) in serum-free medium for 24 hours, no alteration in $PGE_2$ production was detected at concentrations ranging form $10^{-6}$—$10^{-4}$ M. In these experiments 100 U/ml of hr IL-1α increased $PGE_2$ production approximately three fold. Statistical analysis of the results from 4 experiments for SP and five experiments for SP-(7-11) revealed that incubation

**Figure 3.10. Preincubation with hr IL-1α does not alter the dose response to SP in bovine chondrocyte $PGE_2$ secretion.**

Chondrocytes were preincubated with hr IL-1α (50 U/ml) or medium alone for 24 hours in serum-free medium. Following incubation medium was removed and cell layers were washed with PBS. Chondrocytes were then exposed to SP ($10^{-7}$—$10^{-5}$ M) in serum-free medium for a further 24 hours. Medium was collected and assayed for $PGE_2$ secretion. Data are representative of four separate experiments expressed as mean ± S.E.M. Control in graphs refers to diluent control (without SP).

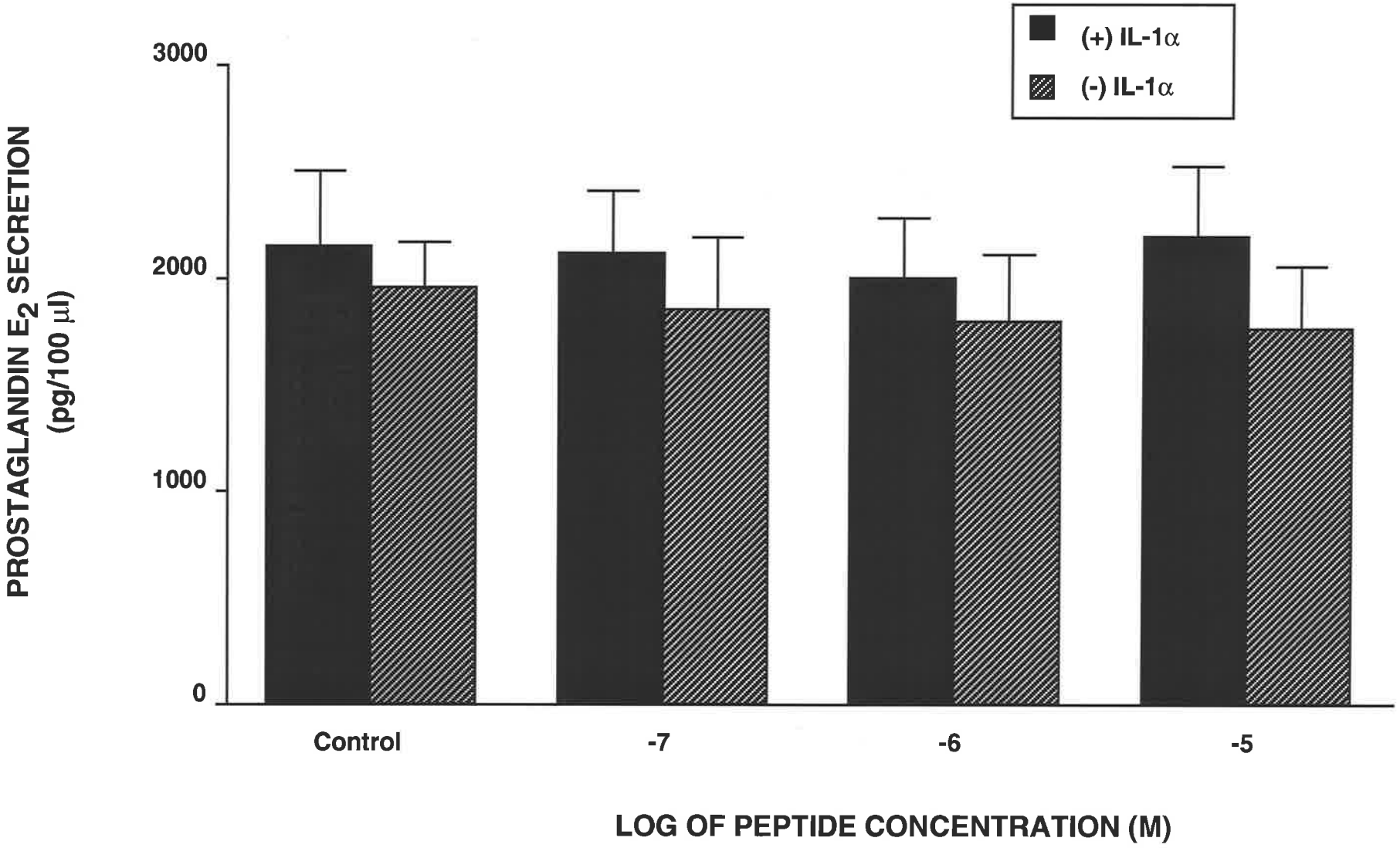

(+) IL-1α
(-) IL-1α
3000
2000
1000
0
PROSTAGLANDIN E2 SECRETION
(pg/100 μl)
Control
-7
-6
-5
LOG OF PEPTIDE CONCENTRATION (M)

**Figure 3.11. Preincubation with hr IL-1α enhances the production of $PGE_2$ in response to SP-(7-11).**

Chondrocytes were preincubated with hr IL-1α (50 U/ml) or medium for 24 hours in serum-free medium. Medium was removed and cell layers were washed with PBS. Chondrocytes were then incubated for 24 hours in the presence of SP-(7-11) ($10^{-7}$— $10^{-5}$ M). Data are representative of four experiments expressed as mean ± S.E.M. Control in the graph refers to diluent control (without SP-(7-11)).

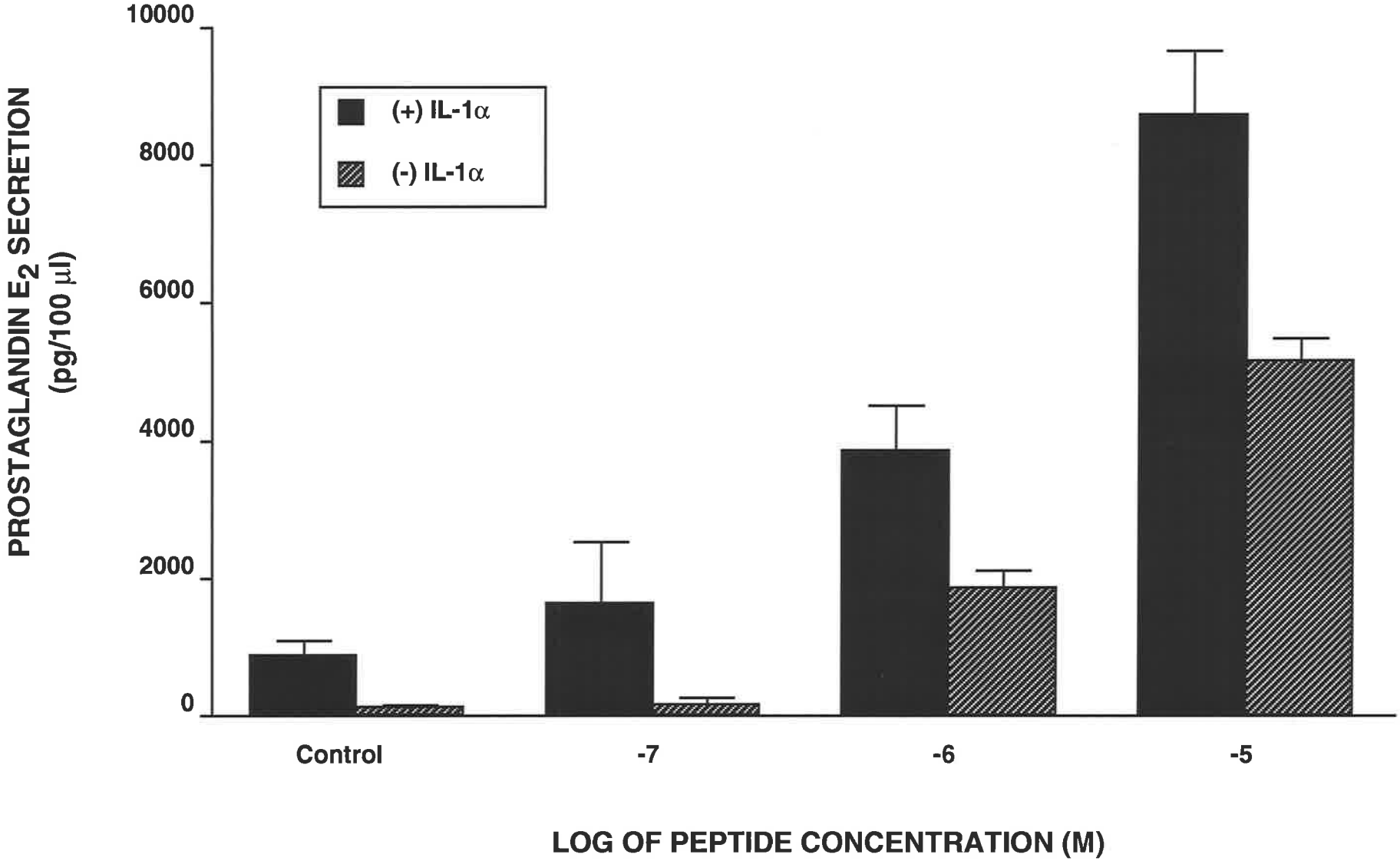
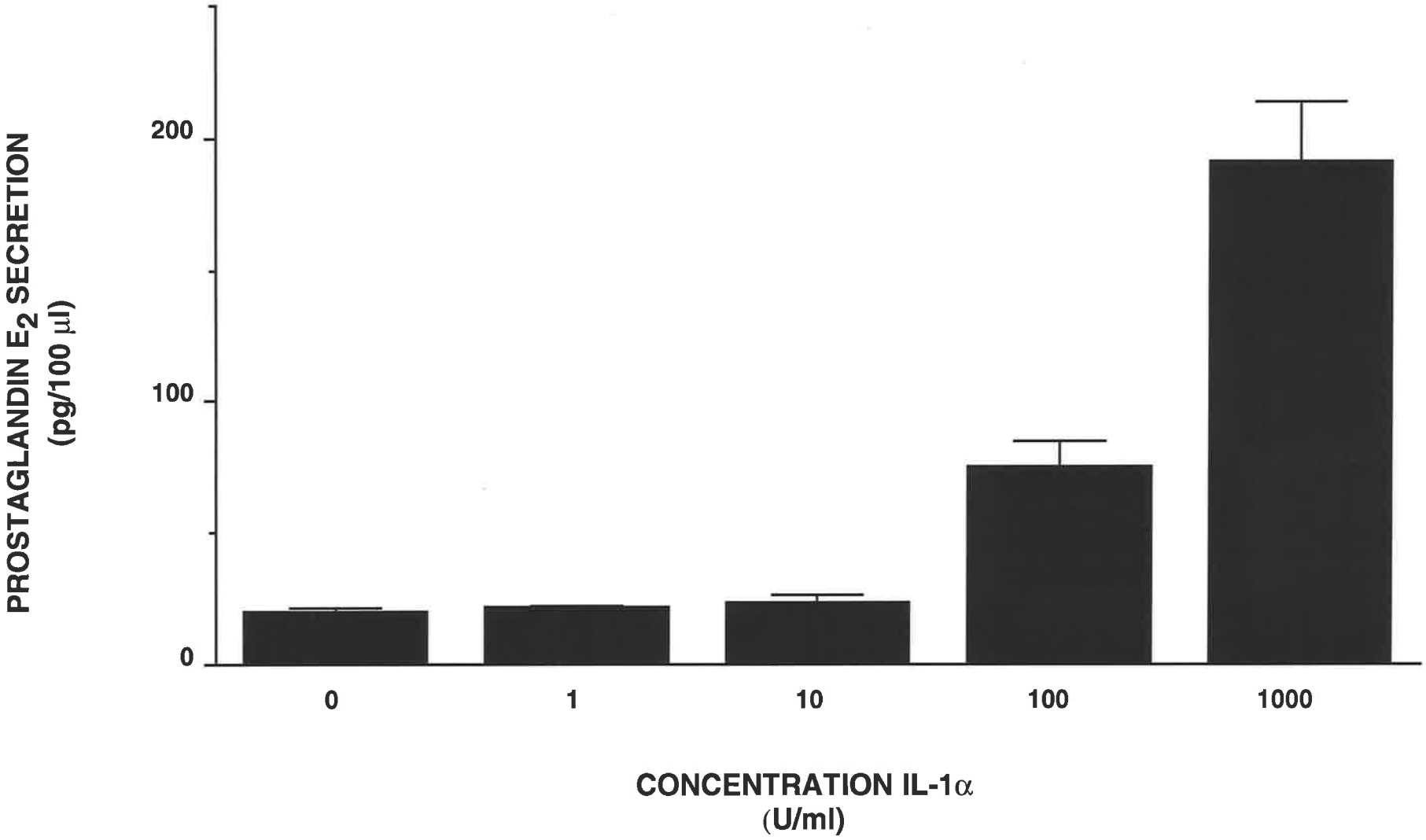

10000
8000
6000
4000
2000
0
PROSTAGLANDIN $E_2$ SECRETION
(pg/100 μl)
(+) IL-1α
(-) IL-1α
Control
-7
-6
-5
LOG OF PEPTIDE CONCENTRATION (M)

**Figure 3.12. Effect of hr IL-1α on $PGE_2$ production in synoviocytes isolated from patients with rheumatoid arthritis.**

Confluent synoviocytes were incubated with hr IL-1α (1—1000 U/ml) in 250 µl medium for 24 hours. Supernatants were collected and stored at $-20^0$ C until assayed. Values represent mean ± S.E.M. Data are from a single experiment which is representative of five other experiments.


PROSTAGLANDIN $E_2$ SECRETION
(pg/100 µl)
200
100
0
0
1
10
100
1000
CONCENTRATION IL-1α
(U/ml)

with these peptides did not alter $PGE_2$ production (table 3.1). Concentrations of SP between $10^{-10}$— $10^{-6}$ M were also tested, however at these concentrations SP did not alter $PGE_2$ production either.

Bovine synoviocyte cultures were established and the effect of SP, NKA and NKB ($10^{-8}$—$10^{-5}$ M) was tested on $PGE_2$ production. Statistical analysis of these results revealed that tachykinins did not affect $PGE_2$ production (table 3.2).

The N-terminal fragment SP-(1-4) and C-terminal fragment of SP, SP-(7-11) were tested on bovine synoviocytes. Table 3.3 shows that neither the N- or C- terminal fragments affected $PGE_2$ production, hr IL-1$\alpha$ was used as a positive control in these experiments and consistently stimulated $PGE_2$ synthesis 3-4 fold

Synoviocytes, pre-exposed to hr IL-1$\alpha$ for 24 hours were washed and exposed to SP ($10^{-10}$—$10^{-6}$ M) for 24 hours. The data from four separate experiments are summarized in table 3.4. Statistical analysis revealed that following pre-exposure to hr IL-1$\alpha$ , human synoviocytes did not respond to SP.

## D. DISCUSSION

The effect of tachykinins and SP metabolites on bovine chondrocyte and human and bovine synoviocyte, $PGE_2$ production has been determined. Only chondrocytes responded to the SP-(7-11) fragment. This response could be augmented by preincubating chondrocytes with hr IL-1$\alpha$. The effect could be significantly blocked by indomethacin or by the addition of extracellular EGTA. Chondrocytes did not respond to SP, NKA or NKB nor to the N-terminal fragments SP-(1-4) and SP-(1-6) nor the C-terminal fragments SP-(8-11) and SP-(9-11). Furthermore human rheumatoid synoviocytes did not respond to SP or SP-(7-11). Bovine synoviocytes also failed to respond to SP, NKA, NKB and SP-(1-4) and SP-(7-11).

The finding that only the C-terminal pentapeptide fragment of SP is biologically active and that this effect can be blocked by both pertussis toxin and CP-96,345 raises several interesting points. Firstly the absence of any effect of SP on

**TABLE 3.1**

**EFFECT OF SP AND SP-(7-11) ON RA SYNOVIOCYTES PROSTAGLANDIN $E_2$ SECRETION.**

**$PGE_2$ SECRETION (MEAN ± S.E.M.)**

| TREATMENT | % CONTROL* | S.E.M. |
|---|---|---|
| **SUBSTANCE P** | | |
| 100 Units/ml IL-1α + 0.1 % FCS | 321 | 26.3 |
| $10^{-6}$ M SP | 92 | 10.2 |
| $10^{-5}$ M SP | 89 | 33.4 |
| $10^{-4}$ M SP | 105 | 20.2 |
| **SUBSTANCE P-(7-11)** | | |
| $10^{-6}$ M SP-(7-11) | 117.9 | 24.4 |
| $10^{-5}$ M SP-(7-11) | 132.6 | 47.9 |
| $10^{-4}$ M SP-(7-11) | 95.2 | 19.9 |

Confluent synovial fibroblasts from patients with RA were cultured for 24 hours in 250 µl culture medium containing neuropeptide + 1% L-glutamine. Values represent data from 4 separate experiments.

* Control $PGE_2$ release 24.6 ± 4.7 ng/ml.

TABLE 3.2

**EFFECT OF TACHYKININS ON BOVINE SYNOVIOCYTE $PGE_2$ PRODUCTION.**

| | $PGE_2$ SECRETION (MEAN ± S.E.M.) | |
|---|---|---|
| **TACHYKININ** | **% CONTROL*** | **± S.E.M.** |
| **SUBSTANCE P (M)** | | |
| $10^{-8}$ | 82.2 | 17.9 |
| $10^{-7}$ | 120.4 | 19.8 |
| $10^{-6}$ | 110.3 | 6.4 |
| $10^{-5}$ | 79.6 | 17.2 |
| **NEUROKININ A** | | |
| $10^{-8}$ | 122.5 | 21.2 |
| $10^{-7}$ | 117.8 | 10.3 |
| $10^{-6}$ | 111.5 | 9.8 |
| $10^{-5}$ | 114.7 | 13.1 |
| **NEUROKININ B** | | |
| $10^{-8}$ | 109.0 | 22.1 |
| $10^{-7}$ | 74.1 | 11.8 |
| $10^{-6}$ | 77.8 | 7.8 |
| $10^{-5}$ | 79.7 | 20.9 |

Confluent bovine synoviocytes were exposed to the indicated concentrations of tachykinins in the absence of serum in 250 µl DMEM for 24 hours. Data represent mean ± S.E.M. of 3-5 separate experiments.

* Control $PGE_2$ release, 23.2 ± 3.6 ng/ml

TABLE 3.3

**EFFECT OF SP-(1-4) ANDSP-(7-11) ON $PGE_2$ PRODUCTION BY BOVINE SYNOVIOCYTE *IN VITRO*.**

**$PGE_2$ SECRETION (MEAN ± S.E.M.)**

| TACHYKININ FRAGMENT | % CONTROL* | ± S.E.M. |
|---|---|---|
| **SP-(1-4) (M)** | | |
| $10^{-8}$ | 113.9 | 14.1 |
| $10^{-7}$ | 67.2 | 19.0 |
| $10^{-6}$ | 94.6 | 16.1 |
| $10^{-5}$ | 140.0 | 57.2 |
| **SP-(7-11)** | | |
| $10^{-8}$ | 84.0 | 23.1 |
| $10^{-7}$ | 89.0 | 7.1 |
| $10^{-6}$ | 99.2 | 16.6 |
| $10^{-5}$ | 99.0 | 19.8 |

Confluent bovine synoviocytes were exposed to N- and C- terminal fragments of SP in 250 µl serum free medium for 24 hours. Data represents mean ± S.E.M. of 3-5 separate experiments.

* Control $PGE_2$ release, 12.6 ± 0.6 ng/ml

TABLE 3.4

**PRE-INCUBATION WITH hr IL-1α DOES NOT ALTER THE RESPONSE TO SUBSTANCE P ON HUMAN RHEUMATOID SYNOVIOCYTES *IN VITRO***

**$PGE_2$ SECRETION (MEAN ± S.E.M.)**

| TREATMENT | % CONTROL* | ± S.E.M. |
|---|---|---|
| SUBSTANCE P (M) | | |
| $10^{-10}$ | 139.2 | 30.0 |
| $10^{-9}$ | 69.9 | 10.2 |
| $10^{-8}$ | 93.0 | 23.1 |
| $10^{-7}$ | 108.5 | 29.9 |
| $10^{-6}$ | 116.0 | 33.3 |

Confluent human synovial fibroblasts were pre-exposed to hr IL-1α, 50 U/ml for 24 hours. Medium was removed and cells were washed with PBS and replaced with fresh medium containing SP. Values are expressed as % control ± S.E.M. of four separate experiments.

* Control $PGE_2$ secretion, 22.0 ± 4 ng/ml.

$PGE_2$ production warrants further explanation. It is known that the C-terminal region of SP is responsible for binding to the extracellular domain of the NK-1 receptor (Fong et al 1992). Possibly the number of receptors on cultured chondrocytes is low and in this situation, SP-(7-11) may be a more potent agonist than SP or alternatively chondrocytes may express an NK-1 receptor subtype which responds more readily to the C-terminal fragment. A recent study of this type has been reported by Mitsuhashi et al (1992) in which the cDNA for the NK-1 receptor was transfected into rat KNRK kidney cells. It was found that the C-terminal fragment SP-(7-11) increased levels of cAMP in a manner comparable to SP-(1-11) whereas the N-terminal fragment SP-(1-7) failed to modulate cAMP.

Extracellular calcium appears to be involved in the stimulation of $PGE_2$ synthesis by SP-(7-11) since the fragment does not increase $PGE_2$ in the presence of extracellular EGTA. Indeed the enzyme phospholipase $A_2$, which releases arachidonic acid from phospholipids is regulated by the intracellular, calcium binding protein, calmodulin (Moskowitz et al 1983). Calmodulin ( Means and Dedman 1980; Klee, et al 1980) is an ubiquitous, highly conserved intracellular $Ca^{2+}$ receptor. Calmodulin has four high affinity binding sites for $Ca^{2+}$ and undergoes conformational change when it binds intracellular $Ca^{2+}$. A number of cellular proteins are regulated by calmodulin after it binds to $Ca^{2+}$, including phospholipase $A_2$, adenylate cyclase and phosphorylase kinases. Elevation in intracellular calcium therefore appears to be a mechanism for regulation of $PGE_2$ synthesis by SP-(7-11) in bovine chondrocytes.

The inhibition of $PGE_2$ production by ADP ribosylation of the $\alpha$ subunit of G proteins by pertussis toxin, in response to SP-(7-11) suggests that pertussis toxin-sensitive G proteins may have a fundamental role in the signal transduction mechanisms stimulated by this fragment. It has been previously stated that prostaglandin synthesis can be induced by the cytokine hr IL-1$\alpha$ in chondrocytes. Possibly the augmentation of the SP-(7-11) effect may be due to hr IL-1$\alpha$ inducing expression of NK-1 receptors during the pre-exposure period. This could result in an enhanced SP-(7-11) response due to increased NK-1 receptor numbers.

These results on the effect of SP on $PGE_2$ secretion by synoviocytes differ from those reported by Lotz et al (1987). The failure of tachykinins and their fragments to increase $PGE_2$ synthesis, both in human and bovine synoviocytes, may be due to one of several reasons. Firstly, the culture conditions which were used in the study by Lotz et al (1987) were different from our study and therefore this may have affected the outcome of our experiments. Our culture conditions were serum-free at the time of neuropeptide addition to synoviocytes whereas Lotz added 0.05% FCS and incubated synoviocytes with SP for 48 hours. Possibly 0.05% FCS present in culture may have prevented SP receptor shedding, however this possibility has not been investigated in detail.

Secondly, the maximal induction of $PGE_2$ synthesis was found at $10^{-8}$ M in the study by Lotz et al (1987) and the effect of SP on $PGE_2$ production could be inhibited by spantide a relatively non-specific SP receptor antagonist. These data suggest that the effect of SP on rheumatoid synoviocyte $PGE_2$ production is receptor mediated. However binding studies with $^{125}$I-SP on RA and OA synovium (Walsh et al 1992) showed binding only to endothelial cells but not to synoviocytes. Similar results were demonstrated *in vitro*, no $^{125}$I-SP binding was demonstrated on OA or RA synoviocytes (Walsh et al 1992).

Thirdly, Bathon et al (1992) reported that SP was rapidly metabolized by NEP on both OA and RA synoviocytes *in vitro*. It is therefore likely that metabolites of SP are rapidly generated *in vitro* and that SP may be inactivated upon exposure to synoviocytes. Lotz et al (1987) did not investigate this possibility, however in our study SP-(1-4), SP-(1-6) and SP-(7-11) did not induce $PGE_2$ synthesis in bovine synoviocytes.

Whether synoviocytes *in vitro* synthesize NK-1 receptors in response to factors present in FCS has not been determined. The presence of FCS in the medium at the time of SP incubation with rheumatoid synoviocytes may be a critical factor in determining whether SP has any effect of synoviocyte function. However the reason

why SP and its fragments did not increase $PGE_2$ synthesis in synoviocytes remains to be determined.

## E. SUMMARY

Chondrocytes increased the synthesis of $PGE_2$ in response to the SP fragment SP-(7-11). This stimulation could be blocked by indomethacin, extracellular EGTA, pertussis toxin and CP-96,345. These results suggest that extracellular calcium and possibly G-proteins coupled to NK-1 receptors may be important in mediating the effect of SP-(7-11) in bovine chondrocytes. Preincubation of chondrocytes with hr IL-1α augmented the effect of SP-(7-11). Both human and bovine synoviocytes failed to increase the synthesis of $PGE_2$ in response to SP. Preincubation of synoviocytes with hr IL-1α did not result in increased $PGE_2$ synthesis in response to SP. These studies indicate that metabolism of SP in the joint is an important process which has not been appreciated and may be of significance in determining the biological effect(s) of tachykinins in the joint.

# CHAPTER IV

# THE EFFECT OF TACHYKININS AND THEIR FRAGMENTS ON BOVINE ARTICULAR CHONDROCYTE METALLOPROTEINASE (MMP-I) AND TISSUE INHIBITOR OF METALLOPROTEINASE PRODUCTION.

# A. INTRODUCTION

The regulation of the synthesis and activation of metalloproteinases is a fundamental biological event in a number of normal and pathological processes. *In vitro,* synoviocytes and chondrocytes synthesize and secrete both latent and active collagenase (MMP-I). MMP-I is particularly important in the pathogenesis of arthritis since it can cause dissolution of articular cartilage via degradation of type II collagen which is essential for formation of the extracellular matrix with aggrecan. Cartilage degradation by metalloproteinases in the joint may result from the stimulation of matrix metalloproteinases in synoviocytes and chondrocytes by inflammatory cytokines such as IL-1$\alpha$ and TNF-$\alpha$ (Saklatvala 1986; McCachren et al 1989). Alternatively inflammatory mediators can alter TIMP synthesis (Lefebvre et al 1990). For a full appreciation of the total process the balance between both systems needs to be investigated. The possibility that tachykinins may be directly involved in chondrocyte-mediated cartilage degradation stems from a study by Levine et al (1984) in the adjuvant arthritis model in the rat. They found that direct infusion of SP into the knee joint of rats with adjuvant arthritis resulted in increased joint inflammation and cartilage erosions. Injection of the SP receptor antagonist, D-SP had no effect on the severity of joint inflammation but was able to ameliorate SP-induced cartilage erosions. However similar studies have not been performed in normal rats which had not been inoculated with Freund's adjuvant. In the rat adjuvant arthritis model it is likely that when SP was infused it was rapidly metabolized into fragments by NEP. SP metabolites produced in this way may stimulate metalloproteinase production in chondrocytes and/or synoviocytes.

For these reasons the effect of SP and the C-terminal fragment SP-(7-11) was tested on bovine articular chondrocyte MMP-I and TIMP production.

The questions posed in this study were therefore:-

1. Do tachykinins influence MMP-I (collagenase) production in bovine articular chondrocytes *in vitro* ?
2. Do tachykinins influence TIMP-1 production in bovine articular chondrocytes?
3. Is MMP-I secreted in a latent or active form?
4. Do tachykinins induce *de novo* synthesis of MMP-I and/or TIMP?

These studies were performed in monolayer cultures of bovine articular chondrocytes. MMP-I activity was assayed in culture supernatants on the basis of its ability to degrade rat tail type I acid-soluble collagen. TIMP was inactivated by reduction and alkylation methods and production was assessed by reverse zymography.

## B. MATERIALS AND METHODS

### B.1. *Materials*

Chemicals used in the preparation of acid-soluble type I collagen and the collagenase assay were purchased from either Sigma (St. Louis, Mo, USA) or Ajax Chemicals (Sydney, Australia). Tissue culture materials were the same as in chapter II. Assay wells (Nunc-Immunomodule maxisorp), for coating with purified type I collagen were purchased from Nunclon, Denmark. Human recombinant IL-1$\alpha$ was from the same source as the studies on proteoglycan production. The TIMP inhibitors dithiothreitol and iodoacetamide were purchased from Biorad (Sydney, Australia) and Sigma respectively. The BC-1 cell line was a gift from Professor Robert O' Grady, University of Technology, Sydney, Australia. Electrophoresis reagents and equipment were purchased from Biorad, Sydney, Australia.

## B.2. *Methods*

### *a. Preparation of acid-soluble Type I collagen*

Acid-soluble Type I collagen was prepared from rat tail tendons using the method of Bazin and Delaunay (1976). Rat tails (12) were collected and stored frozen. The tendons were dissected from the tails and placed into ice cold, 0.2 M NaCl. They were rinsed three times in cold, 0.2 M NaCl before freeze fracturing.

### *b. Freeze Fracturing*

A stainless steel mortar and pestle were pre-chilled overnight in a $-70^0$ C freezer. Rat tendons were snap frozen in liquid nitrogen in the mortar. The pestle was added and tendons were shattered with the use of a sledge hammer. More liquid nitrogen was added and the process was repeated until the tendons were reduced to a powder.

The resultant powder was stirred in 0.5 M acetic acid at $4^0$ C for 48 hours. This solution was ultracentrifuged at 20, 000 g for 2 hours at $4^0$ C. The supernatant was dialysed overnight against 0.02 M phosphate buffer, pH 7.8. After dialysis, the material was dissolved in 0.5 M acetic acid for 18 hours at $4^0$ C. This solution was lyophilised in a vacuum freeze-drier for 48 hours. The dry weight of this material was determined and dissolved in 0.5 M acetic acid to give a concentration of 2 mg/ml.

### *c . Salt Fractionation of collagen*

To prepare native monomeric collagen with intact non-helical ends, differential salt precipitation by the method of Chandrakasan et al (1976) was used. The collagen concentration was adjusted to a final concentration of 3% (w/v) by adding a stock solution of 25% NaCl. This solution was stirred for a further 18 hours at $4^0$ C. The precipitate from the '3% NaCl cut' was removed by centrifugation for 2 hours at 10,000 g. The supernatant was collected and re-precipitated at 4% NaCl and the pellet was collected after centrifugation at 10,000 g for 45 minutes. This '3-4% NaCl cut'

represents the native type I collagen. Collagen was dissolved in 0.5 M acetic acid and dialysed against 0.2% acetic acid for 24 hours.

The concentration of the purified collagen was quantified by adding 0.5 ml of sample with 0.5 ml of 2 M $CaCl_2$ in 0.2% acetic acid. The absorbance at 230 nm was measured using 1 M $CaCl_2$ in 0.2% (v/v) acetic acid as the blank. The collagen concentration was calculated by using the following equation :-

[Collagen] mg/ml = $A_{230}$ X 0.5

***d. Collagenase Assay***

*d.1. Preparation of collagen coated wells*

Collagen (20 µg) was plated into microwell modules on ice in the following buffers. Collagen, (stock concentration 2 mg/ml in 0.2% v/v acetic acid) in neutralising buffer (100 mM Tris/HCl, 200 mM NaCl, 0.04 % $NaN_3$, pH 7.8) was gelled to the microwells by incubation for 16 hours at $30^0$ C under humidified conditions, followed by a further 24 hours incubation under dry conditions. The wells were washed in distiled water and allowed to dry at room temperature. Collagenase activity, ie MMP-I, in the culture medium was assayed using the spectrophotometric method of Nethery et al (1986). Samples were mixed with a one-tenth volume of 1 M Tris, 0.2% $NaN_3$, pH 7.5. Latent collagenase was either activated by incubation at $35^0$ C for 10 minutes with either 25 µg/ml trypsin in assay buffer (50 mM Tris, 100 mM NaCl, 10 mM $CaCl_2$, 0.2% $NaN_3$, pH 7.5) or with 1 mM 4-aminophenylmercuricacetate (APMA). Trypsin activity was inhibited with a 5-fold molar excess of soy bean trypsin inhibitor. Assays were performed for 18 hours at $35^0$ C, after which wells were washed with deionised water and allowed to dry. The wells were stained with 100 µl /well Coomassie Brilliant Blue R250, (0.25 mg/ml in 50% methanol, 10% acetic acid, 40% water) for 25 minutes at room temperature. Wells were rinsed, allowed to dry and the absorbance was read at 590 nm on a spectrophotometer (Titertek Multiskan). Each assay contained the following controls: 25 µg/ml trypsin (measure of native collagen) in assay buffer, assay buffer alone (zero digestion) and conditioned medium from BC-1 cells, a rat

mammary carcinoma cell line with high spontaneous tissue collagenase (MMP-I) activity ( to act as a positive control for collagenolytic activity). The collagenolytic activity of each sample was expressed as units/ml, with one unit of activity being defined as that amount of enzyme required to degrade 1 μg of collagen per minute/ ml of sample at $35^0$ C.

**B4.** ***Cell culture***

Bovine chondrocytes were isolated by the same method as outlined in chapter II and neuropeptides were handled in the same fashion. When chondrocytes reached confluence, the medium containing 10% FCS was removed. Cell layers were washed twice with PBS and fresh DMEM containing the appropriate neuropeptide concentration was added and the cells incubated for 18 hours. Hr IL-1α + 0.01% FCS was used as a positive control for MMP-I production and chondrocytes were incubated with this cytokine for the same period of time as with tachykinins. At the completion of each experiment, cell supernatants were collected and stored at $-20^0$ C until assayed for collagenase activity.

**B5** ***Inactivation of TIMP by reduction and alkylation***

To determine whether tachykinins could induce the synthesis of TIMP-I, the activity of TIMP-I in cell supernatants was inactivated by the method of Dean et al (1989). In brief, cell supernatants were incubated with 2 mM dithiothreitol at $37^0$ C for 30 minutes, followed by further treatment with 5 mM iodoacetamide for 30 minutes at $37^0$ C. The samples were then dialysed against a buffer containing 50 mM Tris-HCl, 10 mM $CaCl_2$, 0.2 M NaCl, 0.05 % Brij 35 and 0.02 % $NaN_3$, pH 7.4 at $4^0$ C for 18 hours. Control samples were treated with PBS under the same conditions as samples which had been TIMP-inactivated. After dialysis the samples were assayed for collagenase activity.

### B6. *Reverse Zymography*

Polyacrylamide gel electrophoresis was performed on a Protean II dual slab cell. The sample buffer contained 10 % glycerol (v/v), 2.3 % SDS (w/v) and 0.001% bromophenol blue (w/v) in 62.5 mM Tris, pH 6.8 (non-reducing buffer). The electrophoresis running buffer was 25 mM Tris, 192 mM glycine , 0.1 % SDS at pH 8.3. The electrophoresis was run at 15 mA during the stacking phase and 30 mA during the separation phase. Gelatin was incorporated into polyacrylamide gels at a final concentration of 1 mg/ml. Samples for reverse zymography were incubated in non-reducing buffer (without 2-mercaptoethanol) at room temperature. The SDS-PAGE gelatin-incorporated gel had a 7.5 % resolving gel and a 4 % stacking gel. Following electrophoresis of the samples the gels were incubated with gentle shaking in 2.5 % Triton X-100 for 30 minutes at room temperature. The gels underwent further washing in Triton X-100 and were then placed into a solution of BC-1 conditioned medium containing collagenase and incubated at $35^0$ C overnight. Following digestion of gelatin by BC-1-derived collagenase, gels were stained with 0.25 % Coomassie blue R250 in 50 % methanol, 10 % acetic acid and destained using 50 % methanol, 10 % acetic acid. The presence of TIMP in samples could be visualised as blue bands (representing TIMP bound to collagenase) which remained after staining with Coomassie Brilliant Blue R250.

### B7. *Statistics*

Data are expressed as means ± S.E.M. of 3-4 separate experiments. Student's t-test was used to test for significance between means.

## C. RESULTS

### *c.1. SP-(7-11) increases collagenase activity in bovine articular chondrocytes*

Chondrocytes were incubated with neuropeptides ($10^{-7}$ M—$10^{-4}$ M) in serum-free medium for 24 hours after which the medium was assayed for collagenolytic

activity. SP had no effect on collagenase production in bovine articular chondrocytes, at any of the concentrations tested (Figure 4.1). However SP-(7-11) increased ($p < 0.05$; n = 4) collagenase levels significantly at concentrations greater than $10^{-6}$ M in a dose-dependent manner. The response was maximal at $10^{-4}$ M (control versus $10^{-4}$ M SP-(7-11), 13.8 ± 4.1 and 60.8. ± 6.2 mUnits/ml respectively, $p < 0.01$, n = 4) and was approximately 4 fold higher than control values. The N-terminal fragments SP-(1-4) and SP-(1-6) did not induce collagenase synthesis. The related tachykinins NKA and NKB also did not alter collagenase synthesis. Only samples which had been exposed to SP-(7-11) and then activated with APMA or trypsin contained elevated collagenolytic activity (Figure 4.2). Cycloheximide, a protein translation inhibitor, completely abolished the stimulatory effect of $10^{-4}$ M SP-(7-11) (Control 14.8 ± 3.5 mUnits/ml; $10^{-4}$ M SP-(7-11) alone, 62.4 ± 8.2 mUnits/ml; SP-(7-11) plus 10 µg/ml cycloheximide 3.2 ± 0.1 mUnits/ml ).

### *c.2. TIMP-1 production following exposure to SP-(7-11)*

Following TIMP inactivation both treated and untreated media which had been exposed to SP-(7-11) contained increased collagenolytic activity, both with and without TIMP inactivation. However the amount of collagenase activity was reduced by approximately half in those supernatants which had undergone TIMP inactivation. This result was unexpected as TIMP inactivation should have increased the amount of collagenolytic activity detected by the assay. Nevertheless in both groups collagenase activity was increased in response to SP-(7-11) (Figure 4.3).

Reverse zymograms revealed that TIMP levels in supernatants from monolayer and articular cartilage-slice cultures which had been exposed to SP-(7-11) ($10^{-7}$—$10^{-4}$ M), did not alter when compared to control expression (Figure 4.4). Bands of metalloproteinase inhibition were seen at both 19 kDa and 30 kDa in supernatants from monolayers and articular cartilage-slices. However it was noted that an intermediate TIMP band of approximately 25 kDa was present only in the articular

**Figure 4.1. Effect of SP and SP-(7-11) on collagenase secretion by bovine articular chondrocytes.**

Confluent chondrocytes were incubated for 24 hours with medium (control) or SP or SP-(7-11). TIMP activity was inactivated following reduction and alkylation with 2 mM dithiothreitol and 5 mM iodoacetamide for 30 minutes at $37^0$ C. Samples were subsequently dialysed as outlined in the methods section. Latent collagenase was activated with 1 mM APMA or 25 μg/ml trypsin followed by inactivation with SBTI. Collagenase activity was measured as outlined in the methods. Values represent means ± S.E.M. for four separate experiments expressed as % control. Control values were 13.8 ± 4.1 mUnits/ml (*$p < 0.05$; **$p$ <0.01).

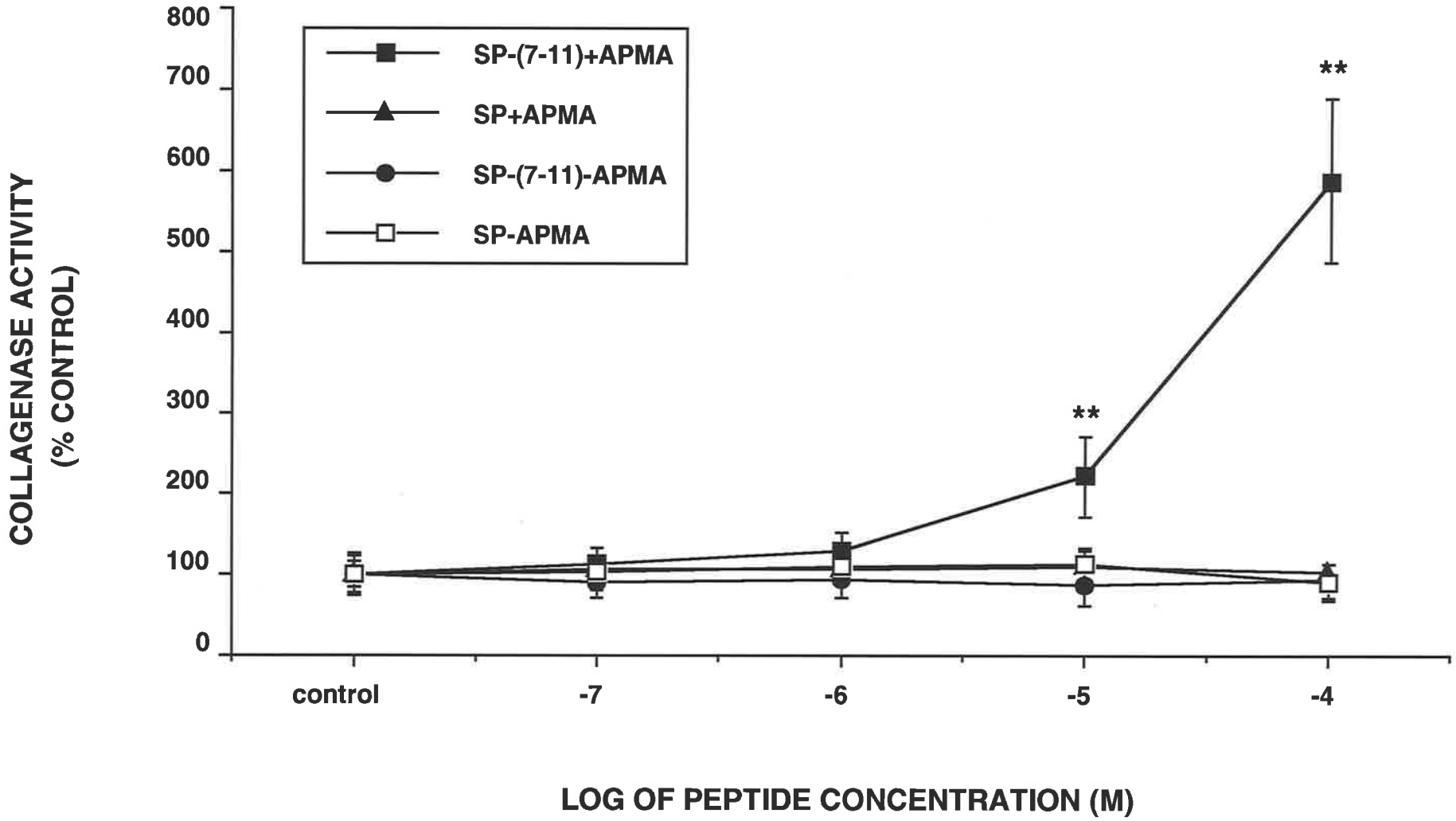

SP-(7-11)+APMA
SP+APMA
SP-(7-11)-APMA
SP-APMA
COLLAGENASE ACTIVITY
(% CONTROL)
800
700
600
500
400
300
200
100
0
**
**
control
-7
-6
-5
-4
LOG OF PEPTIDE CONCENTRATION (M)

**Figure 4.2. Latent collagenolytic activity stimulated by SP-(7-11) can be activated by trypsin or APMA.**

Confluent bovine chondrocytes were incubated with SP-(7-11) ($10^{-7}$—$10^{-4}$ M) for 24 hours. Supernatants were either assayed without metalloproteinase activation (no activation); treated with 1 mM APMA (APMA) or 25 µg/ml trypsin for 10 minutes followed by 125 µg/ml SBTI. Treated supernatants were then assayed for collagenase activity by the procedure outlined in the methods section. Values represent means ± S.E.M. for 4 separate experiments expressed as a % control (*$p< 0.05$)

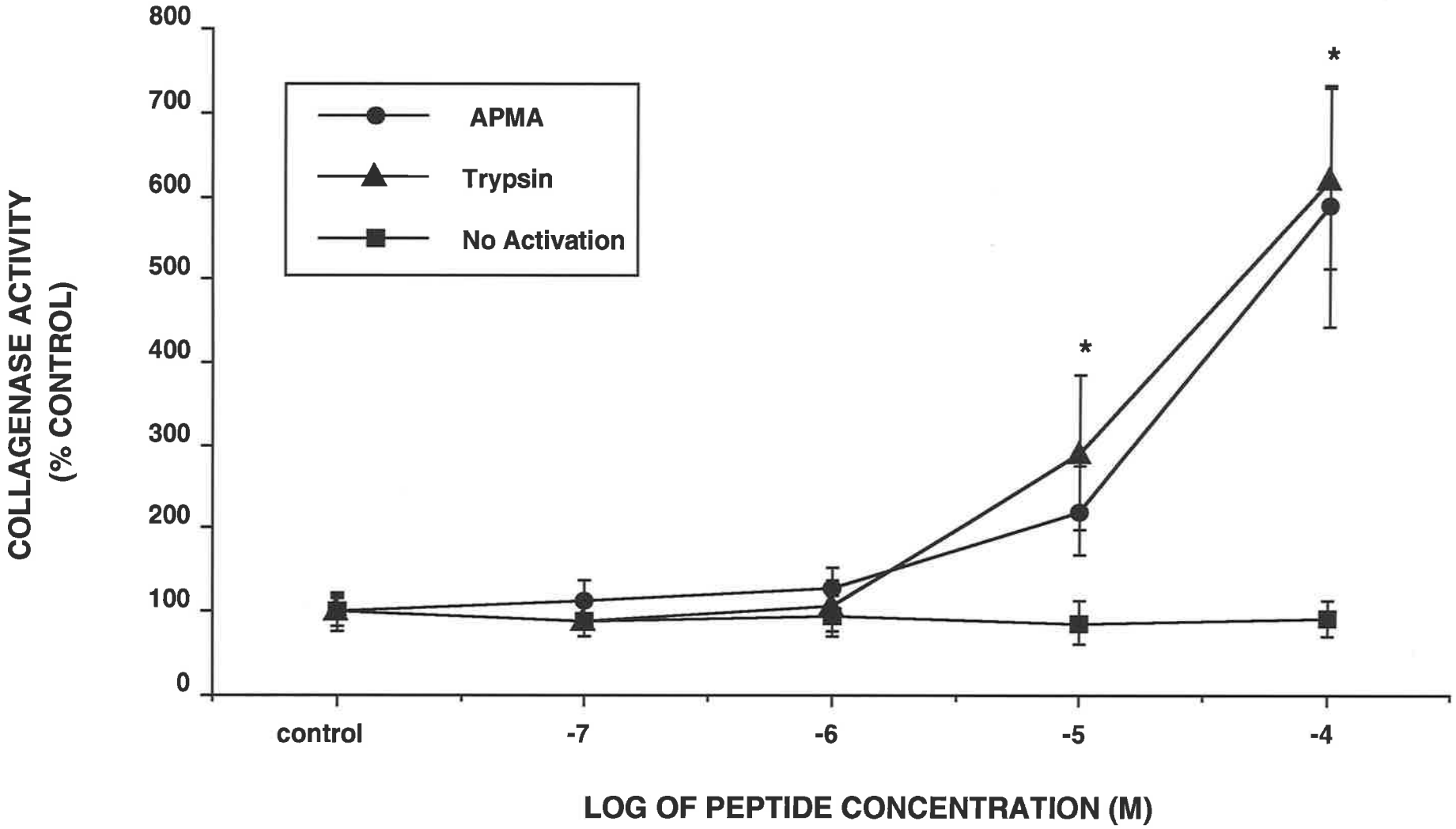

COLLAGENASE ACTIVITY
(% CONTROL)
800
700
600
500
400
300
200
100
0
APMA
Trypsin
No Activation
*
*
control
-7
-6
-5
-4
LOG OF PEPTIDE CONCENTRATION (M)

**Figure 4.3. TIMP inactivation reduces the amount of collagenase activity detected by the collagenase assay.**

Chondrocytes were incubated with SP-(7-11), ($10^{-7}$—$10^{-4}$ M), or medium (control) for 24 hours in culture. Hr IL-1$\alpha$ + 0.01% FCS was used as a positive control for collagenase stimulation. Supernatants were divided into TIMP inactivated (+) and not TIMP inactivated (-) groups. TIMP inactivated supernatants were incubated with 2 mM dithiothreitol (reduction) at $37^0$ C for 30 minutes followed by incubation with 5 mM iodoacetamine (alkylation) at $37^0$ C for a further 30 minutes. Samples were then dialysed against 50 mM Tris-HCl, 10 mM $CaCl_2$, 0.2 M NaCl, 0.05% Brij 35 and 0.02 % $NaN_3$, pH 7.4 for 18 hours at $4^0$ C in microdialysis tubes as described by (Overall 1987). Control samples (-) TIMP inactivation were treated with equal volumes of PBS under the same conditions as (+) TIMP inactivation samples. Samples were then assayed for collagenase activity. Values are mean ± S.E.M. of three separate experiments.

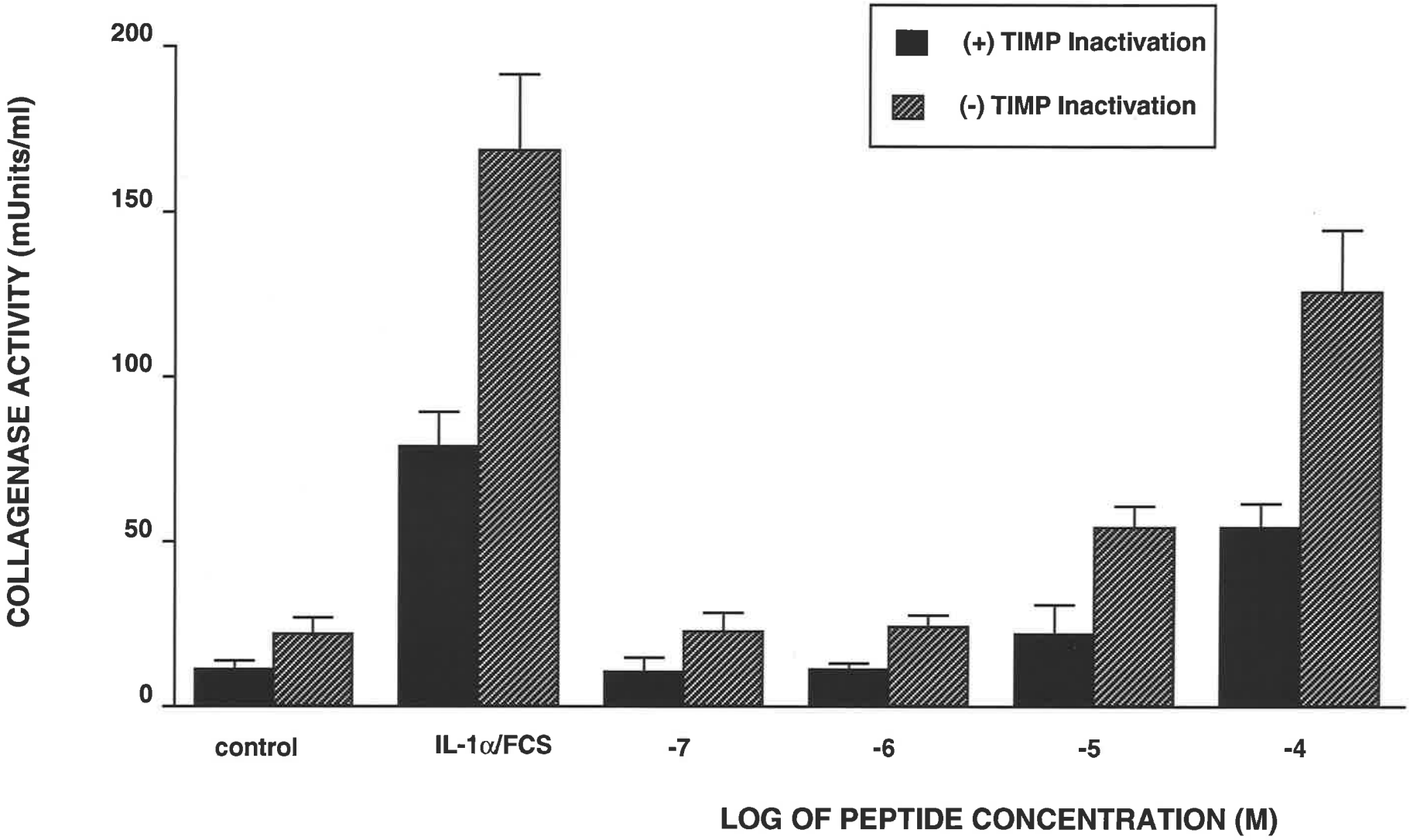
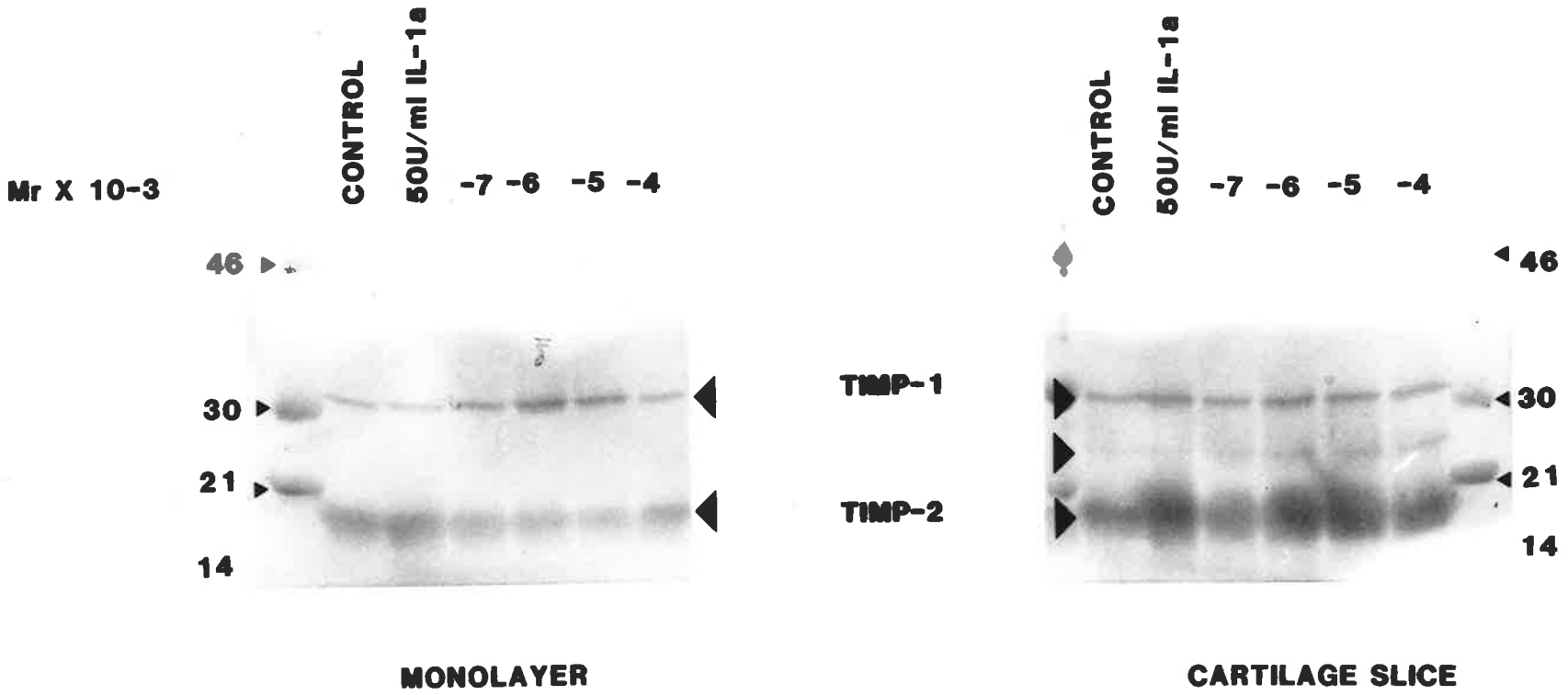

(+) TIMP Inactivation
(-) TIMP Inactivation
COLLAGENASE ACTIVITY (mUnits/ml)
200
150
100
50
0
control
IL-1α/FCS
-7
-6
-5
-4
LOG OF PEPTIDE CONCENTRATION (M)

**Figure 4.4. Reverse zymograms of supernatants from chondrocyte monolayers (A) and articular cartilage slices (B) following exposure to SP-(7-11).**

Chondrocytes in monolayer or articular cartilage slices were exposed to SP-(7-11) ($10^{-7}$—$10^{-4}$ M) for 24 hours and supernatants were collected. Samples were treated as outlined in the methods section and separated on gelatin impregnated SDS gels. Bands indicate the molecular weight of TIMP present in the supernatant and the amount produced following exposure to SP-(7-11) .


Mr X 10-3
CONTROL
50U/ml IL-1a
-7 -6 -5 -4
46
30
21
14
TIMP-1
TIMP-2
MONOLAYER
CARTILAGE SLICE

cartilage-slice supernatants. The amount of TIMP present in the supernatants was not altered following exposure to SP-(7-11).

## D. DISCUSSION

The effect of mammalian tachykinins and the C-terminal fragment of SP, SP-(7-11) was studied on MMP-I (collagenase) production in bovine articular chondrocytes *in vitro*.. The effect of SP-(7-11) on TIMP production was also assessed.

Only the C-terminal fragment SP-(7-11) induced MMP-I synthesis but not TIMP synthesis. Substance P, NKA, NKB and the N-terminal fragments, SP-(1-4) and SP-(1-6), did not alter MMP-I synthesis. Furthermore the secreted enzyme was present in a proenzyme or latent form, requiring either trypsin or APMA activation. These findings are consistent with the studies on $PGE_2$ synthesis, in which $PGE_2$ synthesis was specifically increased in response to SP-(7-11) but not SP. It appears that SP-(7-11) stimulates the *de novo* synthesis of MMP-I since addition of cycloheximide to cell cultures completely abolished MMP-I activity in chondrocyte cell supernatants. SP-(7-11) therefore does not stimulate the release of a possible intracellular pool of preprocollagenase.

The reduction and alkylation of TIMP in chondrocyte supernatants revealed that SP-(7-11) did not alter the synthesis of TIMP. A suppression of TIMP synthesis for example by SP-(7-11) would be detected by the collagenase assay as an increase in overall collagenolytic activity. Since both treated and untreated supernatants contained increased collagenolytic activity in response to SP-(7-11) it is likely that SP-(7-11) has no effect on TIMP synthesis and the increase in collagenolytic activity is due only to the induction of MMP-I gene synthesis following stimulation by SP-(7-11).

Reverse zymography revealed that chondrocytes cultured in both monolayer and articular cartilage-slices release TIMPs into their supernatants The band seen at approximately 19 kDa is probably TIMP-2 which has been isolated from articular cartilage and is found as a 21 kDa protein (Bunning et al 1984). The higher molecular

weight band seen at approximately 30 kDa is TIMP-1 which is the most common form of TIMP found in most connective tissues ( Docherty et al 1985). The intermediate TIMP seen at approximately 25 kDa only in the articular cartilage slice supernatants may be a glycosylated form of the 21 kDa TIMP-2. The relative amount of TIMP present in each supernatant following exposure to SP-(7-11) did not alter which confirmed the reduction and alkylation experiments which suggested that TIMP synthesis was not altered in response to SP-(7-11).

MMP-I synthesis therefore appears to be specifically increased by SP-(7-11). Since TIMP synthesis was not stimulated by SP-(7-11) it is possible that SP-(7-11) may induce expression of preprocollagenase gene. The control of collagenase gene expression by SP-(7-11) was not investigated in this study, however the regulation of matrix metalloproteinase genes by other pro-inflammatory mediators such as IL-1$\alpha$ and TNF-$\alpha$ has been investigated in some detail. Possibly similarities exist between the intracellular events which occur in cells following neuropeptide and cytokine stimulation. For example, studies on the 5' region of the collagenase gene in fibroblasts has revealed an 8 base pair TPA (12-O-tetradecanoyl-phorbol-13-acetate) Responsive Element (Angel et al 1987) called TRE. Transactivation of the TRE sequence can occur after binding of a complex consisting of a Fos/Jun heterodimer with the AP-1 site (Allegretto et al 1990, Chiu et al 1988). In human fibroblasts TNF-$\alpha$ stimulates the transcription of c-fos and c-jun (gene) which results in the translation of the nuclear proteins Fos and Jun. These two proteins form a heterodimer and bind to the TRE responsive element and this is followed by the induction of collagenase expression (Brenner et al 1989). In rabbit synovial fibroblasts (Chin et al 1985, Frisch et al 1987) and rabbit articular chondrocytes (Murphy et al 1986), collagenase and stromelysin synthesis are coordinately regulated at the level of transcription. Therefore although MMP-3 was not measured in response to SP-(7-11) it would be likely that MMP-3 could also be increased in response to SP-(7-11). However this type of gene regulation may not occur in chondrocytes. Studies by Mitchell and Cheung (1991) demonstrated that the degree of TNF-$\alpha$ stimulation of collagenase did not correlate with c-fos or c-jun

expression. In these studies epidermal growth factor (EGF) induced significant accumulation of c-fos and c-jun but very little collagenase or stromelysin was produced. The authors suggest that post-transcriptional and post-translational events, such as phosphorylation may be necessary for functional Fos/Jun transactivating complexes. Alternatively the stimulation of collagenase in chondrocytes may act via a c-fos, c-jun independent pathway. A c-fos independent pathway has been demonstrated for stromelysin induction by EGF in NIH 3T3 fibroblasts (Kerr et al 1988). Understanding the cis and trans-activating factors which operate in bovine chondrocytes during the induction of collagenase and stromelysin may resolve this finding.

The metabolism of SP in the joint to SP-(7-11) also appears to be important for the stimulation of collagen degradation by MMP-I. The observation that SP-(7-11) specifically induces MMP-I synthesis has not been previously considered and suggests that neuropeptide metabolites may be mediators of cartilage destruction.

## E. SUMMARY

These studies demonstrated that bovine articular chondrocytes spontaneously secrete latent MMP-I *in vitro.* and increase this secretion several fold following exposure to SP-(7-11) The proteinase released by chondrocytes could be activated by either trypsin or the organomercurial compound APMA, suggesting that the protein was a metalloproteinase. Furthermore the increase in MMP-I activity by SP-(7-11) was not due to suppression of TIMP-1 synthesis, suggesting that SP-(7-11) may directly induce the synthesis of MMP-I These results suggest SP-(7-11) may be a novel factor in chondrocyte-mediated articular cartilage destruction of the joint.

# CHAPTER V

# THE EFFECT OF TACHYKININS AND THEIR FRAGMENTS ON THE INTRACELLULAR CALCIUM CONCENTRATION IN BOVINE CHONDROCYTES

# A. INTRODUCTION

The concentration of intracellular (cytosolic) free calcium $[Ca^{2+}]_i$ is critical in the control of many cellular functions. Changes in $[Ca^{2+}]_i$ can control such diverse functions as cellular excitability, exocytosis, metabolism and gene expression. In most cells the change in $[Ca^{2+}]_i$ is due to either $Ca^{2+}$ entering the cell through calcium channels present in cell membranes, release of $Ca^{2+}$ from intracellular stores or both processes occuring simultaneously.

Increases in $[Ca^{2+}]_i$ trigger the activation of phospholipase C which leads to the rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate and the production of two intracellular messenger molecules, diacylglycerol (DAG) and 1,4,5-inositol trisphosphate (1,4,5 $IP_3$) (see Berridge 1984). Diacylglycerol activates protein kinase C (Nishizuka 1986), whereas $IP_3$ leads to calcium mobilization from intracellular stores (Kishimoto et al 1980; Bonis et al 1986).

Many of the biological activities of SP are mediated via alterations of $[Ca^{2+}]_i$ and subsequent production of DAG and $IP_3$ (Handley et al 1980, Holzer and Lippe 1985). Since SP-(7-11) was shown to increase the production of both $PGE_2$ and collagenase it may be possible that these changes were brought about by changes in $[Ca^{2+}]_i$.

The following experiments therefore had the following aims:-

**1.** To determine which mammalian tachykinins and SP fragments are able to alter $[Ca^{2+}]_i$.

**2.** To determine if the change in $[Ca^{2+}]_i$ is due to an influx of $Ca^{2+}$ from an extracellular source, intracellular stores or both.

Intracellular calcium changes were measured with the use of the fluorescent probe FURA 2 AM in suspensions of freshly isolated bovine articular chondrocytes.

# B. MATERIALS AND METHODS

## *B.1. Materials*

FURA-2 AM was purchased from Calbiochem (San Diego, California USA). HBSS was purchased from Gibco ( Gaithersburg, MD USA) (10X stock) and diluted to the appropriate concentration. The peptide, *f*ormyl-Methionyl-Leucyl-Phenylalanine (*f*MLP) and the calcium ionophore A23187 were purchased from Sigma Chemical Co USA. All other chemicals were from either Sigma or AJAX Chemicals, Australia. The fluorimeter for recording changes in $[Ca^{2+}]_i$ was a Perkin-Elmer LS-50

## *B.2. Methods*

### *a. Measurement of Intracellular Free Calcium$[Ca^{2+}]_i$*

Bovine chondrocytes, isolated by collagenase digestion, were washed and incubated in 7 mM HBSS containing 1.3 mM $CaCl_2$, 0.3 mM $KH_2PO_4$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 138 mM NaCl, 4 mM $NaHCO_3$ and 0.3 mM $Na_2HPO_4$, pH 7.3. Chondrocytes were loaded with 1 mM FURA-2 AM for 30 minutes at $37^0$ C. After incubation, excess and non-hydrolysed FURA-2 AM was removed by washing twice with HBBS. Chondrocytes were resuspended in HBSS at a concentration of $1x10^6$ cells/ml and kept in a waterbath at $37^0$ C. Chondrocytes in HBSS were placed into glass cuvettes and placed in a Perkin-Elmer LS-5 fluorospectrophotometer using excitation and emission wavelengths of 340 nm and 510 nm respectively; slit widths were both 10 mm. Maximal fluorescence (Fmax) was determined by the addition of 0.1% Triton X-100. Minimum fluorescence (Fmin) was determined by the addition of 2 mM EGTA and 25 mM Tris/HCl simultaneously. The change in $[Ca^{2+}]_i$ was calculated by the formula described by Grynkiewicz et al (1985) using the dissociation constant ($K_d$) for FURA- 2 AM of 220 nM.

*b. Neuropeptides*

Stock solutions of neuropeptides were made fresh for each series of experiments as previously described.

## C. RESULTS

### *c.1. Chondrocytes respond to A23187*

The calcium ionophore A23187 was used at the beginning of all FURA-2 AM experiments to determine whether chondrocytes respond to a stimulus. In all experiments, chondrocytes responded to $10^{-6}$ M A23187 as shown in figure 5.1. The addition of A23187 resulted in an immediate rise in $[Ca^{2+}]_i$ which peaked within 10 seconds after the initial stimulus. The mean change in $[Ca^{2+}]_i$ was 295 ± 24 nM (n = 4). Intracellular calcium concentrations did not return to baseline levels but remained elevated after stimulation with A23187.

### *c.2. Intracellular calcium only increases in response to SP-(7-11)*

Tachykinins were freshly dissolved in $10^{-3}$ M acetic acid before being added to FURA-2 AM loaded chondrocytes. To demonstrate that chondrocytes do not respond to a pH change induced by acetic acid, several pilot experiments were performed. Chondrocytes did not alter baseline $[Ca^{2+}]_i$ in response to acetic acid at final concentrations between $10^{-6}$—$10^{-3}$ M (figure 5.2).

Tachykinins were reconstituted with $10^{-3}$ M acetic acid immediately before use and added to chondrocytes at a final concentration of $10^{-5}$ M. Figure 5.3 shows a representative experiment which demonstrates that SP did not alter $[Ca^{2+}]_i$ . Likewise, NKA (figure 5.4) and NKB ( figure 5.5) at similar concentrations had no direct effect on $[Ca^{2+}]_i$. These experiments were repeated on several different occasions using different chondrocyte preparations. The concentration of neuropeptides ranged from $10^{-6}$ M to $10^{-4}$ M and no increase in $[Ca^{2+}]_i$ was observed.

**Figure 5.1 Effect of the calcium ionophore A23187 on intracellular calcium concentration in bovine chondrocytes.**

FURA-2 AM loaded chondrocytes were exposed to $10^{-6}$ M A23187 to determine whether cells were adequately loaded with the fluorescent dye and able to respond to a known stimulus.

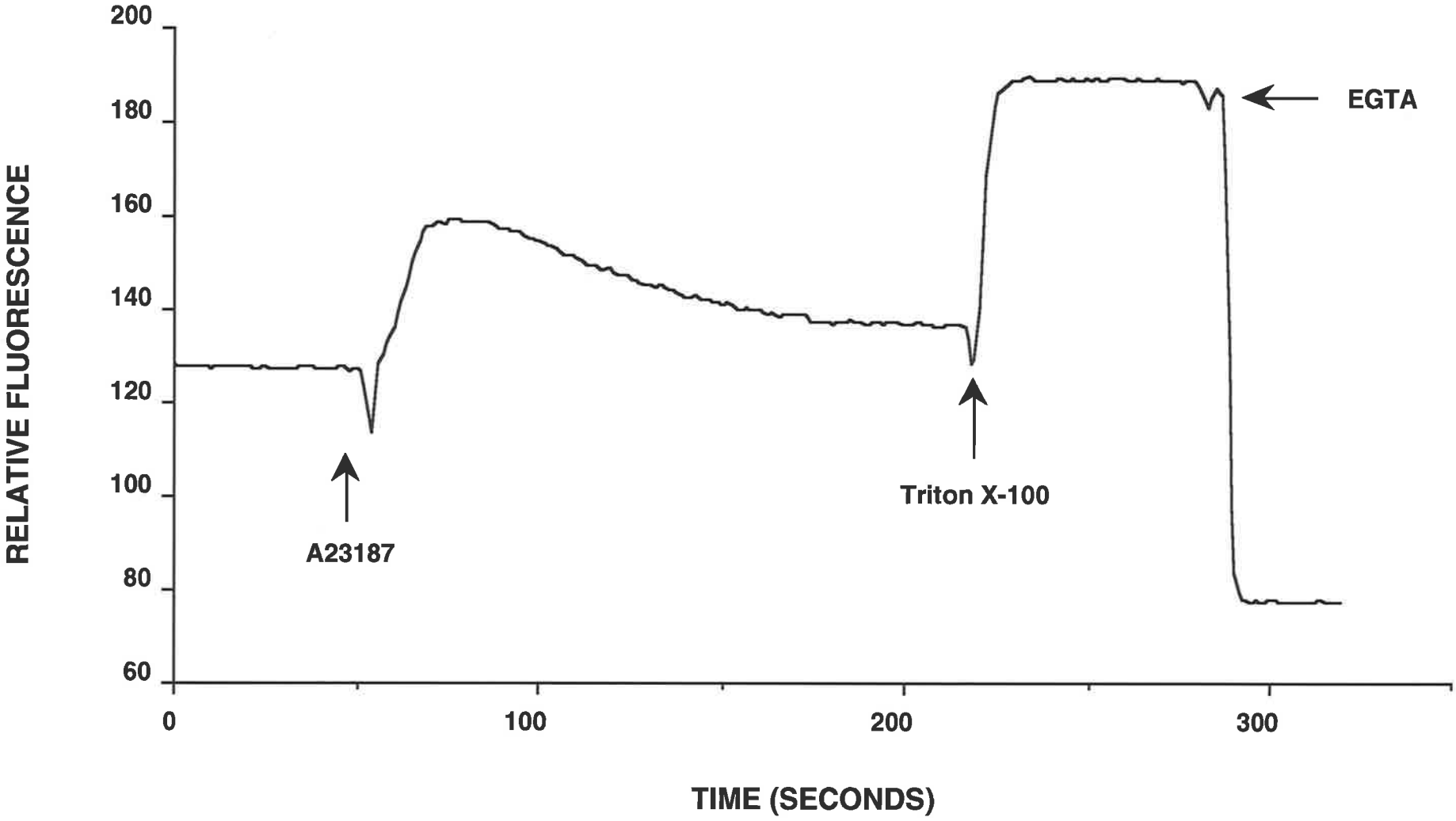

RELATIVE FLUORESCENCE
200
180
160
140
120
100
80
60
0
100
200
300
TIME (SECONDS)
A23187
Triton X-100
EGTA

**Figure 5.2. Acetic acid does not alter intracellular calcium levels.**

Acetic acid at concentrations between $10^{-6}$ M to $10^{-3}$ M were added to FURA-2 AM loaded chondrocytes. This figure is representative of adding $10^{-4}$ M acetic acid alone.

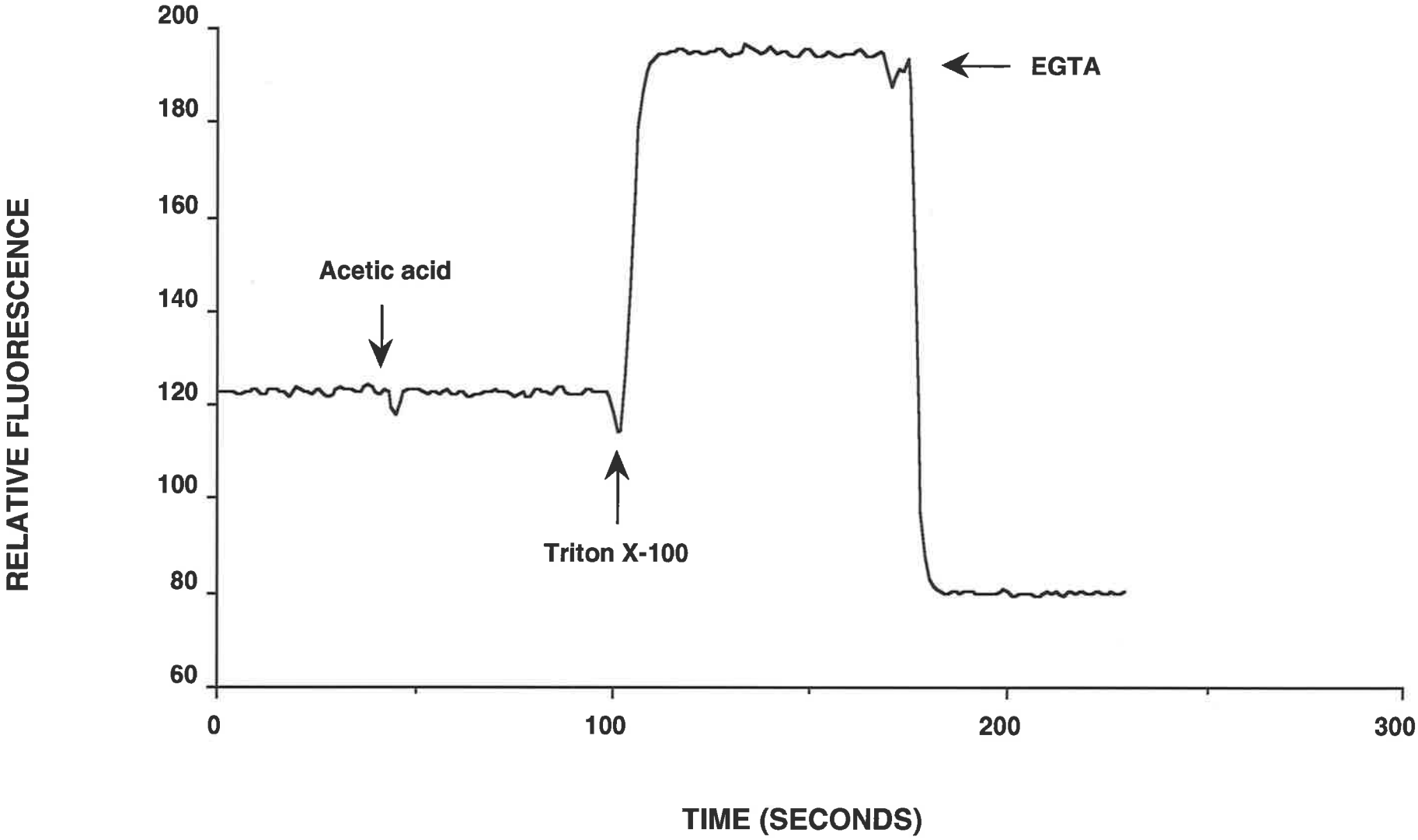

RELATIVE FLUORESCENCE
200
180
160
140
120
100
80
60
0
100
200
300
TIME (SECONDS)
Acetic acid
Triton X-100
EGTA

**Figure 5.3. Substance P does not influence intracellular calcium levels in bovine chondrocytes.**

The effect of SP at concentrations ranging from $10^{-7}$—$10^{-5}$ M was tested on chondrocyte intracellular calcium concentration. This figure is representative of $10^{-5}$ M SP added 60 seconds after baseline levels were established.

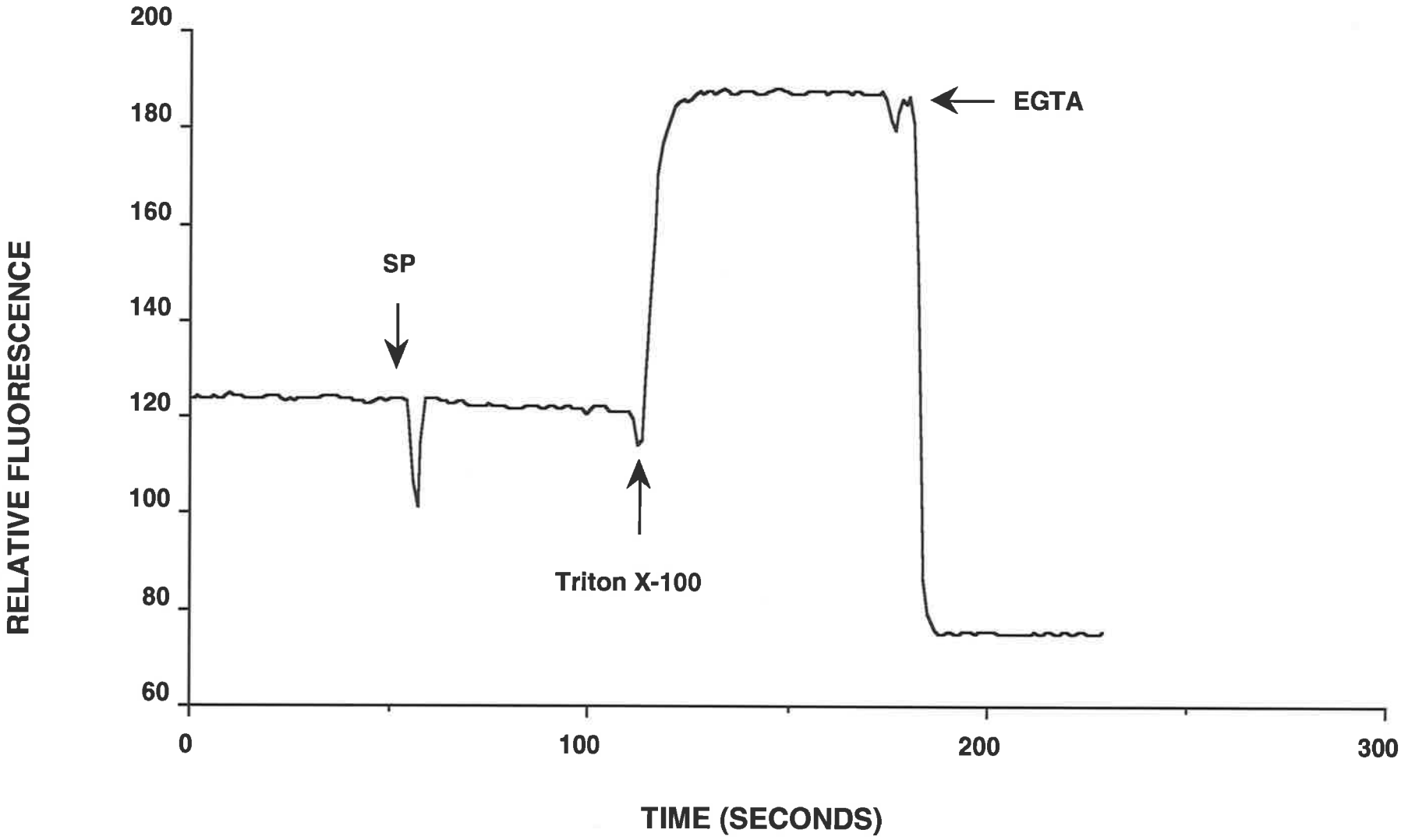

RELATIVE FLUORESCENCE
200
180
160
140
120
100
80
60
SP
Triton X-100
EGTA
0
100
200
300
TIME (SECONDS)

**Figure 5.4. Chondrocytes do not alter intracellular calcium concentration in response to NKA.**

This figure is a typical representative experiment in which $10^{-5}$ M NKA was added to FURA-2 AM loaded chondrocytes.

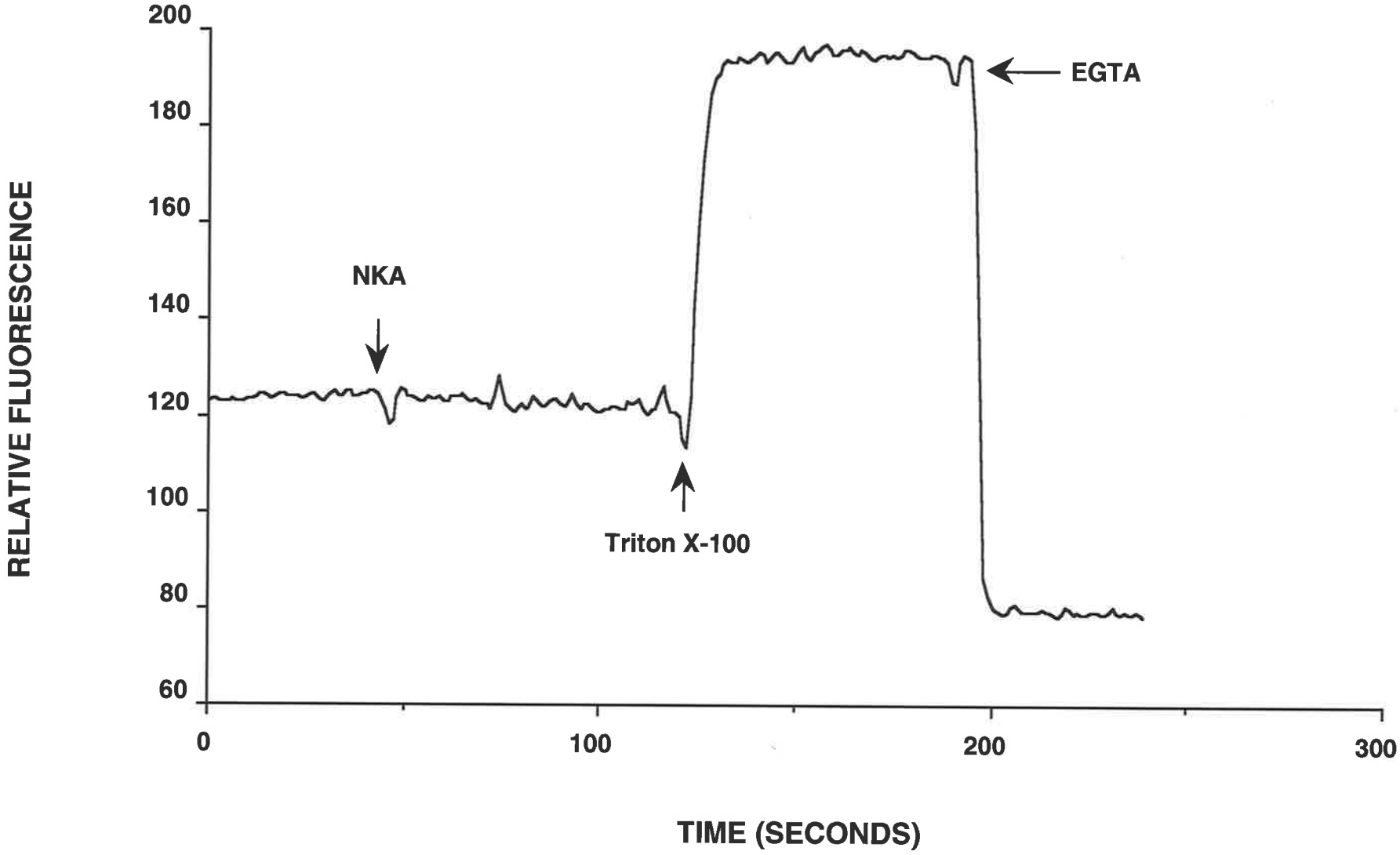
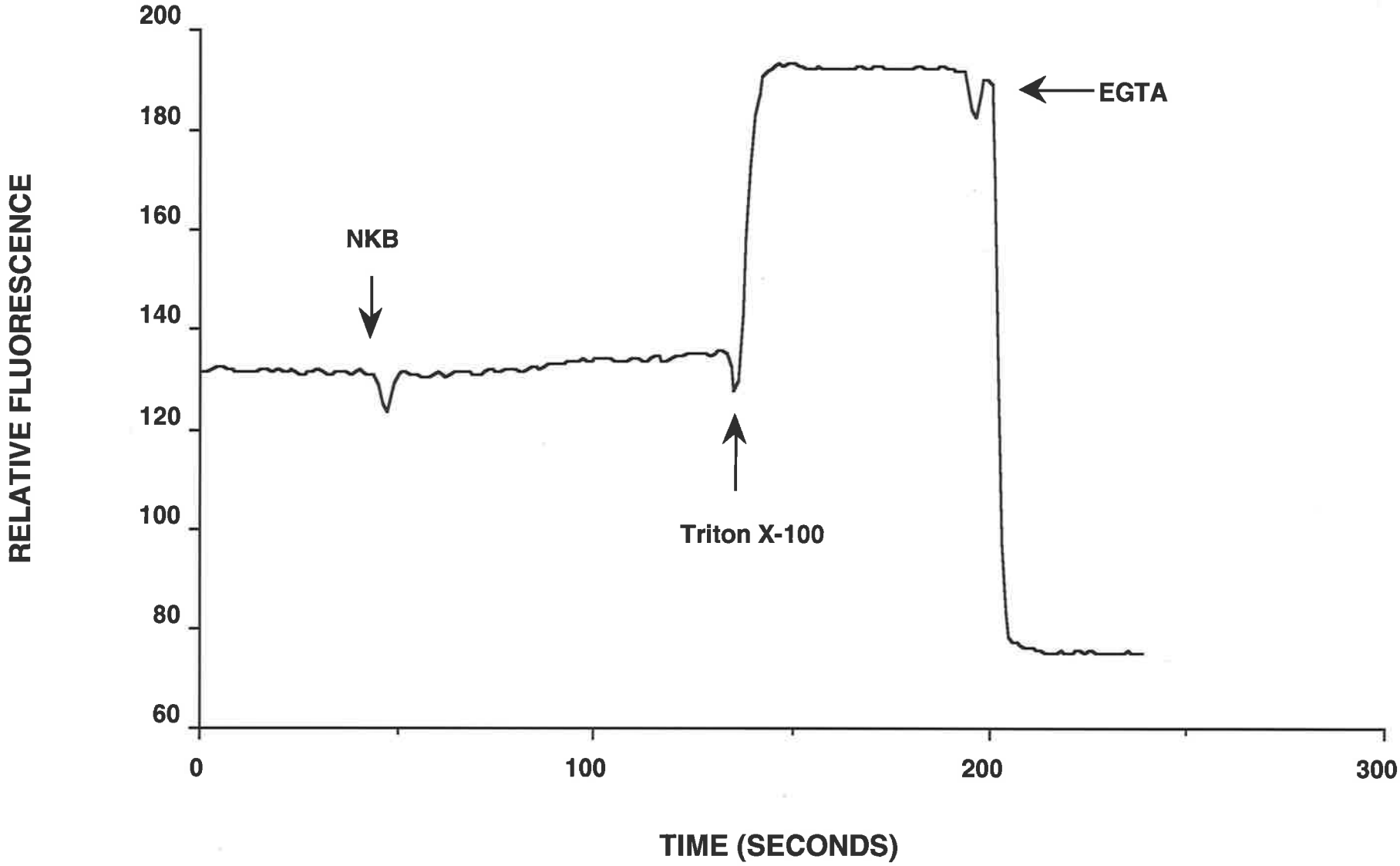

RELATIVE FLUORESCENCE
200
180
160
140
120
100
80
60
0
100
200
300
TIME (SECONDS)
NKA
Triton X-100
EGTA

**Figure 5.5. Neurokinin B does not alter intracellular calcium in bovine chondrocytes.**

This figure represents addition of $10^{-5}$ M NKB to FURA-2 AM loaded chondrocytes. Chondrocytes did not alter intracellular calcium at NKB concentrations ranging from $10^{-6}$ to $10^{-4}$ M.


RELATIVE FLUORESCENCE
200
180
160
140
120
100
80
60
0
100
200
300
TIME (SECONDS)
NKB
Triton X-100
EGTA

Since SP is rapidly metabolized by NEP into a number of fragments, the N-terminal SP-(1-4) and SP-(1-6), and C-terminal SP-(7-11), SP-(8-11) and SP-(9-11) fragments were tested for any effect on $[Ca^{2+}]_i$.

The addition of $10^{-5}$ M SP-(1-4) to chondrocytes did not alter $[Ca^{2+}]_i$ and neither did SP-(1-6) (figure 5.6). However the C-terminal fragment SP-(7-11) significantly increased $[Ca^{2+}]_i$ when added at a concentration of $10^{-5}$ M. The maximal change in $[Ca^{2+}]_i$ was calculated as 140 ± 30 nM (n = 4). However in some preparations this response was as great as 406 nM as recorded in figure 5.7. The addition of SP-(7-11) to chondrocytes resulted in a rapid increase in $[Ca^{2+}]_i$ which peaked approximately 30 seconds after the initial stimulus. Unlike A23187, after SP-(7-11), $[Ca^{2+}]_i$ returned to baseline levels within 60 seconds after the initial stimulus. This response was also refractory to further stimulation with SP-(7-11) following a 2 minute rest interval (figure 5.8). This experiment was repeated on several different preparations of chondrocytes and in each experiment, the response of chondrocytes to a second stimulation with SP-(7-11), was refractory.

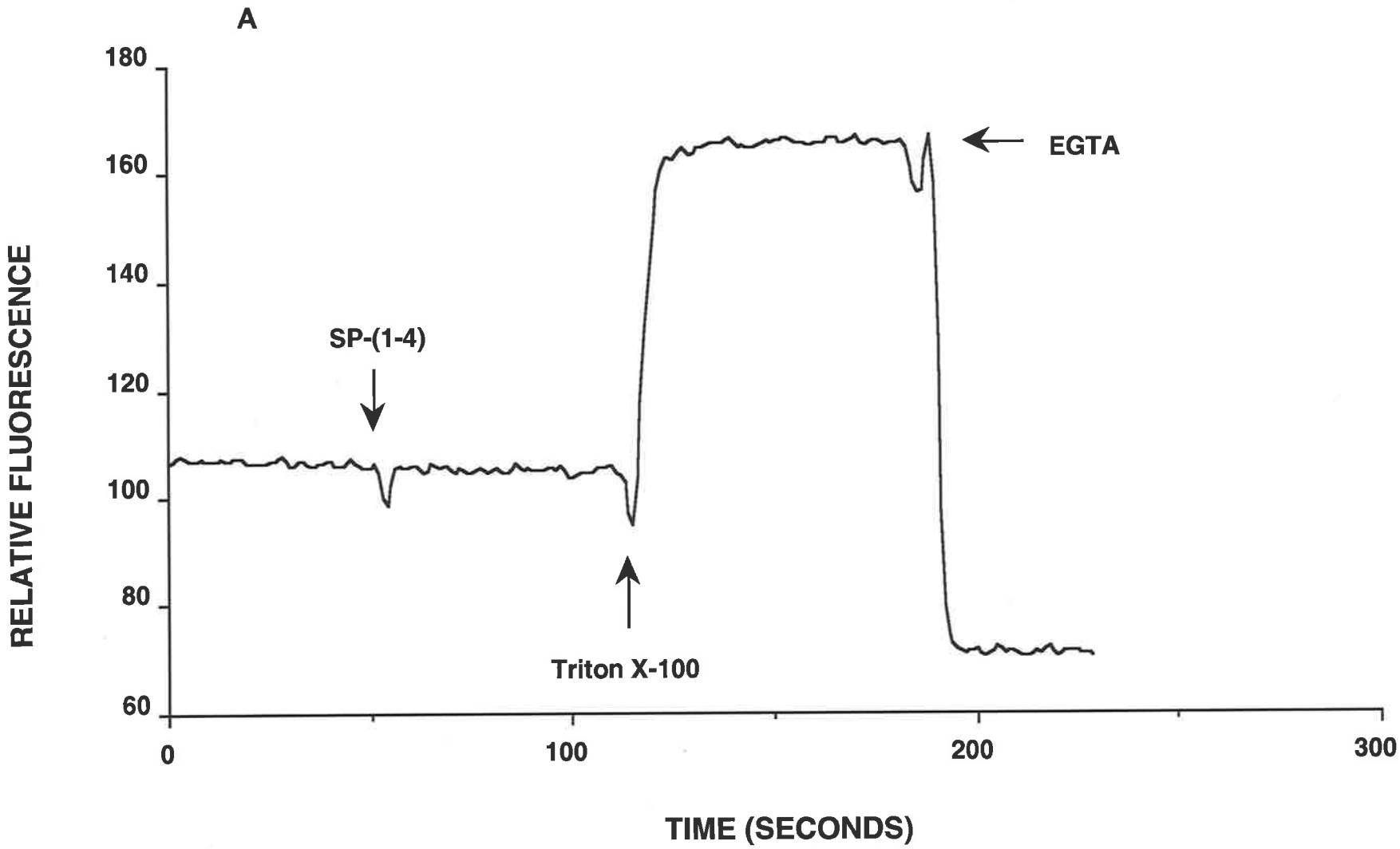
To determine the length of the SP fragment necessary to elicit this response, the peptides SP-(8-11) and SP-(9-11) at a concentration of $10^{-5}$ M were added to chondrocytes . Neither of these fragments significantly altered $[Ca^{2+}]_i$ (figure 5.9). These results indicated that only the sequence Phe-Phe-Gly-Leu-Met-$NH_2$ could induce increases in $[Ca^{2+}]_i$ in bovine chondrocytes.

### 3. *Blockade of SP-(7-11) induced $[Ca^{2+}]_i$ increases by extracellular EGTA*

To determine whether the rise of $[Ca^{2+}]_i$ was due to an influx of $Ca^{2+}$ from the extracellular medium or via mobilization from intracellular stores such as the endoplasmic reticulum, 2 mM EGTA was added 1 minute before $10^{-5}$ M SP-(7-11). EGTA specifically chelates calcium ions and inhibited completely the stimulatory effect of SP-(7-11). EGTA alone did not alter $[Ca^{2+}]_i$ at any concentration tested (100 mM—2 mM). When $10^{-5}$ M SP-(7-11) was added 50 seconds after 2 mM EGTA the rise in $[Ca^{2+}]_i$ induced by SP-(7-11) was completely inhibited (Figure 5.10). This

**Figure 5.6. Effect of N-terminal fragments SP-(1-4) and SP-(1-6) on intracellular calcium concentration.**

(A) $10^{-5}$ M SP-(1-4) was added to FURA-2 AM loaded chondrocytes. (B) SP-(1-6) added at a similar concentration.


A
180
160
140
120
100
80
60
RELATIVE FLUORESCENCE
SP-(1-4)
Triton X-100
EGTA
0
100
200
300
TIME (SECONDS)

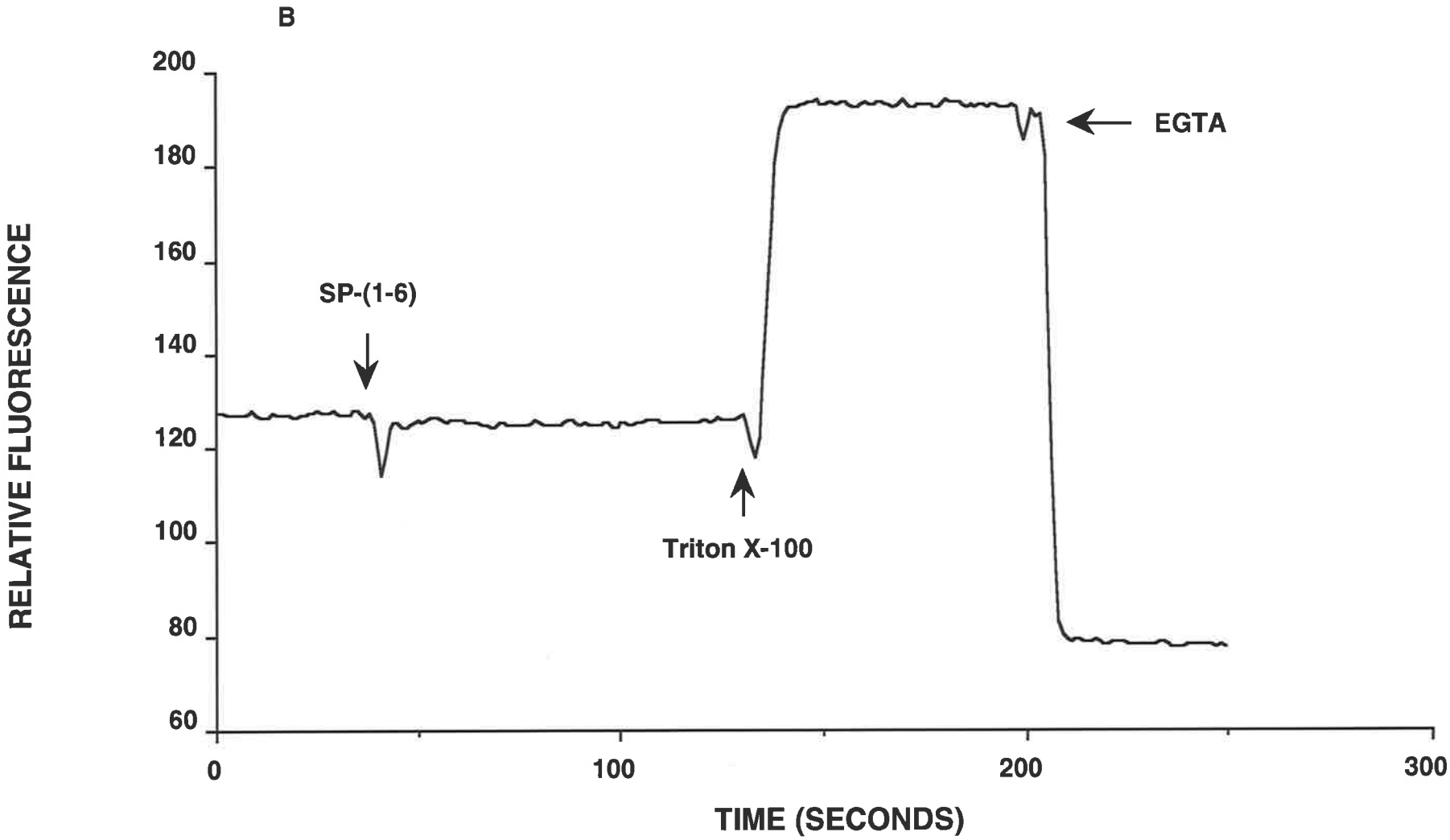

B
200
180
160
140
120
100
80
60
RELATIVE FLUORESCENCE
SP-(1-6)
Triton X-100
EGTA
0
100
200
300
TIME (SECONDS)

**Figure 5.7. Effect of SP-(7-11) on intracellular calcium concentration.** FURA-2 AM loaded chondrocytes were exposed to $10^{-5}$ M SP-(7-11). This figure is representative of several different preparations of chondrocytes and SP-(7-110 concentrations.

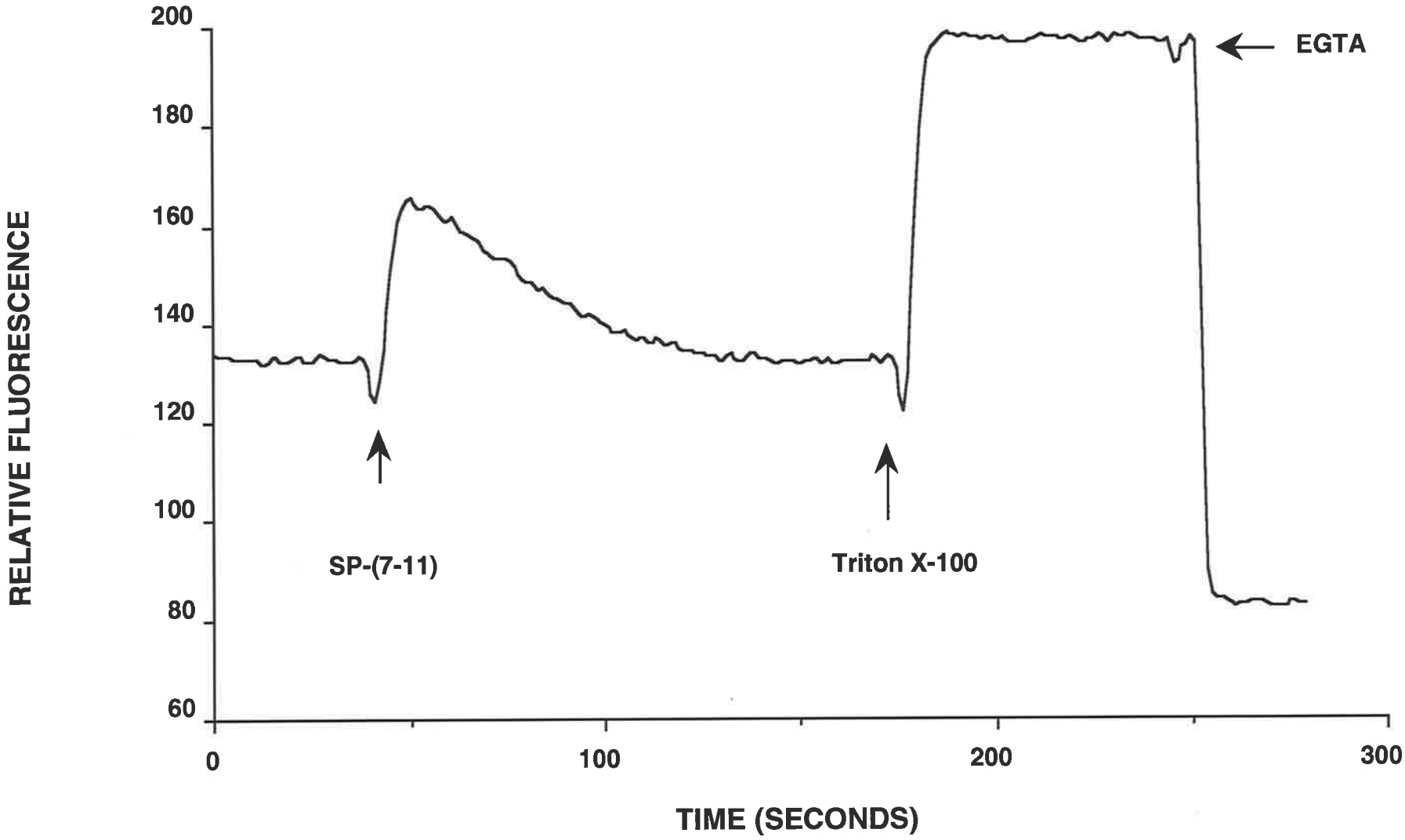

RELATIVE FLUORESCENCE
200
180
160
140
120
100
80
60
0
100
200
300
TIME (SECONDS)
SP-(7-11)
Triton X-100
EGTA

**Figure 5.8. Chondrocytes are refractory to a second stimulus of SP-(7-11).**

Following the initial stimulus of SP-(7-11) ($10^{-6}$ M) at 45 seconds, the subsequent addition of SP-(7-11) at 150 seconds did not alter intracellular calcium concentration.

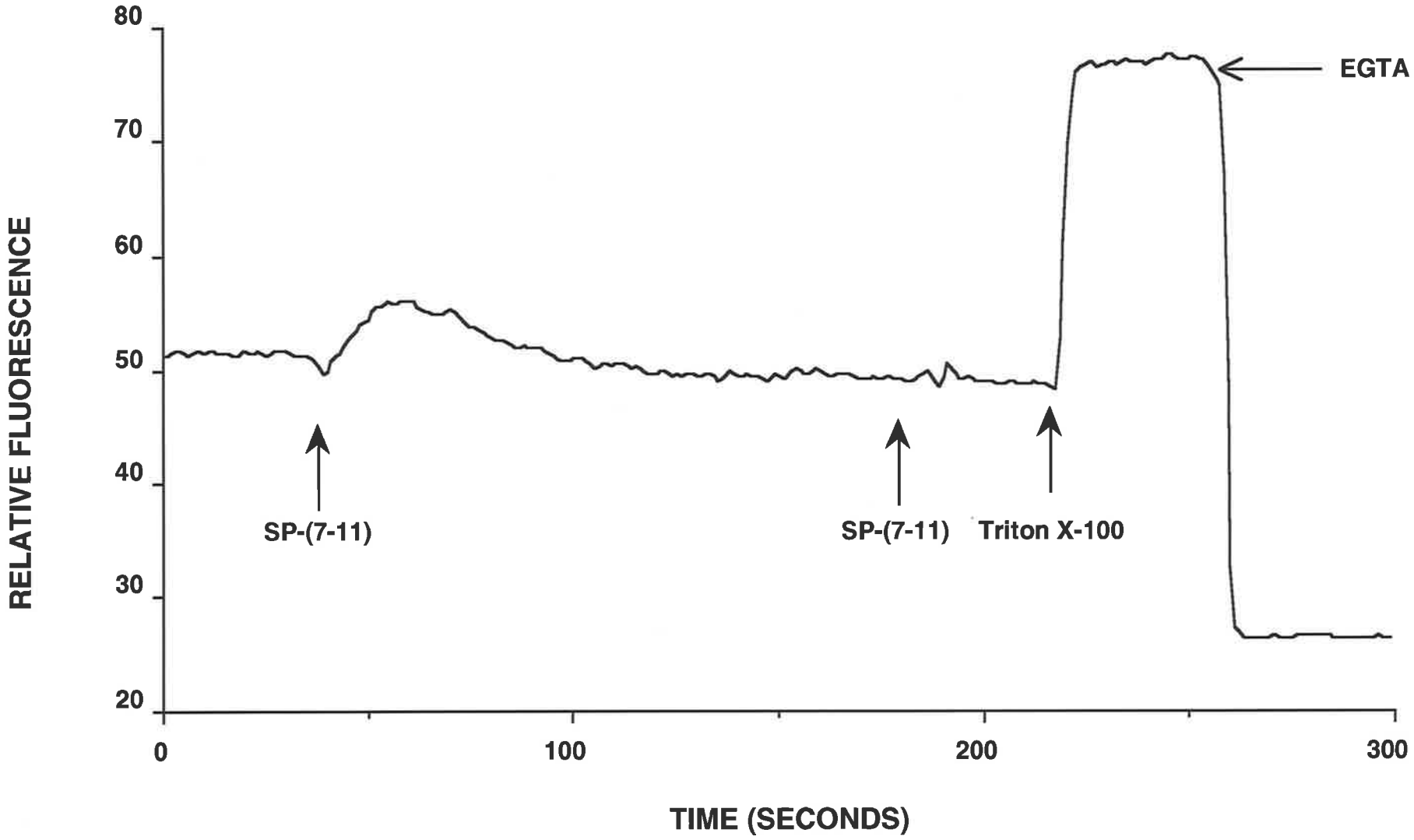
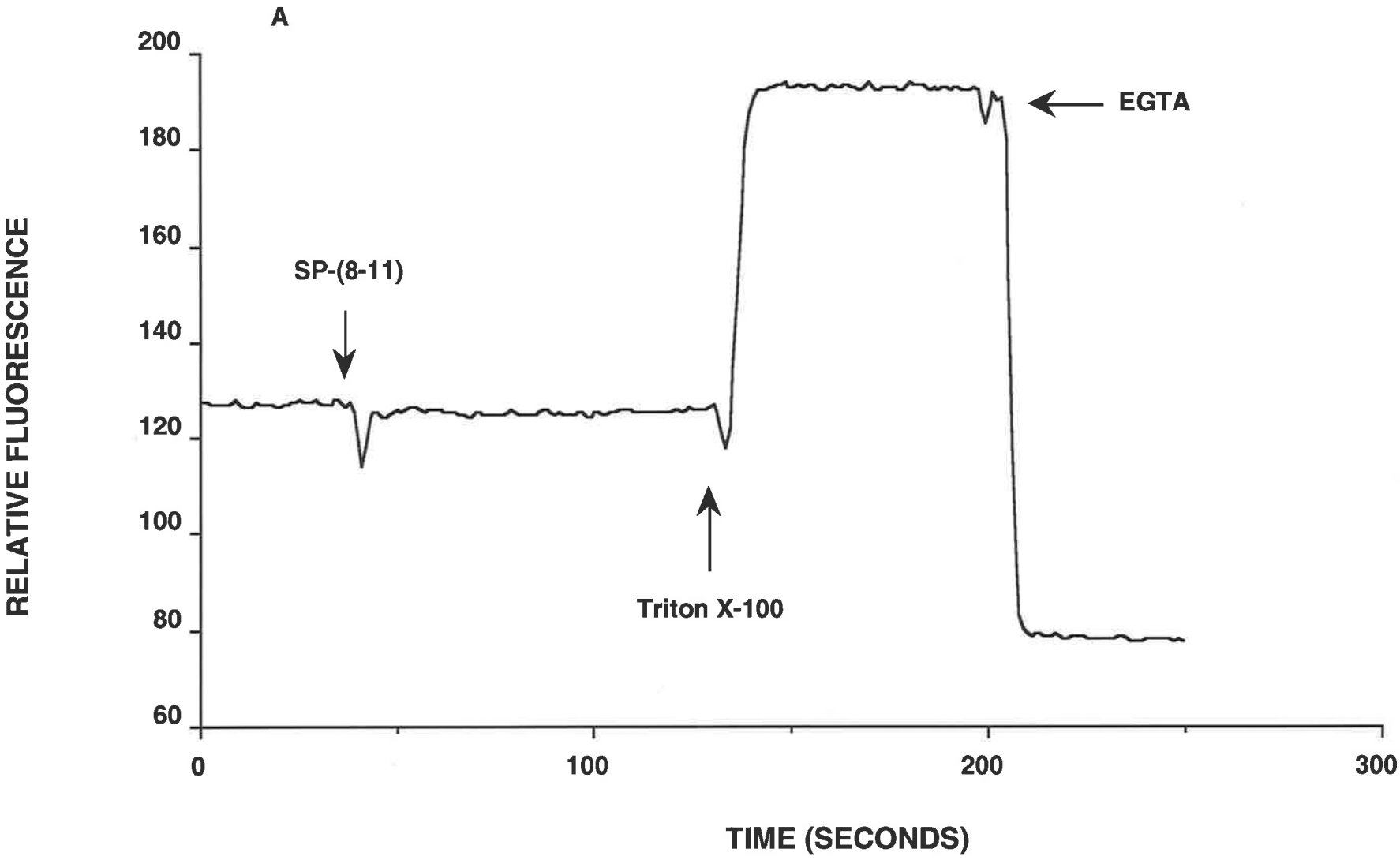

80
70
60
50
40
30
20
RELATIVE FLUORESCENCE
0
100
200
300
TIME (SECONDS)
SP-(7-11)
SP-(7-11)
Triton X-100
EGTA

**Figure 5.9. Effect of the C-terminal peptides SP-(8-11) and SP-(9-11) on intracellular calcium concentration.**

**(A)** $10^{-5}$ M SP-(8-11) and **(B)** $10^{-5}$ M SP-(9-11). Figures are representative of several peptide concentrations and chondrocyte preparations.


A
200
180
160
140
120
100
80
60
RELATIVE FLUORESCENCE
SP-(8-11)
Triton X-100
EGTA
0
100
200
300
TIME (SECONDS)

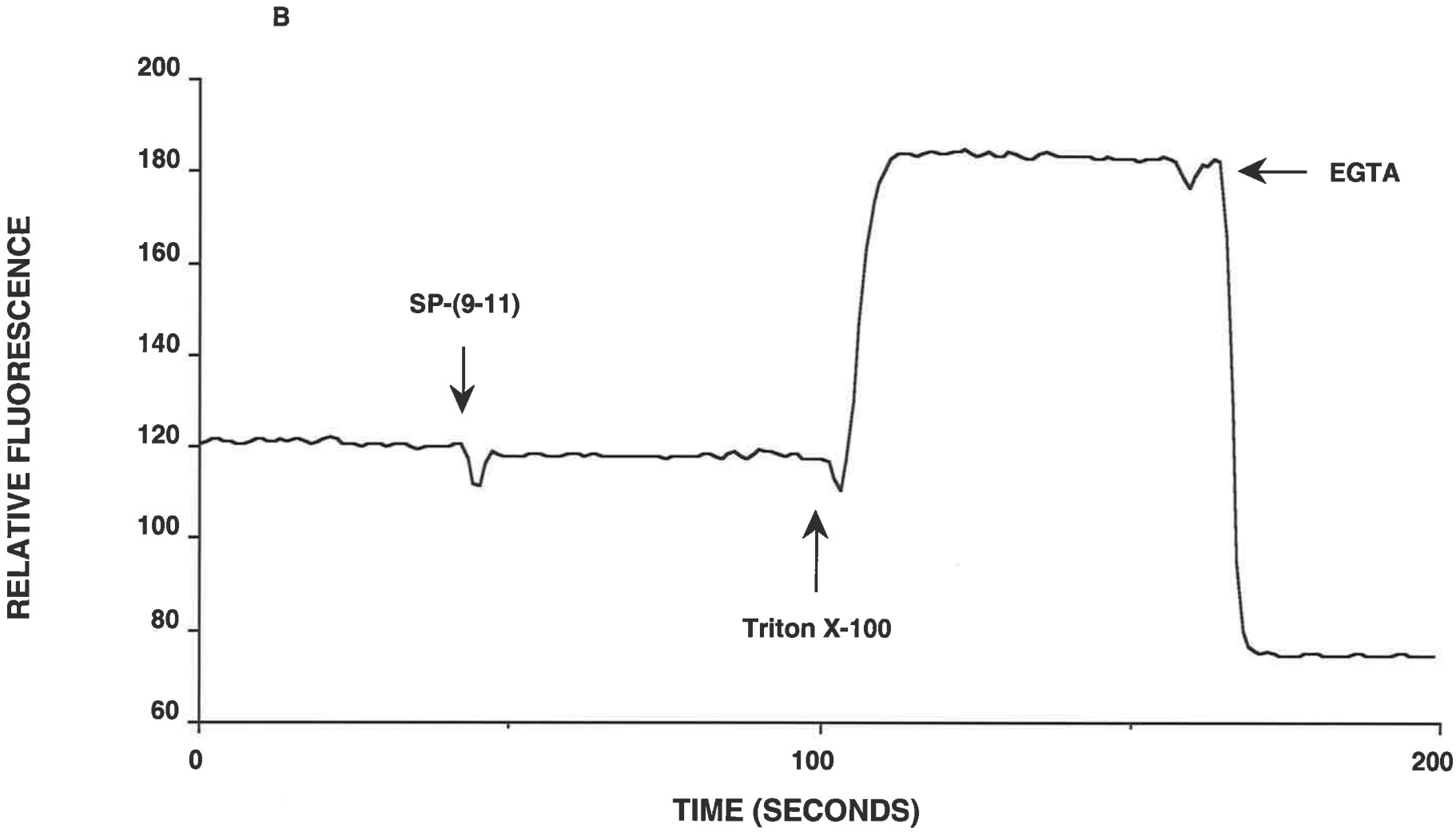

B
200
180
160
140
120
100
80
60
RELATIVE FLUORESCENCE
SP-(9-11)
Triton X-100
EGTA
0
100
200
TIME (SECONDS)

**Figure 5.10. Preincubation with EGTA abolishes the effect of SP-(7-11) on intracellular calcium.**

FURA-2AM loaded chondrocytes were preincubated for 1 minute in the presence of 2 mM EGTA before the addition of $10^{-5}$ M SP-(7-11).

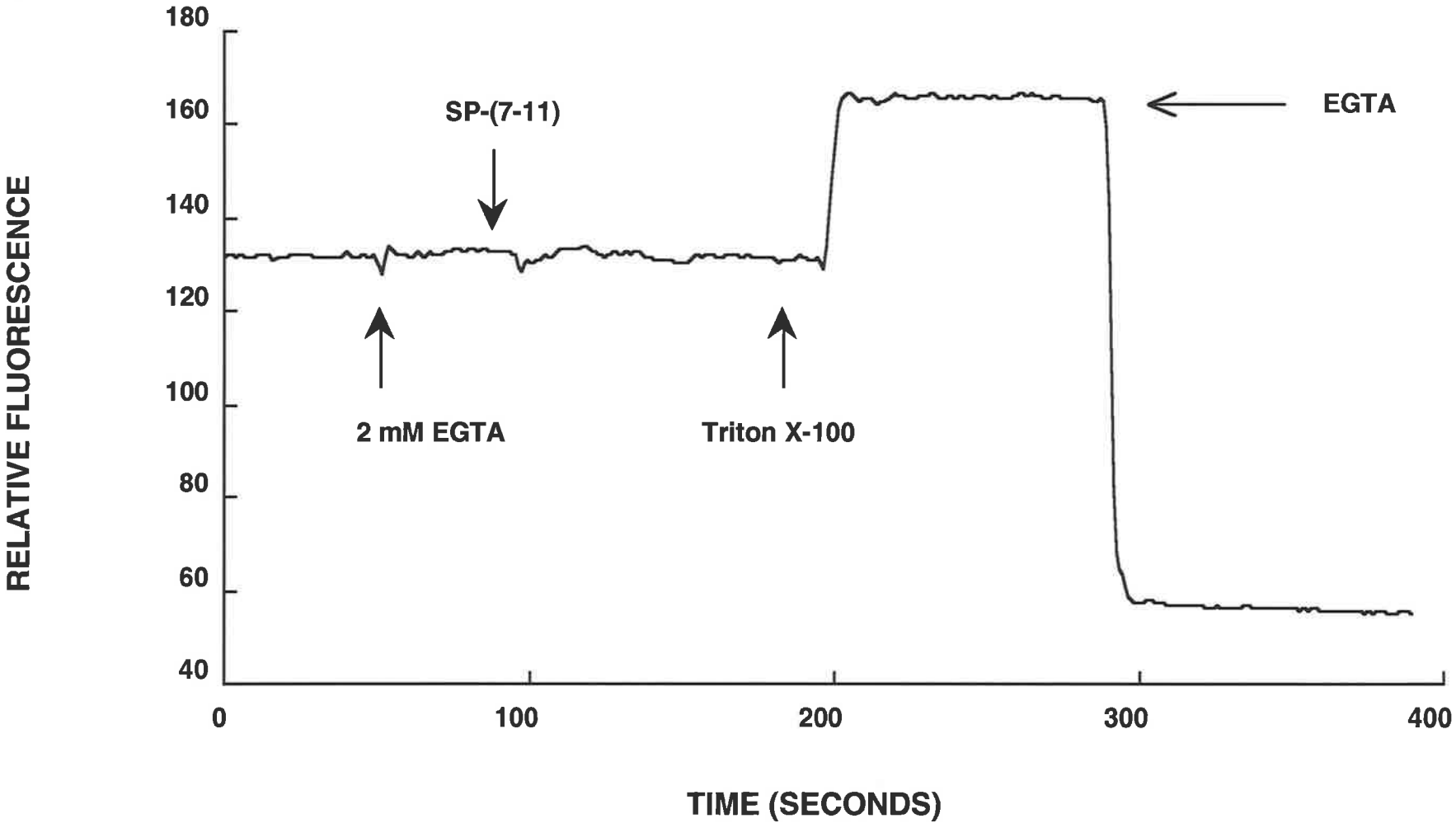

RELATIVE FLUORESCENCE
180
160
140
120
100
80
60
40
0
100
200
300
400
TIME (SECONDS)
SP-(7-11)
EGTA
2 mM EGTA
Triton X-100

**Figure 5.11. *f*MLP does not alter intracellular calcium concentration in bovine chondrocytes.**

The addition of $10^{-6}$—$10^{-4}$ M *f*MLP to chondrocytes failed to alter intracellular calcium concentration. This figure represents stimulation with of $10^{-6}$ M *f*MLP only.

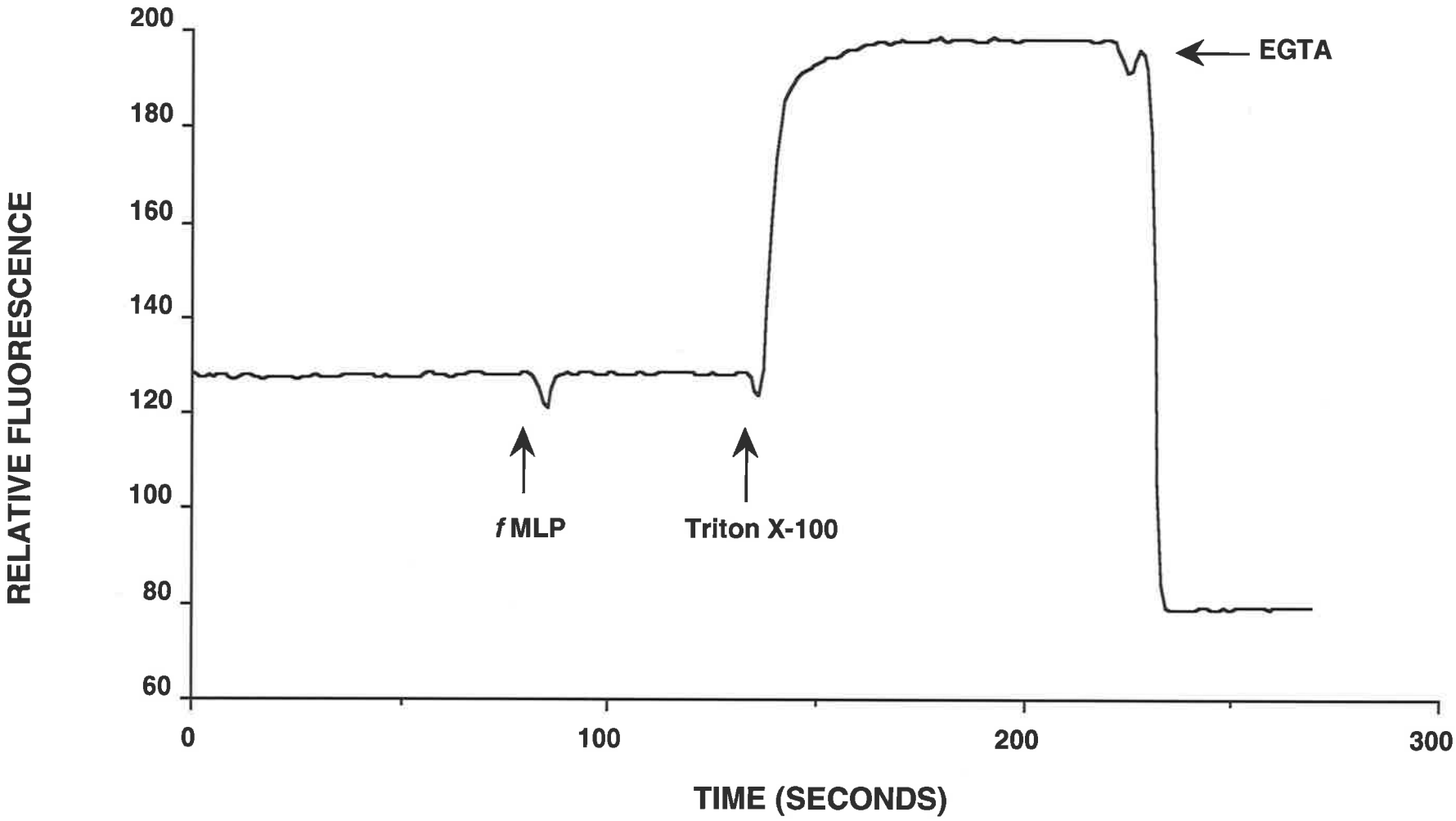
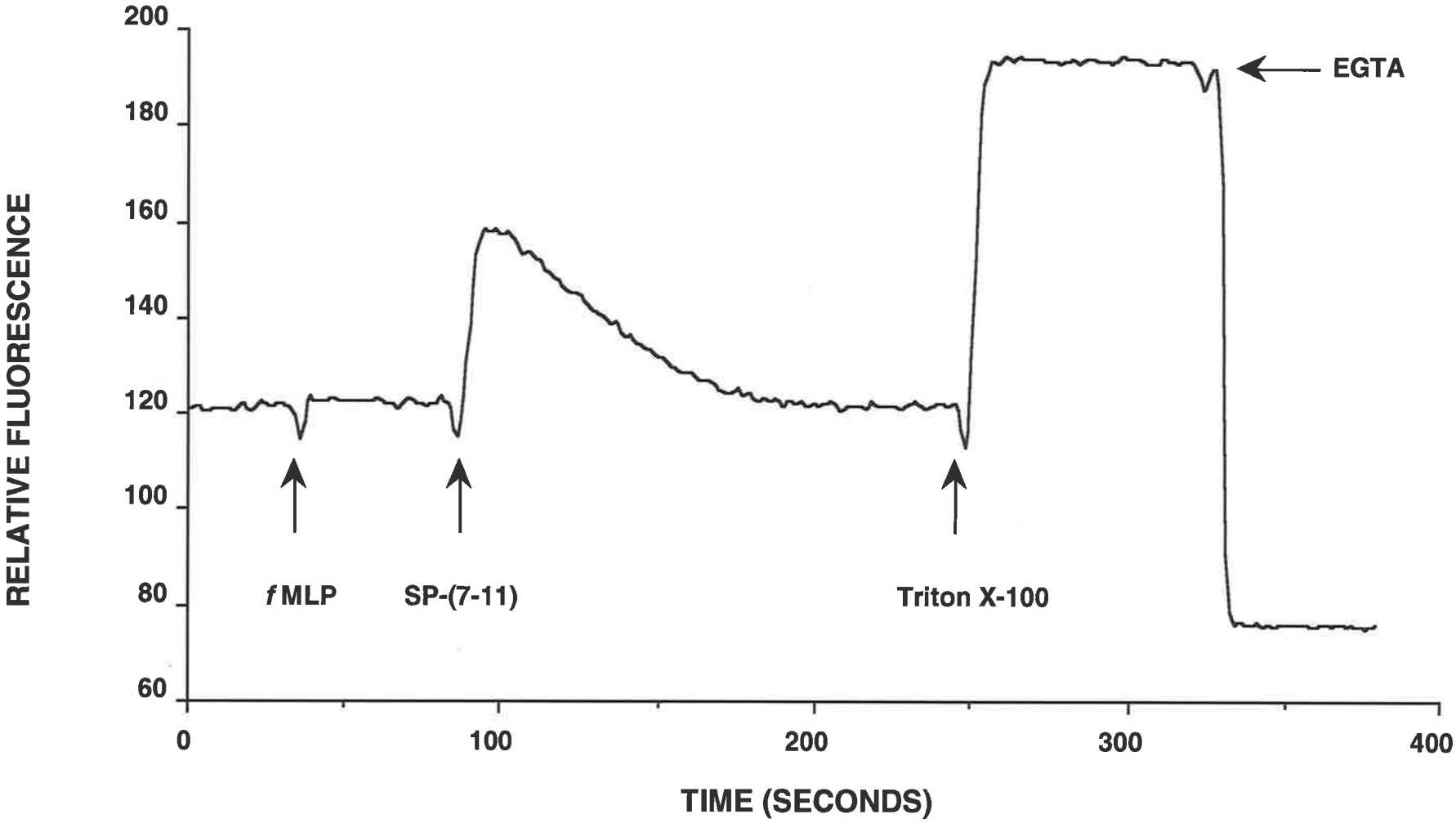

RELATIVE FLUORESCENCE
200
180
160
140
120
100
80
60
0
100
200
300
TIME (SECONDS)
EGTA
fMLP
Triton X-100

**Figure 5.12. SP-(7-11) does not mediate its effect on $[Ca^{2+}]_i$ via the *f*MLP receptor.**

No change in intracellular calcium was observed when *f*MLP ($10^{-6}$ M) was added to FURA -2 AM loaded chondrocytes. However the chondrocytes responded maximally to $10^{-5}$ M SP-(7-11).


RELATIVE FLUORESCENCE
200
180
160
140
120
100
80
60
0
100
200
300
400
TIME (SECONDS)
EGTA
fMLP
SP-(7-11)
Triton X-100

effect was not due to a toxic effect of EGTA on chondrocytes as further experiments demonstrated that chondrocytes which had been exposed to 2 mM EGTA for 24 hours could still exclude trypam blue and synthesize prostaglandins (data not shown).

### *4. Could SP-(7-11) mediate its effect through the fMLP receptor?*

Since SP-(7-11) and *f*MLP share several amino acids the possibility that SP-(7-11) could mediate its effect on $[Ca^{2+}]_i$ via *f*MLP receptors was examined. When $10^{-5}$ M *f*MLP was added to FURA-2 AM loaded chondrocytes no significant change in $[Ca^{2+}]_i$ was detected (figure 5.11). Changes in the concentration of *f*MLP did not alter this lack of response. The sane chondrocytes still responded to $10^{-5}$ M SP-(7-11) following addition of *f*MLP and a rest period of 50 seconds (Figure 5.12). The same batch of *f*MLP increased $[Ca^{2+}]_i$ in human neutrophils by several fold at $10^{-5}$ M (Murphy, G. Rheumatology Unit, Queen Elizabeth Hospital, Adelaide, South Australia, personal communication), suggesting that the lack of effect was not due to an absence of biological activity of *f*MLP.

## D. DISCUSSION

The studies with the fluorescent probe FURA-2 AM demonstrate that chondrocytes alter $[Ca^{2+}]_i$ in response to the C-terminal fragment of SP, SP-(7-11). This response consists of an immediate rise in $[Ca^{2+}]_i$ which is extracellular in origin since it was completely inhibited in the presence of extracellular EGTA.

These studies on $[Ca^{2+}]_i$ changes, provide a mechanism through which SP-(7-11) may increase the production of both $PGE_2$ and MMP-I. The production of $PGE_2$ is dependent on the enzyme phospholipase $A_2$ which releases arachidonic acid from phospholipids. Phospholipase $A_2$ activity is regulated by calmodulin (Moskowitz et al 1983). Calmodulin activation is regulated in turn, by intracellular calcium. It has been demonstrated that incubating chondrocytes in the presence of extracellular EGTA and SP-(7-11) abolishes the effect on $PGE_2$ production. Therefore the effect of SP-(7-

11) on $PGE_2$ production in chondrocytes appears to be coupled with increased $[Ca^{2+}]_i$ .

Collagenase production also appears to be linked with intracellular calcium changes in rabbit articular chondrocytes (Nolan et al 1988). Inhibitors of intracellular calcium movement prevented the synthesis of collagenase suggesting that alteration in $[Ca^{2+}]_i$ were linked to collagense synthesis. Whether this phenomenon was receptor-mediated or resulted from the direct interaction of SP-(7-11) with G proteins was not determined. However it appears that the amino acid sequence Phe-Phe-Gly-Leu-Met-$NH_2$ is important for this effect on collagenase, $PGE_2$ and $[Ca^{2+}]_i$. It is possible that the full length sequence of SP may interfere with the interaction of the peptide with a putative SP-(7-11) receptor molecule. Further studies to determine the effect of specific NK-1 receptor antagonists such as CP-96,345 on the biological effects of SP-(7-11) will help to determine whether SP-(7-11) acts via NK-1 receptors.

The possibility that G protein activation may be involved in the regulation of calcium channel activity in bovine chondrocytes warrants further investigation. G protein activation has been implicated in a number of intracellular events including alterations to $[Ca^{2+}]_i$ (Tsien 1992). Indeed the a subunit of the G protein complex has been shown to directly interact with voltage-dependent calcium channels (Tsien 1992; Sternweis and Pang 1990). Whether further intracellular signalling is required for modulation of calcium channel activity or simply the dissociation of the a subunit from the G protein complex and its subsequent interaction with proteins of the calcium channel complex, remains to be determined. G protein regulation by SP-(7-11) would be consistent with the observation that pertussis toxin, which ADP-ribosylates G proteins, was able to inhibit the increase in $PGE_2$ secretion induced by SP-(7-11).

## E. SUMMARY

These studies demonstrated that chondrocytes respond rapidly to the C-terminal fragment SP-(7-11) by altering their $[Ca^{2+}]_i$ . This effect could be blocked by the removal of extracellular calcium, demonstrating that the source of calcium was

from outside the cell and not from mobilization from intracellular stores. The observation that the effect of SP-(7-11) was refractory to a second stimulus is consistent with similar studies on tachykinin receptors and suggests that the effect may be linked to G-protein activation.

# CHAPTER VI

# GENERAL DISCUSSION

# GENERAL DISCUSSION

The aims of the experiments described in this thesis were to investigate the effects of mammalian tachykinins, particularly SP and its metabolites on connective tissue cells of the joint, namely chondrocytes and to a lesser extent synoviocytes. In this study there are several major findings which have led to the formation of a new hypothesis for the role of SP in the joint.

The first major observation was that intact tachykinins had no effect on either chondrocyte or synoviocyte function. Substance P, NKA and NKB had no effect on bovine chondrocyte proteoglycan, $PGE_2$ and MMP-I secretion or $[Ca^{2+}]_i$. Furthermore these tachykinins had no effect on bovine synoviocyte $PGE_2$ synthesis. Although the results have not been presented in this thesis, chondrocytes isolated from ovine and porcine sources failed to alter their $PGE_2$ production in response to intact SP. Thus this lack of response to SP appears to hold true across several species.

The second major observation was that a C-terminal metabolite of SP, the SP-(7-11) fragment increased bovine articular chondrocyte $PGE_2$ and MMP-I secretion and $[Ca^{2+}]_i$ in a dose-dependent manner. The N-terminal fragments, SP-(1-4) and SP-(1-6) had no direct effect on $PGE_2$ secretion or $[Ca^{2+}]_i$. Furthermore the C-terminal fragments SP-(8-11) and SP-(9-11) were also without effect on chondrocyte intracellular calcium concentration. The technique for measuring $[Ca^{2+}]_i$ enabled direct comparison of the effect of different neuropeptides on the same cell-preparation under identical conditions. In both the $PGE_2$ assay and the intracellular calcium assay these fragments were not biologically active. These results suggest that chondrocytes specifically respond to the SP-(7-11) fragment and not to the full length peptide. The primary structure of SP and its metabolites both active and inactive are given in figure 6.1.

The observation that the SP-(7-11) fragment is biologically active whereas the full length molecule is not, requires further explanation. Classically the biological activity of SP is terminated by hydrolysis primarily by NEP. Differential effects of

**Figure 6.1. Amino acid sequences of SP and related mammalian tachykinins studied in this thesis and SP fragments achieved by neutral endopeptidase cleavage.**

Bold underlining indicates ability to induce chondrocyte $PGE_2$ and collagenase secretion and increase $[Ca^{2+}]_i$.

———— **ACTIVE**

---------- **INACTIVE**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Substance P | Arg | Pro | Lys | Pro | Gln | Gln | Phe | Phe | Gly | Leu | Met | $NH_2$ |
| SP-(1-4) | Arg | Pro | Lys | Pro | OH | | | | | | | |
| SP-(1-6) | Arg | Pro | Lys | Pro | Gln | Gln | OH | | | | | |
| SP-(7-11) | | | | | | Phe | Phe | Gly | Leu | Met | $NH_2$ | |
| SP-(8-11) | | | | | | | Phe | Gly | Leu | Met | $NH_2$ | |
| SP-(9-11) | | | | | | | | | Gly | Leu | Met | $NH_2$ |
| NKA | His | Lys | Thr | Asp | Ser | Phe | Val | Gly | Leu | Met | $NH_2$ | |
| NKB | Asp | Met | His | Asp | Phe | Phe | Val | Gly | Leu | Met | $NH_2$ | |

C- and N-terminal SP fragments have been reported on neutrophil and mast cell function. For example the N-terminal peptide of SP enhances human neutrophil phagocytosis (Bar-Shavit et al 1980; Shanahan et al 1986) and the C-terminal peptide induce the respiratory burst (Wozniak et al 1989; Serra et al 1988). Furthermore SP and the C-terminal fragments SP-(4-11) and SP-(6-11) induced an increase in $[Ca^{2+}]_i$, $O_2^-$ generation and chemotaxis in human neutrophils in a dose-dependent manner. There were also differences in the potencies of SP-(6-11) and SP-(4-11). SP-(6-11) was found to be 7.7 fold more potent in increasing $[Ca^{2+}]_i$ concentration than SP-(4-11) whereas SP-(1-4) was 16.6 fold more potent in inducing $O_2^-$ generation that SP-(6-11) (Serra et al 1988). Therefore there are differential potencies in the biological activities of various C-terminal fragments of SP. The N-terminal fragments SP-(1-9) and SP-(1-4) were inactive in inducing any of these responses except phagocytosis (Iwamoto et al 1990) although SP-(1-4) was able to modulate the neutrophil response to SP-(7-11) (Wozniak et al 1993). Clearly these studies including my own, demonstrate that SP fragments may have greater agonist potencies than intact SP.

Whether the effect of SP-(7-11) is mediated by a receptor-dependent or receptor-independent mechanism needs further clarification. The possibility that cationic neuropeptides including SP have the ability to traverse the cell membrane lipid bilayer and interact directly with GTP-binding proteins has been proposed by Mousli et al (1990) based on several lines of evidence. Firstly, this model has been proposed to account for the effect of the N-terminal fragment SP-(1-4) on rat peritoneal mast cell histamine release in the absence of any specific $^{125}$I-SP binding to these cells (Devillier et al 1985). Secondly, in rat peritoneal mast cells, the release of histamine can be blocked by pretreating mast cells with pertussis toxin or benzalkonium chloride (Bueb et al 1990) which are specific inhibitors of $G_i$ of the G protein complex. Lastly it was shown that the concentration of SP-(1-4) required to trigger histamine release and increase $[Ca^{2+}]_i$ was greater than 1 μM (Mousli et al 1989). Studies by Fewtrell et al (1982) demonstrated that SP, not only released calcium from intracellular stores, but also induced an influx of calcium from outside the cell and furthermore, that this step

was essential for histamine release. The effects of SP-(1-4) on mast cells resembles the effects of SP-(7-11) on bovine articular chondrocyte function, ie SP-(7-11) increases chondrocyte $[Ca^{2+}]_i$ , the effect can be blocked by pertussis toxin and the concentration of SP-(7-11) needs to be greater than 1 μM.

The charge requirements for the putative receptor-independent actions of SP on mast cells are at least two positively charged amino acids in the N-terminus of SP. These charged amino acids interact with negatively charged components of the cell membrane (Mousli et al 1989) and allow penetration of SP through the lipid bilayer and subsequently the interaction of these positive charges with the C-terminal domain of the G protein α subunit. However SP-(7-11) does not possess these positively charged amino acids. The C-terminal of SP is thought to be important in the binding of SP to NK-1 receptors ( Regoli et al 1988 a & b ; Fong et al 1992,). The third intracellular cytoplasmic loop of the NK-1 receptor is thought to interact with the C-terminal domain of the α subunit of G-proteins (Huang et al 1990). Therefore G protein involvement can be invoked in both receptor-dependent and receptor-independent actions of SP.

Since NK-1 receptor activation is dependent on the C-terminal domain of SP, it may be possible that SP-(7-11) is simply a more potent agonist than intact SP and binds to putative NK-1 receptors on chondrocyte membranes. This could be possible since the non-peptide NK-1 antagonist, CP-96,345, blocks the effect of SP-(7-11) but not IL-1α on $PGE_2$ production. Therefore, although SP-(7-11) shares similar biological effects on chondrocytes as SP-(1-4) does on mast cells, the mismatch of charge and structural requirements of SP-(7-11), suggests a possible receptor mediated action of SP-(7-11) on bovine articular chondrocytes.

If NK-1 receptors are present on chondrocytes why does SP itself have no effect on chondrocyte function? The interaction of SP with other molecules or binding proteins in serum or in tissue culture is largely unknown. Possibly, *in vitro* , the action of SP is hindered by interactions with negatively charged groups on connective tissue molecules surrounding chondrocytes or by interaction with other proteins secreted by

chondrocytes which inhibit receptor /ligand interaction. Possibly SP-(7-11) has higher affinity for the NK-1 receptor than SP. Furthermore, due to its lack of positive charge SP-(7-11) is not sterically hindered by either matrix molecules or molecules which bind to the SP receptor. However these latter types of interactions, are unlikely to be responsible, since SP-(7-11) caused an immediate increase in $[Ca^{2+}]_i$ in freshly isolated chondrocyte suspensions. In these circumstances chondrocytes do not have the extracellular matrix which surrounds them *in vivo* and *in vitro* after a period in culture. Thus non-specific binding of tachykinins to these extracellular matrix proteins and sugars seems unlikely.

A second alternative is that NK-1 receptor numbers may be very low on chondrocytes and SP-(7-11) may simply have higher receptor affinity than SP. Therefore the hypothesis that SP-(7-11) interacts with NK-1 receptors on chondrocyte should be investigated in more detail.

## A NEW HYPOTHESIS

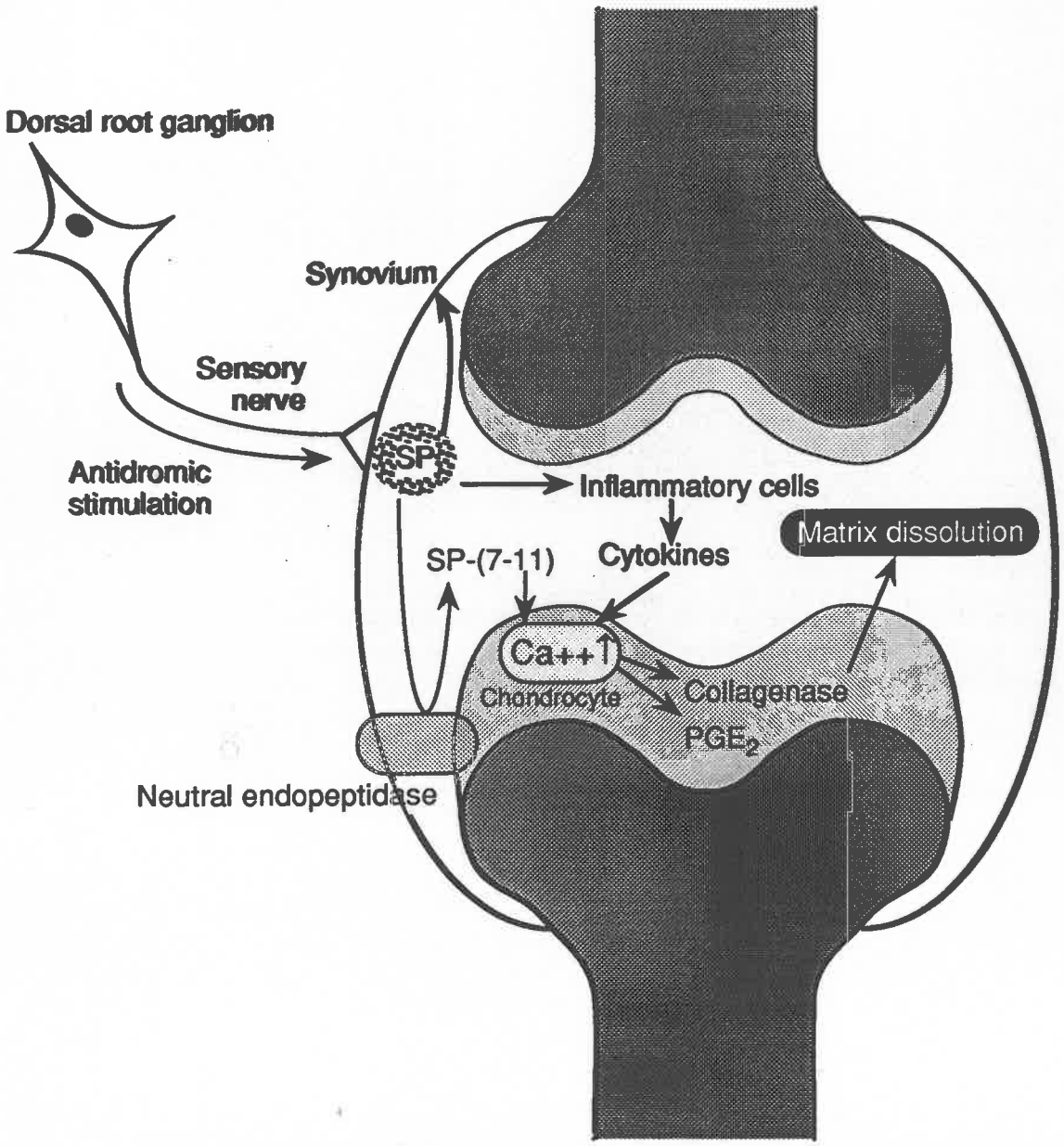
The findings in this thesis direct attention to the importance of SP metabolism in the joint. Most studies in the field of tachykinins and the joint have concentrated on the role of SP in the joint at the expense of SP fragments. As a result of my studies the production of SP-(7-11) in the joint warrants further analysis.

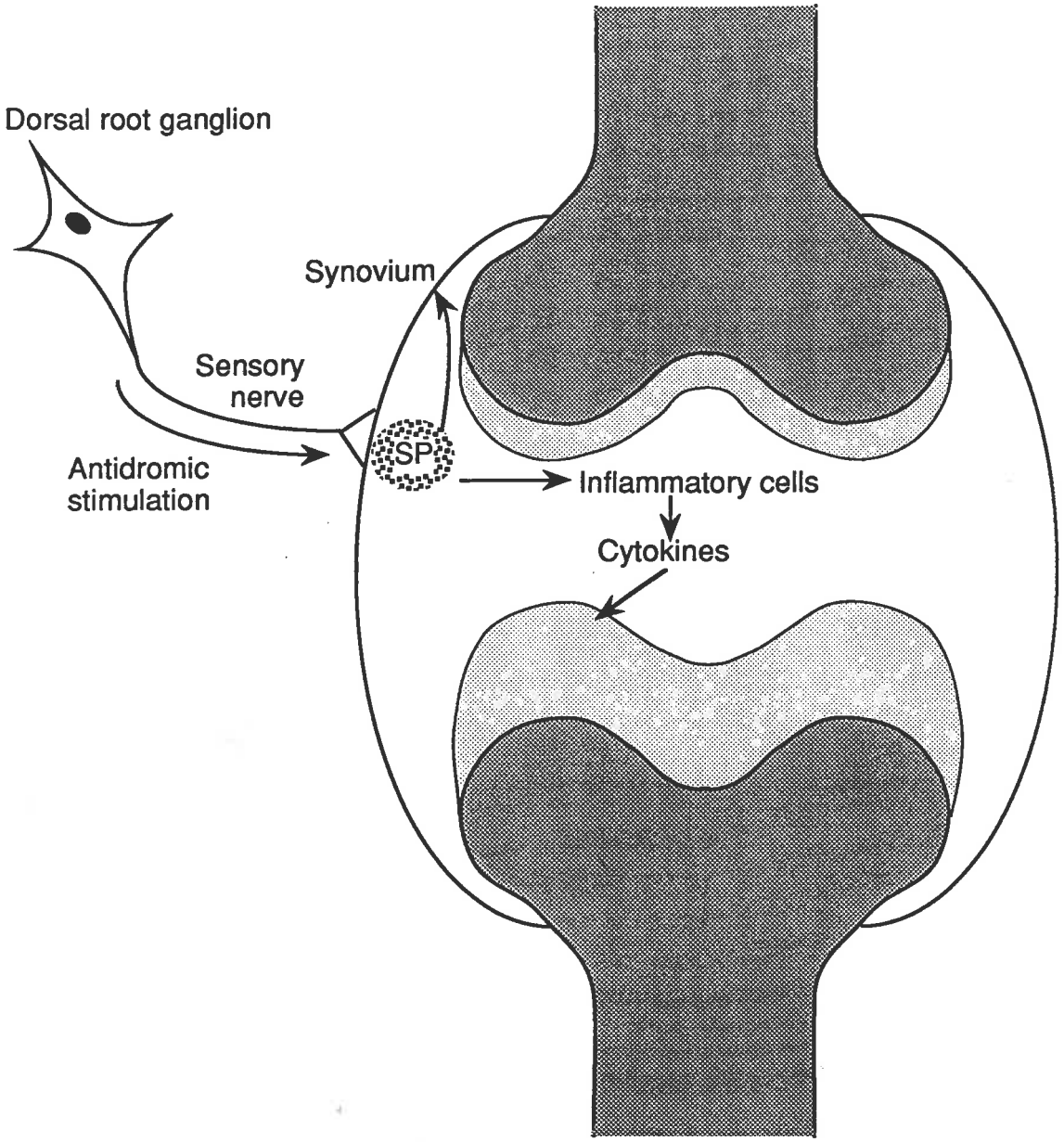
The new hypothesis formed as a result of this thesis is outlined in figure 6-2. The first figure relates to a model for the role of tachykinins in the joint before these studies (Figure 6.2a). Substance P is synthesized in the dorsal root ganglion and is released via antidromic stimulation of sensory nerves into the joint and interacts with synoviocytes to increase $PGE_2$, metalloproteinase and synovial proliferation to accelerate the formation of pannus (Lotz et al 1987). In this model SP metabolism was not considered. Furthermore I could not replicate these results.

The second diagram outlines a new concept for the role of tachykinins in the joint (Figure 6.2b). In this model SP is synthesized in the dorsal root ganglion and

**Figure 6.2. Comparison of old and new hypothesis for the role of SP in the joint**

(6a.) Diagram of role of SP in the joint before this study. (6b) Overlay sheet shows the hypothesis which has been generated as a result of this study. SP is rapidly cleaved by synoviocyte associated NEP, NEP in the synovial fluid and possibly NEP on the cell surface of chondrocytes, to generate SP-(7-11). SP-(7-11) then interacts with chondrocytes to increase intracellular calcium concentrations and subsequently MMP-I and $PGE_2$ production.


Dorsal root ganglion
Synovium
Sensory nerve
Antidromic stimulation
SP
Inflammatory cells
Cytokines
Matrix dissolution
SP-(7-11)
Ca++↑
Chondrocyte
Collagenase
PGE2
Neutral endopeptidase


Dorsal root ganglion
Synovium
Sensory nerve
SP
Antidromic stimulation
Inflammatory cells
Cytokines

upon release from sensory nerves would come into contact with NEP present on synovial cells in the synovium. NEP would rapidly degrade SP released locally into the synovium and it is likely that any SP in the synovial fluid would also be rapidly cleaved into a number of fragments. To date the metabolites of SP in synovial fluid have not been quantified or fully characterised. However, cleavage of SP by NEP between Gln-6 and Phe-7 would be expected, with generation of SP-(7-11). Other sites of SP metabolism in the joint would be the synovial fluid and possibly within the cartilage at the surface of the articular chondrocytes. Human neutrophils which are present in high numbers in synovial fluid possess cell-membrane bound NEP as well as high concentrations of intracellular cathepsin G (Malfroy et al 1978). Cathepsin G is capable of cleaving SP, however the cleavage site is between Phe-7 and Phe-8 generating the fragments SP-(1-7) and SP-(8-11), neither of which are biologically active on bovine articular chondrocytes. Therefore in this model it is suggested that regulation of NEP production in the joint may be a critical factor in the formation of the SP-(7-11) fragment. Whether SP-(7-11) is the predominant metabolite formed in the joint is currently not known.

The observation that NEP levels are elevated in synovial fluid from patients with RA (Matucci-Cerinic 1992) compared with OA suggests that NEP synthesis may be under the control of inflammatory mediators which are present in the RA fluids. The synthesis of NEP can be induced by several extracellular factors including growth factors, tumour promoters and cytokines (Herron et al 1986). The synthesis of NEP on synoviocytes may be increased by cytokines such as IL-1$\alpha$ present in inflamed synovial fluids Articular chondrocytes have not been examined as a source of NEP in the joint and this possibility should be investigated since it would provide an important site at which SP could be rapidly cleaved into SP-(7-11).

The low molecular weight of SP-(7-11) is below the exclusion limit of articular cartilage and therefore this peptide would have access to and could interact with chondrocytes. The local concentrations of SP-(7-11) in the extracellular environment of chondrocytes has not been determined, however my studies indicate that if the

concentration is greater that 1 μM, then SP-(7-11) could induce chondrocytes to produce MMP-I and possibly MMP-3 and $PGE_2$ but without effect on proteoglycan production *in vivo*. Therefore SP-(7-11) would contribute to the loss of the articular cartilage matrix seen in rheumatoid arthritis by the production of metalloproteinases, and would contribute to the exacerbation of joint inflammation by stimulating $PGE_2$ production. The cytokine IL-1α may enhance this response and possibly other cytokines which have not been investigated here, may interact with neuropeptides at a number of different levels in joint pathology. The production of $PGE_2$ and collagenase appears to be regulated in chondrocytes by alterations in $[Ca^{2+}]_i$. This does not appear to be the case with proteoglycan synthesis as SP-(7-11) had no effect on proteoglycan production.

**Further questions arising from these studies**

The observation that SP-(7-11) alters chondrocyte function suggests that the biological activity of SP in the joint may be regulated by NEP which can generate this fragment.

These studies have led to the formation of the following questions concerning the role of SP in the joint:-

1. Can SP-(7-11) be found in synovial fluid and does the quantity of this fragment increase in inflamed synovial fluids?
2. What are the sources of NEP in the joint? Do chondrocytes synthesize and express NEP on their cell surfaces or do chondrocytes secrete NEP?
3. What extracellular factors regulate NEP production in chondrocytes and/or synoviocytes?
4. Are tachykinin receptors expressed on chondrocytes and synoviocytes and which factors regulate this expression?

Although NEP has been localised in many tissues and its enzymic activities have been characterized in detail, the regulation of NEP expression is poorly understood and there is no information in regard to the connective tissues of the joint. The role of neuropeptide metabolites may therefore have wider biological implications in the joint than their non active 'precursors'.

## CONCLUDING REMARKS

The work described in this thesis outlines a novel mechanism of chondrocyte-mediated cartilage destruction induced by neuropeptides. Pro-inflammatory mediators derived from cells of the immune system, have been the focus of studies concerned with the modulation of both chondrocyte and synoviocyte function inflammatory joint disease. Therapies based on the inhibition of cytokine production or inhibition of cytokine/receptor interaction have received a great deal of attention. However since tachykinins now represent a new group of pro-inflammatory mediators in arthritis it is appropriate to investigate the effects of the new non-peptide tachykinin receptor antagonists on the outcomes of joint inflammation. Furthermore since the metabolites of SP are active whereas the native molecule is not, the metabolism of tachykinins, in particular SP, should be investigated in more detail. Tachykinin synthesis, metabolism and tachykinin receptor expression may be areas for intervention in the future treatment of the inflammatory joint diseases.

# APPENDIX A

## Composition of tissue culture medium

### A. TISSUE CULTURE MEDIUM FOR CHONDROCYTE CULTURE

Dulbecco's Modified Eagle's Medium (DMEM)
20 mM Hepes Buffer
20 mM L-Glutamine (added fresh)
1 % Penicillin/Streptomycin
10 % Heat Inactivated Foetal Calf Serum
(heat inactivated by incubating FCS at $56^0$ C for 30 minutes)
pH 7.4., 5% $CO_2$ at $37^0$ C

### B. TISSUE CULTURE MEDIUM FOR SYNOVIOCYTE CULTURE

Dulbecco's Modified Eagle's Medium (DMEM)
20 mM Hepes Buffer
20 mM L-Glutamine (added fresh)
1 % Penicillin/Streptomycin
1 % Fungizone
10 % Heat Inactivated Foetal Calf Serum
pH 7.4., 5% $CO_2$ at $37^0$ C

### C. PHOSPHATE BUFFERED SALINE ( x 10 stock)

| | |
|---|---|
| NaCl | 80 g/l |
| $KH_2PO_4$ | 2 g/l |
| KCl | 2 g/l |
| $Na_2HPO_4.12H_2O$ | 29 g/l |

pH 7.4

# APPENDIX B

## CALCULATIONS FOR DETERMINATION OF CHANGE IN $[Ca^{2+}]_i$

$$\text{Change in } [Ca^{2+}]_i \text{ (F1)} = \frac{F1-F0 \times 220\ nM}{F\ max - F1}$$

**where :** $F_1$ = Basal or resting $[Ca^{2+}]_i$

$F_2$ = Maximal stimulated $[Ca^{2+}]_i$

$F_0$ = $[Ca^{2+}]_i$ following addition of 2 mM EGTA

$F_{max}$ = Maximum calcium fluorescence following addition of 0.1% Triton X-100

$$\text{Change in } [Ca^{2+}]_i \text{ (F2)} = \frac{F_2 - F_0 \times 220\ nM}{F_{max} - F_2}$$

**Total Change in $[Ca^{2+}]_i$ nM = Change in $[Ca^{2+}]_i$ ($F_2$) — Change in $[Ca^{2+}]_i$ (F1)**

# APPENDIX C

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# ERRATUM

**1.** Table of contents I.B.1.,

"Innovation of the joint" should read "Innervation of the joint"

**2.** Page 77 line 9, "sane' should read "same".

**3.** A number of references which have been cited as abstracts have now been publishd in scientific journals and are listed below;

**P.92.** Blount,P. and Krause,J.E. (1993) The roles of the putative third cytoplasmic loop and cytoplasmic carboxyl tail of NK-1 and NK-2 receptors in agonist induced cAMP responses in stably transfected CHO cells. *Regulatory Peptides* 46(1-2), 447-449.

**P.107.** Krause,J.E., Takeda,Y., Blount,P.,Sachais,B.S., Raddatz,R., Bu,J-Y.,Chou,K.B. and Takeda,J. (1993) Structure, expression and second messenger mediated regulation of rat and human substance P receptors and their genes. *Regulatory Peptides* 246 (1-2), 59-66.

**P.112.** McLean,S., Ganong,A.,Seymour,P.A., Snider,R.M., Desai,M.C., Rosen,T., Bryce,D.K., Longo,K.P., Reynolds,L.S., Robinson,G., Schmidt,A.W., Siok,C. and Heym,J. (1993) Pharmacology of CP-99, 994; A non-peptide antagonist of the tachykinin NK-1 receptor. *J. Pharmacol. Exp.Ther.* 2687(1), 472-479.

**P. 112.** Menkes,C.L., Mauborgne,A., Laoussadi,S., Renoux, M., Bruxelle,J. and Cesselin,F. (1993) Substance P levels in synovial tissue and synovial fluid from rheumatoid arthritis and osteoarthritis patients. *J. Rheumatol.* 20(4), 714-717.

**P.113.** Mochizuki-Oda,N., Nakajima,Y., Nakanishi,S. and Ito,S. (1993) Substance P induced elevation of intracellular calcium in transfected Chinese hamster ovary cells: role of inositol trisphosphate. *Regulatory Peptides* 46(1-2), 450-452.

**P.115.** Nakanishi,S., Nakajima,Y. and Yokota,Y. (1993) Signal transduction and ligand binding domains of the tachykinin receptors. *Regulatory Peptides.* 46(1-2), 37-42.

**P.123.** Walsh,D.A., Salmon,M. and Polak,J.M. (1993) Autoradiographic localization and characterization of substance P binding sites in rat knee. *Regulatory Peptides* 46(1-2), 189-192.