The Role of Neuropeptides in Inflammatory Disease

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

by

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Awarded 1993

The thesis is dedicated to my wife Majka and my children, Michal, Monika and the smallest one, who we will welcome into our family very soon.

Declaration

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

Andrzej Wozniak (January 1992)

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Acknowledgements

This thesis would not be possible without the help of many people. I am most grateful to my main supervisors, Dr Henry Betts and Dr Ralph Scicchitano, for their encouragement, support and criticism throughout the time of experimental work and writing of this thesis. I have enjoyed very much the discussions we had, which inspired my thinking and guided me through the difficult parts of my research.

My sincere thanks to Dr Betts for correcting my writing and making the thesis much more pleasant to read.

I also wish to thank my third supervisor Dr G McLennan for his encouragement and advise.

Many thanks to Geraldine Murphy, for her encouragement and wonderful support throughout the difficult times and the good times, for helping me with some of my mammoth experiments, and for her excellent technical assistance with the leukotriene and intracellular calcium studies.

I also wish to thank the other people I have been working with, in particular Sinoula Apostolou from the Dept. of Thoracic Medicine and Neville DeYoung, and Eric Smith from the Dept. of Surgery in Royal Adelaide Hospital for their support and help. I have enjoyed every minute of my time working in the Rheumatology Research Laboratory. My warm thanks to Mary Rokicinski, Yi Ziang, for their technical assistance, and George Burgiel, Margaret Arstall, Lan Lan Zhang, Marina Sew Hoy, Janet Williams, for keeping the lab alive and nice to work in. Many thanks to Dr Bruce Kirkham for interesting and exciting discussions.

My thanks to the Renal Transplantation Immunology staff, for being both very friendly and helpful.

My special thanks to the blood donors, who include all the people mentioned above, and my wife, and many others, to whom I apologise for not mentioning here. I hope, I will not be remembered as the lab vampire. Thank you once more, without your kind donations this work would not be possible.

I would like to thank my wife Majka, and my children Michal and Monika for their love, encouragement and support.

During the last two years I have been the recipient of the Mary Paxton Gibson Scholarship awarded by the Arthritis Foundation of Australia, to whom I am very grateful.

I also would like to thank The Queen Elizabeth Hospital Research Foundation and the Joint Research Advisory Committee for their support.

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WOZNIAK A, McLENNAN G, BETTS WH, MURPHY G & SCICCHITANO R. Activation of human neutrophils by substance P: Effect on FMLP-stimulated oxidative and arachidonic acid metabolism and on antibody dependent cell-mediated cytotoxicity. *Immunology* 1989; **63**:359

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Summary

This thesis examines the contribution of tachykinins, in particular Substance P (SP), to the processes of inflammation. SP is a potent regulator of mature, human neutrophil function, and it increased neutrophil cytotoxic activity towards an antibody-coated target cells in a dose-dependent manner. The level of cytotoxic activity induced by SP was comparable to that described by a number of cytokines. In addition, SP facilitated *formyl*-methionyl-leucyl-phenylalanine (fMLP)-, phorbol myristate acetate (PMA)- and platelet activating factor (PAF)-stimulated neutrophil superoxide anion production (O_2^-) in a dose-dependent manner, but did not have an effect when the cells were stimulated with opsonised zymosan (OPZ) or calcium ionophore (A23187). SP also enhanced leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE) production by fMLP-stimulated neutrophils. In contrast to its stimulatory effects on ADCC, O_2^-, LTB_4 and 5-HETE production, SP did not have any stimulatory effect on neutrophil exocytosis and adhesion to human umbilical vein endothelial cells (HUVEC).

Neurokinin A (NKA), neurokinin B (NKB) and eledoisin (E) but not kassinin (K) have similar effects to SP in priming neutrophils for increased O_2^- production in response to fMLP. This similarity in activity was found to be due to the carboxy amino acid terminal end of these tachykinins being highly conserved. SP fragment 7-11 ($SP_{7\text{-}11}$) had the same priming effect as the whole molecule, whereas, the amino end fragment 1-4 ($SP_{1\text{-}4}$) inhibited the response to fMLP. All tachykinins studied increased neutrophil ADCC. In contrast to their effects on fMLP-induced O_2^- production, both SP fragments, $SP_{1\text{-}4}$ and $SP_{7\text{-}11}$, stimulated neutrophil ADCC and had a synergistic effect when used together.

SP increased intracellular free calcium concentration ($[Ca^{++}]_i$) in neutrophils, by mobilising calcium from both intracellular stores and by inducing a calcium influx. The increase in calcium was dose-dependent and occurred in the same range of SP concentrations that primed neutrophils for O_2^- production. Moreover, SP carboxy terminal fragment, $SP_{7\text{-}11}$, but not the amino terminal, $SP_{1\text{-}4}$, also stimulated an increase in $[Ca^{++}]_i$. These observations suggest, that calcium may play an important role in the priming phenomenon. In addition, SP enhanced protein kinase C translocation in fMLP-stimulated neutrophils in some experiments, but the importance of this finding to SP priming mechanisms remains unclear.

Bolton-Hunter labelled ^{125}I-SP bound specifically to neutrophils at 4°C and the binding was dose-dependent and saturable. Scatchard analysis of the data obtained from saturation isotherms indicated a single type of a binding site (132 sites per cell) with an apparent Kd of 5.4 x 10^{-10} M. However, the concentration of unlabelled SP required to assay the non-specific binding was very high (50μM), and the non-specific binding accounted for about 50% of the total binding. Therefore, the data suggest, that a possible receptor for SP may exist on human neutrophils, but it does not exclude the possibility of non-specific interactions of SP with the cell membrane.

Finally, the effect of SP on ADCC by neutrophils and eosinophils isolated from peripheral blood of normal and asthmatic subjects was examined. The baseline (unstimulated) ADCC was significantly higher in asthmatics than normals. SP-stimulated ADCC was greater in asthmatic subjects than normals. The net increase in ADCC (*net*-ADCC) was significantly greater in asthmatics than normals confirming that the higher stimulated ADCC in asthmatics was not due solely to the higher baseline ADCC.

In support of the hypothesis, that enhanced ADCC response in asthmatic subjects was due to the modulation of neutrophil function by cytokines *in vivo*, synergism between SP and recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) was demonstrated *in vitro*. Moreover, a modest correlation ($r = 0.5$, $p < 0.025$, $n = 20$) was demonstrated between SP stimulated ADCC and bronchial hyperresponsiveness assessed as the PC_{20} (bronchial hyperresponsiveness to inhaled histamine). SP failed to stimulate eosinophil ADCC although these cells were responsive to rhGM-CSF.

In addition, the effects of recombinant human interleukin-8 (rhIL-8), and the interactions between SP and rhIL-8, on neutrophil O_2^- production were investigated. rhIL-8 did not stimulate neutrophil O_2^- production on its own, but like SP, primed neutrophils for an enhanced response to other stimuli, such as fMLP, PMA and PAF. rhIL-8 increased $[Ca^{++}]_i$ by mobilising calcium from internal stores and by increasing calcium influx. The increase in $[Ca^{++}]_i$ was dose dependent and occurred in the same range of rhIL-8 concentrations that primed neutrophils for O_2^- production. In addition, rhIL-8 enhanced the fMLP-stimulated increase in $[Ca^{++}]_i$. However, neither SP nor IL-8 enhanced each others priming effect when used together to prime the O_2^- response of fMLP-stimulated neutrophils, suggesting, that there is no interaction between these two mediators.

Abbreviations

5-HETE:	5-hydroxyeicosatetraenoic acid
A23187	calcium ionophore
AA:	arachidonic acid
ADCC:	antibody-dependent cell-mediated cytotoxicity
BCM:	bladder carcinoma cell line U5637 conditioned medium
BHR:	bronchial hyperresponsiveness
BSA:	bovine serum albumin
DG:	diacylglycerol
DPBS:	modified Dulbecco's phosphate-buffered saline
E:	eledoisin
EL:	elastase
FCS:	fetal calf serum
fMLP:	*formyl*-methionyl-leucyl-phenylalanine
rhGM-CSF:	recombinant human granulocyte-macrophage colony-
stimulating	factor
HUVEC:	human umbilical vein endothelial cells
rhIL-8:	recombinant human interleukin-8
IP_3:	inositol 1, 4, 5-trisphosphate
$[Ca^{++}]i$	intracellular free calcium concentration
K:	kassinin
LPS:	lipopolysaccharide
LTB_4:	leukotriene B_4
MPO:	myloperoxidase
NKA:	neurokinin A
NKB:	neurokinin B
O_2^-:	superoxide anion
PAF:	platelet activating factor
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC:	protein kinase C
PLC:	phospholipase C
PMA:	phorbol myristate acetate
SOD:	superoxide dismutase
SP:	substance P
SP_{x-y}	substance P fragment x-y
TNF:	tumour necrosis factor
TNP:	trinitrophenyl

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Chapter 1

Introduction

1.1 Neuropeptides and Inflammation: The Contribution of the Nervous System to Inflammatory Disease

In the past few years, considerable progress has been made on the immunomodulatory effects of neuropeptides and their potential role in inflammation. Neuropeptides are found in the sensory neurons, particularly in small unmyelinated, primary afferent fibres, innervating a wide variety of peripheral tissues such as skin, gastrointestinal tract, respiratory tract and joints. It has been postulated that neuropeptides could be released locally into inflammatory sites by axonal reflexes, following injury-induced activation of afferent fibres, where they may modulate immune function. Accumulating evidence from both *in vitro* and *in vivo* studies, suggests that the release of neuropeptides from peripheral sensory nerves, may contribute to many inflammatory diseases, such as rheumatoid arthritis, asthma and inflammatory bowel disease.

Of the many biologically active neuropeptides that are found in primary afferent fibres, most attention has focused on substance P (SP). This eleven amino acid peptide is a member of the tachykinin family of neuropeptides, and is found in high concentrations in afferent fibres innervating specific tissues, including lung mucosa and joint synovium, where it may be released by axonal reflexes. Once released into tissues, SP causes smooth muscle contraction or relaxation, vascular dilatation and plasma extravasation, all of which often accompany inflammatory processes. More importantly, however, SP has been shown to modulate immune responses, by recruiting and activating inflammatory cells. These findings suggest, that SP is a pro-inflammatory mediator and may contribute to the increased joint injury in rheumatoid arthritis and pathogenesis of asthma.

This review, provides an introduction to the role of tachykinins in inflammation and inflammatory disease. The first few sections describe the inflammatory process, with specific emphasis placed on the role and function of phagocytic cells in inflammation, in particular neutrophils, which are the main research interest of this thesis. The major functions (e.g., oxy radical production) and regulation of phagocytic cells by specific cytokines and mediators of inflammation, are discussed. The next section describes the up to date understanding of the signal transduction mechanisms in neutrophils, especially the intracellular pathways leading to the activation of NADPH oxidase and superoxide anion production. The following section on the role of neutrophils in inflammatory disease, rheumatoid arthritis and asthma, stresses their importance in tissue damage and exacerbation of disease. Finally, the family of tachykinins is discussed, their origin, tissue distribution, enzymatic control of their activity and most importantly, their role and contribution to inflammation and inflammatory disease, in particular rheumatoid arthritis and asthma.

1.2 Inflammation

Inflammation is a localised immune response induced by injury, that aims to eliminate the injurious agents and repair the damaged tissue. In the acute form (short duration), inflammation is characterised by a marked redness, swelling, heat and pain [Hurley 1983]. These symptoms are caused by the release of vasoactive substances from tissue cells that result in a dilatation of blood vessels, increased blood flow and exudation of fluid and plasma proteins into the surrounding tissue as well as the release of various destructive chemicals from the injurious agents and infiltrating leukocytes, predominantly neutrophils. Chronic inflammation is of longer duration, with prominant lymphocyte and macrophage infiltration in addition to neutrophils, and in some special circumstances, such as asthma, eosinophils. Marked proliferation of blood vessels and connective tissue is another feature of chronic inflammation. Whereas acute inflammation may resolve without scarring, a chronic inflammatory process often leads to substantial tissue damage and loss of function.

The reasons for perpetuation of the inflammatory process in various inflammatory diseases such as rheumatoid arthritis, asthma, psoriasis or inflammatory bowel disease, are still far from clear. Slow growing bacteria, retroviruses and impaired immune recognition (recognition of self as non-self) have been all postulated to be responsible for the persistence of the immune response [reviewed by Rook and Stanford, 1992]. However, in the search for the answer as to what causes chronic inflammation, we have learned a great deal about the pathology and the regulatory mechanisms of inflammation.

The immunologic reactions that occur in the early stages of inflammation, whether acute or chronic are probably similar, in that they both begin with the recognition of foreign antigens by specific antibodies and sensitised T lymphocytes. The initial reaction of antigen with specific T cells leads to the production of soluble mediators (cytokines), which are capable of

recruiting more inflammatory cells. These cytokines can regulate all stages of the inflammatory process by controlling cell influx, and by initiating, amplifying or inhibiting cell activation. For example, tumour necrosis factor released from activated monocytes (or tissue macrophages) and interleukin-8 from infiltrating neutrophils [Strieter et al, 1992], augment accumulation of neutrophils at inflammatory sites . These two cytokines increase the adherence [Gamble et al, 1985; Moser et al, 1989] of circulating neutrophils to vascular endothelium and promote their migration [Detmers et al, 1991; Leonard et al, 1991] to the local inflammatory site by chemotaxis. Other factors (e.g. granulocyte-macrophage colony stimulating factor) also activate neutrophil effector function [Lopez et al, 1986].

Under normal circumstances, the inflammatory process resolves when the injurious agents and damaged tissues are removed. The activity of cells at the inflammatory focus is inhibited and the influx of inflammatory cells from the circulation stops. This process is very complex and may involve interactions between a number of different mediators and modulators, whose activity may be regulated by many tissue-specific factors. An additional complexity arises from the observation that some mediators of inflammation are both pro- and anti-inflammatory, depending on time and a place of their action. For example, IL-8 can either promote or inhibit neutrophil influx into tissue sites by regulating their adherence to vascular endothelial cells, depending on wether it is released locally at tissue sites or into blood circulation [Leonard et al, 1991; Huber et al, 1991; Gimbrone et al, 1989; Hechtman et al, 1991].

1.3 The Role of Phagocytic Cells in Inflammation

The major phagocytic cells in man are the neutrophil leukocytes in the myeloid series and the monocyte and macrophages, fixed or free, in the mononuclear phagocyte series. Eosinophils are also phagocytic, although this may not be their major function.

1.3.1 *Neutrophils*

Neutrophils are "end cells" incapable of cell division and are the principal effector cells in the inflammatory reaction. They are particularly effective against bacterial and fungal infections, but also play a role in neoplasia [Baggiolini, 1984]. For example, patients with abnormal neutrophil oxidative metabolism suffer from recurrent life-threatening bacterial infections [Tauber, 1983]. Their short life is devoted primarily to migration to sites of inflammation, where they perform their phagocytic and digestive functions, through an interaction with antibody, complement, chemotactic factors and various cytokines [Jamuar and Cronkite, 1980].

Neutrophils are produced in the bone marrow, at a rate of about 10^{11} cells/day [Cartwright et al, 1964]. The *in vitro* production of neutrophils from bone marrow stem cells requires at least two factors, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [Metcalf, 1985a,b] . However, it is anticipated that *in vivo* many other stimulatory and inhibitory factors are involved.

As mentioned above, human neutrophils are the primary effector cells in inflammation and they are recruited in large numbers from the bloodstream to the inflammatory site [Jamuar and Cronkite, 1980]. The first events that occur involve neutrophil adherence to locally activated vascular endothelial

cells through specialised receptors with subsequent transendothelial migration [Atherton and Born, 1972; Hoover et al, 1978; Tonnesen et al, 1982, 1984; Butcher 1990]. Although these processes are not fully understood, a number of adherence molecules present on neutrophils and endothelial cells have been implicated. The expression of these molecules, the CD11/CD18 complex on neutrophils and ICAM-1, ICAM-2 and E-selectins on endothelial cells, is regulated by chemotactic factors and cytokines [Gamble et al, 1985; Moser et al, 1989; Furie and McHugh, 1989; Smith et al, 1988, 1989]. When they reach the inflammatory site, neutrophils phagocytose immune complexes and bacteria, opsonised by IgG immunoglobulins or complement fragments C3b and C3bi, via Fcγ, CR1 and CR3 receptors respectively [Ahearn and Fearon, 1989; Springer 1990; Ravetch and Kinet, 1991]. The final destruction process takes place inside the neutrophil through the release of toxic oxy radicals and the discharge of lytic enzymes into the phagocytic vacuole, the phagosome [Hirsch and Cohn, 1960; Weissmann et al, 1971]. Degranulation of enzymes to the external milieu may play a role in neutrophil-mediated killing of other cells, tissue destruction in chronic inflammatory disease and may be important in neoplasia [Falloon and Gallin, 1986; Gallin, 1985; Trush et al, 1985]. There are two major types of granules found in human neutrophils, the azurophilic and specific granules, and their contents are listed in Table 1.1.

The neutrophil story would not be complete, without mentioning their potential afferent immune and inflammatory function [reviewed by Lloyd and Oppenheim, 1992]. Recent demonstrations that neutrophils can synthesise MHC class 1 molecules [Neuman et al, 1992] as well as a range of immunomodulating cytokines, including IL-1-α and -β [Lindemann et al, 1988; Goh et al, 1989; Lord et al, 1991], TNFα [Dubravec et al, 1990], IL-6 [Cicco et al, 1990], IL-8 [Bazzoni et al, 1991; Strieter et al, 1992], and INF-γ [Shirafuji et al, 1990], suggest that they can modulate granulocyte, monocyte/macrophage, and T- and B-cell activities.

Azurophilic granules	Specific granules
Microbicidal enzymes	
Myeloperoxidase	Lysozyme
Lysozyme	
Neutral proteinase	
Elastase	
Cathepsin G	
Proteinase 3	
Acid hydrolases	
β-Glycerophosphatase	Collagenase
β-Glucuronidase	
N-Acetyl-β-glucosaminidase	
α-Mannosidase	
Cathepsin B	
Cathepsin D	
Other	
Cationic proteins	Lactoferin
Defensins	Vitamin B_{12}-binding proteins
Bactericidal permeability increasing proteins (BPI)	Plasminogen activator
	Histaminase
Azurophil-derived bactericidal factors (ADBF)	Receptors
	fMLP
	CR3

Table 1.1 Constituents of azurophil and specific granules from human neutrophils. (Adopted from Bainton, 1988).

1.3.1.1 *Oxidative metabolism in human neutrophils*

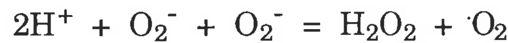
Activated human neutrophils produce oxy radicals that are essential for optimal microbicidal [Babior et al, 1973; Vel et al, 1984; Baggiolini 1984] and cytotoxic [Murray and Cohn, 1987] activity, and which play an important role in host defence. This is clearly demonstrated in patients with chronic granulomatous disease, which is characterised by a decreased or absent phagocytic oxidative burst (i.e. production of oxy radicals) [Tauber et al, 1983]. Patients with this disease suffer from recurrent bacterial infections. However, release of oxy radicals can also induce substantial tissue damage in the host [Sacks et al, 1978; Niwa et al, 1982; Weiss and LoBuglio, 1982] and perhaps could be even linked to carcinogenesis [Trush et al, 1985]. In addition to this, there are numerous reports that implicate oxy radicals in the regulation of inflammatory responses. For example, oxidation of lipids can produce effector molecules [Stossel et al, 1974]. Furthermore, oxy radicals can inactivate [Ossanna et al, 1987] or activate [Burkhardt et al, 1986; Shah et al, 1986] enzymes, and modulate the proliferation [Zoschke et al, 1984] and activity [El-hag and Clark, 1984] of other cells.

Many different stimuli can induce oxy radical generation in neutrophils, particularly, phagocytosis of opsonised bacteria (via CR1 and Fcγ receptors), immune complexes, aggregated IgG, as well as various chemotactic factors including the bacterial product formyl methionyl-leucyl-phenylalanine (fMLP), complement fragment C5a, platelet activating factor (PAF) and leukotriene B_4 (LTB_4) [Weening et al, 1975; Dewald and Baggiolini, 1985; Gerard et al, 1986; Till et al, 1982; Palmblad et al, 1984]. In addition, cytokines such as TNFα, TNFβ and GM-CSF, are also able to induce prolonged production of oxy radicals [Kapp and Zeck-Kapp, 1990] or to enhance the response to another stimulus [Lopez et al, 1986; Weisbart et al, 1987; Berkow et al 1987].

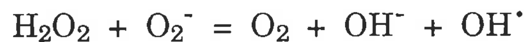
Stimulation of neutrophils activates a membrane-associated NADPH oxidase complex which converts oxygen to superoxide anion (O_2^-)-:

$$NADPH + 2O_2 = NADP^+ + H^+ + 2O_2^-$$

The O_2^- produced in this reaction is very unstable and may spontaneously dismutate to hydrogen peroxide (H_2O_2), which may be further converted (Haber-Weiss reaction) to hydroxyl radical ($OH^\bullet$):

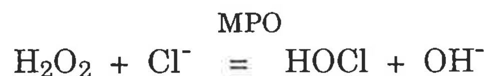


$$2H^+ + O_2^- + O_2^- = H_2O_2 + {}^\cdot O_2$$



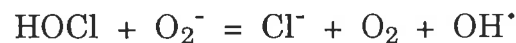
$$H_2O_2 + O_2^- = O_2 + OH^- + OH^\bullet$$

Whereas $OH^\bullet$ is a powerful oxidant and therefore microbicidal agent, the direct toxicity of O_2^- and H_2O_2 is controversial [Fee, 1980]. However, O_2^- and H_2O_2 could act as substrates for other oxy radicals that are more toxic. For example, H_2O_2 can oxidize Cl^- to form highly toxic hypochlorite (HOCl) in a myeloperoxidase (MPO) catalyzed reaction, that is directly very toxic to cells:



$$H_2O_2 + Cl^- \overset{MPO}{=} HOCl + OH^-$$

HOCl may then be further converted to $OH^\bullet$:



$$HOCl + O_2^- = Cl^- + O_2 + OH^\bullet$$

1.3.1.2 *5-lipoxygenase pathway in human neutrophils: synthesis of leukotriene B_4 and 5-hydroxyeicosatetraenoic acid*

The cell membrane phospholipids of inflammatory cells are particularly enriched in arachidonic acid, which is the substrate for eicosanoid and PAF synthesis. Arachidonic acid is released from cell membrane by the action of membrane phospholipase A_2, that is activated upon cell stimulation.

Fig. 1.1 illustrates the metabolic pathways leading to the synthesis of LTB_4 and 5-hydroxyeicosatetraenoic acid (5-HETE), and the enzymes involved [Samuelsson and Funk, 1989]. The next enzyme in the cascade of reactions is Ca^{++}-dependent 5-lipoxygenase. In stimulated neutrophils, 5-lipoxygenase is translocated to the cell membrane [Rouzer and Kargman, 1988] where it is fully activated by 5-lipoxygenase-activating protein (FLAP) located in the cell membrane [Miller et al, 1990; Dixon et al, 1990]. The product of the action of 5-lipoxygenase on arachidonic acid is 5-hydroxyperoxyeicosatetraenoic acid (5-HPETE), which is metabolised further to 5-HETE, by peroxidase, or to LTA_4, by the action of the activated 5-lipoxygenase. Subsequently, LTA_4 hydrolase converts LTA_4 to LTB_4, the major neutrophil eicosanoid product which is either released from the cell or further metabolised to form 20-OH- and 20-COOH-derivatives. Neutrophils do not form the peptide containing leukotrienes LTC_4, LTD_4 and LTE_4.

Oxygenated derivatives of arachidonic acid have potent biological effects. LTB_4 has been shown to have mainly immunomodulating effects, stimulating the cytotoxic activity of natural killer cells [Rola-Pleszczynski et al, 1983], expression of activation markers, proliferation and immunoglobulin production in B-cells [Yamaoka et al, 1989; Dugas et al, 1990] and enhancing effector functions of neutrophils [Dewald and Baggiolini, 1985]. LTB_4 is one of the most potent neutrophil chemotactic factors both *in vitro* and *in vivo* [Goetzl and Pickett, 1981; Lindbom et al, 1982] and also activates neutrophil aggregation and granule secretion [Ford-Hutchinson et al, 1980]. 5-HETE is chemotactic for neutrophils and eosinophils [Goetzl et al, 1977; Goetzl and Pickett, 1980]. In addition, LTB_4 has an indirect immunomodulatory role as it simulates the release of two potent cytokines, IL-1 and TNF, from monocytes [Rola-Pleszczynski and Lemaire, 1985].

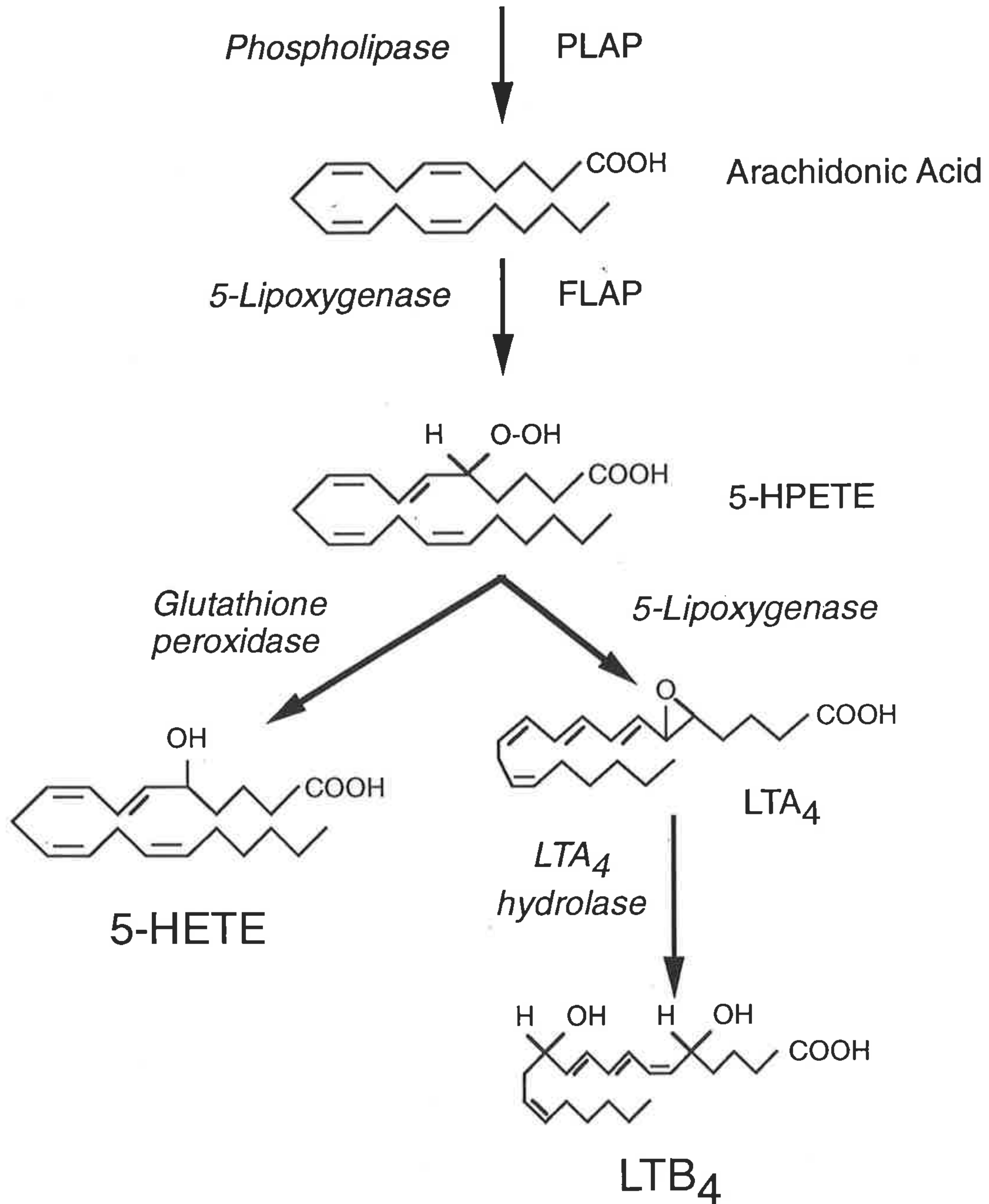


Figure 1.1 Formation of 5-Lipoxygenase products of arachidonic acid metabolism. FLAP=5-lipoxygenase-activating protein; PLAP=phospholipase-activating protein.

1.3.2 *Macrophages and monocytes*

Macrophages are motile and phagocytic cells. Unlike granulocytes, which are end cells with little regenerative capacity, macrophages are capable of cell division. Tissue specific macrophages, such as peritoneal macrophages [Spector et al, 1965], alveolar macrophages [Blusse van Oud Alblas and Van Furth, 1979], Kupffer cells [Gale et al, 1978] and splenic macrophages [Van Furth and Diesselhoff-den Dulk, 1984], are all derived from blood monocytes. Specific recruitment of monocytes to various tissues is in part mediated by newly characterised macrophage chemotactic cytokines such as macrophage chemotactic protein (MCP) and members of the Rantes family [Schall, 1991].

The developmental potential of macrophages is diverse and depends on the particular signals received. Stimulated macrophages may divide rapidly and form various cell lineages with a long life (e.g. monocyte in inflammatory response in the rat liver) [Bouwens and Wisse, 1985]. Activated macrophages are the primary defence against intracellular bacterial infections, protozoa, fungi and metazoan parasites. *In vitro*, macrophages are capable of inhibiting or killing tumour cells [Decker et al, 1987; Cannistra et al, 1988; Coleman et al, 1988; Adams and Hamilton, 1988]. Most importantly, macrophages have been shown to play an essential role in the initiation and regulation of immune responses, and to secrete various potent mediators and cytokines [Gordon et al, 1992].

Macrophages are versatile cells and have not just one, but many roles to play in inflammation. They are protective and destructive cells. At inflammatory sites, they can initiate immune responses by presenting antigen to T-cells and regulating T- and B-cell activity via the secretion of IL-1 and IL-6 [Gordon et al, 1992]. Once an inflammatory response is initiated, they can amplify the response by attracting and activating granulocytes and monocytes by releasing IL-1, IL-8, MCP and GM-CSF. They can also limit

the magnitude of the response by releasing IL-1 receptor antagonists and IL-10 [Gordon et al, 1992; Adams and Hamilton, 1984,1988]. Finally, macrophages remove cellular debris and toxic material, and participate in tissue repair by stimulating growth of other cells including fibroblasts, and production of extracellular matrix components [Nathan and Cohn, 1980; Adams and Hamilton, 1984,1988; Gordon et al, 1992].

1.3.3 *Eosinophils*

Eosinophils, like neutrophils, circulate in the blood for only a short period, then migrate into the tissues, where the majority of the body's eosinophils are found. They are associated with allergic inflammatory reactions and parasitic infections. Although the role of eosinophils in parasitic infections is well established, their function at inflammatory sites is still controversial. For some time eosinophils were thought to exert a negative feedback control on allergic inflammatory responses by removing or neutralising various proinflammatory mediators [Mann 1969; Weller and Goetzl, 1980]. However, more recent findings suggest otherwise [Borish, 1987]. There is now evidence that eosinophils are important proinflammatory cells in asthma [reviewed by Kay, 1988]. The presence of eosinophils in blood, sputum and airways is characteristic of asthma. The accumulation of eosinophils and eosinophil products such as major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) in bronchoalveolar lavage of asthmatics is increased after antigen challenge [de Monchy et al, 1985]. Large number of eosinophils and high concentrations of MBP are present in the lungs of asthmatics dying in "status asthmaticus" and the reduction in lung eosinophils is related to clinical improvement [Diaz et al, 1984],

Eosinophils express CR1, CR3, Fcγ and Fcε receptors on their surface [Anwar and Kay, 1977; Fischer et al, 1986]. Secretion and phagocytosis may be stimulated via these receptors but eosinophils can also be activated by non-specific phagocytosis of particles. Activated eosinophils release granule proteins, of which MBP is particularly toxic, into bronchial epithelium and it has been suggested that the resulting epithelial damage is responsible for the increased bronchial hyperresponsiveness, which is also characteristic of asthma [Gleich, 1986].

In addition, activated eosinophils synthesise leukotrienes C_4 and D_4 (which are potent bronchoconstrictors) and PAF, which is of particular relevance to asthma as it induces bronchoconstriction, bronchial hyperresponsiveness, microvascular leakage, airway secretion and activates a number of inflammatory cells [Barnes et al, 1988]. PAF is also a potent chemotactic factor for eosinophils, although its role in recruiting eosinophils into human lung remains to be established [Wardlaw et al, 1986].

1.4 Regulation of Phagocytic Cells

A large number of phagocytic cell functions (e.g. chemotaxis, phagocytosis, cytotoxicity, exocytosis, release of oxy radicals and other mediators) can be induced by various chemotactic factors, cytokines or agents such as immune complexes or IgG aggregates. Furthermore, responses to one factor can be inhibited or enhanced by another one, depending on the control mechanisms involved and the specific tissue site. A lot of attention has been devoted over many years to determine exactly what factors in tissues attract, activate and modulate leukocyte function. These factors, many of which have now been identified, may be divided into three groups: (i) exogenous factors, (ii) cell-derived substances and (iii) extracellular matrix proteins and products of non-specific tissue injury.

1.4.1 *Exogenous factors*

A number of bacteria have been shown to release factors capable of leucocyte chemotaxis and stimulation. Bacterial products like endotoxin and fMLP [Shiffman et al, 1975] have marked effects on the respiratory and enzymatic activities of neutrophils, eosinophils and macrophages.

1.4.2 *Cell-derived substances*

It is known that lymphocytes (on contact with antigen or mitogens) release a number of factors classified as lymphokines. In recent years, several other cell types, not necessarily cells of the immune system, have been shown to release cytokines, bioactive peptides and other mediators, that attract and activate phagocytic cells [reviewed by: Harvath, 1991; Cerami, 1992; Kroemer and Martinez-A, 1991; Barnes et al, 1988]. Many of these, although important in both acute and chronic inflammation, will not be discussed here because they were not part of this study, or in the case of neuropeptides, will be discussed later in this chapter. Instead, I have chosen two cytokines and one lipid mediator, GM-CSF, IL-8 and PAF, that are regulators of many neutrophil functions, and may have an important role to play in chronic inflammation, in both asthma and rheumatoid arthritis.

1.4.2.1 *Granulocyte-macrophage colony-stimulating factor*

A family of regulators called colony-stimulating factors (CSFs), exhibiting multiple biological activities, have been derived from a multitude of sources (e.g. serum, urine and nearly all tissues and several cell lines) [Metcalf, 1985a,b; Nicola and Vadas, 1984]. There are four classes of CSFs: multi-CSF, macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF) and GM-CSF. These regulators are active

at extremely low concentrations ($10^{-11} - 10^{-13}$ M) and have been shown to act as stimuli for hemopoietic cells, but in some cases they also stimulate biologically important mature cell functions [Nicola and Vadas, 1984]. M-CSF stimulates proliferation of macrophages [Stanley et al, 1976], production and release of IL-1, interferon-γ (INFγ), TNF, myeloid CSF [Moore et al, 1982; Warren and Ralph, 1986], and plasminogen activator [Hamilton et al, 1980], and enhances the ability of macrophages to kill tumour cells [Wing et al, 1982]. G-CSF can prolong survival of mature granulocytes and enhances antibody-dependent killing of tumour cells [Vadas et al, 1983a]. GM-CSF can also stimulate some of the above functions and in addition it enhances the killing of intracellular parasites by macrophages [Metcalf, 1985a], Fc-dependent phagocytosis, and monocyte and macrophage cytotoxicity [Coleman et al, 1988; Cannistra et al, 1988; Grabstein et al 1986]. Many phagocytic cell functions are enhanced in response to a second stimulus rather than directly activated by CSFs, a phenomenon referred to as "priming". For example, *in vitro* pretreatment of neutrophils or eosinophils with GM-CSF or G-CSF enhances production of oxy radicals, degranulation, release of arachidonic acid metabolites and cytokines in response to fMLP [Lopez et al, 1986; Weisbart et al, 1987; Lindemann et al, 1989]. GM-CSF also stimulates cell to cell adherence, phagocytosis and antibody-dependent cell-mediated cytotoxicity [Lopez et al, 1986; Arnaout et al, 1986; Weisbart et al, 1987; DiPersio et al, 1988; Kapp et al, 1988]. Finally, GM-CSF prolongs neutrophil and eosinophil survival [Owen et al, 1987; Lopez et al, 1986], induces histamine release from basophils and mast cells [Haak-Frendscho et al, 1988; Dy et al, 1987] and stimulates macrophages and neutrophils to produce IL-1 and TNFα [Lindemann et al, 1988, 1989]. Since GM-CSF is produced by many different cell types, including human endothelial cells, fibroblasts, T-cells and monocytes [Kaushansky et al, 1988; Broudy et al, 1987; Munker at al, 1986; Lu et al, 1988], it probably plays an important role in inflammation.

1.4.2.2 *Interleukin-8*

Another important cytokine, IL-8, also known as neutrophil activating peptide 1, is produced by a variety of different tissue cells including: stimulated T-lymphocytes, monocytes, keratinocytes, fibroblasts, mesangial cells, endothelial cells, epithelial cells, basophils, alveolar macrophages, synovial cells and chondrocytes. The action of IL-8 is pleiotropic in nature [reviewed by Schroder 1992]. For example, *in vitro* it has been shown to (i) be chemotactic for neutrophils and lymphocytes [Yoshimura et al, 1987; Larsen et al, 1989], (ii) stimulate the migration of T helper/inducer, T suppressor/cytotoxic and B cells [Ross et al, 1991], (iii) degranulate neutrophils [Willems et al, 1989], (iv) augment fMLP-stimulated release of PAF by neutrophils, [v] enhance arachidonic acid release from phospholipids, (vi) enhance neutrophil O_2^- production [Daniels et al, 1992], (vii) enhance neutrophil phagocytosis and (viii) increase neutrophil CR1 and CR3 receptor expression [Detmers et al, 1991]. IL-8-stimulated, neutrophil-mediated cartilage degradation has also been reported [Elford and Cooper, 1991], suggesting its involvement in the pathogenesis of rheumatoid arthritis.

Some of the above *in vitro* effects of IL-8 have been confirmed *in vivo*. For example, intradermal administration of IL-8 into human subjects causes time-dependent neutrophil and monocyte infiltration at the injection site [Leonard et al, 1991; Swensson et al, 1991] and intraperitoneal injection into rabbits results in rapid accumulation of neutrophils.

There is increasing evidence for a role of IL-8 in the pathogenesis of a number of inflammatory diseases including rheumatoid arthritis and asthma. For example, raised levels of IL-8 have been found in synovial fluid of patients with rheumatoid arthritis [Peichl et al, 1991; Brennan et al, 1990] and gout [Terkeltaub et al, 1991] but not in patients with systemic lupus erythematosus [Peichl et al, 1991]. In rheumatoid arthritis there was a close correlation between the concentration of synovial fluid IL-8 and levels of C-

reactive protein and synovial neutrophils [Peichl et al, 1991]. Furthermore, spontaneous production of IL-8 from cultured synovial cells [Brennan et al, 1990], peripheral blood mononuclear cells (PBMC) [Seitz et al, 1991] and synovial fluid mononuclear cells (SFMC) from patients with rheumatoid arthritis [but not normal controls] has been demonstrated. In particular, the production of IL-8 from PBMC was 3-10 fold higher in rheumatoid arthritis patients than normal controls, and production from rheumatoid arthritis SFMC 10 fold greater than in controls [Seitz et al, 1991]. A variety of cultured cells can also be induced to synthesise IL-8. For example, Seitz at al, showed that LPS, rheumatoid factor-containing complexes, zymosan and IL-1 were highly effective in inducing IL-8 production in mononuclear cells from both peripheral blood and synovial fluid [Seitz et al, 1991]. Cultured synovial fibroblasts and articular chondrocytes synthesize IL-8 following induction with IL-1β and TNF-α [Van Damme et al, 1991] and the expression of IL-8 mRNA is increased in cultured synovial cells (from both rheumatoid arthritis and osteoarthritis patients) treated with IL-1α and β. Of even greater significance, is the demonstration that phagocytosing neutrophils *in vitro* express high levels of IL-8 mRNA and releases large amounts of IL-8 into the culture medium [Bazzoni et al, 1991]. If these also occurred *in vivo*, it would provide positive amplification for the inflammatory response of neutrophils.

Sequestration of neutrophils in the lung is characteristic of many acute and chronic pulmonary diseases, including asthma, and may be caused by a variety of chemotactic factors found in respiratory tracts of patients with respiratory diseases [Hayashi et al, 1990]. IL-8 is a chemotactic factor that can potentially induce specific influx of neutrophils into the lung, since it is produced by alveolar macrophages [Rankin et al, 1990; Sylvester at al, 1990; Standiford et al, 1991], lung fibroblasts [Rolfe et al, 1991] and type II pulmonary epithelial cells [Standiford et al, 1990; Kunkel et al, 1991]. A cascade of interactions between alveolar macrophages and lung fibroblasts

and type II pulmonary epithelial cells has been proposed for the rapid generation of IL-8 and neutrophil recruitment [Kunkel et al, 1991]. Furthermore, IL-8 has been shown to induce LTB_4 production from neutrophils from atopic patients [Neuber et al, 1991]. LTB_4 is a very potent chemotactic factor and may further enhance neutrophil influx to the lung. This above findings suggest that this cytokine plays a central role in lung inflammation.

1.4.2.3 *Platelet activating factor*

PAF is involved in the regulation of a variety of normal biological processes as well as acute and chronic inflammation. It is synthesized rapidly by stimulated inflammatory cells such as neutrophils, eosinophils, monocytes, tissue macrophages, basophils, platelets, natural killer cells, endothelial cells and mast cells. PAF is a potent activator not only of inflammatory cells, but also of muscle, endothelial and epithelial cells. PAF's role in inflammation and asthma has been extensively reviewed by Barnes et al [1988].

In vitro, PAF is chemotactic for neutrophils [O'Flaherty et al, 1981], eosinophils [Wardlaw et al, 1986] and monocytes [Czarnetzki 1983]. It also induces neutrophil and monocyte aggregation, granule release and oxy radical production [O'Flaherty et al, 1981, Shaw et al, 1981; Yasaka et al 1982]. PAF induces LTC_4 production and oxy radical production in eosinophils, and enhances IgE-dependent eosinophil activation [Bruijnzell et al, 1986; Capron et al, 1988]. PAF receptors are coupled to G proteins in both neutrophils and eosinophils, [Agrawal et al, 1992] and the binding of PAF to its receptor on neutrophils activates protein kinase C and induces tyrosine phosphorylation [O'Flaherty 1992; Gomez-Cambronero et al, 1991].

Intradermal administration of PAF *in vivo*, causes local accumulation of neutrophils and monocytes [Archer et al, 1985] and in atopic patients, eosinophils [Henocq and Vargaftig, 1986]. In addition to cellular infiltration, PAF increases vascular permeability [Humphrey et al, 1982] and elicits a classical acute wheal and flare response in human skin [Archer et al, 1984; Michel et al, 1987].

Several of the biological effects of PAF may be relevant to its role in rheumatoid arthritis and asthma. In addition to the above, in asthma PAF induces an inflammatory response in human airways [Wardlaw et al, 1991], increases airway vascular permeability [Evans et al, 1987], causes respiratory mucosal damage [Hisamatsu et al, 1991] and stimulates mucus secretion [Wirtz et al, 1986]. PAF is a potent bronchoconstrictor [Cuss et al, 1986] and induces long-lasting bronchial hyperresponsiveness [Cuss et al, 1986; Rubin et al, 1987; Kaye and Smith, 1990]. Finally, PAF has been detected in bronchoalveolar lavage from patients with asthma [Stenton et al, 1990]. Thus, there is large body of *in vitro* and *in vivo* evidence for the role of PAF in inflammation, particularly in asthma.

1.4.3 *Extracellular matrix proteins and products of non-specific tissue injury*

Factors generated in clotted blood, certain fibrinopeptides, denatured proteins from injured tissue, or serum and tissue proteins, in a conformationally altered state, can induce biological activities in phagocytic cells. Fibronectin, commonly present in extracellular matrix, has been shown to mediate phagocytosis in monocytes and macrophages and to enhance significantly phagocytosis in fMLP-stimulated neutrophils [Pommier et al, 1984].

1.5 Receptors on Phagocytic Cells

Although many different type of mediators have been reported to attract and activate phagocytic cells, until recently, not much has been known about the receptors involved and the metabolic changes that occur after activation. However for those receptor that have been studied, it has been demonstrated, that following activation, either the number of receptors expressed on the cell membrane is changed or the receptor itself is chemically modified, so as to become functional [Changelian and Fearon, 1986]. fMLP increases the surface expression of C3bi receptors (CR3) on neutrophils, by causing translocation of intracellular receptor proteins in the granules to the cell surface membrane [Richerson et al, 1985]. GM-CSF and TNF-α modulate the number and affinity of fMLP receptors on neutrophils, and increase their responsiveness to this agonist [Weisbart et al, 1986; Atkinson et al, 1988a]. Phorbol myristate acetate (PMA), is known to activate protein kinase C, which is involved in the phosphorylation of many receptors in different types of cells, causes phosphorylation and subsequent downregulation of C3b receptor (CR1) on B-cells, monocytes and neutrophils [Changelian and Fearon, 1986]. The biochemical pathways that are activated following binding of an agonist to its receptor, that lead to the changes in affinity and expression of other receptors, are currently being investigated by many research groups.

1.6 Signal Transduction in Phagocytic Cells

Phagocytes are activated upon binding of chemoattractants or cytokines to specific receptors on the cell surface. These receptors are usually constituted of three domains: an extracellular domain that binds a ligand and determines the specificity of the receptor, a hydrophobic domain that spans the cell membrane and forms an anchor, and an intracellular domain that is

coupled to a specific signal transduction pathway. Following ligand-receptor interaction, one or more signals from the activated receptor triggers specific biochemical pathways that lead to activation of a specific cell function.

In recent years, much effort has been made to understand the complex biochemical changes which couple receptor activation to various effector functions in phagocytes. As a result of these studies, a number of guanosine triphosphate binding proteins (G proteins) have been identified, that couple receptors to cellular effectors by direct activation of enzymes (e.g. adenylyl cyclase, phospholipase C) and regulation of ionic currents. The discovery of agonist-induced stimulation of phospholipid metabolism by activation of phospholipase C, D and A_2, and the subsequent formation of so called second messengers, diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3) and their role in activation of protein kinase C (PKC) and calcium mobilisation, has helped enormously in our understanding of signal transduction pathways.

1.6.1 *G proteins*

GTP-binding proteins, also referred to as G proteins, utilise GTP as a source of energy required for signal transfer from activated receptors to intracellular effector molecules. Although there are several GTP binding proteins of yet unknown functions, the term G proteins usually refers to a family of receptor-coupled proteins made up of three subunits: α, β and γ [reviewed by Weingarten and Bokoch, 1990]. The α-subunits contain two GTP binding sites as well as a site for ADP ribosylation by cholera and/or pertussis toxin. Depending on the particular G protein, ribosylation may lead to stimulation or an inhibition of cell function. Although there are several types of α-subunits (Gs, Gi, Go, Gz, Tn in human neutrophils), there appear

to be only one type of β and γ subunit, which are non-covalently bound to each other.

Upon ligand (L)-induced receptor (R) activation, the α-subunit releases bound GDP (probably due to a conformational change) which is replaced immediately by the more abundant GTP (Fig. 1.2). The binding of GTP causes dissociation of the α-subunit from the intracellular domain of the receptor and the βγ subunits, apparently due to a change in affinity of α-GTP subunit. The βγ subunits dissociate from the α-GTP subunit, but remain bound together troughout the whole cycle. The α-GTP subunit interacts with effector molecules (E) of a specific activation pathway. GTP is converted to GDP during this interaction and the α-subunit associates again with βγ subunits and the receptor, closing the activation circle. In addition the α-subunit contains some intrinsic GTPase activity, and an α-GTP subunit that has not interacted with effector molecules could be converted slowly to α-GDP and reassociate with βγ subunits. Although a number of experiments support the above model of activation [reviewed by Weingarten and Bokoch, 1990], it should be noted that the exact order of events has not been clearly demonstrated.

The first G proteins described were shown to mediate the activation of adenylate cyclase by hormonal receptors [Ross and Gilman, 1977a,b]. However, subsequent studies demonstrated that G proteins can also directly activate phospholipase C, thus implicating them in other major cellular transduction pathways and mechanisms [Snyderman et al, 1986]. Some neutrophil receptors (e.g. GM-CSF receptor) are coupled to inositol phospholipids hydrolysis by G proteins. Addition of nonhydrolyzable GTP analog, GTPγS, to permeabilized cells, stimulates inositide hydrolysis and enhances agonist stimulated hydrolysis.

Furthermore, there is evidence accumulating that G proteins can regulate ion currents, either via activation of the secondary intracellular

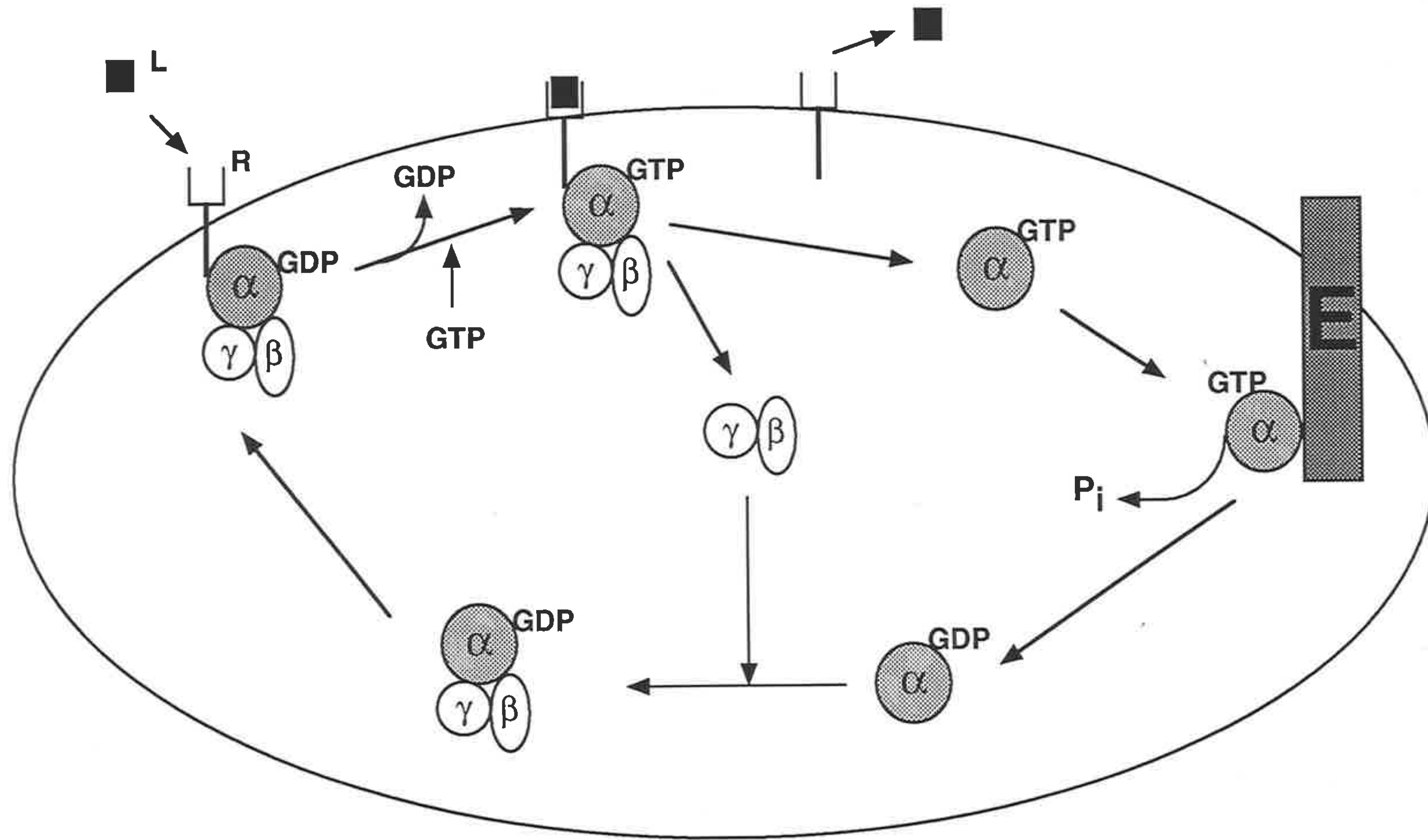


Fig. 1.2 The G protein cycle of activation. Upon ligand binding and receptor activation, α subunit releases bound GDP and binds GTP. This causes dissociation of the α-subunit and the βγ subunits. The α-GTP subunit interacts with effector molecule of a specific activation pathway. GTP is converted to GDP and the α-subunit associates again with the βγ subunits and the receptor closing the activation circle. R = Receptor; L = Ligand; α, β, γ, G protein subunits; E = Effector.

effector molecules or through direct interactions with ion channels. The mechanisms of action involved in ion currents regulation are highly complex and have been reviewed by Sternweis and Pang [1990].

1.6.2 *Phospholipid metabolism in neutrophil signal transduction*

Hydrolysis of inositol phospholipids by phospholipase C (PLC), formation of diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3), and the subsequent activation of protein kinase C (PKC) in response to a variety of extracellular agonists, constitutes one of the major intracellular mechanisms of cell activation [Nishizuka 1984, 1986, 1988]. Moreover, hydrolysis of choline phospholipids by phospholipase D and phospholipase A_2 yields products that can enhance or sustain PKC activation, an interaction that is essential for some cellular responses to occur [reviewed by Nishizuka, 1992].

In 1975, Michell proposed that hydrolysis of phosphoinositides is one of the early biochemical events in cell stimulation. In 1983, Berridge demonstrated that activation of PLC results in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) that is a source of two second messengers, lipophilic DG, an activator of PKC, and IP_3 that is hydrophilic and dissociates into the cytosol and is responsible for mobilisation of Ca^{++} from intracellular stores (Fig. 1.3) [Berridge 1983; Berridge 1984; Berridge and Irvine, 1984]. Both messengers are metabolised very rapidly in the cell and are converted further in a cycle of reactions to reform PIP_2.

The activation of PKC requires the enzyme to be bound to the phospholipid cofactors, such as phosphatidyl serine, in a calcium dependent or independent manner. Transfer of PKC from the cytosol to the cell membrane increases its availability to membrane-associated DG, and therefore is important in PKC activation. The binding of DG to PKC induces

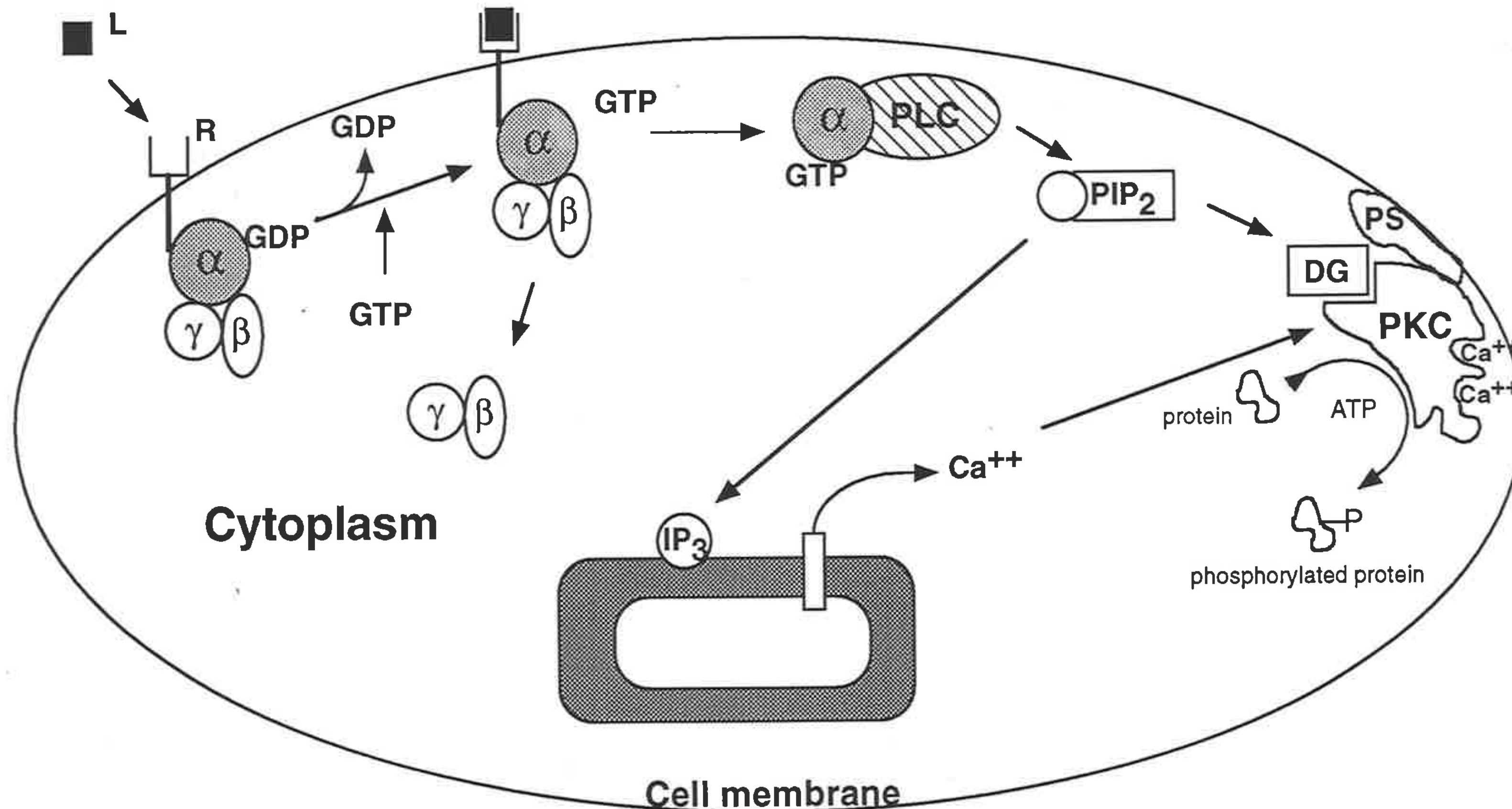


Fig. 1.3 Model for receptor-mediated stimulation of phospholipase C and generation of second second messengers, DG and IP_3. Binding of a ligand to its receptor releases a-subunit that activates PLC. Activation of PLC results in hydrolysis of PIP2 that is a source of lipophilic DG, an activator of PKC , and hydrophilic IP_3 that releases Ca^{++} from intracellular storers, which also binds to PKC. Activated PKC phosphorylates cytosolic proteins which then become activated. R = Receptor; L = Ligand; α, β, γ, G protein subunits; PLC = Phospholipase C; PIP_2 = Phosphatidylinositol 4,5-bisphosphate; DG = Diacylglycerol; IP_3 = inositol 1,4,5-trisphosphate; PKC = Protein kinase C; PS = Phospholipids.

a conformational change and activates the enzyme by dislocation of a pseudosubstrate from its active site [Bell and Burns 1991]. Activated PKC controls activity of other kinases by phosphorylating them on serine and threonine residues. Thus protein phosphorylation can be regarded as a central event in the control of cell activity.

Several PKC enzymes that are regulated by phospholipids and DG have been identified to date [Bell and Burns, 1991]. They have different tissue distribution, intracellular localization and structure-function relationship studies suggest that these enzymes have slightly different activities. However, their role in controlling specific cellular activities in distinct tissues or subcellular compartments remains to be determined [Nishizuka 1988; Bell and Burns 1991].

Agonist-induced hydrolysis of other membrane phospholipids, in particular choline phospholipids, by phospholipase D and phospholipase A_2, may be important in activation of PKC (Fig. 1.4). Hydrolysis of phosphatidylcholine by phospholipase D results in formation of additional DG. Furthermore, phospholipase D activity can be enhanced by PKC, that has been activated by DG derived from phospholipase C hydrolysis of inositol phospholipids, through a positive feedback mechanism [Nishizuka 1992]. Thus, phospholipase D-derived DG may play an important role in intracellular signalling mechanism by activating or prolonging the activation of PKC.

In addition, the activity of PKC can be enhanced by the products of hydrolysis of phospholipids by phospholipase A_2, in particular *cis* unsaturated fatty acids [Nishizuka 1992]. Phospholipase A_2, that is normally located in the plasma membrane in neutrophils [Franson et al, 1980; Victor et al, 1981], becomes activated following phosphorylation of lipomodulin, which in its unphosphorylated form inhibits the enzyme [Hirata et al, 1980]. Several *cis* unsaturated fatty acids can activate PKC or enhance DG-

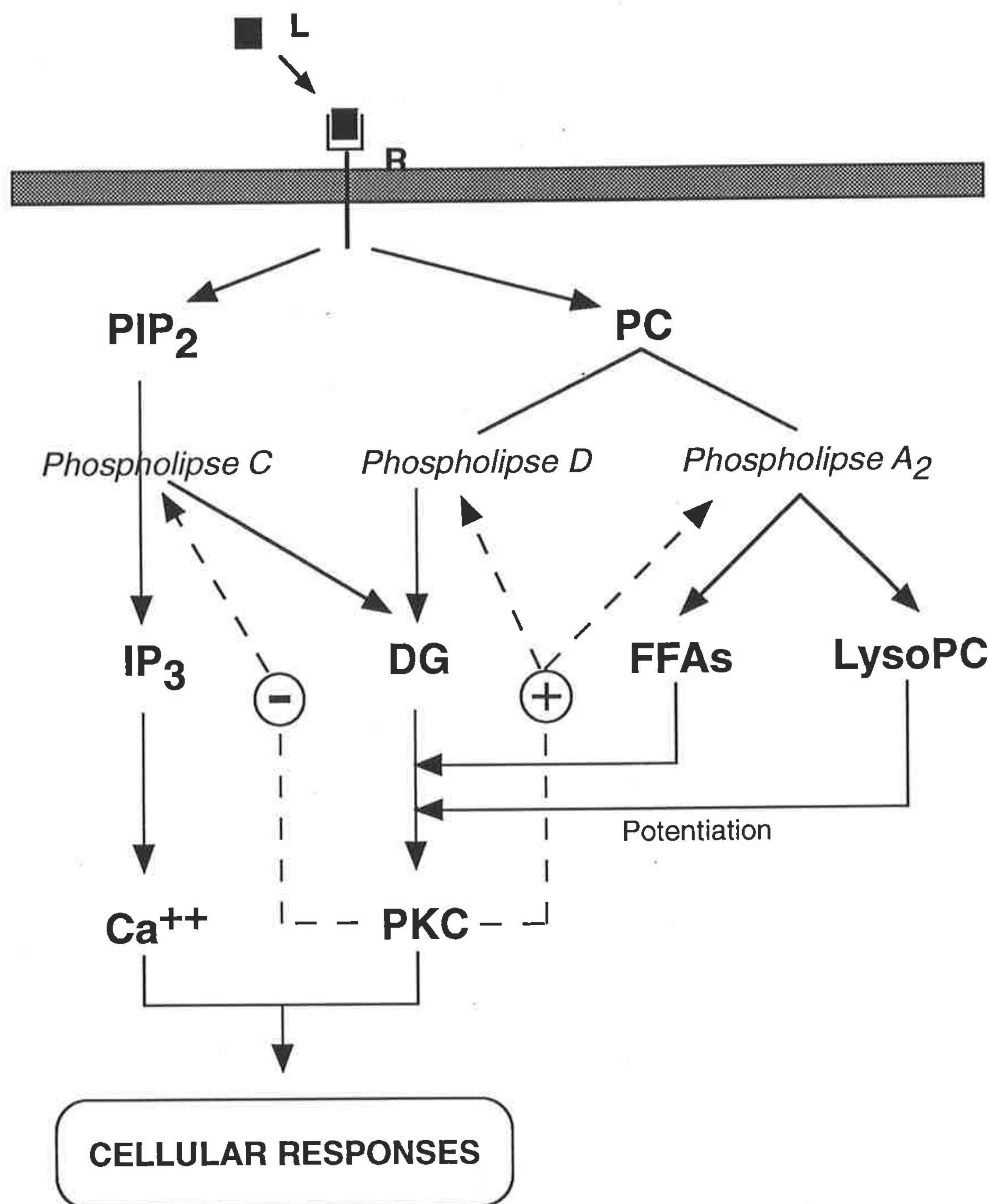


Figure 1.4 Schematic representation of ligand-induced membrane phospholipid degradation for sustained PKC activation. L = Ligand; R = Receptor; PIP_2 = Phosphatidylinositol 4,5-bisphosphate; PC = Phosphatidylcholine; DG = Diacylglycerol; IP_3 = Inositol 1,4,5-trisphosphate; FFAs = Free cis unsaturated fatty acids; LysoPC = Lysophosphatidylcholine (modified from Nishizuka, 1992)

dependent activation of PKC. It was proposed that *cis* unsaturated fatty acids can increase the Ca^{++} sensitivity of PKC, thus keeping the enzyme active, even after the IP_3-induced increases in Ca^{++} concentration have returned to the resting levels. Thus, it is likely, that after cell stimulation, PKC is activated initially by DG and IP_3, but its activity is sustained by DG in the presence of *cis* unsaturated fatty acids.

Lysophosphatidylcholine, a product of phosphatidylcholine hydrolysis by phospholipase A_2, also enhances PKC activity, but unlike *cis* unsaturated fatty acids does not increase Ca^{++} sensitivity of PKC activation.

1.6.3 *Phosphatidylinositol metabolism*

In neutrophils stimulated by chemoattractants, adherence or Fc mediated phagocytosis, PLC activation leads to the hydrolysis of membrane inositol phospholipids and the formation of DG and IP_3 (as discussed above). Whereas DG activates PKC, IP_3 releases Ca^{++} from intracellular stores [Berridge and Irvine 1989]. Phosphoinositide metabolism is cyclic, and DG and IP_3 are eventually converted back to phosphoinositides. IP_3 metabolism is regulated by a number of specific phosphomonoesterases and kinases [Pittet et al, 1992]. Two types of enzymes play a key role in this metabolism: IP_3 5-phosphatase that generates inositol-1,4-bisphosphate and IP_3 3-kinase, that phosphorylates IP_3 to form inositol -1,3,4,5-tetrakisphosphate (IP_4). IP_4 is further metabolised by 5-phosphatase to form inositol-1,3,4-trisphosphate that together with IP_3 is converted by sequential phosphatase action to inositol. The inositol-1,4-bisphosphate is also dephosphorylated to inositol by the action phosphomonoesterases (Fig. 1.5). Although the role of IP_3 in Ca^{++} release from intracellular stores has been well established, the function of other inositol derivatives and the reason for such complex biochemical conversions remains to be determined.

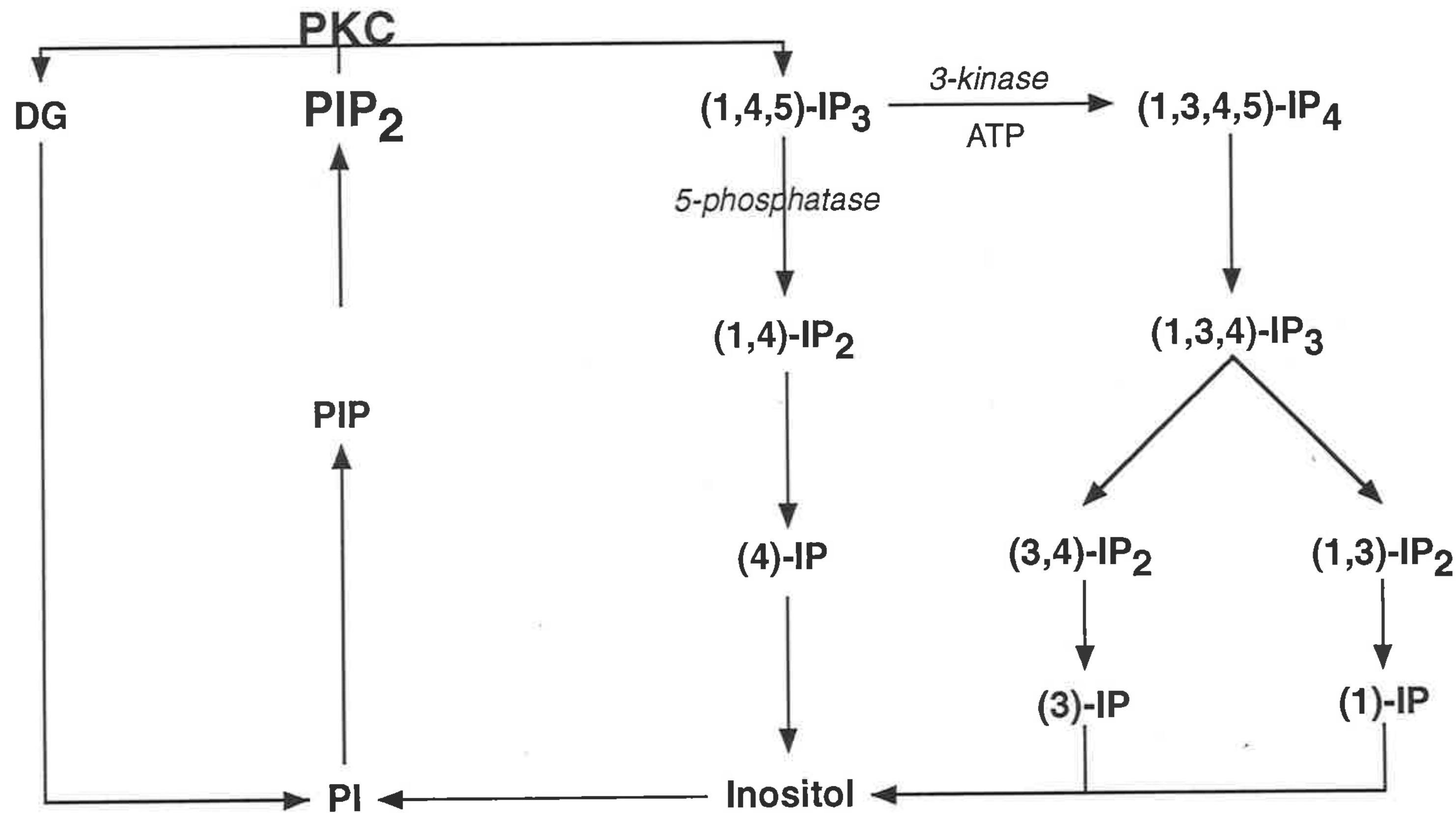


Fig. 1.5 Inositol metabolism in neutrophils. Phosphoinositide metabolism is cyclic. Two enzymes play a key role: IP_3 5-phosphatase that generates IP_2 and IP_3 3-kinase, that phosphorylates IP_3 to IP_4. IP_4 is further metabolised by 5-phosphatase to to form inositol-1,3,4,-trisphosphate that together with IP_3 is converted by sequential phosphatase action to inositol. IP_2 is also dephosphorylated to inositol by the action of phosphomonoesterases. PIP_2 = Phosphatidylinositol 4,5-bisphosphate; DG = Diacylglycerol; IP = inositol phosphate; PKC = Protein kinase C.

1.6.4 *Role of intracellular free calcium in neutrophil activation*

The identities of the intracellular stores from which Ca^{++} is released by the action of IP_3 in neutrophils are still debated. It has been proposed that the majority of Ca^{++} is released from rough endoplasmic reticulum (ER). However neutrophils, that are almost devoid of ER, can release as much Ca^{++} as cells that are rich in ER [Krause et al, 1989], which suggests the existence of several IP_3-sensitive Ca^{++} stores in neutrophils.

In addition to transient Ca^{++} release from intracellular stores, the more prolonged rise in intracellular free calcium concentration ($[Ca^{++}]_i$) in activated neutrophils is sustained by an influx of extracellular Ca^{++} [Meldolesi et al, 1987]. Studies which have measured Ca^{++} release and Ca^{++} influx simultaneously, suggests that both processes are closely related and require similar receptor occupancy [Pittet et al, 1989]. However, the mechanisms that activate and regulate calcium channels located in the neutrophil plasma membrane are not fully understood. A role for second messengers and G proteins in activating those channels has been proposed [Meldolesi et al, 1987; Sternweis and Pang 1990; Baritt 1992].

The activity of PLC as well as PKC are dependent on $[Ca^{++}]_i$ levels. However, although PLC can be fully activated at resting $[Ca^{++}]_i$ resting levels, the PKC activity is enhanced substantially by increasing $[Ca^{++}]_i$. This increased PKC activity is thought to be mediated by transfer of PKC from the cytosol to the plasma membrane. Elevated $[Ca^{++}]_i$ causes cytosolic PKC to associate with the cell membrane, where it binds DG and leads to cell activation through phosphorylation of various substrates. For example, PAF or the calcium ionophore, A23187, increase $[Ca^{++}]_i$, PKC translocation and prime neutrophils for enhanced responses to a second stimuli [French et al, 1987; Dewald and Baggiolini, 1985; O'Flaherty et al, 1990a,b]. However, the mechanism involved with these stimuli are different. Studies from Ca^{++}-depletion and -repletion in neutrophils indicate that, whereas A23187

requires Ca^{++} for PKC translocation, stimuli like PAF (or fMLP) do not. Thus A23187 was ineffective in Ca^{++}-depleted cells. Stimulation of neutrophils by fMLP and PAF resulted in a biphasic effect on PKC translocation, with the early (Ca^{++}-dependent) phase being absent when Ca^{++}-depleted cells were used. Therefore in neutrophils, Ca^{++}-dependent and Ca^{++}-independent mechanisms regulate the translocation of PKC to the cell membrane [O'Flaherty et al, 1990a,b].

The increased $[Ca^{++}]_i$ caused by Ca^{++} release from intracellular stores and Ca^{++} influx is transient, and this response is then terminated even though the process of cell activation may continue for prolong periods of time. The receptor itself is probably responsible for activating inhibitory mechanisms which terminate the rise in $[Ca^{++}]_i$. Chemotactic receptors, for example, can induce phospholipid hydrolysis, elevation in $[Ca^{++}]_i$ and cyclic AMP formation in neutrophils, thus providing activating and inhibitory signals. However multiple inhibitory mechanisms are likely to be involved.

1.6.5 *Signal transduction mechanisms leading to the activation of NADPH oxidase*

The neutrophil NADPH oxidase system is composed of many cytosolic and membrane components that assemble at the cell membrane upon cell activation. Activated NADPH oxidase transfers electrons from NADPH to molecular oxygen to form superoxide anion molecules (O_2^-). Components of the NADPH oxidase include a flavoprotein, membrane bound cytochrome b_{558}, two cytosolic factors, p47-*phox* and p67-*phox*, and small GTP binding proteins $p21^{rac1}$ and Rap1A (Fig. 1.6). The cytochrome b_{558} complex is an iron containing heme protein capable of reducing molecular oxygen. It consists of two glycosylated protein subunits, a 22 kD α-subunit and 91 kD β-subunit. The cytosolic components, p47-*phox* and p67-*phox* are

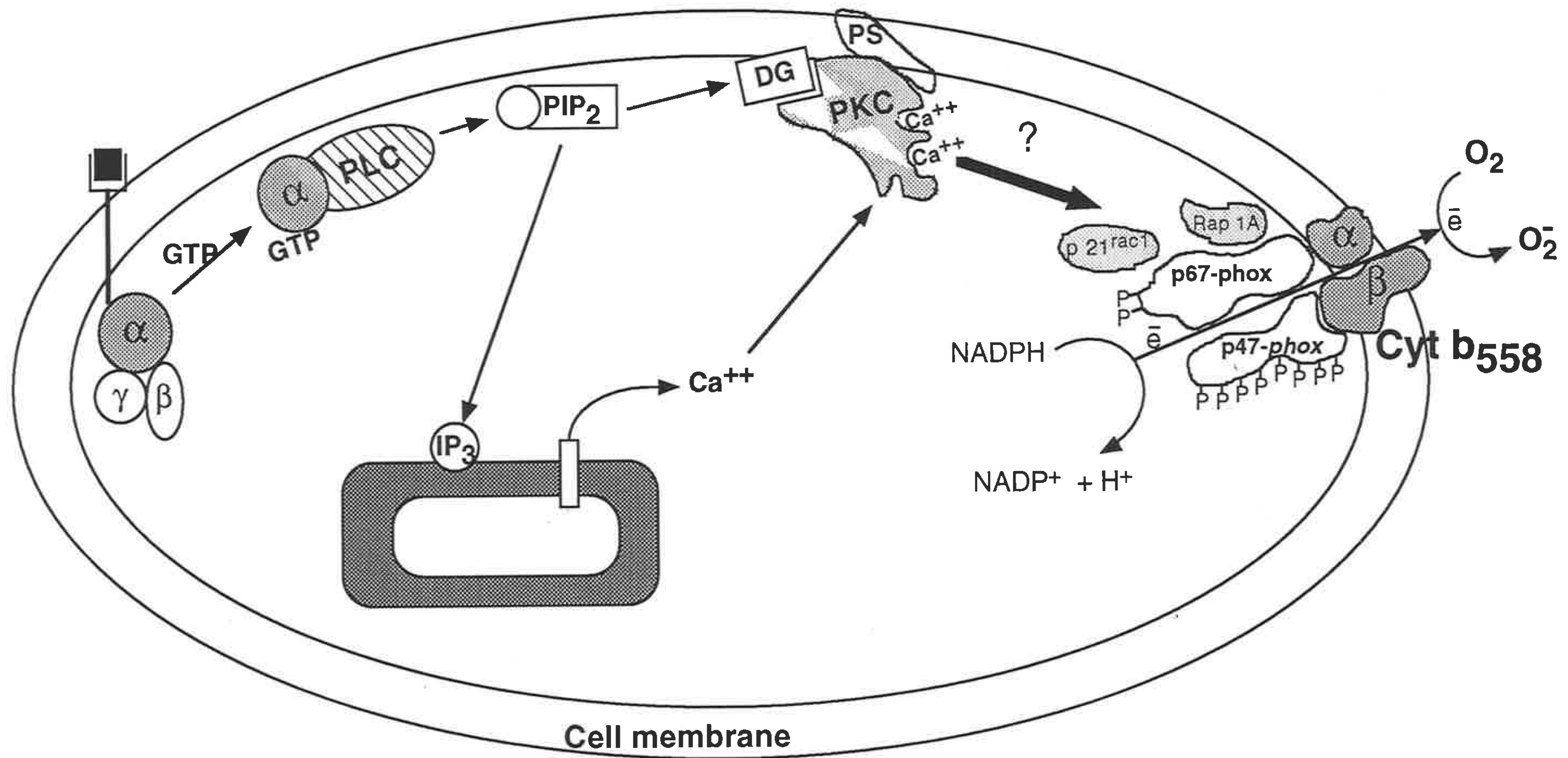


Fig. 1.6 Model for receptor-mediated stimulation of NADPH oxidase. Binding of a ligand to its receptor activates PKC (Fig. 1.3). PKC phosphorylates cytosolic componen-ts of the NADPH oxidase, p47-*phox* and p67-*phox*, which translocate to the cell membrane where with other components for the active NADPH oxidase complex. α, β = componen-ts of cytochrome b_{558}; p47-phox, p67-phox = cytosolic components; Rap 1A, $p21^{rac1}$ = small GTP-binding proteins. Other abbreviations as in Fig. 1.3.

phosphorylated during neutrophil activation and subsequently translocated to the membrane, where they become associated with cytochrome b_{558}. The translocation of p67-*phox* to the cell membrane is dependent on the phosphorylation and translocation of p47-*phox* but not vice versa. This phosphorylation process occurs stepwise and depends on the association of p47-*phox* with cytochrome b_{558}. Initial phosphorylation of p47-*phox* occurs in the cytosol, but additional phosphorylation takes place only on interaction with membrane components [Clark et al, 1990; Rotrosen and Leto, 1990; Nauseef et al, 1991]. This stepwise phosphorylation changes the protein from highly basic to highly acidic, a change that may facilitate its association with membrane components.

There is no clear answer as to why additional phosphate groups can be added to p47-*phox* after interaction with membrane components. It has been suggested that kinases different to those in the cytosol may be activated at the cell membrane, or specific cofactors become available or simply more sites for phosphorylation become exposed as a result of conformational change induced by interactions with membrane [Rotrosen and Leto, 1990].

In addition, p67-*phox* remains and p47-*phox* becomes associated with the cytoskeleton upon activation. The significance of this association, however, is unclear at this moment.

The mechanism by which GTP binding proteins $p21^{rac1}$ and Rap1A contribute to the formation of the active NADPH oxidase complex is not clear. Unlike $p21^{rac1}$, Rap1A does not seem to enhance the NADPH oxidase activity in cell-free systems. Nonetheless, both proteins could play a part in the assembly of the different components of NADPH oxidase or their association with the cytoskeleton [Abo et al, 1991; Bokoch et al, 1991].

Synthetic DG analogs, such as PMA, that bind to and activate PKC, are powerful activators of the NADPH oxidase complex and stimulate prolonged

O_2^- production in neutrophils. This demonstrates that activation of PKC alone is sufficient to render the complex fully active. However, the NADPH oxidase complex may be also activated by PKC-independent mechanisms. For example, stimulation of neutrophils by fMLP induces both, PKC-dependent and -independent activation of NADPH oxidase [Watson et al, 1991]. The existence of a PKC-independent pathway has also been suggested by the demonstration that receptor-induced responses in neutrophil occur in the presence of PKC inhibitors and unaltered $[Ca^{++}]_i$ [Cooke and Hallett, 1985; Gerard et al, 1986; Watson et al, 1991]. Although it is not clear at this moment what PKC-independent mechanisms are involved in the activation of NADPH oxidase, it has been suggested that tyrosine phosphorylation, mediated by receptor coupled G proteins, can regulate this activity. Although many groups have attempted to elucidate the mechanism of NADPH oxidase activation [Grinstein and Furuya 1991], the exact details are far from clear.

1.7 The Role of Neutrophils in Rheumatoid Arthritis and Asthma

Rheumatoid arthritis and asthma are both chronic inflammatory diseases characterized by prominent infiltration by immune cells. The presence of large numbers of neutrophils can be readily demonstrated in histological tissue samples or synovial fluid and bronchoalveolar lavage, in rheumatoid arthritis and asthmatic patients respectively. As described above (Chapter 1.2.1), neutrophils can participate in inflammatory response as effector cells and can also synthesize and release cytokines, which may modulate both cellular and humoral immunity. However, for the purpose of this thesis, I will focus most of the attention on the effector function of neutrophils, and their contribution to tissue damage [Weiss 1989] in these two diseases.

1.7.1 *Neutrophils in rheumatoid arthritis*

As many as 1 billion neutrophils could be recruited every day to an arthritic joint of a patient with mild rheumatoid arthritis [Hollingsworth et al, 1967]. The neutrophil influx into the synovium is stimulated by a number of soluble chemoattractants [reviewed by Harvath 1991] of different origins: some, like fMLP, are released from bacteria growing in synovial fluid, others like IL-8, could be produced by resident synoviocytes, synovial fibroblasts and chondrocytes, or inflammatory cells (Chapter 1.3.2.2). In fact, the most potent neutrophil chemoattractants: IL-8, LTB_4 and PAF, are produced and could be released from neutrophils themselves [Strieter et al, 1992; Ford-Hutchinson et al, 1980; Lotner et al, 1980]. Chemotactic factors have been demonstrated in rheumatoid synovium. For example, IL-8 is found in synovial fluid from patients with rheumatoid arthritis and its synovial concentrations correlate well with the levels of synovial neutrophils and C reactive protein (Chapter 1.3.2.2). The synovial fluid concentration of LTB_4, is also higher in rheumatoid arthritis patients than osteoarthritic patients, and correlated well with cell numbers, rheumatoid factor, and immune complexes in synovial fluid of these patients [Ahmadzadeh et al, 1991].

Chronic inflammation in rheumatoid arthritis is associated with the presence of other types of inflammatory cells, including T cells and macrophages, and is characterised by hyperproliferation of synovial lining cells. All of these cells contribute in a different ways to the destruction of articular cartilage and bone. Proliferating synovial cells penetrate the cartilage in the form of a pannus, and the cartilage is destroyed at the contact area. The pannus formation and cartilage destruction are enhanced by IL-1 [Ishikawa et al, 1991], which is found in increased levels in the rheumatoid joint, and which may be produced by neutrophils [Lindemann et al, 1988; Goh et al, 1989; Lord et al, 1991].

In addition, neutrophils are involved more directly in cartilage destruction, as small foci of neutrophils are seen at the pannus/cartilage interface [Mohr and Wessinghage 1978; Mohr et al, 1981; Mohr et al, 1984]. They were found to be associated with immune complexes and complement material deposited on cartilage surface [Ishikawa et al, 1975; Ugai et al, 1979; Mohr et al, 1981]. Immune complexes and complement fragments can activate neutrophils, which contribute to cartilage damage by the release of destructive enzymes [Lowther at al, 1987] and toxic oxy radicals [Bates 1984; Chapter 1.2.1.1]. In addition, a number of cytokines can stimulate or enhance neutrophil-mediated cartilage damage [Kowanko et al, 1990; Kowanko and Ferrante, 1991]. For example, GM-CSF, which is present in rheumatoid synovial fluid [Xu et al, 1989], and which can activate many different neutrophil functions (Chapter 1.3.2.1), auguments neutrophil-mediated cartilage degradation [Kowanko and Ferrante, 1991]. Thus, the evidence that neutrophils play an important role in the pathogenesis of rheumatoid arthritis is compelling.

1.7.2 *Neutrophils in asthma*

Asthma is a disease characterised by reversible airflow obstruction and bronchial hyperresponsiveness.(BHR). Although the underlying cause of asthma is unknown, chronic inflammation of the airway wall is a prominent feature of this disease and is thought to be responsible for the airflow obstruction and BHR [Barnes 1989]. Mucus hypersecretion and shedding of the epithelial lining of the airways, are also present, with mucus plugs containing epithelial cells and inflammatory cells often found blocking small airways.

A number of studies have suggested that neutrophils and eosinophils are the key effector cells in airway inflammation, in asthma, although

multiple cell types and mediators are likely to be involved [Borish, 1987; Kay, 1988; Sibille and Reynolds, 1990; Boschetto et al, 1989]. Although most attention has focused on the role of eosinophils, there is evidence for the involvement of neutrophils. The initial evidence implicating the role of neutrophils in asthma was a demonstration of increased neutrophil numbers in lung tissue sections collected post mortem from patients who had died in "status asthmaticus" [Dunnill et al, 1969; Hayes, 1976]. Similarly, large numbers of neutrophils were found in the bronchoalvealar lavage fluids from patients with asthma particularly after local allergen challenge [Laitinen et al, 1985; Metzger et al, 1986; Metzger et al, 1987]. However the importance of neutrophils was fully appreciated, when it was realised, that the stimuli that induce asthmatic responses, also provoke the release of proinflammatory mediators that prime or activate peripheral neutrophils [Papageorgiou et al, 1983; Moqbel et al, 1986; Carrol et al, 1985; Durham et al, 1984]. For example, O_2^- production is increased in neutrophils isolated from peripheral blood of asthmatic patients, compared to normal controls, and this correlates with disease severity and duration [Kanazawa et al, 1991].

The priming phenomenon may be especially important, as many of the cytokines and mediators that are released into the circulation from inflammed lung, may dramatically enhance neutrophil responses to other stimuli at very low concentrations. For example, TNFα, GM-CSF, IL-8, PAF and LTB_4, have all been shown to facilitate neutrophil responses [Berkow et al, 1987; Weisbart et al, 1987; Daniels et al, 1992; Dewald and Baggiolini, 1985]. In particular the priming of neutrophils by IL-8 may be of great importance, as this cytokine is thought to be the major neutrophil chemotactic factor in the lung [Kunkel et al, 1991], and it exerts its maximal priming effect at concentrations that are optimal for IL-8-induced neutrophil chemotaxis and transendothelial migration [Smith et al, 1991b]. These findings suggest, that neutrophils may be primed while being recruited to the lung by IL-8,.

Activated neutrophils can induce airway damage and BHR [Chung 1986; Weiss 1989], by virtue of their capacity to produce proteolytic enzymes and toxic oxy radicals, and to release mediators such as PAF, prostaglandins and leukotrienes (Chapter 1.2.1). Neutrophils and neutrophil-derived oxy radicals have been implicated in airway obstruction in asthmatic patients [Kanazawa et al, 1991], as well as pulmonary endothelial cell [Weiss et al, 1981; Martin, 1984; Till et al, 1982; Ward, 1991] and epithelial cells [Robbins et al, 1992] and pulmonary type II cell damage [Simnon et al, 1986]. They can increase airway vascular permeability and plasma exudation [Wedmore and Williams, 1981], that results in airway oedema, which may itself contribute to BHR. Plasma exudation enhances shedding of the epithelium, and promotes mucus plug formation. In addition, plasma exudate may attract and activate other inflammatory cells, and thus amplify the inflammatory proccess [reviewed by Persson, 1988].

It is important to note, that neutrophil-mediated bronchial epithelial cell damage [Robbins et al, 1992] can promote the release of neuropeptides, such as tachykinins, into the bronchial lumen. Epithelial damage and the opening of epithelial tight junctions result in the exposure of afferent nerve endings containing tachykinins. Exposed nerve fibres are more easily stimulated and may release neuropeptides locally via axon reflexes.

The possible role of neutrophil-derived PAF in asthma has been described in Chapter 1.3.2.3.

1.8 Tachykinins

Tachykinins are a family of neuropeptides, which are characterised by a similar carboxy terminal pentapeptide sequence of:

$$—Phe—X—Gly—Leu—Met—NH_2$$

where X is an aromatic or aliphatic amino acid. They are found in both the central and peripheral nervous system, where they serve as neurotransmitters and neuromodulators. There are three mammalian tachykinins, substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). In addition, there are amino terminal (neuropeptide K and neuropeptide γ) and truncated (NKA_{3-10}) derivatives of NKA [Helke et al, 1990] A large number of non-mammalian tachykinins has also been isolated, including amphibian tachykinin, kassinin (K) and molluscan tachykinin, Eledoisin (E) (Table 1.2).

In the peripheral nervous system, tachykinins are localised in capsaicin-sensitive, small sensory afferent nerve fibres. These fibres are nociceptive nerve fibres and are involved in transmission of pain signals when stimulated by noxious stimuli (e.g. heat, physical damage). Substance P, and possibly other tachykinins, are putative neurotransmitters in these fibres. As well as mediating nociception, SP has an efferent action, where activation of sensory nerves causes antidromic (reversed) transmission of nerve signals to other branches of the same peripheral sensory fibre (in addition to orthodromic transmission to the spinal cord). This is known as an axon reflex, and it has been demonstrated that SP is released from sensory fibres upon antidromic stimulation [White and Helm, 1985; Garrett et al, 1992]. In 1979, Lembeck and Holzer demonstrated that the stimulation of these fibres induces a local reaction in the airways with all the signs of inflammation, vasodilation, increased vascular permeability and tissue oedema. Moreover, this effect was mimicked by SP administration [Lembeck and Holzer, 1979]. On the other hand, the neurotoxin capsaicin (the active substance in chilli peppers), destroys sensory afferent fibres, greatly reduced the effect of nerve stimulation [Gamse et al, 1980]. This evidence suggests, that tachykinins released from sensory nerves into tissues are responsible for the demonstrated effect, and points to a role for tachykinins in inflammation.

TACHYKININS

Mammalian:

Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met
Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met
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
Neuropeptide γ	Asp-Ala-Gly-His-Gly-Gln-Ile-Ser-Hid- Lys-Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Ler-Met
Neurokinin A_{3-10}	Thr-Asp-Ser-Phe-Val-Gly-Leu-Met
Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met

Amphibian:

Physalaemin	Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met
Uperolein	Pyr-Pro-Asp-Pro-Asn-Ala-Phe-Tyr-Gly-Leu-Met
Phyllomedusin	Pyr-------Asp-Pro-Asn-Arg-Phe-Ile- Gly-Leu-Met
Kassinin	Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met
Hylambatin	Asp-Pro-Pro-Asp-Pro-Asp-Arg-Phe-Tyr-Gly-Leu-Met

Molluscan:

Eledoisin	Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met

Table 1.2 Primary structures of mammalian and non-mammalian tachykinins.

1.8.1 *Genes encoding tachykinins*

Mammalian tachykinins are encoded by two genes: the SP/NKA or preprotachykinin gene I, and the NKB gene or preprotachykinin gene II [Helke et al, 1990; Krause et al, 1992]. The two neuropeptides, SP and NKA, as well as the NKA derivatives, neuropeptide K, neuropeptide γ, and $NKA_{3\text{-}10}$ are produced from mRNA transcribed from the SP/NKA gene, by differential RNA splicing and posttranslational processing (Fig. 1.7). The other tachykinin, NKB, is produced from mRNA transcribed from a separate NKB gene. Transcription of the SP/NKA gene and alternative splicing of the primary transcript, results in formation of three mRNA species: α-, β- and γ-preprotachykinin (PPT) mRNAs. These mRNAs are translated into three precursor peptides, α-PPT, β-PPT and γ-PPT, that are further processed to yield the individual neuropeptides. SP is encoded by a part of exon 3, and because all three precursor PPT peptides have this exon, it can be produced from any of them. NKA is encoded by exon 6. Since α-PPT does not have exon 6, NKA and $NKA_{3\text{-}10}$ are produced from β-PPT and γ-PPT. Neuropeptide K is derived from exons 3-6, and is produced from β-PPT, whereas neuropeptide γ is derived from exons 3, 5 and 6, and is produced from γ-PPT (Fig. 1.7). That multiple tachykinins can be derived by posttranslational processing, has been demonstrated by MacDonald et al [1989], who transinfected cell lines with α-, β-, or γ-PPT encoding vaccinia virus, and show that they were processed to multiple products as described above. All of the products were co-secreted in a biologically active form upon appropriate stimulation. However, further modification of secreted products may take place extracellularly as demonstrated by extraneuronal cleavage of neuropeptide K to neurokinin A [Martling et al, 1987b].

Not surprisingly, since SP and NKA are encoded by the same gene, they are often found to be co-distributed and co-localised in the same tissues [Schmidt et al, 1991]. Little is known, however, about the intracellular

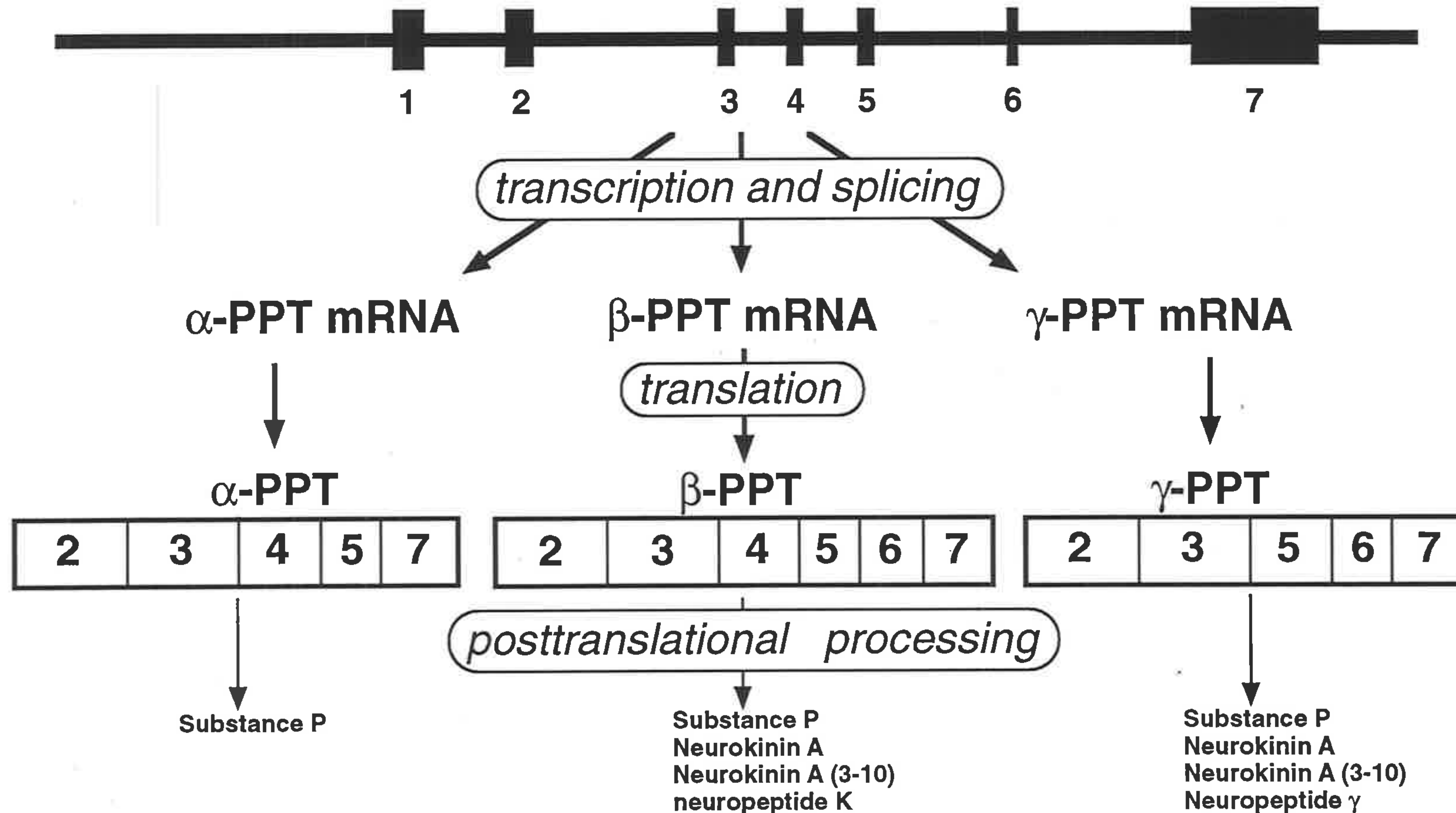


Fig. 1.7 Schematic illustration of the transcription and splicing of the SP/NKA (rat) gene primary transcript, and of the translation and posttranslational processing of the α-, β-, and γ-PPT precursors. The SP/NKA gene is depicted at the top, with the boxes denoting the exons numbered 1-7. Transcription of the gene and splicing of the primary transcript yields α-, β-, and γ-PPT mRNA. The mRNAs are translated with concomitant signal peptide cleavage, and the various peptides derived from each percursor are displayed below each PPT as discussed in the text. The number above each PPT precursor repreent the number of amino acids present in each. (Adopted and modified from Helke et al, 1990).

factors that regulate the SP/NKA gene expression and tissue specific pattern of splicing of the PPT mRNA *in vivo*. Nonetheless, it has been demonstrated the nerve growth factor (NGF) [Lindsay and Harman, 1989], thyroid hormone [Jonassen et al, 1897], dopamine [Bannon et al, 1986], estrogen [Jonassen et al, 1988; Brown et al, 1988] and IL-1 [Freidin and Kessler, 1991] can all be involved in the regulation of SP mRNA levels in peptidergic neurons. Of special interest is NGF, which was shown not only to increase the expression of SP and calcitonin gene related peptide in sensory neurons but also their transport to inflammatory sites *in vivo* [Donnerer et al, 1992]. At the same time, the release of these peptides from neurones supplying the inflammatory sites is increased [Donnerer et al, 1992; Garry and Hargreaves, 1992], although the mechanisms controlling this process are not fully understood. In addition, inflammatory products such as prostaglandin E_2 [Nicol et al, 1992], and low pH [Geppetti et al, 1991; Del Bianco et al, 1991], were shown to enhance the release of SP from neurones by a calcium-dependent mechanism.

1.8.2 *Tachykinin receptors*

At least three mammalian tachykinin receptors exist, neurokinin-1 (NK-1), NK-2 and NK-3 receptors, each defined by the rank order of their potency to bind different tachykinins [Nakanishi, 1991; Krause et al, 1992] in bioassays and in radioligand binding studies. However, provided that the concentration of tachykinins is high enough, all three receptors can be activated by any of tachykinins, as the activation of these receptors is dependent on the common carboxy terminal shared by all tachykinins. The relative receptor potencies for mammalian tachykinins are given below:

NK-1: SP > NP$\gamma \geq$ NKA = NPK > NKB

NK-2: NPK = NP$\gamma \geq$ NKA > NKB > SP

NK-3: NKB > NKA > SP

The NK-1 receptor is expressed by neurones in CNS but also smooth muscle cells, fibroblasts and several types of immune cells including lymphocytes [Payan et al, 1984] and macrophages [Hartung et al, 1986]. The NK-2 receptor is mainly found in peripheral tissues (e.g. smooth muscle of airways, [Frossard and Advenier, 1991]) but is also present on lymphocytes [Casini et al, 1989; Eglezos et al, 1991]. The NK-3 receptor is found mainly in the CNS.

All three human tachykinin receptors have been cloned, sequenced and characterised functionally [Takeda et al, 1991; Girard et al, 1990]. The NK-1 and NK-2 receptor structures deduced from the amino acid sequence, contain several membrane-spanning domains and a sequence similarity to G protein-coupled receptors [Krause et al, 1992; Masu et al, 1987]. A model system for the intracellular signalling involved in the activation of the NK-1 receptor, has been suggested by Krause et al [1992]. He proposed that, binding of SP activates pertussis toxin-insensitive G proteins that mediate PLC-catalysed phosphatidylinositol hydrolysis with subsequent formation of DG and IP_3, and PKC activation. Desensitization would occur by phosphorylation of serine- and threonine-rich intracellular domains of the receptor carboxy end. This model was based on previous observations that showed that activation of NK-1 receptor induced hydrolysis of phospholipids [Hunter et al, 1985; Catalan et al, 1988; Takeda et al, 1991]. However, others have demonstrated that binding of SP to its receptor leads to formation of IP_3 and cyclic AMP formation, and proposed that either, two subtypes of SP receptor exist, one coupled to PLC and the other to adenylate cyclase [Tachado et al, 1991], or a

single SP receptor is in equilibrium with two different G proteins coupled to both pathways [Mitsuhashi et al, 1992].

1.8.3 *Receptor independent action of tachykinins*

An alternative model system for cell activation by tachykinins involves hydrophobic interactions between the neuropeptide and cell membrane, with the neuropeptide spanning of the membrane and directly activating G proteins [Mousli et al, 1990]. For example, SP is attracted to membranes containing anionic lipids, and inserts its carboxy terminus into the hydrophobic compartment as a helix. This receptor-independent model is based on observations, that the hydrophobic residues of amphiphilic peptides (e.g. SP, bradykinin) can penetrate or insert into the hydrophobic core of biological membranes [Hicks et al, 1992; Duplaa et al, 1992]. This type of interaction between the membranes and neuropeptides, changes the peptides conformation from random (present in aqueous solution) to a more defined and rigid structure as the hydrophobic side chains of the peptide become inserted in the hydrophobic core of the membranes. It has been hypothesised that optimal neuropeptide interactions with specific G proteins (and also with its receptor) requires the peptide to have a more defined and stable conformation at the time of binding [Schwyzer, 1991].

This model system for peptide-membrane interaction has gained further support by the demonstration that a number of amphiphilic peptides, including SP, can directly activate G proteins and in this way induce histamine release from mast cells [Mousli et al, 1990]. Whereas the carboxy terminal of SP is involved in the tachykinin interactions with their specific receptors, degranulation of mast cells is dependent on the amino terminus of SP (SP_{1-4}) [Fewtrell et al, 1982; Shibata et al, 1985]. This effect does not appear to be receptor mediated as high concentrations of SP are required to

activate mast cells and there is a lack of convincing evidence from radioligand binding studies to support the existence of a specific receptor. Moreover, although SP_{1-4} is a poor activator of histamine release on its own, when attached to a large hydrophobic chain, not necessarily peptidic, it becomes a potent activator [Mousli et al, 1990], reinforcing the need for a hydrophobic interaction with the cell membrane for optimal activity. Similarly, activation of neutrophil oxidative metabolism requires high concentrations of SP and may involve non-receptor mediated activation of G proteins and subsequent stimulation of PLC-mediated phospholipid hydrolysis [Serra et al, 1988].

Thus, for a direct interaction of a tachykinin with G proteins, the neuropeptide must have a hydrophobic domain that can form α-helix in the membrane, and positively charged domain which can interact with the carboxy terminal of the α subunit of the G protein. This model offers an explanation for activation of many non-neuronal cells by neuropeptides, where the presence of specific receptors on the cell surface has not been demonstrated.

1.9 Tachykinins in Inflammation and Inflammatory Disease

In the last decade, a lot of evidence has accumulated in support of the notion that tachykinins are mediators of both acute and chronic inflammation. It has been demonstrated that tachykinins can modulate immune processes by regulating directly the activity of many inflammatory cells [reviewed by: Stanisz et al, 1987; Payan and Goetzl, 1988; Basbaum and Levine, 1991; Eglezos et al, 1991a; Gilbert and Payan, 1991; Weinstock, 1992], and also by inducing or modulating cytokine production from other cells (e.g. IL-1, IL-2, TNFα, IL-6, INFγ) [Laurenzi eta al, 1990; Calvo et al, 1992; Lotz et al, 1988]. On the other hand, tachykinin activity at an

inflammatory site may be modulated by cytokines released from inflammatory cells, which may regulate the production of these neuropeptides by nerve cells [Freidin and Kessler, 1991]. Thus, the immune system may control or modulate the local activity of the nervous system, integrating both systems through functional interactions under inflammatory conditions. The finding that macrophages and eosinophils have the ability to produce tachykinins [Weinstock et al, 1988; Weinstock and Blum, 1989] and therefore, modulate both the neuronal and immune responses, further substantiates the functional integration of both systems. Thus, tachykinins released from sensory afferent fibres may participate in the regulation of diverse and complex local activities during an inflammatory processes, and therefore may constitute a neuroimmunological contribution to immune function and the pathology of inflammatory diseases.

1.9.1 *The nervous system, tachykinins and inflammatory disease*

A number of clinical observations and results from animal studies are consistent with nervous system involvement in inflammation and inflammatory disease. This is further emphasised by the findings of elevated concentrations of neuropeptides in relation to the development, progression and severity of different diseases, and prevention or limitation of the disease by neuropeptide antagonists in animal models of rheumatoid arthritis and asthma, as described below.

In 1936, Lewis reported that activation of the axon reflex in the afferent fibres of a peripheral nerve produced many of the tissue changes seen in inflammation, namely a wheal and flare response. In rheumatoid arthritis, chronic inflammation of the joints tends to occur with bilateral symmetry [Kidd et al, 1989]. Inflammation in one joint is generally accompanied by inflammatory response in the contralateral joint, implicating the role for

involvement of nervous system. Levine et al [1985] have suggested that this may be explained by the symmetrical innervation of joints by sensory and autonomic nerves and the way in which such nerves are controlled bilaterally by the central nervous system. Support for this suggestion comes from studies in hemiplegic patients, who later develop rheumatoid arthritis: the joints on the paretic side are relatively spared by the inflammatory process [Thompson and Bywaters, 1962], again confirming a role of the nervous system in the inflammatory response. The susceptibility of certain joints to involvement may also be explained by differences in the density of innervation. For example, in the model of adjuvant-induced arthritis in the rat, those joints that were most severely affected (ankles) were more densely innervated by primary nociceptors than those joints less severely involved (knees), and the concentration of the neuropeptide, SP, was higher in the ankle [Levine et al, 1984, 1987]. Similarly, slow infusion of SP into a knee joint considerably increased the severity of the adjuvant-induced arthritis, as assessed by soft tissue swelling, bone loss, osteoporosis and cartilage loss [Levine et al, 1984]. The proinflammatory effects of SP could be inhibited by prior administration of SP antagonists [Lam and Ferrel, 1989a]. These effects of SP are probably receptor mediated as specific SP receptors were shown to be present in rat synovium [Cruwys et al, 1991]. Moreover, Mapp et al [1990b] have demonstrated that there is a large increase in SP receptors in rheumatoid synovium compared with normal synovium. Other studies have shown that in the rat adjuvant model, selective depletion of SP in the fibres innervating the knee joint, by treatment with capsaicin, decreased the inflammatory response [Colpaert et al, 1983]. It has been suggested that in addition to depleting afferent fibresof SP, capsaicin may also deplete SP receptors, and in this way suppress SP-induced joint inflammation [Lam and Ferrel, 1989b]. In rats with adjuvant arthritis, the concentration of SP is increased in peripheral nerves that have branches innervating inflamed joints [Lembeck et al, 1981]. Studies in humans demonstrated that normal

synovium is densely innervated with tachykinin-containing sensory nerves [Mapp et al, 1990a], whereas the innervation and the neuropeptide content of sensory nerves in inflamed synovia from rheumatoid arthritis patients are dramatically decreased [Gronblad et al, 1988; Pereira da Silva and Carmo-Fonseca, 1990]. These results suggested a local release of tachykinins into joint fluid of rheumatoid arthritis patients, which is supported by reports that elevated SP levels are found in synovial fluids from patients with inflammatory arthritis [Devillier et al, 1986; Marshall et al, 1990; Appelgren et al, 1991]. Finally, the onset and exacerbation of rheumatoid arthritis is often preceded by psychological stress [Baker, 1982].

It is apparent from the above studies that SP has a pathophysiological function in rheumatoid arthritis. However, the exact mechanisms of its action on inflammatory processes in inflamed joint, need further investigation. A possible role for SP was suggested by Lotz et al [1987], who demonstrated that SP is capable of stimulating synoviocytes in rheumatoid arthritis. Synoviocyte proliferation leads to panus formation that damages bone and cartilage. Furthermore, SP and NKA increase production of proinflammatory cytokines by synoviocytes (e.g. IL-1, IL-6, TNFα) that have been shown to be associated with many pathological functions in various inflammatory diseases [Lotz et al, 1988].

SP is only one of a number of neuropeptides found in peripheral sensory nerves innervating joints. Those fibres which contain SP are also known to contain other vasoactive peptides, including NKA, NKB and calcitonin gene-related peptide (CGRP)— a potent vasodilator, which acts synergistically with SP in some of its actions [Pereira da Silva and Carmo-Fonseca, 1990; Brain and Williams, 1985]. CGRP is thus an important component of the profile of vasoactive materials released by sensory nerves in damaged or inflamed tissues. Other separate categories of small sensory fibres contain somatostatin, vasoactive intestinal peptide, colecystokinin-like peptides or

bombesin, which add still further to the complexity of the chemical ingredients that nerves may release into inflammatory joints.

Similar clinical observations and experimental approaches have provided evidence for the role of tachykinins in asthma [Casale, 1991]. SP immunoreactivity has been localised to airway nerves of many species including humans. In humans, SP containing nerves were found beneath or within the epithelium, around bronchial blood vessels, within bronchial smooth muscle and around local tracheobronchial ganglion cells [Lundberg et al, 1984b] and more recently in alveolar walls [Nohr and Weihe, 1991]. SP fibres in the airways usually also contain NKA and CGRP [Martling, 1987a]. SP containing nerves are found in highest concentration in the central cartilaginous airways, although they occur throughout the respiratory tree [Lundberg et al, 1983a].

Whether endogenously released or exogenously administered, tachykinins have a number of different physiological effects in the airways, especially those of asthmatics. For example, normal human subjects do not develop airway obstruction after inhalation of SP or NKA, whereas both tachykinins cause broncoconstriction in asthmatic patients [Joos et al, 1989]. NKA is much more potent than SP in provoking bronchoconstriction, suggesting an involvement of NK-2 receptors, a finding further confirmed by studies *in vitro* [Naline et al, 1989]. Other effects induced by exogenously administered tachykinins include tracheobronchial vasodilation, increase in mucosal microvascular permeability and plasma extravasation [Salonen et al, 1988; Lundberg et al, 1984b; MacDonald et al, 1989]. Although all tachykinins (SP, NKA and NKB) increase broncovascular permeability, SP is about eight times more potent than NKA and NKB, suggesting that NK-1 receptors on endothelial cells are involved. Increased airway mucus secretion and plug formation is one of the features of mucosal inflammation and asthma. Tachykinins, SP, NKA and NKB directly stimulate secretion from

brochial submucosal glands in isolated airways of many animals including humans [Borson et al, 1987; Coles et al, 1984; Haxhiu et al, 1991], an effect mediated through NK-1 receptors.

Many of the physiological effects of tachykinins have been demonstrated, when inhibitors of specific enzymes, that degrade tachykinins, were used to potentiate their action *in vivo,* and will be discussed in Chapter 1.9.3.

A recent *in vitro* study showed, that tachykinins may regulate neutrophil recruitment into the lower respiratory tract by inducing the release of neutrophil chemotactic agent from airway epithelial cells [Von Essen et al, 1992]. This effect was encoded in the carboxy-terminal of SP and was probably NK-1 receptor-mediated, as SP was the most potent of all tachykinins. It has been demonstrated previously, that endogenous release of tachykinins causes neutrophil adherence to postcapillary venules [Umeno et al, 1989]. However, since the sequestered neutrophils did not seem to move into the tissue, the significance of this finding remains uncertain [Umeno et al, 1990].

1.9.2 *Effect of SP on cells at inflammatory sites*

Of all the tachykinins studied, SP has received most of the attention and its pro-inflammatory properties have been studied most extensively. SP was originally described by Von Euler and Gaddum in 1931, who reported that extracts from brain and intestine contained a substance that causes atropine-resistant contractions of rabbit intestine. SP is a powerful vasodilator and increases vascular permeability, and in addition degranulates mast cell with release of histamine and serotonin. These mediators contribute to the plasma extravasation and increased local blood flow that accompany inflammation

[Pernow, 1983; Lundberg et al, 1983b]. Moreover, mast cell degranulation by SP and the direct effect of SP on vascular endothelial cells, cause granulocyte (neutrophil and eosinophil) infiltration into tissue sites [Iwamoto et al, 1992]. More importantly, however, SP has been shown to stimulate a variety of immune and inflammatory cells. For example, SP promotes neutrophil chemokinesis, chemotaxis [Wiedermann et al, 1989; Iwamoto et al, 1990; Perianin et al, 1989], lysozymal enzyme release, phagocytosis [Bar-Shavit et al, 1980; Serra et al, 1988; Hafstrom et al, 1989], aggregation, superoxide anion release, leukotriene synthesis and cytotoxicity [Hafstrom et al, 1989; Serra et al, 1988; Brunelleschi et al, 1991; Wozniak et al, 1989; Perianin et al, 1989] and eosinophil enzyme release [Kroegel et al, 1990]. Furthermore, it has effects on lymphocyte function, such as proliferation and immunoglobulin synthesis [Payan et al, 1983; Stanisz at al, 1986; Scicchitano et al, 1987, 1988], macrophage and monocyte chemotaxis, oxy radical production, thromboxane release and cytokine production [Hartung & Toyka, 1983; Ruff et al, 1985; Hartung et al, 1986; Wagner et al, 1987; Lotz et al, 1988; Kimball et al, 1988]. Although its effects on neutrophils and eosinophils are thought not to be receptor-mediated, macrophage and monocyte activation, appear to involve specific receptor for SP [Ruff et al, 1985; Hartung et al, 1986; Brunelleschi et al, 1990b]. However, the presence of SP receptors on lymphocytes is controversial [Roberts, 1992].

Substance P also has a regulatory role by stimulating or enhancing the release of cytokines such as interleukin-1 and -6 (IL-1, IL-6) [Lotz et al, 1988; Kimball et al, 1988; Laurenzi et al, 1990], interferon-gamma (INF-γ) [Wagner et al, 1987] and interleukin-2 [Calvo et al, 1992], as well as inflammatory mediators including histamine [Fewtrell et al, 1982; Foreman & Jordan, 1983; Foreman & Piotrowski, 1984; Shibata et al, 1985; Shanahan et al, 1985] and prostaglandins [Hartung et al, 1986], all of which are important in the inflammatory process.

In addition its proinflammatory activities, SP may regulate tissue repair at inflammatory sites, by enhancing proliferation of smooth muscle cells and fibroblasts [Nilsson et al, 1985; Payan, 1985]. The proliferative effect of SP on smooth muscle cells was blocked by SP antagonists, and appears to be receptor-mediated. Furthermore, SP competed with other polypeptide growth factors, for what is suggested to be a common receptor [Nilsson et al, 1985]. Interestingly, fibroblast polypeptide growth factors, capable of stimulating proliferation, were shown to have amino acid sequence homology with SP and NKA [Gimenez-Gallego et al, 1985].

1.9.3 *Control of tachykinin activity by enzymatic degradation*

Neutral endopeptidase (NEP) is one of the enzymes that are thought to play a major role in degradation of tachykinins. Although other enzymes, such as angiotensin-converting enzyme (ACE) [Skidgel and Erdos, 1987], serine proteases [Hanson and Lovenberg, 1980; Pernow, 1988], mast cell tryptase and chymase [Caughey et al, 1988], cathepsin G [Skidgel et al, 1991], dipeptidyl(amino)peptidase IV (DAP IV), aminopeptidase M [Wang et al, 1991a] and possibly acetylocholinesterase [Chubb et al, 1980] may also cleave tachykinins, most neurogenic inflammatory responses are thought to be modulated by NEP.

Although the specificity of the above enzymes could be either broad or limited, their distribution is usually very broad, and often more than one enzyme is found in any one type of tissue. For example, NEP, that degrades SP and NKA, is found in skin, kidney, brain, gastrointestinal tract and human lung [Johnson et al, 1985; Martins et al, 1990]. ACE is also found in the lung, but unlike NEP, it is specific for SP and not NKA [Hooper et al, 1985]. Aminopeptidase M, DAP IV and ACE, which have been demonstrated

to process (however differently) both SP and NKA, are all found in human plasma [Wang et al, 1991a].

The activity of tachykinins at inflammatory sites may be modulated by enzymatic cleavage with the products (peptide fragments) having inhibitory or enhancing effects on a variety of functions. For example, inhibition of lung NEP dramatically enhances bronchial responsiveness to endogenously released or exogenously administrated tachykinins. Airway responses to either SP or NKA, such as broncoconstriction and smooth muscle contraction [Martins et al, 1990; Shore and Drazen, 1989; Stimler-Gerard, 1987], plasma extravasation [Umeno et al, 1989] and mucus secretion [Borson et al, 1987], are all greatly enhanced when NEP inhibitors are used. It is of interest to note that viral infections [Jacoby et al, 1988; Dusser et al, 1989a] and cigarette smoking [Dusser et al, 1989b] all exaggerate inflammatory responses, and they decrease NEP activity. On the other hand, carboxy-and amino-terminal peptide fragments, resulting from partial digestion of tachykinins, may induce different specific functions in variety of cells. For example, the amino-terminal of SP (but not the carboxy-terminal) stimulates histamine release from mast cells [Fewtrell et al, 1982], whereas the chemotactic responsiveness of monocytes is located in the carboxy terminal [Ruff et al, 1985; Wiedermann et al, 1989]. Moreover, cell responsiveness to tachykinin fragments can be greater than the native peptide (e.g. fragment SP_{4-11} was 26-fold more potent in inducing superoxide generation in neutrophils than SP [Iwamoto et al, 1990]) or inhibited (e.g. SP_{1-4} inhibits superoxide anion production stimulated by fMLP in neutrophils [Wozniak et al, 1992b]) when compared with the whole SP.

The discovery that cathepsin G and NEP which are present on the cell membrane of human neutrophils [Connelly et al, 1985; Painter et al, 1988; Erdos et al, 1989] can metabolise SP [Skidgel et al, 1991], is of crucial importance to inflammatory processes and diseases, in which neutrophils

have been shown to play an important role, including asthma [Barnes, 1986] and rheumatoid arthritis [Levine et al, 1984; Lotz et al, 1987]. The activity of neutrophils at inflammatory site is regulated by a number of mediators; SP also regulates neutrophil effector functions, including chemotaxis, enzyme release, cytotoxicity and oxy radical production (Chapter 1.9.2). Hydrolysis of SP by cathepsin G and NEP present on neutrophils may inactivate the neuropeptide, but equally may result in formation of peptide fragments that can enhance or inhibit various functions of nearby cells, as explained above. Furthermore, it has been demonstrated that activation of PLC and phosphatidylinositol hydrolysis in neutrophils, downregulates the membrane NEP, suggesting that activated cells will have reduced capacity to hydrolyse neuropeptides [Erdos et al, 1989]. This may become significant when circulating neutrophils enter an inflammatory site, and become exposed to cytokines, other activators and SP at the same time. The reduced capacity to hydrolyse SP may help maintain the effects of SP on neutrophils. For example, neutrophil adhesion induced by SP is enhanced by NEP inhibitors [Umeno et al, 1989]. In summary, degradative enzymes can modulate tachykinin activity and this is very relevant to the pathogenesis of inflammation.

1.10 Aims and Outline of Thesis

As reviewed in this chapter, the nervous system is involved in the pathogenesis of many inflammatory diseases, including asthma and rheumatoid arthritis. The close association between the nervous system and immune system has become more apparent in recent years, with the rapid development in the area of neuroimmunomodulation at a cellular and molecular level. However, the mechanisms by which the nervous system modulates immune responses are still not well understood. It is proposed that one way is through the release of endogenous neuropeptides which subsequently act on the cells of the immune system.

This thesis examines the effects of neuropeptides, members of the tachykinin family, on the function of phagocytic cells involved in inflammatory responses. The general aims are to (i) study the effects of SP and other tachykinins on various human neutrophil functions, (ii) investigate the mechanisms by which SP modulates and/or activates neutrophil function, (iii) study the effects of SP on phagocytes derived from asthmatic patients and correlate these with the severity of disease and (iv) investigate possible interactions between SP and proinflammatory cytokines.

In the first experimental chapter (Chapter 3), I have shown that SP is a potent regulator of mature, human neutrophil function. SP activated some neutrophil functions (e.g., ADCC), modulated others (e.g., O_2^- production), but had no effect on neutrophil degranulation and adherence to endothelial cells. Different experimental conditions were evaluated. Time course and dose responses of stimulation were studied to establish conditions required for optimal responses. Additional experiments provided initial information on whether the SP effects on neutrophils are receptor mediated.

Chapter 4 examines the role of other tachykinins and SP fragments in neutrophil stimulation. Experiments were carried out to investigate the

relative potency of SP, NKA, NKB and other non-mammalian tachykinins, E and K, in modulating neutrophil O_2^- production. The terminal fragments of SP were used to locate the activity in the molecule, and provided additional information on possible involvement of tachykinin fragments in inflammation.

Chapters 5 and 6 investigate the intracellular mechanisms involved in SP modulation of O_2^- production and whether the SP responses are receptor mediated. In particular, the role of PKC and calcium in enhancing O_2^- response by SP in neutrophils was studied, and radioligand binding studies were perfomed to characterise the SP binding site on neutrophils.

Finally, the role of SP in inflammatory disease is examined in chapter 7. The effects of SP on cells isolated from asthmatics were studied and the responses correlated with the severity of asthma assesssed by bronchial hyperresposiveness. In addition, the interactions between SP and other inflammatory cytokines (GM-CSF and IL-8), that have been shown to have important role in both asthma and rheumatoid arthritis, were investigated.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 *Peptides and cytokines*

Substance P, NKA, NKB, SP antagonists and fragments were purchased from AUSPEP, Melbourne, Australia. Stock solutions of 1 mM were made up in 1 mM acetic acid and aliquots were stored under nitrogen at -70°C and thawed only once just before use. All peptides were shown to be endotoxin free by the *Limulus* amoebocyte lysate assay.

Recombinant human Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), a gift from Dr Angel Lopez, Institute of Medical and Veterinary Science, Adelaide, was stored at -20°C and used within two weeks.

Recombinant human interleukin-8 (rhIL-8), essentially endotoxin free (less than 0.1 ng/μg of rhIL-8), was purchased from Promega (Sydney, Australia).

The lyophilized rhIL-8 was reconstituted in PBS to a concentration of 10 μg/ml, stored in 50 μl aliquots at -70°C and thawed only once before use.

2.1.2 *Cell lines*

P815 (DBA/2 mastocytoma) and **U5637** (bladder carcinoma) were were kindly supplied by Dr. Angel Lopez, Institute of Medical and Veterinary Science, Adelaide.

2.1.3 *Gradients for cell preparations*

Lymphoprep	NYCOMED AS, Oslo, Norway
Metrizamide (grade C)	NYCOMED AS, Oslo, Norway
Percoll	Pharmacia, Uppsala, Sweden

2.1.4 *Buffers and culture media*

Hank's buffer: 138 mM NaCl, 5 mM KCl, 0.3 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 1.3 mM $CaCl_2$, pH 7.3.

Modified Dulbecco's phosphate buffered saline (DPBS): 138 mM NaCl, 2.7 mM KCl, 16.2 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.5 mM $MgSO_4$, 0.6 mM $CaCl_2$ and 7.5 mM glucose, pH 7.3.

Phosphate Buffer: (i) solution A: 67 mM Na_2HPO_4

(ii) solution B: 67 mM KH_2PO_4

mix A and B to pH 7.0

Posphate Buffered Saline (PBS): 16.2 mM Na_2HPO_4, 4 mM NaH_2PO_4, 120 mMNaCl, pH 7.4.

Posphate Free Buffer: 30 mM Hepes, 100 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM L-glutamine, 10 mM glucose, 2mg/ml BSA, pH 7.4

2X RIPA Buffer: 150 mM NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 5 mM EDTA, 10 mM NaF, 5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM ATP, 10 mM DFP, 2 μM leupeptin, 2 μM pepstatin, 1 μg/ml DNAse, 0.68 M sucrose, 0.2% SDS, 2% NP-40, pH 7.2.

Tris Buffer: 50 mM Tris, 138 mM NaCl, 0.01% Brij, pH 8 (adjusted with HCl)

Tyrode's solution: 138 mM NaCl, 2.7 mM KCl, 0.35 mM Na_2HPO_4, 12 mM $NaHCO_3$, 5.5 mM D-glucose, 60 U/ml DNase and 0.1% gelatin.

RPMI-1640: was always supplemented with 20 mM HEPES, 1 mM sodium pyruvate, 2 mg/ml sodium carbonate, 2 mM L-glutamine, 60 μg/ml penicillin and 8 μg/ml gentamycin. pH was adjusted to 7.3 by bubbling through CO_2.

RPMI/FCS/DNase: RPMI-1640 additionally supplemented with 5% FCS and 60 U/ml DNase.

HUVEC culture medium: RPMI-1640 additionally supplemented with 20% FCS, non-essential amino acids, 25 μg/ml ECGS and heparin 25 μg/ml.

P815 and U5637 culture medium: RPMI-1640 additionally supplemented with 5% FCS and 0.3 μM β-mercaptoethanol.

2.1.5 *Reagents and supplements for culture media*

Reagent	Supplier
β-mercaptoethanol	Sigma Chemicals, USA
Endothelial Cell Growth Supplement (ECGS)	Sigma Chemicals, USA
Fetal Bovine Calf Serum (FCS)	Flow/ICN Biocemicals, NSW Australia
Gentomycin	Delta West, WA, Australia
Heparin (Porcine mucus)	Fison, NSW, Australia
HEPES	Gibco, Grand Island, NY, USA.
L-glutamine (200 mM)	Flow/ICN Biocemicals, NSW Australia
Penicillin	Flow/ICN Biocemicals, NSW Australia
RPMI 1640	Sigma Chemicals, USA
Sodium carbonate (7.5%)	Flow/ICN Biocemicals, NSW Australia
Sodium pyruvate (100 mM)	Flow/ICN Biocemicals, NSW Australia
Non-essential amino acids (100x)	Flow/ICN Biocemicals, NSW Australia

2.1.6 *Other chemicals and reagents*

Adenosine triphosphate (ATP)	Sigma Chemicals, USA
a-DNP (rabbit, polyclonal)	Miles-Yeda, Rehovot, Israel
Bovine Serum Albumin (BSA) (98-99%, Essentially fatty acid free)	Sigma Chemicals, USA
Brij 35 (polyoxyethylene 23-lauryl ether)	Sigma Chemicals, USA
Collagenase	Sigma Chemicals, USA
Cytochalasin B (from *Helminthosporium Dematioideum*)	Sigma Chemicals, USA
Cytochrome C (Cyt C) (type IV, horse heart)	Sigma Chemicals, USA
Dextran T-500	Pharmacia, Uppsala, Sweden
D-glucose	Sigma Chemicals, USA
Di-isopropylfluorophosphate (DFP)	Sigma Chemicals, USA
Dimethyl sulphate	Sigma Chemicals, USA
DNase (Deoxyribonuclease 1, Type II, from bovine pancreas)	Sigma Chemicals, USA
Elastase (HLE) (from human neutrophils)	Elastin Products, Owensville, USA
E-Toxate (Limulus Amebocyte Lysate)	Sigma Chemicals, USA

fMLP (N-formyl-methionyl-leucyl-phenylalanine)	Sigma Chemicals, USA
Fura 2/AM {-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-metholphenoxy)-ethane-N,N,N',N'-tetraacetic acid, pentaacetoxymethyl ester}	Calbiochem, USA
Gelatin	Sigma Chemicals, USA
15-HETE (5(S)-Hydroxy-(5Z,8Z,11Z,13E)-eicosatetraenoic acid)	Sigma Chemicals, USA
Leupeptin	Sigma Chemicals, USA
Luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione)	Sigma Chemicals, USA
Methoxysuccinyl-dialanine-proline-valine- p-nitroanilidine	Sigma Chemicals, USA
Myeloperoxidase (MPO) (from human polymorphonuclear leukocytes, B grade)	Calbiochem, USA
NP-40	Sigma Chemicals, USA
Pepstatin	Sigma Chemicals, USA
12-Phorbol, 12-myristate acetate (PMA)	Sigma Chemicals, USA
Platelet activating factor (PAF)	Sigma Chemicals, USA
p-Phenylodiamine	Sigma Chemicals, USA
Prostaglandin B_2 ([5Z, 13E, 15F]-15-Hydroxy-9-oxoprosta-5, 8[12], 13-trien-1-oic acd)	Sigma Chemicals, USA
Rose Bengal	Ajax Chemicals, Australia
Sodium fluoride	Sigma Chemicals, USA

Sodium pyrophosphate	Sigma Chemicals, USA
Sodium vanadate ($NaVO_4$)	Sigma Chemicals, USA
Sucrose	Sigma Chemicals, USA
Superoxide dismutase (SOD)	Boehringer Mannheim, Germany
Trypsin	Sigma Chemicals, USA
Zymosan A (from *Saccharomyces cerevisiae*)	Sigma Chemicals, USA

2.1.7 *Radiochemicals*

Chromium-51 (10–35 mCi/ml) (sodium chromate in 0.9% NaCl solution)	Amersham, Sydney, Australia
[^{3}H]-phorbol dibutyrate (12.5 Ci/mmol)	New England Neuclear, UK
^{32}P-phosphate (0.25 mCi/ml)	Amersham, Sydney, Australia
^{125}I-Substance P (2200Ci/mmole) (Bolton-Hunter labelled)	New England Neuclear, UK

2.2 Methods

2.2.1 *Cell preparations*

2.2.1.1 *Isolation of human neutrophils by Lymphoprep gradient*●

Neutrophils were isolated from the peripheral blood of normal volunteers that was anticoagulated with 0.09% EDTA (or 10 U heparin/ml blood for ADCC. studies). The leukocyte-rich fraction (buffy coat) was obtained by sedimenting erythrocytes with 5% dextran for 40 min at room temperature. The cells were washed twice by centrifugation at 400g for 10 min. This procedure also removed most of platelets from the buffy coat. Neutrophils were isolated by density-gradient centrifugation using Lymphoprep. 10^8 buffy coat cells in 10 ml RPMI was carefully underlayered with 10 ml of Lymphoprep using 10 ml syringe with 18G needle in a 50 ml conical tubes, and the gradients were centrifuged at 400g for 20 min at 22°C. Neutrophils mixed with erythrocytes formed pellets at the bottom of the tubes. Residual erythrocytes were removed by hypotonic lysis: 5 ml of ice-cold 0.2% NaCl was mixed with each pellet by vortexing for 25 sec, and then 5 ml of 1.6% NaCl was added. The cells were washed twice and resuspended in required buffer of medium. Neutrophils isolated by this method were always > 96% pure, as determined by Grunwald-Giemsa staining, and > 98% viable by trypan blue exclusion.

2.2.1.2 *Isolation of human neutrophils by Percoll gradient*

Neutrophils were isolated from normal blood using Percoll as described previously [McColl et al, 1986]. Blood was anticoagulated with EDTA and the leukocyte-rich fraction was obtained by sedimenting erythrocytes with 5% dextran for 40 min at 37°C, washed twice and resuspended in DPBS. 10 ml of buffy coat cells (10^8) suspended in DPBS were then carefully layered on

● [Boyum 1976]

the top of percoll gradients consisting of two layers of Percoll with different densities. The gradients were prepared by placing 10 ml of 1.092 g/ml Percoll in a 50 ml conical tube and then, using 10 ml syringes with attached 21G needles, overlayering it gently with 10 ml of 1.070 g/ml Percoll. The gradients were centrifuged at 450g for 20 min at 22^oC. Neutrophils were collected from the interface between the two Percoll layers, washed twice in DPBS (Ca^{++}, Mg^{++} free) and resuspended in DPBS to concentration of 1-2 x 10^6/ml.

Occasionally, when small number of red blood cells copurified with the neutrophils, they were removed as described in the Lymphoprep method above. The purity and viability of neutrophils obtained using this method was the same as neutrophils obtained by Lymphoprep gradient.

Percoll solutions.

Percoll 150 was prepared by mixing 9 parts of Percoll stock solution with 1 part (v/v) of 10X DPBS and adjusting the pH to 7.0 with NaOH.

Percoll 1.070 and 1.092 g/ml were prepared by mixing 5.85 ml of Percoll 150 with 4.15 ml of DPBS, and 7.65 ml of Percoll 150 with 2.35 ml of DPBS respectively.

2.2.1.3 *Isolation of human neutrophils and eosinophils by Metrizamide gradient*

This method has been described in detail elsewhere [Vadas et al, 1979]. Blood was anticoagulated with heparin (10 U heparin/ml blood) and the leukocyte-rich fraction was obtained by sedimenting erythrocytes with 5% dextran for 40 min at room temperature. The buffy coat cells were then washed twice by centrifugation at 400g for 10 min. Metrizamide gradients were prepared as follows: dilutions consisting of 18, 20, 22, 23 and 24%

metrizamide were made from a stock solution (30% metrizamide in Tyrode's solution) using Tyrode's solution. Gradients were prepared by carefully layering of 2 ml of each dilution, in order of decreasing densities, into a 15 ml Falcon conical tubes. 5-10 x 10^7 cells resusupended in 2 ml of RPMI/FCS/DNase was loaded on the top of each gradient and the cells were then centrifuged at 1200g for 45 min at 22°C (acceleration to 500 rpm over 2 min, brake off). Neutrophils were collected from the 20/22% interface and were > 96% pure, and > 99% viable. Eosinophils were collected from the 22/23% and 23/24% interfaces, and were mixed for the experiments. The purity varied and was 85–95% and the cells were > 99% viable. Cell purity was determined by Grunwald-Giemsa staining and cell viability by trypan blue exclusion.

2.2.1.4 *Isolation of human umbilical cord vascular endothelial cells*

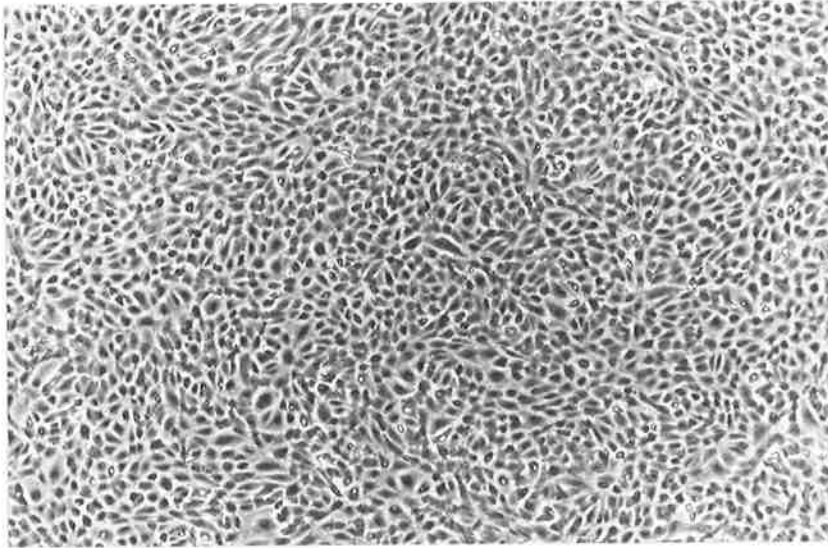
HUVEC were isolated and cultured according to the technique described by Jaffe et al [1973], with minor modifications. HUVEC were cultured in tissue culture flasks which had been coated with sterile 0.1% gelatin.

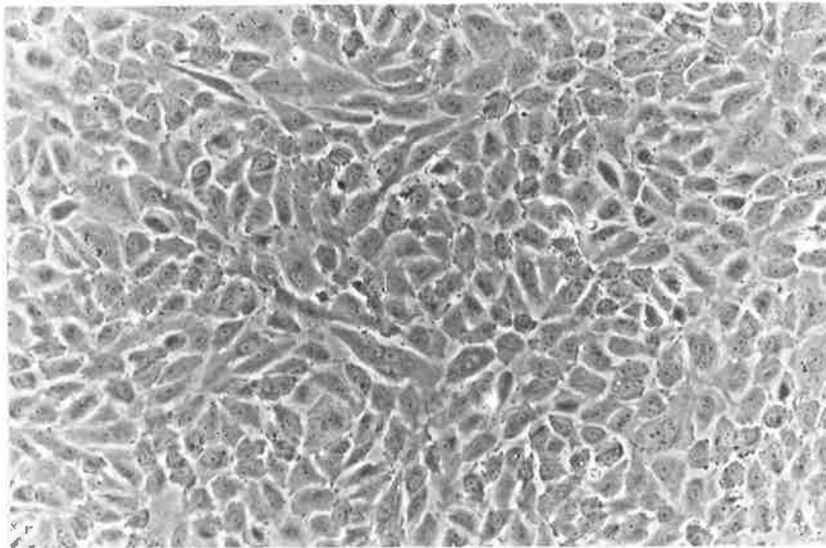
Fresh human umbilical cords were collected from the maternity section of the Queen Victoria Hospital, and stored in sterile Hank's buffer at 4°C until use. The cords were examined for clamp marks and only undamaged portions of 15 cm or longer were used, as clamping damages the interior of the vein increasing the risk of fibroblasts contamination. The cord was trimmed on both sides and the umbilical vein was cannulated a 21G winged infusion set, that was secured using surgical silk. The lumen of the vein was washed thoroughly by perfusion with 50 ml of warm, sterile Hank's buffer. Then, the other end of the vein was cannnulated with a sterile, steel T-tap and secured with surgical silk. With the T-tap closed, the vein was filled with 5-10 ml of 0.1% collagenase in sterile Hank's. The clamped cord filled with

collagenase was then incubated for 25 min in sterile Hank's, in the water bath at 37°C. After the incubation the vein was gently flushed with 50 ml of warm 5% FCS RPMI. FCS present in RPMI inactivates the collagenase and prevents damage to the endothelial cells. The cells were pelleted by spining them at 1500 rpm for 7 min, resuspended in 5 ml of HUVEC culture medium and plated in a gelatin-coated 25 cm^2 tissue culture flasks. After overnight incubation the non adherent cells (mostly red blood cells) and debri were removed by washing with warm Hank's buffer, and 10 ml of fresh medium was added. The cells were grown to confluency at 37°C in a CO_2 incubator (5% CO_2).

After the cells attained confluency, they were removed from the culture flask by detaching with 0.1% EDTA/0.125% trypsin solution and by gently tapping the flask against the palm of a hand. The flask was observed under a microscope to ensure that all the cells dislodged from the bottom. Detached cells were washed with 5% FCS RPMI (FCS inactivates trypsin) by centrifugation at 1500 rpm for 7 min, and passaged to a 75 cm^2 tissue culture flask. Subsequent passages were done by dividing cells from one confluent flask into two 75 cm^2 flasks, using the same technique.

The adherent cells were identified as HUVEC by their typical "cobblestone" morphology which readily distinguishes them from contaminating fibroblasts or smooth muscle cells (see photograph below). Their identity was confirmed by electron microscopy by demonstrating the presence of the Weibel-Palade bodies, that are characteristic of endothelial cells.





Human umbilical vein endothelial cells. Magnification: 60x and 150x, top and bottom photograph respectively.

2.2.2 *Functional responses of neutrophils*

2.2.2.1 *Antibody dependent cell-mediated cytotoxicity*

The method has been described in detail previously [Vadas et al, 1983]. All experiments were performed in triplicate, and SD's within an experiment were always < 10%. The assay was performed in RPMI-1640 suplemented with 0.1% BSA.

Preparation of P815 target cells

Cells used for every experiment were passaged the day before by transfering 1 ml of confluent P815 cell suspension to a 25 cm^2 tissue culture flask containing 10 ml of fresh culture medium. The cells (usually 5-8 x 10^6) were then pelleted by centrifugation at 1500 rpm for 5 min, and the pellet suspended in 100 µl of culture medium to which 200 µCi of ^{51}Cr was added. After 1 hour incubation at 37°C (water bath) with occasional mixing, the cells were washed once in PBS, centrifuged at 1500 rpm for 5 min, and the cell pellet suspended in 400 µl of PBS. The ^{51}Cr-labeled cells were then opsonised with TNP by mixing them with 100 µl of 10 mg/ml TNP and incubating for 20 min at room temperature. As addition of TNP changes the pH of the buffer to acidic, the pH was adjusted back to neutral with 0.1 M NaOH, in the presence of 1 drop of 0.5% phenol red. After the incubation, the cells were washed in 10 ml of 5% FCS RPMI and resuspended in 1 ml of culture medium. Then, they were carefully underlayed with 1 ml of FCS and centifuged at 100 rpm for 10 min. After centrifugation, the supernatant and FCS was carefully removed, the cells resuspended in 0.1% BSA RPMI to a concentration of 10^5 cells/ml and used immediately.

The ADCC assay

40 µl (4x10^3) of ^{51}Cr-labelled TNP-coupled P815 target cells were mixed with 80 µl (1.2x10^5) neutrophils (eosinophils) as effector cells, and 24 µl of

rabbit IgG anti-DNP antibody (cross-reacting with TNP) and 16 µl of the appropriate peptide (stimulus) in V-bottomed 96 well microtitre plates. The final assay volume was 160 µl. After incubation of the reaction mixture for 2.5 hr at 37°C, 80 µl of the supernatant were carefully removed and the radioactivity counted using a gamma counter (LKB, 1282 Commugamma, Turku, Finland). Percentage cytotoxicity was calculated as follows:-

$$\% \text{ cytotoxicity} = \frac{\text{experimental c.p.m. - spontaneous release c.p.m.}}{\text{total c.p.m. - spontaneous release c.p.m.}}$$

where spontaneous release was the ^{51}Cr released from P815 cells in the presence of medium alone and the total count was the ^{51}Cr released from the P815 cells lysed by the addition of 4% Triton X-100. Conditioned medium (BCM) from the bladder carcinoma cell line U5637 was used as a positive control, since it contains a number of colony stimulating factors (CSFs) known to enhance human neutrophil ADCC [Vadas et al, 1983]

2.2.2.2 *Superoxide anion production*

Neutrophil O_2^- production was measured as SOD-inhibitable reduction of ferricytochrome C (Cyt C).● This was determined by the addition of 10 µl of 2 mg/ml SOD to duplicate samples in all preliminary experiments. Measurements were made in real time to enable determination of rates of production of O_2^- and by end point, to enable calculation of total O_2^- production by any given stimulus/manipulation as described below. All end point experiments were performed in triplicate. The final concentration of cells in all experiments was adjusted to 10^6/ml.

●[Weening et al, 1975]

In the initial dose-response experiments, neutrophils (10^6) were incubated in triplicate with 100 μM Cyt C in DPBS (± SOD) containing the varying concentrations of peptides (0.01 μM to 100 μM) in a final volume of 1.0 ml. Since the peptides were dissolved in acetic acid, our medium control included the highest possible concentration (0.1 mM) of acetic acid. This did not have any effect on O_2^- production stimulated by any stimuli used. Cells were incubated for 30 min at 37°C and then either fMLP (0.1 μM), PMA (10 ng/ml) or medium was added and the mixture incubated for a further 6 min. The reaction was stopped by addition of SOD (10 μl of 2 mg/ml) and by placing tubes on ice. The cells were pelleted by centrifugation at 4°C and O_2^- production was quantified in cell supernatants by changes in absorption at 550 nm [Weening et al, 1975] and O_2^- production determined using an extinction coefficient of 21.1 mM^{-1} cm^{-1} [Van Gelder and Slater, 1962].

In the time-course experiments, and in experiments when the effect of SP on the dose-response to fMLP and PMA was studied, neutrophils (5 x 10^6 cell/ml) were incubated with medium or the stated concentration(s) of peptides for indicated time at 37°C. After incubation, 200 μl of cells (10^6) were transferred to a tube containing 800 μl of a warm mixture of 100 μM Cyt C and different concentration(s) of fMLP, PMA, OPZ or medium as a control. The mixture was incubated for a further 10 min at 37°C. The reaction was stopped by addition of SOD and by immersion of the tubes in ice. The cells were pelleted by centrifugation at 4°C and the O_2^-production was quantified in cell supernatants as described above.

The kinetics of O_2^- production were measured by following O.D. changes continuously in a thermostated (37°C) spectrophotometer. Neutrophils (5 x 10^6 cells) were preincubated with stated concentration of studied neuropeptide, or medium as a control, for 10 min at 37°C, then 200 μl of cells (10^6) transferred to cuvettes in the spectrophotometer containing 800 μl of prewarmed mixture containing 100 μM Cyt C and a stimulus (e.g., fMLP or

PMA), and changes in O.D. at 550 nm were monitored. The OD measurement were converted to nmols of O_2^- using an extinction coefficient as explained above.

In all experiments in which OPZ was used as a stimulus, the tubes containing neutrophils and OPZ had to be continuously mixed to prevent OPZ sedimentation. The control cells, to which OPZ was not added, were treated in the same way to control for neutrophil mixing. The mixing had no effect on neutrophil O_2^- production (Chapter 3, Fig. 3.13).

Opsonising zymosan

Zymosan was opsonised as follows: 20 mg of Zymosan A was resusupended in 2 ml of normal saline, boiled for 10 min in water bath and than centrifuged for 7 min at 200g. The supernantant was removed and zymosan suspended in 5 ml of fresh human plasma, and incubated for 30 min at 37°C. The opsonised zymosan (OPZ) was washed twice in normal saline and than resuspended to 10 mg/ml and stored in 0.1 ml aliquotes at -70°C until used

2.2.2.3 *Measurements of oxy radical production by luminol-enhanced chemiluminescence*

Luminol-enhanced chemiluminescence (CL) was employed as another method to assess respiratory burst (NADPH oxidase activity) using a Packard Pico Lite detector.● Before each experiment, luminol (20 µM) was mixed with prewarmed PMN (250 µl, 0.5 x 10^6 cells) and the mixture was preincubated for 7 min at 37°C. In the dose response experiments, background CL was

●[DeChatelet et al, 1982]

measured for 100 sec before stimulant was added and subsequent changes in CL were measured over 5 min. In the priming experiments, the cells were preincubated for 10 min with medium or 10 ng/ml of rhIL-8 and were then stimulated with 100 nM fMLP for an additional 5 min. In these experiments, background CL was measured for 100 sec before addition of fMLP.

2.2.2.4 *Leukotriene and 5-HETE production*

LTB_4 and 5-HETE production was measured by high performance liquid chromatography (HPLC) as described previously by McColl et al [1986], with minor modification. All experiments were performed in quadruplicates using neutrophils that were isolated on Percoll gradient.

The assay was performed in 13 ml glass tubes that had been chromic acid washed before each experiment. 0.9 ml neutrophils suspended in DPBS (1-2 x 10^6 cells/ml) were transferred into the tubes and prewarmed to 37^oC for 5 min, in a water bath. SP (100µl) was added to the prewarmed cells to a final concentration of 0.1 or 50 µM. DPBS (100µM) containing appropriate concentrations of acetic acid was added to the control samples, the cells were mixed and incubated for an additional 15 min at 37^oC. Two minutes before the end of the incubation time, 5 µl of 2 mM AA (10 µM final) was added to the samples in which LTB_4 and 5-HETE production was studied in the presence of exogenous AA and 5 µl of methanol (AA diluent) to the others. After 15 min of incubation with SP, the cells were then stimulated with 5 µM A23187 or 0.1 µM fMLP for an additional 5 min at 37^oC and the reaction terminated by addition of 100 µl 100 mM citric acid. Addition of citric acid lowers the pH of the aqueous phase to 3, which is necessary for the subsequent extraction of LTB_4 and 5-HETE into an organic phase. This is very important and usually the pH of several samples was checked to ensure that pH ≤ 3. At this point in the assay, 30 ng of prostaglandin B_2 and

124,5 ng of 15-HETE were added to each tube as the internal standards for LTB_4 and 5-HETE respectively and the samples mixed. LTB_4 and 5-HETE (and the internal standards) were extracted with 5 ml of chloroform/methanol (7:3). The tubes were vortexed vigorously for 1 min and then centrifuged for 10 min at 2000 rpm to separate the aqueous and the organic phases. The lower chloroform layer (containing leukotrienes and HETEs) was transferred to 5 ml borosilicate glass tubes and the chloroform evaporated under vaccum, at room temperature, using a centrifugal evaporator (Savant, Hicksville, NY, USA). The samples were reconstituted in 100 μl of the LTB_4 mobile phase, transferred to Waters low volume inserts and anlysed by HPLC.

Mobile phases

LTB_4:	67% Methanol/33% H_2O/0.08% Acetic acid (pH adjusted to 6.2 with ammonium hydroxide)
5-HETE:	77% Methanol/23% H_2O/0.08% Acetic acid (pH adjusted to 6.2 with ammonium hydroxide)

HPLC conditions

Injection volume:	25 μl
Wavelength:	270 nm (LTB_4), 235 nm(5-HETE)
Flow rate:	1 ml/min
Column and guard pack:	C_{18} Nova Pak (Waters Millipore)
Chart speed:	0.25 cm/min

Retention times

LTB_4:	8.8 min
Prostaglandin B_2	4.4 min
5-HETE:	11.5 min
15-HETE:	8.4 min

2.2.2.5 *Neutrophil degranulation*

Neutrophil degranulation was assesed by the activity of elastase (EL) and myeloperoxidase (MPO) in supernantants from stimulated cells. The assay for measurement of EL has been described previously by Walsh et al (1992). All experiments were performed in triplicate. The final concentration of cells in all experiments was adjusted to 10^6/ml. All measurements were performed on a Cobas Bio centrifugal analyser.

In the initial experiments, neutrophils were incubated with medium or cytochalasin B (5 µg/ml) for 5 min at 37°C and were then stimulated with various concentrations of SP or diluent control for additional 15 min. The reaction was stopped by pelleting cells (13,000 rpm, 30 sec) and placing tubes on ice. The supernantants were collected and used for measurements of EL and MPO.

In the priming experiments, neutrophils were preincubated for 5 min with cytochalasin B, then were incubated with medium or various concentrations of SP for 15 min and were then stimulated with various concentrations of fMLP or its diluent for an additional 10 min. The reaction was stopped and supernantants collected as described above.

The assay for the measurement of EL activity used methoxysuccinyl-dialanine-proline-valine-p-nitroanilide as substrate in the presence of 0.01% Brij. It was prepared as 0.1 M stock solution in dimethyl sulfoxide and diluted withTris buffer (pH 8.0) to 4 mM for the start reagent. Human neutrophil EL was used to calibrate the assay. The response was linear between 0 to 250 International Units (IU), over 5 min and a calculating factor of 140 was used as 140 Units of EL resulted in change of OD=1.

For the measurement of MPO activity, 0.2% p-phenylondiamine in phospate buffer (pH 7.0) was used as substrate. The substrate and the start reagent, 3 mM H_2O_2, were prepared fresh on each day. As for EL, human neutrophil MPO was used to calibrate the assay. The response was linear for at least 1 min and the conversion factor of 2 was used as 1 Unit of MPO resulted in change of OD=0.5.

Cobas Bio Settings

		EL	MPO
1.	Units	International Units (IU)	IU
2.	Calculation factor	140	2
3.	Standard 1 conc	0	0
4.	Standard 2 conc	0	0
5.	Standard 3 conc	0	0
6.	Limit	0	0
7.	Temperature (^oC)	25	25
8.	Type of analysis	1	6
9.	Wavelength (nm)	405	485
10.	Sample volume (µl)	50	50
11.	Diluent volume (µl)	0	0
12.	Reagent volume (µl)	200	150
13.	Incubation time (sec)	0	10
14.	Start reagent volume (µl)	0	20
15.	Time of first reading (sec)	15	0.5
16.	Time interval (sec)	30	10
17.	Number of readings	10	4
18.	Blanking mode	1	1

2.2.2.6 *Neutrophil adherence to HUVEC*

The adherence of neutrophils to cultured HUVEC monolayers was quantified by a method described previously [Gamble & Vadas, 1988]. In this assay, neutrophils and HUVEC were stimulated simultaneously with various concentrations of SP. However, for some experiments, HUVEC monolayers were incubated with 100 ng/ml LPS for 12 hr prior to the assay.

The HUVEC monolayers were prepared by harvesting HUVEC with trypsin-EDTA and plating them onto gelatin-coated flat-bottomed 96 well microtitre plates at 2 x 10^4 cell/well in 200 μl of HUVEC culture medium. The cells were grown overnight to confluence, and depending on the experiments were then either additionally stimulated with LPS or washed in 5% FCS RPMI and used immediately for the adherence assay.

Neutrophils isolated on Lymphoprep gradient were resuspended in 5%FCS RPMI at a concentration of 5 x 10^6 cell/ml, and 100 μl of the cells suspension (5 x 10^5 cells) was added to each well containing HUVEC. Stimulant was added to the wells and the plates were incubated for 30 min at 37°C in the 5% CO_2 incubator. After this incubation, supernatant was removed and 200 μl of the Rose Bengal dye (0.25% in PBS) was added to each well for and the plates were allowed to stand for 5 min at room temperature. The wells were carefully washed twice to remove the non-adherent neutrophils and the HUVEC monolayers inspected under an inverted phase contrast microscope to ensure that the monolayers were intact. 200 μl of ethanol:PBS mixture (1:1) was added to each well to fix the cells, and 60 min later the absorpion at 570 nm (OD_{570}) measured, using ELISA plate reader. The neutrophil adherence was quantified by subtracting the OD_{570} of wells containing HUVEC alone from the experimental OD_{570}. A linear relationship exists between the OD_{570} and the number of adherent neutrophils [Gamble & Vadas, 1988], and this was confirmed when various concentrations of LPS

was used as stimulus. The maximum adherence was determined by measuring absorption of 5 x 10^5 neutrophils that were stained and fixed as described above, and the results were expressed as a percentage of maximum adherence.

2.2.3 *Methods for studying signal response coupling in neutrophils*

2.2.3.1 *Measurements of intracellular free calcium concentration ($[Ca^{++}]_i$)*

Changes in intracellular free calcium ($[Ca^{++}]_i$) were measured using FURA-2 loaded PMN.● After separation 10^7 PMN were incubated with 2 μM FURA-2-AM for 30 min at 37°C in Hank's buffer. During this time the FURA-2-AM entered the cells where it was hydrolysed to the acid form FURA-2 and trapped inside the cells. The cells were washed twice with Hank's buffer to remove any unesterified FURA-2-AM. For our studies the cells (10^6/ml) were then placed in a spectrophotofluorimeter (Perkin Elmer LS-50). The various test components were added and changes in fluorescence (excitation 340 nm, emission 510 nm, slit widths 10 nm) monitored continuously. Levels of intracellular calcium were then calculated using the formula developed by Tsein and colleagues [Tsien et al, 1982; Rink and Pozzan, 1988]:-

$$[Ca^{++}]_i \quad = \quad K_d(F - F_{min})/(F_{max} - F)$$

where F is the fluorescence of the cell sample, F_{max} the maximum fluorescence signal (obtained at the end of each measurement by releasing all intracellular calcium by treatment of the cell suspension with excess Triton, (0.1%), and F_{min} the minimum fluorescence value (the value obtained when there is no calcium bound to FURA-2), obtained by the addition of excess

●[Rink and Pozzan 1988]

EGTA (2 mM) (buffered with 25 mM Tris) to the above (Fig 2.2). K_d is the dissociation constant for FURA-2 (220 nM).

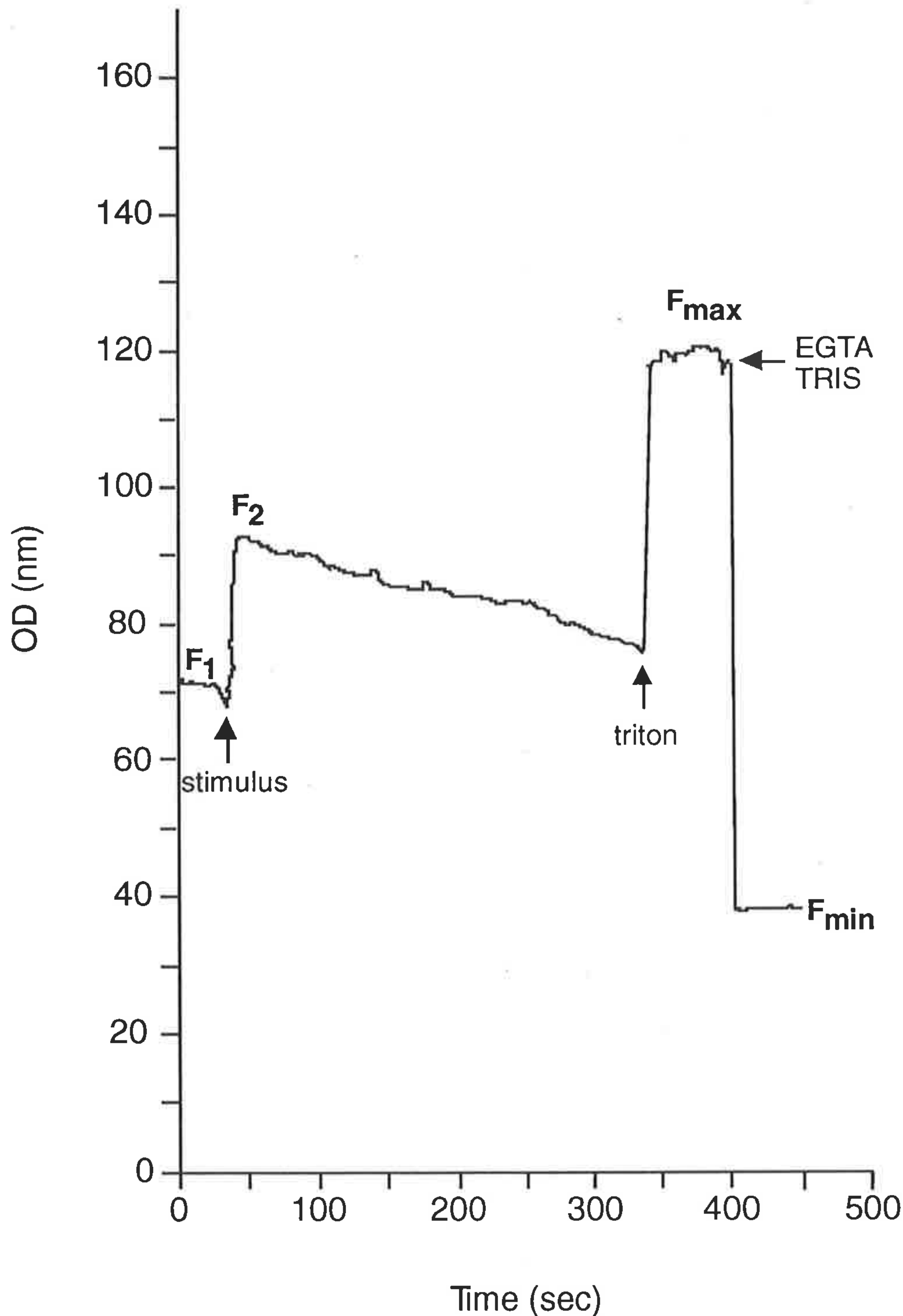


Figure 2.1 Fluorescence trace from FURA-2 loaded fMLP-stimulated human neutrophils measured in presence of 2mM EGTA. F_{max} and F_{min} were determined in each experiment. F_1 is used to determine the basal level of calcium concentration in the cells, and F_2 the concentration to which it increases after stimulation.

2.2.3.2 *PKC translocation: ^{3}H-PDBu binding assay*●

^{3}H-PDBu binds specifically to neutrophil PKC, and was used to study PKC translocation from cytosol to plasma membrane. Neutrophils isolated on Lymphoprep gradient were resuspended in 0.1%BSA DPBS at a concentration of 1.1 x 10^7 cell/ml and equilibrated with 10 nM ^{3}H-PDBu for 10 min at 37°C. After that time, stimulant (50 µM SP or medium control) was added to the cells, and at a desired time (1,3,6 and 9 min) 100 µl of the cells suspension (10^6 cells) was removed, transfered to a 96 well plate and harvested immediately onto filters (designed for receptor studies) using Titertek cell harvester, with two cycles of washing with 0.1% BSA PBS. This technique could accommodate large numbers of individual binding assays in a short space of time. Replicate samples containing a 100 fold excess of PMA in addition to ^{3}H-PDBu were set up for each stimulus to determine non-specific binding. PMA and not PDBu was used for determination of non-specific binding for economic reasons, however, similar results were obtained when PDBu was used instead of PMA. In the priming experiments, remaining cells had been allowed to incubate for additional 6 min (total 15 min with medium or SP) and then were stimulated with 100 nM fMLP (or medium). 100 µl (10^6) cells were removed at 1, 3, 6, and 9 min and harvested as above. All samples were performed in triplicate. The papers were dried for 30 min at 60°C, and the radioactivity counted using liquid scintillation counter. The specific binding was defined as the fraction of total label bound by cells incubated with 10 nM ^{3}H-PDBu minus that bound by cells incubated with 10 nM ^{3}H-PDBu + 1 µM PMA.

2.2.3.3 *Measurement of protein phosphorylation*●●

Neutrophils isolated on Lymphoprep were depleted of intracellular phosphate by incubation in phosphate-free buffer for 30 min at 37°C, and

● [O'Flaherty et al, 1990a,b]

●● [Changelian and Fearon 1986]

washed once. The cells (5 x 10^7/ml) were then resuspended in phosphate-free buffer, containing ^{32}P-phosphate and were incubated for 30 min at 37°C with occasional mixing. After the incubation, the cells were washed twice in RPMI, centrifuged at 1500 rpm for 5 min, and the cell pellet resuspended to 5 x 10^6 cell/ml. 200 µl (10^6) of cells was then incubated with A23187 (or methanol control) for 10 min at 37°C and were then stimulated with PMA for 5 min. Phosphorylation was stopped by addition of an equal volume of ice cold lysis buffer (2x RIPA) and placing tubes on ice. The lysate was incubated for 30 min on ice, and then the insoluble material removed by centrifugation at 21,000 rpm for 30 min at 4°C. 100 µl aliquots of supernatant were taken and electrophoresed (under reducing conditions) on 10% SDS gel. The gels were then stained, dried and autoradiographed. The autoradiographs were scanned on a densitometer and the relative densities of each band are presented.

2.2.4 *^{125}I-Substance P binding assay*●

The binding assays were performed in low adsorption polypropylene tubes. The binding medium contained 0.5% BSA and 10 mM sodium azide, to prevent non-specific adherence and internalization of ^{125}I-SP, and 10µM phosphoramidone, to inhibit possible degradation of ^{125}I-SP by neutrophil endopeptidase. Neutrophils (5 x 10^6 — 2 x 10^7 in 130 µl) were incubated with ^{125}I-SP (10 µl) and medium or unlabelled SP (10 µl) for 75 min at 4°C in most of the assays. The non specific binding was assessed in the presence of 50 µM SP, except in saturation experiment where 5 x 10^5 fold excess of unlabelled SP was used for each ^{125}I-SP concentration used. After incubation with ^{125}I-SP, neutrophils were separated from the free ^{125}I-SP by centrifuging the cells (13,000 rpm, 30 sec) through a layer of 200 µl cold FCS, and cutting of the tip of the tube, that contained the cell pellet. The radioactivity of the total ^{125}I-SP added, the cell pellet (the tip) and the free ^{125}I-SP left in the supernatant

●[Scatchard 1949; Payan et al, 1986; Hartung et al, 1986]

was counted using a gamma counter (LKB, 1282 Commugamma, Turku, Finland). Specific binding was defined as the total amount of ^{125}I-SP bound to the cells minus corresponding value of non-specific binding. All binding assays were done in triplicate. This technique was simple, reproducible and allowed processing of many samples in a short time. Further specific description of conditions for each assay performed and the description of Scatchard analysis are given in the results section of chapter 6.

2.2.5 *Histamine Provocation*

2.2.5.1 *Subjects*

Normal subjects were volunteers not suffering from allergic (seasonal) rhinitis or asthma, and asthmatic subjects who were recruited from patients attending the outpatient service of the Department of Thoracic Medicine, Royal Adelaide Hospital. Asthma was diagnosed according to the criteria of the American Thoracic Society [1987]. In particular reversible airflow limitation was documented objectively in all subjects. None had suffered from an upper respiratory tract infection in the preceding 6 weeks and all were studied at a time when their asthma was stable but symptomatic, usually on daily medications. In a subgroup of asthmatics, bronchial hyperresponsiveness (BHR) was measured as outlined below. All asthmatics were studied after abstinence from medications as per the protocol for assessment of BHR (see below).

2.2.5.2 *Histamine provocation test*

Histamine provocation was carried out as described by Cockroft et al [1977]. Prior to the test, the subjects abstained from medications for the

following times: aerosol bronchodilators, 8 hrs; theophylline preparations, 24 hrs; sodium cromoglycate, 24 hrs and antihistamines, 48 hrs. The challenge was carried out between 0900 and 1000 hrs in the following way. Forced expiratory volumes (FEV) were measured using an Ohio 840 Spirometer. Baseline spirometry was carried out and the subject was given isotonic saline to inhale for 2 min using a Wright's nebulizer (aerodynamic mass median diameter 1.0-1.5 μm) driven by oxygen at 10 l/min. The subjects then inhaled doubling concentrations of histamine acid phosphate in saline (beginning at 0.03 mg/ml) for 2 mins. Forced expiratory volumes (in one second, FEV_1 were measured at 0.5, 1.5 and 3 mins. The procedure was terminated when the FEV had fallen by 20% from the saline control. PC_{20} histamine, defined as the provocation dose which resulted in a fall of ≤20% in FEV_1, was obtained by interpolation from a plot of the decrease in FEV_1 versus the log of the dose of histamine and expressed as mg/ml. For analysis of data involving PC_{20}, the data were initially log-transformed. The highest concentration of histamine given was 8 mg/ml.

2.2.6 *Data Analysis*

Data are expressed as means ± SEM (n = number of subjects). Differences between means were assessed for statistical significance using Student's t-test. and ANOVA. The Wilcoxon rank sum test was used when appropriate. All calculations were carried out with the Statview II™ program using a Macintosh computer.

Chapter 3

Activation of Human Neutrophils by Substance P

3.1 Summary

The neuropeptide, substance P (SP), is a potent regulator of mature, human neutrophil function. SP increased neutrophil cytotoxic activity towards an antibody-coated target cells in a dose-dependent manner. This effect was not due to the toxic effect of SP to the target cells and was antibody dependent. The level of cytotoxic activity induced by SP was comparable to that described by a number of cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), under identical assay conditions. SP-induced cytotoxicity was 73 ± 6% of that produced by an optimum concentration bladder carcinoma cell line U5637 conditioned medium (BCM) known to contain a number of cytokines which activate human neutrophils. In addition, SP facilitated *formyl*-methionyl-leucyl-phenylalanine (fMLP)-, phorbol myristate acetate (PMA)- and platelet activating factor (PAF)-stimulated neutrophil superoxide anion production (O_2^-) in a dose-dependent manner, but did not have an effect when the cells were stimulated with

opsonised zymosan (OPZ) or calcium ionophore (A23187). This priming effect of SP was rapid in onset (<15 min.) and was maximal from 15 to 60 min, after which it declined. It was not reversed by washing the cells and was temperature dependent. SP did not shift the dose-response curves to fMLP or PMA to the left, and exerted its effect over a wide range of concentrations of both stimuli. Moreover, SP enhanced leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE) production by fMLP-stimulated but not A23187-stimulated neutrophils. In contrast to its positive effects on ADCC, O_2^-, LTB_4 and 5-HETE production, SP did not have any stimulatory effect on elastase (EL) or myeloperoxidase (MPO) release from unstimulated neutrophils, and neither did it increase fMLP-stimulated release of these destructive enzymes. Similarly, neutrophil adhesion to either unstimulated or LPS-stimulated human umbilical vein endothelial cells (HUVEC) was unaffected by SP.

Therefore, the results presented in this chapter provide evidence that SP regulates a number of neutrophil functions in a complex manner and suggest a mechanism whereby the nervous system may affect the immune response. Furthermore, the regulatory effects of SP on some neutrophil functions appear to be similar to those of a number of cytokines that have been previously implicated in inflammation.

3.2 Introduction

As discussed in Chapter 1.3.1., neutrophils play an important role in immunity and inflammation, but may contribute to substantial tissue destruction in chronic inflammatory diseases such as rheumatoid arthritis (Chapter 1.7.1) or asthma (Chapter 1.7.2). Therefore, the demonstration that neutrophil functions could be regulated by neuropeptides, such as SP, may be of great importance to the understanding of the nervous system modulation

of the inflammatory processes. Of particular interest is the finding that SP causes granulocyte infiltration into tissue sites mediated by both mast cells degranulation [Matsuda et al, 1989] and direct activation of endothelial cells [Iwamoto et al, 1992]. There are also several reports from *in vitro* studies which have demonstrated that SP is chemotactic for granulocytes (Chapter 1.9.2). Substance P has been shown to be directly chemotactic for human neutrophils [Iwamoto et al, 1990] and monocytes [Wiedermann et al, 1989; Ruff et al, 1985], and to facilitate neutrophil chemotaxis induced by other chemotactic stimuli [Perianin et al, 1989]. The chemotactic response on monocytes was shown to be mediated via a specific SP receptor.

In addition to its chemotactic activities, SP also stimulates other neutrophil functions. For example, SP promotes oxy radical production, granule exocytosis and neutrophil aggregation [Serra et al, 1988; Hafstrom et al, 1989; Brunelleschi et al, 1991]. Release of oxy radicals and destructive enzymes are particularly important neutrophil effector functions in combating infectious disease, but also in tissue damage occurring in chronic inflammatory diseases (Chapter 1.3.1.1 and 1.7).

The experiments described in this chapter investigate the possible proinflammatory effects of SP on mature human neutrophil function. In particular, it was hypothesised that SP activates or modulates the major neutrophil effector functions, such as ADCC, oxy radical production and exocytosis. In addition, it has been postulated that SP may cause neutrophil infiltration into tissues by stimulating or enhancing the release of LTB_4, one of the most potent neutrophil chemotaxins, and neutrophil adherence to vascular endothelial cells. It has also been proposed, that SP effects on neutrophils are mediated via a specific receptor. The major objective of the work described in this chapter, was to provide further insight into the role of SP in inflammation.

3.3 Results

3.3.1 *The effect of substance P on antibody-dependent cell-mediated cytotoxicity*

SP stimulated neutrophil cytotoxicity dose-dependently (in the range of 1 to 100 μM) at all antibody (α-TNP) concentrations tested (Fig. 3.1). Optimal antibody concentration was 3 μg/ml. At higher concentrations, high levels of cytotoxicity were seen in the presence of antibody alone (medium control) in some individuals. At this antibody concentration, a significant effect was seen at an SP concentration of 25 μM, when SP increased ADCC from 4.7 ± 0.9 (medium control) to 11.1 ± 1.6% ($p < 0.02$). Maximal mean cytotoxicity with 100 μM SP was 33.4 ± 10.3% ($p < 0.05$), which represents *net*-ADCC of 28.7% (calculated as ADCC in presence of stimulus minus spontaneous ADCC in the presence of medium alone). Although, in one subject maximum cytotoxicity (37.5%) was seen with 50 μM SP, 100 μM SP was required for maximal effect in another three subjects (34, 39 and 55%). Since the magnitude of the response to SP differed between individuals, data were also calculated as a percentage of ADCC stimulated by an optimal dilution (1:10) of BCM, used as a positive control. In these experiments, mean maximal ADCC induced by SP was 73 ± 5.8% (range 64.2—88.7%) of that induced by BCM.

At antibody concentrations of 0.1 and 1.0 μg/ml a small but significant effect was seen at an SP concentration of 50 μM compared to medium control (4.5 ± 0.6 and 10.9 ± 1.9% versus 2.4 ± 1.0 and 3.4 ± 0.2%, $p < 0.02$ and $p < 0.04$ respectively). Maximum effect with 100 μM SP at these antibody concentrations was 8.1 ± 1.4 and 20.6 ± 2.2% respectively ($p < 0.05$ and $p = 0.005$) .

In the absence of SP or BCM, the spontaneous ADCC was alway less than 7% at any antibody concentration tested. SP had no direct cytotoxic

effect on the target cells (e.g., in the absence of antibody and neutrophils) nor stimulated any significant cytotoxicity in the absence of antibody.

3.3.2 *The effect of substance P on fMLP-stimulated superoxide anion production*

3.3.2.1 *The dose-response effect of substance P*

In the initial experiments, neutrophils (10^6) were incubated with cytochrome C and various concentrations (0.001-100 μM) of SP or its diluent control for 30 min at 37°C. Following this incubation, the cells were stimulated with 0.1 μM fMLP or its diluent for an additional 6 min at 37°C, and the reaction was stopped by addition of SOD. Over the 36 min reaction time, SP alone did not induce any significant O_2^- production (Fig. 3.2A). However, preincubation of neutrophils with SP greatly facilitated fMLP-stimulated O_2^- production in a dose-dependent fashion (Fig. 3.2A). A small priming effect was seen at 10 μM SP (11.5 ± 1.8 nmol $O_2^-/10^6$ cells, $p < 0.005$) and the maximum was seen at 75 μM SP (25.0 ± 2.8 versus 9.2 ± 1.6 nmol O_2^- $/10^6$ cells for SP and its diluent control respectively, $p = 0.0001$).

Because of the donor-dependent variability of fMLP-stimulated O_2^- production (responses in cells stimulated with fMLP alone ranged from 1.1 to 22.4 nmol $O_2^-/10^6$ cells, see Table 3.3), the data for each donor was normalised and expressed as a percentage of the control fMLP response for that donor (Fig. 3.2B). Priming of neutrophils with the optimum concentration of 75 μM SP increased the fMLP-stimulated O_2^- production by 286 ± 76.5% (range 53–982%, $p < 0.005$).

3.3.2.2 *The effect of preincubation time on substance P priming*

The priming effect of SP was dependent on the incubation time. When neutrophils were preincubated with various concentrations of SP or its diluent for 20 sec before stimulation with 0.1 μM fMLP, SP did not have any effect on the fMLP response (Fig. 3.3). In these experiments, SP increased fMLP-stimulated O_2^- production in the control cells that were preincubated with 75 μM SP for 30 min at 37°C. Preincubation of neutrophils for 30 min in the buffer alone resulted in smaller fMLP response, than when the cells were preincubated for 20 sec (Fig. 3.3).

A minimum incubation of 1 min was required for SP to exert an effect, although when the cells were preincubated for 1 min only, the dose-response pattern of SP priming was different to that demonstrated previously (Fig. 3.2). As illustrated in Fig. 3.4, after 1 min incubation with SP, the maximum effect occurred at 50 μM rather than 75 μM SP, the response declined at 75 μM, and preincubation with 100 μM SP had no effect at all. In the same experiment, 30 min preincubation with SP increased the fMLP response from 10.7 ± 0.2 to 18.0 ± 0.5 nmol $O_2^-/10^6$ cells. However, there was a small decrease in the fMLP response when the cells were incubated for 30 min at 37°C in medium alone.

In order to study the kinetics of the priming effect of SP, neutrophils (5×10^6) were preincubated with 50 μM SP or diluent for 1—120 min at 37°C. At each time point, 10^6 cells were transferred to prewarmed tubes containing 0.1 μM fMLP and cytochrome C and were incubated for an additional 6 min (Fig. 3.5). When cells were preincubated with diluent, the responsiveness of neutrophils to fMLP declined gradually and at 120 min was 44.3% of the initial control response at time 0. The SP induced enhancement of fMLP-stimulated O_2^- production was already evident at 1 min, became significant at 5 min, plateaued between 15 and 60 min ($0.003 < p < 0.05$) and then

declined slowly, but was still significant at 120 min (18.3 ± 2.8 versus 8.1 ± 1.4 nmol $O_2^-/10^6$ cells, $p < 0.02$, for SP and diluent control respectively). In these experiments there was no significant O_2^- production when neutrophils were stimulated with SP alone for 6 min.

3.3.2.3 *The effect of substance P on the dose-response curve to fMLP*

Subsequently, the effect of SP on the neutrophil responsiveness to fMLP was investigated (Fig. 3.6). SP (50 μM) enhanced the response to fMLP at concentrations between 0.01 to 1 μM ($0.02 < p < 0.02$) but did not shift the fMLP dose-response curve to the left. Thus SP did not make neutrophils more sensitive to lower fMLP concentrations. The maximum effect was seen at 0.05 and 0.1 μM fMLP when O_2^- production increased from 6.3 ± 2.7 to 27.1 ± 8.7 and from 6.2 ± 2.5 to 27.2 ± 8.3 nmol $O_2^-/10^6$ cells respectively.

3.3.2.4 *The effect of temperature on substance P priming*

To test the temperature dependence of the priming effect of SP, neutrophils were incubated with 50 μM SP or diluent for 30 min at 4, 24 or 37°C. Neutrophils were then washed twice in buffer (DPBS), allowed to equilibrate to 37°C, and stimulated with 0.1 μM fMLP for an additional 6 min. Results are shown in Fig. 3.7. At 4°C SP did not have any priming effect on fMLP-stimulated O_2^- production of neutrophils. However neutrophils incubated with SP at 24 and 37°C, showed an enhanced response to fMLP that increased from 12.3 ± 3.1 to 15.4 ± 2.3 ($p < 0.05$) and from 10.7 ± 2.6 to 21.3 ± 4.3 ($p < 0.015$) nmol $O_2^-/10^6$ cells respectively. Furthermore, the effect of SP was not reversed by washing the cells after incubation. When cells were incubated with SP at 37°C and washed, the

response to fMLP after washing did not differ to that when cells were stimulated without washing (Fig. 3.7).

3.3.2.5 *The effect of substance P antagonists, spantide and SP* (D-Arg^1, D-Pro^2, D-Phe^7, D-Trp^9, Leu^{11}) on SP facilitation*

The SP antagonists, spantide and SP* (see Table 3.1 for amino acid sequences), have been shown previously to bind to SP receptor on lymphocytes and macrophages, and to inhibit SP-mediated activation of these cells.● Do these antagonists also bind to SP receptors on neutrophils, block subsequent SP binding and inhibit the facilitating effect of SP ? Before that could be done, however, the direct effect of these antagonists on neutrophil fMLP-stimulated O_2^- production had to be determined. Neutrophils were preincubated with various concentrations of spantide or SP* for 30 min at 37°C, and were then stimulated with 0.1 μM fMLP for an additional 6 min. As shown in Fig. 3.8, both antagonists enhanced the fMLP O_2^- response in a dose-dependent manner over the same range of concentrations as did SP, and therefore were not "true antagonists", and could not be used to inhibit the priming effect of SP by blocking SP binding sites on neutrophils.

3.3.3 ***The effect of substance P on PMA-, OPZ-, A23187- and PAF-stimulated superoxide anion production***

Four other agonists were used to stimulate O_2^- production in neutrophils that had been primed with SP to find out whether SP exclusively facilitates fMLP response or whether it can also enhance responses induced by other stimuli. The stimuli that were chosen stimulate O_2^- production by acting on different neutrophil receptors or different components of the transduction signal pathway leading to this response, and could provide

●[Payan et al, 1984; Hartung et al, 1986; Kimball et al, 1988; Lotz et al, 1988; Calvo et al, 1992]

additional information on how SP may exert the priming effect. PMA was used, that induces O_2^- production by binding to and activating protein kinase C, PAF that has a specific receptor coupled to G-proteins (as does fMLP), A23187 that releases calcium from intracellular stores and OPZ, that like fMLP and PAF works through a specific receptor, but has slower kinetics, indicating different mechanism of activation (see Chapter 1.6 on signal transduction in phagocytes). It was found that in addition to fMLP responses, SP also facilitated PMA- and PAF-stimulated O_2^- production but had no significant effect on OPZ and A23187 stimulated neutrophils (Fig. 3.9). Preincubation of neutrophils with various concentrations of SP for 30 min at 37°C, increased the PMA response in a dose-dependent manner (Fig. 3.9A). A maximum effect was observed at 100 μM SP, when O_2^- production increased from 19.6 ± 2.0 to 30.4 ± 1.8 nmol $O_2^-/10^6$ cells (160 ± 9% control, range 149–183%), in response to 10 ng/ml PMA (6 min).

This priming effect was rapid in onset but required more than 20 sec preincubation time with SP. Preincubation of neutrophils for 20 sec with 50 μM SP had no effect on the PMA response (Fig. 3.10). However, when neutrophils were preincubated for more than 1 min with 50 μM SP, the PMA response was dramatically enhanced (Fig. 3.11). A maximum effect was seen after 3 min preincubation with SP, when O_2^- production increased from 26.0 ± 0.2 to 41.0 ± 0.4 nmol $O_2^-/10^6$ cells. The facilitating effect of SP was long lasting and still present at 120 min (15.9 ± 0.6 versus 22.1 ± 0.1 nmol $O_2^-/10^6$ cells, medium vs SP preincubated cells).

As seen previously for fMLP, SP facilitated the PMA-stimulated O_2^- production over a range of different PMA concentrations (Fig. 3.12). In these experiments neutrophils were incubated with 50 μM SP for 30 min at 37°C and were then stimulated with various PMA concentrations for an additional 6 min. Here, the absolute increase in O_2^- production was the same (11.5 ± 0.5 nmol $O_2^-/10^6$ cells) at each PMA concentration used.

As illustrated in Fig. 3.9B, SP also enhanced PAF-stimulated O_2^- production. In these experiments, neutrophils were incubated with diluent or 50 μM SP for 30 min at 37°C and were then stimulated with 0.1 μM PAF for an additional 10 min. O_2^- production increased from 2.3 ± 0.4 to 8.4 ± 1.5 nmol $O_2^-/10^6$ cells ($p < 0.008$). This priming effect of SP increased PAF-stimulated O_2^- production to 365 ± 65% of control.

Preincubation of neutrophils with various concentrations of SP did not have any significant effect on subsequent 30 min stimulation with 1 mg/ml OPZ (Fig. 3.9A). The stimulation time of 30 min was chosen to yield suboptimal O_2^- production for the above OPZ concentration, but the response to OPZ was unaffected over 90 min (Fig. 3.13). Similarly, preincubation with 50 μM SP did not facilitate A23187-stimulated O_2^- production in neutrophils (Fig. 3.9B).

3.3.4 *The effect of substance P on LTB_4 and 5-HETE production*

The effect of SP on the production of the metabolites of the 5-lypoxygenase pathway, LTB_4 and 5-HETE, was studied by incubating neutrophils with medium or SP (0.1 or 50 μM) for 15 min at 37°C followed by stimulation with 0.5 μM A23187 or 0.1 μM fMLP for additional 5 min in the presence or absence of 10 μM exogenous AA. As can be seen from Table 3.2, SP did not significantly affect the production of LTB_4 and 5-HETE by neutrophils stimulated with A23187, but did increase the response to fMLP. LTB_4 production increased from 3.5 ± 0.8 to 7.0 ± 1.4 ng/10^6 neutrophils ($p < 0.05$, $n = 3$) after preincubation with 50 μM SP and stimulation with 0.1 μM fMLP in the presence of 10 μM AA. Under the same experimental conditions, 5-HETE production increased from 23.4 ± 10.7 to 43.1 ± 12.8 ng/10^6 cells. SP alone did not stimulate any LTB_4 or 5-HETE production.

3.3.5 *The effect of substance P on neutrophil degranulation*

To study the effect of SP on the release of elastase (EL) and myeloperoxidase (MPO), neutrophils were preincubated with medium or various concentrations of SP (in the presence or absence of 5 μg/ml cytochalasin B) for 15 min at 37°C and were then stimulated with different concentrations of fMLP or its diluent for an additional 10 min. The reaction was stopped by cooling samples on ice and pelleting the cells. Supernatants were then collected and analysed for the activity of particular enzyme using automated Cobas Bio system, as explained in "Materials and Methods" (Chapter 2.2.2.4). The results from three different experiments, each done in triplicate are shown in Fig. 3.14 and Fig. 3.15. Exocytosis of both enzymes occurred only in the presence of cytochalasin B. In all experiments, a dose dependent increase in EL and MPO was seen when the cells were stimulated with 0.001, 0.01 and 0.1 μM fMLP alone. SP alone did not stimulate release of EL or MPO at any concentration tested. No increase in EL or MPO release was observed, when neutrophils that had been preincubated with SP were subsequently stimulated with different concentrations of fMLP. In fact, higher concentrations of SP inhibited MPO but not EL release. For example, 100 μM SP decreased the 0.1 μM fMLP response from 0.82 ± 0.19 to 0.40 ± 0.18 mIU/ml (n = 3, p = 0.06). Because of the donor-dependent variability of fMLP-stimulated enzyme release and the small number of experiments performed, this effect was not statistically significant. However, within each experiment the SD's were less then 5%, and the inhibition that varied varied from 30 to 70% was significant.

3.3.6 *The effect of substance P on neutrophil adherence to endothelial cells*

The effect of SP on neutrophil adhesion to endothelium was investigated by co-incubating neutrophils with endothelial monolayers in the presence of 0.00001-100 μM SP for 30 min. The monolayers were either unstimulated or stimulated with 100 ng/ml LPS for 12 hr prior to the addition of SP. PMA (10 ng/ml) was used as a positive control and increased neutrophil adherence to unstimulated endothelium from 7.0 ± 4.2 to 55 ± 11% (n = 2). However, no increase in adherence to unstimulated endothelium was observed at any SP concentration tested. Preincubation of monolayers with LPS increased the neutrophil adherence to 32 ± 16%, but no additional increase was seen after stimulation with SP.

3.4 Discussion

The above results show that SP activates some important human neutrophil functions in a complex and selective manner. It enhanced neutrophil effector activity by directly stimulating ADCC. Preincubation of neutrophils with SP facilitated the production of O_2^- in response to a second stimulus such as fMLP, PMA or PAF, and inflammatory mediators LTB_4 and 5-HETE in response to fMLP. These neutrophil metabolites have an important role in the pathogenesis of inflammation. Therefore, on the basis of these studies, and the work of others, which demonstrated that SP is able to induce or facilitate neutrophil chemotaxis [Roch-Arveiller et al, 1986; Perianin et al, 1989; Wiedermann et al, 1989; Iwamoto et al, 1990], oxy radical production, aggregation and exocytosis [Serra et al, 1988; Hafstrom et al, 1989; Iwamoto et al, 1990; Brunelleschi et al, 1991], SP appears to be a potential proinflammatory agent. Furthermore, these effects of SP on neutrophil function are qualitatively and quantitatively similar to those of a

number of cytokines such as GM-CSF, TNF-α and IL-8 under the same assay conditions [Lopez et al, 1986; Weisbart et al, 1987; Berkow et al, 1987; Vadas et al, 1983; Atkinson et al, 1988a; Daniels et al, 1992]. For example, Lopez et al [1986] reported that GM-CSF increased neutrophil ADCC from 6% to 32%, comparable to the levels induced by SP. More recently, Daniels et al [1992] and work done in our laboratory have demonstrated that preincubation of neutrophils with IL-8 enhances fMLP-stimulated O_2^- production just as does SP.

SP stimulated ADCC by human neutrophils. This effect was not due to the toxic effect of SP on the target cells and was dependent on the antibody concentration. At the highest concentrations used SP did not affect neutrophil viability (under the different experimental conditions) as demonstrated by a negative effect on trypan blue exclusion and by phase contrast microscopy. The mechanism by which neutrophils, and more importantly SP-stimulated neutrophils, lyse antibody-coated target cells is uncertain. However a contact between the effector and target cells is necessary as spontaneous cytotoxicity (in the absence of antibody) was not enhanced by SP. A possible mechanism by which SP may stimulate ADCC is to increase the expression of the Fcγ receptors or to degranulate neutrophils (Chapter 1.3.1). Neutrophil degranulation has been shown to play an important role in antibody-mediated cell lysis [Dallegri et al, 1987]. Although SP did not have any effect on enzyme exocytosis (EL and MPO) (Chapter 3.3.5), it is possible that binding of antibody to the Fcγ receptor in SP-stimulated neutrophils may trigger degranulation to assist cell killing. In other studies, Serra et al [1988] have demonstrated β-glucuronidase and vitamin B_{12}-binding protein secretion after stimulating neutrophils with 300 μM SP, whereas Hafstrom et al [1989] showed facilitation of fMLP-stimulated lysozyme and β-glucoronidase by the carboxy end of SP rather than the whole molecule. Degranulation of other inflammatory cells, including mast cells,

macrophages and eosinophils by SP have also been previously reported [Shibata et al, 1985; Hartung et al, 1986; Kroegel et al, 1990].

Production of destructive oxy radicals (Chapter 1.3.1.1) may also be important in the lysing of antibody-coated target cells by neutrophils. Although SP alone did not seem to induce O_2^- production in neutrophils, it is possible that SP primes the cells for O_2^- response to Fc stimulation. Several other studies have shown that SP affects neutrophil opsonin-dependent phagocytosis and degranulation [Bar-Shavit et al, 1980].

SP enhanced the O_2^- production of neutrophils stimulated with fMLP, PMA and PAF, and also the production of LTB_4 and 5-HETE stimulated with fMLP. This effect was not caused by endotoxin contamination as SP was shown to be endotoxin free by the *Limulus* amoebocyte lysate assay. In addition, SP neither directly stimulated neutrophil O_2^- production nor had any direct effect on AA metabolism via the 5-lipoxygenase pathway. Serra et al [1988] reported that SP directly stimulated neutrophils to produce H_2O_2, but this effect was only seen at a SP concentration greater than 100 μM.

Using different detection systems, others have reported SP facilitating effects on neutrophil oxy radical production at much lower SP concentrations than those required to demonstrate a SP effect in my experiments. For example, Perianin et al [1989] showed that 0.01 μM SP facilitated fMLP- and C5a-stimulated H_2O_2 production and Hafstrom et al [1989] reported SP facilitation of fMLP-induced luminol-enhanced chemiluminescence at the same SP concentration. The different results obtained from these studies could be explained by the various degree of specificity and sensitivity of the detection system used in each study. Luminol, for example, is probably more sensitive than Cyt C but is less specific and may be oxidised by many different oxidants in addition to oxy radicals. Certainly, this system does not reflect the activity of NADPH oxidase alone, as it is often acclaimed. As

demonstrated recently by Wang et al [1991b, 1992], nitric oxide (NO) produced by nitric oxide synthase, can also oxidise luminol and increase luminol-enhanced chemiluminescence, especially in the presence of H_2O_2 [Kikuchi et al, 1992]. Consequently, interpretation of results from experiments using luminol-enhanced chemiluminescence as detection system is more difficult, as luminol may be oxidised by many radicals and oxidants. On the other hand, the Cyt C system that I have used is very specific for the measurements of O_2^-, the immediate product of the NADPH oxidase (Chapter 1.3.1.1). This enzyme is the major source of neutrophil-derived oxy radicals, but perhaps not the only one. Therefore, if there were cell enzymes producing oxy radicals other then O_2^-, the Cyt C system would not detect them but chemiluminescence would. Because of these differences, results obtained from my experiments and those demonstrated by others may not be reflecting the same neutrophil activities, making them difficult to compare.

The mechanism by which SP facilitates O_2^- responses in neutrophils is uncertain. Marasco et al [1981] reported that in rabbit neutrophils, SP stimulated chemotaxis and caused degranulation, most likely by binding to the fMLP receptors. Results from my experiments suggest that this is an unlikely mechanism because: (i) SP enhanced O_2^- production in response to fMLP and (ii) the effect of SP was not second stimulus specific as in addition to fMLP it facilitated PMA and PAF responses. If SP was binding to the human fMLP receptor, this would be expected to interfere with the subsequent response to fMLP and, as a consequence, to inhibit rather than facilitate the response. Furthermore, Serra et al [1988] showed that SP acts via a biochemical pathway which differs from that induced by fMLP.

The priming effect of SP on O_2^- production was rapid in onset (< 15 min) and did not change neutrophil sensitivity to the activating stimulus. Neither fMLP nor PMA dose-response curves were shifted to the left by SP, rendering the cells responsive to concentrations that are lower from those normally

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required to trigger the O_2^- response. This shows that SP can not lower the "activation threshold" of neutrophils, and that it may only enhance a response that has been initiated by appropriate signal.

A possible mechanism by which SP may exert its effect may be to increase the intracellular free calcium concentration ($[Ca^{++}]_i$) with subsequent translocation of protein kinase C (PKC) (Chapter 1.6, 1.6.4. and 1.6.5). SP has been shown to increase phosphatidylinositol turnover in brain [Watson & Downes, 1983; Catalan et al, 1988] and human neutrophils [Serra et al, 1988], and also to increase $[Ca^{++}]_i$ in neutrophils [Serra et al, 1988] and smooth muscle cells [Payan, 1985]. This could have the effect of increasing the intrinsic responsiveness to fMLP and PAF, as receptors for both stimuli are coupled through G-regulatory proteins (Chapter 1.6.1) to the phospholipase C transduction pathway [Allen et al, 1988; O'Flaherty et al, 1987, 1989]. This enzyme (phospholipase C) hydrolyses phospholipids to form inositol phosphates and diacylglycerol (DAG), with the latter binding to and activating PKC. PMA is a synthetic analog of DAG, and stimulates O_2^- production in neutrophils by binding to and directly activating PKC (Chapter 1.6). Since O_2^- production induced by all stimuli above depends on PKC activity, SP could, for example, increase PKC activity by translocating it to the cell membrane. This may be regulated by increasing $[Ca^{++}]_i$ or by a calcium-independent mechanism as demonstrated by O'Flaherty et al [1990a]. This aspect is investigated further in chapter 5.

There are several questions that need to be addressed concerning the role of SP in regulating neutrophil effector function, including specificity, and whether the effects are receptor mediated, and the physiological relevance *in vivo*. To date these questions still remain largely unanswered. The finding that some neutrophil functions are stimulated directly by SP (e.g., ADCC), while others are facilitated (e.g., O_2^- production) or not affected at all (e.g., adhesion to HUVEC, degranulation), suggests a specific effect. This is

further substantiated by the finding that SP facilitated O_2^- production by some (e.g., fMLP, PMA, PAF) but not all stimuli tested (e.g., OPZ, A23187). Moreover, SP facilitated one but not the other of the two different neutrophil functions stimulated by the same agonist: fMLP. It enhanced fMLP-stimulated O_2^- and LTB_4 production but had no effect on fMLP-stimulated EL and MPO exocytosis. Results from these experiments argue for a specific and complex modulatory effect of SP. It is not known whether these effects of SP were mediated via a specific SP receptor on neutrophils, as the SP antagonists used had an agonist effect on O_2^- response and could not be used to block the effect of SP.

Granulocyte adherence to endothelial lining of blood vessels and subsequent infiltration into tissue sites, are the very first steps of the process of inflammation. Several studies have demonstrated SP-induced granulocyte infiltration *in vivo* (Chapter 1.3.1). Since SP stimulates mast cell degranulation, it has been subsequently demonstrated that SP-induced granulocyte infiltration is mast cell dependent [Matsuda et al, 1989; Yano et al, 1989]. However, SP also acts on vascular endothelial cells and increases vascular permeability [Lembeck & Holzer, 1979; Pernow, 1985; Iwamoto & Nadel, 1989; Kowalski et al, 1990]. Studies by Iwamoto et al, [1992] demonstrated that the increased vascular permeability results in greater granulocyte infiltration in addition to mast cell degranulation, and that this effect is mediated by the carboxy end of SP and not by the amino end that activates mast cells. Based on these studies, the question was asked; whether SP can stimulate neutrophil adherence to vascular endothelial cells and in this way enhance neutrophil infiltration into specific tissue sites. Binding of neutrophils to unstimulated or LPS activated endothelial monolayers was unaffected by SP, suggesting that SP, although chemotactic for neutrophils, does not affect their infiltration by directly increasing cell adherence to endothelium. It is likely that SP acts on other tissue cells *in*

vivo, which when stimulated release factors promoting neutrophil adherence and infiltration. For example, Von Essen et al [1992] have demonstrated that SP and other tachykinins stimulate bronchial epithelial cells to produce neutrophil chemotactic substance, and suggested that this may be the mechanism for neutrophil recruitment to inflamed lung.

SP ANTAGONISTS

Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met
Spantide	D-Arg-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-Leu
SP*	D-Arg-D-Pro-Lys-Pro-Gln-Gln-D-Phe-Phe-D-Trp-Leu-Leu

Table 3.1 Primary structures of substance P and its antagonist: spantide and SP*.

Conc. of SP (μM)	Stimulus	AA	5-HETE (ng/10^6 cells)	LTB_4 (ng/10^6 cells)
0	A23187	-	29.8 ± 4.2	7.3 ± 1.0
0		+	104.7 ± 13.2	12.3 ± 1.4
0.1	A23187	-	30.6 ± 8.7	6.0 ± 1.2
0.1		+	110.1 ± 11.6	12.1 ± 2.0
50	A23187	-	43.2 ± 12.1	8.7 ± 2.0
50		+	117.8 ± 10.7	14.5 ± 2.7
0	fMLP	-	ND	ND
0		+	23.4 ± 6.2	3.5 ± 0.8
0.1	fMLP	-	ND	ND
0.1		+	26.7 ± 6.8	4.3 ± 1.1
50	fMLP	-	ND	ND
50		+	43.1 ± 7.4*	7.0 ± 1.4*

Table 3.2 The effect of SP on LTB_4 and 5HETE production by neutrophils stimulated with fMLP and A23187. Neutrophils (10^6) were incubated with 0.1 or 50 μM SP for 15 min. at 37°C and were then stimulated with 0.1μM fMLP or 5 μM A23187 for an additional 5 min., in the presence or absence of 10 μM AA. Values are means of three experiments performed in triplicates.
*, indicates values which differ significantly from the corresponding diluent control ($p<0.05$).
±, refers to presence or absence of exogenous AA.
ND, non detectable.

Exp. No.	100 nM fMLP	75 µM SP/100 nM fMLP
1.	1.1	12.5
2.	1.4	6.5
3.	6.6	23.3
4.	15.7	39.7
5.	3.1	28.1
6.	6.2	25.2
7.	7.6	13.8
8.	11.5	29.0
9.	12.9	33.6
10.	4.1	9.3
11.	14.1	34.7
12.	13.0	30.2
13.	9.5	32.6
14.	22.0	31.2
MEAN	9.2 ± 1.6	25.0 ± 2.8

Table 3.3 Donor-dependent variability of the fMLP-stimulated O_2^- production by unprimed and SP (75 µM) primed neutrophils. The neutrophils were stimulated as explained in Chapter 3.3.2.

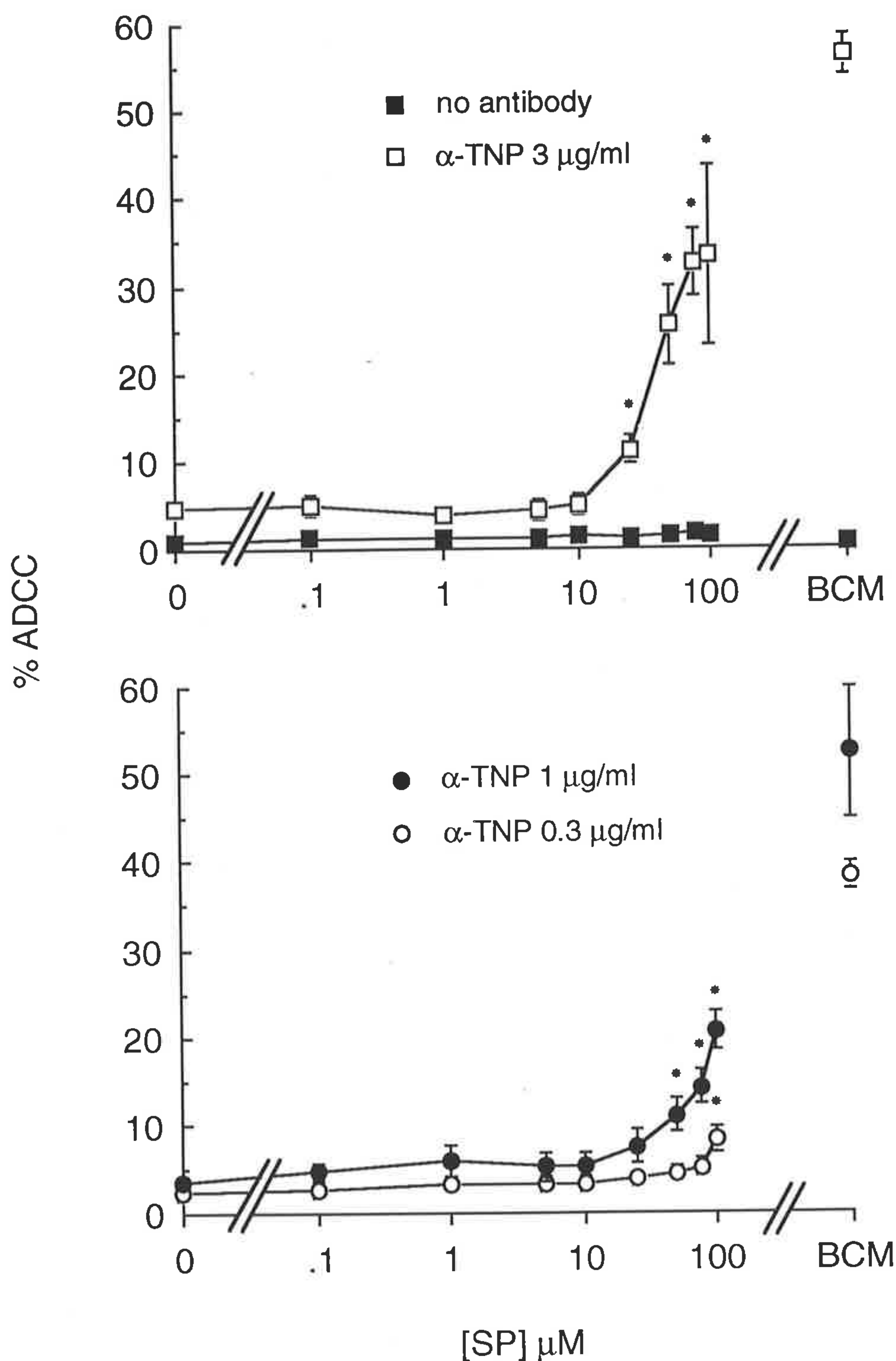


Figure 3.1 Effect of SP on neutrophil ADCC. Neutrophils and target cells were incubated together with various concentrations of SP (or medium control) for 2.5 hrs at 37°C and indicated concentrations of antibody. Bladder carcinoma cell line U5637 conditioned medium (BCM) was used as a positive control. Data are means of three experiments. * Indicates values (in presence of SP) which differ significantly from the corresponding medium control ($0.01 < p < 0.05$)

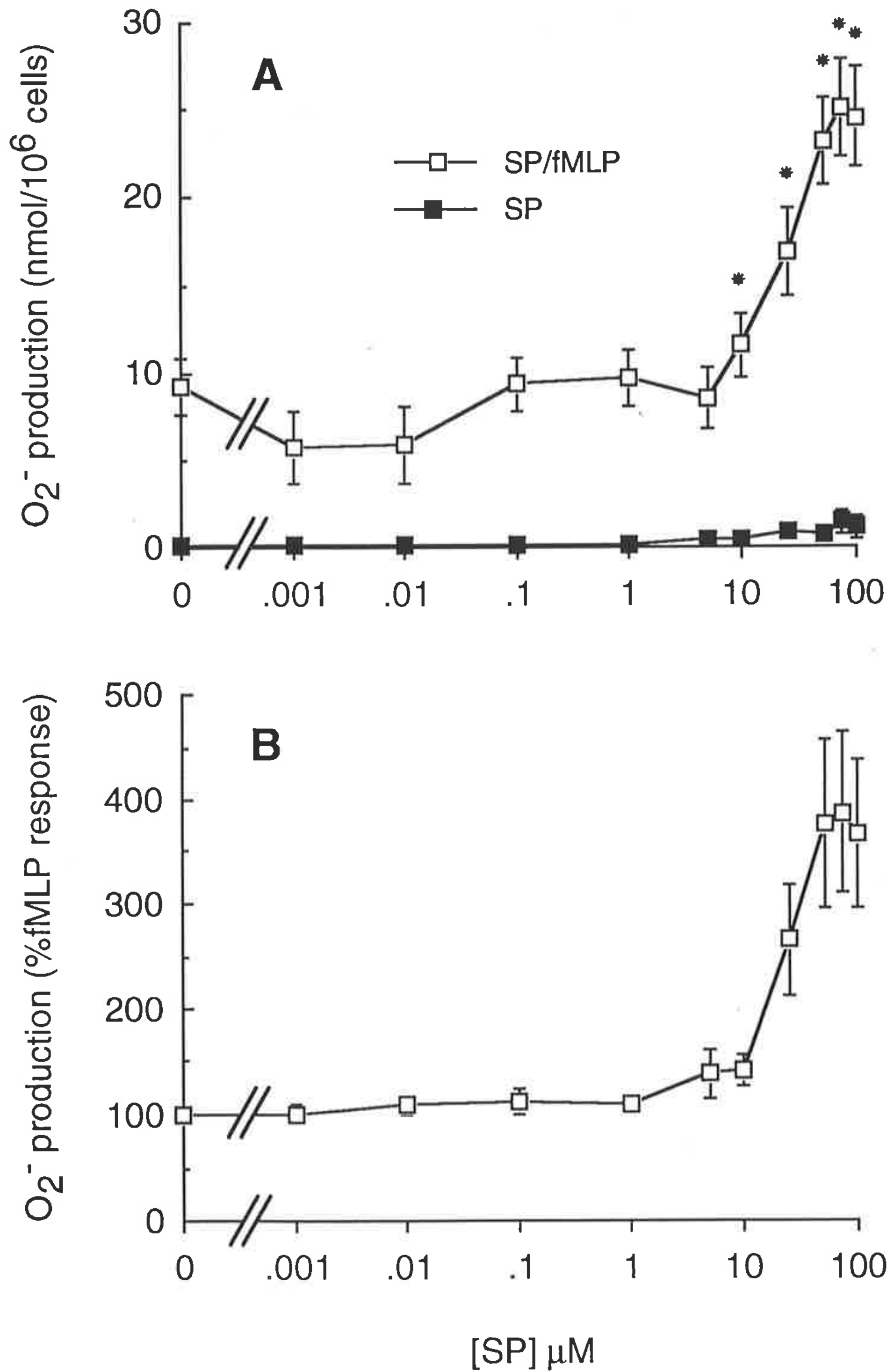


Figure 3.2 (**A**) Effect of SP on fMLP-stimulated O_2^- production. Neutrophils were preincubated with medium or the stated concentrations of SP for 30 min at 37°C and were then stimulated with 0.1 μM fMLP (—□—) or diluent (—■—) for an additional 6 min. Values represent means of fifteen experiments. (**B**) Effect of SP on fMLP-stimulated O_2^- production shown as % fMLP response.

* Indicates values which differ significantly from the corresponding medium control ($p < .001$).

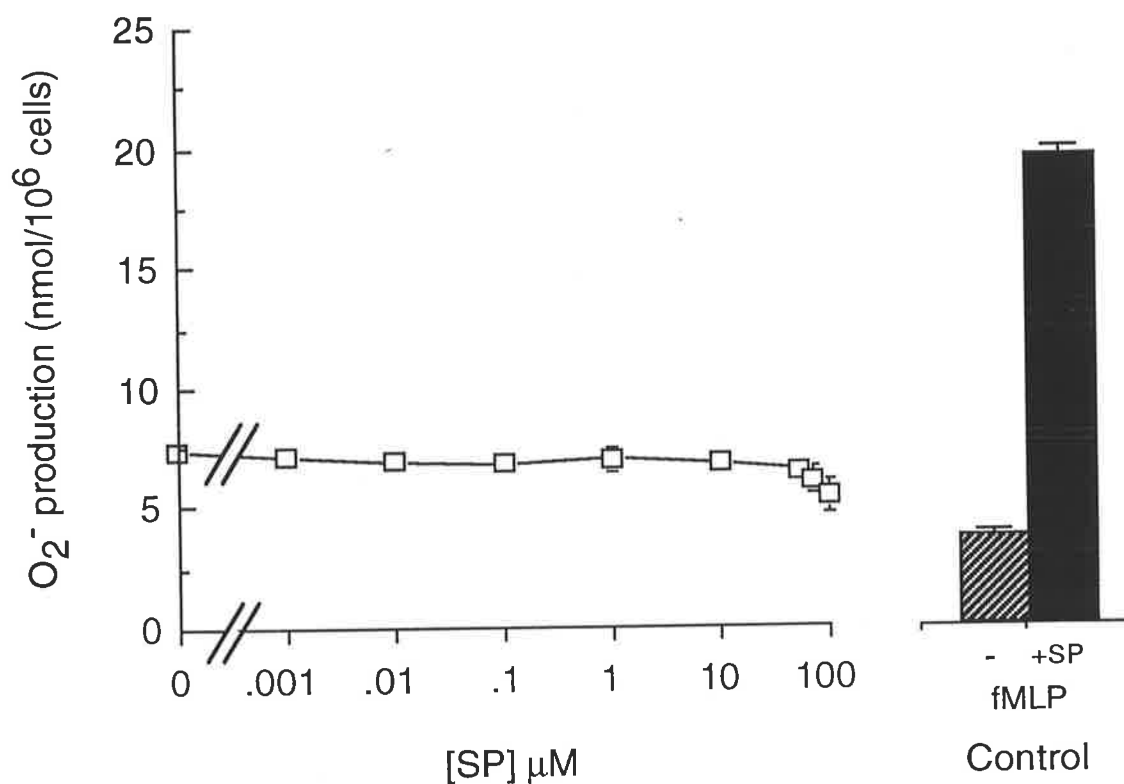


Figure 3.3 Effect of 20 sec preincubation with SP on fMLP-stimulated O_2^- production. Neutrophils were preincubated with medium or the stated concentrations of SP for 20 sec at 37°C and were then stimulated with 0.1 μM fMLP for an additional 6 min. In the same experiments, control neutrophils were preincubated with medium or 75 μM SP for 30 min at 37°C and were then stimulated as above. The response of the control cells preincubated with medium for 30 min is lower than those preincubated for 20 sec, as the O_2^- production declines with time (see Fig. 3.5). Values represent means from two experiment.

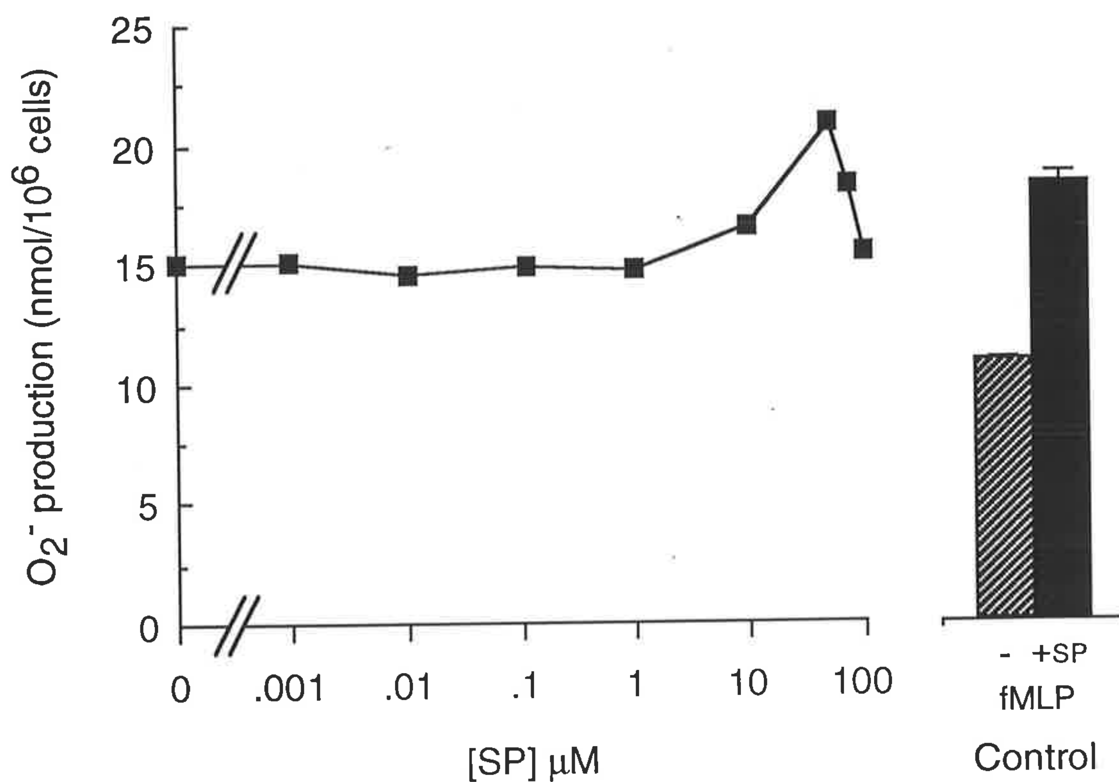


Figure 3.4 Effect of 1 min preincubation with SP on fMLP-stimulated O_2^- production. Neutrophils were preincubated with medium or the stated concentrations of SP for 1 min at 37°C and were then stimulated with 0.1 µM fMLP for an additional 6 min. In the same experiment, control neutrophils were preincubated with 75 µM SP or diluent for 30 min at 37°C and were then stimulated as above.

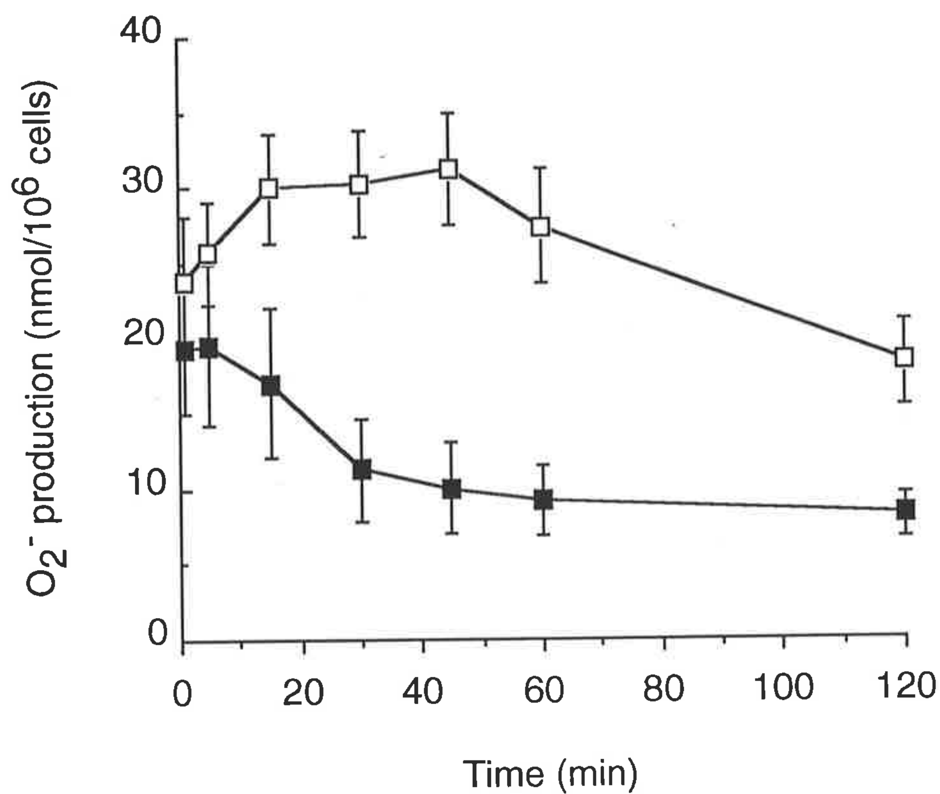


Figure 3.5 Effect of SP on fMLP-stimulated O_2^- production as a function of time. Neutrophils (5 x 10^6/ml) were incubated with 50 μM SP (—□—) or diluent (—■—) for indicated times at 37°C. At each time point 10^6 cells were removed and stimulated with 0.1 μM fMLP for 6 min. Data are means of four experiments.

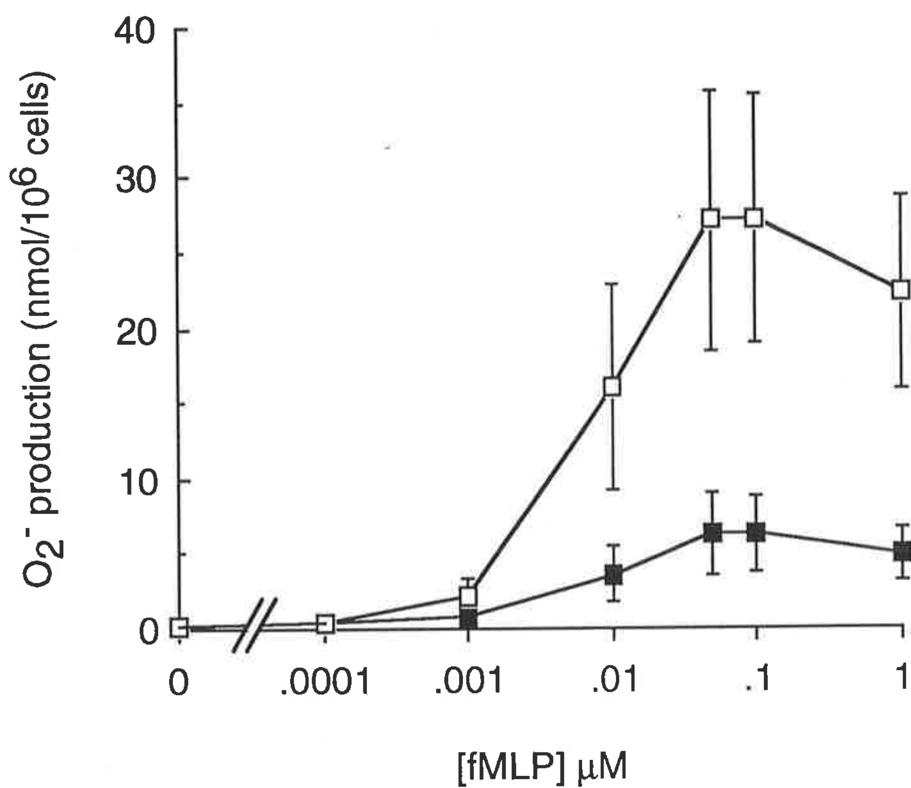


Figure 3.6 Effect of SP on O_2^- dose-response to fMLP. Neutrophils were incubated with 50 µM SP (—□—) or diluent (—■—) for 30 min at 37°C and were then stimulated with stated concentrations of fMLP for an additional 6 min. Values are means of four experiments.

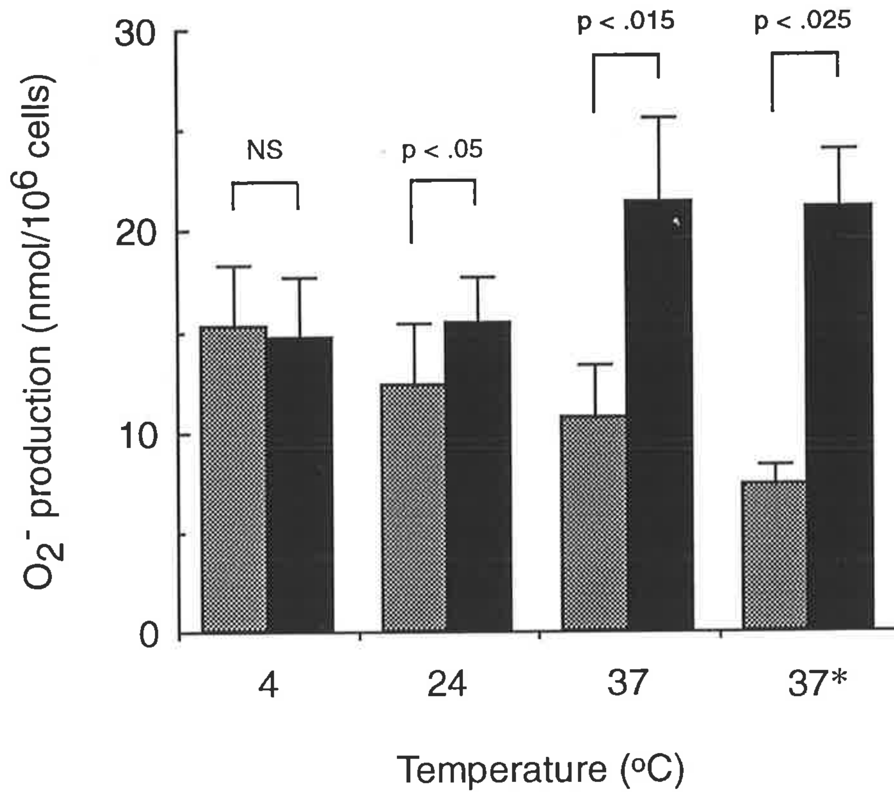


Figure 3.7 Temperature dependence of SP on fMLP-stimulated O_2^- production. Neutrophils were incubated with 50 μM SP (■) or diluent (▩) for 30 min at the stated temperature, washed twice and were then stimulated with 0.1 μM fMLP for an additional 6 min at 37°C. Values are means of four experiments. 37* refers to cells which were incubated with SP or diluent at 37°C but were not washed before stimulation with fMLP. Values are means of three experiments. Statistical significance is indicated by "p" values. NS, not significant.

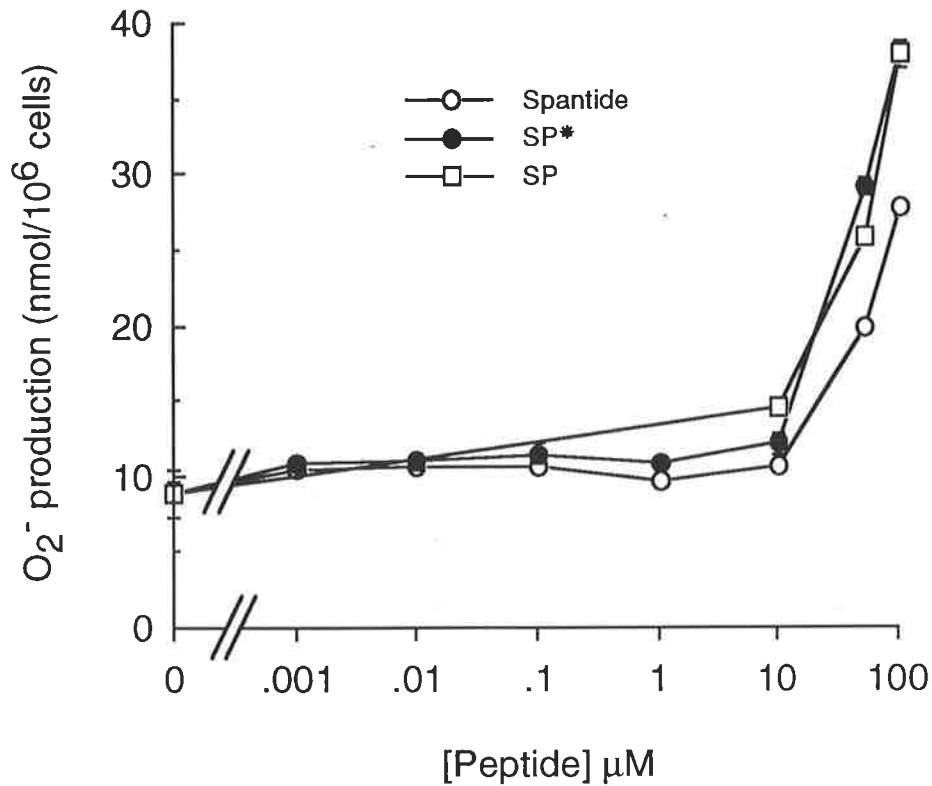


Figure 3.8 Effect of Substance P antagonists on fMLP-stimulated O_2^- production. Neutrophils were preincubated with diluent or indicated concentrations of spantide, SP* or SP (as control) for 30 at 37°C min and were then stimulated with 0.1 μM fMLP for an additional 6 min. Values represent means from one experiment, SD's < 5%.

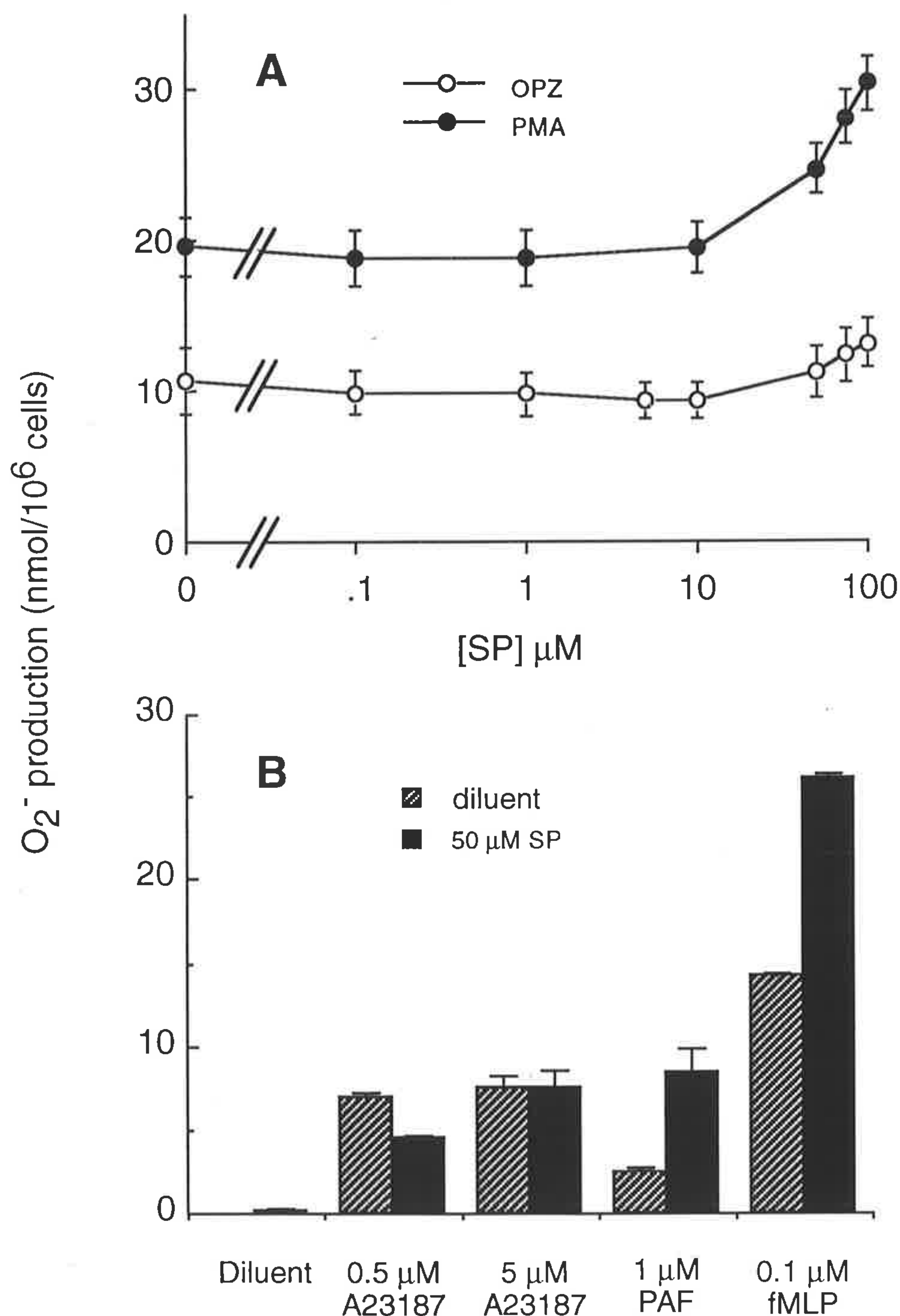


Figure 3.9 Effect of SP on OPZ-, PMA-, A23187- and PAF-stimulated O_2^- production. (**A**) Neutrophils were preincubated with diluent or various concentrations of SP for 30 min at 37°C and were then stimulated with 1 mg/ml OPZ or 10 ng/ml PMA for an additional 30 and 6 min respectively. (**B**) Neutrophils were preincubated with diluent or 50 μM SP for 30 min at 37°C and were then stimulated with 0, 0.5, 5 μM A23187 or 1μM PAF for an additional 10 min (for comparison, results are shown for cells that were stimulated with 0.1 μM fMLP for 6 min). Values represent means of eight experiments in **A**, and two (A23187) and four (PAF) experiments in **B**.

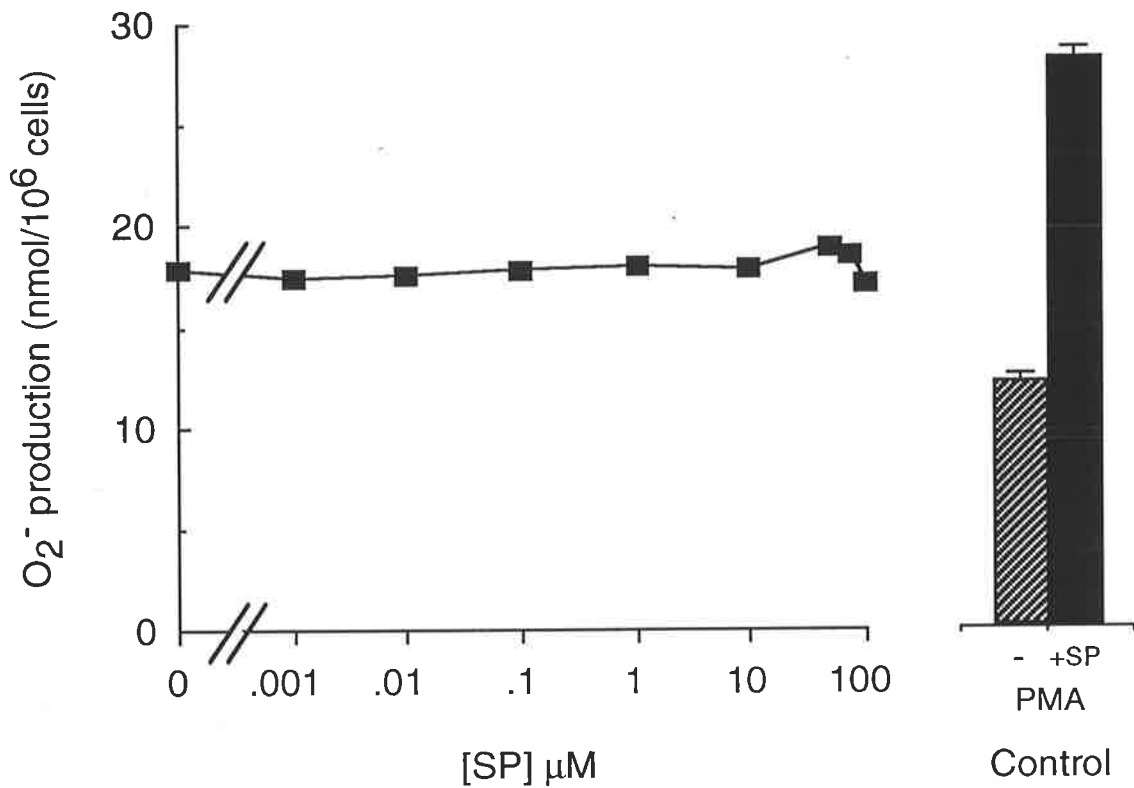


Figure 3.10 Effect of SP on PMA-stimulated O_2^- production. Neutrophils were preincubated with medium or the stated concentrations of SP for 20 sec at 37°C and were then stimulated with 10 ng/ml PMA for an additional 6 min. In the control, neutrophils were preincubated with 75 μM SP for 30 min at 37°C and were then stimulated as above. Values represent means from one experiment.

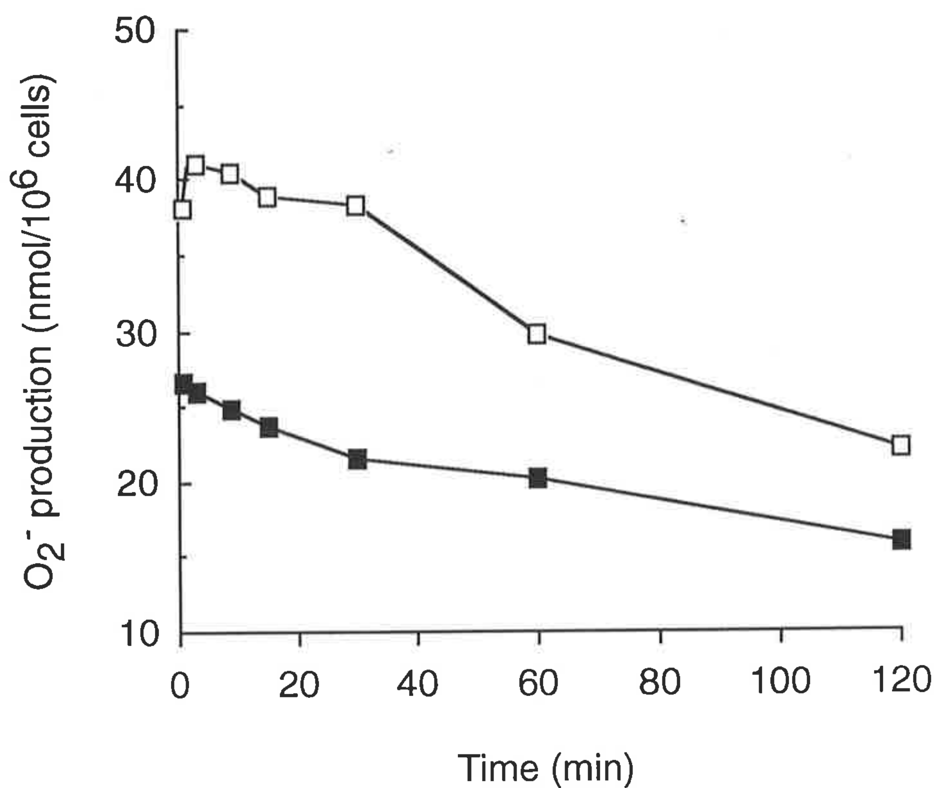


Figure 3.11 Effect of SP on PMA-stimulated O_2^- production as a function of time. Neutrophils (5 x 10^6/ml) were incubated with 50 μM SP (—□—) or diluent (—■—) for indicated times at 37°C. At each time point 10^6 cells were removed and stimulated with 10 ng/ml PMA for 6 min. Data are means of two experiments each performed in triplicate.

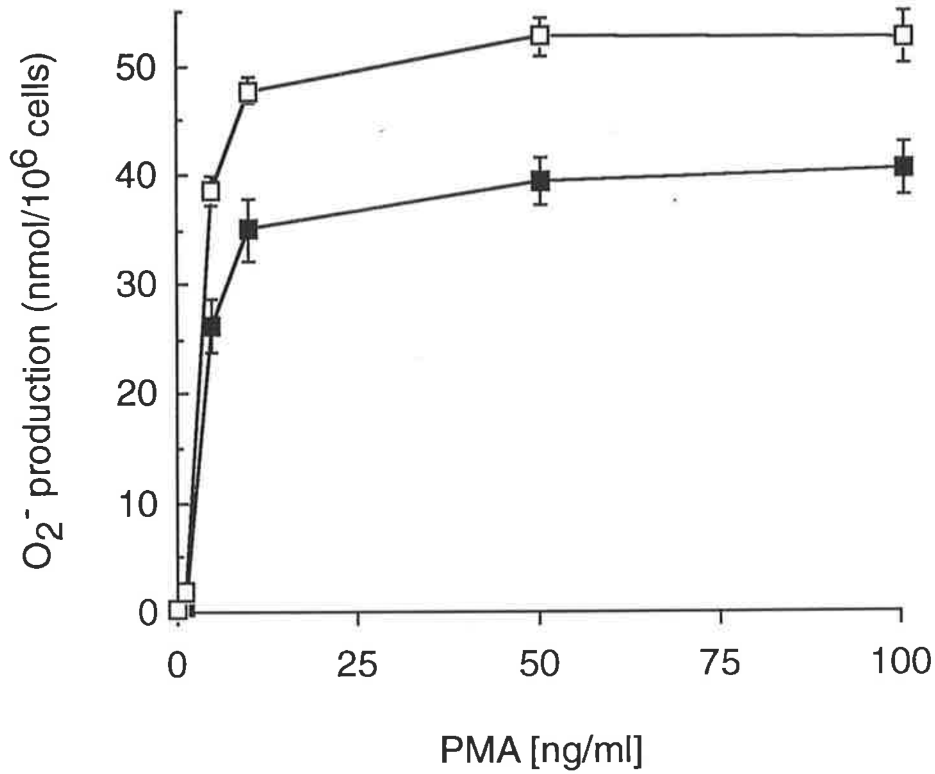


Figure 3.12 Effect of SP on O_2^- dose-response to PMA. Neutrophils were incubated with 50 µM SP (—□—) or diluent (—■—) for 30 min at 37°C and were then stimulated with stated concentrations of PMA for an additional 6 min. Values are means of three experiments each performed in triplicate.

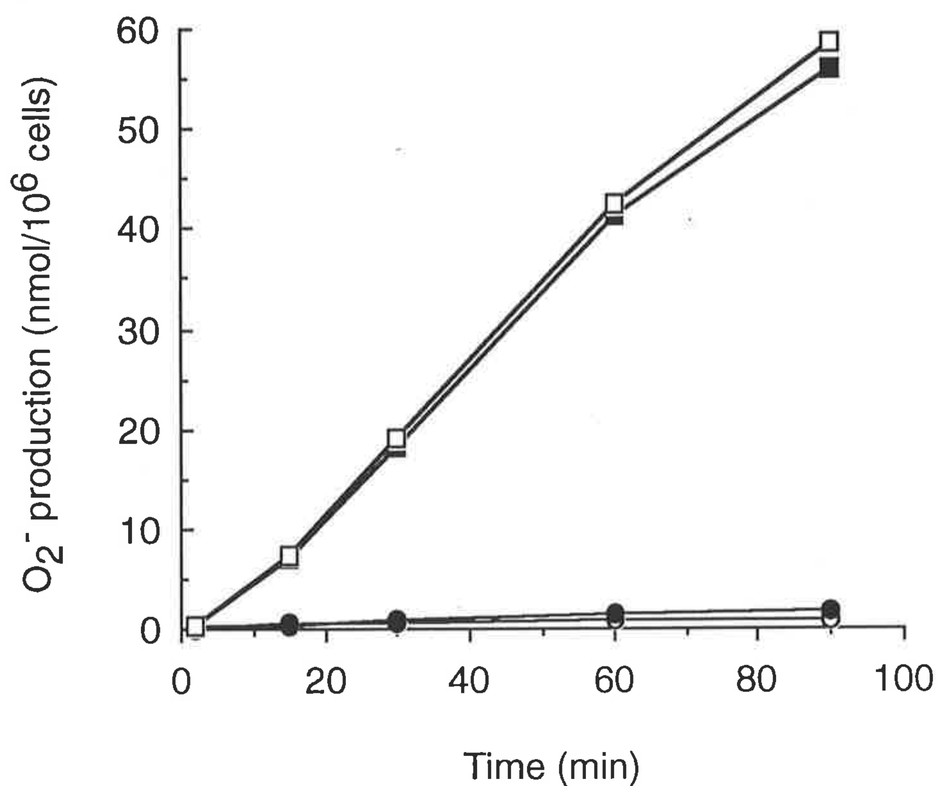


Figure 3.13 Effect of SP on OPZ-stimulated O_2^- production. Neutrophils were incubated with 50 μM SP (—□—) or diluent (—■—) for 30 min at 37°C and were then stimulated with 1 mg/ml of OPZ for indicated period of time. Control tubes contained cells that were preincubated with SP (—●—) or diluent (—○—) as above, to which diluent instead of OPZ was added but otherwise were treated the same as tubes with added OPZ (see Materials and Methods). Values are means of one experiment performed in triplicate.

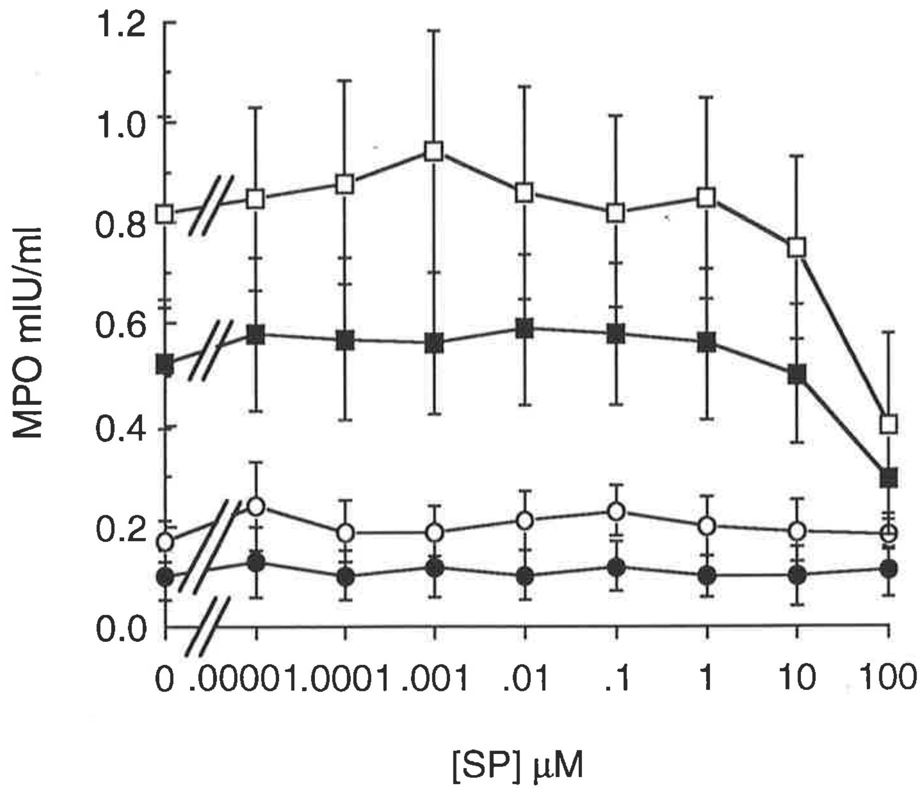


Figure 3.14 Effect of SP on fMLP-stimulated MPO release. Neutrophils were preincubated with medium or the stated concentrations of SP for 15 min at 37°C and were then stimulated with diluent (—●—) or 0.1, 0.01, 0.001 μM fMLP (—□— , —■—, —○—) for an additional 10 min. Values represent means of three experiments.

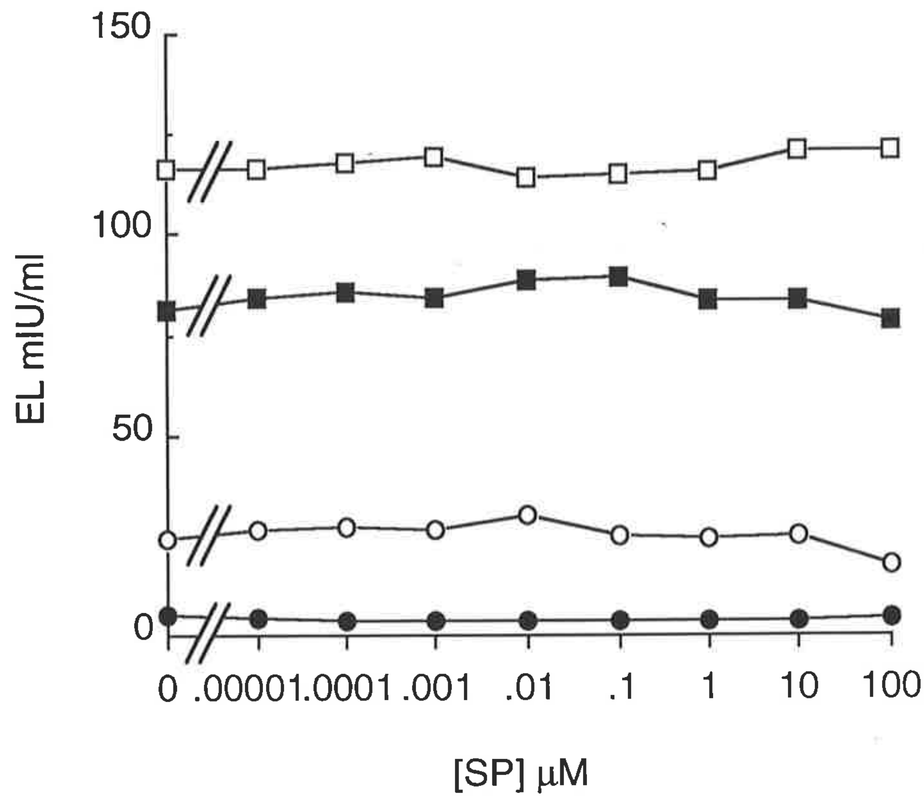


Figure 3.15 Effect of SP on fMLP-stimulated EL release. Neutrophils were preincubated with medium or the stated concentrations of SP for 15 min at 37°C and were then stimulated with diluent (—●—) or 0.1, 0.01, 0.001 μM fMLP (—□— , —■— , —○—) for an additional 10 min. Values represent means of three experiments. SEM are not shown for better graph clarity.

Chapter 4

Activation of Human Neutrophils by Other Tachykinins and Substance P fragments

4.1 Summary

This study examines the contribution of tachykinins other than SP to the processes of inflammation. Neurokinin A (NKA), neurokinin B (NKB) and eledoisin (E) but not kassinin (K) have similar effects to SP in priming neutrophils for increased O_2^- production in response to fMLP. This similarity in activity may be due to the carboxy amino acid terminal end of these tachykinins being highly conserved. This was confirmed by demonstrating that SP fragment 7-11 (SP_{7-11}) had the same priming effect as the whole molecule, whereas, the amino end fragment 1-4 (SP_{1-4}) inhibited the response to fMLP. The priming effect of tachykinins was not confined to a single stimulus, such as fMLP, since NKA, NKB and SP also enhanced O_2^- production stimulated by PAF. In addition, all the tachykinins studied increased neutrophil ADCC towards opsonised target cells. In contrast to their effects on fMLP-induced O_2^- production, both SP fragments, SP_{1-4} and

SP_{7-11}, stimulated neutrophil ADCC and had a synergistic effect when used together.

4.2 Introduction

The tachykinins are a family of neuropeptides (Chapter 1.8) defined by a common C-terminal amino acid sequence of phe-X-gly-leu-met-NH_2, where X is an aliphatic or aromatic amino acid, and by an ability to cause rapid contraction of a variety of smooth muscle types. Three mammalian tachykinins have been described to date; SP, NKA and NKB. A number of non-mammalian tachykinins have also been isolated including E and K. Kassinin is the homologue of NKA as both have identical C-terminal pentapeptide sequences. The primary structures of the tachykinins investigated in this study are shown in Table 4.1.

In the previous chapter, I demonstrated that SP primes human neutrophils to produce increased amounts of O_2^- and leukotriene B_4 in response to the synthetic tripeptide fMLP and stimulates neutrophil ADCC. Information on the effects of the other tachykinins on inflammatory cells is limited [Roch-Arveiller et al, 1986; Brunelleschi et al, 1991], and their potential role in neurogenic inflammation is largely unknown. Since several of tachykinins may colocalize in sensory afferents, it is possible that some of the actions ascribed to SP from *in vivo* studies may in fact be due to other peptides (Chapter 1.9). For example, SP and NKA were shown to be codistributed and colocalized in pig gastrointestinal tract [Schmidt et al, 1991], NKA and NKB in human gastrointestinal tract, pancreas and gall bladder [Kishimoto et al, 1991] and SP, NKA and NKB in rat spinal cord but only SP and NKA in rat peripheral tissues [Moussaoui et al, 1992]. Moreover, both SP and NKA were found in synovial fluid above plasma level in patients with rheumatoid arthritis [Appelgren et al, 1991]. The

colocalization and codistribution of SP and NKA is not surprising, as both neuropeptides are produced from a single preprotachykinin gene as a result of differential RNA splicing and posttranslational processing [Helke et al, 1990; Agro et al, 1990; Chapter 1.8.1] and are co-secreted upon appropriate stimulation [MacDonald et al, 1989; Vedder and Otten, 1991; Schmidt et al, 1992]. Capsaicin has been widely used to deplete SP containing nerves in experimental animals, were in fact it depletes afferent sensory nerves containing several tachykinins. Therefore, results from experiments using capsaicin should be interpreted with caution, since peptides other than SP could be responsible for the biological effects (or lack of them) observed after capsaicin treatment.

Furthermore, there are several enzymes which metabolise SP to smaller fragments (Chapter 1.9.3), whose effects on neutrophils may be either stimulatory or inhibitory [Hafstrom et al, 1989]. What fragments are generated from SP and other tachykinins at specific tissue sites and their relative importance in modulating inflammatory responses is still not well understood. Since tachykinins, after their release from afferent nerves, are rapidly metabolised to many fragments, it is extremely difficult to study the effects of particular fragments *in vivo*. Specific enzyme inhibitors were used successfully to study localised degradation of exogenously administered tachykinins by tissue enzymes [Stimler-Gerard, 1987; Shore and Drazen, 1989; Martins et al, 1990; Chapter 1.9.3], but the *in vivo* effect of specific tachykinin fragments have not been studied.

The role of SP fragments on inflammatory cell function have been studied *in vitro* using isolated cell populations. Subsequently it was demonstrated that the amino and carboxy end of SP have distinct activities (Chapter 1.9.3). For example, the carboxy end of SP is chemotactic for monocytes [Ruff et al, 1985] whereas the amino end is essential for histamine release from mast cells [Fewtrell et al, 1982].

To test the hypothesis that tachykinins other than SP, as well as SP metabolic fragments, regulate human neutrophil function, their effects on neutrophil O_2^- production and ADCC were studied. The primary structures of SP fragments studied are shown in Table 4.2.

4.3 Results

4.3.1 *The effect of tachykinins on fMLP-stimulated superoxide anion production*

As demonstrated in Chapter 3.3.2, SP had no direct effect on neutrophil O_2^- production alone, but primed them for an increased O_2^- production in response to fMLP. None of the tachykinins (0.01 to 100 μM) studied stimulated significant O_2^- production on its own. To determine, whether other tachykinins could influence fMLP-stimulated O_2^- production, neutrophils were incubated with each tachykinin for 30 min., and then stimulated with 0.1 μM fMLP. Preincubation of neutrophils with either SP, NKA, NKB or E enhanced the response to fMLP in a dose-dependent manner (Fig. 4.1). A maximum effect was observed at 100 μM for SP, NKA and E, when O_2^-production increased from 12.7 ± 3.1 to 28.8 ± 5.1 ($p < 0.01$), 23.3 ± 3.7 ($p < 0.02$) and 21.1 ± 3.6 ($p < 0.02$) nmol/10^6 cells respectively. Kassinin did not have any effect on neutrophil fMLP-stimulated O_2^- production (Fig. 4.1A). Although NKB significantly enhanced the response to fMLP at lower concentrations than the other tachykinins, the effect was reduced at 100 μM (Fig. 4.1B). For example 5 μM NKB increased O_2^- production from 15.3 ± 2.1 to 27.9 ± 1.7 nmol/10^6 cells ($p < 0.001$), had its maximum effect at 50 μM (36.8 ± 5.1; $p < 0.02$), and a reduced effect at 100 μM (20.5 ± 4.2; $p < 0.04$).

4.3.2 *The effect of tachykinins on PAF-stimulated superoxide anion production*

The effect of tachykinins was not confined to the fMLP as stimulus . As illustrated in Fig. 4.2, the tachykinins tested also enhanced PAF-stimulated O_2^- production. Here, cells were incubated for 30 min with medium or 50 μM NKA, SP or NKB and then stimulated with 1 μM PAF (for 6 min.). O_2^- production increased from 2.3 ± 0.4 to 5.8 ± 0.7 ($p < 0.002$) for NKA, to 8.4 ± 1.5 ($p < 0.008$) for SP and 17.5 ± 3.9 ($p < 0.02$) nmol/10^6 cells for NKB. This priming effect of NKB was to increase O_2^- production to 766 ± 116% of control.

4.3.3 *The effect of tachykinins on neutrophil antibody-dependent cell-mediated cytotoxicity*

As shown in the Chapter 3.3.1, SP directly stimulated neutrophil ADCC in a dose-dependent fashion. Here, the effects of other tachykinins, using an antibody concentration of 1 μg/ml, which were shown previously to be suboptimal for SP stimulation (see Chapter 3.3.1) were studied. As illustrated in Fig. 4.3, in the presence of antibody alone (medium control), neutrophils killed 15-19% of target cells. Although the tachykinins had no direct cytotoxic effect on the target cells (e.g., in the absence of either neutrophils or antibody), they stimulated ADCC dose-dependently within the range of 1-100 μM. The maximal responses at 100 μM were compared and the results are shown in Fig. 4.4, expressed as *net*-ADCC (calculated as ADCC in presence of stimulus minus spontaneous ADCC in the presence of medium alone). NKB stimulated neutrophil *net*-ADCC was 31.4 ± 1.0%. NKA- and E-induced *net*-ADCC was 12.9 ± 1.8 and 12.8 ± 2.6% respectively, which was similar to the value for SP (15.5 ± 2.5%). Kassinin was the weakest stimulus of all tachykinins and *net*-ADCC was only 6.3 ± 2.8%.

4.3.4 *The effect of SP fragments on fMLP-stimulated superoxide anion production*

The activities of SP_{1-4}, SP_{1-6} and SP_{7-11} on fMLP-induced neutrophil O_2^- production are compared in Fig. 4.5. SP and SP_{7-11} had similar effects in that they primed for an enhanced response in a dose-dependent fashion. For example, SP and SP_{7-11} increased O_2^- production maximally from 10.7 ± 1.6 to 25.0 ± 4.7 ($p < 0.03$) and to 25.6 ± 5.2 ($p < 0.03$) nmol/10^6 cells respectively. In contrast, SP_{1-4} at concentrations above 10 μM decreased the response by as much as 77%, to 2.5 ± 1.2 nmol/10^6 cells ($p < 0.01$) and SP_{1-6} had no significant effect on the response (Fig. 4.5).

4.3.5 *The effect of SP fragments on neutrophil antibody-dependent cell-mediated cytotoxicity*

In the next series of experiments, the effects of SP, SP_{7-11} and SP_{1-4} on neutrophil ADCC were compared. Again, a suboptimal concentration of antibody of 1 μg/ml was used. Both fragments stimulated a dose-dependent increase in ADCC in the range of 1 to 100 μM, and exerted their maximal effect at 100 μM (Fig. 4.6). As shown in Fig. 4.7, *net*-ADCC with 100 μM SP was 12.7 ± 2.2%. *Net* -ADCC with 100 μM SP_{7-11} was 16.7 ± 1.2%, which was not significantly different from that of SP. SP_{1-4} (100 μM) increased ADCC to a lesser extent (*net*-ADCC was 3.3 ± 1.0% $p < 0.03$).

However, when cells were stimulated with both fragments simultaneously, there was a synergistic increase in *net*-ADCC in that their combined effect was greater than the sum of each fragment (e.g., *net*-ADCC was 26.2 ± 2.5 compared to 20.0 ± 1.5 ($p < 0.04$).

4.4 Discussion

The present study provides evidence for a role of tachykinins in the modulation of different neutrophil functions. The results extend the earlier findings on the priming and direct effects of SP on neutrophil function, and demonstrate that other mammalian (e.g., NKA and NKB) and non-mammalian tachykinins (e.g., eledoisin but not kassinin) and SP fragments have similar modulating effects. For example, it was demonstrated that although NKA, NKB and E had no direct effect on neutrophil O_2^- production, they did facilitate the response to fMLP. Although NKB produced its effects at slightly lower concentrations than the other tachykinins, at the highest concentration tested there was a reduction in the stimulation, which was not observed with the other tachykinins. Similarly, SP, NKA and NKB facilitated neutrophil O_2^- production in response to PAF, with NKB being the most potent. All the tachykinins tested increased neutrophil ADCC towards opsonised target cells, and again NKB was the most potent.

The stimulatory effect of NKB on neutrophil function is not in agreement with Brunelleschi et al [Brunelleschi et al, 1991], who found NKB to be inactive in priming human neutrophils for increased responses to both fMLP and PAF. There may be several reasons for this discrepancy. Firstly, in the experiments of Brunelleschi and coworkers the cells were primed with the tachykinins for only 3 min compared to 30 min in the experiments described here, and previous work in our laboratory has shown, that longer preincubation times are required for SP [Wozniak et al, 1989] and other tachykinins to exert their effects. Secondly, Brunelleschi et al, used NKB that was dissolved in DMSO rather than water or dilute acetic acid. Although low concentration of DMSO (0.1%) does not interfere with fMLP- or PAF-stimulated O_2^- production, it is possible that DMSO inhibit the priming activity of NKB.

A common feature of all tachykinins is their highly conserved carboxy terminal [Chapter 1.8] and it has been hypothesised that the carboxy terminal determines the priming activity observed. For example, the carboxy terminal appears to be largely responsible for the stimulatory effect of SP on lymphocyte proliferation [Payan et al, 1983]. Similarly the carboxy end of SP has been found to induce chemotaxis, increase cytosolic free calcium concentration and oxidative metabolism in neutrophils [Serra et al, 1988; Iwamoto et al, 1990]. In these experiments, SP_{7-11} (10-100 μM) but not SP_{1-4} or SP_{1-6}, enhanced O_2^- production by neutrophils stimulated with 0.1 μM fMLP in the same manner as did SP_{1-11}. Similarly, Hafstrom et al [1989] demonstrated that the carboxy end of SP (SP_{7-11}) but not the amino end (SP_{1-4}) stimulated neutrophil oxidative metabolism (as measured by chemiluminescence) and aggregation. Thus, the priming activity of tachykinins on neutrophils appears to be associated with the common carboxy end.

However, the inability of kassinin to prime neutrophils as well as the different potencies of the other tachykinins tested, suggests an additional role for the amino terminus in modulating this activity. This was further confirmed by the observation that SP_{1-4} inhibited the fMLP stimulated neutrophil O_2^- production. Previous report by Serra et al [1988], who demonstrated that the fragment SP_{1-4} alone is inactive and that SP_{4-11} is more effective than the entire molecule in direct activation of neutrophil respiratory burst and enzyme secretion, provide additional evidence for the modulatory effect of the amino end on the carboxy end activity. Similarly, the finding that both SP_{7-11} and SP_{1-4} stimulated ADCC (as well as the facilitation observed with these fragments together) suggests that tachykinin activity is not solely determined by carboxy end alone.

However, it should be noted that, in contrast to the above findings, degranulation of mast cells has been shown to be determined by the amino

terminus of SP (e.g., SP_{1-4} was active but not SP_{7-11}), and that deletion of two amino acids from the carboxy terminal modulated the activity of SP – reducing the activity by 30% [Fewtrell et al, 1982; Shibata et al, 1985].

The diverse effects of SP fragments on fMLP-stimulated O_2^- production and ADCC, suggest alternative mechanisms by which tachykinins may modulate inflammation. In addition, their potential effects on inflammatory cells may further be modified by cytokines and other cell agonists that are released during inflammation (Chapter 1.4). Although this study and others [Bar-Shavit et al, 1980; Hafstrom et al, 1989; Iwamoto et al, 1990] have evaluated the effects of SP fragments on neutrophil function, enzymes that are capable of cleaving the other tachykinins have been identified on neutrophils and their effects remain to be studied (Chapter 1.9.3).

With the exception of the effect of 100 μM NKB on neutrophil O_2^- production, no biphasic responses (e.g., stimulation followed by inhibition at higher concentrations) of any of the tachykinins tested were seen. This is in contrast to the results of Hafstrom [Hafstrom et al, 1989] who observed that concentrations of SP and SP_{7-11} greater than 10 μM inhibited the priming effect on neutrophil O_2^- production, an effect that was overcome by increasing fMLP concentration. These differences may result from the different techniques used to quantify oxy radical production. In the studies of Hafstrom, total oxy radical production rather than O_2^- production, was monitored by luminol-amplified chemiluminescence that is very sensitive to minor changes in pH and alterations in buffer components (e.g. antioxidants and albumin). This method is also non-specific, as luminol may be oxidised by many different oxy radicals including H_2O_2, $^1O_2$, NO and oxidants such as hypochlorous acid [DeChatelet et al, 1982; Brestel, 1985; Muller-Peddinghaus, 1984], as discussed in the chapter 3.4.

Since many tachykinins have been shown to colocalise in the same sensory fibres, and SP and NKA and its derivatives are products of the same

gene and arise by alternative RNA splicing and post-translational modification (Chapter 1.8.1), the *in vivo* tachykinin effect may be a result of a complex multiple peptide stimulation on neutrophil function and activity. This, as I have shown, may be further complicated by tissue enzymes capable of generating peptide fragments of specific activities. Therefore, future availability of very specific enzyme inhibitors and tachykinin receptor antagonists should yield a more accurate assessment of the role of tachykinins in inflammation.

TACHYKININS

Substance P	Arg-Pro-Lys-Pro-Gln-Gln-**Phe**-Phe-**Gly-Leu-Met**
Neurokinin A	His-Lys-Thr-Asp-Ser-**Phe**-Val-**Gly-Leu-Met**
Neurokinin B	Asp-Met-His-Asp-Phe-**Phe**-Val-**Gly-Leu-Met**
Eledoisin	Pro-Ser-Lys-Asp-Ala-**Phe**-Ile-**Gly-Leu-Met**
Kassinin	Asp-Val-Pro-Lys-Ser-Asp-Gln-**Phe**-Val-**Gly-Leu-Met**

Table 4.1 Primary structures of tachykinins. The common amino acid residues of the carboxy end are shown in bold face.

SUBSTANCE P FRAGMENTS

SP	Arg-Pro-Lys-Pro-Gln-Gln-**Phe**-Phe-**Gly-Leu-Met**
SP_{1-4}	Arg-Pro-Lys-Pro-
SP_{1-6}	Arg-Pro-Lys-Pro-Gln-Gln-
SP_{7-11}	-**Phe**-Phe-**Gly-Leu-Met**

Table 4.2 Primary structures of substance P fragments. The common amino acid residues of the carboxy end are shown in bold face.

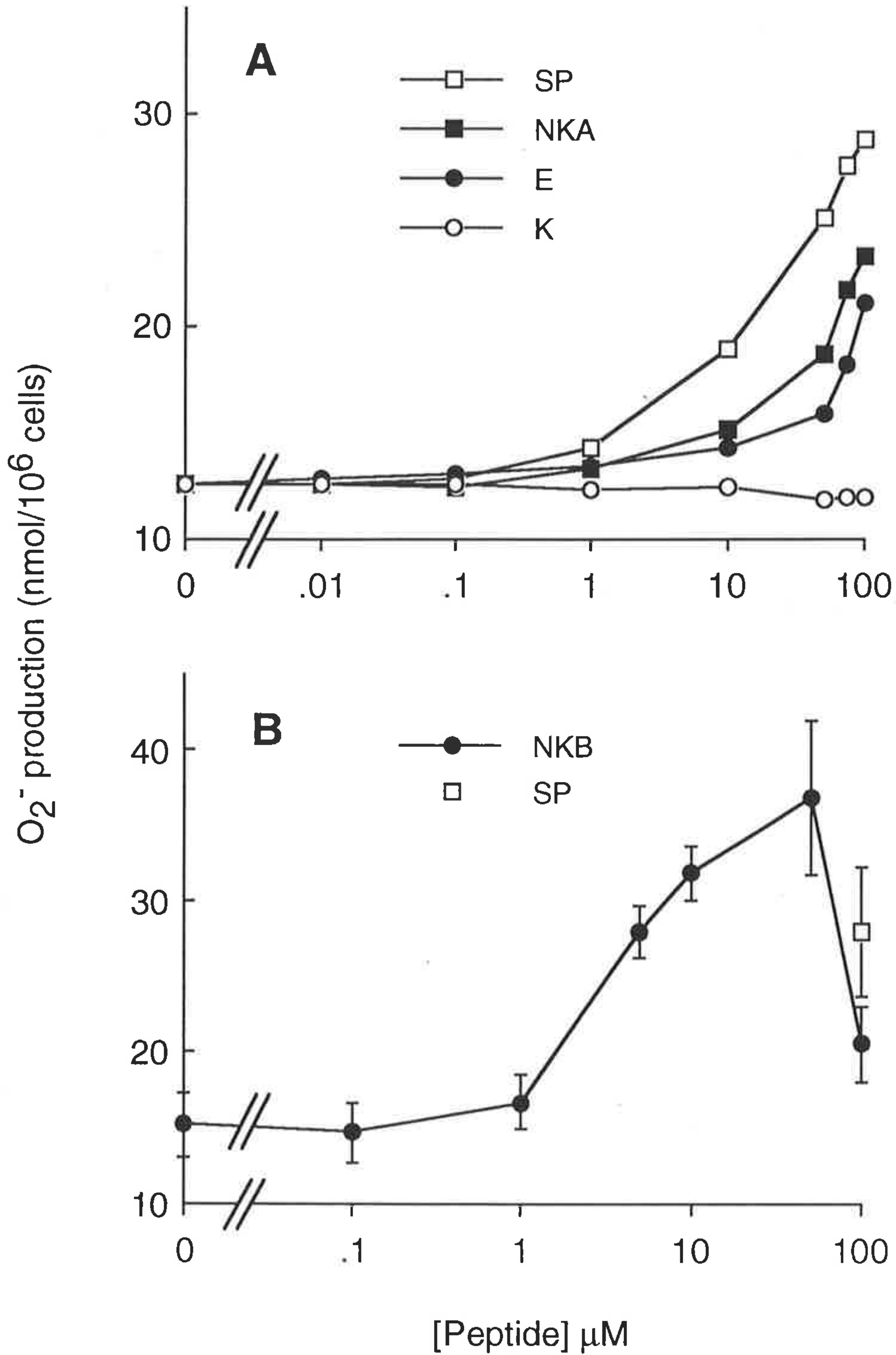


Figure 4.1 Effect of tachykinins (**A**) and NKB (**B**) on fMLP-stimulated O_2^- production. Neutrophils were preincubated with medium or tachykinins for 30 min at 37°C and were then stimulated with 0.1 μM fMLP for an additional 6 min. Values represent means of five experiments in **A** and three experiments in **B**. SEM are not shown in **A** for better graph clarity.

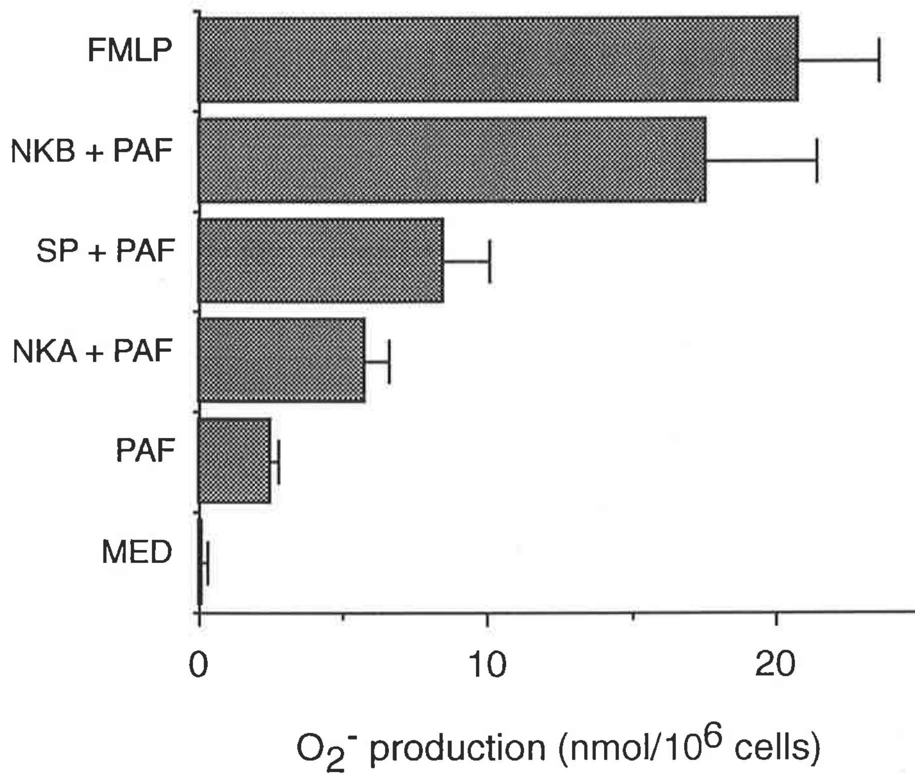


Figure 4.2 Effect of tachykinins on PAF-stimulated O_2^- production. Neutrophils were preincubated for 30 min with medium or 50 µM NKA, SP or NKB and then stimulated for an additional 6 min with 1 µM PAF. For comparison, results are shown for cells that were stimulated with 0.1 µM fMLP alone. Values represent means of four experiments.

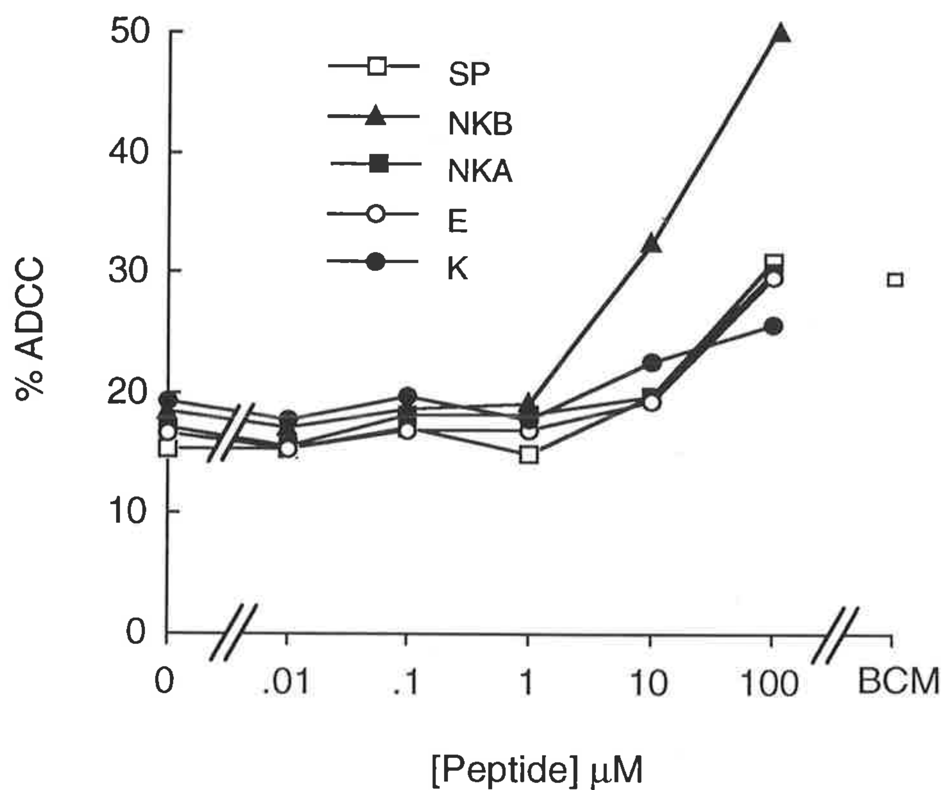


Figure 4.3 Effect of tachykinins on neutrophil ADCC. Neutrophils were incubated for 2.5 hrs with target cells, antibody (1 μg/ml) and various concentrations of tachykinins. Bladder carcinoma cell line U5637 conditioned medium (BCM) was used as a positive control. Data are means of three experiments.

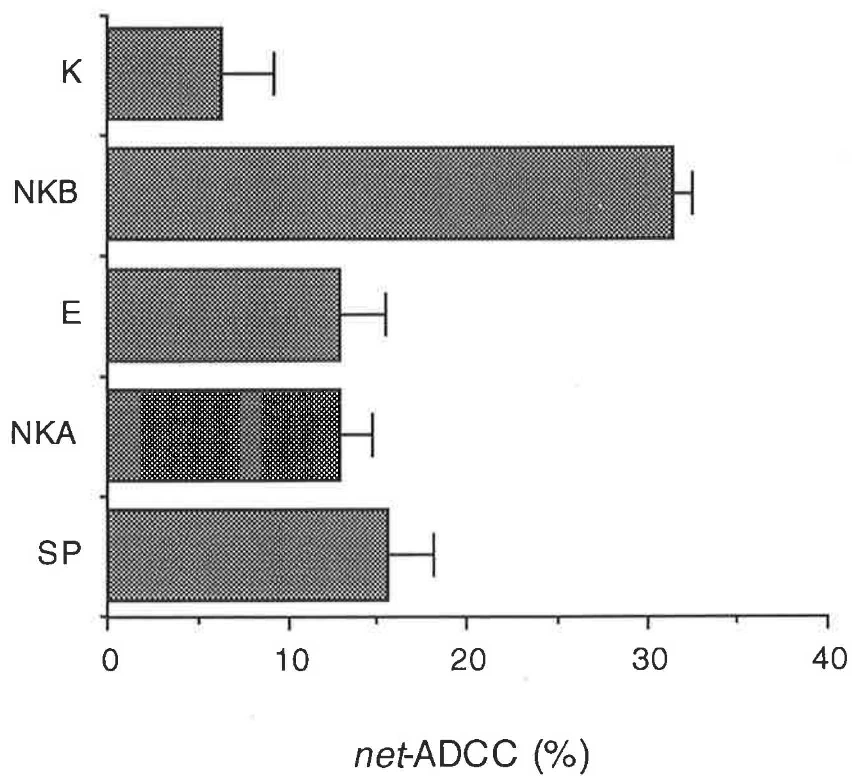


Figure 4.4 Effect of tachykinins (100 μM) on *net*-ADCC (stimulated minus baseline responses). Values are derived from **Fig. 4.3**, and are means of three experiments.

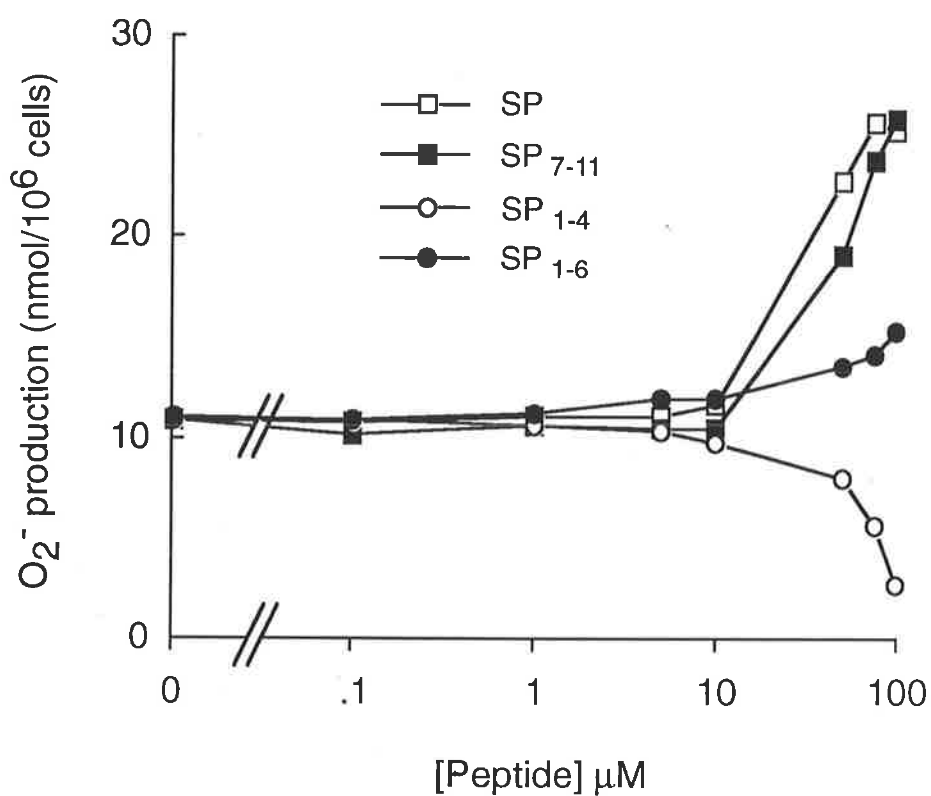


Figure 4.5 Effect of Substance P fragments on fMLP-stimulated O_2^- production. As in Fig. 4.1, cells were preincubated with SP_{1-4}, SP_{1-6}, SP_{7-11} or SP for 30 at 37°C min before fMLP stimulation. Values represent means of three experiments.

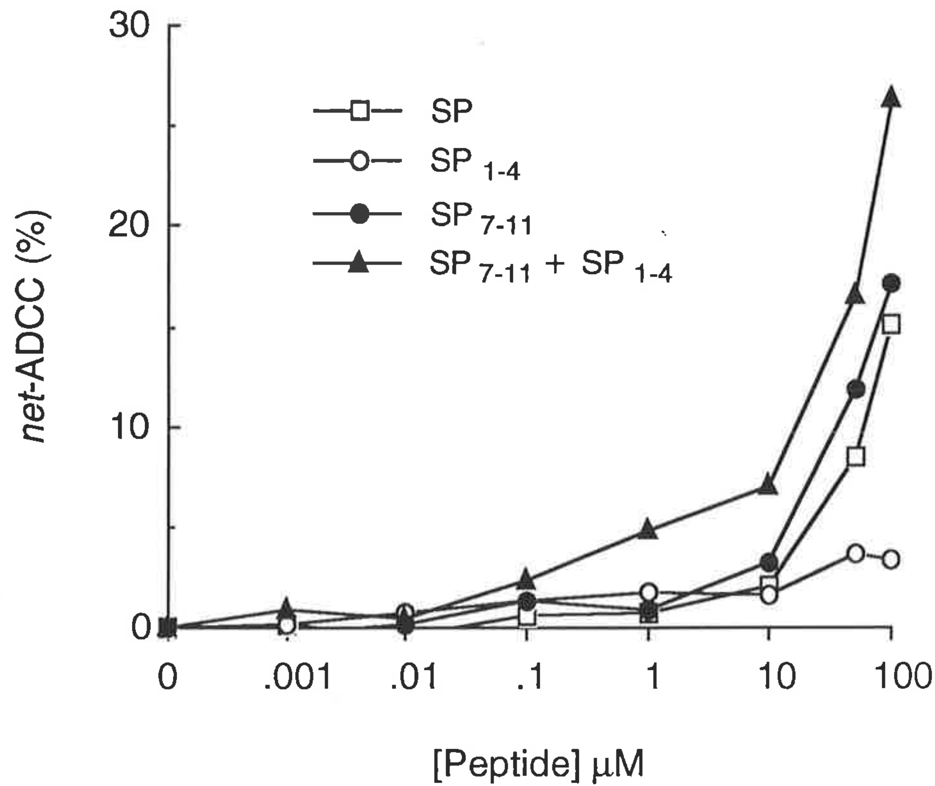


Figure 4.6 Effect of substance P fragments on neutrophil ADCC. Neutrophils were incubated for 2.5 hrs with target cells, antibody (1μg/ml) and various concentrations of SP, SP_{1-4}, SP_{7-11} added separately or together. Values represent net-ADCC (stimulated minus baseline responses) and are means of four experiments

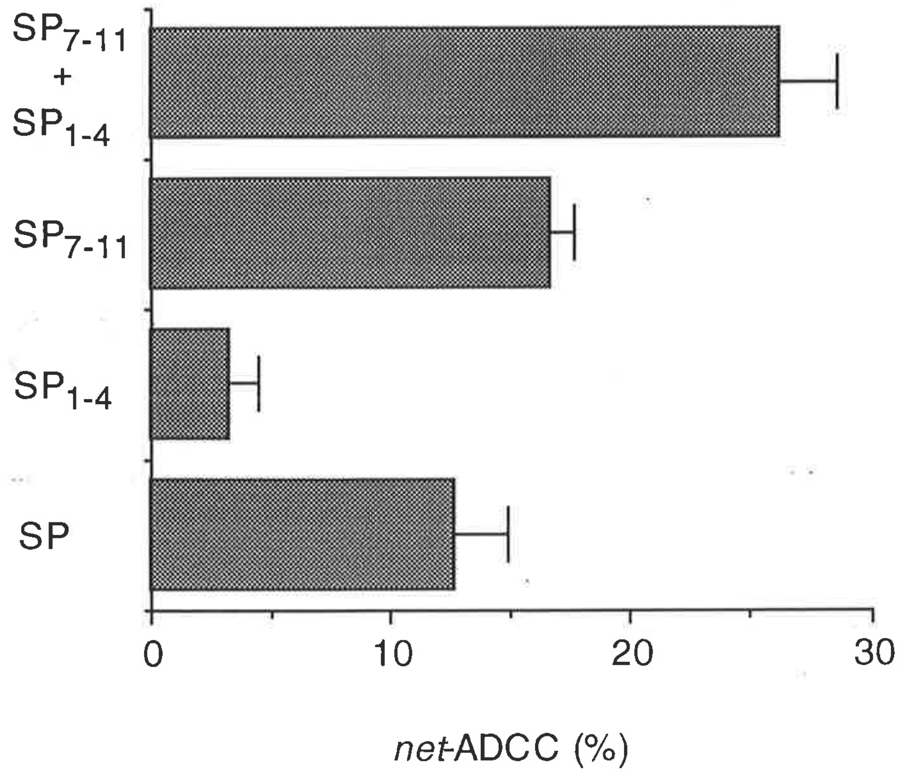


Figure 4.7 Effect of SP_{7-11} and SP_{1-4} on neutrophil *net*-ADCC. Cells were incubated with 100 μM SP_{7-11} or SP_{1-4} added separately or together. SP was used as a positive control. Values are means of five experiments.

Chapter 5

The Mechanism of Priming of Human Neutrophils for Enhanced Superoxide Production by Substance P

5.1 Summary

This study investigates the mechanisms by which SP facilitates neutrophil O_2^- production. SP increased the rate of O_2^- production in PMA stimulated neutrophils, but also shortened the lag period before the onset of the response. Stimulation of neutrophils with SP increased intracellular free calcium concentration ($[Ca^{++}]_i$) by mobilising calcium from both intracellular stores and by inducing a calcium influx. The increase in calcium was dose-dependent and occurred in the same range of SP concentrations that primed neutrophils for O_2^- production, and so the calcium alone could be responsible for this effect of SP. Moreover, SP carboxy terminal fragment, SP_{7-11} (but not the amino terminal, SP_{1-4}), that was shown to enhance O_2^- production, also stimulated an increase in $[Ca^{++}]_i$. These observations suggest, that calcium may play an important role in the priming phenomenon. In addition, SP

caused translocation of protein kinase C to the cell membrane, a phenomenon usually associated with increased cell activity and O_2^- production in neutrophils, but this did not happen every time the O_2^- response was enhanced and therefore the importance of this finding to SP mechanisms remains unclear. Finally, it is proposed that the facilitation of O_2^- production in neutrophils may occur through increased phosphorylation of the p47-*phox* and p67-*phox* components of NADPH oxidase, and may be a calcium-mediated effect, as calcium ionophore (A23187) pretreatment of neutrophils increased phosphorylation of 47 kD and 67 kD proteins in PMA stimulated neutrophils.

5.2 Introduction

In the two previous chapters, it was shown that SP, SP fragments and other tachykinins prime human neutrophils for enhanced O_2^- production in response to a second stimulus. The SP effect was not second stimulus-specific, and it facilitated O_2^- production in neutrophils stimulated by fMLP, PMA and PAF, implying, that it must exert its effect on a component of the stimulus-response pathway, that is common to these three stimuli.

Although many cytokines and proinflammatory mediators (e.g. TNFα, GM-CSF, PAF, LTB_4) have been shown to prime human neutrophils for enhanced O_2^- production, the mechanisms involved in neutrophil priming are still debatable and far from clear. Whereas some studies suggested that receptor changes may explain the priming effect (as seen for fMLP receptors in cells primed with TNFα and GM-CSF) [Weisbart et al, 1986, Atkinson et al, 1988], others have shown, that priming can not be explained by changes in receptor numbers alone [O'Flaherty et al, 1991], and that priming involves changes in the activity of components of the signal transduction pathway that are independent and distal to receptors [McColl et al, 1990].

The hydrolysis of membrane phospholipids by phospolipase C (PLC), and subsequent formation of diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3), is considered to be the major intracellular mechanism of neutrophil activation (Chapter 1.6.2). DG and IP_3 act as second messengers in the cell, leading to rises in cytosolic calcium and PKC activation. The activity of PKC is enhanced by elevated $[Ca^{++}]_i$ and by DG, both of which cause increased PKC translocation from cytosol to cell membrane, and increased binding to phosphatidyl serine in the membrane. Once activated, PKC phosphorylates other cellular proteins and enzymes and in this way controls the activity of many processes in the cell. Some components of the NADPH oxidase complex (p47-*phox* and p67-*phox*) are phosphorylated by PKC or other kinases (e.g. tyrosine kinases, [Grinstein and Furuya, 1991]) prior to the activation of the complex (Chapter 1.6.2).

The experiments outlined in this chapter were designed to test the hypothesis that SP primes human neutrophils for enhanced O_2^- production by direct interaction with the components of signal transduction pathway leading to NADPH oxidase activation. In particular, it was hypothesised that SP priming elevates $[Ca^{++}]_i$ and consequently increases PKC translocation to the cell membrane, as demonstrated by increased PDBu (a DG anolog) binding and activation of PKC, and results in enhanced NADPH oxidase activation. The latter may occur by phosphorylation of p47-*phox* and p67-*phox* NADPH components [Nauseef et al, 1991; Clark et al, 1990].

5.3 Results

5.3.1 *The effect of substance P on the kinetics of PMA-stimulated superoxide anion production*

To study the kinetics of the priming effect of SP, neutrophils (5 x 10^6) were preincubated with 50 μM SP for 15 min at 37°C and then 10^6 cells were

transferred to prewarmed cuvettes containing cytochrome C and 10 ng/ml or 50 ng/ml PMA. The O_2^- production was measured continuously and the results from a representative experiment are shown in Fig. 5.1. SP increased the rate of O_2^- production and decreased the lag phase before its onset. The PMA-stimulated rate of O_2^- production increased from 30 ± 8 to 78 ± 28 pmol/10^6 cells/sec and from 65 ± 11 to 115 ± 21 pmol/10^6 cells/sec (n = 3) for PMA concentrations of 10 ng/ml and 50 ng/ml respectively. Although SP facilitated the PMA-stimulated O_2^- production over a range of different concentrations (Fig. 3.12), it did not shift the dose response to PMA.

5.3.2 *The effect of substance P on $[Ca^{++}]_i$*

To determine whether enhancement of O_2^- production was associated with changes in $[Ca^{++}]_i$, FURA-2 loaded neutrophils were stimulated directly with SP, SP_{7-11} and SP_{1-4}. Both, SP and SP_{7-11} stimulated a rise in $[Ca^{++}]_i$, but SP_{1-4} did not (Fig. 5.2, data for SP_{1-4} not shown). SP_{7-11} was a better stimulus than SP, and a concentration just above 1 μM SP_{7-11} substantially increased $[Ca^{++}]_i$ levels, whereas higher concentrations of SP were required to produce smaller effect. The effect was rapid, reaching a maximum at approximately 20 sec after SP addition, and disappeared within 5 min (data not shown).

SP increased $[Ca^{++}]_i$ in a dose-dependent manner as illustrated in one of the representative experiments (Fig. 5.3). Neutrophils stimulated with 50 μM SP increased $[Ca^{++}]_i$ levels by 418 nM. However, when neutrophils were stimulated in calcium-free buffer, the increase in $[Ca^{++}]_i$ was reduced to 132 nM, but the effect could be reversed by supplementing the buffer with 1.25 mM $CaCl_2$ (425 nM at SP 50 μM). These results suggest that the increase in $[Ca^{++}]_i$ induced by SP was caused by both an influx of extracellular calcium and release of calcium from intracellular stores.

5.3.3 *The effect of substance P on protein kinase C translocation*

PKC translocation can be studied by cell-disruptive techniques or by ^{3}H-PDBu binding to whole cells. The cell-disruptive methods require lengthy cell processing and exposure of cell fractions to different buffers, that may affect the PKC attachment to the cell membrane and therefore may fail to detect translocation of PKC [O'Flaherty et al, 1990]. The second technique is utilising the special property of ^{3}H-PDBu that binds directly to the DG receptors on PKC, but does not fully penetrate the cell membrane. Therefore the ^{3}H-PDBu binding depends on the availability of PKC in the membrane and is used to assess accurately the movement of cytosolic PKC to the membrane.

5.3.3.1 *The effect of fMLP on neutrophil binding of ^{3}H-PDBu*

To determine the optimal concentration of fMLP that induces maximal ^{3}H-PDBu binding to neutrophils, the cells were incubated with 10 nM ^{3}H-PDBu for 30 min at 37°C and were then stimulated with medium or 0.001, 0.01, 0.1 or 1 μM fMLP for an additional 6 min. A maximum effect was seen at 0.1 μM fMLP and the response declined slightly when 1 μM fMLP was used (Fig. 5.4). This dose-response pattern of ^{3}H-PDBu binding to fMLP-stimulated neutrophils resembled closely the dose-response curve of O_2^- production stimulated by fMLP (Fig. 3.6).

5.3.3.2 *The effect of temperature on neutrophil binding of ^{3}H-PDBu*

The binding kinetics of any ligand to its receptor on the cell depends on the temperature at which an experiment is carried out. The temperature

effect on the kinetics of ^{3}H-PDBu binding to unstimulated neutrophils was studied by preincubating neutrophils (10^{7}/ml) for 15 min at 4, 22 or 37°C followed by addition of 10 nM ^{3}H-PDBu (Fig. 5.5a, 5.5b and 5.5c). At each time point, 100 μl (10^{6} cells) were removed and harvested in a cell harvester with three washings. Non specific binding was assessed by having replicates that contained 100 fold excess of cold PMA (1 μM), and the fraction of ^{3}H-PDBu bound to the cells specifically calculated. At 4°C, the maximal specific binding was reached after 120 min of incubation and remained unchanged for the next 120 min measured (Fig. 5.5a). The specific binding of ^{3}H-PDBu at 22°C showed similar pattern (Fig. 5.5b), although the kinetics were different. The maximum specific binding was reached by 15 min and did not decrease over the next 45 min. The binding to neutrophils at 37°C was very rapid, reached a maximum 1 min after addition of ^{3}H-PDBu, declined slowly over the next 30 min, and more slowly thereafter (Fig 5.5c).

5.3.3.3 *The effect of substance P on neutrophil binding of ^{3}H-PDBu*

SP alone significantly stimulated translocation of PKC to the cell membrane as shown by increased ^{3}H-PDBu binding (Fig. 5.6). The translocation was rapid, and in most experiments lasted less then 3-6 min. This effect parallelled the increase in $[Ca^{++}]_i$ and it is likely that calcium was associated with PKC translocation to the cell membrane. Since the SP induced increase in association of PKC with cell membrane was short lasting, it is difficult to see how it could enhance superoxide production in cells that had been preincubated with SP for 30 min or longer prior to fMLP stimulation. However, cells that had been preincubated with SP increase fMLP-stimulated ^{3}H-PDBu binding. The experimental procedure for this series of experiment is illustrated by an example in Figure 5.7a. In these experiments, neutrophils (10^{7} cell/ml) were first equilibrated with 10 nM ^{3}H-PDBu for 30 min at 37°C and then 75 μM SP or medium control was added.

Cells were sampled before addition of SP and 1, 3, 6, 9 and 13 min after SP addition, and the specific binding determined. 15 min after addition of SP (or medium control), the neutrophils were further stimulated with 0.1 μM fMLP, and 10^6 cells withdrawn at 1, 3, 6 and 9 min for measurement of specific ^{3}H-PDBu binding. As previously, the non-specific binding was assayed in the presence of 1 μM PMA.

Preincubation with SP enhanced significantly fMLP-stimulated ^{3}H-PDBu binding in six out of nine experiments performed. The increase was very rapid (less than 1 min) and lasted less than 3 min. At 1 min, SP enhanced fMLP-stimulated ^{3}H-PDBu binding increased from 0.0134 ± 0.0010 to 0.0172 ± 0.0009 fraction ^{3}H-PDBu bound ($n = 6$; $p = 0.0001$) (Fig. 5.7b).

In addition, combined sets of experiments were performed, in which the SP enhancement of fMLP-stimulated ^{3}H-PDBu and O_2^- production were assessed in the same experiment. These experiments were performed in a similar way to those described above. Neutrophils (10^7 cell/ml) that had been equilibrated with 10 nM ^{3}H-PDBu were preincubated with 75 μM SP or medium for 15 min at 37°C. Then, a 100 μl (10^6) of cells was transferred to prewarmed cuvettes containing cytochrome C and 0.1 μM fMLP in which O_2^- production was measured over 5 min at 37°C, and another 100 μl of cells was stimulated with 0.1 μM fMLP for 1 min at 37°C, and the cells were harvested and processed for assessment of ^{3}H-PDBu binding. As shown in Table 5.1, in 6 out of 9 experiments performed an increase in ^{3}H-PDBu binding (PKC translocation) was paralleled by increase in O_2^- production. In experiments 7 and 8, O_2^- production was increased but not ^{3}H-PDBu binding, and in experiment 9 there was a small decrease in ^{3}H-PDBu binding but large enhancement of O_2^- production. In the six experiments that showed increased ^{3}H-PDBu binding after preincubation with SP, there was no correlation ($r^2 = 0.402$) between the increase in binding and O_2^- production.

5.3.4 *The effect of calcium ionophore (A23187) on protein phosphorylation in neutrophil*

Neither, A23187 that increases $[Ca^{++}]_i$ nor PMA that binds and activates PKC, are naturally occurring cell agonists, but are good pharmacological tools for studying intracellular processes. To determine whether changes in $[Ca^{++}]_i$ alone could enhance O_2^- production by increasing PKC-mediated protein phosphorylation, ^{32}P-loaded neutrophils were preincubated with 0.1 µM A23187 for 10 min at 37°C, and were then stimulated with 3 nM PMA for an additional 5 min. Then, the cells were lysed and the supernatants containing phosphorylated proteins studied by SDS PAGE and autoradiography. Preincubation of neutrophils with 0.1 µM A23187 alone, had no effect on protein phosphorylation. The activation of neutrophils with 3 nM PMA slightly increased the overall phosphorylation in the cells, but preincubation of neutrophils with 0.1 µM A23187 enhanced substantially this response, in particular phosphorylation of 47 kD and 67 kD proteins (Fig. 5.8). In addition, neutrophils that were stimulated with 0.1 µM PMA (positive control) also showed enhanced phosphorylation of these proteins.

5.4 Discussion

The findings described in Chapters 3 and 4 laid the groundwork for subsequent investigations of the intracellular mechanisms involved in neutrophil priming and activation by SP. In particular, it was demonstrated that (i) SP enhances neutrophil O_2^- production in response to many different stimuli, (ii) SP induced facilitation of O_2^- production occured at suboptimal as well as optimal concentrations of the stimulus used, and (iii) the priming activity was located in the carboxy terminal of SP. In this study the effect of SP on two intracellular components of the neutrophil activation pathway,

$[Ca^{++}]_i$ and PKC translocation, was investigated in order to understand the intracellular mechanism involved in SP priming for enhanced O_2^- production in human neutrophils.

Changes in $[Ca^{++}]_i$ and PKC activity have been associated with a variety of neutrophil responses ranging from enzyme degranulation through O_2^- generation to synthesis of proinflammatory mediators and induction of cytokine production (Chapter 1.6). However, the mechanisms by which SP or cytokines (shown previously to facilitate O_2^- production in human neutrophils) exert their priming effect on neutrophil O_2^- production are uknown. Since the demonstration few years ago, that GM-CSF and TNF-α enhance neutrophil oxy radical production, there has been considerable interest in mechanisms underlying the priming effect. Subsequent investigations have shown that preincubation of neutrophils with GM-CSF or TNF-α modulates the number and affinity of fMLP receptors [Weisbart et al, 1986; Atkinson et al, 1988] and suggested that the receptor changes may explain the priming effect. However, studies by McColl et al [1990] challenged this hypothesis by demonstrating that GM-CSF or TNF-α prime neutrophils by inducing changes in the signal transduction pathways, which are independent of and distal to the fMLP receptor. This was further supported by O'Flaherty et al [1991] who demonstrated that receptor numbers alone cannot explain the priming phenomenon by showing that, although TNF-α enhanced neutrophil degranulation responses to fMLP, LTB_4 and PAF, it increased the number of fMLP receptors but downregulated LTB_4 receptors and only transiently upregulated PAF receptors. An alternative mechanism of priming, whereby the cells could be primed by their own metabolites, was proposed by Bauldry et al [1991], who showed that TNF-α enhances neutrophil phospholipase A_2 activity, thus increasing arachidonic acid release, LTB_4 production (Chapter 1.3.1.2) and PAF synthesis. Since these neutrophil metabolites have been shown previously to prime neutrophil

oxidative metabolism [Dewald and Baggiolini, 1985], they could prime the cells in an autocrine fashion. As shown in chapter 3, SP increases LTB_4 production and release from fMLP-stimulated neutrophils (Chapter 3.3.4), and therefore SP may indeed enhance phospholipase A_2 activity in these cells. In addition to LTB_4 production, the products of hydrolysis of membrane phospholipids by phospholipase A_2, *cis* unsaturated fatty acids and lysophosphatidylcholine, may directly enhance PKC activity by potentiating the action of DG (Chapter 1.6.2; Fig. 1.4) and therefore enhance O_2^- production in human neutrophils.

Although no single satisfactory explanation provides an answer as to what causes cell priming, the above studies suggest that changes in activity of components of intracellular signal transduction pathways leading to oxy radical production must take place following cell exposure to the priming agent. These changes most probably occur in a part of the pathway that is common to the different stimuli, as I have shown that SP affects fMLP, PMA and PAF responses. Furthermore, these changes were associated with the rate of O_2^- production in both PMA- and fMLP-stimulated neutrophils. PMA stimulates O_2^- production in neutrophils by binding to and directly activating protein kinase C (PKC) [Bell and Burns, 1991; Chapter 1.6.2 and 165]. In fMLP-stimulated cells, two sequential pathways leading to oxy radical production have been recognised: a PKC-independent pathway that initiates and a PKC-dependent pathway that sustains the response [Watson et al, 1991]. Since O_2^- production induced by both stimuli depends on PKC activity, and the binding of DG or PMA to PKC occurs in association with cell membrane, SP could, for example, increase PKC activity by translocating it to the cell membrane where it would be more accessible for DG or PMA binding [O'Flaherty et al, 1991]. ^{3}H-PDBu has been used to assess PKC translocation to the cell membrane. Neutrophil membrane PKC is considered to be the receptor for ^{3}H-PDBu, as it has been shown to bind to human neutrophils

rapidly and reversibly, compete for binding with diacylglycerol, and its binding inhibitable by sphinganine, a blocker of PKC-phospholipids interaction [O'Flaherty et al, 1990]. ^{3}H-PDBu does not appear to cross the cell membrane and therefore does not bind to cytosolic PKC. However, the temperature at which binding assays are conducted seems to be very important. I have shown that at 4°C the kinetics of ^{3}H-PDBu binding are slower but once the receptors became saturated, the binding equilibrium remained unchanged for long time. At 37°C, however, there was a sharp decline in ^{3}H-PDBu binding within the first 30 min. Therefore in all subsequent binding experiments neutrophils were equilibrated with ^{3}H-PDBu for 30 min before any stimulation. Although SP enhanced PKC translocation in fMLP-stimulated neutrophils in some experiments, in others an increase in O_2^- production occurred without any apparent additional PKC translocation. This finding suggests that SP enhancement of PKC translocation is not a critical element of SP-induced neutrophil priming, implying that O_2^- production is enhanced by another mechanism.

Alternatively, SP could increase PKC activity by elevating $[Ca^{++}]_i$ (Chapter 1.6.4) and accelerate the catalytic activity of NADPH oxidase by PKC-dependent phosphorylation of its component(s) (Chapter 1.6.5). Calcium is involved in formation of a bridge between the PKC and membrane phospholipids, and also enhances DG binding to PKC, and therefore PKC activity. NADPH oxidase consists of many cytosolic and membrane associated components that are assembled together upon activation (Chapter 1.6.5). Since the assembly of NADPH oxidase is dependent on the phosphorylation of its componenets [Clark et al, 1990], it is possible that SP speeds up the assembly process by elevating $[Ca^{++}]_i$ that enhances PKC phosphorylation and redistribution of the cytosolic components to the cell membrane, resulting in increased rate of oxy radical production. Both, SP and its carboxy terminal, SP_{7-11}, enhanced O_2^- production but the amino terminal, SP_{1-4}, did not (Chapter 4.3.1 and 4.3.4). Here, it was shown that SP

and SP_{7-11} but not SP_{1-4} elevates $[Ca^{++}]_i$. Interestingly, SP stimulated increases in $[Ca^{++}]_i$ over the same concentration range (Fig 5.2) as it facilitated O_2^- production of fMLP-stimulated PMN (Fig. 3.2). These observations suggest that elevations in $[Ca^{++}]_i$ may play an important role in the SP priming effect. It has been shown previously, that elevations in $[Ca^{++}]_i$ can facilitate oxy radical production in neutrophils stimulated with PMA or fMLP [Dale and Penfield, 1984; French et al, 1987]. This effect was thought to be mediated by increased PKC activity, resulting from PKC translocation to the cell membrane as well as increased affinity for phorbol esters. More recently, activated PKC has been implicated in phosphorylation-dependent translocation to the cell membrane of cytosolic oxidase factors p47-*phox* and p67-*phox*, which are essential for NADPH oxidase assembly and activation [Nauseef et al, 1991; Rotrosen and leto, 1990].

Calcium ionophore (A23187) was used to assess the contribution of calcium to the priming of neutrophils. A23187 releases calcium from intracellular stores, and is a widely used pharmacological tool for studying intracellular calcium effects. A23187 was shown previously to enhance phorbol ester-stimulated oxy radical responses, by shortening of the lag time and increasing the rate of O_2^- production in human neutrophils [French et al, 1987], an effect similar to that of SP. Preincubation of ^{32}P-labeled neutrophils with 0.1 μM A23187 enhanced phosphorylation of 47 kD and 67 kD proteins in PMA (3 nM) stimulated cells (Fig. 5.8). Furthermore, these proteins were heavily phosphorylated in cells activated with 100 nM PMA, a concentration of PMA that causes maximal production of O_2^- and has been used as a positive control. This suggests that the calcium enhancement of PKC activity in these cells, leads to the enhanced O_2^- production, as demonstrated by French et al [1987], probably via increased phosphorylation of the p47-*phox* and p67-*phox* proteins, that facilitate assembly of the active

NADPH oxidase complex. Unfortunately, because of the lack of specific antibody, it was not possible to confirm the identity of the 47 kD and 67 kD to be p47-*phox* and p67-*phox* proteins.

It is of interest to note that, although GM-CSF and TNF-α prime PMN for enhanced oxy radical production by fMLP, the preincubation time required for their optimal effect is much longer than that required by SP Furthermore, neither of these cytokines can prime neutrophils for the direct protein kinase C activator PMA, or increase $[Ca^{++}]_i$ in these cells [Weisbart et al, 1987; Naccache et al, 1988; Yuo et al, 1989]]. Therefore, it is anticipated that other mechanisms may regulate neutrophil priming. These may include an effect of SP on (i) the number of NADPH oxidase molecules that are being assembled and activated by a second stimulus [Hirai et al, 1992], (ii) the affinity of NADPH oxidase for its substrate, or (iii) substrate availability by as yet unknown mechanism. Further studies are required to determine the exact effect of SP on modulation of the intracellular pathways involved in oxy radical production in human neutrophils.

Exp. No:	**increase in ^{3}H-PDBu binding** (%)	**increase in O_2^- production** (%)	(nmol/10^6 cells)
1	20.5	91.4	12.8
2	20.0	205.0	15.6
3	11.5	25.0	4.0
4	17.7	110.0	6.0
5	14.7	160.0	21.4
6	29.9	191.0	9.0
7	0.0	23.0	6.7
8	0.0	67.0	12.0
9	-6.0	336.0	9.4

Table 5.1 The effect of SP on fMLP-stimulated ^{3}H-BDBu binding and O_2^- production in human neutrophils (see text for details). Values are from nine separate experiments performed in triplicates.

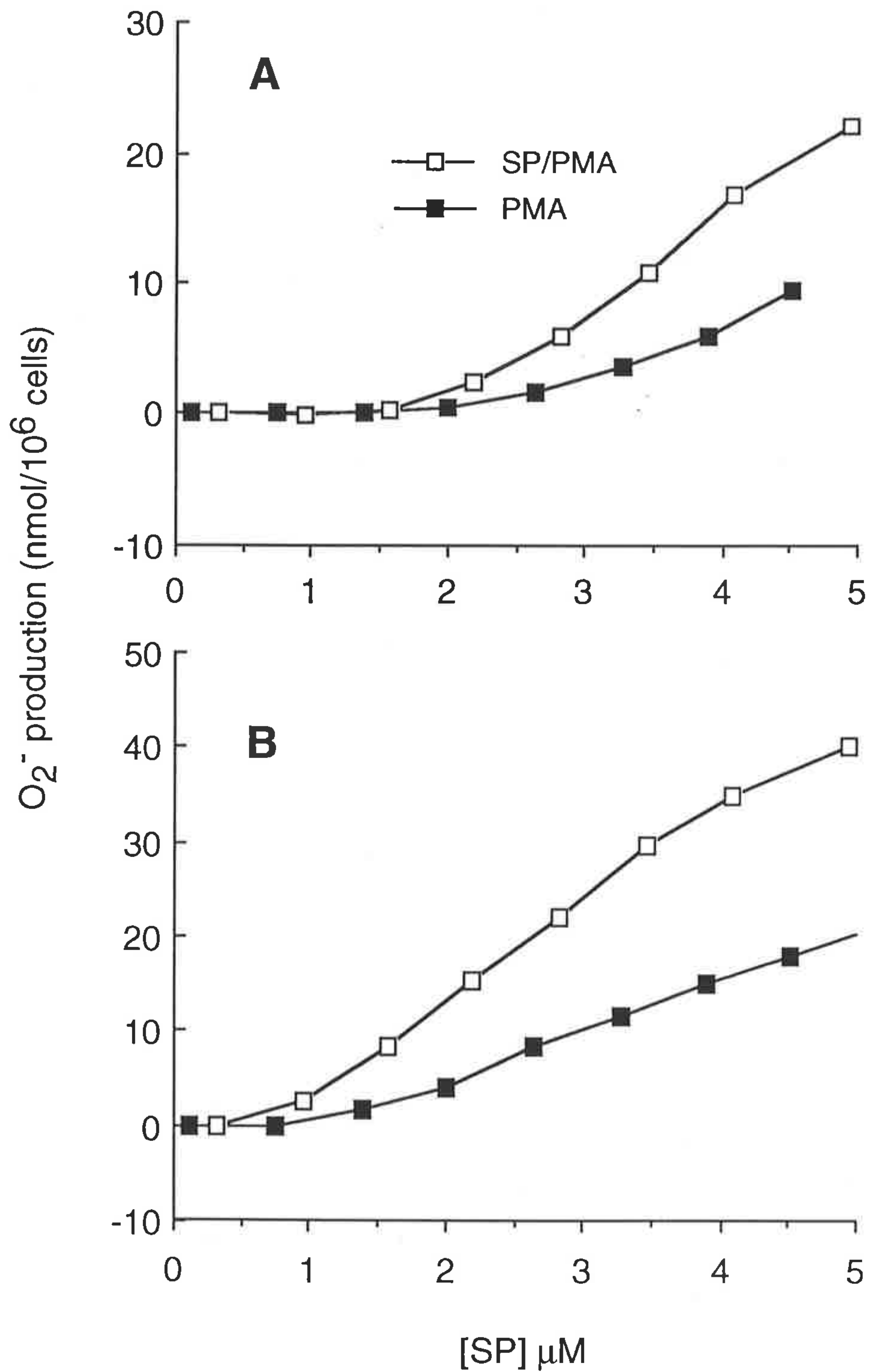


Figure 5.1 Effect of SP on PMA-stimulated O_2^- production. Neutrophils (5×10^6) were preincubated with medium or 50 μM SP for 15 min at 37°C and then 10^6 cells were transfered to prewarmed cuvettes containing (**A**) 10 ng/ml PMA or (**B**) 50 ng/ml PMA in which the O_2^- production was measured continuously. The results are from one experiment representative of three.

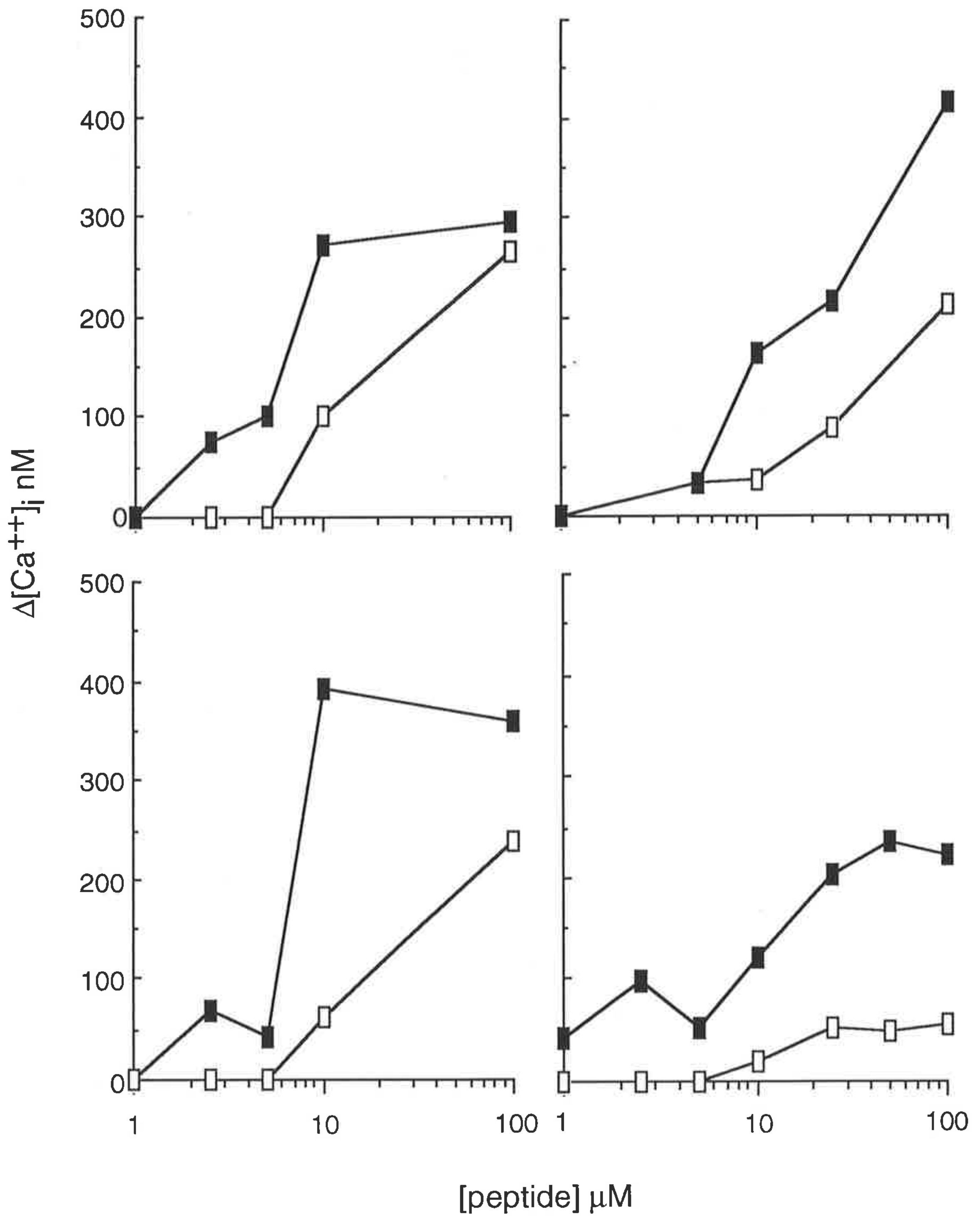


Figure 5.2 Effect of SP and SP_{7-11} on $\Delta[Ca^{++}]_i$. For the study of changes in $\Delta[Ca^{++}]_i$, FURA-2 loaded neutrophils were stimulated with various concentrations of SP (—□—) or SP_{7-11} (—■—). The results are from four experiments representative of six.

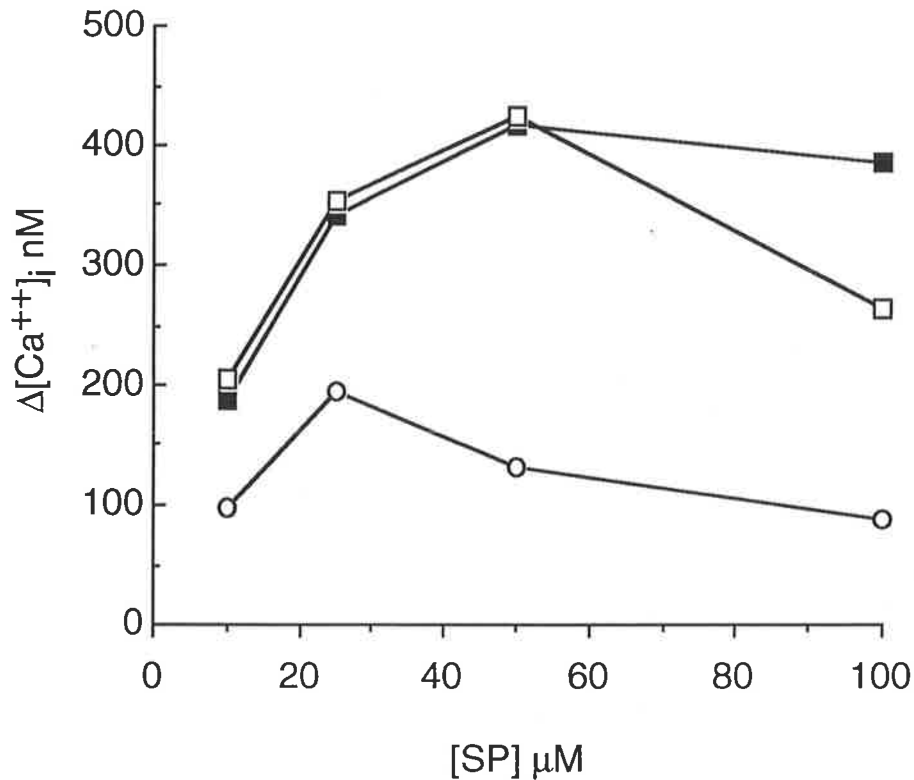


Figure 5.3 Effect of extracellular calcium on SP-stimulated $\Delta[Ca^{++}]_i$. Neutrophils prepared as outlined in "Materials and Methods" were loaded with FURA-2 in complete Hanks buffer and divided into two preparations. Each was then washed and resuspended in either Hanks buffer (—■—) or calcium-free Hanks buffer (—o—). 10^6 cells from each preparation were then stimulated with the stated concentrations of SP. In addition, neutrophils that were resuspended in calcium-free Hanks were repleted with 1.25 mM $CaCl_2$ (—□—) Values represent means of three experiments.

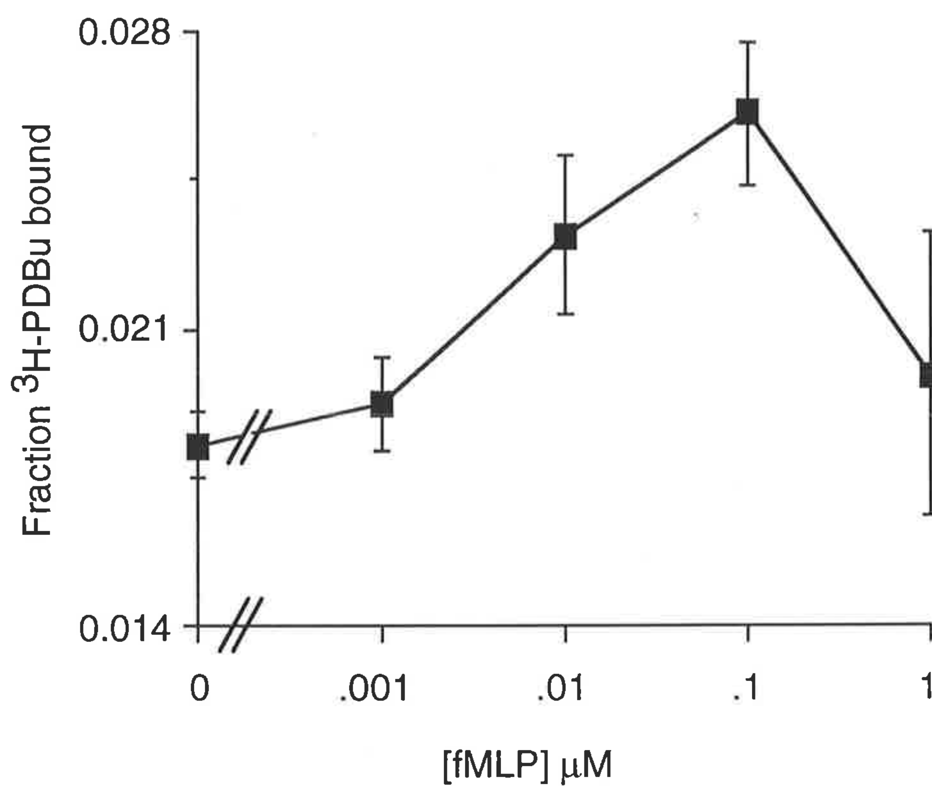


Figure 5.4 Effect of fMLP on ^{3}H-BDBu binding to neutrophils. Neutrophils were incubated with ^{3}H-PDBu for 30 min at 37°C and were then stimulated with stated concentrations of fMLP for an additional 6 min. Specific binding was calculated as explained in "Matherials and Methods" (Chapter 2.2.3.2).

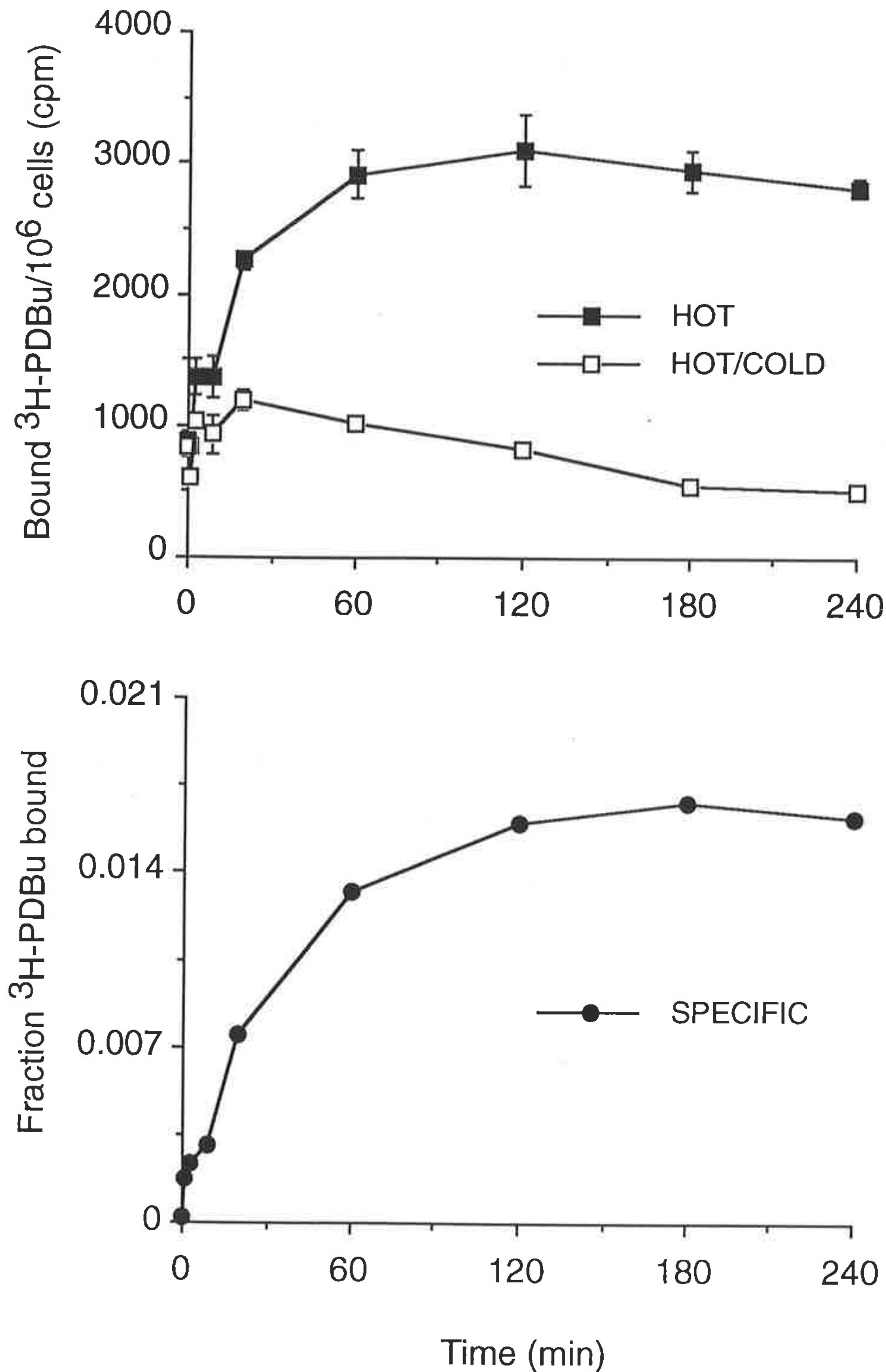


Figure 5.5a Time course of ^{3}H-PDBu binding to neutrophils at 4°C. Neutrophils (10^7) were preincubated for 15 min at 4°C and 10 nM ^{3}H-PDBu was added. At indicated times, 10^6 cells were transfered to 96 well plate and harvested onto special filters using cell harvester. The samples were processed, counted and the specific binding calculated as explained in "Materials and Methods" (Chapter 2.2.3.2). The results are from one experiment representative of four.

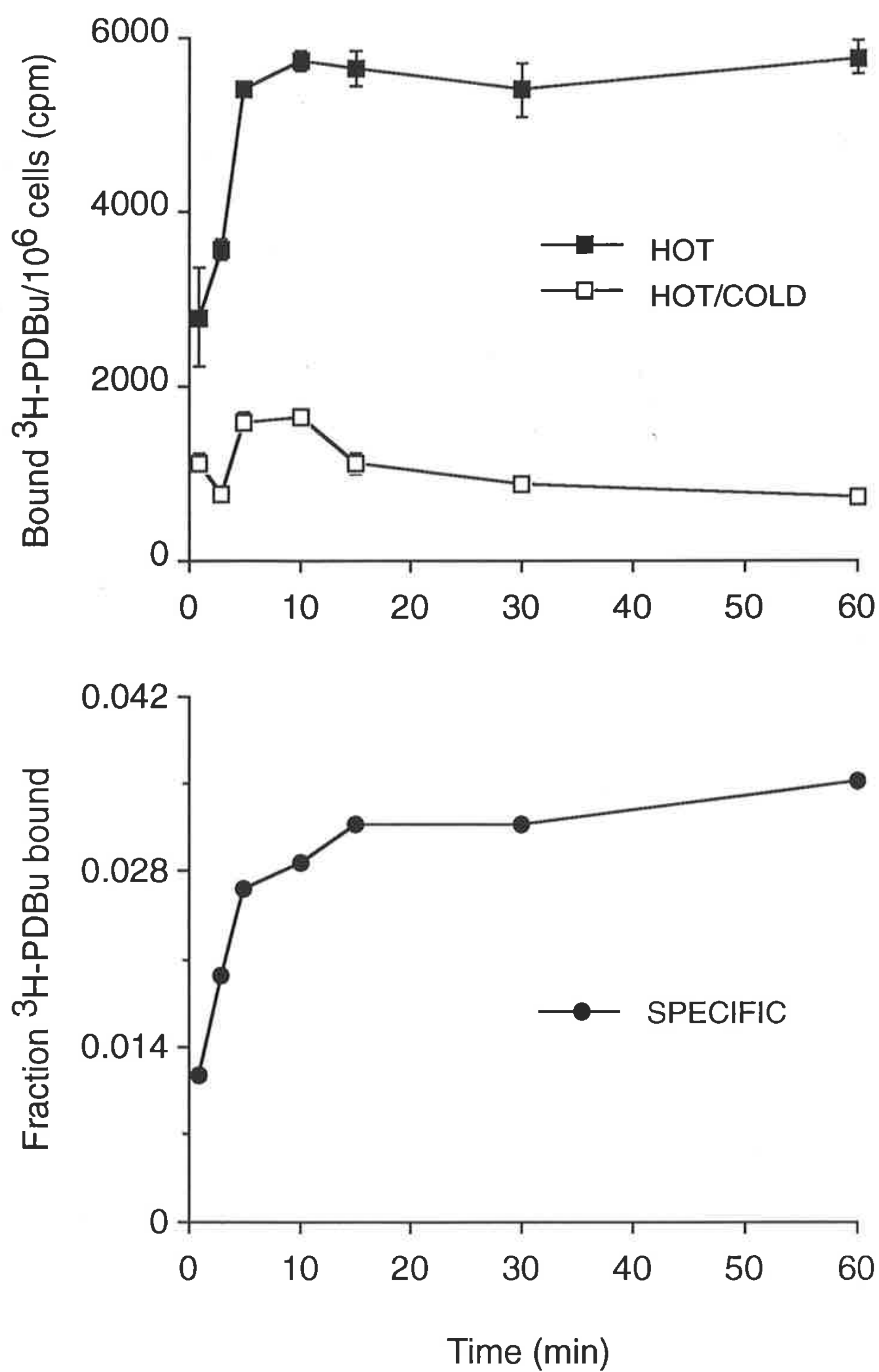


Figure 5.5b Time course of ^{3}H-PDBu binding to neutrophils at 22°C. The results are from one experiment representative of two.

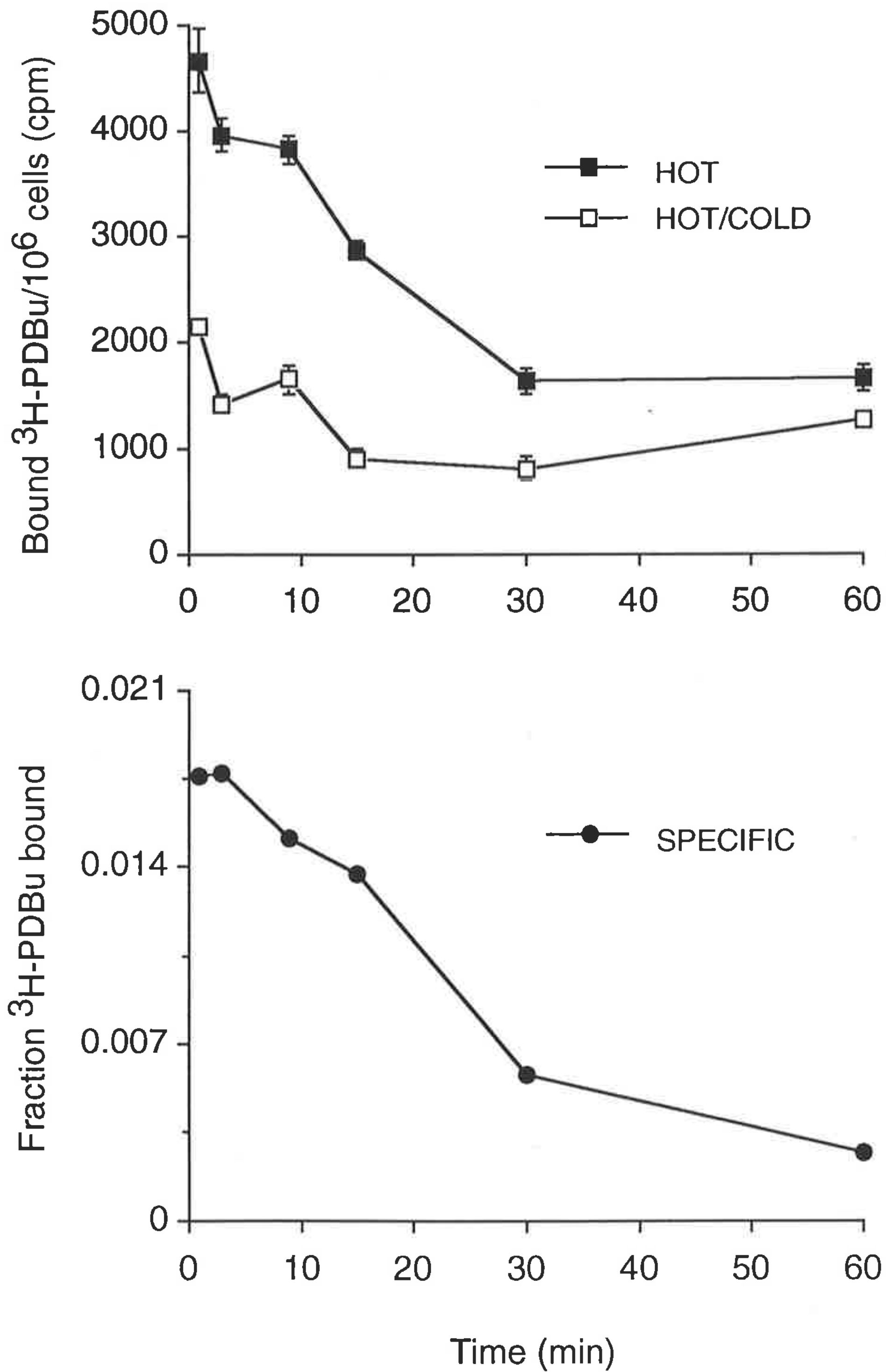


Figure 5.5c Time course of ^{3}H-PDBu binding to neutrophils at 37^oC. The results are from one experiment representative of six.

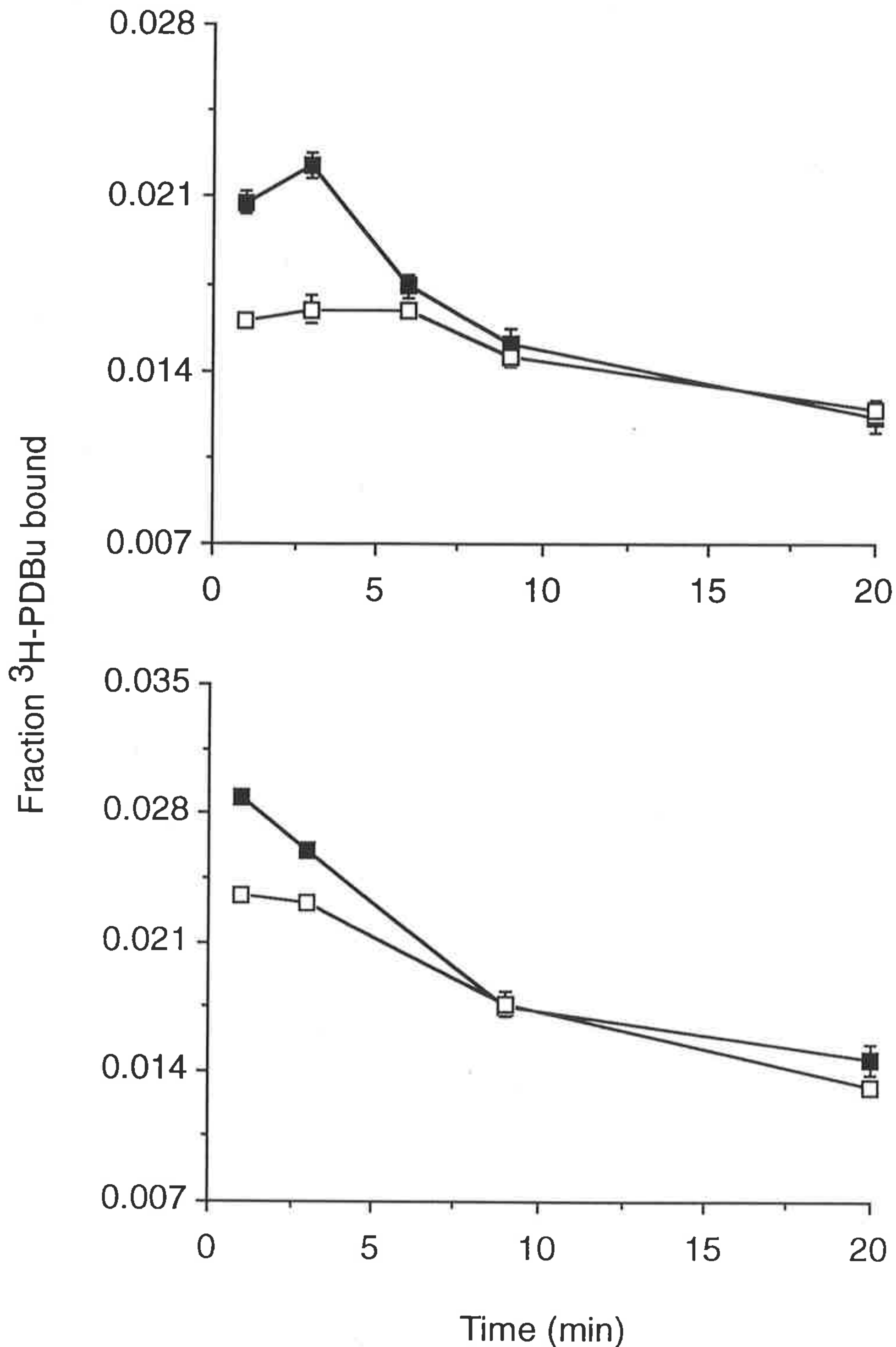


Figure 5.6 Effect of SP on ^{3}H-PDBu binding to neutrophils at 37°C. Neutrophils were preincubated with 10 nM ^{3}H-PDBu for 30 min at 37°C and were then stimulated with 75 μM SP (—■—) or medium control (—□—) at 37°C for indicated periods of time. At each time pont 10^6 cells were transferred to the 96 well plate and processed as explained before. The results are from two experiments representative of eight.

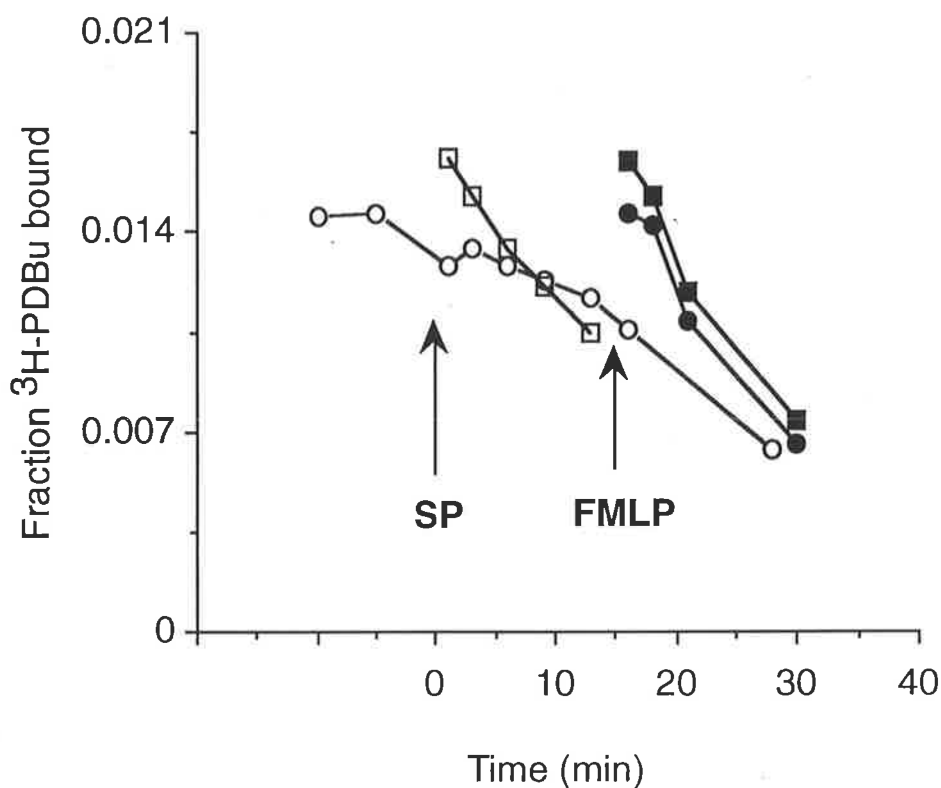


Figure 5.7a Effect of SP on fMLP-stimulated ^{3}H-BDBu binding to neutrophils: an illustration of experimental procedure. Neutrophils were equilibrated with 10 nM ^{3}H-PDBu for 30 min at 37°C and were then preincubated with 75 μM SP (—□—) or medium control (—○—) for an additional 15 min. The SP preincubated cells were then stimulated with 0.1 μM fMLP (—■—). The medium preincubated cells were stimulated with 0.1 μM fMLP (—●—) or its diluent control (—○—). Non-specific binding was assayed in the presence of 1 μM PMA. Specific binding was calculated as explained in "Materials and Methods" (Chapter 2.2.3.2).

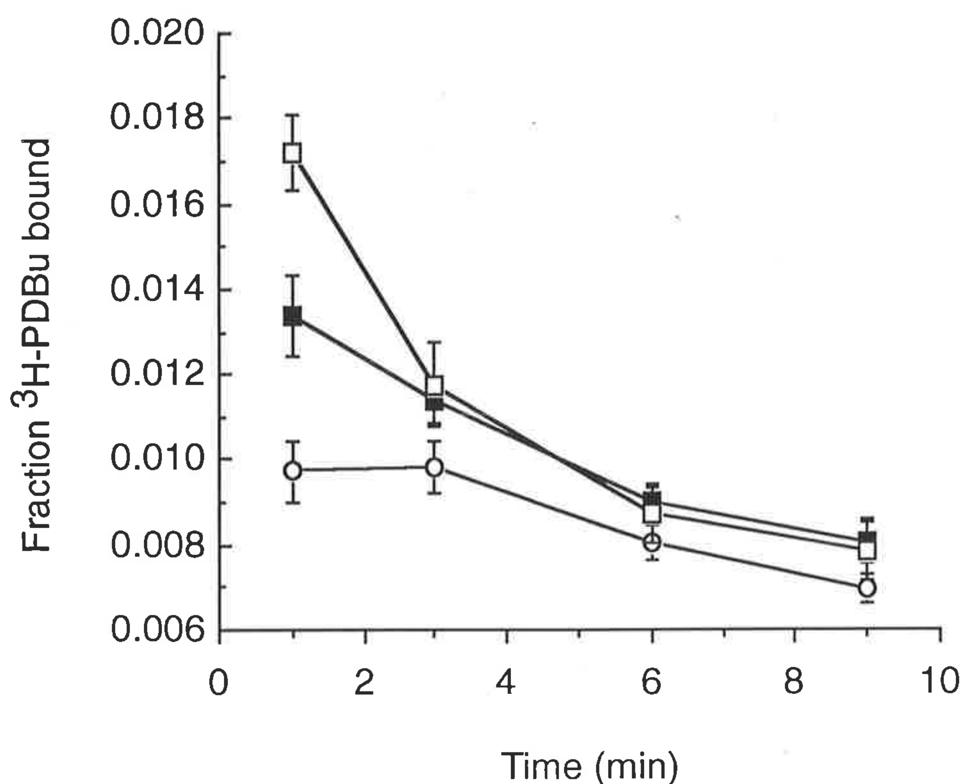


Figure 5.7b Effect of SP on fMLP-stimulated ^{3}H-PDBu binding to neutrophils. Neutrophils were equilibrated with 10 nM ^{3}H-PDBu for 30 min at 37°C and were then preincubated with 75 μM SP or medium control for an additional 15 min. The medium preincubated cells were stimulated with 0.1 μM fMLP (—■—) and its diluent control (—o—). The SP preincubated cells were then stimulated with 0.1 μM fMLP (—□—). Non-specific binding was assayed in the presence of 1 μM PMA. Specific binding was calculated as explained in "Materials and Methods" (Chapter 2.2.3.2). Values represent means of seven experiments.

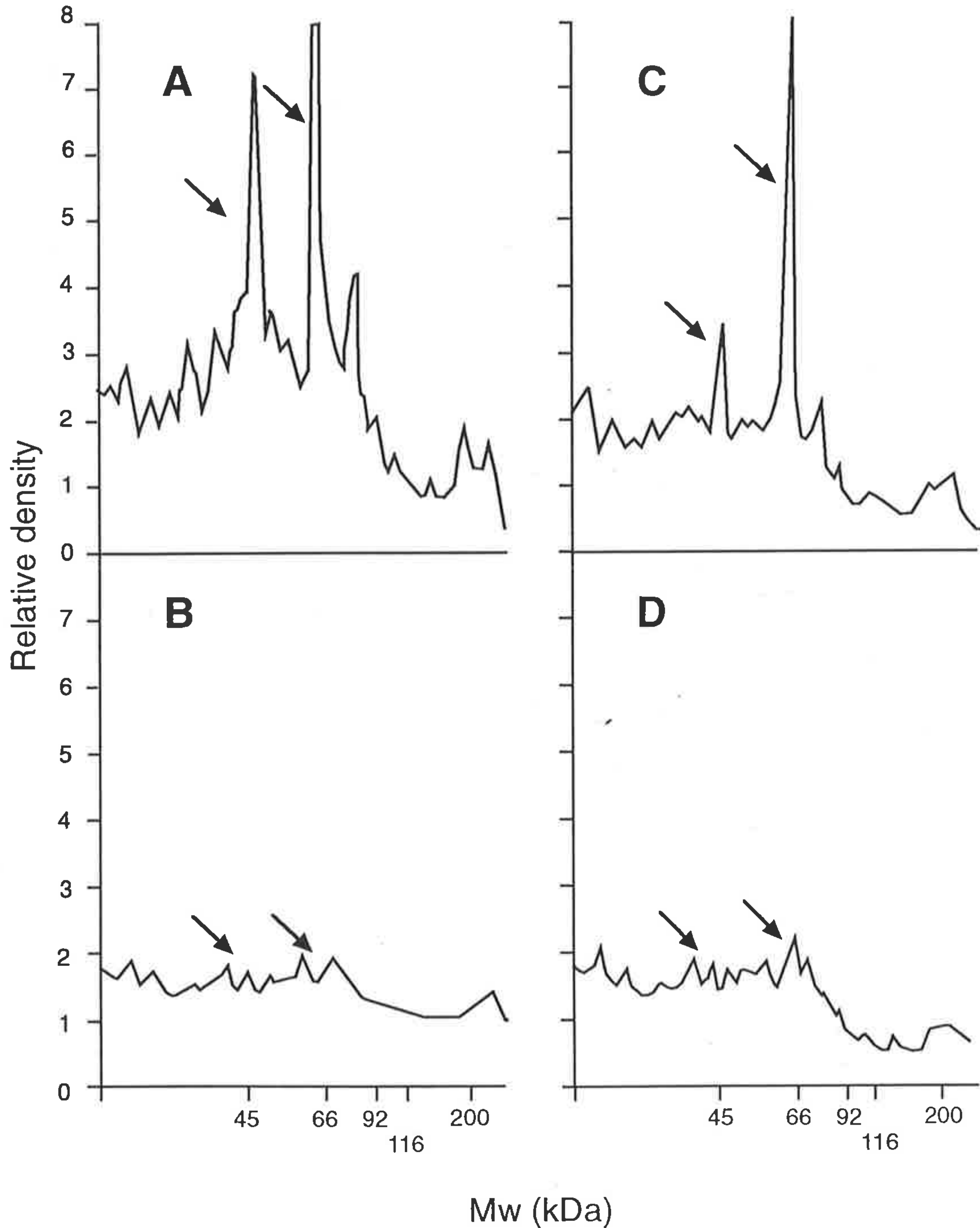


Figure 5.8 Effect of A23187 on PMA-stimulated protein phosphorylation. Neutrophils were stimulated with (**A**) medium and 0.1 µM PMA, (**B**) 0.1 µM A23187 and medium, (**C**) 0.1 µM A23187 and 3 nM PMA and (**D**) medium and 3 nM PMA. The reaction was stopped and the samples processed as explained in "Materials and Methods" (Chapter 2.2.3.3). The arrows point to the 47 kD and 67 kDa proteins.

Chapter 6

Substance P Receptor Study

6.1 Summary

Although SP has been shown to activate and modulate many functions of human neutrophil, it is not known if the effects are mediated through specific cell surface receptors. In this chapter I have investigated the binding of SP to neutrophils, and have shown that possible specific binding sites for SP exist on neutrophils. SP bound specifically to neutrophils at $4^{0}C$, although the concentration of unlabelled SP required to assay the non-specific binding was very high (50μM), and even then, the non-specific binding accounted for about 50% of the total binding. The maximal binding capacity of the Bolton-Hunter labelled ^{125}I-SP was determined to be 42%, and its binding was linear over a range of neutrophil concentrations (10^{7}-2.2×10^{7}). Finally, it was demonstrated that the specific binding was dose-dependent and saturable. Scatchard analysis of the data obtained from saturation isotherm indicated a single type of a binding site with an apparent Kd of 5.4×10^{-10} M. The binding capacity was calculated to be about 132 sites per cell. The validity of the assay is discussed.

6.2 Introduction

Substance P has been shown to activate and modulate many neutrophil effector functions such as chemotaxis, oxy radical production, degranulation aggregation and cytotoxicity (Chapter 1.9.2). Although SP has been shown to exert all these effects on human neutrophils, and specific receptors for SP have been identified on some inflammatory cells, (Chapter 1.8.2 and 1.9.1), up to now, no specific SP receptor has been demonstrated on neutrophils. It has been suggested, however, that these effects of SP are mediated by a specific receptor, possible the fMLP receptor [Marasco et al, 1981], as the carboxy terminal of SP is similar to the fMLP. More recent observations do not support this hypothesis, and argue for a more specific, although undefined, SP binding site, that is different to fMLP receptor. Serra et al [1988], demonstrated several differences between SP and fMLP stimulation of neutrophils. For example, SP activates pig neutrophils which do not possess receptors for fMLP, and in human neutrophils, SP-induced respiratory burst can be only partially inhibited by pertussis toxin, which completely inhibits fMLP-stimulated cells. In addition, I have demonstrated that SP facilitates O_2^- in neutrophils stimulated by optimal concentrations of fMLP (Fig. 3.6), indicating that SP must exert its effect elsewhere in the stimulus-response pathway.

The major receptor for SP is the NK-1 receptor, however, provided that the concentration of SP is high enough, SP can also bind to NK-2 and NK-3 receptors, that are more specific for the other tachykinins, NKA and NKB respectively (Chapter 1.8.2). It is unclear, at this moment, what signal transduction mechanisms are triggered by the activated SP receptor. It has been shown, that activation of SP receptor may lead to G protein mediated PLC-catalysed phosphatidylinositol hydrolysis [Hunter et al, 1985; Catalan et al, 1988]. However, others demonstrated that SP activates adenylate cyclase and promotes cyclic AMP formation [Tachado et al, 1991]. It is anticipated,

that the signal transduction induced by SP receptor may be not the same in different cells, as G proteins with different specificity may be involved in the receptor coupling.

Although specific binding sites for SP have been demonstrated on other phagocytic cells (e.g. macrophages) [Hartung et al, 1986], the existence of specific SP receptors on neutrophils has been questioned because of the high concentration of SP required to activate or modulate neutrophil functions. Instead, a model system was proposed whereby SP, by spanning cell membrane, can directly interact with G proteins and activate intracellular pathways (Chapter 1.8.3). This receptor independent model is based on the observations that SP can insert itself into the core of artificial membranes, and is further supported by the demonstration that it directly interacts and activates G proteins [Mousli et al, 1990].

The objective of the experiments described in this chapter was to investigate the binding of ^{125}I-SP to human neutrophils. The binding assays described aimed to (i) determine the saturability and affinity of ^{125}I-SP binding to human neutrophils and (ii) show that the binding is specific.

6.3 Results

6.3.1 *The kinetics of ^{125}I-SP binding*

The specific binding of ^{125}I-SP to neutrophils was dependent on the temperature and incubation time. In the initial experiments neutrophils (5 x 10^6 - 2 x 10^7) were washed in the binding buffer and incubated with 200 pM ^{125}I-SP from 30 to 360 min at 22°C or 4 ^{o}C. The non-specific binding was assayed in the presence of 50 μM SP, and was subtracted from the total binding to give the specific binding. At 22°C no specific binding of ^{125}I-SP to neutrophils was detected. However, when the cells were incubated with ^{125}I-

SP at 4^0C, specific binding was observed (Table 6.1). Under these conditions, in three experiments performed, the maximum binding occurred after 60 to 90 min incubation of neutrophils with ^{125}I-SP, and then declined slowly but it was still significant at 180 min. Results from one of the experiments are shown in Fig. 6.1. In this particular experiment, the maximal binding occurred at 90 min.

6.3.2 *Titration of ^{125}I-SP versus unlabelled SP*

To determine the concentration of cold SP required to assess the non-specific binding, neutrophils (2 x 10^7) were incubated with 200 pM ^{125}I-SP and increasing concentrations of cold SP for 75 min at 4^0C. The data from this binding experiment were plotted as a percentage of bound ^{125}I-SP at each concentration of cold SP, and are illustrated in Fig. 6.2. The total binding declined rapidly at first, between 1 and 25 μM of cold SP, and then doubling the concentration of cold SP to 50 μM, did not have much effect. Even at this highest concentration of cold SP used (50 μM) the non-specific binding accounted for about 50% of the total ^{125}I-SP bound. The concentration of 50 μM of cold SP was decided to be used for all subsequent experiments.

6.3.3 *Determination of maximal binding capacity (bindability)*●

Several alterations induced in the radioactive ligand molecule by, for example, radiolabelling, improper storage or bacterial degradation, may prevent the ligand from binding to its receptor completely, or substantially reduce the ligand-receptor interaction. Since ligand molecules that are unable to bind specifically to the receptor do not participate in ligand-receptor interactions, they should be excluded when calculating the total pool of ligand

● [Cuatrecasas and Hollenberg 1975]

provided for binding. Therefore, when performing the binding assay, it was very important to establish what proportion of the radioligand used was not capable of binding specifically to its receptor, and the total free ligand concentration should be accordingly corrected for the maximal binding capacity, or bindability, prior to data analysis.

To determine the maximal binding capacity of ^{125}I-SP, neutrophils (1.5 x 10^7) were incubated with 10 nM of ^{125}I-SP for 75 min at 4^oC. Non-specific binding was determined in the presence 50 μM SP. After this incubation, the cells were centrifuged and the specific binding determined. The supernatant from cells incubated with ^{125}I-SP alone (no unlabelled SP added) was transferred to tubes containing fresh cells (a sample of this supernatant was taken to determine what portion of initial counts had been transferred and added to the cells). The amount of supernatant transferred to each subsequent incubation was proportional to the cell number to ensure that the cell concentration remained constant for each incubation. The cells were incubated under the same conditions as described for the first incubation, and the whole procedure repeated again. The data was normalised to account for the cell number in each incubation. The specific binding for each incubation was calculated and plotted against the amount of ligand that was transferred from the previous incubation. When 100% of a ligand is capable of binding to its receptor, data plotted in this manner should result in a straight line with a slope of 1 and intercepts of 0,0 and 100,100.

When neutrophils were incubated with ^{125}I-SP, and the cells and supernatants processed as explained above, in two experiments performed the X intercept (extrapolated) was found to be exactly 58% (Fig. 6.3). Therefore, 58% of ^{125}I-SP preparation, for unknown reasons, was incapable of binding specifically to whatever constitutes the binding sites on neutrophils, or in other words, the bindability of ^{125}I-SP was 42%.

6.3.4 *Titration of ^{125}I-SP binding versus cell number*

In the initial experiments, various concentrations of neutrophils were used to establish the binding assay conditions as it was very important to determine the range of cell concentrations that were suitable for use in a binding assay. This was done by titrating radioligand versus increasing cell numbers. By plotting cell numbers against the specific binding of the ligand, a range of cells concentrations can be determined for which the binding forms a linear part of the plot, the use of cell concentrations beyond the linear portion of the graph, may result in abnormalities, such as unusually high non-specific binding or anomalous results in saturation equilibrium biding assays. In the following experiments, increasing neutrophil concentrations were incubated with 200 pM ^{125}I-SP for 75 min at 4°C under equilibrium binding conditions. The non-specific binding at each cell concentration was determined by incubating the cells in the presence of 50 μM SP. When cell concentrations were plotted versus specific binding at each concentration, the results yielded a curve with a linear portion between 10^7 and 2.2×10^7 cell concentration (Fig. 6.4). Cell concentrations within this range were determined to be suitable for ^{125}I-SP binding, and were used in subsequent binding assays.

6.3.5 *Equilibrium binding studies: saturation isotherm and Scatchard analysis*

In order to examine the binding capacity, fresh neutrophils (1.2×10^7) were incubated with increasing concentrations of ^{125}I-SP (from 10 pM to 300 pM, corrected for bindability) for 75 min at 4°C. The non-specific binding was determined in the presence of 5×10^5 fold excess of SP. The results of a representative experiment are shown in Fig. 6.5. The binding of ^{125}I-SP was specific, dose dependent and saturable. The Scatchard analysis of the results,

a plot of [bound ligand]/[free ligand] versus [bound ligand], yielded points that could be fitted by a straight line (regression line: $y = -.1843x + 3.25729$, confidence intervals 95 and 90%), indicating that a single type of binding site is involved. The apparent dissociation constant (Kd) and the number of binding sites per neutrophil were derived from this plot. The slope of the line is -1/Kd. The Kd for the ^{125}I-SP binding site was 5.4×10^{-10} M. The intercept with the X axis gave a B_{max} value of 17.5 pM that was used to calculate the number of binding sites per single neutrophil. The calculation was as follows:

$$\text{molecules bound/cell} = B_{max} \times 6.023 \times 10^{23} \times \text{volume (l)/cell number}$$

Assuming 1:1 ligand:receptor stoichiometry,

$$\text{binding sites/neutrophil} = 17.5 \times 10^{-12} \times 6.023 \times 10^{23} \times 1.5 \times 10^{-4}/1.2 \times 10^{7}$$

$$= 132$$

6.4 Discussion

Substance P exerts many of its actions via specific receptors. Three receptors for SP and other tachykinins, NK-1, NK-2 and NK-3, have been found on non-inflammatory type cells, and have been cloned and characterised functionally (Chapter 1.8.2). Specific SP receptors have also been demonstrated on inflammatory cell, including lymphocytes [Payan et al, 1984] and macrophages [Hartung et al, 1986], but they have not been cloned and it is not clear whether they are the same as the receptors found on non-immune cells. Although many research groups have reported SP effects on human neutrophils, a receptor or a binding site for SP has not been demonstrated on these cell, and the way in which SP interacts with neutrophils remains speculative.

High concentrations of SP are required to demonstrate an effect on neutrophil function [Serra et al, 1988; Wozniak et al, 1989]. This has prompted many to believe that SP interactions with neutrophils are not receptor mediated, but rather the peptide interacts directly, in a non-specific manner, with the cell membrane. Moreover, it was demonstrated that SP and other amphiphilic peptides can insert themselves into the hydrophobic core of artificially-made membranes in a non-specific manner (Chapter 1.8.3). To explain how SP (and other amphiphilic peptides) may activate cells by the peptide-cell membrane interaction, a hypothetical model was proposed that involves membrane spanning by the peptide and direct interaction and activation of G proteins that are located at the inner surface of the cell membrane [Mousli et al, 1990]. The model is further supported by the finding that SP and other amphiphilic peptides directly activate G proteins *in vitro*. Although very attractive, this model does not fully explain some effects of SP. For example, why high and not low peptide concentrations are required to span the membrane and activate G proteins? If, as suggested by Mousli et al [1990], this is the positively charged amino terminal of SP that binds to and activates negatively charged G protein subunits in mast cells, how could the carboxy terminal alone cause activation of several neutrophil functions? I have shown clearly in chapter 4, that the enhancement of O_2^- production by neutrophils is mediated by the carboxy terminal SP_{7-11}, although the amino terminal SP_{1-4} also had a modulatory effect (SP but not K increased O_2^- production). There are additional questions that would have to be answered before accepting the model for neutrophil activation. Is the carboxy terminal, SP_{7-11}, long enough to span the cell membrane and bind to G proteins? Why does SP affect some of neutrophil functions but not others? Are there SP specific G proteins in neutrophils that are not coupled to any receptors and what is their role?

On the other hand, some of the experiments described in the chapter 3, would suggest that SP induced neutrophil activation is receptor-mediated.

For example, it was shown that SP facilitation of O_2^- production is dose- and time-dependent, temperature-dependent and could not be reversed by washing (Figs. 3.2, 3.5 and 3.7). The most commonly used assay to study membrane receptors is the saturation equilibrium binding assay. The limited amount of information obtained from this assay can be used to calculate the number of binding sites and their dissociation constant, which is a measure of the receptor affinity (Scatchard analysis, [Scatchard 1949]). However, at least two assumptions about the radiolabelled ligand are being made when analysing experimental data. First, that the radioligand has an equal biological activity, and upon binding to the receptor, can trigger the same response as the unlabelled ligand, and second, that the affinities of both the labelled and unlabelled ligands for the receptor are the same. These assumptions presuppose that the radioligand is interchangeable at the receptor level with the unlabelled ligand.

Experimentally, identical cell numbers are incubated with increasing concentrations of radioligand, and non-specific binding is determined at each concentration in the presence of an excess of unlabelled ligand. A plot of specifically bound ligand versus concentration of ligand reach a plateau when the receptors become saturated. Experimental data, even from very low concentrations of radioligand, can be extrapolated mathematically after linear transformation, to obtain the estimates of the apparent number of binding sites and their affinity.

Investigations, using ^{125}I-SP, suggest that SP binding sites may exist on human neutrophils. The binding of ^{125}I-SP at 4^{0}C to neutrophils was shown to be specific, dose- dependent and saturable. Scatchard analysis of the data derived from saturation binding experiment, imply a single binding site (132 sites/cell) with a Kd = 0.44 nM. However, the concentration of unlabelled SP that displaced 50% of bound ^{125}I-SP was shown to be very high (50 μM), and therefore great caution should be employed in the interpretation of the above

findings. The exact reason as to why 100 or 1000 fold excess of unlabelled SP is not sufficient to compete for binding with ^{125}I-SP, is not known. Possible explanations may include (i) the presence of altered (increased) binding affinity of ^{125}I-SP, (ii) an intramembrane receptor with a two step binding process, the first being a non-specific interaction with the cell membrane followed by specific interaction with the receptor binding site, and (iii) non-specific but saturable binding sites.

The data presented in this chapter suggest, that a possible receptor for SP may exist on human neutrophils, but it does not exclude the possibility of non-specific interactions of SP with the cell membrane. In fact, non-specific interactions of SP with the cell membrane may be a requirement for the specific interaction with its receptor, which may be located in the hydrophobic core of the cell membrane. Therefore, further studies are required to prove the existence of SP receptor on neutrophils.

Time (min)	No. of cells	^{125}I-SP (cpm) (SD)	^{125}I-SP+cold SP (cpm) (SD)	specific (cpm)
Temperature:				**4°C**
60	5 x 10^6	563 (32)	403 (9)	**160**
	10^7	823 (69)	550 (43)	**273**
120	5 x 10^6	695 (51)	308 (43)	**387**
	10^7	1310 (91)	653 (28)	**657**
180	5 x 10^6	612 (32)	423 (30)	**189**
	10^7	1175 (61)	759 (17)	**416**
360	5 x 10^6	586 (58)	345 (18)	**360**
	10^7	1083 (40)	664 (22)	**419**
O/N	5 x 10^6	400 (72)	372 (30)	**28**
	10^7	775 (231)	618 (8)	**157**
Temperature:				**22°C**
60	5 x 10^6	398 (14)	406 (4)	—
	10^7	670 (37)	723 (101)	—
120	5 x 10^6	411 (16)	365 (27)	**46**
	10^7	641 (40)	582 (64)	**59**
180	5 x 10^6	382 (23)	379 (21)	**3**
	10^7	756 (27)	693 (36)	**63**
360	5 x 10^6	370 (3)	412 (31)	—
	10^7	666 (68)	599 (105)	**67**

Table 6.1 ^{125}I-SP binding to human neutrophils: the effect of temperature on the binding kinetics and specific binding (see text for details). Values are from one experiment performed in quadruplicate.

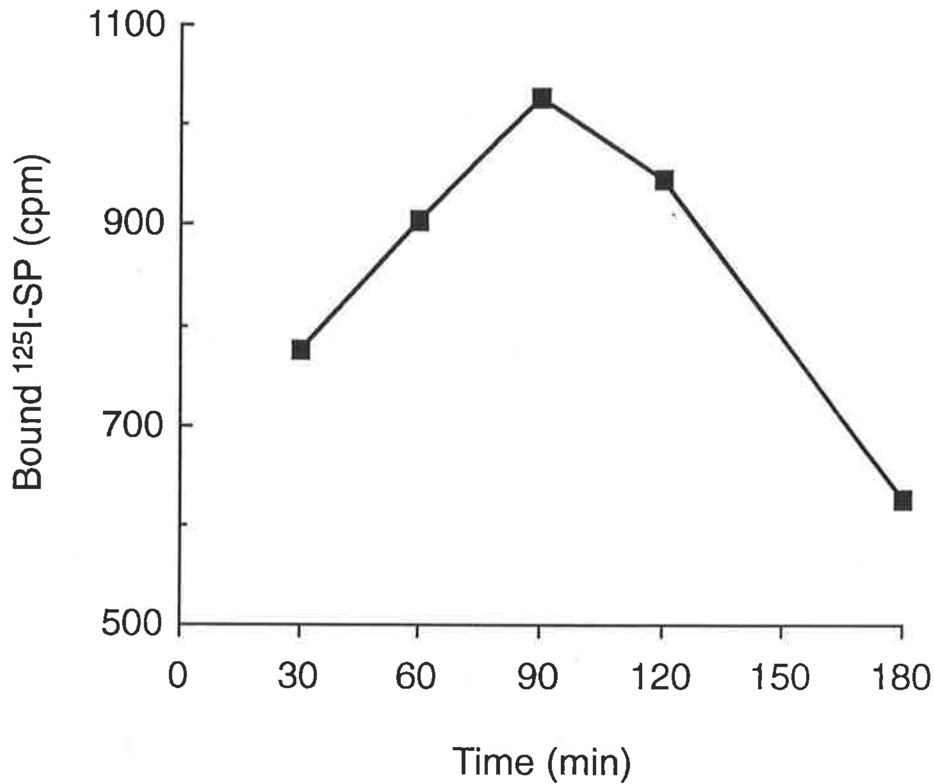


Figure 6.1 Effect of time on ^{125}I-SP binding to human neutrophils. Neutrophils (2 x 10^7) were incubated with 200 pM ^{125}I-SP ± 50 μM SP for indicated times at 4°C. At each time point the cells were removed and separated from the unbound ^{125}I-SP by centrifugation over FCS at 13,000 rpm 30 sec. The cells were processed and specific binding calculated as explained in "Materials and Methods", Chapter 2.2.5. The results are from one experiment representative of three.

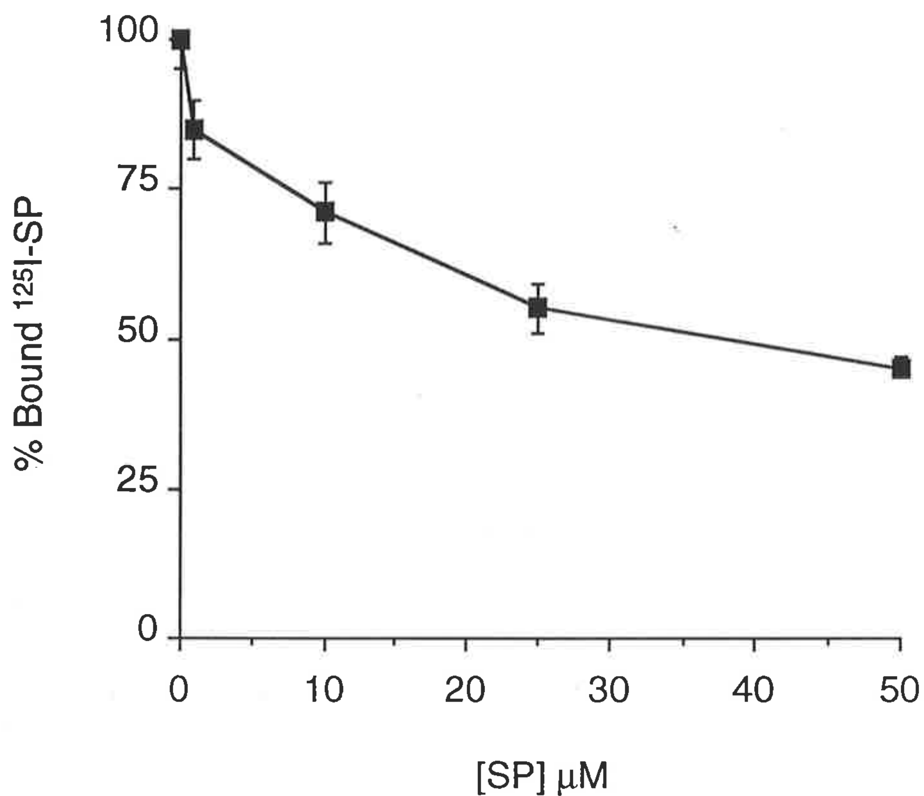


Figure 6.2 Titration of ^{125}I-SP binding versus unlabelled SP. Neutrophils (2 x 10^7) were incubated with 200 pM ^{125}I-SP and then indicated concentrations of SP were added. The incubation was stopped and the cells separated from unbound ^{125}I-SP by centrifugation over FCS. The cells were processed and specific binding calculated as explained in previous figure.

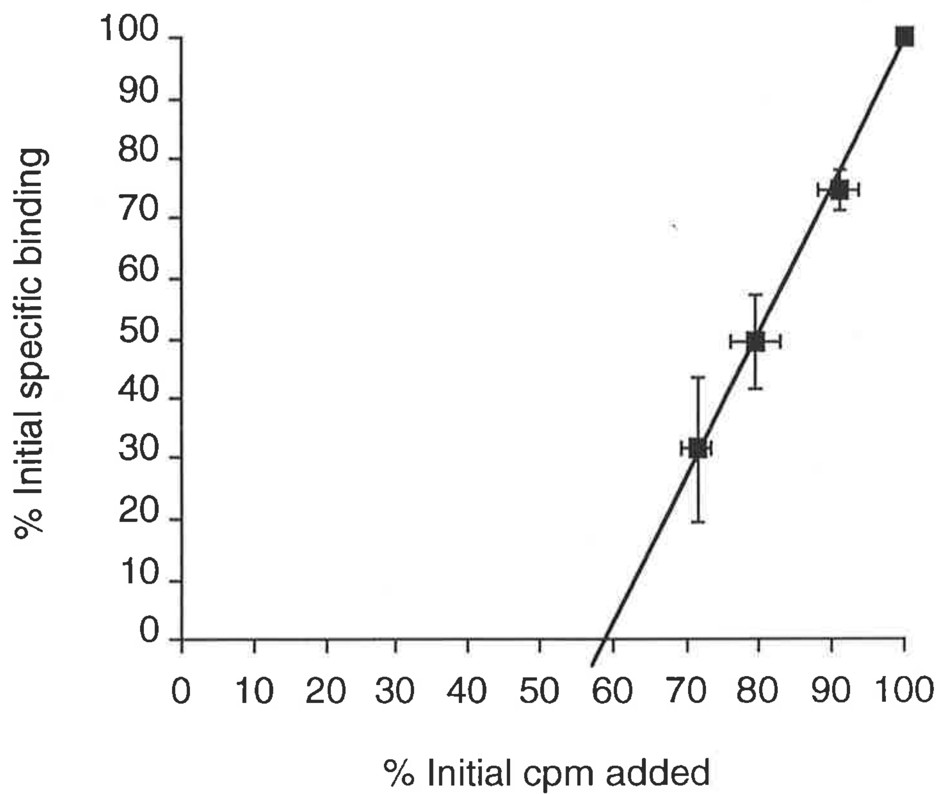


Figure 6.3 Determination of maximal binding capacity (bindability) of ^{125}I-SP to neutrophils. The binding conditions were as specified in previous experiments: neutrophils (1.5 x 10^7) were incubated with 200 pM ^{125}I-SP ± 50 μM SP for 75 min at 4°C. See text for the explanation of experimental procedure. Values represent mean of two experiments.

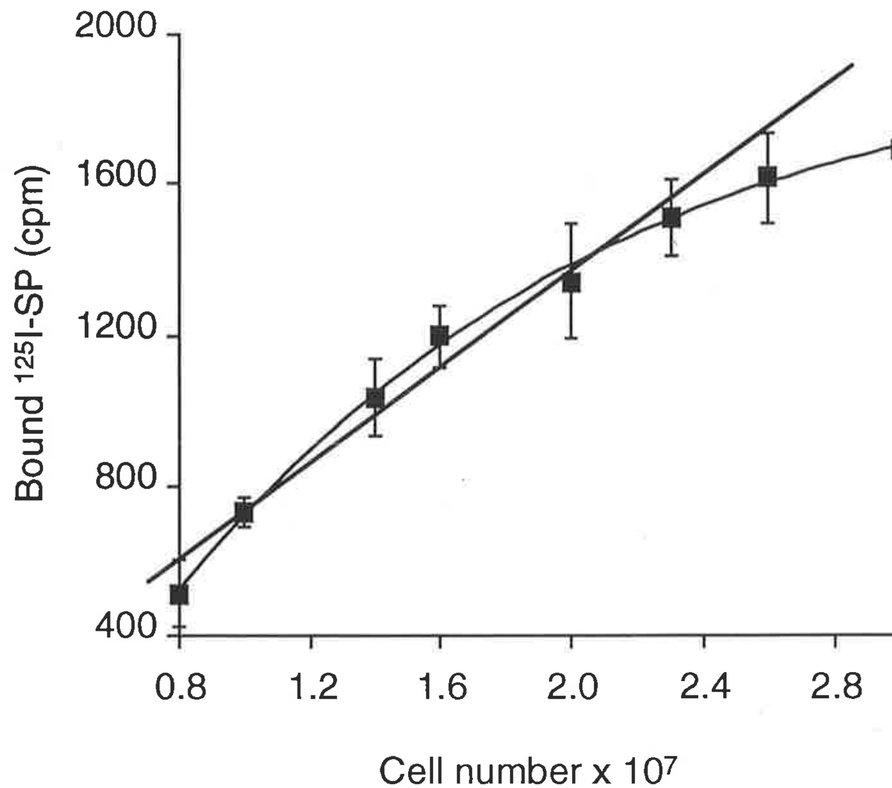


Figure 6.4 Titration of ^{125}I-SP binding versus cell number. Indicated numbers of neutrophils were incubated with 200 pM ^{125}I-SP ± 50 μM SP for 75 min at 4°C. The cells were separated from the unbound ^{125}I-SP and processed as explained before. The results are from one experiment representative of two.

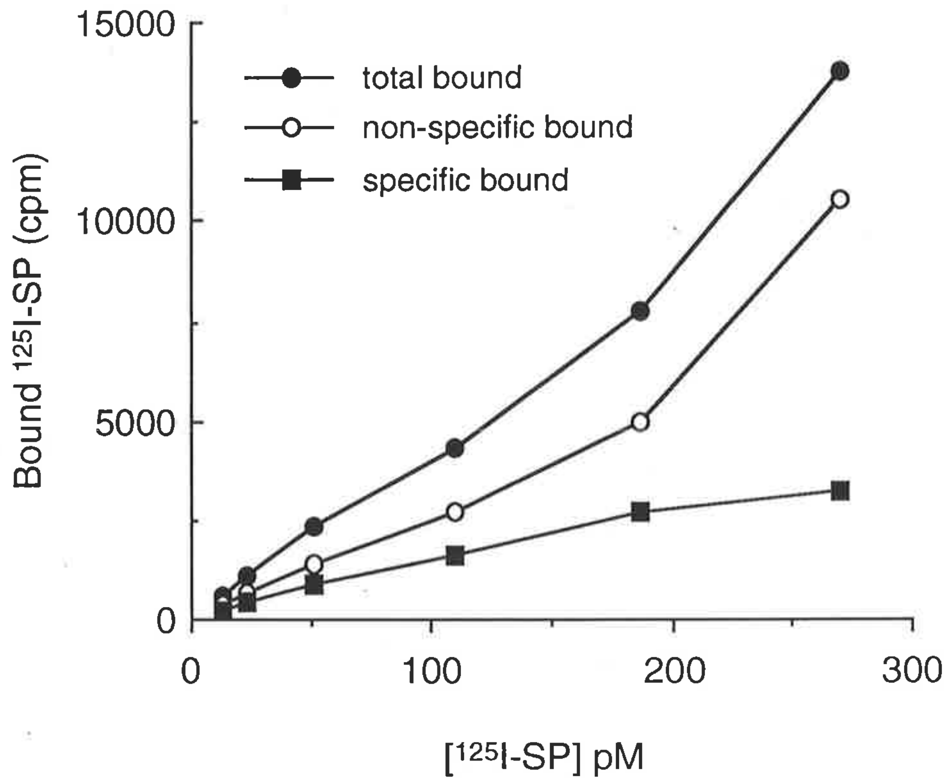


Figure 6.5 Saturation equilibrium binding of ^{125}I-SP to human neutrophils. Neutrophils (1.2 x 10^7) were incubated with indicated concentration of ^{125}I-SP in the presence or absence of 5 x 10^5 fold exess SP for 75 min at 4°C. At each time point the cells were removed and separated from the unbound ^{125}I-SP by centrifugation over FCS. The cells and supernatants were counted and the specific binding, calculated as explained previously.

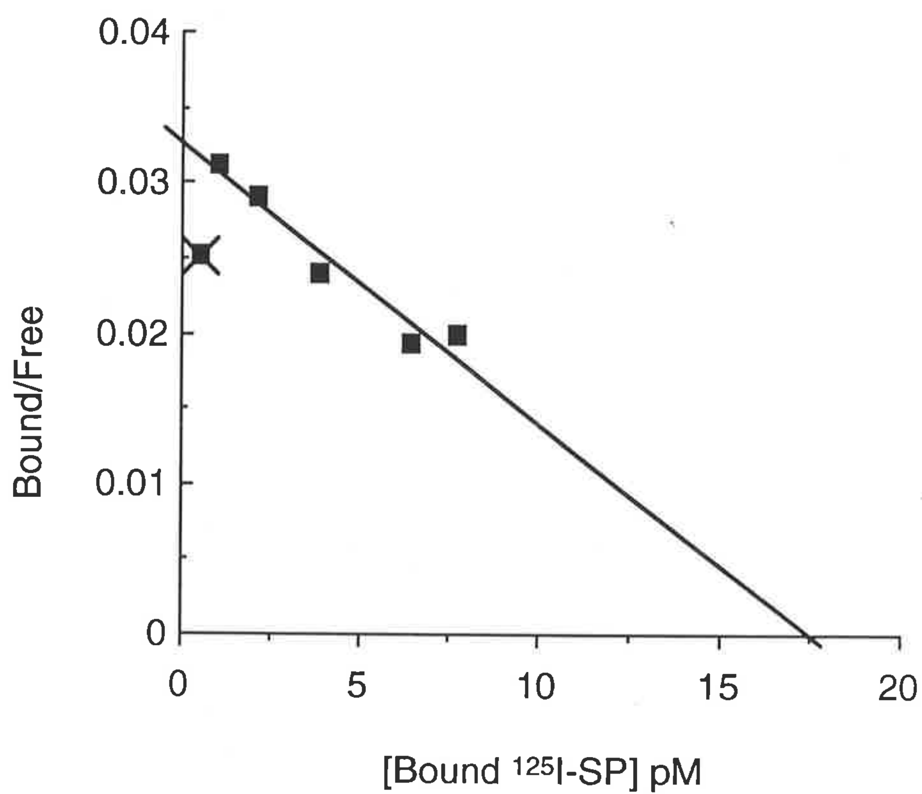


Figure 6.6 Scatchard plot of ^{125}I-SP saturation binding data. Equilibrium saturation binding data from Fig. 6.5 were used for Scatchard analysis. After exclusion one of the points a regression line was plotted. The single straight line indicate a probable single class of binding sites, with an apparent Kd=5.4 x 10^{-10} M and 132 binding sites per cell.

Chapter 7

The Role of Substance P in Inflammatory Disease: Interaction with GM-CSF and IL-8

7.1 Summary

This study examines the contribution of SP to the processes of inflammatory disease, and in particular, the role of SP in the pathogenesis of asthma. The effect of SP on antibody-dependent cell-mediated cytotoxicity (ADCC) by neutrophils and eosinophils isolated from peripheral blood of normal and asthmatic subjects was examined. The baseline (unstimulated) ADCC was significantly higher in asthmatics (18.7 ± 2.7%) than normals (8.2 ± 1.4%, $p < 0.01$). SP stimulated neutrophil ADCC in a dose-dependent manner with a maximal response at an SP concentration of 100 μM. SP-stimulated ADCC was greater in asthmatic subjects (39.4 ± 3.4%) than normals (21.0 ± 2.1%, $p < 0.0001$). The net increase in ADCC (*net*-ADCC, calculated as ADCC in presence of stimulus minus spontaneous ADCC in the presence of medium alone) was significantly greater in asthmatics (17.6 ± 1.9%) than normals (7.0 ± 0.8%, $p < 0.0001$) confirming that the higher

stimulated ADCC in asthmatics was not due solely to the higher baseline ADCC.

In support of the hypothesis, that enhanced ADCC response in asthmatic subjects was due to the modulation of neutrophil function by cytokines *in vivo*, synergism between SP and recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) was demonstrated *in vitro*. *Net*-ADCC was 44.0 ± 4.0% with 0.1 μM SP plus 10 ng/ml rhGM-CSF compared to 31.0 ± 5.2% with 10 ng/ml GM-CSF alone ($p < 0.05$). Moreover, a modest correlation ($r = 0.5$, $p < 0.025$, $n = 20$) was demonstrated between SP stimulated ADCC and bronchial hyperresponsiveness assessed as the PC_{20} (bronchial hyperresponsiveness to inhaled histamine), which is the *in vivo* measure of asthma severity. In contrast to its effects on neutrophils, SP failed to stimulate eosinophil ADCC although these cells were responsive to rhGM-CSF.

In addition, the effects of recombinant human interleukin-8 (rhIL-8), and the interactions between SP and rhIL-8, on neutrophil O_2^- production were investigated. rhIL-8 (1-10 ng/ml) did not stimulate neutrophil O_2^- production on its own, but like as SP, primed neutrophils for an enhanced response to other stimuli, such as fMLP, PMA and PAF. The priming effect of rhIL-8 was dose dependent, rapid and long lasting. rhIL-8 increased both the maximal rate and the total O_2^- production. Stimulation of neutrophils with rhIL-8 increased $[Ca^{++}]_i$ by mobilising calcium from internal stores and by increasing calcium influx. The increase in $[Ca^{++}]_i$ was dose dependent and occurred in the same range of rhIL-8 concentrations that primed neutrophils for O_2^- production. In addition, rhIL-8 enhanced the fMLP-stimulated increase in $[Ca^{++}]_i$. However, neither SP nor IL-8 enhanced each others priming effect when used together to prime the O_2^- response of fMLP-stimulated neutrophils, suggesting, that there is no interaction between these two mediators.

7.2 Introduction

Although the studies described in this chapter are mostly concerned with the inflammatory process in asthma, it is anticipated that the basic findings may also be important in other inflammatory diseases, in which it has been suggested that neutrophils and tachykinins have an important role (Chapters 1.7 and 1.9).

Airways inflammation is a prominent feature of asthma and is thought to be responsible for the airflow obstruction and bronchial hyperresponsiveness (BHR) which characterise this disease clinically [Barnes, 1989]. A number of studies have suggested that neutrophils and eosinophils are key effector cells in this inflammatory process, although multiple cell types and mediators are also likely to be involved (Chapter 1.7.1).

There is evidence to implicate the sensory nervous system in the pathogenesis of asthma (Chapter 1.9.1). In the respiratory tract, sensory nerves are located in the epithelium and smooth muscle, and around mucus glands and blood vessels [Lundberg et al, 1984b; Nohr and Weihe, 1991]. These nerves contain a number of neurotransmitters, including tachykinins, which colocalise to the same nerves and are released locally by antidromic stimulation [Casale, 1991]. In asthma, it has been postulated that SP and other tachykinins are released locally by axonal reflexes when sensory nerves are stimulated following epithelial damage [Barnes, 1986] and that they contribute to the pathophysiology of asthma by causing bronchoconstriction, mucus secretion and plasma extravasation [Lundberg et al, 1983a, b].

As discussed in Chapter 1.9.2, SP has a number of proinflammatory effects on effector functions of various immune cells and the production of cytokines and other mediators of inflammation. Most of the above effects of SP have been defined using cells isolated from normal individuals. However,

in asthma there is leucocyte activation *in vivo* [Melewicz et al, 1981; Meltzer et al, 1989; Fukuda et al, 1989; Radeau et al, 1990], probably as a result of the local production and release into the circulation of cytokines. These cytokines are capable of recruiting, activating and priming circulating cells for an enhanced response to a variety of stimuli [Howell et al, 1989; Wilkinson et al, 1989; Weisbart et al, 1987; Atkinson et al, 1988b; Daniels et al, 1992].

I hypothesised that peripheral blood neutrophils and eosinophils derived from asthmatics would show enhanced responses to SP which would correlate with asthma severity. To test this hypothesis, the effect of SP on neutrophils and eosinophils isolated from normal and asthmatic subjects was investigated and their *in vitro* function examined in an antibody-dependent cell-mediated cytotoxicity (ADCC) assay. To provide a clinical correlation, neutrophil responsiveness to SP was correlated with bronchial reactivity to inhaled histamine. Finally, it was hypothesised that the enhanced responsiveness of the cells isolated from asthmatic subjects is caused by their *in vivo* priming by various cytokines. Thus the role of cytokines in activating and priming neutrophils for enhanced responses to SP was investigated.

7.3 Results

7.3.1 *The effect of substance P on neutrophil antibody-dependent cell-mediated cytotoxicity in normal and asthmatic subjects*

ADCC was assayed in the presence of 0, 0.3 or 1 μg/ml antibody, and SP in the concentration range 1 nM - 100 μM or medium control. In normal subjects ($n = 20$), SP stimulated neutrophil ADCC in a dose-dependent manner (Fig. 7.1A). With 1 μg/ml antibody, 100 μM SP stimulated ADCC from 8.2 ± 1.4% to 21.0 ± 2.1% ($p < 0.0001$). SP did not stimulate cytotoxicity significantly in the absence of antibody (2.6 ± 0.6% versus 1.8 ± 0.4% for

100 μM SP and medium control respectively). An optimal dilution of 10% BCM, stimulated ADCC to 26.9 ± 3.6% which was not significantly different from 100 μM SP.

In asthmatic subjects (n = 34), 100 μM SP increased ADCC from 18.7 ± 2.7% to 39.4 ± 3.4 % (p = 0.0001; 1.0 μg/ml antibody). The response to BCM was 39.1 ± 2.7%, which was not significantly different from 100 μM SP (Fig. 7.1B). SP did not stimulate cytotoxicity in the absence of antibody (3.2 ± 0.6 versus 2.5 ± 0.3% for medium).

Spontaneous cytotoxicity in the absence of antibody and stimulus was 1.8 ± 0.4% for normals and 2.5 ± 0.3% for asthmatic subjects. At 1 μg/ml antibody, ADCC was significantly greater in asthmatics than normals for medium ($p < 0.01$), all concentrations of SP ($0.0001 \le p \le 0.01$) and BCM ($p < 0.01$). With 0.3 μg/ml antibody, ADCC with medium or SP, but not BCM, was significantly greater in asthmatics than normals ($0.005 \le p \le 0.05$).

The greater stimulatory effect of SP on neutrophils isolated from asthmatics did not merely reflect the higher baseline ADCC in this population, as the *net*-ADCC was also significantly greater in asthmatics (Fig. 7.2). For example, with 50 μM SP, *net*-ADCC was 7.0 ± 0.8% in normals versus 17.6 ± 1.9% in asthmatics (p = 0.0001). The *net*-ADCC stimulated by BCM, on the other hand, did not differ significantly between normals and asthmatics.

7.3.2 *The effect of SP on eosinophil antibody-dependent cell-mediated cytotoxicity*

The effect of SP on ADCC for eosinophils and neutrophils isolated from the peripheral blood of 5 asthmatics was compared and the results are

illustrated in Fig. 7.3. Although SP stimulated some eosinophil ADCC, the response was not statistically significant. With 100 μM SP, ADCC increased from 2.7 ± 1.0% to 8.8 ± 5.7% (1 μg/ml antibody; $p > 0.05$). In the same experiments, rhGM-CSF (10 ng/ml), used as a positive control,● increased eosinophil ADCC to 15.1 ± 3.5% ($p < 0.05$) demonstrating that it was possible to stimulate eosinophil ADCC under these experimental conditions. Furthermore, under the same assay conditions SP-stimulated neutrophil ADCC increased from 21.9 ± 6.8% to 44.9 ± 5.7% ($p < 0.05$; Fig. 7.3). Even when the antibody concentration was increased to 3 μg/ml, SP did not stimulate eosinophil ADCC significantly (5.5 ± 3.1% with 100 μM SP). The corresponding eosinophil response to rhGM-CSF was 19.4 ± 3.2% ($p < 0.002$)

Although SP did not stimulate mean eosinophil ADCC, it did increase ADCC for eosinophils isolated from the peripheral blood of one subject (30.8% at 100 μM SP, Fig. 7.4). In addition, SP also stimulated eosinophils isolated from the pleural effusion fluid of another individual with lung carcinoma, although higher effector:target ratios were needed for the latter (Fig. 7.5).

7.3.3 *Correlation of neutrophil antibody-dependent cell-mediated cytotoxicity and bronchial hyperresponsiveness*

Neutrophil ADCC and histamine reactivity were measured in a subgroup of 20 asthmatics whose clinical details are shown in Table 7.1. Most subjects suffered from moderate or severe asthma judged on symptoms (usually daily) and medication requirements. The effect of SP and BCM on *net*-ADCC was similar to the total asthmatic group (Fig. 7.6)

There were modest but significant correlations between ADCC and PC_{20} (Fig. 7.7). For example, for 100 μM SP the correlations were $r = 0.45$ ($p < 0.05$) and $r = 0.5$ ($p < 0.025$) for 1 μg/ml and 0.3 μg/ml antibody respectively. *Net*-ADCC with 100 μM SP and 0.3 μg/ml antibody, also

●[Lopez et al, 1986]

correlated with PC_{20} ($r = 0.51$, $p < 0.025$). There were no significant correlations between PC_{20} and baseline ADCC or BCM-stimulated cytotoxicity (data not shown).

7.3.4 *The effect of SP on rhGM-CSF-stimulated neutrophil antibody-dependent cell-mediated cytotoxicity*

Neutrophils isolated from normal subjects were preincubated with medium or 0.1 nM - 1 μM SP for 30 min at 37°C, prior to the ADCC assay, and were then stimulated with the same concentration of SP or rhGM-CSF (0, 1, 5 or 10 ng/ml). Data from these experiments are summarised in Fig. 7.8. Neutrophils, preincubated with SP and stimulated with rhGM-CSF demonstrated enhanced ADCC compared to cells that were preincubated with medium alone. For example, *net*-ADCC for neutrophils preincubated with 0.1 μM SP and stimulated with 10 ng/ml rhGM-CSF) was 44.0 ± 4.0% compared to 31.0 ± 5.2% for cells that had been preincubated with medium alone ($p < 0.05$). SP (0.1 nM - 1 μM) alone did not stimulate ADCC.

7.3.5 *The effect of rhIL-8 on neutrophil superoxide anion production*

Incubation of neutrophils with concentrations of up to 1 μg/ml rhIL-8 alone had no effect on O_2^- production (data not shown). To determine whether rhIL-8 was able to prime neutrophils for enhanced O_2^- production in response to fMLP, the cells were preincubated for 10 min at 37°C in the presence of 1-50 ng/ml rhIL-8 (or medium as a control), and subsequently, stimulated with various concentrations of fMLP. Because of the donor-dependent variability of fMLP-stimulated neutrophil O_2^- production, the data was normalised and expressed as a percentage of the control fMLP

response. rhIL-8 enhanced the response to fMLP in a dose-dependent manner (Fig. 7.9) with the maximum effect seen at 10 ng/ml rhIL-8.

rhIL-8 also enhanced PAF-stimulated O_2^- production. Neutrophils were preincubated with medium or 10 ng/ml rhIL-8 and then stimulated with 1 μM PAF (for 6 min). In these experiments, O_2^- production was enhanced from 6.1 ± 1.0 to 8.2 ± 1.3 nmol/10^6 cells or by 37.1 ± 2.2% ($p = 0.02$, n = 3).

The priming effect of rhIL-8 was dependent on the incubation time (Fig. 7.10). When cells were incubated with medium alone, the responsiveness of neutrophils to fMLP declined gradually and at 2h was 51% of the initial control response at time 0. The rhIL-8 induced enhancement of the fMLP-stimulated O_2^- production was already evident at 1 min, reached maximum at 6 min and was still present at 2h.

In order to study the kinetics of the priming effect of rhIL-8, neutrophils were preincubated with 10 ng/ml rhIL-8 or medium for 10 min at 37°C and then 10^6 cells were transferred to prewarmed cuvettes containing Cyt C and 10 ng/ml PMA or 100 nM fMLP. The O_2^- production was measured continuously and the results from a representative experiment are shown in Fig. 7.11. rhIL-8 increased the maximal rate of O_2^- production. The PMA-stimulated rate of O_2^- production increased from 83 ± 16 to 107 ± 16 pmol/10^6 cell/sec ($p < 0.001$, n = 5) and the fMLP from 145 ± 10 to 220 ± 13 pmol/10^6 cell/sec ($p < 0.005$, n = 9). These results were confirmed by the luminol-enhanced chemiluminescence (CL) assay (Fig. 7.12). The peak of CL represents the maximum rate of oxy radical production. Preincubation of neutrophils with 10 ng/ml rhIL-8 increased the rate of fMLP-stimulated oxy radical production twofold.

To determine whether, as for SP, enhancement of O_2^- production by rhIL-8 was associated with changes in $[Ca^{++}]_i$, FURA-2 loaded neutrophils were stimulated directly with rhIL-8 concentrations from 1 to 25 ng/ml (Fig. 7.13). rhIL-8 stimulated an increase in $[Ca^{++}]_i$, which paralleled closely the results

obtained from O_2^- experiments. For example, the concentration of rhIL-8 (10 ng/ml), shown to be optimal for the facilitation of O_2^- response, induced maximal increase in $[Ca^{++}]_i$. Neutrophils stimulated with 10 ng/ml rhIL-8 increased $[Ca^{++}]_i$ levels by 573 ± 100 nM. When neutrophils were stimulated in calcium free buffer, the $[Ca^{++}]_i$ increased to 221 ± 43 nM and even less (129 ± 32 nM) with 2 mM EGTA in the buffer. The rhIL-8 response was restored to 71% of the original response when calcium was added to the cells suspended in the calcium-free buffer (Fig. 7.14). These results suggest that the increase in $[Ca^{++}]_i$ induced by rhIL-8 is caused by both an influx of extracellular calcium and release of calcium from intracellular stores.

Furthermore, preincubation of neutrophils with rhIL-8 facilitated the fMLP-stimulated increase in $[Ca^{++}]_i$. In these experiments neutrophils were preincubated with 10 ng/ml rhIL-8 or medium for the indicated times and were then stimulated with 100 nM fMLP. The maximal facilitating effect of rhIL-8 was seen at 5 min when the fMLP-stimulated elevation in $[Ca^{++}]_i$ was increased by 1027 ± 80 nM (220 ± 43 % fMLP response) and gradually declined to 308 ± 136 nM (140 ± 20 % fMLP response) at 15 min (Fig. 7.15).

7.3.6 *Combined effects of SP and rhIL-8 on stimulation of neutrophil superoxide anion production*

To determine whether O_2^- production can be induced by SP in neutrophils that had been primed by IL-8, or vice versa, the cells were preincubated with one of the mediators and subsequently stimulated with the other. SP (1 nM - 50 μM) did not stimulate any O_2^- production in neutrophils, that had been preincubated with 10 ng/ml rhIL-8 for 10 min at 37°C (data not shown). In the same experiment, fMLP-stimulated O_2^- production (14.3 ± 0.8 nmole/10^6 cells) was increased by preincubation with IL-8 to 22.6 ± 0.3 nmole/10^6 cells (an increase of 158%). Similarly, rhIL-8 (10

ng/ml) had no effect on induction of O_2^- production in SP (1 nM - 50 μM) primed neutrophils (data not shown).

In subsequent experiments, neutrophil O_2^- response was examined after priming the cells with both IL-8 and SP together. Initially, neutrophils had been preincubated sequentially, first with 10 ng/ml rhIL-8 or medium (10 min at 37°C) and then with SP (0, 1 nM - 50 μM, 6 min at 37°C), before they were stimulated with 0.1 μM fMLP. Preincubation with SP alone (cells incubated with medium followed by SP) enhanced the fMLP response in a dose-dependent manner. rhIL-8 also enhanced the fMLP response, but the combined effect was neither additive nor synergistic (Fig. 7.16A). Then, the order of the preincubations was changed, and the cells were preincubated first with SP (0, 1 nM - 50 μM, 6 min at 37°C), and subsequently rhIL-8 (10 ng/ml, 10 min at 37°C), and were then stimulated with 0.1 μM fMLP for an additional 6 min. As in the previous experiments, preincubation with SP or IL-8 alone facilitated the fMLP-stimulated O_2^- response, but there was no synergistic or combined effect seen when both mediators were used simultaneously (Fig. 7.16B).

7.4 Discussion

Chronic inflammation is a prominent feature of asthma, and is thought to cause airway damage and bronchial hyperresponsiveness (BHR) (Barnes 1989). Neutrophils may play an important role in the airways inflammation of asthma (Chapter 1.7.2), since they are found in large numbers in the airways and lavage fluids of asthmatic patients [Laitinen et al, 1985], especially after allergen challenge [Metzger et al, 1986, 1987]. Peripheral blood neutrophils become activated after allergen- [Carrol et al, 1985] and exercise-induced [Moqbel et al, 1986] asthmatic responses and neutrophil-activating factors are present in the circulation. It is particularly relevant to

these studies, which used an ADCC assay to assess neutrophil function, that enhanced cytotoxicity to complement coated targets has also been demonstrated in exercise-induced asthma [Papageorgiou et al, 1983]. It is likely therefore, that neutrophils contribute significantly to the pathogenesis of asthma, even though the exact mechanisms by which they cause tissue damage are unknown.

In asthma, there is evidence that circulating neutrophils and eosinophils are activated and have enhanced responses to a number of stimuli [Kanazawa et al, 1991]. Since it has been postulated that SP plays a role in the pathogenesis of asthma, it seemed pertinent to study its effects on neutrophils and eosinophils obtained from asthmatic subjects. It could be expected that in asthma, neutrophil responsiveness to SP would be increased.

In this chapter neutrophil cytotoxicity against an antibody coated target cells was investigated. Dallegri et al [1987] reported that neutrophil ADCC involves exocytosis, particularly of primary granules, and production of oxy radicals. SP does not stimulate superoxide anion production directly to any significant degree, but, as shown previously, it stimulates neutrophil ADCC and primed neutrophils for enhanced superoxide anion production in response to fMLP [Chapter 3.3.1 and 3.3.2; Wozniak et al, 1989]. Since the target cells used in this assay are sensitive to eosinophil and neutrophil-mediated cytotoxicity, SP effects on both cell populations could be compared.

The studies dealing with the effects of SP on human neutrophils (Chapter 1.9.2 and 3.3), used cells obtained from the peripheral blood of normal subjects. However, in asthma there is increasing evidence that neutrophils and eosinophils are activated and primed by cytokines, including GM-CSF, which are potentially able to be produced locally in airways [Vancheri et al, 1989; Lopez et al, 1986; Weisbart et al, 1987].

In normal subjects, SP stimulated neutrophil ADCC in a dose-dependent manner with the maximum effect noted at 100 μM SP. It did not stimulate cytotoxicity in the absence of antibody and its effect was dependent on the antibody concentration used (Chapter 3.3.1). In asthmatics, SP also stimulated neutrophil ADCC. However, there were differences between these two groups that led to the following conclusions: (i) baseline (or unstimulated) ADCC was significantly higher in asthmatics. This is consistent with a number of other studies which have shown that in asthma, peripheral blood neutrophils are activated and show increased responses to a number of stimuli including fMLP. (ii) ADCC was significantly higher in asthmatics for all the concentrations of SP tested. It could be argued that this merely reflects the higher baseline ADCC in this group. However, when *net*-ADCC (stimulated minus baseline) was considered, it became apparent that SP has a stimulatory effect on asthmatic neutrophils beyond that expected from the greater baseline activation. (iii) The increased responsiveness appeared to have some selectivity for SP since responses to BCM did not differ significantly between the two groups. This could indicate that neutrophils are already stimulated *in vivo*, however this should not be the case in normal subjects.

Although these *in vitro* experiments indicate potential differences between asthmatics and normals, they cannot be extrapolated entirely to the situation *in vivo*, where the SP may be acting in concert with other cytokines and/or mediators of inflammation. As can be seen from the data presented in this chapter, SP in low concentrations may act synergistically with other neutrophil stimulating factors such as GM-CSF. The reasons for the enhanced reactivity of neutrophils to SP in asthma are unknown. Since the ADCC assay used here is dependent on the presence of Fcγ receptors, it is possible that the differences may represent increased expression of these receptors in asthma and there is some evidence to support this [Walsh and Kay, 1984].

Some of the results presented here contribute to the increasing evidence for a role of cytokines, in particular GM-CSF and IL-8, in this disease. When neutrophils obtained from normal subjects were incubated with SP for a short time (30 min), and then with GM-CSF plus SP, there was a synergistic effect on ADCC. The effect is synergistic, because, SP at the concentrations used in these experiments (0.1 nM - 1μM), SP did not stimulate ADCC in normals or asthmatics. These experiments were performed with normal subjects to ensure the neutrophils had not been maximally stimulated *in vivo*. The concentration of GM-CSF chosen was in the range previously shown to prime neutrophils and stimulate ADCC [Lopez et al, 1986; Weisbart et al, 1987].

In contrast to its effect on neutrophils, SP had no effect on eosinophil ADCC under identical experimental conditions (except in one subject in whom it caused stimulation). In contrast, De Simmone et al [1987] reported that SP stimulated eosinophil ADCC using chicken red blood cells as a target and an IgG antibody to opsonise the cells; SP also increased the expression of Fc receptors. The reasons why SP failed to stimulate eosinophils, are uncertain. The target cells used are sensitive to both eosinophil and neutrophil ADCC and it was possible to stimulate eosinophil ADCC with GM-CSF. The isolation procedure for eosinophils most likely yielded a normodense population, but in asthma there is an increased population of hypodense eosinophils, which are more responsive to a number of stimuli [Fukuda et al, 1989]. Therefore, it is quite possible that an effect of SP on eosinophils would have been detected if hypodense cells had been used.

In population studies of asthmatics, bronchial hyperresponsiveness (BHR) correlates with the severity of asthma as assessed by medication requirements, symptoms and degree of airflow obstruction and variability [Woolcock and Jenkins, 1990]. A modest but significant correlation between SP-induced ADCC, *net*-ADCC and BHR, assessed as PC_{20} histamine, was

demonstrated. This suggests that the neutrophil responses to SP reflects partially the clinical severity of asthma: This is the first time such a relationship has been demonstrated.

IL-8 is one of the most potent neutrophilic chemotactic factors, and its role in lung inflammation has been recently recognised. Il-8 has been acclaimed to be the major chemotactic factor in the lung [Kunkel et al, 1991], and a cytokine network of interactions between lung fibroblasts and alveolar macrophages has been proposed for IL-8 production and neutrophil recruitment to the lung [Rolfe et al, 1991]. Il-8 does not stimulate neutrophil ADCC, but has been implicated in activating other important neutrophil functions, including enzyme exocytosis and O_2^- production (Chapter 1.4.2.2). Recent studies by You et al [1991] and Daniels et al [1992] have shown that IL-8 primes neutrophils for enhanced O_2^- production in response to fMLP, but other investigators have failed to demonstrate an effect of IL-8 in the same assay system [Djeu et al, 1990]. Since activated neutrophils [Chung 1986; Weiss 1989] and neutrophil-released oxy radicals [Fisher and Malik, 1991; Kanazawa et al, 1991] may lead to severe local tissue damage in the lung (Chapter 1.7.2), it was important to assess the role of IL-8 on neutrophil oxy radical production before its interactions with SP could be studied.

The finding that IL-8 may facilitate fMLP-stimulated neutrophil O_2^- production was confirmed in the present study, using the Cyt C reduction and the luminol-enhanced CL assays. In addition, IL-8 enhanced neutrophil O_2^- production induced by two other stimuli, PMA and PAF. The priming response of rhIL-8 reached maximum at 10 ng/ml of IL-8, and it is therefore likely that IL-8 released locally in the lung from alveolar macrophages, type II cells [Standiford et al, 1991] or fibroblasts [Rolfe et al, 1991], may enhance neutrophil oxy radical production and contribute to airways damage. Alternatively, neutrophils may be primed when exposed to low chemotactic levels of IL-8 during migration into the lung.

The mechanisms by which IL-8 exerts its priming on neutrophil O_2^- production is unknown. However, the finding that it primed neutrophils for fMLP-, PMA- and PAF-stimulated responses, suggests that similar mechanisms to those induced by SP may be involved. Moreover, the IL-8 priming response was associated with increases in $[Ca^{++}]_i$, caused by calcium mobilisation from intracellular stores and calcium influx, which were also demonstrated for SP. Although Walz et al [1991] demonstrated that neutrophils primed with low concentrations of concanavalin A, could stimulate IL-8 induced O_2^- production, priming with either SP or IL-8 was not able to preactivate neutrophils for induction of this response to the other mediator respectively. Furthermore, preincubation of neutrophils with both mediators did not result in additive or synergistic effects, when stimulated with a third stimulus fMLP. Thus, although SP and IL-8 have both proinflammatory effects on neutrophils and may use similar mechanisms for priming the cells for enhanced O_2^- production, when combined, they do not synergise each others effects.

	Mean ± SEM (range), n = 20
AGE (yrs)	42.4 ± 3.3 (16-67)
M:F	14:6
FEV_1 (L)	3.0 ± 0.3 (1.2-5.3)
FEV_1 (% predicted)	81.9 ± 6.5 (32-86.5)
FVC (L)	3.8 ± 0.3 (1.8-7.3)
FVC (% predicted)	82.9 ± 5.0 (37.4-126.8)
FEV_1/FVC (%)	77.1 ± 3.0 (58.0-98.4)
PC_{20} (mg/ml)	0.93 ÷/x 1.43
Bronchodilators	17
Aerosol corticosteroids	17
Systemic corticosteroids	Nil
Smokers	2

Table 7.1 Clinical details of asthmatic subjects in whom histamine reactivity was correlated with ADCC.

FEV_1 forced expiratory volume in one second
FVC forced vital capacity
PC_{20} provocation concentration for 20% fall in FEV_1
ADCC antibody-dependent cell-mediated cytotoxicity

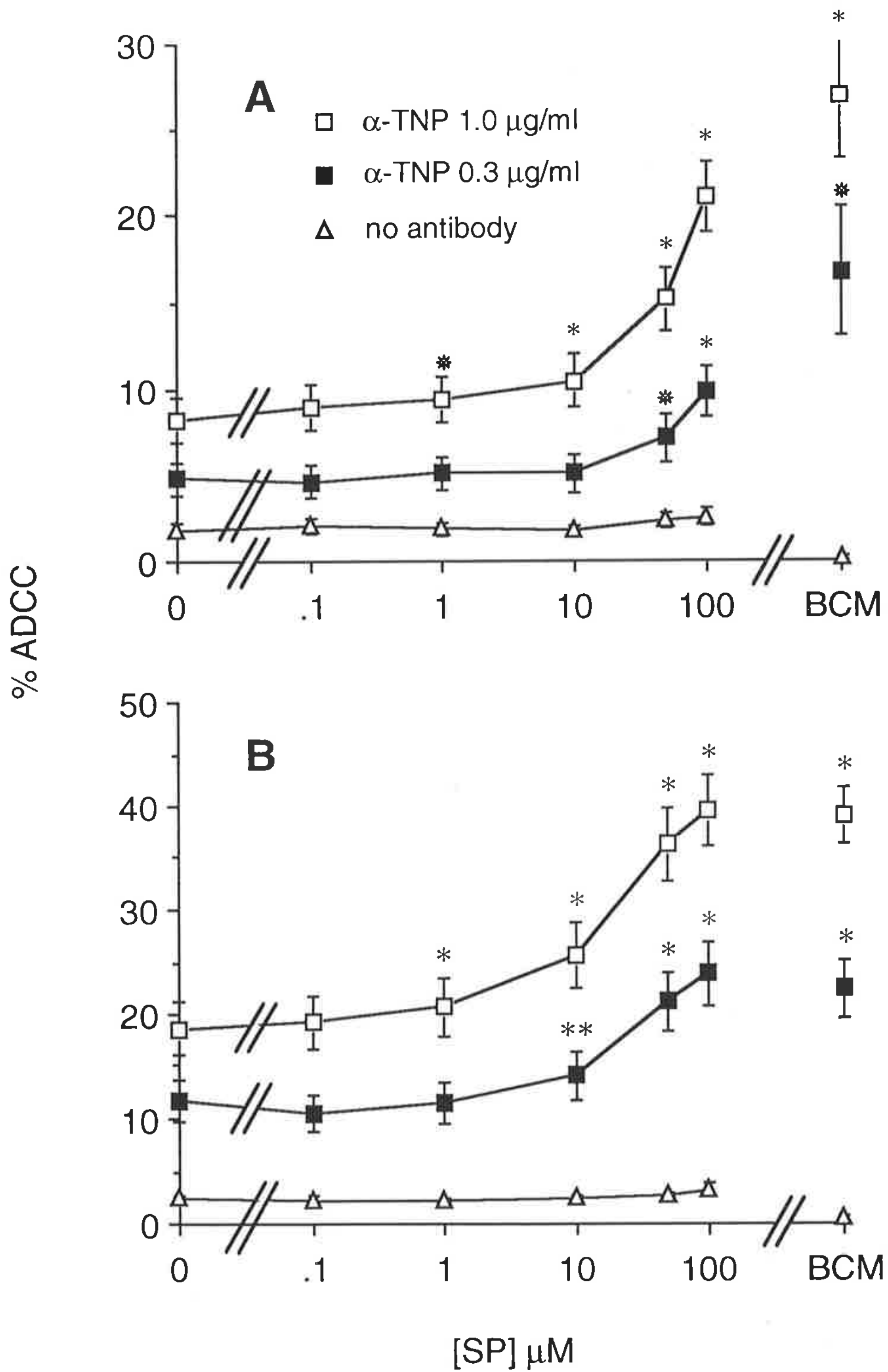


Figure 7.1 Effect of SP on neutrophil ADCC in (**A**) normal and (**B**) asthmatic subjects . Neutrophils and target cells were incubated together with various concentrations of SP (or medium control) for 2.5 hrs at 37°C and indicated concentrations of antibody. Bladder carcinoma cell line U5637 conditioned medium (BCM) was used as a positive control. * $p < 0.0001$; ** $p < 0.002$; ⁎ $p < 0.02$: indicates values which differ significantly from the medium control.

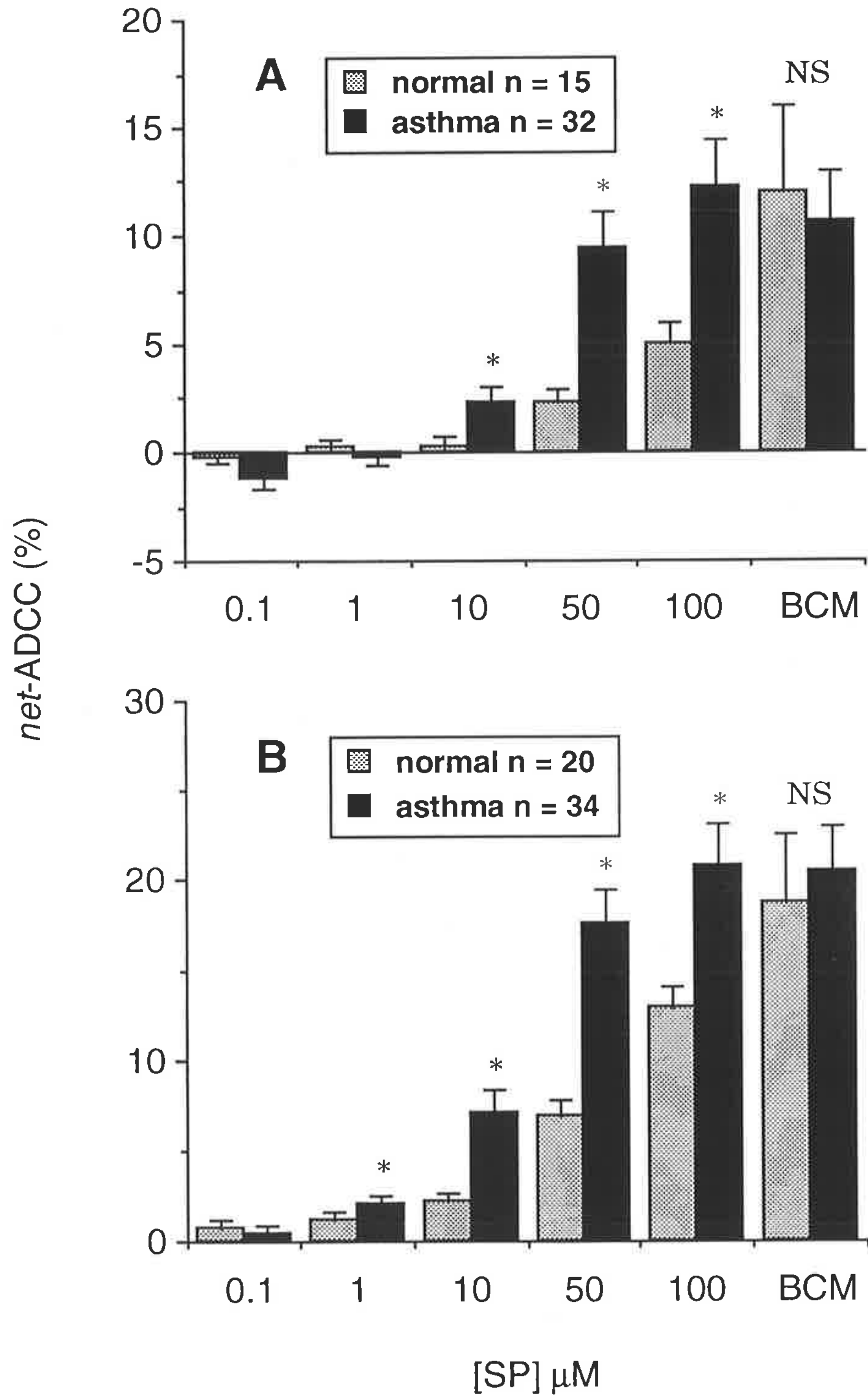


Figure 7.2 *net*-ADCC in normal and asthmatic subjects. (**A**) 0.3 μg/ml antibody and (**B**) 1.0 μg/ml antibody. Data are from subjects shown in Fig. 7.1. Normal versus asthmatics: * $0.0001 \leq p \leq 0.05$. NS = not significant.

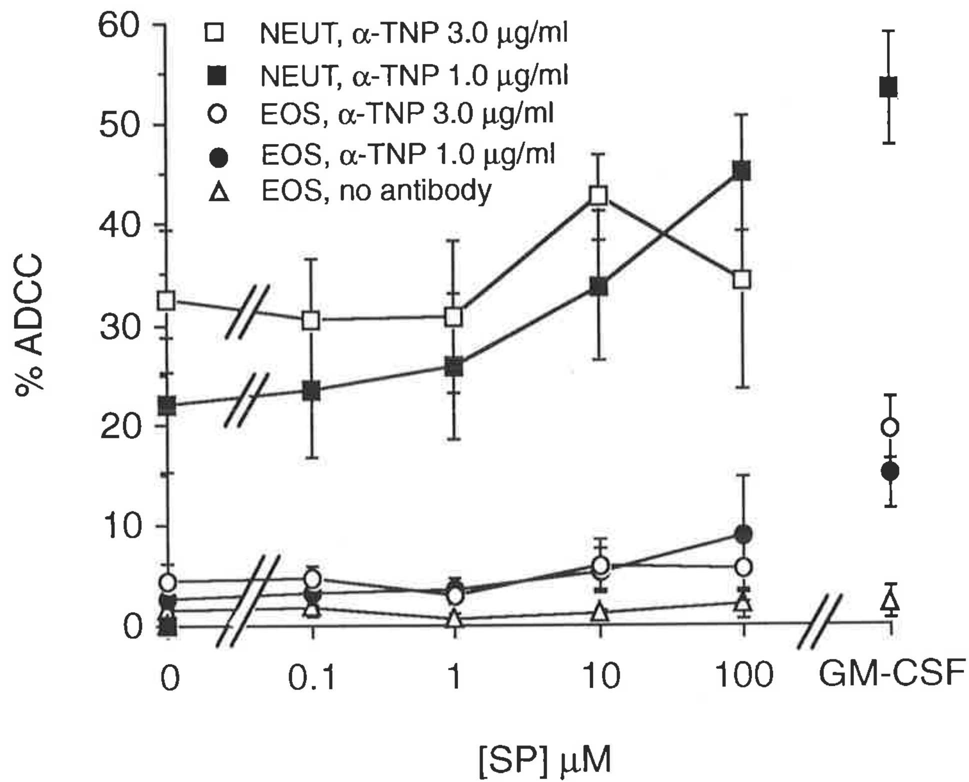


Figure 7.3 Effect of SP on eosinophil (EOS) and neutrophil (NEUT) ADCC in 5 asthmatic subjects. Neutrophils and eosinophils were incubated with target cells, antibody, and SP (0.1—100 μM), GM-CSF (10 ng/ml) or medium for 2.5 hr at 37°C. Neutrophil ADCC was significanly greater than eosinophil ADCC for medium, all concentrations of SP and GM-CSF at $0.002 < p < 0.05$. Data are means of five experiments .

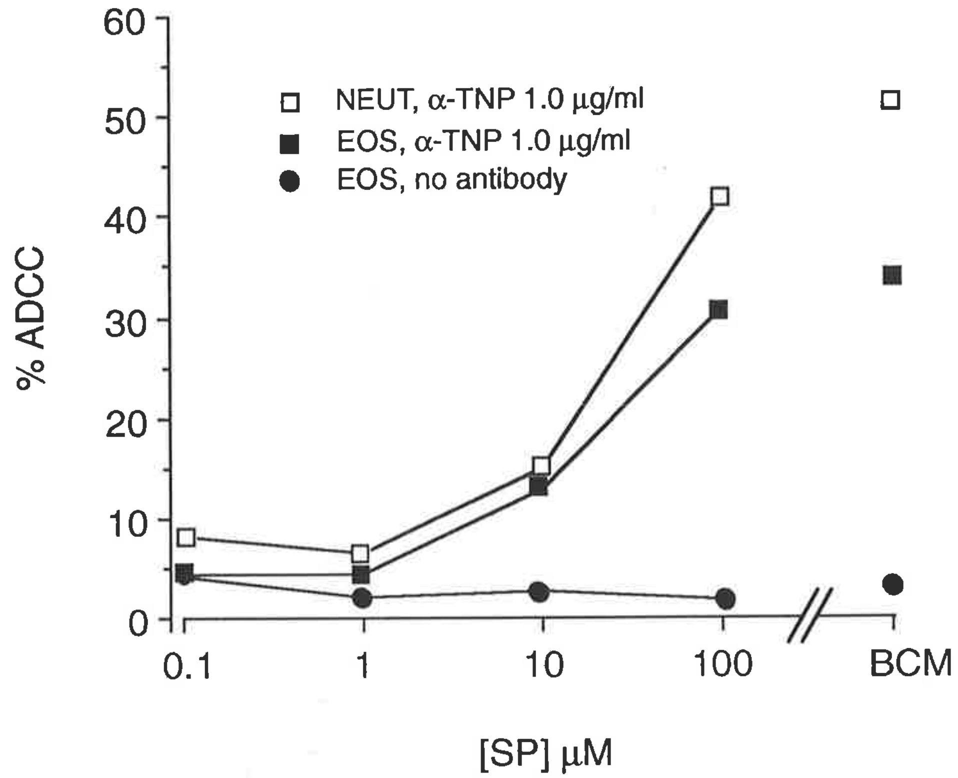


Figure 7.4 Effect of SP on eosinophil (EOS) and neutrophil (NEUT) ADCC in subject 17. Data are from one experiment done in triplicate.

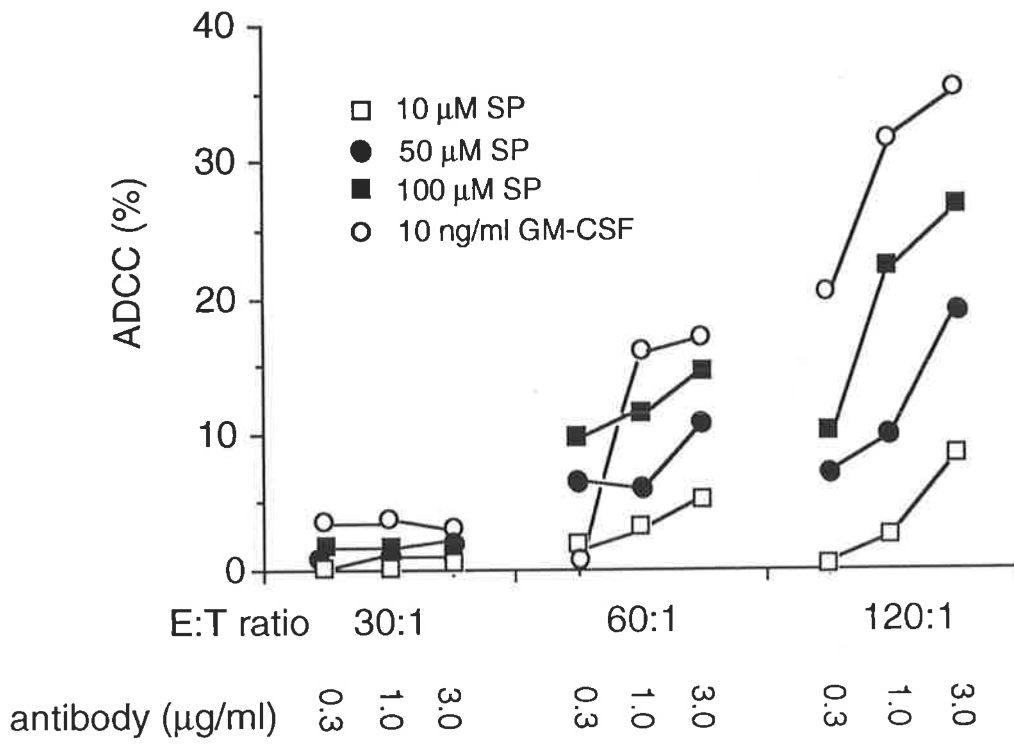


Figure 7.5 Effect of SP on eosinophil ADCC of a lung carcinoma patient. Data are from one experiment done in triplicate.

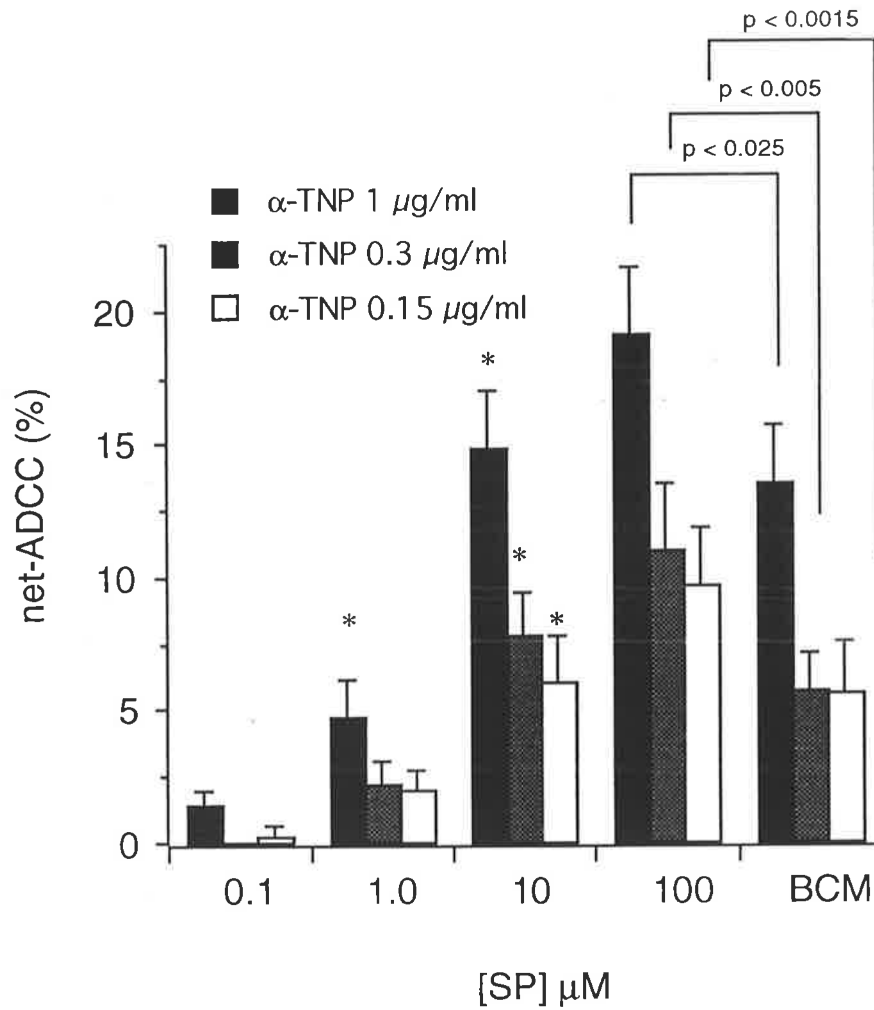


Figure 7.6 Effect of SP on neutrophil ADCC in the subgroup of asthmatics in whom histamine reactivity was assessed. Data are from twenty subjects; each experiment done in triplicate. *, significantly greater than the spontaneous cytotoxicity at $0.0001 \leq p \leq 0.025$.

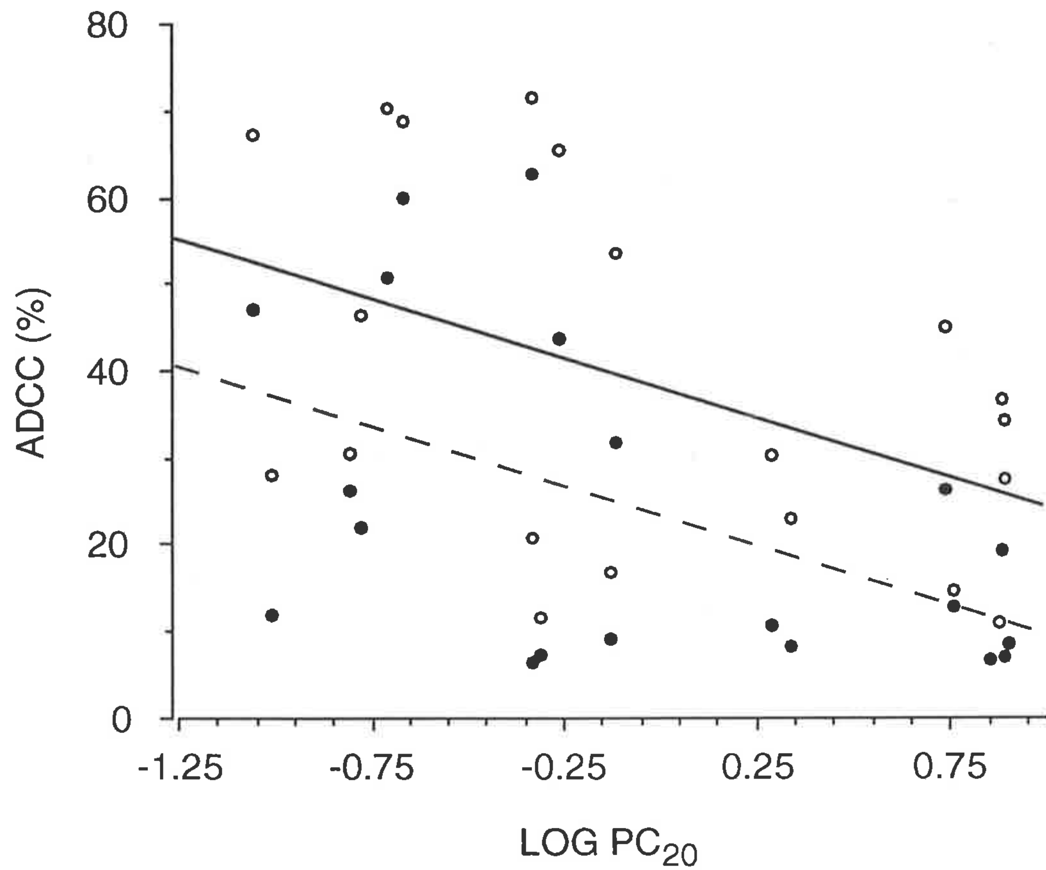


Figure 7.7 Correlation of neutrophil cytotoxicity with bronchial hyperresponsiveness. Cytotoxicity (as %) was correlated with histamine reactivity (PC_{20} in mg/ml) at 100 μM SP for 1 μg/ml antibody (open circles, solid line identity) and 0.3 μg/ml antibody (closed circles, dashed line identity).

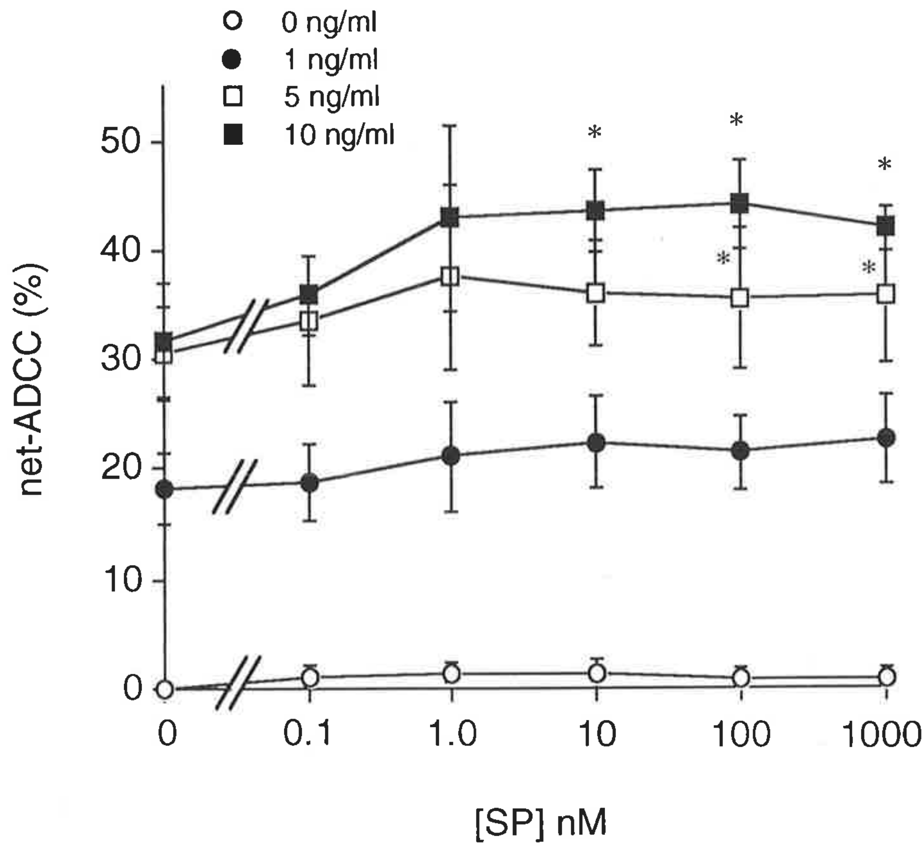


Figure 7.8 Effect of SP and GM-CSF on neutrophil *net*-ADCC. Cells were incubated with indicated concentrations of SP and GM-CSF (concentrations in the legend). The response to 100 μM SP alone was 16.8 ± 3.7%. Data are means from 4—7 subjects, each experiment performed in triplicate.
*, indicates $0.015 \leq p \leq 0.05$ compared to GM-CSF alone (no SP).
Baseline ADCC used to calculate *net*-ADCC was 4.7 ± 2.1%.

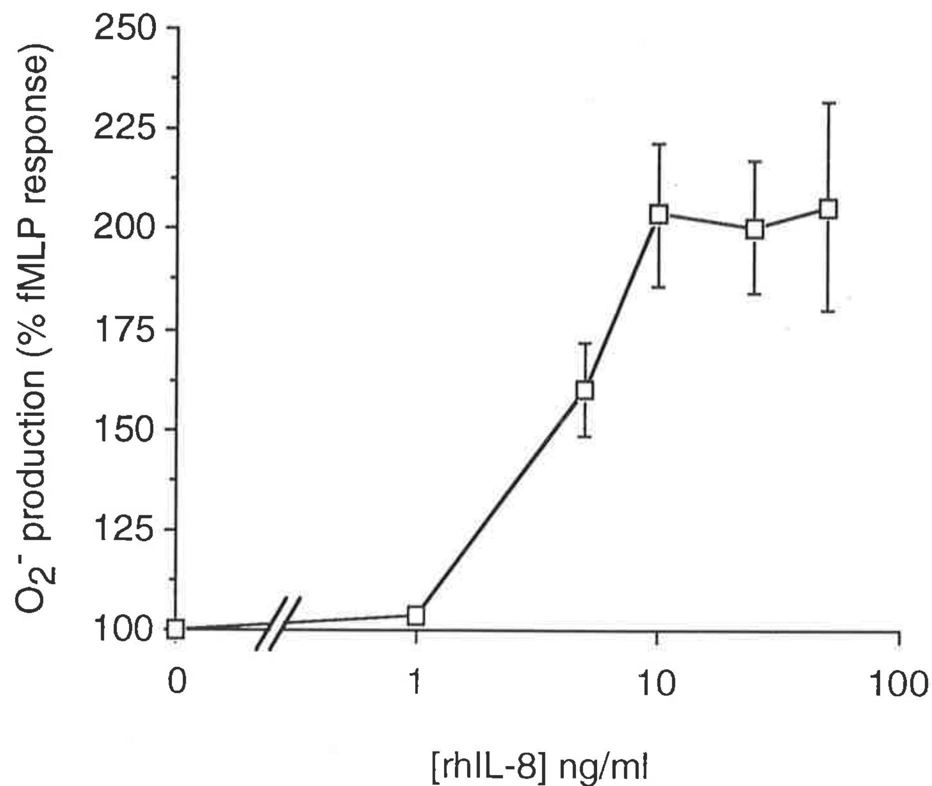


Figure 7.9 Dose-response effect of rhIL-8 on fMLP-stimulated O_2^- production. Neutrophils were incubated with medium or stated concentration of rhIL-8 for 10 min at 37°C and were then stimulated with 0.1 μM fMLP for an additional 6 min. Results are normalised and expressed as a percentage of the control fMLP response. The response of control cells preincubated with medium alone and then stimulated with 0.1 μM fMLP was 12.0 ± 2.3 nmol/10^6 cells. Values are means of four experiments performed in triplicate.

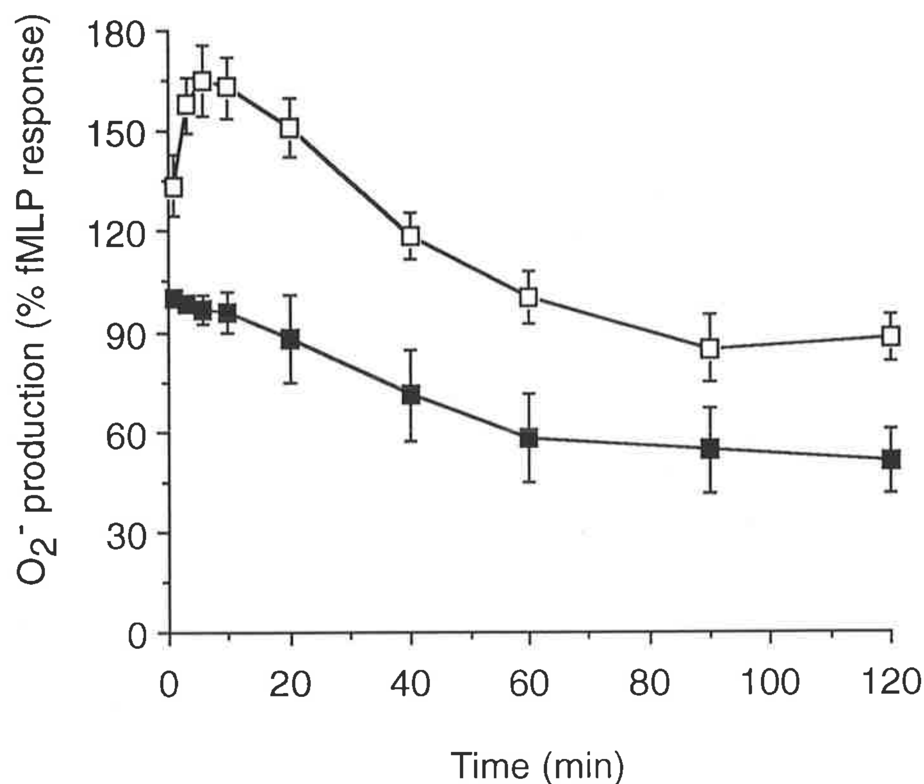


Figure 7.10 Effect of rhIL-8 on fMLP-stimulated O_2^- production as a function of time. Neutrophils (5 x 10^6/ml) were incubated with 10 ng/ml rhIL-8 (—□—) or diluent (—■—) for indicated times at 37°C. At each time point 10^6 cells were transferred to tubes containing cytochrome C and 0.1 μM fMLP for an additional 6 min stimulation. Results are normalised and expressed as a percentage of the control fMLP response. Data are means of seven experiments performed in triplicate.

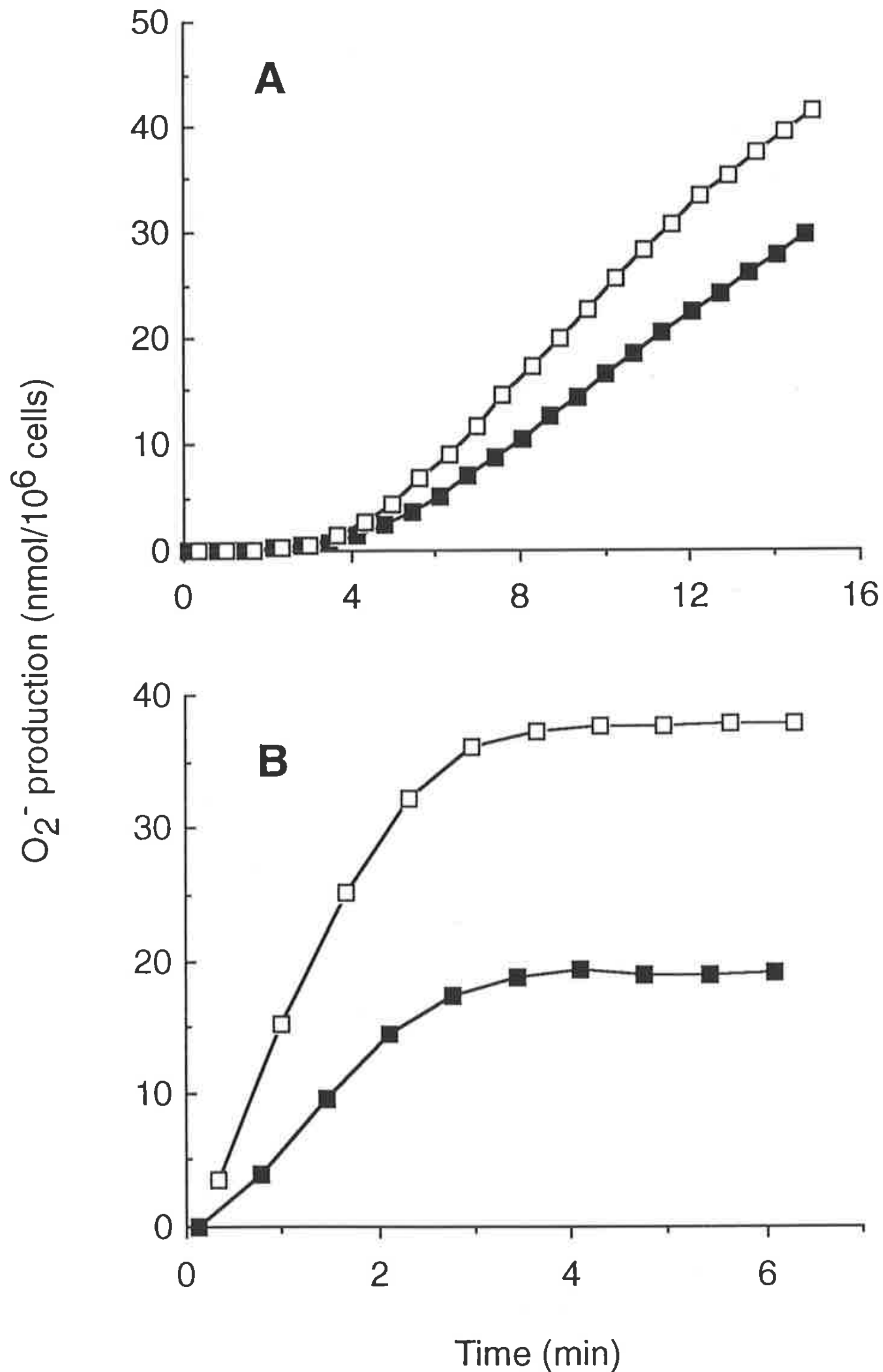


Figure 7.11 Effect of rhIL-8 on PMA- and fMLP-stimulated O_2^- production. Neutrophils were preincubated with medium (—■—) or 10 ng/ml rhIL-8 (—□—) for 10 min at 37°C and were then stimulated with (**A**) 10 ng/ml PMA or (**B**) 0.1 μM fMLP . O_2^- production was measured continuously. The results are from one experiment representative of five (**A**) and nine (**B**).

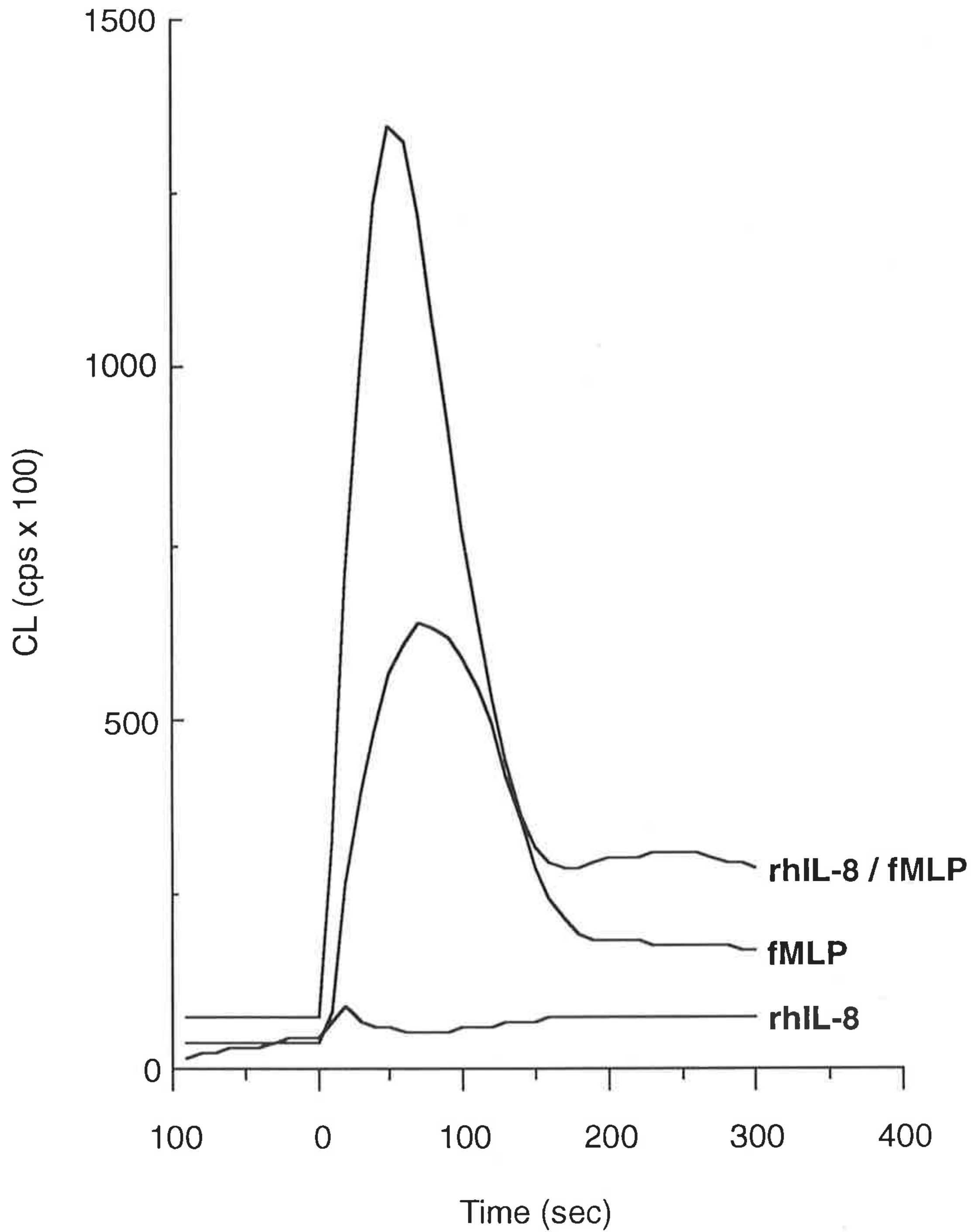


Figure 7.12 Luminol-enhanced CL response of rhIL-8 primed neutrophils. Neutrophils were preincubated with 10 ng/ml rhIL-8 or medium for 10 min at 37°C and were then stimulated with 0.1 μM fMLP. The peak of CL corresponds to the maximum rate of oxy radical production (NADPH oxidase activity). The results are from one experiment representative of four.

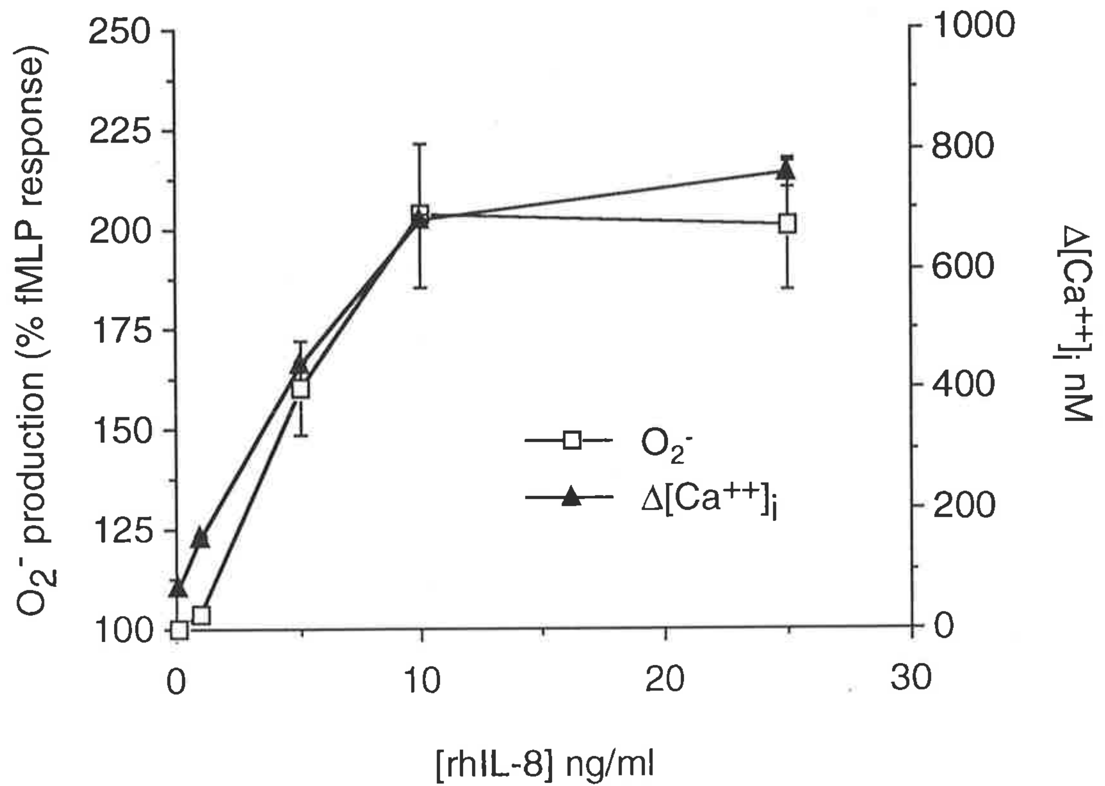


Figure 7.13 Effect of rhIL-8 on $\Delta[Ca^{++}]i$ and fMLP-stimulated superoxide production. For the study of changes in $\Delta[Ca^{++}]i$, FURA-2 loaded neutrophils were stimulated with various concentrations of rhIL-8. Values represent means of four experiments. O_2^- production as in Fig. 7.9.

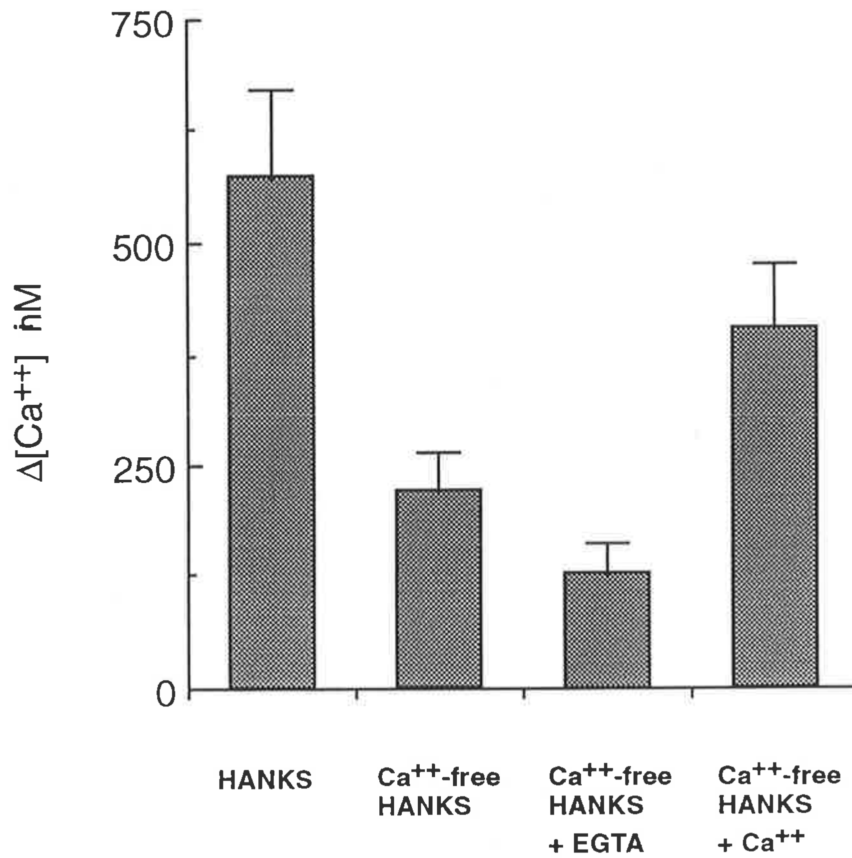


Figure 7.14 Effect of the extracellular calcium on the rhIL-8-induced $\Delta[Ca^{++}]i$. Neutrophils were loaded with FURA-2 in complete Hanks buffer and divided into two preparations. Each was then washed and resuspended in either Hanks buffer or calcium-free Hanks buffer. Cells from each preparation were then stimulated with 10 ng/ml rhIL-8. In addition, neutrophils that were resuspended in calcium-free Hanks, were either repleted with 1.25 mM Ca^{++} or treated with 2 mM EGTA for 5 min at $37^{0}C$ before stimulation with rhIL-8. Values represent means from four experiments.

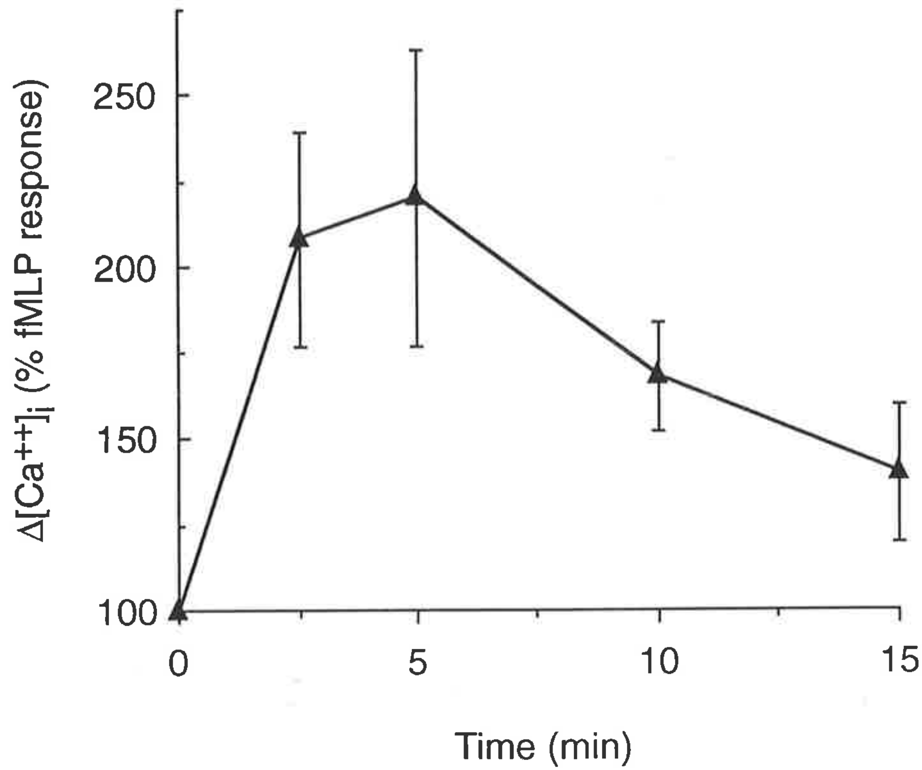


Figure 7.15 Effect of rhIL-8 on fMLP-stimulated $[Ca^{++}]i$ as a function of time. Neutrophils were preincubated with medium or 10 ng/ml rhIL-8 at 37^oC for indicated times and were then stimulated with 0.1 μM fMLP. Results are expressed as a percentage of the fMLP response. Values represent means of five experiments.

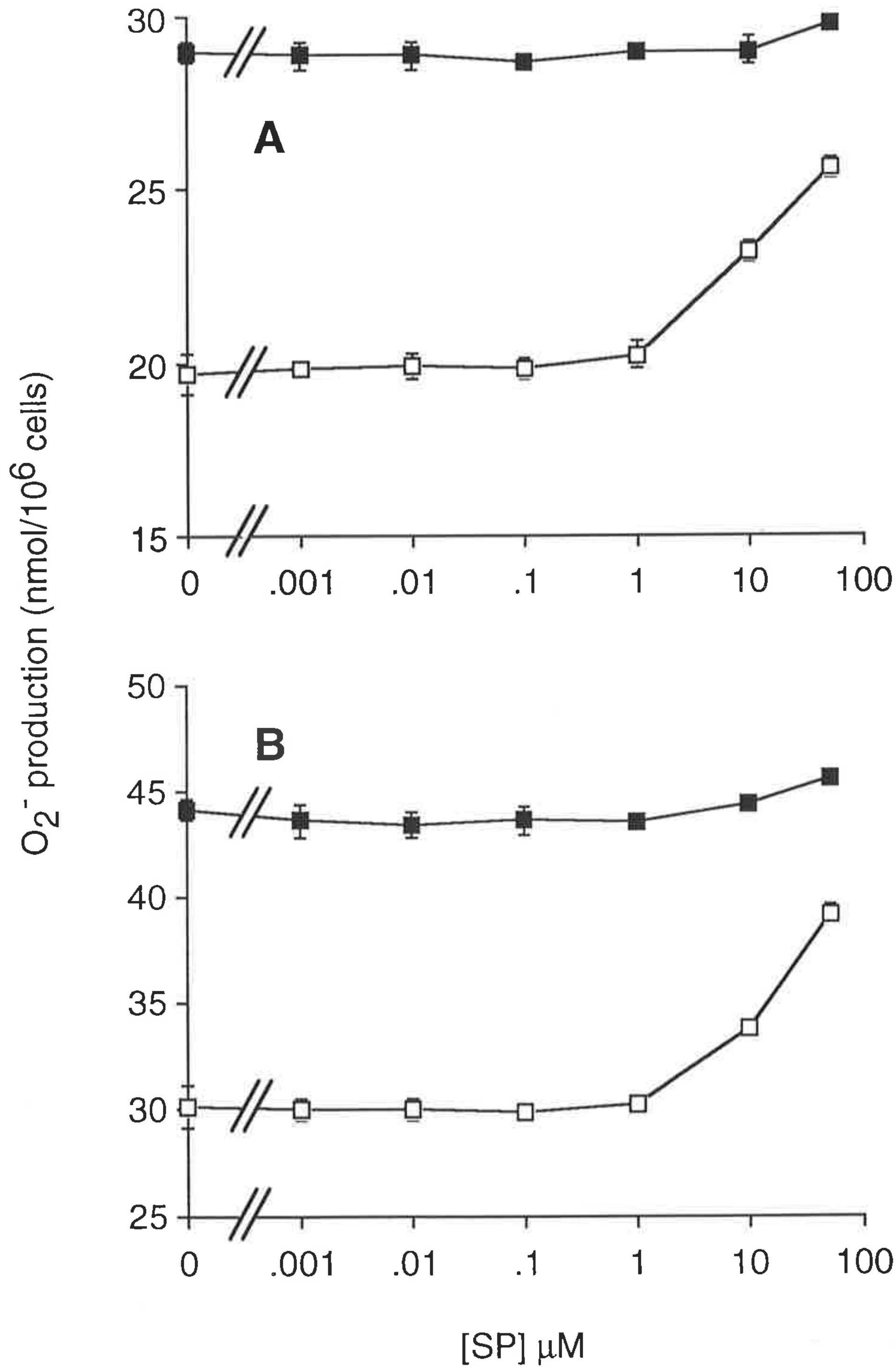


Figure 7.16 Combined effect of rhIL-8 and SP on fMLP-stimulated O_2^- production. Neutrophils were preincubated with either (**A**) 10 ng/ml rhIL-8 (—■—) or medium (—□—) for 10 min and then with shown concentrations of SP for 6 min or (**B**) SP (0-50 μM) for 6 min and then with 10 ng/ml rhIL-8 (—■—) or medium (—□—) for 10 min, and were then stimulated with 0.1 μM fMLP. Refer to text for details (Chapter 7. 3.6).

Chapter 8

Conclusions and Future Studies

Advances in the understanding of the interaction between the nervous and the immune systems have resulted in the creation of a new, rapidly growing scientific field of neuroimmunomodulation. In particular, considerable progress have been made towards the role of neuropeptides in inflammation. A number of neuropeptide receptors have been cloned, and neuropeptide effects on the function of individual cells of immune system described, demonstrating that there is still a great deal to be learned about the fundamental aspects of the interactions between these two systems. The major aims of the experiments described in this thesis were to investigate the effects of tachykinins on neutrophil function and to assess the possible biological significance of these interactions in inflammatory disease. Although many details of the interactions between tachykinins and neutrophils still remain to be defined, and little is yet known about the biochemical mechanisms involved in these processes, these studies provide a conceptual framework in which to explore the molecular basis of these interactions.

8.1 *Advantages and limitations of these studies*

The experiments in these studies were performed using freshly purified human neutrophils, isolated from blood of a number of different volunteers. The freshly isolated cells offer many advantages over systems that use cell lines, as these are normal, unaltered cells, which reflect the biological diversity normally seen within the population of normal subjects (as seen by the different magnitude of a particular response studied), providing more "real" and meaningful results.

The *in vitro* systems used to demonstrate the effects of tachykinins on different neutrophil function have been well established, and require only slight modification to optimise the experimental conditions. The neutrophil responses to many physiological mediators (GM-CSF, fMLP, PAF) and synthetic agonists (A23187, PMA), which were used here, either as positive controls or second stimuli, have been well defined. This was very helpful, as the functional responses of neutrophils studied could be compared to those induced by other cytokines and mediators, and assessed against a background of well documented research data published by others.

The major limitation of these studies was the lack of suitable tachykinin antagonists and anti-tachykinin receptor-blocking antibodies, whose availability would have enabled the much sought answers to the specificity of tachykinin action to be found. Instead, the ability of a number of different tachykinins to induce a particular response was compared, or the ability of one tachykinin to induce various neutrophil functions was tested, in the hope to get some information on the specificity of these neuropeptides. Although the results from these experiments suggest specificity of action of tachykinins, the ultimate answer may be only provided by true antagonists, and as such, the issue needs further investigation.

8.2 *The effect of substance P, substance P fragments and other tachykinins on neutrophil function*

The main objective of these studies was to determine whether tachykinins, in particular SP, can activate or modulate human neutrophil function. This was achieved by demonstrating that SP stimulate neutrophil ADCC, and enhances LTB_4 synthesis and O_2^- production in response to a second stimulus. Other mammalian tachykinins, NKA and NKB had similar effects to SP and also stimulated ADCC and facilitated O_2^- production. The action of tachykinins was concluded to be specific for four reasons: (i) whereas some of the neutrophil functions were activated by SP (ADCC), others were not (e.g., adherence to endothelial cells). It would be expected that if a tachykinin effect was due to non specific activation of cells, then multiple functions would also be affected, (ii) SP primed neutrophils for enhanced fMLP-stimulated O_2^- production but it did not enhance enzyme release induced by the same agonist fMLP, (iii) SP primed neutrophils for enhanced fMLP-, PMA- and PAF- but not A23187- or OPZ-stimulated O_2^- production, and (iv) the tachykinins studied demonstrated different potencies in their ability to activate neutrophils, NKB being twice as potent as SP or NKA, and Kassinin having no effect at all on any function tested, even though, it has the same carboxy pentapeptide sequence as NKA.

The other aim of these studies, was to identify the part of tachykinin molecules, that causes the activity. It was expected, that the activity resides in the carboxy terminal, which is common to the tachykinin family. Since the carboxy terminal of SP, SP_{7-11}, was as active as the whole molecule in facilitation of O_2^- production and stimulation of ADCC, it was concluded that the activity is indeed located in the carboxy end. However, the inhibitory effect of the amino end of SP, suggested that it may have a role in determining both the different potencies of tachykinins and their specificity.

Since many tachykinins are not only codistributed but also colocalised to the same neurone fibres, and may be released together upon appropriate stimulation of these fibres , [Schmidt et al, 1991; Kishimoto et al, 1991; Moussaoui et al, 1992; Appelgren et al, 1991], it would of considerable interest to study their combined effects on neutrophil function as this may have great implication for their role *in vivo*. The possibility that tachykinin effects on neutrophil function may synergise with other neuropeptides or neurotransmitters should also be considered. For example, calcitonin gene related peptide is often found in the same small sensory afferent nerve fibres as SP, and the expression, transport to peripheral fibres and release of both neuropeptides is increased under inflammatory conditions [Donnerer et 1992, Garry and Hargreaves, 1992]. Therefore, it could be expected that in inflammation, the action of both SP and calcitonin gene-related peptide is harmonised. Even more interesting is the finding that nitric oxide, a neurotransmitter and smooth muscle relaxing factor [Lancaster 1992], is released from stimulated nonadrenergic, noncholinergic nerves [Snyder 1992] which are also known to contain tachykinins. Nitric oxide reacts with O_2^- to form the toxic peroxynitrite radical ($ONOO^-$), which can diffuse into tissues before decomposing to form two further potent cytotoxic oxidants, hydroxyl radical and nitrogen dioxide [Beckman 1991]. Thus, it is possible that when nitric oxide and tachykinins are released together, they may act in concert, the nitric oxide potentiating the tachykinin-enhanced neutrophil (superoxide)-mediated tissue injury.

In addition, since tachykinins are rapidly metabolised *in vivo* (chapter 1.9.3), it is important to determine the effects of tachykinin fragments on specific neutrophil function. The studies in this thesis provide clear evidence that not only tachykinins, but also the products of the enzymatic degradation of tachykinins, may play a role in activating or modulating neutrophil function (Chapter 4.3). Moreover, although different SP terminal fragments

demonstrated opposing modulatory effects on neutrophil O_2^- production (Chapter 4.3.4), they had synergistic effects when used together to stimulate neutrophil ADCC (Chapter 4.3.5). This shows, that the activity of one fragment may be modulated by another, and implies that, in any future study, the possible interactions of biologically stable tachykinin fragments with other fragments or tachykinins should be considered, when analysing effects of tachykinins *in vivo*.

Further understanding of the biological significance of the role of tachykinins and their fragments in activating and modulating neutrophil function, requires characterization of the specificity of binding of these neuropeptides to cells. SP antagonists, derived by chemical modification of amino acids that comprise SP, were used in this thesis, but had agonist effects, and therefore were rendered unsuitable (Chapter 3.3.2.5). These antagonists, although used successfully to inhibit substance P binding to lymphocytes [Payan et al, 1984] and SP effects on macrophages [Hartung et al, 1986], are generally thought to possess limited activities and may be easily metabolised by neutrophil enzymes (Chapter 1.9.3). However, in the last year, a number of non-peptide tachykinin receptor antagonists have become available, which promise a new era in tachykinin research [Watling 1992]. These are characterized by both high affinity and selectivity, and, unlike peptide antagonists, are metabolically more stable.

Although the focus of these studies was on effector function activation and the cytotoxic potential of these cells, it is anticipated that other neutrophil functions, such as cytokine production, may also be modulated by tachykinins. Neutrophils have been shown to produce a number of proinflammatory cytokines, including TNFα, IL-1α and β, INFγ and IL-8, which may contribute to inflammation by regulating function of other inflammatory or immune cells [reviewed by Lloyd and Oppenheim, 1992]. Since neutrophils are often the first cells at an inflammatory site and also the

most abundant, they may contribute significantly to the induction and regulation many effector functions of other cells. Substance P and NKA have been shown to stimulate or enhance cytokine production from monocytes and synoviocytes [Lotz et al, 1988; Kimball et al, 1988; Laurenzi et al, 1990; Wagner et al, 1987; Calvo et al, 1992] but their effects on neutrophil cytokine production have not been studied.

8.3 *Involvement of calcium and PKC in substance P priming for enhanced superoxide production*

Although PKC-independent mechanisms have been implicated in neutrophil O_2^- production [Gerard et al, 1986; Watson et al, 1991], the demonstration that DG analogs, such as PMA, that bind and activate PKC may also stimulate O_2^- production, point to the direct involvement of PKC in this process (Chapter 1.6.5). Most of the agonists that stimulate O_2^- production in neutrophils have been shown to induce hydrolysis of membrane phosphatidylinositol, to produce DG and IP_3, which activate PKC (Chapter 1.6.2 and 1.6.3). These studies tested the hypothesis, that SP primes neutrophils by increasing $[Ca^{++}]i$, which induces PKC translocation to the cell membrane and enhances its activity, and that the subsequent facilitation of activation of NADPH oxidase is caused by increased PKC-mediated phosphorylation of its two components, p47-*phox* and p67-*phox*. SP was shown to increase $[Ca^{++}]i$ and that this increase was associated with the facilitation O_2^- production. More information on calcium involvement in the priming phenomenon may be derived from experiments in which calcium-depleted cells are used during the priming phase, that then are calcium repleted for the stimulation with a second stimulus. Depleting cells of calcium, even for a short time, affects the capacity of the cells to produce O_2^-,

but this is highly reversible and should not be a problem in the assessment of SP-induced priming.

In addition, SP alone caused translocation of PKC to cell membrane, as assessed by increased ^{3}H-PDBu binding, however, the SP enhancement of fMLP-stimulated PKC translocation was not essential for enhancement of O_2^- production. This suggests, that SP may increase PKC activity, for example, by increasing PKC affinity for DG or its substrates, and this could be tested using purified PKC preparations. There is also a possibility that SP may affect the PKC-independent pathway leading to O_2^- production, and this could be studied in neutrophils pretreated with PKC inhibitors before stimulating them with a second stimulus.

I have proposed that SP-stimulated increase in $[Ca^{++}]i$ may cause enhanced phosphorylation of p47-*phox* and p67-*phox* components of NADPH oxidase system. Phosphorylation of 47 kD and 67 kD proteins, was demonstrated indirectly using calcium ionophore, thus implicating $[Ca^{++}]i$ in this process. Although this approach did not involve SP directly, and the identity of the 47 kD and 67 kD proteins to be p47-*phox* and p67-*phox* components of NADPH oxidase system was not confirmed (due to lack of a specific antibody), there is a good possibility that SP-induced increases in $[Ca^{++}]i$ may have similar effect and this needs to be pursued further.

8.4 *Are the effects of substance P on neutrophils receptor-mediated?*

This question still remains unanswered, and no firm conclusions can be drawn until the matter is studied further. Although binding of SP to neutrophils has been shown to be time- and dose-dependent, and saturable, the high concentration of unlabelled SP required to demonstrate specific

binding, questions the validity of such studies. On the other hand, if a SP receptor is localised within the membrane hydrophobic core, and the binding of SP to this receptor must be preceded by a non-specific interaction of SP with cell membrane in order to gain an access to the receptor, then the assay system used was inappropriate. One way to identify the presence of such unusual receptors on neutrophils, is to perform SP binding on solubilised cell membranes, that would expose the receptors, or on purified membrane proteins. Other techniques that could be used, may involve cross-linking SP and its receptor on SP affinity columns.

8.5 *Relevance of these studies to the inflammatory process in vivo*

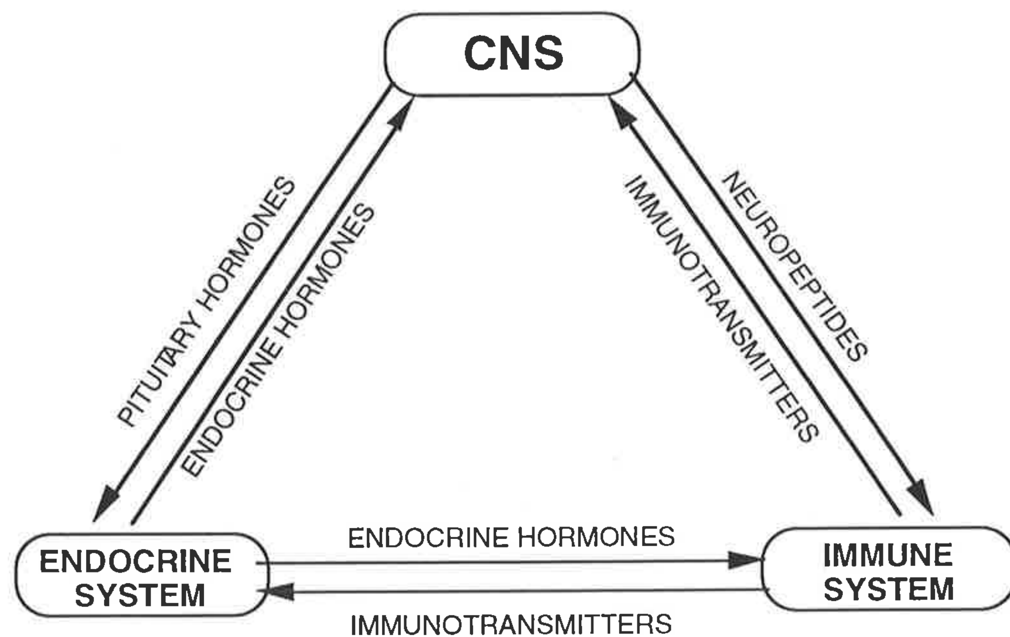
A major unresolved question is whether the effects of tachykinins on neutrophil function occur *in vivo*.

It was found that high concentrations of SP are needed to demonstrate its effects on neutrophils and it is uncertain at the moment if neutrophils are exposed to such concentrations *in vivo*. However, neutrophils may be exposed to high concentrations of tachykinins as they migrate through inflamed innervated tissue (e.g. synovial membrane). This is supported by evidence that in rheumatoid disease, levels of synovial fluid SP as high as 0.2 μM have been measured [MacDonald et al, 1989]. Since synovial fluid SP is originally released from the nerve terminals within the synovial membrane, concentrations considerably higher than 0.2 μM must occur within these inflamed synovial membranes. Furthermore, the migration of neutrophils through the synovial membrane to the synovial fluid coincides with the release of neuropeptides, both of which are early events in the establishment of an inflammatory response. Similarly in asthma, airways inflammation is characterised by cellular infiltration by neutrophils and eosinophils (Chapter 1.7.2) and the SP produced locally by axonal reflexes, and also by eosinophils

and mast cells, may reach sufficient concentrations to affect the function of migrating neutrophils (Chapter 1.9). Furthermore, the concentrations needed to demonstrate an *in vitro* effect under these assay conditions, are similar to those needed to degranulate mast cells *in vitro* [Fewtrell et al, 1982; Shanahan et al, 1985; Lowman et al, 1988].

On the other hand, the results presented in Chapter 7 clearly suggest that SP and other tachykinins may work in concert with other cytokines at inflammatory sites, at concentrations much lower than those shown to be required for a direct effect of each alone on neutrophil effector function, and thus may modulate inflammatory response at concentrations regarded as physiological. As only two cytokines, GM-CSF and IL-8, were studied and no work has been published on other cytokine-tachykinin interactions, this field may be regarded as completely unexplored, but certainly worth studying. After all, inflammation could be defined as a process of very complex interactions between many different cells via adherence and a number of released mediators. Some people argue, and for good reasons, that the action of many of these mediators should be studied and analysed taking into account the extracellular milieu of the responding cells, as there is evidence that the response of cells to cytokines can be markedly affected by the extracellular matrix in which most cells are normally embedded [Nathan and Sporn, 1991]. Further support for the cytokine-neuropeptide interactions come from the studies of cytokine-induced SP expression [Freidin and Kessler, 1991] and cytokine regulation of SP receptor numbers [Johnson and Johnson, 1991].

The interactions between the nervous and the immune systems form only a part of a greater network of interactions, which also involve the endocrine system, as illustrated below.



Reprinted from Khansari et al, 1990.

There is now a considerable evidence for the involvement of the endocrine system in stress-induced immunosuppression. It has been known for many years that patients suffering from psychological stress or psychiatric illness are more susceptible to disease [Jankovic et al, 1987]. These observations have led to considerable research into the mechanisms responsible for stress-induced immunosuppression. A variety of stresses (e.g. fear, cold, heat or physical injury) result in stimulation of the adrenal glands and the release of adrenocorticoid hormones. Subsequent studies demonstrated that endogenous corticosteroids may play a role in limiting the magnitude of an immune response and preventing it from reaching a level that is potentially damaging to the host [Bernton et al, 1987; Sapolsky et al, 1987; Berkenbosch et al, 1987]. Convincing evidence was presented in support of this idea by the demonstration that adrenalectomized, but otherwise normal rats, immunised with Freud's complete adjuvant died within 24-48 hours, but survived if normal levels of corticosterone were maintained by intravenous injection [MacPhee et al, 1989]. Overexpression

of IL-1 and TNFα was thought to be the cause of death of the adrenalectomized rats, as injection of these cytokines was shown to be fatal for these animals [Bertini et al, 1988]. These studies clearly demonstrated that: (i) an uncontrolled immune response is lethal to the host, (ii) an immune response is recognised by the host as stress, (iii) neuroendocrine feedback mechanisms exist that prevent at least some immune responses reaching potentially damaging levels and (iv) corticosteroids have potential immunomodulating properties.

A number of studies have demonstrated the presence of specific receptors for neuroendocrine factors on immune cell. For example, ACTH, growth hormone, steroid hormones, catecholamines, acetylocholine and a variety of hormone releasing factors. Opioid receptors have been found on lymphocytes, granulocytes, monocytes and platelets. The interaction of these factors with their receptors on immunocompetent cells could lead to either cell activation, modulation of the cell responses to other agonists or changes in cytokine production, which together may have profound effects not only on the magnitude of the responses but also on the type of the response. This has been illustrated in the rat model of experimental allergic encephalomyelitis (EAE) [Mason 1991]. Using this model, evidence was presented suggesting that corticosteroids not only induce immunosuppression in favour of the animal, but also induce a change from cell-mediated immune responses to humoral ones. In support of this observation, others have also demonstrated that corticosteroids induce IL-4 and inhibit IL-2 production [Daynes and Araneo, 1989], a cytokine profile that would promote humoral response, and that corticosteroids promote antibody production [Akahoshi et al, 1988].

In addition, a number of hormones and neurotransmitters have been shown to be produced by cells of immune system (e.g. that eosinophils

synthesise SP), and it has been suggested that these factors act as kinins, transmitting signals between immune cells.

Thus the nervous system, the immune and the endocrine system interact with each other in a complex manner. The experimental results presented in this thesis, provide support for the existence of one arm of this interactions - that between the nervous system and the immune system.

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NOTE:

This publication is included in the print copy
of the thesis held in the University of Adelaide Library.

Wozniak, A., Scicchitano, R., Betts, W. H. & McLennan, G. (1992). The effect of Substance P on neutrophil function in normal and asthmatic subjects. *Annals of the New York Academy of Sciences, 650*, 154–159.

NOTE:
This publication is included in the print copy
of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:
http://dx.doi.org/10.1111/j.1749-6632.1992.tb49113.x

ACTIVATION OF HUMAN NEUTROPHILS BY TACHYKININS: Effect on *formyl*-methionyl-leucyl-phenylalanine- and platelet activating factor-stimulated superoxide anion production and antibody-dependent cell-mediated cytotoxicity.

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SUMMARY

This study examines the contribution of tachykinins to the processes of inflammation. Neurokinin A (NKA), neurokinin B (NKB) and eledoisin (E) but not kassinin (K) have similar effects to substance P (SP) in priming neutrophils for increased superoxide anion (O_2^-) production in response to *formyl*-methionyl-leucyl-phenylalanine (fMLP). This similarity in activity may be due to the carboxy amino acid terminal end of these tachykinins being highly conserved. This was confirmed by demonstrating that SP fragment 7-11 (SP_{7-11}) had the same priming effect as the whole molecule, whereas, the amino end fragment 1-4 (SP_{1-4}) inhibited the response to fMLP. The priming effect of tachykinins was not confined to a single stimulus, such as fMLP, since NKA, NKB and SP also enhanced O_2^- production stimulated by platelet activating factor, an important mediator of inflammation but a weak stimulus of O_2^- production on its own. In addition, all the tachykinins studied increased neutrophil antibody-dependent cell-mediated cytotoxicity (ADCC) towards opsonised target cells. In contrast to their effects on fMLP-induced O_2^- production, both SP fragments, SP_{1-4} and SP_{7-11}, stimulated neutrophil ADCC and had a synergistic effect when used together.

INTRODUCTION

The tachykinins are a family of neuropeptides, which are putative neurotransmitters in the central and peripheral nervous systems. They are defined by a common C-terminal amino acid sequence of phe-X-gly-leu-met-NH_2, where X is an aliphatic or aromatic amino acid, and an ability to cause rapid contraction of a variety of smooth muscle types. Three mammalian tachykinins have been described to date; substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). In addition, there are amino terminal (neuropeptide K and neuropeptide γ) and truncated (NKA_{3-10}) derivatives of NKA [1]. A number of non-mammalian tachykinins have also been isolated including eledoisin (E) and kassinin (K). Kassinin is the homologue of NKA as both have identical C-terminal pentapeptide sequences.

Substance P, the first of these peptides described [2], has a variety of physiological actions such as smooth muscle contraction, vascular dilatation and plasma extravasation [3], and has been implicated as a mediator of neurogenic inflammation. This conclusion is supported by a number of studies which have shown that SP has stimulatory effects on a variety of immune and inflammatory cells including lymphocytes [4-6], macrophages and monocytes [7-11], mast cells [12-14] and neutrophils [15-22]. Previously, we have shown that SP primes human neutrophils to produce increased amounts of superoxide anion (O_2^-) and leukotriene B_4 in response to the synthetic tripeptide *formyl*-methionyl-leucyl-phenylalanine (fMLP) and stimulates neutrophil antibody-dependent cell-mediated cytotoxicity (ADCC) [20]. Information on the effects of the other tachykinins on inflammatory cells is limited [22,23], and their potential role in neurogenic inflammation is largely unknown. Since several of these tachykinins colocalize in sensory afferents, it is possible that some of the actions ascribed to SP from *in vivo* studies may in fact be due to other peptides. Furthermore, there are several en-

zymes which metabolise SP to smaller fragments [24], whose effects on neutrophils may be either stimulatory or inhibitory [17].

To test the hypothesis that tachykinins other than SP, as well as SP metabolic fragments, may regulate human neutrophil function, we studied their effects on neutrophil O_2^- production and ADCC. We report that mammalian tachykinins, NKA and NKB as well as SP, directly stimulate human neutrophil ADCC or prime neutrophils for an enhanced response to a second stimulus such as fMLP or platelet activating factor (PAF). We also show that SP metabolic fragments have diverse effects and can either prime, activate or inhibit neutrophils depending on the cell function and fragment tested.

MATERIALS AND METHODS

Peptides

All peptides were purchased from AUSPEP, Melbourne, Australia. Stock solutions of 1 mM were made up in 10 mM acetic acid and aliquots were stored under nitrogen at -70°C and thawed only once just before use.

Isolation of human neutrophils

Neutrophils were isolated from the peripheral blood of normal volunteers after informed consent. A leucocyte fraction was obtained by sedimenting the erythrocytes with dextran (Dextran T-500; Pharmacia, Uppsala, Sweden). Neutrophils were isolated by density-gradient centrifugation (400 g, 20 min, 22°C) using Lymphoprep (NYCOMED AS, Oslo, Norway) and hypotonic lysis of residual erythrocytes. The cells were always >96% pure, as determined by Grunwald-Giemsa staining, and >98% viable by trypan blue exclusion. Neutrophils were resuspended either in RPMI-1640 medium supplemented with 20 mM HEPES (Gibco, Grand Island, NY), 2 mM L-glutamine, 60 µg/ml penicillin and 8 µg/ml gentamycin for the ADCC assay, or in a modified Dulbecco's phosphate buffered saline (DPBS) containing 138 mM NaCl, 2.7 mM KCl, 16.2 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.5 mM $MgSO_4$, 0.6 mM $CaCl_2$ and 7.5 mM glucose, pH 7.3, for O_2^- measurement.

Superoxide anion production

Generation of O_2^- was assessed as superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C. This was determined by the addition of 10 µl of 2 mg/ml SOD (Boehringer Mannheim, GMbH, West Germany) to duplicate samples in all experiments. Neutrophils (10^6) were incubated in triplicate with 100 µM cytochrome C (Calbiochem) in medium containing the varying concentrations of peptides (0.01 µM to 100 µM) in a final volume of 1.0 ml. Since the peptides were dissolved in acetic acid, our medium

control included the highest possible concentration (1 mM) of acetic acid. This did not have any effect on fMLP-stimulated O_2^- production. Cells were incubated for 30 min at 37°C and then either fMLP (0.1 µM final concentration), PAF (1 mM final) or medium was added and the mixture incubated for a further 6 min. The reaction was stopped by addition of SOD (10 µl of 2 mg/ml) and by placing tubes on ice. The cells were pelleted by centrifugation at 4°C and O_2^- production was quantified in cell supernatants by changes in absorption at 550 nm [25].

Antibody dependent cell-mediated cytotoxicity

The method has been described in detail previously [26]. All experiments were performed in triplicate, and SD's within an experiment were < 10%. The assay was performed in RPMI-1640 containing 0.1% bovine serum albumin. Briefly, 40 µl (4×10^3) of ^{51}Cr-labelled trinitrophenyl (TNP)-coupled P815 target cells (DBA/2 mastocytoma) were mixed with 80 µl (1.2×10^5) neutrophils as effector cells, and 24 µl of rabbit IgG anti-TNP antibody (Miles-Yeda, Rehovot, Israel) and 16 µl of the appropriate peptide in V-bottomed microtitre plates. The final assay volume was 160 µl. After incubation of the reaction mixture for 2.5 hr at 37°C, 80 µl of the supernatant were removed and the radioactivity counted using a gamma counter (LKB, 1282 Commugamma, Turku, Finland). Percentage cytotoxicity was calculated as follows:-

$$\%\ \text{cytotoxicity} = \frac{\text{experimental c.p.m. - spontaneous release c.p.m.}}{\text{total c.p.m. - spontaneous release c.p.m.}}$$

where spontaneous release was the ^{51}Cr released from P815 cells in the presence of medium alone and the total count was the ^{51}Cr released from the P815 cells lysed by the addition of 4% Triton X-100. Conditioned medium (CM) from the bladder carcinoma cell line U5637 was used as a positive control, since it contains factors which maximise human neutrophil ADCC [20].

Tachykinins (at the highest concentrations used) did not affect neutrophil viability under the different experimental conditions as demonstrated by a negative effect on trypan blue exclusion. Furthermore, all tachykinins were shown to be endotoxin free by the *Limulus* amoebocyte lysate assay.

Statistics

Data were analysed by Student's t-test for paired data and ANOVA, using a Macintosh computer and the Statview II^{TM} statistics package. Experiments were performed in triplicate, and the number of experiments is indicated in the figure legends. Results are expressed as mean ± SEM.

RESULTS

The effect of tachykinins on fMLP-stimulated superoxide anion production.

As previously reported by us [20], SP had no direct effect on neutrophil O_2^- production alone, but primed them for an increased O_2^- production in response to fMLP. To determine, whether other tachykinins could influence fMLP-stimulated O_2^- production, neutrophils were incubated with each tachykinin for 30 min., and then stimulated with 0.1 μM fMLP. None of the tachykinins (0.01 to 100 μM) studied stimulated significant O_2^- production on its own (data not shown). Preincubation of neutrophils with either SP, NKA, NKB or E enhanced the response to fMLP in a dose-dependent manner (Figs. 1A and 1B). A maximum effect was observed at 100 μM for SP, NKA and E , when production increased from 12.7 ± 3.1 to 28.8 ± 5.1 ($p < 0.01$), 23.3 ± 3.7 ($p < 0.02$) and 21.1 ± 3.6 ($p < 0.02$) nmol/10^6 cells respectively. Kassinin did not have any effect on neutrophil fMLP-stimulated O_2^- production (Fig. 1A). Although NKB significantly enhanced the response to fMLP at lower concentrations than the other tachykinins, the effect was reduced at 100 μM (Fig.1B). For example 5 μM NKB increased O_2^- production from 15.3 ± 2.1 to 27.9 ± 1.7 nmol/10^6 cells ($p < 0.001$), had its maximum effect at 50 μM (36.8 ± 5.1; $p < 0.02$), and a reduced effect at 100 μM (20.5 ± 4.2; $p < 0.04$).

The effect of SP fragments on fMLP-stimulated superoxide anion production.

The activities of SP_{1-4} and SP_{7-11} on fMLP-induced neutrophil O_2^- production are compared in Fig. 2. SP and SP_{7-11} had similar effects in that they primed for an enhanced response in a dose-dependent fashion. For example, SP and SP_{7-11} increased O_2^- production maximally from 10.7 ± 1.6 to 25.0 ± 4.7 ($p < 0.03$) and from 25.6 ± 5.2 ($p < 0.03$) nmol/10^6 cells respectively. In contrast, SP_{1-4} at concentrations above 10 μM (Fig. 2) decreased the response by as much as 77%, to 2.5 ± 1.2 nmol/10^6 cells ($p < 0.01$).

The effect of tachykinins on PAF-stimulated superoxide anion production.

As illustrated in Fig. 3, tachykinins also enhanced PAF-stimulated O_2^- production. Here, cells were incubated for 30 min with medium or 50 μM NKA, SP or NKB and then stimulated with 1 μM PAF (for 6 min.). O_2^- production increased from 2.3 ± 0.4 to 5.8 ± 0.7 ($p < 0.002$) for NKA, to 8.4 ± 1.5 ($p < 0.008$) for SP and 17.5 ± 3.9 ($p < 0.02$) nmol/10^6 cells for NKB. This priming effect of NKB was to increase O_2^- production to 766 ± 116% of control.

The effect of tachykinins on neutrophil antibody-dependent cell-mediated cytotoxicity.

We have previously reported that SP directly stimulates neutrophil ADCC in a dose-dependent fashion [20]. Here, we studied the effect of other tachykinins, using an antibody concentration of 1 μg/ml, which we found previously to be suboptimal for SP stimulation. As illustrated in Fig. 4A, in the presence of antibody alone (medium control), neutrophils killed 15-19% of target cells. Although the tachykinins had no direct cytotoxic effect on the target cells (e.g., in the absence of either neutrophils or antibody), they stimulated ADCC dose-dependently within the range of 1-100 μM. We compared the maximal responses at 100 μM and the results are shown in Fig. 4B, expressed as *net*-ADCC (calculated as ADCC in presence of stimulus minus spontaneous ADCC in the presence of medium alone). NKB stimulated neutrophil *net*-ADCC was 31.4 ± 1.0%. NKA- and E-induced *net*-ADCC was 12.9 ± 1.8 and 12.8 ± 2.6% respectively, which was similar to the value for SP (15.5 ± 2.5%). Kassinin was the weakest stimulus of all tachykinins and *net*-ADCC was only 6.3 ± 2.8%.

The effect of SP fragments on neutrophil antibody-dependent cell-mediated cytotoxicity.

In the next series of experiments, we compared the effects of SP, SP_{7-11} and SP_{1-4} on ADCC. Again we used a suboptimal concentration of antibody of 1 µg/ml. Both fragments stimulated a dose-dependent increase in ADCC in the range of 1 to 100 µM, and exerted their maximal effect at 100 µM (data not shown). As shown in Fig. 5, *net*-ADCC with 100 µM SP was 12.7 ± 2.2%. *Net* ADCC with 100 µM SP_{7-11} was 16.7 ± 1.2%, which was not significantly different from that of SP. SP_{1-4} (100 µM) increased ADCC to a lesser extent (net ADCC was 3.3 ± 1.0% $p < 0.03$).

When cells were stimulated with both fragments simultaneously, there was a synergistic increase in *net*-ADCC in that their combined effect was greater than the sum of each fragment (e.g., *net*-ADCC was 26.2 ± 2.5 compared to 20.0 ± 1.5 ($p < 0.04$).

Discussion

The present study provides evidence for a role of tachykinins in the modulation of different neutrophil functions. Our results extend our earlier findings [20] on the priming and direct effects of SP on neutrophil function, and demonstrate that other mammalian (e.g., NKA and NKB) and non-mammalian tachykinins (e.g., eledoisin but not kassinin) and SP fragments have similar modulating effects. For example, we demonstrated that although NKA, NKB and E had no direct effect on neutrophil O_2^- production, they did facilitate the response to fMLP. Although NKB produced its effects at slightly lower concentrations than the other tachykinins, at the highest concentration tested there was a reduction in the stimulation, which was not observed with the other tachykinins. Similarly, SP, NKA and NKB facilitated neutrophil O_2^- production in response to PAF, with NKB being the most potent. All the tachykinins tested increased neutrophil ADCC towards opsonised target cells, and again NKB was the most potent.

The stimulatory effect of NKB on neutrophil function is not in agreement with Brunelleschi et al [23], who found NKB to be inactive in priming human neutrophils for increased responses to both fMLP and PAF. There may be several reasons for this discrepancy. Firstly, in the experiments of Brunelleschi and coworkers the cells were primed with the tachykinins for only 3 min compared to our 30 min, and we have shown that longer preincubation times are required for SP [20] and other tachykinins to exert their effects (data not shown). Secondly, they dissolved NKB in DMSO rather than water or dilute acetic acid. Although DMSO does not interfere with fMLP- or PAF-stimulated O_2^- production, it is possible that low concentrations of DSMO (0.1%) inhibit the priming activity of NKB.

We found that high concentrations of tachykinins are needed to facilitate neutrophil responses. However, neutrophils may be ex-

posed to high concentrations of tachykinins as they migrate through inflamed innervated tissue (e.g. synovial membrane). This is supported by evidence that in rheumatoid disease, levels of synovial fluid SP as high as 0.2 μM have been measured [27]. Since synovial fluid SP is originally released from the nerve terminals within the synovial membrane, concentrations considerably higher than 0.2 μM must occur within these inflamed synovial membranes. Furthermore, the migration of neutrophils through the synovial membrane to the synovial fluid coincides with the release of neuropeptides, both of which are early events in the establishment of an inflammatory response. Similarly in asthma, airways inflammation is characterised by cellular infiltration by neutrophils and eosinophils and the SP produced locally by axonal reflexes, and also eosinophils and mast cells, may reach sufficient concentrations to affect the function of migrating neutrophils. Furthermore, the concentrations needed to demonstrate an *in vitro* effect under our assay conditions, are similar to those needed to degranulate mast cells *in vitro* [28].

A common feature of all tachykinins is their highly conserved carboxy terminal and it has been hypothesized that the carboxy terminal determines the priming activity observed. For example, the carboxy terminal appears to be largely responsible for the stimulatory effect of SP on lymphocyte proliferation [6]. Similarly the carboxy end of SP has been found to induce chemotaxis, increase cytosolic free Ca^{2+} concentration and oxidative metabolism in neutrophils [16, 21]. In our study, SP_{7-11} (10-100 μM) but not SP_{1-4}, enhanced O_2^- production by neutrophils stimulated with 0.1 μM fMLP in the same manner as did SP_{1-11}. Similarly, Hafstrom et al [17] demonstrated that the carboxy end of SP (SP_{7-11}) but not the amino end (SP_{1-4}) stimulated neutrophil oxidative metabolism (as measured by chemiluminescence) and aggregation. Thus, the priming activity of tachykinins on neutrophils appears to be associated with the common carboxy end.

However, the inability of kassinin to prime neutrophils as well as the different potencies of the other tachykinins tested, suggests an additional role for the amino terminus in modulating this activity. This was further confirmed by the observation that SP_{1-4} inhibited the fMLP stimulated neutrophil O_2^- production. Similarly, the finding that both SP_{7-11} and SP_{1-4} stimulated ADCC (as well as the facilitation observed with these fragments together) suggests that tachykinin activity is not solely determined by carboxy end alone.

However, it should be noted that, in contrast to the above findings, degranulation of mast cells has been shown to be determined by the amino terminus of SP (e.g. SP_{1-4} was active but not SP_{7-11}), and that deletion of two amino acids from the carboxy terminal modulated the activity of SP – reducing the activity by 30% [12,13].

The diverse effects of SP fragments on fMLP-stimulated O_2^- production and ADCC, suggest alternative mechanisms by which tachykinins may modulate inflammation. In addition, their potential effects on inflammatory cells may further be modified by cytokines and other cell agonists that are released during inflammation. Although this study and others [15,17,21] have evaluated the effects of SP fragments on neutrophil function, enzymes that are capable of cleaving the other tachykinins have been identified on neutrophils and their effects remain to be studied.

With the exception of the effect of 100 μM NKB on neutrophil O_2^- production, we observed no biphasic responses (e.g., stimulation followed by inhibition at higher concentrations) of any of the tachykinins tested. This is contrast to the results of Hafstrom [17] who observed that concentrations of SP and SP_{7-11} greater than 10 μM inhibited the priming effect on neutrophil O_2^- production, an effect that was overcome by increasing fMLP concentration. These differences and other differences may result from the different

techniques used to quantify oxy radical production. In the studies of Hafstrom, total oxy radical production rather than O_2^- production, was monitored by luminol-amplified chemiluminescence. This method is non-specific as luminol may be oxidised by many different oxy radicals including H_2O_2, and $^1O_2$ and oxidants such as hypochlorous acid [29-31]. Furthermore, the chemiluminescence assay is very sensitive to minor changes in pH and alterations in buffer components (e.g. antioxidants and albumin). In contrast, the assay system we used, the superoxide dismutase-inhibitable reduction of ferricytochrome C, is specific for O_2^-.

Since many tachykinins have been shown to colocalise in the same sensory fibres, and SP and NKA and its derivatives are products of the same gene and arise by alternative RNA splicing and post-translational modification [1, 32], it would be of great interest to study the effects of multiple peptide stimulation on neutrophil function and activity. These, we believe, would provide important new information for assessment of the role of tachykinins in inflammation.

ACKNOWLEDGEMENTS

This work was supported by National Health and Medical Research Council of Australia, Royal Adelaide Hospital Special Purposes Fund, The Queen Elizabeth Hospital Research Foundation and Arthritis Foundation of Australia.

ABBREVIATIONS

ADCC:	antibody-dependent cell-mediated cytotoxicity
E:	eledoisin
fMLP	*formyl*-methionyl-leucyl-phenylalanine
K:	kassinin
NKA:	neurokinin A
NKB:	neurokinin B
PAF:	platelet activating factor
SP:	substance P
O_2^-:	superoxide anion

FIGURE LEGENDS

Fig. 1 Effect of tachykinins (A) and NKB (B) on fMLP-stimulated O_2^- production. Neutrophils were preincubated with medium or tachykinins for 30 min at 37°C and were then stimulated with 0.1 μM fMLP for an additional 6 min. Values represent means of five experiments in A and three experiments in B. SEM are not shown in Fig. 1A for better graph clarity.

Fig. 2 Effect of Substance P fragments on fMLP-stimulated O_2^- production. As in Fig. 1, cells were preincubated with SP_{1-4}, SP_{7-11} or SP for 30 min before fMLP stimulation. Values represent means of three experiments.

Fig. 3 Effect of tachykinins on PAF-stimulated O_2^- production. Neutrophils were preincubated for 30 min with medium or 50 μM NKA, SP or NKB and then stimulated for an additional 6 min with 1 μM PAF. For comparison, results are shown for cells that were stimulated with 0.1 μM fMLP alone. Values represent means of four experiments.

Fig. 4 (A) Effect of tachykinins on neutrophil ADCC. Neutrophils were incubated for 2.5 hrs with target cells, antibody and various concentrations of tachykinins. Bladder carcinoma cell line U5637 conditioned medium (CM) was used as a positive control. Data are means of three experiments.
(B) Effect of tachykinins (100 μM) on *net*-ADCC (stimulated minus baseline responses). Values are derived from Fig. 4A, and are means of three experiments.

Fig. 5 Effect of SP_{7-11} and SP_{1-4} on neutrophil *net*-ADCC. Cells were incubated with 100 μM SP_{7-11} or SP_{1-4} added separately or together. SP was used as a positive control. Values are means of three experiments.

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Figure 1

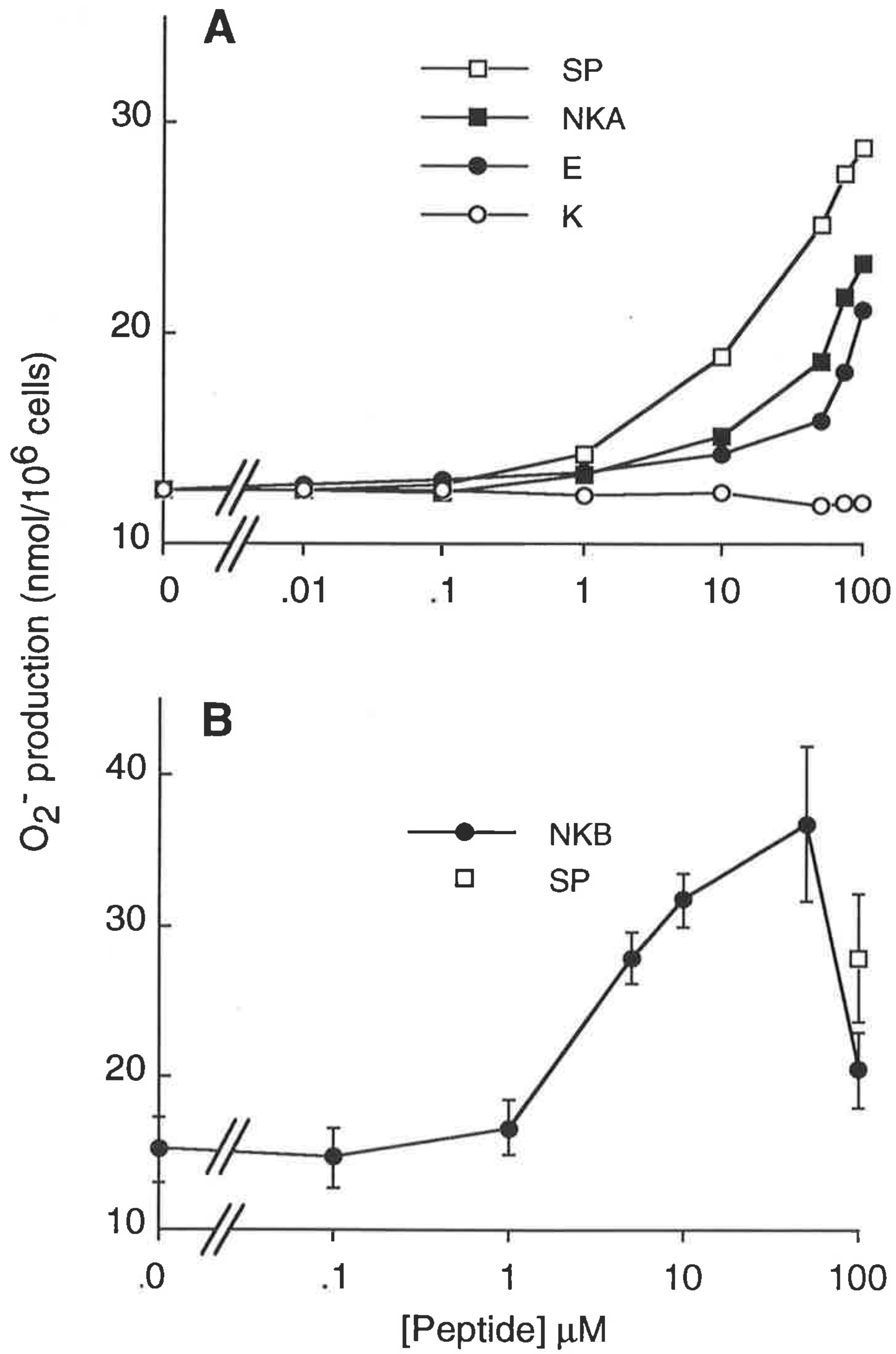


Figure 2

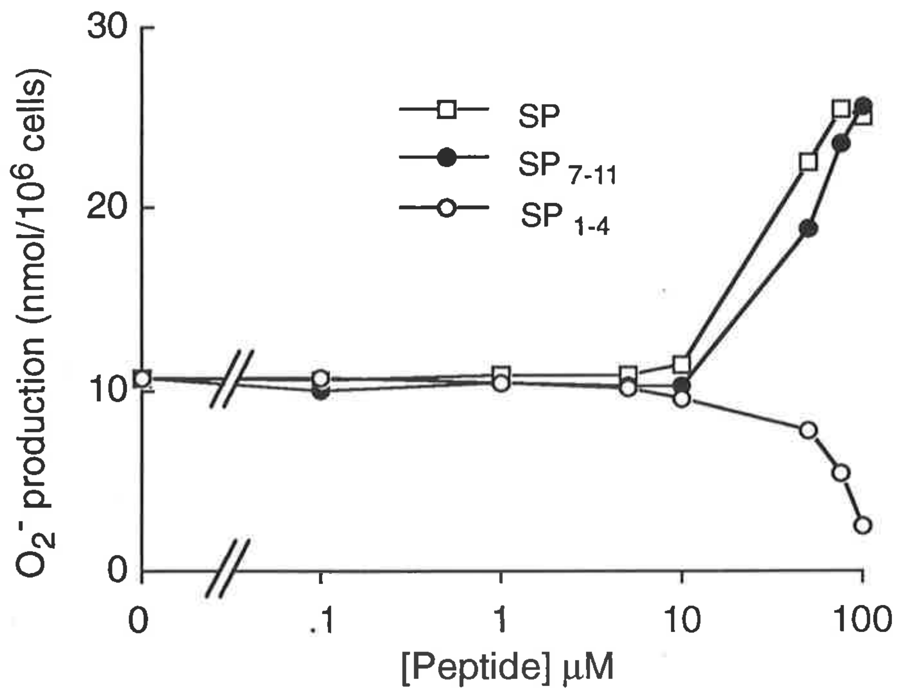


Figure 3

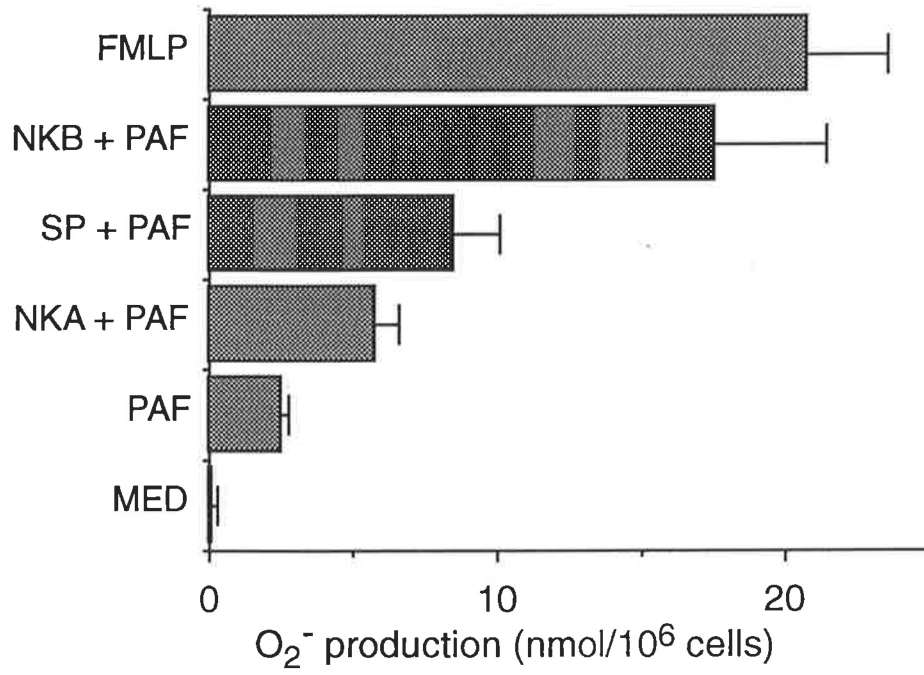


Figure 4A

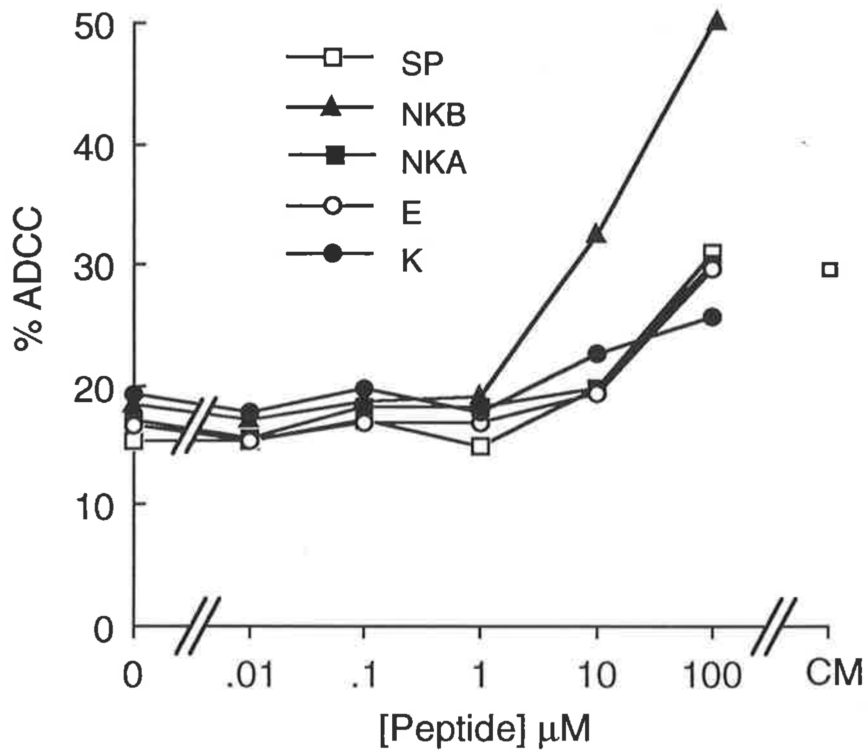


Figure 4B

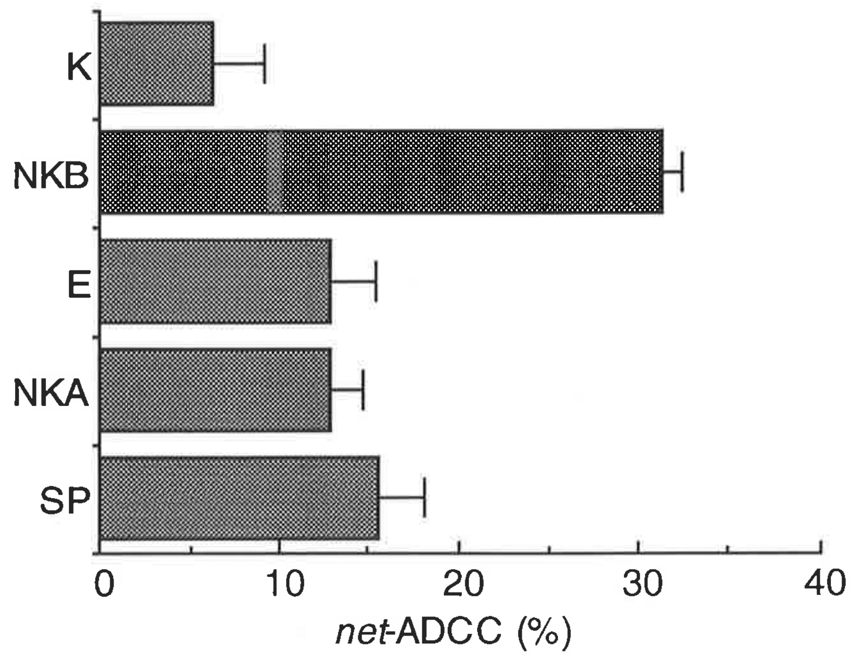
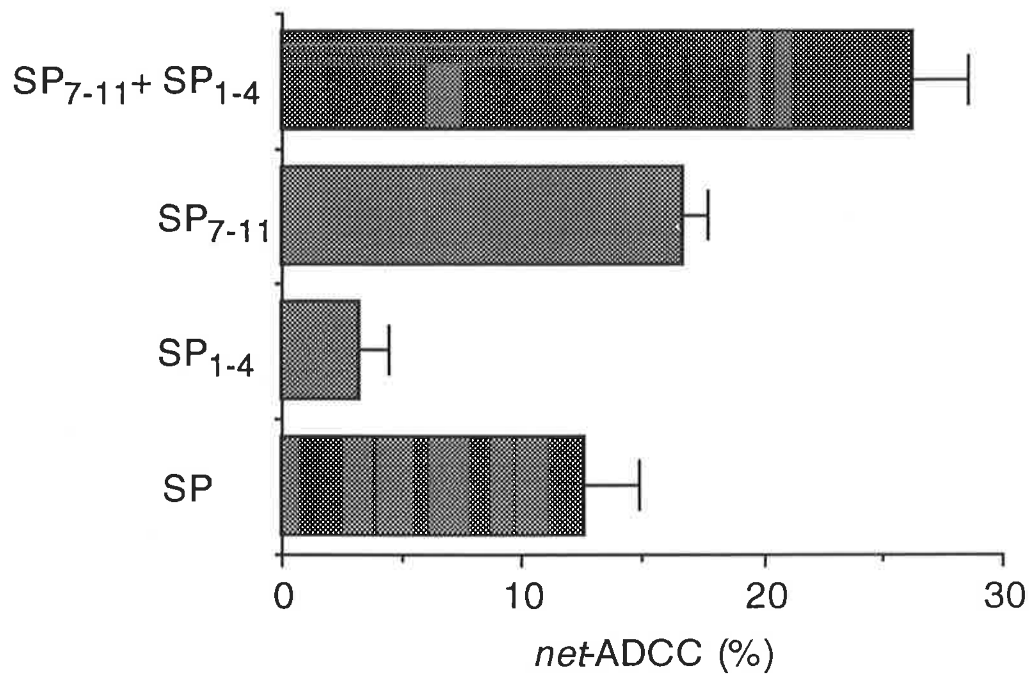


Figure 5



INTERLEUKIN-8 PRIMES HUMAN NEUTROPHILS FOR ENHANCED SUPEROXIDE ANION PRODUCTION

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The running title: **IL-8 enhances oxy radical production by human neutrophils**

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Summary

Interleukin-8, a novel chemotactic cytokine, has been shown to play an important role in inflammation. In this study, we investigated the effect of recombinant human interleukin-8 (rhIL-8) on superoxide (O_2^-) production by neutrophils. We found that rhIL-8 (1-10 ng/ml) did not stimulate neutrophil O_2^- production on its own, but primed neutrophils for an enhanced response to other stimuli, such as N-*formyl*-methionyl-leucyl-phenylalanine (fMLP), phorbol myristate acetate (PMA) and platelet activating factor (PAF). The priming effect of rhIL-8 was dose dependent, rapid and long lasting. rhIL-8 increased both the maximal rate and the total O_2^- production, but did not prolong the response to fMLP. Stimulation of neutrophils with rhIL-8 increased intracellular free calcium concentration ($[Ca^{2+}]_i$) by mobilizing calcium from internal stores and by increasing calcium influx. The increase in $[Ca^{2+}]_i$ was dose dependent and occurred in the same range of rhIL-8 concentrations that primed neutrophils for O_2^- production. In addition, rhIL-8 enhanced the fMLP-stimulated increase in $[Ca^{2+}]_i$. These observations suggest that calcium may play an important role in the priming phenomenon. Although, the role of IL-8 in inflammatory processes remains to be defined, elevated levels of IL-8 found in synovial fluids of patients with rheumatoid arthritis (RA) and our present findings collectively suggest that IL-8 may contribute to the pathogenesis of RA by increasing synovial neutrophils oxy radical production.

Introduction

An elevated number of neutrophils in synovium is a common feature of inflamed synovial joints of patients with rheumatoid arthritis (RA). Increased activity of neutrophils, in particular enhanced oxy radical generation and release of lysosomal enzymes, contributes to the joint destruction. A novel chemotactic cytokine, interleukin-8 (IL-8), capable of attracting neutrophils to the joints and activating their specific functions, may play a significant role in neutrophil-mediated tissue damage in RA.

There is increasing evidence for a role of IL-8 in the pathogenesis of RA. Elevated levels of IL-8 have been found in the synovial fluid of patients with RA [1,2], and gout [3] but not in patients with systemic lupus erythematosus [2]. In the RA patient group there was a close correlation between the synovial fluid IL-8 concentration, the levels of C-reactive protein and synovial neutrophil numbers [2]. Furthermore, spontaneous production of IL-8 from cultured synovial cells [1], peripheral blood mononuclear cells, and synovial fluid mononuclear cells from patients with RA (but not normal controls) has been demonstrated [4].

The action of IL-8 is pleiotropic in nature. For example, *in vitro* it has been shown to (i) be chemotactic for neutrophils and lymphocytes [5,6], (ii) degranulate PMN [7], (iii) augment N-*formyl*-methionyl-leucyl-phenylalanine (fMLP)-stimulated release of platelet activating factor (PAF) by neutrophils, (iv) enhance arachidonic acid release from neutrophil phospholipids, (v) enhance neutrophil superoxide (O_2^-) production [8,9], (vi) enhance neutrophil phagocytosis and (vii) increase neutrophil CR1 and CR3 receptor expression [10]. It should be noted that many of these

effects are not seen with IL-8 alone, but in conjunction with a second stimulus of cell activity.

Neutrophil oxidative metabolism plays an important role in host defence system but exaggerated or prolonged production of oxy radicals may lead to substantial tissue damage, as is the case of the rheumatoid joint. Oxy radical production by activated neutrophils may be enhanced by various cytokines and mediators. For example, granulocyte-macrophage colony stimulating factor (GM-CSF) [11,12], tumour necrosis factor (TNF) [13] and more recently IL-8 [8,9] have all been shown to prime neutrophils for enhanced oxy radical production. The facilitating effect of IL-8 is of particular importance as, unlike GM-CSF and TNF, it causes rapid and prolonged neutrophils infiltration into tissue sites [14,15] and thus may further increase oxy radical production at inflammatory sites by increasing the numbers of cells.

However, the priming effect of IL-8 remains controversial, as some investigators have failed to demonstrate an effect of rhIL-8 [16]. In view of these conflicting reports, we investigated the priming effect of rhIL-8 on neutrophil O_2^- production in response to fMLP, PMA and PAF, and have shown that rhIL-8 enhances O_2^- release by all of the above stimuli. Furthermore, we have demonstrated that rhIL-8 facilitates the fMLP-stimulated increase in intracellular free calcium, suggesting a further mechanism for the rhIL-8 priming.

Materials and Methods

Materials. Recombinant human interleukin-8 (rhIL-8), essentially endotoxin free (less than 0.1 ng/µg), was purchased from Promega (Sydney, Australia). The lyophilized rhIL-8 was reconstituted in PBS to a concentration of 10 µg/ml and stored in 50 µl aliquots at -70°C and thawed only once before use. Cytochrome C (type IV, horse heart), N-formyl-methionyl-leucyl-phenylalanine, phorbol myristate acetate, platelet activating factor, luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione) and RPMI 1640 (without phenol red) were purchased from Sigma (Sigma Chemicals, USA). Dextran T-500 was purchased from Pharmacia (Uppsala, Sweden), Lymphoprep from NYCOMED AS (Oslo, Norway), HEPES from Gibco (Grand Island, NY), Fura 2 AM from Calbiochem (Calbiochem, USA) and superoxide dismutase (SOD) from Boehringer Mannheim (Mannheim, Germany).

*Isolation of human neutrophils*Peripheral blood was obtained from normal volunteers and was anticoagulated with EDTA. The leukocyte-rich fraction was obtained by sedimenting the erythrocytes with 5% dextran for 40 min at room temperature. Neutrophils were isolated by density-gradient centrifugation (400 g, 20 min, 22°C) using Lymphoprep followed by hypotonic lysis of residual erythrocytes. The cells were always >96% pure, as determined by Grunwald-Giemsa staining, and >98% viable by trypan blue exclusion. Neutrophils were resuspended in RPMI-1640 medium supplemented with 20 mM HEPES, 2 mM L-glutamine, 60 µg/ml penicillin and 8 µg/ml gentamycin.

Superoxide anion production. Neutrophil O_2^- production was measured as SOD-inhibitable reduction of ferricytochrome C (Cyt C). This was determined by the addition of 10 µl of 2 mg/ml SOD to duplicate samples in all experiments. Measurements were made in real time to enable determination of rates of production of O_2^-, and by end point, to enable calculation of total O_2^- production by any given stimulus/manipulation as described below. All end point experiments were performed in triplicate. The final concentration of cells in all experiments was adjusted to 10^6/ml.

In the initial dose-response experiments, neutrophils (5 x 10^6 cell/ml) were incubated with medium or the stated concentration of rhIL-8 (1-50 ng/ml) for 10 min at 37°C. 200 µl of cells were transferred to a tube containing 800 µl of 100 µM Cyt C and medium or different concentrations (1, 5, 10, 100 nM) of fMLP. The mixture was incubated for a further 10 min. The reaction was stopped by addition of SOD and by immersion of the tubes in ice. The cells were pelleted by centrifugation at 4°C. O_2^-production was quantified in cell supernatants by changes in absorption at 550 nm [17] and O_2^- production determined using an extinction coefficient of 21.1 mM^{-1} cm^{-1} [18].

In the time-course experiments, PMN were incubated with 10 ng/ml rhIL-8 or medium at 37°C, and 200 µl (10^6 cells) was removed at stated times for stimulation with 100 nM fMLP, as in the dose-response experiments. The reaction was stopped and the measurements performed as outlined above.

The kinetics of O_2^- production were measured by following O.D. changes continuously in a thermostated (37°C) spectrophotometer. neutrophils (5 x 10^6 cells) were preincubated with 10 ng/ml rhIL-8 or medium as control, for 10 min at 37°C, then 200 µl of cells (10^6)

transferred to cuvettes in the spectrophotometer containing 800 µl of prewarmed mixture containing 100 µM Cyt C and a stimulus (e.g., fMLP or PMA), and changes in O.D. at 550 nm were monitored.

Chemiluminescence. Luminol-enhanced chemiluminescence (CL) was employed as another method to assess respiratory burst (NADPH oxidase activity) using a Packard Pico Lite chemiluminometer. Before each experiment, luminol (20 µM) was mixed with prewarmed neutrophils (250 µl, 0.5 x 10^6 cells) and the mixture was preincubated for 7 min at 37^oC. In the rhIL-8 dose response experiments, background CL was measured for 100 sec before rhIL-8 was added and subsequent changes in CL were measured over 5 min. In the priming experiments, the cells were preincubated for 10 min with medium or 10 ng/ml of rhIL-8 and were then stimulated with 100 nM fMLP for an additional 5 min. In these experiments, background CL was measured for 100 sec before addition of fMLP.

Intracellular calcium. Changes in intracellular free calcium ($[Ca^{2+}]_i$) were measured using FURA-2 loaded neutrophils. After separation 10^7 neutrophils were incubated with 2 µM FURA-2-AM for 30 min at 37^oC in Hank's buffer. During this time the FURA-2-AM entered the cells where it was hydrolysed to the acid form FURA-2 and trapped inside the cells. The cells were washed twice with Hank's buffer to remove any unesterified FURA-2-AM. For our studies the cells (10^6/ml) were then placed in a spectrophotofluorimeter (Perkin Elmer LS-50). The various test components were added and changes in fluorescence (excitation 340 nm, emission 510 nm, slit widths 10 nm) monitored continuously.

Levels of intracellular calcium were then calculated using the formula developed by Tsein and colleagues [19,20]:-

$$[Ca^{2+}]_i \quad = \quad K_d(F - F_{min})/(F_{max} - F)$$

where F is the fluorescence of the cell sample, F_{max} the maximum fluorescence signal (obtained at the end of each measurement by releasing all intracellular calcium by treatment of the cell suspension with excess Triton, 0.1%), and F_{min} the minimum fluorescence value (the value obtained when there is no calcium bound to FURA-2), obtained by the addition of excess EGTA (2 mM) (buffered with 25 mM Tris) to the above. K_d is the dissociation constant for FURA-2 (220 nM).

Statistics Data were analysed by Student's t-test for paired data, using a Macintosh computer and the Statview II^{tm} statistics package. Each experiment was performed in triplicate and the results are expressed as mean ± SEM (where n = number of experiments).

Results

The effect of rhIL-8 on fMLP-stimulated superoxide anion production. Incubation of neutrophils with concentration of up to 1 μg/ml rhIL-8 alone had no effect on O_2^- production (data not shown). To determine whether rhIL-8 was able to prime neutrophils for enhanced O_2^- production in response to fMLP, the cells were preincubated for 10 min at 37°C in the presence of 1-50 ng/ml rhIL-8 (or medium as a control), and subsequently, stimulated with various concentrations of fMLP (Fig. 1). Because of the donor-dependent variability of fMLP-stimulated neutrophil O_2^- production, the data was normalised and expressed as a percentage of the control fMLP response. rhIL-8 enhanced the response to fMLP in a dose-dependent manner. The maximum effect was seen at 10 ng/ml rhIL-8 for each fMLP concentration tested (204 ± 13, 210 ± 28, 215 ± 17 and 204 ± 18.0% of fMLP response at 1, 5, 10 and 100 nM fMLP respectively).

The priming effect of rhIL-8 was dependent on the incubation time (Fig. 2). When cells were incubated with medium alone, the responsiveness of neutrophils to fMLP declined gradually and at 2h was 51% of the initial control response at time 0. The rhIL-8 induced enhancement of the fMLP-stimulated O_2^- production was already evident at 1 min, reached maximum at 6 min and was still present at 2h.

The effect of rhIL-8 on PAF-stimulated superoxide production. rhIL-8 also enhanced PAF-stimulated O_2^- production. Neutrophils were incubated for 10 min with medium or 10 ng/ml of rhIL-8 and then stimulated with 1 μM PAF (for 6 min). In these experiments, O_2^- production was enhanced from 6.1 ± 1.0 to 8.2 ± 1.3 nmol/10^6 cells (*p*

= 0.02, n = 3). Thus rhIL-8 priming of neutrophils increased PAF-stimulated O_2^- production by 37.1 ± 2.2%.

The effect of rhIL-8 on the rate of superoxide production. In order to study the kinetics of the priming effect of rhIL-8, neutrophils were preincubated with 10 ng/ml rhIL-8 or medium for 10 min at 37 ^oC and then 10^6 cells were transferred to prewarmed cuvettes containing Cyt C and 10 ng/ml PMA or 100 nM fMLP. The O_2^- production was measured continuously and the results from a representative experiment are shown in Fig. 3. rhIL-8 increased the maximal rate of O_2^- production induced by both PMA and fMLP. The PMA-stimulated rate of O_2^- production increased from 83 ± 16 pmol/10^6 cell/s to 107 ± 16 pmol/10^6 cell/s ($p < 0.001$, n = 5) and the fMLP-stimulated rate of O_2^- production increased from 145 ± 10 pmol/10^6 cell/s to 220 ± 13 pmol/10^6 cell/s ($p < 0.005$, n = 9). The termination of the fMLP response in rhIL-8 treated or untreated cells occurred at the same time. There was a high correlation ($r^2 = 0.91$; n = 9) between the increase in the maximal rate and the total O_2^- production induced by rhIL-8 in fMLP-stimulated cells.

The effect of rhIL-8 on chemiluminescence. The priming effect of rhIL-8 and the kinetics of the fMLP response were further investigated using luminol enhanced CL. The peak of CL represents the maximum rate of oxy radical production. Preincubation of neutrophils with 10 ng/ml rhIL-8 increased the rate of fMLP-stimulated oxy radical production twofold. Maximum CL was reached at 60-70s after addition of fMLP in the control cells but occurred 20s earlier in the rhIL-8 primed cells (Fig. 4). In addition, rhIL-8 alone at 10 ng/ml produced a small but significant CL that reached a maximum at 10-20s and reverted to the baseline by 60s

(Fig. 4 insert). This direct CL response of rhIL-8 was dose dependent. At the highest concentration tested (500 ng/ml) the rhIL-8 induced CL response was 15% of that induced by fMLP alone (data not shown).

The effect of rhIL-8 on intracellular free calcium concentration. To determine whether enhancement of O_2^- production was associated with changes in $[Ca^{2+}]_i$, FURA-2 loaded neutrophils were stimulated directly with rhIL-8 concentrations from 1 to 25 ng/ml (Fig. 5). rhIL-8 stimulated an increase in $[Ca^{2+}]_i$, which paralleled closely the results obtained from O_2^- experiments. For example, the concentration of rhIL-8 (10 ng/ml), shown to be optimal for the facilitation of O_2^- response, induced maximal increase in $[Ca^{2+}]_i$. Neutrophils stimulated with 10 ng/ml rhIL-8 increased $[Ca^{2+}]_i$ levels by 573 ± 100 nM. However, when neutrophils were stimulated in calcium free buffer, the increase in $[Ca^{2+}]_i$ was reduced to 221 ± 43 nM and even more (129 ± 32 nM) when 2 mM EGTA was added to the buffer. The rhIL-8 response was restored to 71% of the original response when calcium was added to the cells suspernded in the calcium-free buffer (Fig. 6). These results suggest that the increase in $[Ca^{2+}]_i$ induced by rhIL-8 is caused by both an influx of extracellular calcium and release of calcium from intracellular stores.

Furthermore, preincubation of neutrophils with rhIL-8 facilitated the fMLP-stimulated increase in $[Ca^{2+}]_i$ and this effect of rhIL-8 was dependent on preincubation time. In these experiments neutrophils were preincubated with 10 ng/ml rhIL-8 or medium for the indicated times and were then stimulated with 100 nM fMLP. The maximal facilitating effect of rhIL-8 was seen at 5 min when the

fMLP-stimulated elevation in $[Ca^{2+}]_i$ was increased by 1027 ± 80 nM (220 ± 43 % fMLP response) and gradually declined to 308 ± 136 nM (140 ± 20 % fMLP response) at 15 min (Fig. 7). This time response pattern complements the time-course of the priming response for fMLP-stimulated O_2^- production (Fig. 2).

Discussion.

Activated neutrophils produce oxy radicals that are essential for their microbicidal and cytotoxic activities and play an important role in the host defence system. This is clearly demonstrated in chronic granulomatous disease, which is characterised by decreased oxidative burst. Patients with this disease suffer from recurrent bacterial infections. However, in chronic inflammatory diseases, such as RA, release of oxy radicals from activated neutrophils may lead to severe local tissue damage and exacerbation of disease.

There have been conflicting reports regarding the role of IL-8 in the enhancement of oxy radical production by neutrophils. Recent studies by You et al [8] and Daniels et al [9] have shown that IL-8 primes neutrophils for enhanced O_2^- production in response to fMLP, but other investigators have failed to demonstrated an effect of IL-8 in the same assay system [16]. In the present study, we confirmed the finding that IL-8 facilitates fMLP-stimulated neutrophil O_2^- production using the Cyt C reduction assay and also demonstrated this effect in a luminol-enhanced CL assay. We also demonstrated that IL-8 enhances neutrophil O_2^- production to two other stimuli, PMA and PAF. The priming response of rhIL-8 was rapid, long lasting and reached maximum at 10 ng/ml of IL-8. Since IL-8 concentrations of up to 15 ng/ml have been found in synovial fluids of patients with RA [2], it is likely that IL-8 may enhance synovial neutrophil oxy radical production and contribute to the joint destruction. Alternatively, neutrophils may be primed when exposed to low chemotactic levels of IL-8 during migration before entering the synovium. Similar concentrations of IL-8 to those required for maximal O_2^- priming effect have been reported to be optimal for IL-8 induced neutrophil chemotaxis and transendothelial

migration [21,22]. In contrast, circulating neutrophils are less likely to be primed, as IL-8 released to the blood could be absorbed by red blood cells [23] or its activity neutralised by circulating a-IL-8 antibody [24].

The mechanisms by which IL-8 and other cytokines exert their priming effect on neutrophil oxy radical production are unknown. Since the demonstration that GM-CSF and TNF-a enhance neutrophil oxy radical production, there has been considerable interest in mechanisms underlying the priming effect. Subsequent investigations have shown that preincubation of neutrophils with GM-CSF or TNF-a modulates the number and affinity of fMLP receptors [11,25] and suggested that the receptor changes may explain the priming effect. However, studies by McColl et al [26] challenged this hypothesis by demonstrating that GM-CSF or TNF-a prime neutrophils by inducing changes in signal transduction pathways that are independent of and distal to the fMLP receptor. This was further supported by O'Flaherty et al [27] who demonstrated that receptor numbers alone cannot explain the priming phenomenon by showing that, although TNF-a enhanced neutrophil degranulation responses to fMLP, leukotriene B_4 (LTB_4) and PAF, it increased the number of fMLP receptors but downregulated LTB_4 receptors and only transiently upregulated PAF receptors. An alternative mechanism of priming, whereby the cells could be primed by their own metabolites, was proposed by Bauldry et al [28], who showed that TNF-a enhances neutrophil phospholipase A_2 activity, thus increasing arachidonic acid release, LTB_4 production and PAF synthesis. Since these neutrophil metabolites have been shown previously to prime neutrophil oxidative metabolism [29], they could prime the cells in an autocrine fashion.

Although no single satisfactory explanation provides an answer as to what causes cell priming, the above studies suggest that changes in activity of components of intracellular signal transduction pathways leading to oxy radical production must take place following cell exposure to the priming agent. These changes most probably occur in a part of the pathway that is common to the different stimuli, as we have shown that rhIL-8 affects fMLP, PMA and PAF responses. Furthermore, these changes are associated with the rate of O_2^- production rather than the duration of the response. For example, the rhIL-8-increased rate of O_2^- production in both PMA- and fMLP-stimulated neutrophils correlated closely with the total O_2^- production. In fMLP-stimulated cells, where the O_2^- response is normally terminated after 2-4 min, IL-8 did not prolong but slightly shortened the duration of the response. PMA stimulates O_2^- production in neutrophils by binding to and directly activating protein kinase C (PKC) [30]. In fMLP-stimulated cells, two sequential pathways leading to oxy radical production have been recognised: a PKC-independent pathway that initiates and a PKC-dependent pathway that sustains the response [31]. Since O_2^- production induced by both stimuli depends on PKC activity, IL-8 could, for example, increase PKC activity by translocating it to the cell membrane [32]. Alternatively, IL-8 could accelerate the catalytic activity of NADPH oxidase in PKC-independent manner, by acting on component(s) of the signal transduction pathway that are more proximal to this enzyme than PKC. Since NADPH oxidase consists of many cytosolic and membrane associated components that are assembled together upon activation [33,34,35,36], it is possible that IL-8 speeds up the assembly process by inducing a redistribution of the cytosolic components to the cell membrane [37]. Further mechanisms may include an effect of IL-8 on (i) the number of

NADPH oxidase molecules that are being assembled and activated by a second stimulus [38], (ii) the affinity of NADPH oxidase for its substrate, or (iii) substrate availability by as yet unknown mechanism.

It is of interest to note that, although GM-CSF and TNF-a prime neutrophils for enhanced oxy radical production by fMLP, the preincubation time required for their optimal effect is much longer than that required by IL-8. Furthermore, neither of these cytokines can prime neutrophils for the direct protein kinase C activator PMA, or increase $[Ca^{2+}]_i$ in these cells [12,39,40]. We have shown that rhIL-8 stimulates increases in $[Ca^{2+}]_i$ over the same concentration range as it facilitates O_2^- production of fMLP-stimulated neutrophils. In addition, fMLP-stimulated $[Ca^{2+}]_i$ increase was enhanced further when cells were preincubated with rhIL-8 prior to fMLP stimulation. These observations suggest that elevations in $[Ca^{2+}]_i$ may play an important role in the IL-8 priming effect. It has been shown previously, that elevations in $[Ca^{2+}]_i$ can facilitate oxy radical production in neutrophils stimulated with PMA or fMLP [41,42]. This effect was thought to be mediated by increased PKC activity, resulting from PKC translocation to the cell membrane as well as increased affinity for phorbol esters. More recently, activated PKC has been implicated in phosphorylation-dependent translocation to the cell membrane of cytosolic oxidase factors p47-phox and p67-phox, which are essential for NADPH oxidase assembly and activation [34,43]. Although our present studies did not aim to address the role of IL-8-induced $[Ca^{2+}]_i$ rises in NADPH oxidase assembly, they alerted us to this possibility.

In agreement with a previous report by Daniels et al [9], who was also unable to demonstrate a direct effect of rhIL-8 on O_2^-

production using the Cyt C assay, we did not detect any O_2^- production in neutrophils that were stimulated with up to 1 μM rhIL-8 alone. However, we demonstrated a direct effect of rhIL-8 in the luminol-enhanced CL assay, which was very weak. The rhIL-8 concentration of 10 ng/ml, found to have maximal priming effect on neutrophils only slightly increased CL, and even concentrations as high as 500 ng/ml induced a response that was less than 15 % of the fMLP response. The direct effect of rhIL-8 on luminol-enhanced CL has been previously demonstrated [44], and it was suggested that the apparent discrepancy between the results from the two different assays was due to a greater sensitivity of the CL assay [45]. However, a further explanation may be the different specificity of these assays. Cyt C is specific for O_2^- and therefore reflects the activity of NADPH oxidase alone, whereas luminol may be oxidised by many different oxy radicals and oxidants, and thus may reflect the activity of other radical-generating enzymes. This is relevant since, Wang et al [46,47] have recently demonstrated that nitric oxide, produced by nitric oxide synthase, increases luminol-enhanced CL. Whatever the explanation, the high IL-8 concentrations required to directly stimulate neutrophil oxy radical release and the low magnitude of this response question its physiological significance, in contrast to the priming effect, where very low concentrations of IL-8 can dramatically enhance neutrophil responses.

Thus, we believe that our studies clearly demonstrate that IL-8 facilitates neutrophil oxy radical production and provide important new information for the assessment of IL-8 in inflammation. On the molecular level, this study alerted us to the possible role of cytosolic calcium in neutrophil priming with particular reference to NADPH oxidase assembly and activation. However, further studies are

required to determine the exact mechanism by which IL-8 exerts its priming effect on neutrophils as we anticipate that many intracellular pathways may be involved.

ACKNOWLEDGMENTS

We wish to thank Dr. Yi Zhang for her assistance with chemiluminescence experiments.

This work was supported by The Queen Elizabeth Hospital Research Foundation and Arthritis Foundation of Australia.

ABBREVIATIONS

rhIL-8	recombinant human interleukin-8
$[Ca^{2+}]_i$	intracellular free calcium concentration
CL	chemiluminescence
fMLP	*formyl*-methionyl-leucyl-phenylalanine
PAF	platelet activating factor
PMA	phorbol myristate acetate
O_2^-	superoxide anion

Figure Legends

Figure 1. The effect of rhIL-8 on fMLP-stimulated O_2^- production. Neutrophils (10^6) were preincubated for 10 min at 37°C in the presence of 1-50 ng/ml rhIL-8 (or medium as a control) and were then stimulated with 1, 5, 10 or 100 nM fMLP for an additional 6 min. Results are normalised and expressed as a percentage of the control fMLP response (The responses of control cells, preincubated with medium alone and then stimulated with 1, 5, 10 and 100 nM fMLP were 1.8 ± 0.7, 2.1 ± 0.7, 8.4 ± 2.6 and $12. \pm 2.3$ nmol O_2^- /10^6 cells respectively). Values represent means of four experiments performed in triplicate.

Figure 2. The effect of rhIL-8 on fMLP-stimulated O_2^- production as a function of time. Neutrophils (5×10^6/ml) were incubated with medium (—■—) or 10 ng/ml rhIL-8 (—□—) at 37°C for indicated periods of time. At each time point 10^6 cells were transferred to tubes containing cytochrome C and 100 nM fMLP for an additional 6 min stimulation. Results are normalised and expressed as a percentage of the control fMLP response. Values represent means of seven experiments performed in triplicate.

Figure 3. The effect of rhIL-8 on the kinetics of PMA- and fMLP-stimulated O_2^- production. Neutrophils (5×10^6/ml) were preincubated with medium (—■—) or 10 ng/ml of rhIL-8 (—□—) for 10 min at 37°C and then 10^6 cells were transferred to prewarmed cuvettes containing Cyt C and (A) 10 ng/ml PMA or (B) 100 nM fMLP in which the O_2^- production was measured continuously. The results are from one experiment representative of five (A) and nine (B).

Figure 4. Luminol-enhanced CL response induced by 10 ng/ml rhIL-8 (see also insert) in neutrophils that were preincubated with medium or 10 ng/ml of rhIL-8 for 10 min at 37°C and were then stimulated with 100 nM fMLP. The peak of CL corresponds to the maximum rate of oxy radical production (NADPH oxidase activity). The results are from one experiment representative of four.

Figure 5. The effect of rhIL-8 on D$[Ca^{2+}]_i$ (—▲—) and fMLP-stimulated O_2^- production (—□—). For the study of changes in $[Ca^{2+}]_i$, FURA-2 loaded neutrophils were stimulated with various concentrations of rhIL-8. Values represent means of four experiments. For O_2^- production the cells were treated as in Fig. 1 and stimulated with 100 nM fMLP.

Figure 6. The effect of extracellular calcium on the rhIL-8-induced D$[Ca^{2+}]_i$. Neutrophils prepared as outlined in "Materials and Methods" were loaded with FURA-2 in complete Hanks buffer and divided into two preparations. Each was then washed and resuspended in either Hanks buffer or calcium-free Hanks buffer. 10^6 cells from each preparation were then stimulated with 10 ng/ml rhIL-8. In addition, neutrophils that were resuspended in calcium-free Hanks, were either repleted with 1.25 mM Ca^{2+} or treated with 2 mM EGTA for 5 min at 37°C before stimulation with rhIL-8. Values represent means of four experiments.

Figure 7. The effect of rhIL-8 on fMLP-stimulated $[Ca^{2+}]_i$ as a function of time. Neutrophils ($10^{6)}$ were preincubated with medium

or 10 ng/ml of rhIL-8 at 37°C for indicated times and were then stimulated with 100 nM fMLP. Results are expressed as a percentage of the fMLP response. Values represent means of five experiments.

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Fig. 1

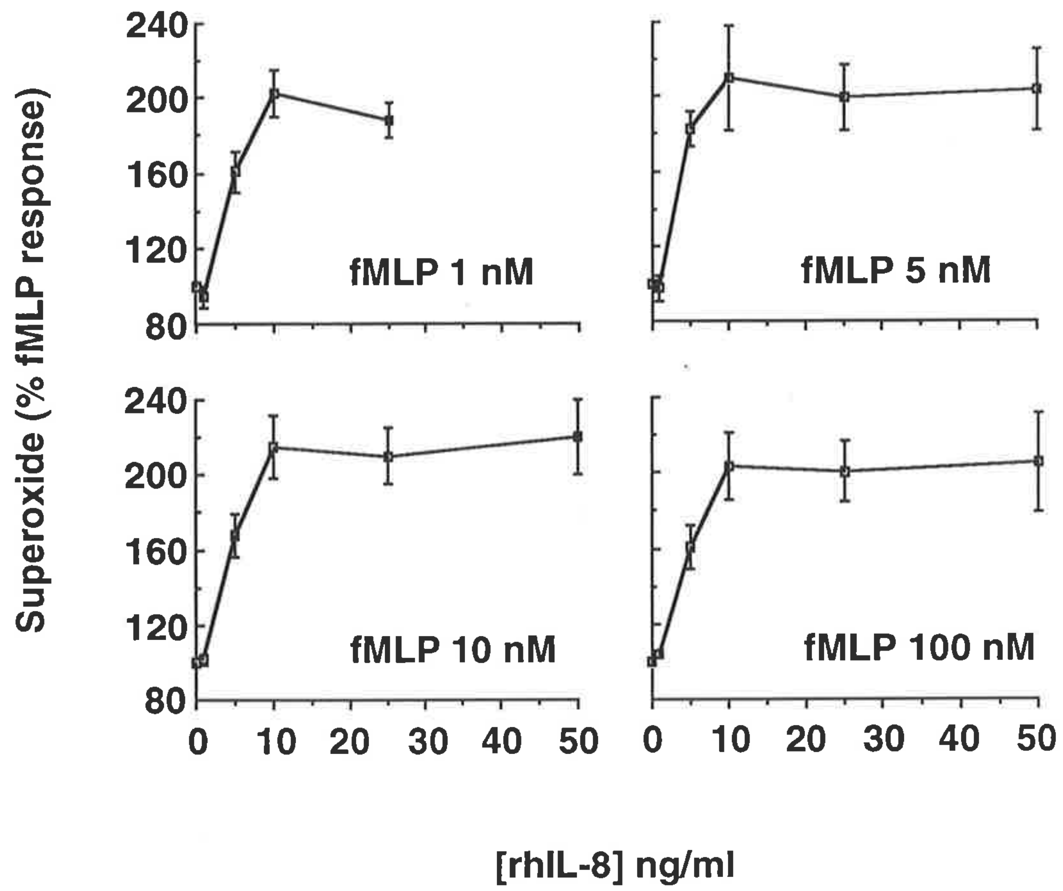


Fig. 2

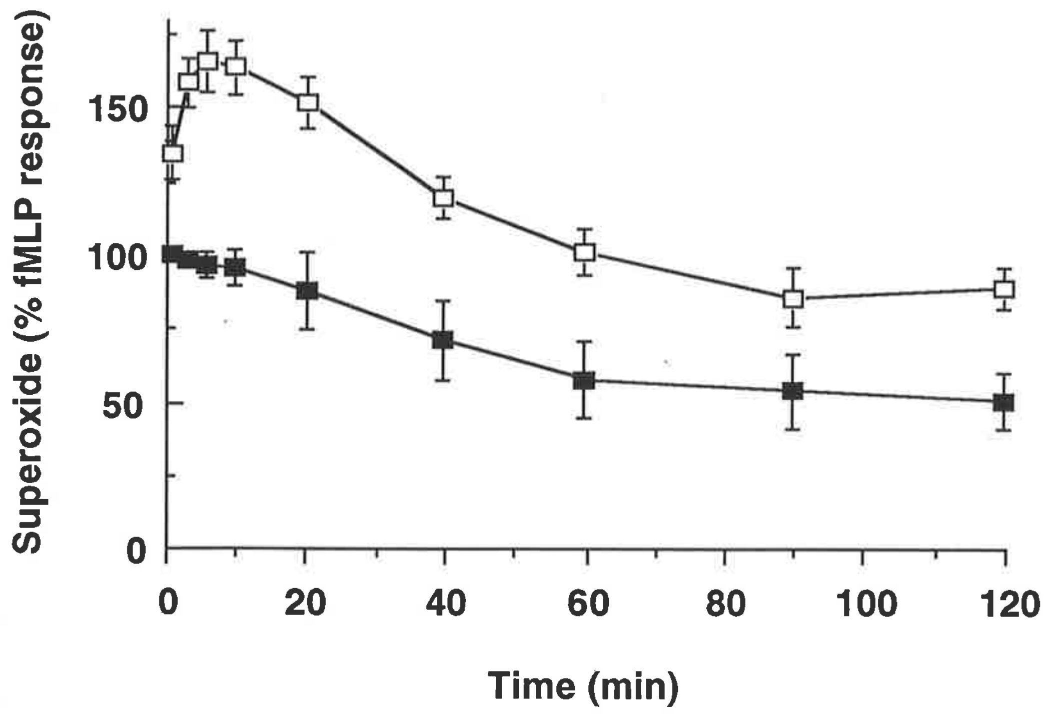


Fig. 3

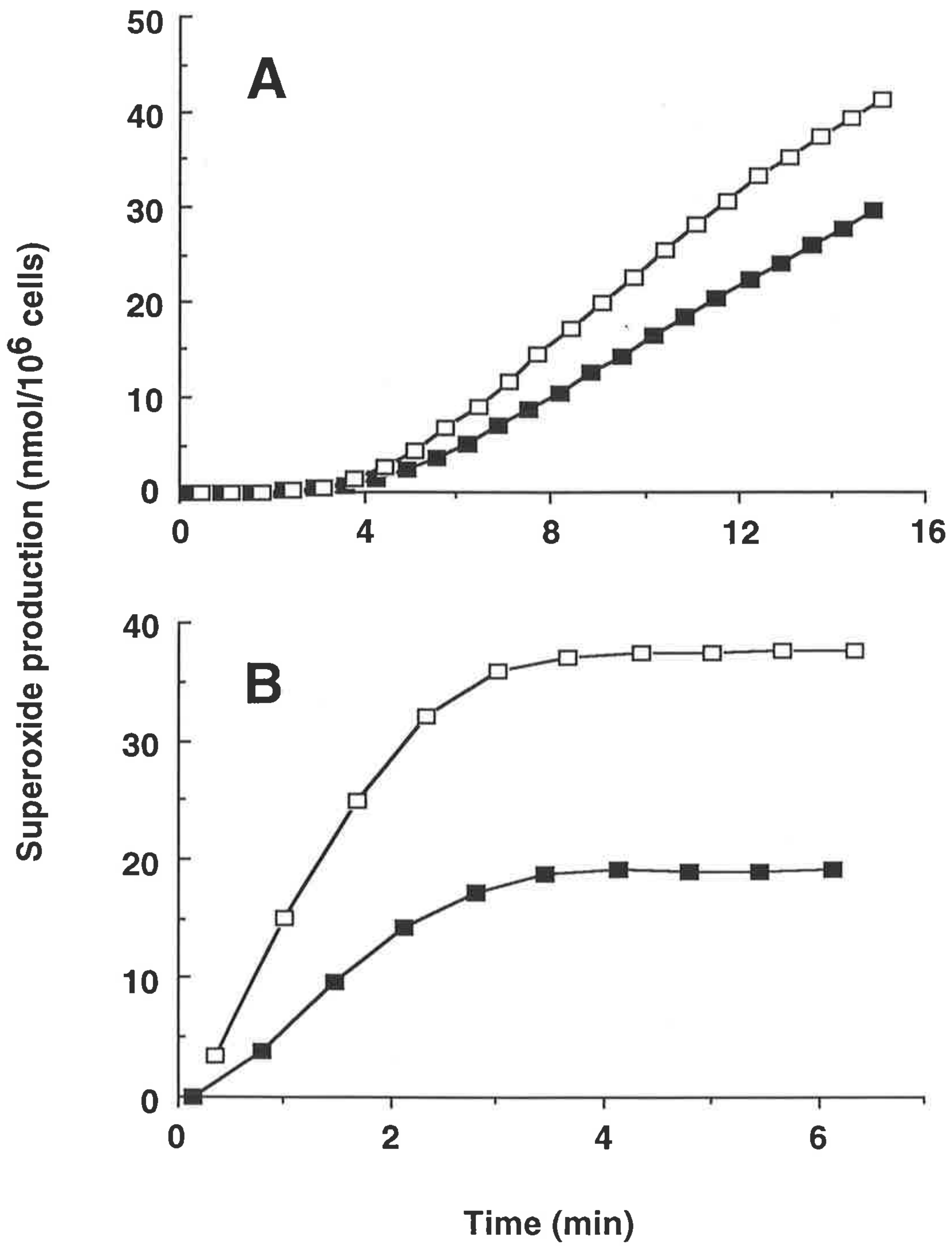


Fig. 4

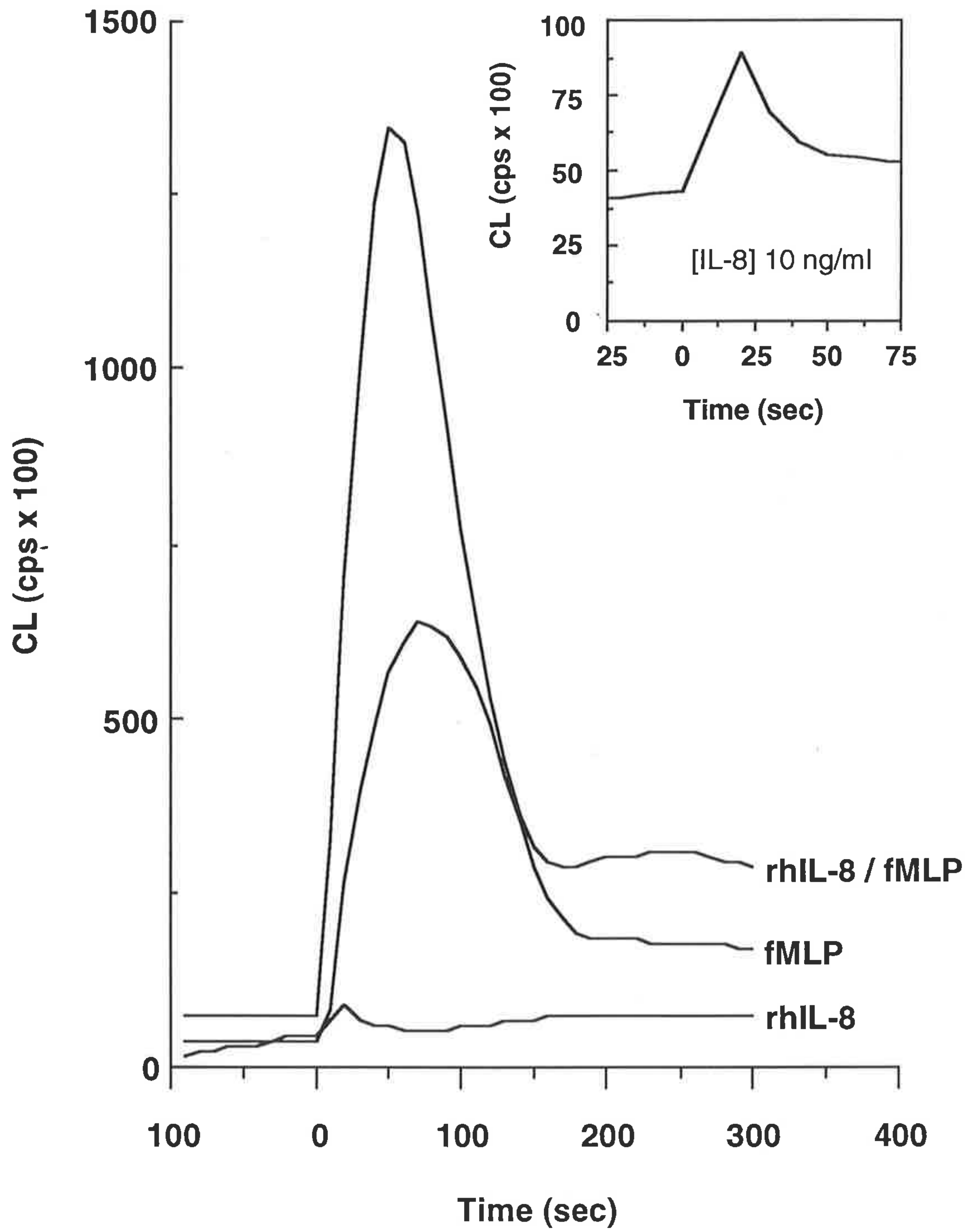


Fig. 5

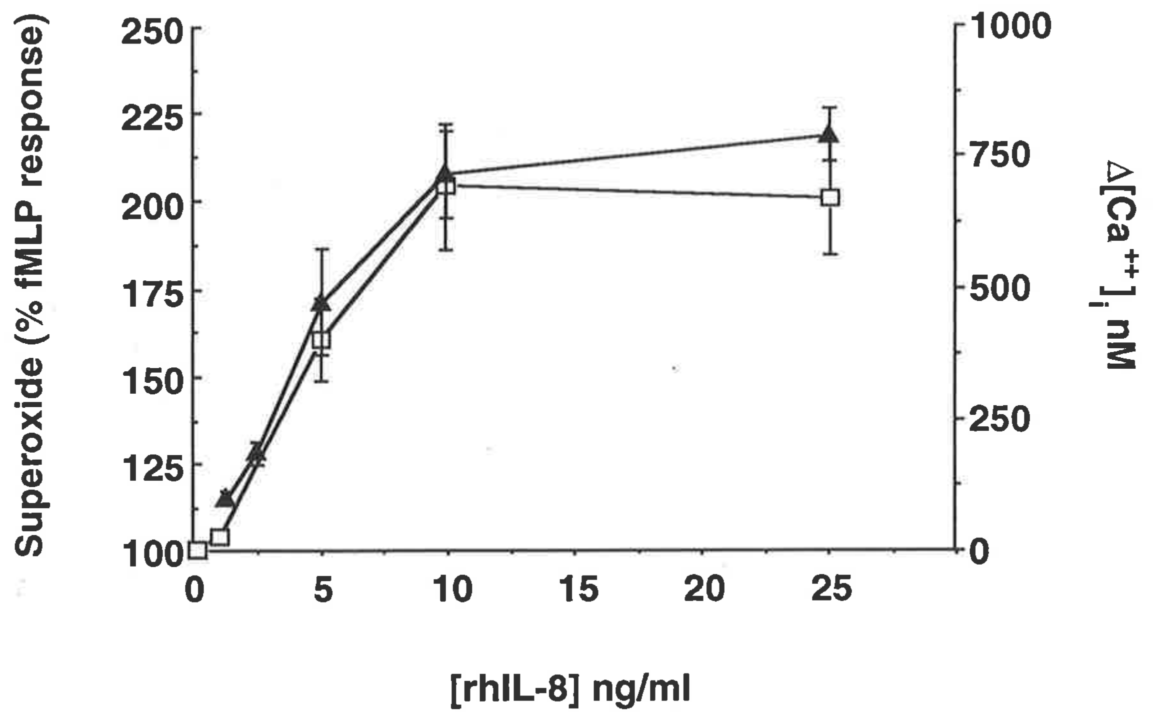


Fig. 6

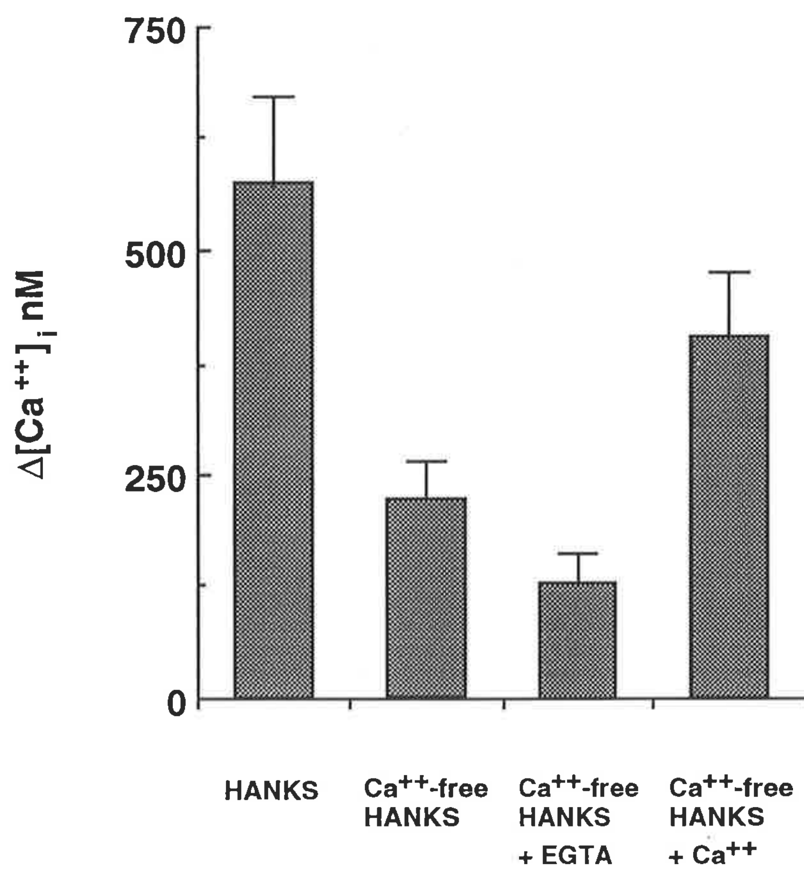
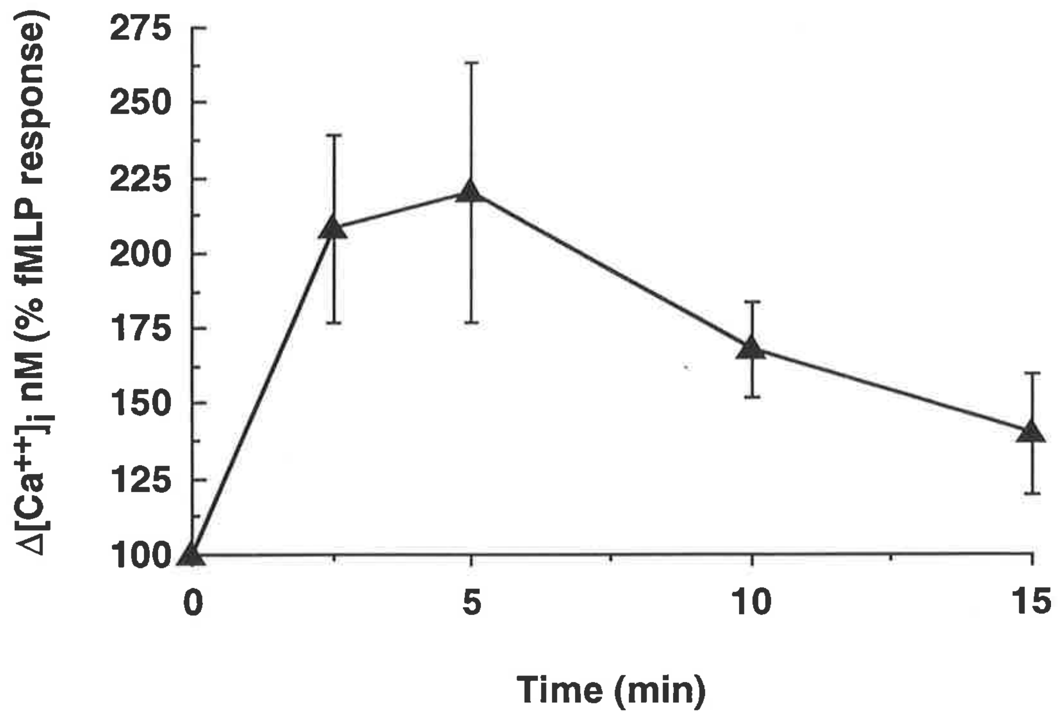


Fig. 7



PERIPHERAL BLOOD NEUTROPHILS FROM ASTHMATIC SUBJECTS ARE PRIMED FOR ENHANCED RESPONSES TO SUBSTANCE P: ASSESSMENT WITH ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

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Running Title: Neutrophils from asthmatic subjects are hyperresponsive to substance P

Keywords: substance P, asthma, neutrophils, eosinophils, tachykinins, cytotoxicity, granulocyte-macrophage colony-stimulating factor

ABSTRACT

The sensory neuropeptide substance P (SP) is a mediator of neurogenic inflammation and is thought to play a role in the pathogenesis of asthma. We examined the effect of SP on antibody-dependent cell-mediated cytotoxicity (ADCC) by neutrophils and eosinophils isolated from peripheral blood of normal (n = 20) and asthmatic subjects (n = 34) .

Baseline (unstimulated) ADCC was significantly higher in asthmatics (18.7 ± 2.7%) than normals (8.2 ± 1.4%; $p < 0.01$). SP stimulated neutrophil ADCC in a dose-dependent manner, peaking at an SP concentration of 100 µM. SP-stimulated ADCC was greater in asthmatic subjects (39.4 ± 3.4%) than normals.(21.0 ± 2.1%; $p < 0.0005$). The net increase in ADCC (stimulated minus the baseline) was significantly greater in asthmatics (17.6 ± 1.9%) than normals (7.0 ± 0.8%; $p < 0.0001$) confirming that the higher, stimulated ADCC in asthmatics was not due solely to the higher baseline ADCC.

We hypothesised that this enhanced response is due to the modulation of neutrophil function by cytokines in vivo. In support of this hypothesiswe demonstrated synergism between SP and granulocyte-macrophage colony-stimulating factor (GM-CSF) in vitro. The net increase in ADCC was 44.0 ± 4.0% with 0.1 µM SP plus 10 ng/ml GM-CSF compared to 31.0 ± 5.2% with 10 ng/ml GM-CSF alone ($p < 0.05$). In addition, in a subgroup of asthmatics, we demonstrated a modest correlation between SP stimulated ADCC and bronchial hyperresponsiveness assessed as the PC_{20} histamine ($r = 0.5$, $p < 0.025$, n = 20). In contrast to its effects on neutrophils, SP failed to stimulate eosinophil ADCC although these cells were responsive to GM-CSF (19.4 ± 3.2%, $p < 0.002$).

Our findings suggest that in asthma, SP plays a role in airways inflammation and that this may be mediated in part by its effects on neutrophil ADCC. Importantly our data provide further evidence for the role of the nervous system in the pathogenesis of asthma.

INTRODUCTION

Airways inflammation is a prominent feature of asthma and is thought to be responsible for the airflow obstruction and bronchial hyperresponsiveness (BHR) which characterise this disease clinically [1]. Eosinophils are key effector cells in this inflammatory process, although multiple cell types including neutrophils are also involved [2].

There is evidence which implicates the sensory nervous system in the pathogenesis of asthma [3]. In the respiratory tract, sensory nerves are located in the epithelium and smooth muscle, and around mucus glands and blood vessels [4]. These nerves contain a number of neurotransmitters, including tachykinins, that colocalise to the same nerves and are released locally by antidromic stimulation [3]. Tachykinins are a family of peptides which are defined by a common Cterminal amino acid sequence of phe-X-gly-leu-met-NH_2 (where X is an aliphatic or aromatic amino acid) and the ability to rapidly contract a variety of smooth muscle types [5]. The tachykinin substance P (SP) is widely distributed in the peripheral and central nervous systems where it acts as a neurotransmitter [6]. In asthma, it has been postulated that SP and other tachykinins are released locally by axonal reflexes when sensory nerves are stimulated following epithelial damage [7]. Consequently the contribute to the pathophysiology of asthma by causing bronchoconstriction, mucus secretion and plasma extravasation [8, 9].

SP also has a number of pro-inflammatory effects: it stimulates neutrophils [10-13], mast cells [14], macrophages, monocytes [15-17] and lymphocytes [18-19] and it induces cell chemotaxis, exocytosis of granule contents and production of cytokines and other mediators of inflammation [20-22]. Most of the above effects of SP have been defined using cells isolated from normal individuals. However, in asthma there is leucocyte activation in vivo [23-26], probably as a result of the local production of cytokines in the airways and they release into the circulation . These cytokines are capable of recruiting, activating and 'priming' circulating cells for an enhanced response to a variety of stimuli [27, 28.

We hypothesised that peripheral blood neutrophils and eosinophils derived from asthmatics would show enhanced responses to SP which would correlate with asthma severity. To investigate this hypothesis, we examined the effect of SP on neutrophils and eosinophils isolated from normal and asthmatic subjects, and assessed their in vitro function in an antibody-dependent cell-mediated cytotoxicity (ADCC) assay. To provide a clinical correlation, we related neutrophil responsiveness to SP to bronchial reactivity as measured by an inhaled histamine dose-response curve. Finally to assess the role of cytokines in priming inflammatory cells for enhanced responses to SP, we have examined the effect of macrophage-granulocyte colony-stimulating factor (GM-

CSF) on the neutrophil responses to SP.

MATERIALS AND METHODS

Subjects

All subjects were volunteers and informed consent was obtained. The project was approved by the Human Ethics Committee of the Royal Adelaide Hospital. Normal subjects did not suffer from allergic (seasonal) rhinitis or asthma. Asthmatic subjects were recruited from patients attending the outpatient service of the Department of Thoracic Medicine, Royal Adelaide Hospital. Asthma was diagnosed according to the criteria of the American Thoracic Society [29] and in particular reversible airflow obstruction had been documented objectively in all subjects. None had suffered from an upper respiratory tract infection in the preceding 6 weeks and all were studied at a time when their asthma was stable but symptomatic, usually on daily medications. In a subgroup of asthmatics, BHR was measured as outlined below. All asthmatics were studied after abstinence from medications as per the protocol for assessment of BHR (see below).

Isolation of Human Neutrophils and Eosinophils

Neutrophils were isolated from peripheral blood as follows. A leucocyte fraction was obtained by sedimenting the erythrocytes with dextran (Dextran T-500; Pharmacia, Uppsala, Sweden). Neutrophils were isolated by density-gradient centrifugation (400g, 20 min, 22° C) using Lymphoprep (NYCOMED AS, Oslo, Norway) and hypotonic lysis of residual erythrocytes. The cells were always >94% pure, as determined by Grunwald-Giemsa staining, and >98% viable by trypan blue exclusion.

In the studies using eosinophils, neutrophils and eosinophils were isolated from the same blood sample using metrizamide density-gradient centrifugation as described previously [30]. Briefly, blood was dextran sedimented and the leucocyte fraction was washed in RPMI-1640 medium containing 2% foetal calf serum. The cells were then centrifuged at 1200g for 45 min at 22° C over a gradient consisting of 18, 20, 22, 23 and 24% metrizamide (NYCOMED AS, Oslo, Norway). Neutrophils collected from the 20/22% interface were >96% pure and >99% viable. Eosinophils were collected from the 22/23% and 23/24% interfaces, and pooled for the experiments. The purity was >94% and the cells were >99% viable.

Antibody-Dependent Cell-Mediated Cytotoxicity

The method has been described in detail previously [31]. The assay was performed in RPMI-1640 medium containing 0.1% bovine serum albumin. Briefly, 40 µl ($4x10^3$) of ^{51}Cr-labelled trinitrophenyl (TNP)-coupled P815 target cells (DBA/2 mastocytoma) were mixed with 80 µl ($1.2x10^5$) neutrophils or eosinophils as effector cells, 24 µl of rabbit anti-TNP antibody (ab; Miles-Yeda, Rehovot, Israel) and 16 µl of the appropriate concentration of SP (Auspep, Melbourne, Australia) or human recombinant GM-CSF, in V-bottomed microtitre plates. The final assay volume was 160 µl. After incubation of the reaction mixture for 2.5 h at 37° C, 80 µl of the supernatants were removed and the radioactivity counted using a gamma counter (LKB, 1282 Compugamma, Turku, Finland). Percentage cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{experimental cpm - spontaneous release cpm}}{\text{total cpm - spontaneous release cpm}} \times 100$$

where spontaneous release was the ^{51}Cr released from P815 cells in the presence of medium alone and the total count was the ^{51}Cr released from P815 cells lysed by the addition of 4% Triton X-100. *Net*-ADCC was calculated as cytotoxicity in the presence of stimulus minus the baseline cytotoxicity in the presence of antibody alone. All experiments were performed in triplicate and SD's were ≤10% in each experiment. We used conditioned medium (CM) from the bladder carcinoma cell line, U5637 as a positive control since it contains factors known to enhance human neutrophil and eosinophil ADCC [31].

Histamine Provocation

Histamine provocation was performed as described by Cockroft et al [32]. Prior to the test, the subjects abstained from medications as follows: aerosol bronchodilators, 8 h; theophylline preparations, 24 h; sodium cromoglycate, 24 h and antihistamines, 48 h. The challenge was performed between 09.00 and 10.00 h. The procedure was terminated when the FEV_1 had fallen by 20% from the saline control or at 8 mg/ml of histamine. The provocation concentration which resulted in a fall of ≤20% in FEV1 (PC_{20}), was obtained by interpolation from a plot of the decrease in FEV1 versus the log of the dose of histamine and was expressed as mg/ml. For analysis, the data were initially log-transformed.

Data Analysis

Data are expressed as means ± SEM (n = number of subjects). Differences between means were assessed for statistical significance using Student's t-test and ANOVA. The Wilcoxon rank sum test was used when appropriate. All calculations were carried out with the Statview II™ program using a Macintosh computer.

RESULTS

The effect of SP on neutrophil ADCC

ADCC was assayed in the presence of 0, 0.3 or 1 µg/ml ab and SP in the concentration range 1nM - 100 µM or medium control. In normal subjects, SP stimulated neutrophil ADCC in a dose-dependent manner (Fig 1). With 1 µg/ml ab, 100 µM SP stimulated ADCC from 8.2 ± 1.4% to 21.0 ± 2.1% ($p < 0.0001$). SP did not stimulate cytotoxicity significantly in the absence of ab (2.6 ± 0.6% versus 1.8 ± 0.4% for 100µM SP and medium control respectively). An optimal dilution of 10% CM, stimulated ADCC to 26.9 ± 3.6% which is not significantly different from 100 µM SP.

In asthmatic subjects, 100 µM SP increased ADCC from 18.7 ± 2.7% to 39.4 ± 3.4 % ($p = 0.0001$; 1.0 µg/ml ab). The response to CM was 39.1 ± 2.7%, not significantly different from 100 µM SP (Fig 2). SP did not stimulate ADCC in the absence of antibody (3.2 ± 0.6 versus 2.5 ± 0.3% for medium).

Spontaneous ADCC in the absence of antibody or any stimulus was 1.8 ± 0.4% for normals ($n = 20$) and 2.5 ± 0.3% for asthmatic subjects ($n = 34$; $p < 0.05$). SP itself did not kill target cells. At 1 µg/ml ab, ADCC was significantly greater in asthmatics than normals for medium ($p < 0.01$), all concentrations of SP ($0.0001 \leq p \leq 0.01$) and CM ($p < 0.01$). With 0.3 µg/ml ab, ADCC with medium or SP but not CM was significantly greater in asthmatics than normals ($0.005 \leq p \leq 0.05$).

The greater stimulatory effect of SP on neutrophils isolated from asthmatics did not merely reflect the higher baseline ADCC in this population. For SP, *net*- ADCC was also significantly greater in asthmatics (Fig 3). For example, with 50 µM SP, *net*-ADCC was 7.0 ± 0.8% in normals versus 17.6 ± 1.9% in asthmatics ($p = 0.0001$). The net increase in ADCC produced by CM did not differ significantly between normals and asthmatics.

The Effect of SP on Eosinophil ADCC

We compared the effect of SP on ADCC for eosinophils and neutrophils isolated from the peripheral blood of 5 asthmatics (Fig 4). SP did not stimulate eosinophil ADCC significantly. With 100 µM SP, ADCC increased from 2.7 ± 1.0% to 8.8 ± 5.7% (1 µg/ml ab; $p > 0.05$). GM-CSF (10 ng/ml) stimulated eosinophil ADCC to 15.1 ± 3.5% ($p < 0.05$) demonstrating that it was possible to stimulate eosinophil ADCC under our assay conditions. Under the same conditions SP stimulated neutrophil ADCC from 21.9 ± 6.8% to 44.9 ± 5.7% ($p < 0.05$; Fig 4). Even when the

ab concentration was increased to 3 μg/ml, SP did not stimulate eosinophil ADCC significantly (5.5 ± 3.1% with 100 μM SP). The corresponding response to GM-CSF was 19.4 ± 3.2% ($p < 0.002$)
Although SP did not stimulate mean eosinophil ADCC, it did stimulate eosinophils isolated from the peripheral blood of one subject (30.8%, 100 μM SP).

The Effect of SP and GM-CSF on neutrophil ADCC

We examined the effect of SP together with GM-CSF on neutrophil ADCC. Neutrophils isolated from normal subjects were pre-incubated with medium or 0.1 nM - 1 μM SP for 30 min. ADCC was then assessed by incubating the reaction mixture with the same concentrations of SP plus GM-CSF (0, 1, 5 or 10 ng/ml). Data are summarised in Fig 5. Neutrophils, incubated with both SP and GM-CSF demonstrated enhanced ADCC compared to GM-CSF alone. For example, *net*-ADCC was 44.0 + 4.0% with 0.1 μM SP plus 10 ng/ml GM-CSF compared to 31.0 ± 5.2% with GM-CSF alone ($p < 0.05$). SP (0.1 nM - 1 μM) alone did not stimulate ADCC.

Correlation of Neutrophil ADCC and BHR

Neutrophil ADCC and histamine reactivity were measured in a subgroup of 20 asthmatics whose clinical details are shown in Table I. Most subjects suffered from moderate or severe asthma judged on symptoms (usually daily) and medication requirements. The effect of SP and CM on *net*-ADCC was similar to the total asthmatic group (data not shown)

There were modest but significant correlations between ADCC and PC_{20} (Fig 6.): $r = 0.45$, $p < 0.05$ for 100 μM SP at 1 μg/ml ab and $r = 0.5$, $p < 0.025$ (100 μM SP, 0.3 μg/ml ab). *Net*-ADCC with 100 μM SP and 0.3 μg/ml ab, also correlated with PC_{20}: $r = 0.51$, $p < 0.025$. There were no significant correlations between PC_{20} and baseline ADCC or CMstimulated cytotoxicity (data not shown).

DISCUSSION

The effector cell which has received the most attention a propos of the pathogenesis of asthma, is the eosinophil. Neutrophils may also play an important role in the airways inflammation in asthma [33]. Bronchoalveolar lavage neutrophilia occurred 48 h after local endobronchial allergen or aerosol challenge with plicatic acid [34, 35] or 8 h after isocyanate challenge [36]. Neutrophils are capable of altering airways function.in animal models of asthma. Peripheral blood neutrophils become activated after allergen- and exercise-induced asthmatic responses [37, 38] and neutrophil-activating factors are present in the circulation. It is particularly relevant to our studies which used an ADCC assay to assess neutrophil function, that after exercise-induced asthma, neutrophils were found to be primed for enhanced cytotoxicity [38]. It is likely therefore that neutrophils contribute significantly to the pathogenesis of asthma although this remains controversial.

In asthma, there is evidence that circulating neutrophils and eosinophils are activated and demonstrate enhanced responses to a number of stimuli [39]. Since it has been postulated that SP plays a role in the pathogenesis of asthma, it seemed pertinent to compare its effects on neutrophils and eosinophils obtained from normal and asthmatic subjects. We expected that in asthma, effector cell responsiveness to SP would be increased

We studied cytotoxicity against an antibody coated target. Neutrophil ADCC involves exocytosis particularly of primary granules and production of oxy radicals [40]. SP does not stimulate superoxide anion production directly to any significant degree but we have shown previously that it stimulates neutrophil ADCC and primes neutrophils for enhanced superoxide anion reproduction in response to fMLP [11]. Since the target in our assay is sensitive to both eosinophil- and neutrophil-mediated cytotoxicity, we could compare the effects of SP on both cell populations.

The studies dealing with the effects of SP on human neutrophils, used cells obtained from the peripheral blood of normal subjects. However, in asthma there is increasing evidence that neutrophils and eosinophils are activated and primed by cytokines, including GM-CSF [41], which are potentially able to be produced locally in the airways [42 - 44].

In normals, SP stimulated neutrophil ADCC in a dose-dependent manner with the maximum effect noted at 100 μM SP. It did not stimulate cytotoxicty in the absence of antibody and its effect was dependent on the antibody concentration used. In asthmatics SP also stimulated neutrophil ADCC. However, we found a number of differences between these two groups and make the following conclusions: (a) Baseline or unstimulated ADCC was significantly higher in asthmatics. This is consistent with a number of other studies which have shown that in asthma peripheral blood neutrophils

are activated and show increased reponses to a number of stimuli including fMLP. (b) ADCC was significantly higher in asthmatics for all the concentrations of SP tested. It could be argued that this merely reflects the higher baseline ADCC in this group. However when we considered *net*-ADCC (stimulated minus baseline) it became apparent that SP has a stimulatory effect on asthmatic neutrophils beyond that expected from the greater baseline activation. (c) The increased responsiveness appeared to have some selectivity for SP since responses to CM did not differ significantly between the two groups. This could indicate that neutrophils are already maximally stimulated in vivo, however this should not be the case in normal subjects. The reasons for the enhanced reactivity of neutrophils to SP in asthma are unknown. Since the ADCC assay used here is dependent on the presence of Fcγ receptors, it is possible that the differences we report may represent increased expression of these receptors in asthma and there is some evidence in support of this [45].

We found that high concentrations of SP are needed to stimulate ADCC and it is uncertain at the moment if neutrophils are exposed to such concentrations in vivo. To date, this must remain speculative. The SP concentration in the microenvironment is not known but it may be quite high: Agro et al reported SP concentrations as high as 0.2 μM in synovial fluid in rheumatoid arthritis [46]. Increased levels of SP were detected in bronchoalveolar lavage fluid after allergen challenge [47]. One can draw parallels with the effect of SP on mast cell histamine release in vitro and in vivo. The concentrations which we needed to demonstrate an effect on neutrophils in vitro are similar to those which are required to degranulate mast cells in vitro. However, nerve stimulation in vivo produces a cutaneous wheal and flare response a component of which is due to histamine release from mast cells by SP and other tachykinins. Although our in vitro experiments indicate potential differences between asthmatics and normals, one cannot extrapolate entirely to the situation in vivo where the SP may be acting in concert with other cytokines and/or mediators of inflammation. As can be seen from our own data, SP in low concentrations may act synergistically with other neutrophil-stimulating factors such as GM-CSF and we provide indirect evidence for this

When neutrophils obtained from normal subjects were incubated with SP for a short time (30 min), and then with GM-CSF plus SP, there was a synergistic effect on ADCC. The effect is synergistic because at the concentrations used in these experiments (0.1 nM - 1μM), SP did not stimulate ADCC in normals or asthmatics. These experiments were performed with normal subjects to ensure the neutrophils had not been maximally stimulated in vivo. The concentration of GM-CSF chosen was in the range previously shown to prime neutrophils and stimulate ADCC [41].

In contrast to its effect on neutrophils, SP had no effect on eosinophil ADCC under identical experimental conditions except in one subject in whom it caused stimulation. In contrast De Simmone et al [48] reported that SP stimulated eosinophil ADCC using

chicken red blood cells as a target and an IgG antibody; SP also increased the expression of Fcε receptors. The reasons why we failed to demonstrate a consistent stimulation of eosinophils by SP are uncertain. The target which we used is sensitive to both eosinophil and neutrophil ADCC and we were able to stimulate eosinophil ADCC with GM-CSF. Our isolation procedure for eosinophils most likely yielded a normodense population; in asthma there is an increased population of hypodense eosinophils which is more responsive to a number of stimuli [24]. Therefore it is quite possible that we may have detected an effect of SP on eosinophils had we used hypodense cells.

In population studies of asthmatics BHR correlates with the severity of asthma assessed by medication requirements, symptoms and the degree of airflow obstruction and its variability [49]. We demonstrated a modest but significant correlation between SP-induced ADCC, *net*-ADCC and BHR, assessed as PC_{20} histamine ie our data suggest that the neutrophil response to SP reflects partially the clinical severity of the asthma, the first time this relationship has been demonstrated.

In summary,our studies support a putative pro-inflammatory role for SP in asthma, as distinct from its role in causing bronchoconstriction and plasma extravasation. The modest correlation of this with BHR suggests that SP may have a greater role in the asthma syndrome than has previously been thought.

ACKNOWLEDGMENTS:

This work was supported by the National Health and Medical Research Council of Australia and the Special Purposes Fund, Royal Adelaide Hospital.

The recombinant human GM-CSF was a gift from Dr. A. Lopez, Institute Medical and Veterinary Science, Adelaide.

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TABLE I

CLINICAL DETAILS OF ASTHMATIC SUBJECTS IN WHOM HISTAMINE REACTIVITY WAS CORRELATED WITH ADCC

	Mean ± SEM (range), n = 20
AGE (yrs)	42.4 ± 3.3 (16 - 67)
M:F	14:6
FEV_1 (L)	3.0 ± 0.3 (1.2 - 5.3)
FEV_1 (% predicted)	81.9 ± 6.5 (32 - 86.5)
FVC (L)	3.8 ± 0.3 (1.8 - 7.3)
FVC (% predicted)	82.9 ± 5.0 (37.4 - 126.8)
FEV_1/FVC (%)	77.1 ± 3.0 (58.0 - 98.4)
PC_{20} (mg/ml)	0.93 ÷/x 1.43
Bronchodilators (n)	17
Aerosol corticosteroids (n)	17
Systemic corticosteroids (n)	Nil
Smokers (n)	2

FEV_1 forced expiratory volume in one second
FVC forced vital capacity
PC_{20} provocation concentration for 20% fall in FEV_1
ADCC antibody-dependent cell-mediated cytotoxicity

FIGURE LEGENDS

Figure 1

The effect of SP on neutrophil ADCC in normal subjects. Neutrophils were incubated with medium (0 SP), 1 nM - 100 µM SP or CM. Antibody concentrations and numbers of subjects are shown in the legend. Values are mean ± SEM, each experiment performed in triplicate.

Significantly greater than medium control (0 SP): * $p < 0.0001$; ** $p < 0.025$; # $p < 0.02$.

100 µM SP versus CM with 0.3 µg/ml ab, $p = 0.01$.

Figure 2

The effect of SP on neutrophil ADCC in asthmatic subjects. Conditions as in Fig 1.

Significantly greater than medium control (no SP): * $p < 0.0001$; ** $p < 0.002$.

100 µM SP versus CM, 0.3 µg/ml ab, $p = 0.01$.

Figure 3

Net-ADCC in normal and asthmatic subjects with 0.3 µg/ml ab (uppper panel) and 1.0 µg/ml ab (lower panel). Data are from subjects shown in Fig 1 and 2.

Normal versus asthmatics: * $0.0001 \leq p \leq 0.05$. NS = not significant.

Figure 4

Comparison of neutrophil and eosinophil ADCC in 5 asthmatic subjects. Cells were incubated with SP (concentrations as shown) or 10 ng/ml GM-CSF .

Significantly greater than medium control (no SP): * $p < 0.05$; ** $p < 0.002$.

Neutrophil ADCC was significantly greater than eosinophil ADCC for medium, all concentrations of SP, CM and GM-CSF at $0.0025 < p < 0.05$.

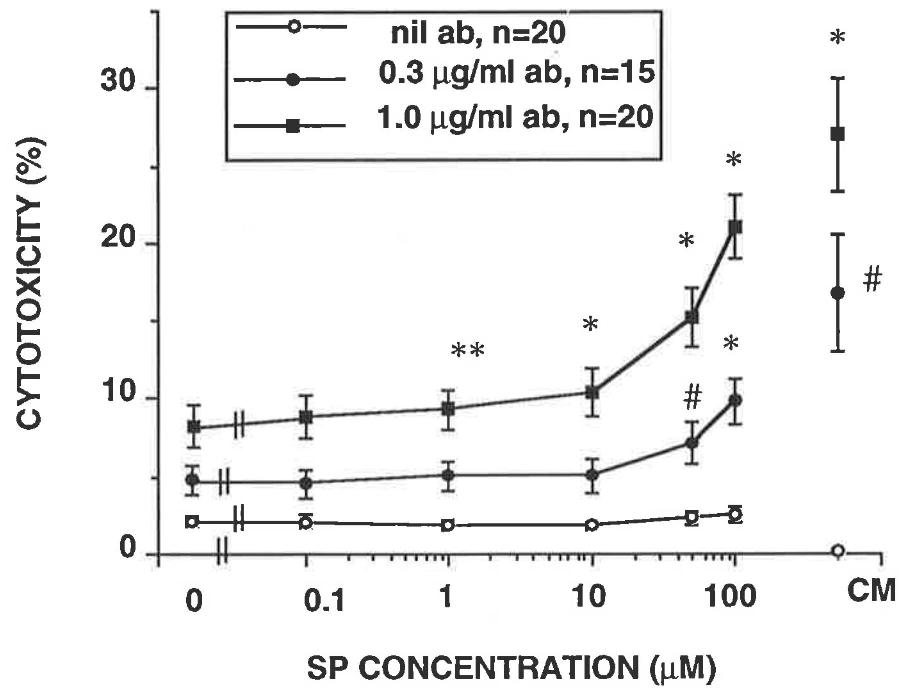
Figure 5

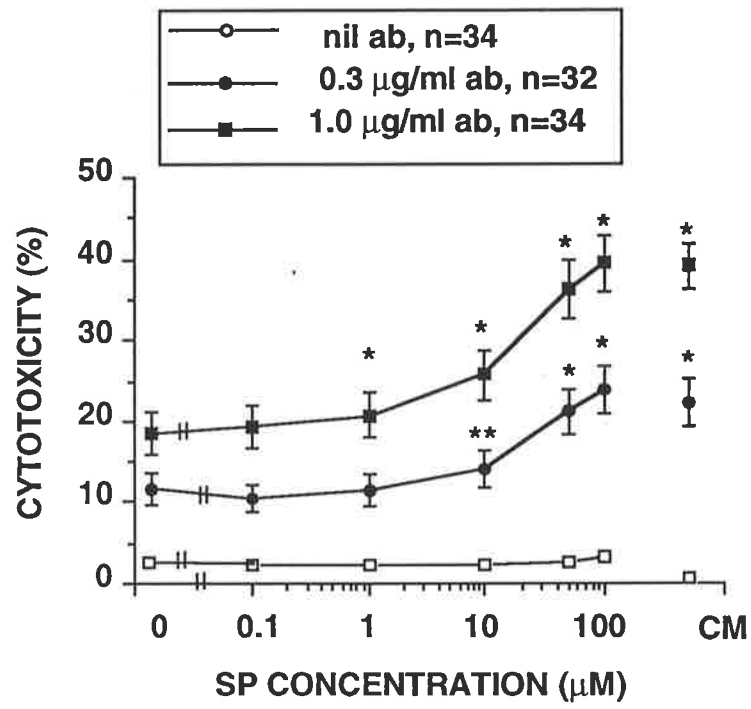
The effect of SP plus GM-CSF on *net*-ADCC. Cells were incubated with SP (concentration shown on abcissa) and GM-CSF (concentrations in legend). The response to 100 µM SP alone was 16.8 ± 3.7%. Data are means of 4-7 subjects, each experiment performed in triplicate.

* $0.015 \leq p. \leq 0.05$; ** $p \leq 0.005$ compared to GM-CSF alone (0 SP). Baseline ADCC used to calculate *net*-ADCC was 4.7 ± 2.1%.

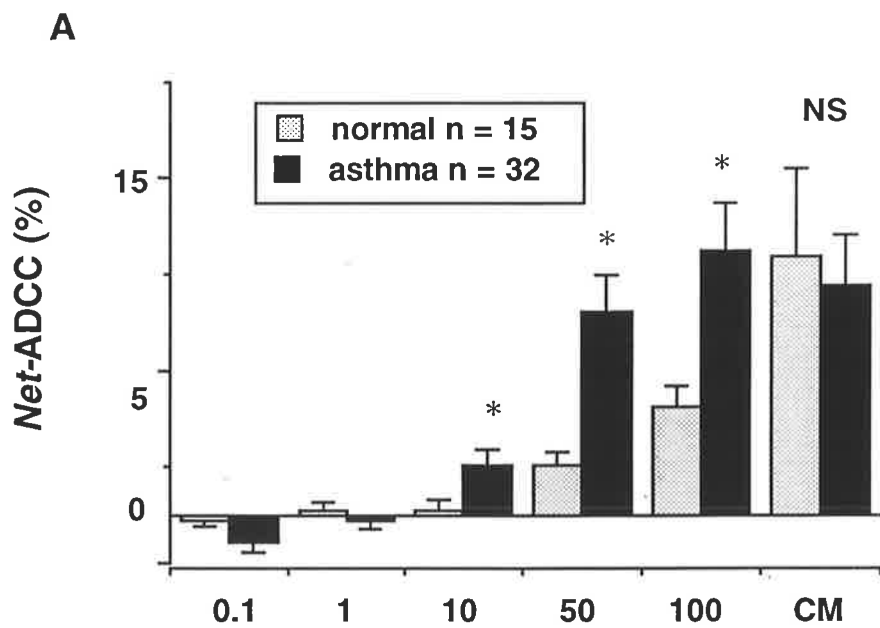
Figure 6

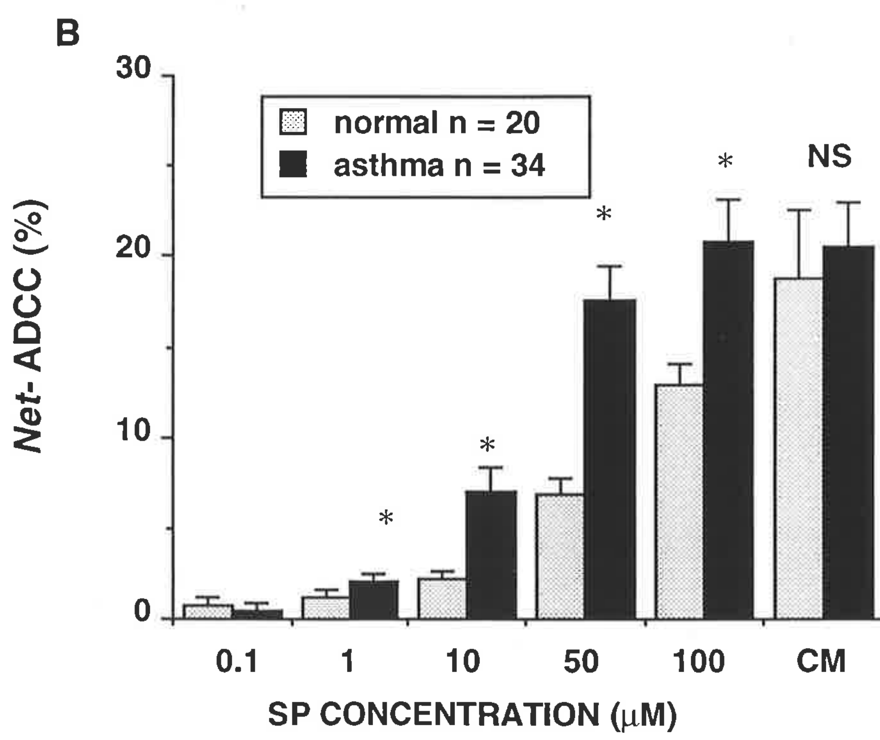
Correlation of cytotoxicity (as %) with histamine reactivity (PC_{20} in mg/ml) at 100 mM SP for 1 μg/ml (open circles, solid regression line) and 0.3 μg/ml ab (closed circles, dashed regression line).

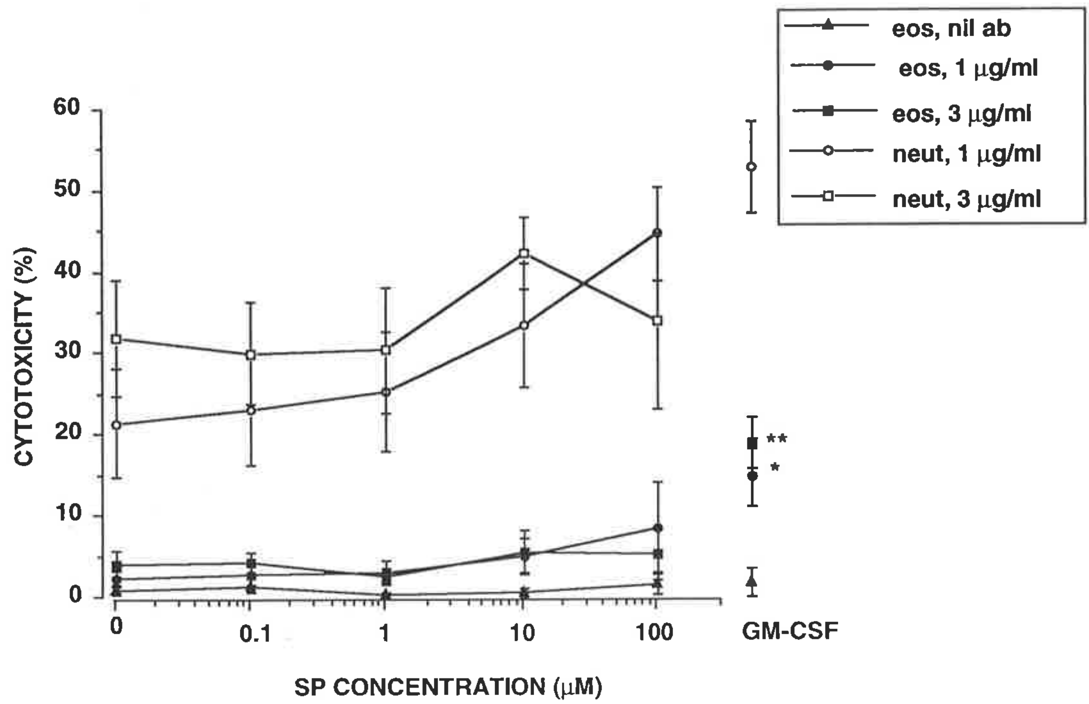

nil ab, n=20
0.3 µg/ml ab, n=15
1.0 µg/ml ab, n=20
CYTOTOXICITY (%)
30
20
10
0
0
0.1
1
10
100
CM
SP CONCENTRATION (µM)

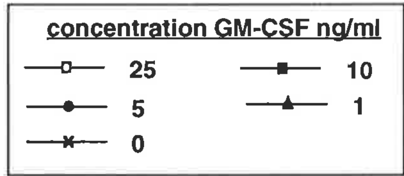


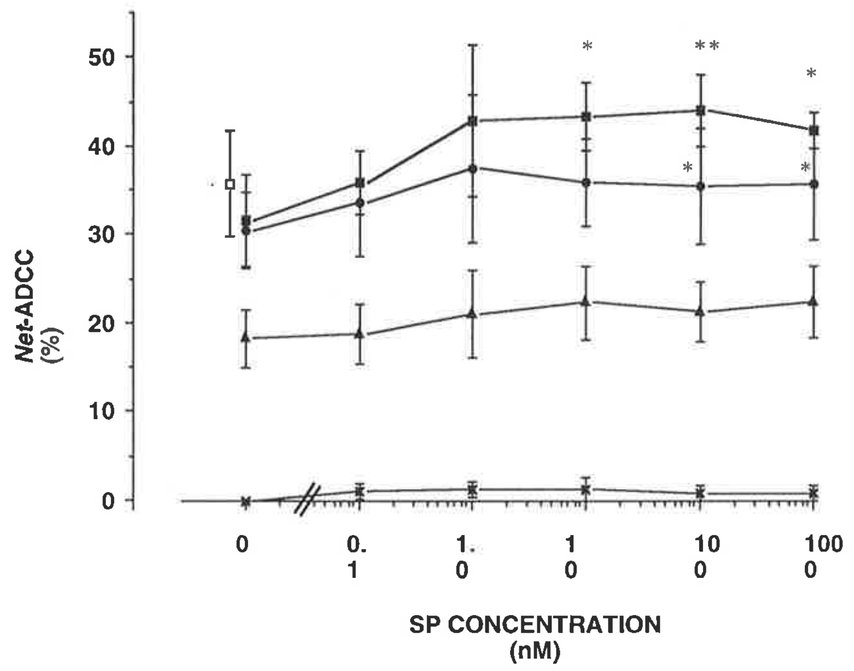
nil ab, n=34
0.3 µg/ml ab, n=32
1.0 µg/ml ab, n=34
CYTOTOXICITY (%)
50
40
30
20
10
0
*
**
0
0.1
1
10
100
CM
SP CONCENTRATION (µM)

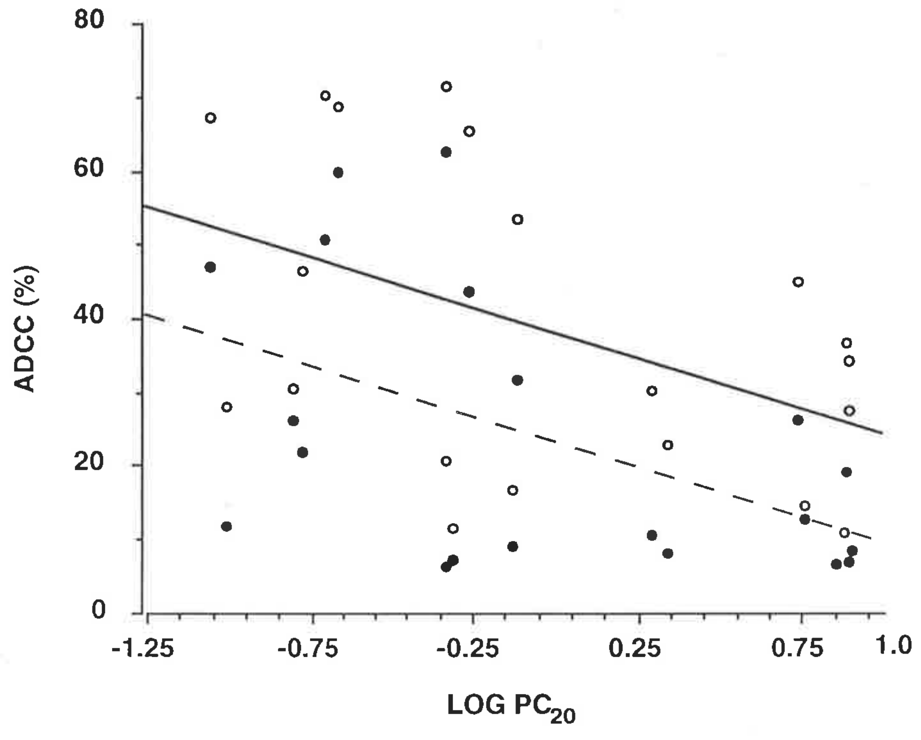

A
normal n = 15
asthma n = 32
NS
Net-ADCC (%)
15
5
0
*
0.1
1
10
50
100
CM


B
normal n = 20
asthma n = 34
NS
Net- ADCC (%)
30
20
10
0
*
0.1
1
10
50
100
CM
SP CONCENTRATION (μM)


eos, nil ab
eos, 1 μg/ml
eos, 3 μg/ml
neut, 1 μg/ml
neut, 3 μg/ml
CYTOTOXICITY (%)
60
50
40
30
20
10
0
**
*
0
0.1
1
10
100
GM-CSF
SP CONCENTRATION (μM)


concentration GM-CSF ng/ml
25
10
5
1
0


Net-ADCC (%)
50
40
30
20
10
0
0
0.1
1.0
10
100
1000
SP CONCENTRATION (nM)
*
**
*
*
*



80
60
40
20
0
ADCC (%)
-1.25
-0.75
-0.25
0.25
0.75
1.0
LOG PC_{20}

THE EFFECT OF SUBSTANCE P ON NEUTROPHIL FUNCTION IN NORMAL AND ASTHMATICS SUBJECTS.

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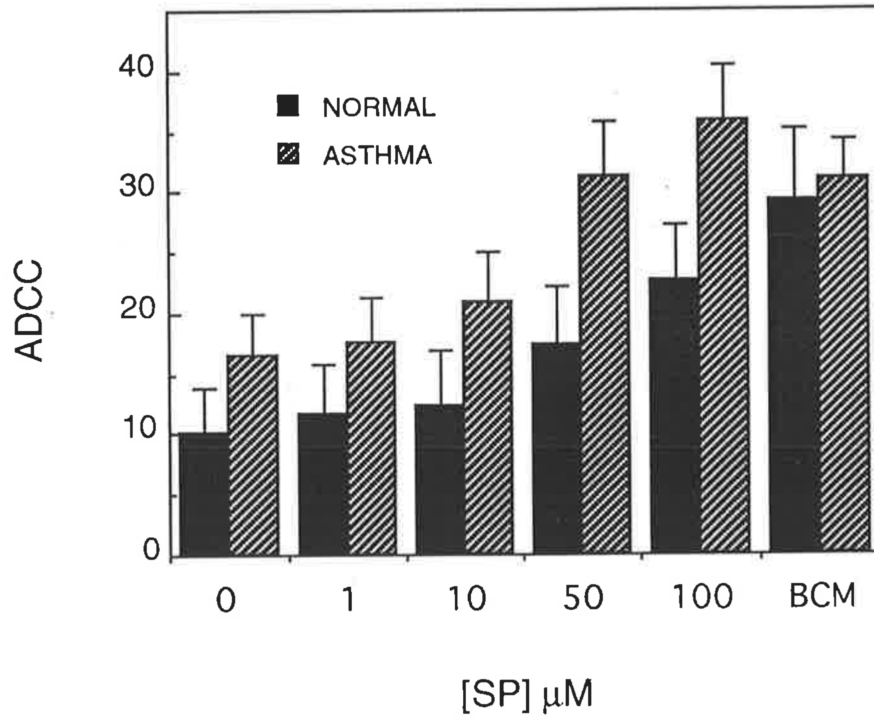
Substance P (SP), is a neurotransmitter found in C-afferent, unmyelinated sensory nerves. In the respiratory tract these nerves innervate epithelium, smooth muscle bundles, mucus glands and blood vessels. In asthma, it has been demonstrated that the number of fibres containing SP is increased when compared to normal airways. Recent evidence suggests that the local release of SP, by axonal reflexes, may induce or modulate airways inflammation and is important in the pathogenesis of asthma.

In this study we examined the ability of SP to activate neutrophils. We examined the direct effect of SP on neutrophil superoxide anion (O_2^-) production, as well as the priming effect for a second stimulus such as fMLP (formyl-methionyl-leucyl-phenylalanine), PMA (phorbol myristate acetate) and OPZ (opsonised zymosan). We also studied the effect of SP on neutrophil antibody-dependent cell-mediated cytotoxicity (ADCC) in normal and asthmatic subjects. We showed that 75 µM SP had only a small direct effect on neutrophil O_2^- production (3.6 ± 1.6 nmol O_2^- /10^6 cells), but primed the cells for an increased O_2^- production in response to 0.1 µM fMLP. A maximum priming effect was observed at 100 µM SP when O_2^- production increased from 10.4 ± 1.5 to 27.4 ± 3.0 nmol O_2^-/10^6 cells (301 ± 44% of medium control). Priming was rapid in onset (<5 min but required >20 sec), temperature-dependent (optimum at 37°C, absent at 4°C) and was not reversed by the removal of SP by washing after incubation. Similarly, when neutrophils were stimulated with 10 ng/ml PMA for 5 min, SP enhanced O_2^- production from 19.6 ± 2.0 to 30.4 ± 1.8 nmol O_2^- /10^6 cells (160 ± 9% control) by increasing the rate of O_2^- production and decreasing the lag phase before its onset.

In addition, SP stimulated neutrophil ADCC in a dose dependent manner. In the preliminary experiments, we compared the effect of SP on neutrophils isolated from normal and asthmatic subjects

(see figure). Spontaneous (baseline) ADCC was greater in asthmatics than normals (16.4 ± 3.6 versus 10 ± 3.9%). Even allowing for the higher baseline ADCC, SP-induced ADCC was significantly higher in asthmatics than normals. For example, 50 μM SP increased ADCC above the baseline by 14.7% in asthmatics compared to 7.3% in normals. Conditioned medium from a bladder carcinoma cell line (BCM), used as a positive control, induced the same response in normals and asthmatics (29.2 ± 5.8% and 30.9 ± 3.4% respectively).

These data suggest that SP may induce or potentiate airways inflammation by activating neutrophil cytotoxicity or by priming these cells for enhanced oxy radical production, and may play an important proinflammatory role in asthma.



NORMAL
ASTHMA
ADCC
40
30
20
10
0
0
1
10
50
100
BCM
[SP] μM

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AMENDMENTS.

In view of referees comments and suggestions the following changes and additions have been made to this thesis:

1. Where appropriate additional references to some methods and statements have been added. These are marked by a black dots in the text and appear at the bottom on the same pages.

2. Additional references have been added to the "Bibliography".

3. Legend to the figure 7.16 has been rewritten and inserted under the figure.

4. Typographical/spelling mistakes have been corrected in the text.

Due to unrefrenced methodology, one of the referees expressed difficulties in accepting the validity of the ^{3}H-PDBu binding assay used for the studies of PKC translocation in intact cells. This method has been previously validated and published [Shoyab and Todaro, 1980], subsequently reviewed [Janken 1986], and has been extensively used in our laboratory [French et al, 1987] and that of Professor Forbes [Zalewski et al, 1984], Department of Medicine, Queen Elizabeth Hospital. The method is specific for PKC and offers many advantages over other disruptive techniques [Janken 1986; O'Flaherty 1991 et al, 1990a,b; O'Flaherty 1992]. ^{3}H-PDBu binds rapidly and reversibly to human neutrophils. That the PKC constitutes the receptor for ^{3}H-PDBu, has been demonstrated by showing that: a diacylglycerol, dioctanoyl-glycerol, competes with ^{3}H-PDBu for the receptors, and a blocker of PKC-phospholipid interactions, sphinganine, inhibits the binding. Furthermore, studies with calcium-depleted neutrophils and cells that had been activated with calcium ionophore, ionomycin, demonstrated that the binding is dependent on the intracellular calcium concentration [O'Flaherty 1991 et al, 1990a,b].

PDBu does not activate PKC and neutrophils at concentration used as it is much less potent than PMA.

The major advantage of this assay over other disruptive methods is that PDBu binds to the PKC but apparently does not penetrate the membrane of the whole cells [O'Flaherty 1991 et al, 1990a], and thus it measures accurately PKC accumulation in the plasma membrane alone and yields real time results rather then PKC localisation after cell exposure to many agents and lengthy cell processing Since PKC translocation is

very rapid and reversible, it may be missed by cell processing used in the other methods.

It should be noted that although several PKC isoenzymes have been reported [Bell and Burns, 1991], some of which do not bind PDBu (for example PKC-zeta [Liyanage et al, 1992]), it is not the case for the two type of PKC isoenzymes (type II and III) found in human neutrophils [Balazovich et al, 1992].

Thus, the ^{3}H-PDBu binding assay is a valuable tool for studying the movements of PKC in situ.