

**The release and activity of the neuropeptide vasodilator Calcitonin
Gene-Related Peptide (CGRP) in rabbit skin**

A Thesis Submitted for the Degree of

Doctor of Philosophy

by

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1991

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**To my parents
and
Duncan**

ABSTRACT

Vasodilatation and oedema formation are important components of the inflammatory response. This study investigates the release and modulatory actions of the neuropeptide vasodilator calcitonin gene-related peptide (CGRP) *in vivo*, in the rabbit skin.

Microvascular plasma protein leakage and neutrophil accumulation were measured by the accumulation of ^{125}I -albumin and ^{111}In -neutrophils respectively. Increased blood flow was determined by measuring the rate of clearance of ^{133}Xe . CGRP, a potent long-lasting vasodilator, potentiated oedema formation and neutrophil accumulation induced by inflammatory mediators. CGRP alone did not induce neutrophil accumulation or plasma protein leakage, but when CGRP was combined with a long lasting neutrophil dependent mediator (eg. C5a des Arg or IL-1) a protracted inflammatory response was observed. Oedema induced by IL-1 was shown to be protein synthesis dependent but not dependent on the generation of PAF or histamine.

CGRP is localised in the sensory C-fibres of the peripheral nervous system. Capsaicin specifically activates sensory C-fibres resulting in the release of stored neuropeptides and neurogenic inflammation. Capsaicin, when injected intradermally induced oedema formation and vasodilatation. This response was inhibited by ruthenium red, a cationic dye that interferes with Ca^{2+} -dependent neuropeptide release. Ruthenium red had no effect on the actions of other agents known to activate sensory C-fibres (eg bradykinin). Goat anti-human CGRP antibody was used to establish whether CGRP was involved in neurogenic inflammation. Fab fragments of this antibody, produced by papain digestion, were separated over a DEAE-HPLC column. Purified IgG and Fab equally neutralised responses to exogenous CGRP. Capsaicin-induced vasodilatation and oedema formation were both significantly inhibited by intradermal pretreatment for 40mins with the Fab fragment whereas IgG was weaker at neutralising capsaicin-induced responses.

Further investigations into the mechanisms of sensory C-fibre activation and the evaluation of novel inhibitors against either release of stored neuropeptides or their actions will provide the potential for the development of new anti-inflammatory agents.

Acknowledgements

I wish to thank Prof. Tim Williams and Dr. Sue Brain for their guidance, support, encouragement and never ending patience.

I would also like to thank Dr. Peter Jose, Dr. Malcolm Watson, Dr. Paul Collins, Dr. Frances Williams, Dr. Sussan Nourshargh, Dr. Simon Larkin, Elaine Hawkings and Sarah Hughes and everyone in the Department of Pharmacology for their helpful discussions and friendship.

I am extremely grateful to Carol Coker for her expert help in the preparation of this thesis.

The work presented in this thesis was carried out during the tenure of a Medical Research Council Studentship.

I would like to express my sincere thanks to my parents and family for their love and endless support.

Last but not least, I would like to thank my husband, Duncan, for his love, devotion and support particularly during the writing of this thesis.

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List of Abbreviations

min	= minute
h	= hour
SEM	= standard error of the mean
$t^{1/2}$	= half life
i.d.	= intradermally
i.v.	= intravenously
PBS	= phosphate buffered saline
cAMP	= adenosine 3'5'-cyclic monophosphate
cGMP	= guanosine 3'5'-cyclic monophosphate
DRG	= dorsal root ganglia
FMLP	= N-formyl-methionyl-leucyl-phenylalanine
EDRF	= endothelial-derived relaxing factor
PAF	= platelet activating factor
PGE ₂ , PGI ₂	= prostaglandin E ₂ , I ₂
LTB ₄	= leukotriene B ₄
VIP	= vasoactive intestinal peptide
CGRP	= calcitonin gene-related peptide
NKA, NKB	= neurokinin A, B
NGF	= nerve growth factor
IL-1	= interleukin 1
Ca ²⁺	= calcium
AA	= arachidonic acid
ANS	= anti-neutrophil antiserum
ZAP	= zymosan activated plasma

Manuscripts published as part of this thesis

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J.Immunol. in press

Buckley,T.L., Brain,S.D., Rampart,M., Williams,T.J. (1991). Time-dependent synergistic interactions between the vasodilator neuropeptide calcitonin gene-related peptide (CGRP) and mediators of inflammation: potentiation of neutrophil accumulation and oedema formation.

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Buckley,T.L., Brain,S.D., Rampart,M., Williams,T.J. (1988). The potential of calcitonin gene-related peptide as an inflammatory mediator.

Regulatory Peptides 22:393P

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Eur.J.Pharmacol. **183**:1587P

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Br.J.Pharmacol. **99**:94P

1.1 Introduction to Inflammation

The classical signs of inflammation, which play a vital role in host defence, are swelling, redness, heat and pain. The first three are direct consequences of microvascular alterations in response to inflammatory mediators. The last sign is dependent on the activation of the sensory nervous system and the propagation of impulses to the brain leading to the perception of pain. Moreover, release of stored neuropeptides from peripheral sensory nerve terminals (eg substance P and calcitonin gene-related peptide; CGRP), can result in 'neurogenic inflammation' which can contribute to the inflammatory process.

There are many chemical mediators that are responsible for the inflammatory response. These can be broadly classified into those arising from blood, plasma and other tissue fluids and those arising from cells. However mediators are often grouped according to their chemical nature (eg lipid, phospholipid, peptide etc.) as well as their biological actions.

Synergism between mediators of increased microvascular permeability and vasodilators occurs. Interestingly some mediators are capable of inducing both an increase in permeability and vasodilatation in certain species eg histamine (Lewis, 1927). The 'two mediator' hypothesis, proposed by Williams (1977), suggested that vasodilators can potentiate oedema formation induced by mediators that cause plasma protein leakage. It is thought that vasodilators promote arteriolar dilatation and hence increase hydrostatic pressure

downstream within the venule lumen. This synergistic response is seen most markedly in systems that have inherently low basal blood flow rates (eg the rabbit skin).

1.1.1 Direct acting mediators of increased microvascular permeability

These agents induce oedema formation by acting directly on the endothelial cells lining the blood vessels. Thomas Lewis (1927) was the first to describe the 'wheal' response which was caused by plasma protein leakage in response to intradermal histamine. Direct microscopical studies by Majno et al (1961) demonstrated that histamine acts on the small post-capillary venules, opening junctions between adjacent endothelial cells and allowing passage of plasma proteins. Histamine is widely distributed in mammalian tissues; particularly in the human epidermis, lungs, gastro-intestinal mucosa and central nervous system. Histamine is stored in high concentrations in tissue mast cells, circulating basophil leukocytes and platelets of many species. Allergic responses are often associated with histamine release because mast cells and basophils, which store amines, have IgE and IgG antibody receptors on their cell surfaces. However, histamine release can also be induced by other peptides and proteins including substance P. It has also been shown that histamine can initiate the release of substance P (Foreman *et al.*, 1983b). The use of specific histamine antagonists have allowed the sub-classification of histamine H₁ and H₂ receptors (and more recently H₃ receptors). As described by Lewis (Lewis, 1927), the intradermal (i.d.) injection of histamine evokes a wheal and flare response. The flare response was shown to be principally mediated via H₁ receptors (Plaut, 1979; Foreman *et al.*, 1983b; Brain *et al.*, 1986a).

Bradykinin, a nonapeptide is a potent vasodepressor in many species. Bradykinin receptors have also been sub-classified into bradykinin B₁ and B₂ receptors. B₂ receptors mediated plasma protein leakage whereas B₁ receptors facilitated vasodilatation (Regoli & Barabe, 1980). However, some of the dilatory actions of bradykinin appear to be dependent on prostaglandin synthesis (Terragno *et al.*, 1972). B₁ receptors, but not B₂ receptors can be constitutively induced in response to inflammatory stimuli (Regoli & Barabe, 1980). Other mediators that have been reported to act directly on venules to increase vascular permeability include, leukotrienes LTC₄, LTD₄ (Williams & Piper, 1980) substance P, as described later (Lembeck & Holzer, 1979) and PAF (Vargaftig *et al.*, 1981; Wedmore & Williams, 1981). Direct-acting mediators tend to induce inflammatory responses that are rapid in onset and of short duration.

1.1.2 Neutrophil-dependent mediators of increased microvascular permeability

Mediators that induce neutrophil-dependent oedema formation have a different inflammatory profile; they tend to have a slower onset of action but produce a more persistent response. This thesis focuses on oedema formation induced by direct-acting mediators and oedema induced by the accumulation of neutrophils. Neutrophils predominate in the acute phase of inflammation whereas mononuclear cells and other leukocytes are more evident in later phases of inflammation. Neutrophils were first implicated in inflammatory oedema in Arthus reactions as shown in experiments carried out by Stetson *et al* (1951) and Humphrey (1955). In these studies the depletion of circulating neutrophils reduced inflammatory oedema. It was then shown that neutrophil chemoattractants could also induce oedema by a neutrophil-dependent process (Wedmore & Williams, 1981). Further, it was shown that this occurs within a few

minutes, rather than several hours as in previous studies. Neutrophils adhere to endothelial cell membranes, usually in venules, in response to chemoattractants. Following adherence, neutrophils pass through inter-endothelial junctions and penetrate the basement membrane. It is thought that the plasma leakage occurs either in the wake of emigrating leukocytes or is dependent on the release of mediators of increased microvascular permeability from adherent cells.

The chemoattractant activity of complement activated rabbit serum, *in vitro*, was discovered by Boyden (1962). Later, Jose *et al* (1981) reported that the complement fragment C5a and its metabolite C5a des Arg were both potent neutrophil dependent inflammatory mediators. In addition to its well-documented effects on neutrophils, *in vitro* and *in vivo* (chemotaxis, aggregation, enzyme release and accumulation) C5a was reported to release the direct acting mediator histamine from mast cells. However the cleaved product, C5a des Arg had little histamine releasing activity in man (Gerard & Hugli, 1981). The importance of C5a in inflammation was emphasised by detection of C5a, by radioimmunoassay, in many inflammatory exudates, including fluid from inflamed synovial joints (Jose *et al.*, 1990). Other mediators that induce neutrophil-dependent oedema include, the lipxygenase product LTB₄, and the formyl peptide FMLP (Wedmore & Williams, 1981). These mediators induced neutrophil adherence to the venular wall by stimulating a conformational change in the CD11b/CD18 glycoprotein complex on the surface of neutrophils (Sanchez-Madrid *et al.*, 1983; Wallis *et al.*, 1986). Recently the cytokines interleukin-8 (IL-8) and tumour necrosis factor have been shown to induce neutrophil accumulation associated with oedema formation (Rampart *et al.*, 1989; Foster *et al.*, 1989).

1.1.3 Interleukin 1 (IL-1)

IL-1 belongs to the cytokine family of polypeptides. Earlier studies, using fairly crude preparations demonstrated many inflammatory properties of IL-1; these actions have been largely confirmed using recombinant IL-1. The pro-inflammatory actions of IL-1 include; fever, neutrophilia, increase in leukocyte adherence, induction of the acute phase response and prostanoid production (Dinarello, 1988). IL-1 has also been shown to induce neutrophil accumulation, in vivo, in the rabbit skin and synovial cavity (Beck *et al.*, 1986; Pettipher *et al.*, 1986; Movat *et al.*, 1987; Rampart & Williams, 1988). Unlike C5a, IL-1 does not directly stimulate neutrophil chemotaxis, but increases the adhesiveness of the endothelial cell surface for neutrophils by inducing the synthesis of endothelial-leukocyte adhesion molecules (ELAM) and increasing the expression of intracellular adhesion molecules (ICAM) (Pober, 1988; Dustin *et al.*, 1986; Bevilacqua *et al.*, 1989).

There are two forms of IL-1, IL-1 alpha and beta; March *et al* (1985) reported that they had only 26% homology but they shared many inflammatory activities and bound to the same receptors. It has been reported, however, that IL-1 beta can induce hyperalgesia (nociception) in the rat, whereas IL-1 alpha is 3000 times less potent at inducing this activity (Ferreira *et al.*, 1988). IL-1 is synthesised by a wide variety of cells. These include synovial fibroblasts, keratinocytes and Langerhans cells of the skin, vascular endothelium, smooth muscle, monocytes and lymphocytes. It is currently thought that a considerable amount of IL-1 alpha (from whatever source) remains cell associated but the beta form is secreted into the extracellular fluid (Dinarello, 1988). Biologically-active IL-1 has been detected in inflammatory exudates, particularly in synovial fluids taken from patients with rheumatoid arthritis (Nouri *et al.*,

1984; Eastgate *et al.*, 1988; Hopkins *et al.*, 1988). IL-1 appears to be an important mediator in the synovial joint because in addition to inducing cell accumulation, this cytokine may also contribute to cartilage degradation and pain (Pettipher *et al.*, 1986; Pettipher *et al.*, 1988; Krane *et al.*, 1985).

1.1.4 Local blood flow and inflammation

The main physiological role of the microvascular bed is to distribute the blood supply to tissues to provide it with oxygen and nutrients and to remove metabolic waste products. The blood is also the carrier of leukocytes which can release or respond to inflammatory mediators and therefore aid host defence. The local blood flow in the microvessels is tightly controlled by local factors under normal conditions e.g. in response to metabolic demands. In addition, vascular tone is also regulated to maintain systemic blood pressure. However, following an inflammatory stimulus local blood flow can be altered so as to over-ride the physiological factors controlling flow.

There are numerous vasoactive substances that are involved in the regulation of vascular tone. It has been shown that some of these agents act directly on vascular smooth muscle cells, whereas others require the presence of endothelial cells. Endothelial-derived relaxing factor (EDRF; Furchgott *et al.*, 1980), now known to be nitric oxide (Palmer *et al.*, 1987), is an example of an endothelium-dependent vasodilator. Many inflammatory stimuli liberate arachidonic acid leading to the release of a whole host of biologically-active metabolites (PGE₂, PGI₂ thromboxane A₂ etc). PGE₂ and PGI₂, which are cyclooxygenase products, induced equipotent vasodilatation in rabbit skin (Peck & Williams, 1978); the vasodilatory actions of prostaglandins, reported to date, do not necessitate the presence of an intact endothelium and are therefore

classified as direct acting vasodilators. PGE_2 has been detected in wound fluid (Lundberg & Gerdin, 1984) and PGI_2 was generated in an acute inflammatory response induced by an intraperitoneal injection of zymosan in the rabbit (Forrest *et al.*, 1986). In addition, release of both PGE_2 and PGI_2 have been reported in immediate hypersensitivity reactions (Brown *et al.*, 1987). Many cell types are capable of producing prostaglandins including mast cells and neutrophils (Zurier, 1976) as well as vascular endothelium and smooth muscle (Smith, 1986).

Several neuropeptides are also known to have potent effects on blood vessels (eg substance P, vasoactive intestinal peptide (VIP) and CGRP). The actions of these neuropeptides were shown to be endothelium-dependent in certain in vitro systems (Furchgott, 1981; Davies & Williams, 1984; Brain *et al.*, 1985).

1.2 Neurogenic Inflammation

The proposal that sensory nerves are involved in inflammation was first investigated in the 19th century by Stricker (1876). At the turn of the century Bruce (1910) and Breslauer (1919) found that transection of sensory nerves negated the inflammatory responses usually induced by mustard oil in man and other species. The above experiments as well as the classical investigations of Bayliss (1901) and Lewis (1924,1927) led to the assumption that neurogenic inflammation, including the flare response, was dependent on the activation of sensory nerves. Lewis described the visual response to injury in skin, induced by either chemical, mechanical or thermal injury in terms of three separate components. These were the flush (local reddening), the flare and the wheal and together they make up the triple response. The wheal and local reddening were observed at the site of injury; they were dependent on plasma protein leakage and vasodilatation respectively. However the third component, the flare was seen as a less intense erythema that spread for up to several centimeters around the focal point of the inflammatory response. Lewis showed that the flare was dependent on the presence of an intact neural network. It is now believed that the flare is the result of an axon reflex response, which may involve mast cell activation and histamine release (Foreman & Jordan, 1984).

How is the axon reflex response evoked? This is a question that many theories have tried to answer. According to Lewis (Lewis, 1927) the mediator of antidromic vasodilatation was released from a nerve ending which was specialised for the release of a transmitter. It was distinct therefore from the sensory receptor, which was specialised for initiation of afferent impulses. However later experiments by Szolcanyi (1988) and others do not support this hypothesis entirely. Their data suggested that each sensory fibre has a

bidirectional axon reflex arrangement with nerve endings having a dual sensory-efferent function. Szolcanyi concluded that axonal branching or axo-axonal transmission between two polymodal fibres was necessary for the development of a flare reaction. To avoid confusion, in this thesis antidromic stimulation is taken as the transmission of neural impulses from the dorsal ganglia to the peripheral terminals of sensory fibres and orthodromic stimulation is the transmission in the opposite direction, ie. from the peripheral terminals.

In the 1960's attention was drawn to the problem of neurogenic inflammation by Jancso and co-workers. They demonstrated that capsaicin, the pungent principle of Hungarian hot peppers, could desensitise pain receptors to chemical stimuli such as xylene, mustard oil, related acyl amides and capsaicin itself (Jancso & Jancso-Gabor, 1959; Jancso *et al.*, 1961). In 1967 Jancso went further and demonstrated that antidromic stimulation of the saphenous nerve in rats evoked a similar response to capsaicin ie: arteriolar vasodilatation, enhancement of vascular permeability and protein exudation (Jancso *et al.*, 1967). Furthermore, capsaicin desensitisation inhibited the signs of inflammation normally evoked by antidromic stimulation. They concluded that a neurohumoral substance could be released by anti- and/or orthodromic stimulation of pain sensitive nerve terminals (Jancso *et al.*, 1967). These studies laid down the foundations of neurogenic inflammation and investigations, to date, have been trying to answer these two basic questions.

1. What are the exact mechanisms involved in nerve activation?
2. Which substances are responsible for neurogenic inflammation?

While most of the early work on neurogenic inflammation was completed in the skin, it has become increasingly evident that this response could also be elicited in; the mucous membrane (Saria *et al.*, 1983), the respiratory tract (Lundberg & Saria, 1983; Barnes *et al.*, 1988), the gastro-intestinal and urinary tracts (Saria *et al.*, 1983; Maggi & Meli, 1988) and many other systems. In the next section the evidence for the role of neuropeptides in inflammation is described.

1.3 Candidates for mediators of neurogenic inflammation

1.3.1 Tachykinins; Substance P

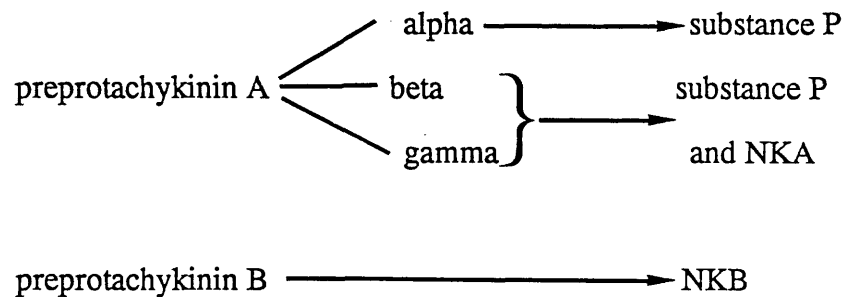
Substance P, an undecapeptide, was first discovered in 1931 by Von Euler and Gaddum (von Euler & Gaddum, 1931). It was subsequently immunohistochemically localised in a distinct population of unmyelinated primary sensory neurons throughout the peripheral and central nervous systems (Hokfelt *et al.*, 1975). However, the possibility that this peptide was involved in neurogenic inflammation was discussed years earlier by Lembeck (1953). Substance P induced plasma extravasation (Lembeck *et al.*, 1977); evidence to suggest that this response had a neural origin was provided by the finding that capsaicin pretreatment decreased the amount of substance P contained in sensory neurons as well as impairing neurogenic plasma extravasation (Gamse *et al.*, 1979). Lembeck and Holzer (1979) also observed mimicry of neurogenic inflammation by exogenous substance P (vasodilatation and oedema formation). In addition they (and others), suggested that substance P, once released could activate mast cells resulting in histamine release because on depletion of mast cell amines with compound 48/80, neurogenic responses were greatly diminished. Later these findings were confirmed when the flare component associated with intradermal substance P, in human skin, was abolished by an H₁ antagonist (Foreman & Jordon, 1984; Hagermark *et al.*, 1977). Foreman *et al.* (1983) investigated the mechanism of mast cell activation by substance P. Their studies suggested that substance P did not activate mast cells via a receptor-mediated mechanism, but rather as a consequence of the presence of basic amino acids at the N-terminal that probably insert a hydrophobic region into the mast cell membrane lipid to cause mast cell activation. However, the pro-inflammatory actions of substance P could not be solely attributed to indirect mechanisms (ie

mast cell activation), but result also from a combination of activities including effects on blood vessels (Foreman *et al.*, 1983b). Recent studies have confirmed that the C-terminal end of this peptide interacts directly with specific vascular receptors (Foreman *et al.*, 1983b). In 1982, the development of substance P antagonists helped confirm the role of this peptide in neurogenic inflammation (Lembeck *et al.*, 1982). In addition, Helme and White (1985) provided direct evidence, by radioimmunoassay, that substance P-like immunoreactivity (LI) was released from sensory nerves. In their studies nerves serving the hind paw of the rat were stimulated and fluid was collected from a thermal injury blister. This fluid was assayed and was found to be positive for substance P-LI. Payan *et al.* (1983) demonstrated that substance P could interact with granulocytes, monocytes and lymphocytes, probably via specific receptors. At high doses ($> 10\mu\text{M}$), substance P also appeared to directly stimulate neutrophils, but these doses are probably not physiologically relevant. At lower doses of substance P (10nM), that did not effect neutrophil function, substance P did however synergistically enhance migration and the respiratory burst of neutrophils stimulated by FMLP and C5a (Payan *et al.*, 1987). The substance P-related peptides physalaemin, eledosin and kassinin also mimicked neurogenic inflammation but these peptides have not been localised in mammals (Gamse *et al.*, 1980).

1.3.1.1 Neurokinin A and B (NKA,NKB)

Substance P was considered to be the only tachykinin present in mammals until NKA (also termed substance K or neuromedin L), NKB (also termed neuromedin K) and neuropeptide K (NPK, a 36 amino acid polypeptide that

contains the NKA sequence in the C-terminal portion) were isolated from the porcine central nervous system (Kimura *et al.*, 1983; Minamino *et al.*, 1984). Two similar genes encode substance P, NKA and NKB.



Gamse *et al* (1985) demonstrated that both NKA and NKB could induce plasma extravasation in rat and human skin. NKA and NKB were shown to possess a common C-terminal with substance P. However, the tachykinins do not possess a common N-terminal and in accord with previous findings demonstrating that this end was responsible for mast cell activation, NKA and NKB did not stimulate histamine release from rat mast cells. In addition Brain and Williams (1989) demonstrated that oedema formation induced by NKA and NKB in the rat skin was not inhibited by pre-treatment with amine antagonists, whereas responses to substance P were clearly attenuated by this pre-treatment regime.

The use of capsaicin has implicated the neurokinins as mediators of neurogenic inflammation because this neurotoxin depleted neurokinin-LI from sensory neurons (Maggio & Hunter, 1984). Furthermore, the mRNA for preprotachykinin A has been localised in sensory nerves which is probably sufficient evidence to postulate a role for NKA as a sensory neuropeptide. However, little evidence is available for the presence of NKB in sensory nerves.

1.3.1.2 Tachykinin receptors

It has been known for many years that there are significant differences between various tachykinins and their fragments in bioassays. This observation is consistent with the existence of multiple types of tachykinin receptors. Lee et al (1982) were among the first to recognise multiple receptors for substance P. Using rank order of potency, the receptors for tachykinins were differentiated into three subtypes as follows :

NK-1: sp > phy > NKA > NKB > ele > kas

NK-2: NKA > NKB > kas > ele > sp > phy

NK-3: NKB > ele > kas > NKA = phy > sp

sp = substance P

ele = eledosin

kas = kassinin

phy = physalaemin

Although it is generally accepted that plasma extravasation in skin, induced by tachykinins is a NK-1-mediated event, there is some evidence to suggest the involvement of NK-3 (Gamse & Saria, 1985). Ward et al (1990) to support the argument for NK-1, demonstrated that GR71251, an NK-1 selective antagonist significantly attenuated extravasation of Evan's blue dye induced by the NK-1 selective agonist GR73632. There is little evidence for NKB, which is most potent at the NK-3 receptor, in peripheral sensory fibres. Therefore if the NK-3 receptor is responsible for neurogenic plasma extravasation then the localisation of NKB in sensory nerves would have to be confirmed.

1.3.2 Vasoactive Intestinal Peptide (VIP)

VIP is a 28 amino acid peptide which was originally isolated from the porcine small intestine (Said & Mutt, 1970) and exhibits potent vasodilator activity in vivo and in vitro, in many vascular beds (Piper *et al.*, 1970; Bryant *et al.*, 1976; Williams, 1982). In some tissues VIP induces vasodilatation via an endothelium-dependent mechanism (Davies & Williams, 1984). It belongs to a family of peptides including secretin, glucagon, corticotropin-releasing factor (CRF), gastrin-inhibitory peptide, growth hormone releasing factor and peptide histidine isoleucine (PHI). VIP has been localised in mammalian skin (Bloom & Polak, 1983) and there is increasing evidence for the localisation of VIP (and CRF) in sensory neurons, in the peripheral nervous system (Fuji *et al.*, 1983; Skofitsch *et al.*, 1984; Bjorklund *et al.*, 1986). To further implicate VIP as a neurogenic mediator, this peptide induced a wheal and histamine-dependent flare response (Anand *et al.*, 1983; Brain & Edwardson, 1989). In addition, central branches of sensory nerves show an increase in VIP-LI following axotomy or crush (McGregor *et al.*, 1984). In addition, Maggi *et al.* (1990), demonstrated that the neurotoxin capsaicin produced a significant release of VIP-like immunoreactivity.

VIP has been also localised, in abundance, in the parasympathetic nervous system. Lundberg (1981), demonstrated that VIP and acetylcholine are released from the same nerve terminals. It is thought that, in this system, VIP allows marked vasodilatation of blood vessels and that it acts as a neuromodulator to substantially enhance the release and effect of acetylcholine.

1.3.3 Somatostatin

Somatostatin was originally isolated from the hypothalamus in 1968 (Krulich *et al.*, 1968) and exists in two forms; 14 and 28 amino acids. Gibbins *et al* (1985) reported that 75% of CGRP positive fibres, in human skin, were also somatostatin positive. In addition this peptide has been detected in neurons of sensory and sympathetic ganglia (Hokfelt *et al.*, 1975; Luft *et al.*, 1978). Like VIP and substance P, somatostatin was shown to be capable of evoking a wheal and flare response (Fjellner & Hagermark, 1985), but in most vascular beds somatostatin induces vasoconstriction. It has been suggested that this peptide has presynaptic actions and is able to alter the release of other transmitters which could give rise to a variety of effects on the post-synaptic neuron. The biological effects of this peptide are not fully understood but it has been postulated that somatostatin could play a neuromodulatory role in neurogenic inflammation.

1.3.4 Endothelin

This 21 amino acid peptide endothelial product was isolated and sequenced by Yanagisawa *et al* (1988). Three subclasses of this peptide have been described (endothelin-1, endothelin-2 and endothelin-3). Endothelin has been shown to have potent constrictor effects on major arteries, *in vitro*, and produces a long lasting pressor response when injected intravenously in the rat (Yanagisawa *et al.*, 1988). Recently, endothelin has been localised in sensory neurons (Hiley, 1989), raising the possibility that endothelin could be a sensory neuropeptide. Brain *et al* (1988) noticed a surprising response when endothelin was injected into human skin; in addition to the expected vasoconstriction they demonstrated that this peptide produced a flare but not a wheal response.

1.4 Calcitonin Gene-Related Peptide (CGRP)

The calcitonin/CGRP gene has been found to encode a family of peptides; calcitonin, CGRP and katacalcin; this thesis concentrates on CGRP.

The existence of a 37 amino acid peptide, CGRP was predicted on the basis of the alternative processing of the calcitonin gene (Amara *et al.*, 1982; Rosenfeld *et al.*, 1983). CGRP was the only member of this family to be detected in the central and peripheral nervous system, particularly in fibres associated with blood vessels (Rosenfeld *et al.*, 1983; Kawai *et al.*, 1985). However, CGRP was also found in the C cells of the thyroid where it was first isolated from human medullary thyroid carcinoma tissue (Morris *et al.*, 1984). In addition to these sources, some CGRP has been localised to the adrenal medulla (Rosenfeld *et al.*, 1983), pancreatic islet cells, pulmonary neuroendocrine cells (Lauweryns & van Ranst, 1987; Wada *et al.*, 1988), the neuromuscular junction (Fontaine *et al.*, 1986) as well as neurons in the autonomic nervous system (Ishida-Yamamoto & Tohyama, 1989). Although the role of CGRP, in the central nervous system, is a very important topic (reviewed by Ishida-Yamamoto and Tohyama, 1989), this thesis focuses on the localisation, release and function of CGRP in the periphery. CGRP, highly concentrated in large dense-core vesicles (Matteoli *et al.*, 1988) is transported by anterograde axoplasmic flow from the neuron cell bodies to the nerve terminals (Kashihara *et al.*, 1989). This flow occurs at about 1mm/hr in sensory (and a few motor) fibres. It has also been reported that the majority of CGRP disappears from terminals after denervation (Kashihara *et al.*, 1989).

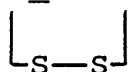
CGRP sequences from different species (human, rat and chicken) were found to be highly conserved (figure 1.1). Two forms of this peptide have been characterised, alpha and beta CGRP (Amara *et al.*, 1985) and a high degree of

Figure 1.1 CGRP IN THE NERVOUS TISSUE

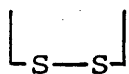
ACNTATCVTHRLAGLLSRSGGMVKSNFVPTNVGSKAF-amide human α -CGRP



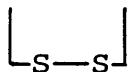
ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF-amide human β -CGRP



SCNTATCVTHRLAGLLSRSGGVVKDNFVPTNVGSEAAF-amide rat α -CGRP



SCNTATCVTHRLAGLLSRSGGVVKDNFVPTNVGSKAAF-amide rat β -CGRP



homology exists between them. The alpha and beta form of human CGRP differed by 3 amino acid, whereas the rat form differed by 1 amino acids (Amara *et al.*, 1985; Steenberg *et al.*, 1985). There appears to be striking differences in the distribution of alpha and beta CGRP. In most sensory nerves, the concentration of alpha CGRP was reported to be 3-6 times that of beta CGRP, whereas in the enteric neurons only beta CGRP mRNA has been detected (Mulderry *et al.*, 1985). However, Amara *et al.* (1985) demonstrated that alpha and beta CGRP could both be present in a single peripheral terminal. Recently, another 37 amino acid peptide has been discovered, amylin amide, which bears 46% structural homology to CGRP (Cooper *et al.*, 1987) and has vasodilator activity in rabbit skin (Brain *et al.*, 1990).

1.4.1 Vasodilator properties of CGRP

The vasodilatory activity of CGRP has been demonstrated in a variety of vascular beds, including; the mesenteric (Kawasaki *et al.*, 1988), coronary (Holman *et al.*, 1986), renal (Lappe *et al.*, 1989), cerebral (Edvinsson *et al.*, 1985), the respiratory tract (Martling *et al.*, 1988) and skin (Brain *et al.*, 1985). It was demonstrated that in the human skin both alpha and beta CGRP were very potent long-lasting vasodilator neuropeptides (Brain & Williams, 1985; Brain *et al.*, 1986b). At a dose of 15 fmol, a threshold erythema was visualised in human skin, which faded about 20 minutes after intradermal injection. At a dose of 10 pmol, CGRP induced reddening (a local blood flow response) up to 6 hours after injection. At this dose pseudopodia were seen radiating from the injection site; this response probably involved the lymphatic system possibly carrying the CGRP away from the site of injection (Brain *et al.*, 1985). A wheal and flare response was only seen when CGRP was injected at much higher doses (>50 pmol) in human skin (Brain *et al.*, 1985; Piotrowski & Foreman, 1986). This response was

inhibited by histamine antagonists suggesting that, at these doses, CGRP could be capable of activating mast cells resulting in histamine release. However, at doses < 10 pmol, CGRP only induced vasodilatation in rabbit and human skin without inducing plasma protein leakage. Direct observation of the microvasculature by intravital microscopy allowed the vasoactive actions of CGRP to be visualised (Brain, 1989). These studies, in the hamster cheek pouch, revealed that arterioles rapidly relaxed in response to topical CGRP (10 pmol) followed by a passive relaxation of the venules.

Although xenon clearance techniques demonstrated the potent vasodilator activity of CGRP in rabbit skin (Brain *et al.*, 1985), it was not until the introduction of the laser Doppler flow meter that this response was directly measured in human skin. Non-invasive studies, using the laser probe, also showed that CGRP increased local blood flow in human skin (Brain *et al.*, 1990). The role of CGRP as a vasodilator was further confirmed when CGRP was administered intravenously; a depressor response was observed (Gennari & Fischer, 1986). As a consequence of its vasodilator activity, CGRP was able to potentiate oedema formation induced by mediators of increased microvascular permeability (Brain & Williams, 1985).

1.4.2 Mechanism of action of CGRP

Endothelium-dependent relaxation induced by CGRP has been demonstrated in a variety of human blood vessels and in rat aorta (Brain *et al.*, 1985; Kubota *et al.*, 1985), but relaxation was independent of an intact endothelium in some vessels (Hanko *et al.*, 1985; Edvinsson *et al.*, 1985). Therefore, it remains controversial as to whether the endothelium is necessary for the relaxation of vessels by CGRP. With reference to the lung however,

McCormack et al (1988), demonstrated that the removal of the epithelium did not diminish the bronchoconstrictor response induced by CGRP. In studies where binding sites for CGRP were found on endothelial cells and smooth muscle, CGRP stimulated cAMP formation (Kubota *et al.*, 1985), but not cGMP formation (Grace *et al.*, 1987). Crossman et al (1987), using cultured human endothelial cells demonstrated that in addition to activation of adenylate cyclase, a concentration-dependent release of prostacyclin was also observed. They concluded that some of the vasodilator properties of CGRP may be explained by the release of vasorelaxant substances such as prostacyclin from endothelial cells. Brain et al (1985) had previously reported that vasorelaxation by CGRP of precontracted aortic strips was partially inhibited by indomethacin. However, they also showed that CGRP-induced vasodilatation in rabbit skin *in vivo* was not indomethacin-sensitive. Thus there appears to be a difference between *in vivo* and *in vitro* responses to CGRP. Crossman and co-workers suggested that this inconsistency could be due to the differing effect of CGRP on arterioles and major arteries. Recently it has been reported that arterial dilatation in response to CGRP involved activation of K^+ channels (Nelson *et al.*, 1990). In this study, Nelson et al (1990), demonstrated that the K^+ channel blockers glibenclamide and barium partially blocked arterial dilatations, hyperpolarisation and the opening of K^+ channels on smooth muscle induced by CGRP.

1.4.3 Inactivation of the vasodilator activity of CGRP

The co-existence of substance P with CGRP in many sensory fibres has been widely reported. Brain and Williams (1989) highlighted two functional reasons for this co-localisation in the rat skin. Firstly, the increase in microvascular permeability induced by substance P was potentiated by CGRP as

a direct consequence of its vasodilator activity; this potentiation was also shown by Gamsé and Saria (1985). In another study, long lasting vasodilatation, induced by CGRP, was converted to a transient response in the presence of substance P (Brain & Williams, 1988). CGRP was inactivated; this metabolism was effected by proteolytic enzymes released from or expressed on mast cells in response to substance P. Unlike substance P, NKA and NKB were unable to attenuate the persistent response induced by CGRP, probably because the neurokinins do not possess similar basic N-terminal sequences to substance P and are much poorer activators of mast cells (Brain & Williams, 1989)). Compound 48/80, a potent activator of skin mast cells did, however, inhibit the vasodilator activity of CGRP. Hence the involvement of mast cells in this response seems highly likely. These investigations suggest that the balance of CGRP and substance P at sites of inflammation could be important and that normally the vasodilator response induced by CGRP was tightly controlled, but in some situations, when CGRP was not degraded, persistent and possibly pathological responses could be observed. Another factor that could contribute to the inactivation of the vasodilator response is that the degraded molecule is likely to be cleared faster than intact CGRP from the microcirculation. However, CGRP does not appear to be metabolised in blood or plasma (Brain *et al.*, 1985). In addition, fragments of CGRP do not possess vasodilator activity. This mechanism for the inactivation of CGRP, outlined in the skin, may also be responsible for neuropeptide metabolism in other systems eg. the respiratory tract, where these neuropeptides have also been co-localised. Additionally, there could equally be interactions between other co-stored neuropeptides. It is therefore of interest to note that CGRP has been co-localised with somatostatin in cutaneous fibres and that somatostatin can activate mast cells (Anand *et al.*, 1983).

1.4.4 Other biological functions of CGRP

In addition to being a potent vasodilator, a variety of other functions of CGRP have been reported, particularly in the urinary system. Unlike in rat intestines, where CGRP caused relaxation (Maggi & Meli, 1986), Tippins *et al* (1984), reported that CGRP induced contraction of the guinea-pig ileum. However, as this response was blocked by hyoscine, an anti-cholinergic compound, the contractile effect of CGRP may be due to activation of cholinergic neurons. CGRP has also been shown to inhibit the motility of rat and guinea-pig ureters (Hua, 1986; Maggi *et al.*, 1987b) and in the uterus and fallopian tubes CGRP inhibited the contraction of smooth muscle (Bek *et al.*, 1988).

Kjartansson and co-workers (Kjartansson & Dalsgaard, 1987a; Kjartansson *et al.*, 1987b) have shown a) reduced healing in skin flaps following denervation with capsaicin b) increased healing potential with CGRP. The dose of CGRP needed to promote healing, in this model, was below the threshold dose for vasodilator activity. Therefore, they postulated that CGRP might promote wound healing by another mechanism, such as acting as a trophic agent in addition to increasing local blood flow.

Recently, it has also been demonstrated that CGRP was involved in the differentiation of dopaminergic olfactory bulb neurons. The number of dopaminergic-cells increased 5 fold when olfactory bulb neurons were co-cultured with CGRP-containing chemosensory neurons. To further implicate CGRP in this process, the induction of dopaminergic phenotypes was abolished by an anti-serum to CGRP (Denis-Donini, 1989).

In the heart, CGRP caused positive inotropic and chronotropic responses (Tippins *et al.*, 1989; Franco-Cereceda & Lundberg, 1985; Franco-Cereceda *et al.*, 1987). Satoh *et al.* (1986) suggested that the positive inotropic response caused by CGRP was mediated by an increase in the influx of Ca^{2+} but cAMP production was not involved (Satoh *et al.*, 1986).

Finally, it has been reported that CGRP was a bronchoconstrictor agent in the lung however, Martling *et al.* (1988) were unable to find any bronchoconstriction in human bronchi, *in vitro*.

1.4.5 Distribution of CGRP in skin

CGRP is only one of the many different neuropeptides localised in mammalian skin; the tachykinins, VIP, somatostatin, opioids, neurotensin, neuropeptide Y, bombesin and galanin have also been shown to be present in sensory fibres. It has been reported that, at least in human skin, CGRP is the most abundant sensory peptide (Wallengren *et al.*, 1987a; Wallengren *et al.*, 1987b). Immunocytochemistry localised CGRP to free nerve endings in the epidermal and dermal layers and around blood vessels (Gibson *et al.*, 1984; Gibbins *et al.*, 1985; Lundberg *et al.* 1985; Ju *et al.*, 1987; Gibbins *et al.*, 1987). Gibbins *et al.* (1987) reported that, in the epidermis, 75% of these CGRP-LI sensory nerves also contained somatostatin, whereas in the remainder CGRP co-existed with substance P. Substance P was not co-stored with somatostatin in human skin. They also reported that very few perivascular nerves contained CGRP-LI + substance P-LI but an abundance of nerves containing CGRP-LI + somatostatin-LI existed. It has been suggested that sensory nerves, containing substance P or somatostatin, project to different layers in the dorsal horn; these

fibres might be functionally distinct (Hokfelt *et al.*, 1976). It was also postulated that nerves containing CGRP + somatostatin conveyed a different modality to nerves containing CGRP + substance P (Kuraishi *et al.*, 1985).

In hairy skin, thin CGRP fibres were found entering the base of each hair follicle. The Meissner corpuscles were also shown to have nerve fibres containing both CGRP and substance P (Alvarez *et al.*, 1988). Binding sites for CGRP have been found using autoradiography on smooth muscles and the endothelium of blood vessels in both veins and arteries (Hanko *et al.*, 1985; Gibbins *et al.*, 1985; Wanaka *et al.*, 1988; Gibbins *et al.*, 1987; Lundberg *et al.*, 1985; Hirata *et al.*, 1988). There could be at least two origins of CGRP in the microvasculature; the perivascular nerves and in the blood.

1.4.6 Distribution of CGRP in the respiratory tract

Substantial amounts of CGRP-LI were detected in all parts of the respiratory tree (range 5-37 pmol/g) (Cadieux *et al.*, 1986). CGRP was localised to neuroendocrine cells and sensory nerve fibres. Endocrine cells containing CGRP were found singly in the trachea and stem bronchi and in groups in the intrapulmonary airways (Uddman *et al.*, 1986; Cadieux *et al.*, 1986; Wada *et al.*, 1988; Martling *et al.*, 1988). Nerve fibres containing CGRP were localised among smooth muscle, seromucous glands, beneath and within the epithelium of the airways and around blood vessels (Cadieux *et al.*, 1986; Uddman *et al.*, 1986). Barnes (1987) concluded that CGRP was mostly associated with the afferent (sensory) innervation of the respiratory tract.

Autoradiography revealed that binding sites for CGRP were widely distributed with particularly dense labelling over bronchial and pulmonary blood vessels and alveolar walls (Mak & Barnes, 1988). The strongest binding was shown to be to small blood vessels (Cadieux *et al.*, 1986). Airway smooth muscle and epithelium of large airways was sparsely to moderately labelled, but no labelling was found on glands. With the exception of the alveolar wall, the placing of CGRP 'receptors' was consistent with the localisation of the network of nerve fibres containing CGRP-LI. Both Umeda and Ansawa (1989) and Carstairs (1987), reported the presence of low and high affinity binding sites for CGRP, whereas Mak and Barnes (1988) reported only high affinity binding sites in the respiratory tract.

1.4.7 Distribution of CGRP in the joint

In the synovium Ralston *et al* (1960) reported a rich innervation of free sensory nerve fibre endings. In contrast, other groups have shown that the synovium, along with the joint capsule, were relatively insensitive to pain (Kellgren & Samuel, 1950). Gronblad *et al* (1988) demonstrated, using specific antisera for the neurofilament and stored neuropeptides, that neural elements were present in the synovial tissue, predominantly in a perivascular location. It was also shown that the fascia, tendons, ligaments and periosteum, which are specific elements in the articular tissue, were innervated. In addition, sensory fibres were also found to be abundant near the epiphyseal plate, in the bone marrow of the patella, and in the epiphysis (Golzman & Mitchell, 1985). Substance P and CGRP have been co-localised to sensory C-fibres in the knee joint (Gronblad *et al.*, 1988).

1.4.8 CGRP in the circulation

CGRP-LI has been found in the plasma of healthy humans (Girgis *et al.*, 1985), patients with medullary thyroid carcinoma (Morris *et al.*, 1984) and rats (Zaidi *et al.*, 1985). It has been reported that the level in plasma from healthy humans was around 25 pmol/l, however, in patients with thyroid carcinoma the level was elevated to around 3.4 nmol/l. When capsaicin was intraperitoneally injected into rats a 15 fold increase was observed in circulating CGRP (Emson & Zaidi, 1989). It has been suggested that there are at least two origins of circulating CGRP in the rat, the thyroid (C cells) and perivascular nerves but the thyroidal origin was only obvious in older rats. If CGRP or related peptides circulate, in biologically active amounts, they could possibly be involved in the regulation of blood flow and the maintenance of vascular tone. In addition the administration of CGRP, at a high dose, was able to lower plasma calcium levels and inhibit bone reabsorption stimulated by parathyroid hormone (Tippins *et al.*, 1984).

1.4.9 Central effects of CGRP localised in peripheral nerves

Rosenfeld *et al* (1983), localised a CGRP fibre plexus in the dorsal horn and trigeminal spinal plexus. Miletic and Tan (1988) have reported that iontophoretic application of CGRP produces a slow and protracted excitation of neurons in the cat dorsal horn. CGRP also potentiated synaptic transmission at the level of the dorsal horn by altering calcium conductance (Ryu *et al.*, 1988). CGRP cells have been localised using immunohistochemistry in the substantia gelatinosa at the level of the spinal cord; this could suggest a relationship between CGRP and the nociception conducting system. However, there has been conflicting evidence to support this hypothesis. Oku *et al* (1987) suggested that CGRP was involved in mechanical nociception, but Gamse and Saria (1985)

failed to detect any effect of this peptide on nociception. Recently, Kuraishi et al (1988) and Kawamura et al (1989) have both reported the anti-nociceptive effect of intrathecally administered antiserum against CGRP in rats. CGRP, as well as being co-localised with substance P in the dorsal horn, increased the release of this tachykinin from the dorsal horn (Oku *et al.*, 1987). In addition to this facilitatory role, CGRP attenuated substance P degradation by inhibiting a substance P endopeptidase isolated from the cerebrospinal fluid (LeGreves *et al.*, 1985).

1.4.10 CGRP and the motor system

There have been many reports of CGRP outside the sensory nervous system, particularly in the motor neuron (Rosenfeld *et al.*, 1983; Gibson *et al.*, 1984; Laufer & Changeux, 1987; Takami *et al.*, 1985), where CGRP and acetylcholine have been co-localised. CGRP, once transported to the neuromuscular junction has been reported to enhance striated muscle contraction (Takami *et al.*, 1985). The first morphological evidence that CGRP was present at the human neuromuscular junction was presented by Mora et al (1989). But this group reported that normally very low concentrations of this peptide existed. However, CGRP, at these levels was able to stimulate the synthesis of acetylcholine receptors and increase the cellular level of the second messenger cAMP (Fontaine *et al.*, 1986; Laufer & Changeux, 1987). It was therefore postulated that low levels of CGRP may normally be required for acetylcholine receptor turnover. In addition to its neuromodulator function, CGRP suppressed terminal sprouting of nerves and therefore seems to have an anti-neurotrophic function (Tsujimoto & Kuno, 1988).

Leighton and Cooper (1988) demonstrated that CGRP inhibited both basal and insulin-stimulated rates of glycogen synthesis in rat soleus muscle. If CGRP modulates glycogen synthesis, then it is not clear whether this peptide originates from the motor end plate, perivascular nerves or circulating CGRP. However, recently a novel 37 amino acid peptide, amylin amide, has been shown to have 46% structural similarity with CGRP (Cooper *et al.*, 1987). It is now thought that the endogenous mediator that regulates glycogen synthesis could be amylin amide. Its localisation, in the beta cells of the pancreas, and the fact that Clark *et al* (1987) reported that it was co-stored with insulin, would strongly support this hypothesis. Interestingly, amylin amide, like CGRP, was shown to have vasodilator activity in the rabbit and perhaps these peptides are able to bind to the same receptors (Brain *et al.*, 1990).

1.4.11 CGRP receptors

Foord and Craig (1987) reported the presence of a 'cardiovascular' CGRP receptor on human placenta. The membrane bound protein had a molecular weight of 240 000 Da as determined by crosslinking ¹²⁵I-labelled alpha CGRP. However studies on solubilised preparations demonstrated a number of CGRP binding subunits of molecular weight 62 000 - 68 000 Da. Silver staining of the purified CGRP preparation showed two distinct doublets, but there appeared to be a single binding site of high affinity. They suggested that the observed heterogeneity of CGRP binding proteins can be interpreted in a number of ways; receptor heterogeneity, variable glycosylation of one of two subunits, or limited proteolysis of a single subunit during purification. In contrast to these findings, CGRP binding proteins from the cerebellum had molecular weights of 13 700 and 50 000 Da (Sigrist *et al.*, 1986), considerably smaller than the Foord and Craig receptor subunits. There have been reports of other differences between

CGRP receptors in the brain and the periphery. Calcitonin cross-reacted with the CGRP receptor complex in the brain (Breimer *et al.*, 1988), although Sigrist *et al.* (1988) could only demonstrate low binding of this gene product to the CGRP receptor. However, the cardiovascular receptor has very low binding affinity for calcitonin. Foord and Craig (1987) concluded that, at physiological concentrations, human calcitonin and CGRP act via different receptors. Yamaguchi *et al.* (1988) reported the presence of CGRP receptors on rat liver plasma membranes. They suggested that the C-terminal portion of CGRP was essential for binding to its receptors, since an N-terminal fragment of human CGRP could not bind to CGRP receptors whereas C-terminal fragments were able to displace labelled binding. In addition Chiba *et al.* (1989), reported that the C-terminal fragments of CGRP, lacking a ring structure with a sulphide bond, may not be sufficient to induce a subsequent intracellular signal transduction event ie adenylate cyclase activation. Denis *et al.* (1989) agreed that the C-terminal portion of CGRP was important and suggested that amino acids 9-37 were important for maintaining appropriate receptor recognition.

To address the possibility of heterogeneity of CGRP receptors, evidence was presented for the dissociation of CGRP receptor sub-types in peripheral and central tissues. Binding data from brain and spleen membrane preparations strongly suggest CGRP receptor heterogeneity (Seifert *et al.*, 1985; Sexton *et al.*, 1986; Dotti-Sigrist *et al.*, 1988). These data and results obtained from agonist potency ratios in a variety of isolated tissue preparations (Tippins *et al.*, 1986; Denis *et al.*, 1990), have led to the proposal of CGRP₁ and CGRP₂ receptor subclasses. CGRP₁ class is most sensitive to the antagonistic properties of the C-terminal fragment hCGRP₁₂₋₃₇, whereas the CGRP₂ subtype demonstrated preferential affinity for the agonist [cys(ACM)^{2,7}]hCGRP (Denis *et al.*, 1990).

The use of the newly developed putative antagonist hCGRP₈₋₃₇ (Chiba *et al.*, 1989) and other antagonists should help determine the receptor subclasses involved in CGRP-mediated events.

1.5 Activation of Sensory C-Fibres

There are numerous agents that are able to activate peripheral sensory fibres resulting in nociception and release of stored neuropeptides. Chemical irritation with capsaicin, mustard oil and resiniferatoxin has been well documented. In addition mediators of allergic reactions like bradykinin and histamine can evoke neurogenic responses. Bradykinin has been shown to be a potent C-fibre activator in the airways, the heart and throughout the cardiovascular system (Kaufman *et al.*, 1980). The responses induced by bradykinin often mimick those induced by capsaicin, further implicating the sensory neuron in bradykinin-evoked events (Manzini *et al.*, 1989). One example of this was presented in the rat trachea where bradykinin and capsaicin induced neuropeptide release resulting in protein extravasation (Lundberg & Saria, 1983; Kaufman *et al.*, 1980). Bradykinin also had potent bronchoconstrictor effects in normal persons suggesting a possible role for bradykinin in the pathogenesis of asthma (Simonssen *et al.*, 1973). Recently second messenger studies using a rat spinal tail preparation have indicated that bradykinin activates nociceptors via a bradykinin B₂ receptors coupled to protein kinase C (Dray & Perkins, 1988). However, Geppetti *et al* (1990) demonstrated that in the guinea-pig atria, bradykinin-induced CGRP release was unaffected by a protein kinase C inhibitor. Therefore the mechanism of action of bradykinin, in activating sensory fibres, is still largely unknown.

Capsaicin-sensitive nerves are activated by mediators released upon mast cell degranulation, most notably histamine (Lundberg & Saria, 1983). In human skin, a histamine-induced flare response was inhibited following topical application of capsaicin (Toth-Kasa *et al.*, 1986).

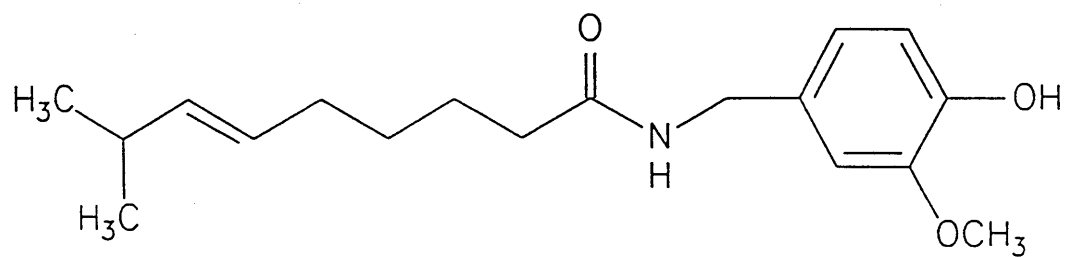
Exogenous irritants, like nicotine in cigarette smoke have also been demonstrated to activate sensory fibres (Lundberg & Saria, 1983). Nicotine induced airways bronchoconstriction and, in addition, pulmonary vascular protein leakage was noted (Kizawa & Takayanagi, 1985).

Recently it has been reported that ischaemic conditions such as those following a myocardial infarction were associated with a prolonged post-occlusive hyperaemia in the guinea-pig heart. Since pre-treatment with capsaicin reduced this increase in flow, it was concluded that the release of sensory peptides after ischaemia may contribute to this phenomenon (Franco-Cereceda & Lundberg, 1989).

1.6 Capsaicin

Capsaicin (a homovanillylic acid, figure 1.2), is the pungent component of red hot peppers. It has been used as a tool to investigate the physiology and pathophysiology of primary afferent neurons. There are two major uses of this substance. One is to damage selectively a subpopulation of sensory neurons by repeated or persistent application, hence depleting stored neuropeptides. Many investigators have used systemic capsaicin pre-treatment in neonates as a means of achieving complete depletion of neuropeptides in many species. The other is to give a single application of capsaicin and monitor neuropeptide release. Together these procedures have allowed the evaluation of the importance of sensory neuropeptides in various responses.

Capsaicin



Resiniferatoxin

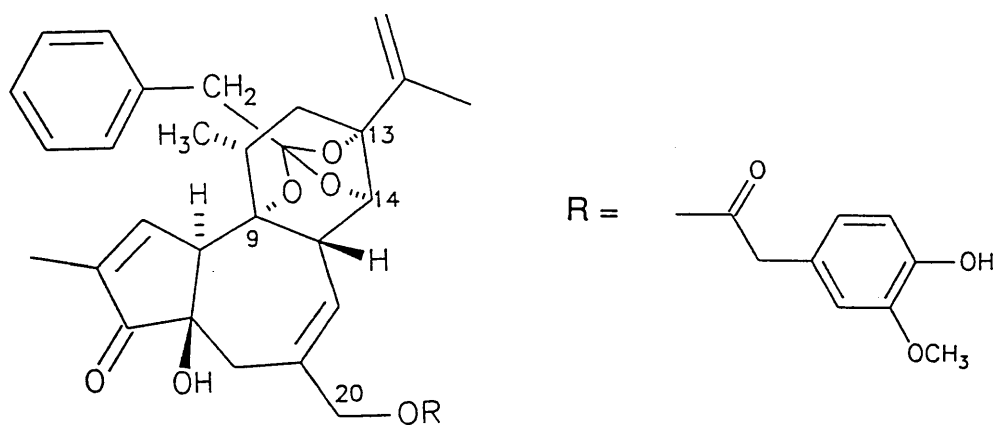


Figure 1.2

Comparison of the Structures of Capsaicin and Resiniferatoxin

The mechanism of action of capsaicin is by no means fully understood. However, it has been reported that capsaicin excites polymodal nociceptive neurons with conduction velocities principally in the C-fibre range, but some A delta-fibres also showed signs of activation. Sensory C-fibres are small ($1\mu\text{m}$ in diameter) and unmyelinated with conduction velocities of 1-2m/sec. A delta-fibres are small ($1-2\mu\text{m}$ in diameter) and myelinated with conduction velocities of approximately 50m/sec. Fibres that are responsive to capsaicin are collectively known as capsaicin-sensitive neurons. Jancso and co-workers (Jancso *et al.*, 1961; Jancso *et al.*, 1967) were the first group to realise the potential importance of this neurotoxin to investigate neurogenic inflammation. They also showed that mustard oil, xylene and other acyl amides belong to the same functional family as capsaicin. Since then many advances have been made, using these substances, in the understanding of neurogenic inflammation. Indeed, capsaicin has been used therapeutically in the treatment of rhinitis and Guillain Barre syndrome (Morgenlander *et al.*, 1990) and there may be more therapeutic uses for capsaicin and its analogues.

1.6.1 Effects of capsaicin on sensory C-fibres

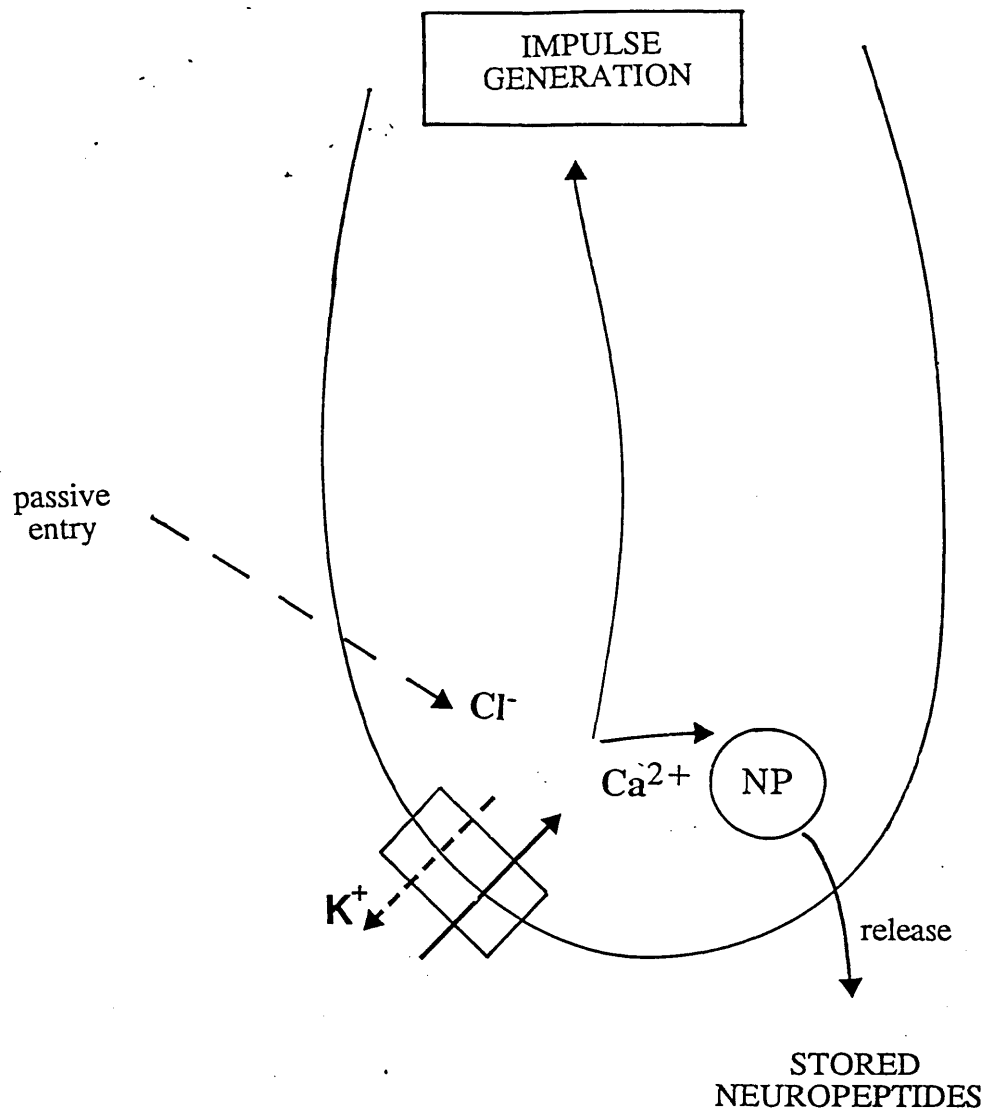
Excitation of sensory C-fibres can lead to both activation of sensory (release of neuropeptides in the central nervous system) and efferent (release of neuropeptides at peripheral sites) functions. There are many reports of the sensory action of capsaicin on pain perception and thermoregulation (Maggi & Meli, 1988). Studies on the antinociceptive effect of systemic capsaicin desensitisation were a major source of enthusiasm to many groups providing the possibility that this neurotoxin could have a therapeutic potential. Szolcsanyi *et al* (1988), provided an important contribution to this topic. They showed that when

capsaicin was administered to adult rats, the antinociceptive effect observed was highly dependent upon the type and intensity of the nociceptive stimulus. The central effects of neuropeptides released by capsaicin activation of peripheral terminals is a very important topic, but this study concentrates on the peripheral release of stored neuropeptides.

A whole host of neuropeptides have been reported to be contained in capsaicin-sensitive neurons (see figure 1.3). Capsaicin, and other agents have been reported to excite sensory C-fibres and subsequently release these stored neuropeptides. At the end of the excitation phase the sensory terminal becomes desensitised to both natural and artificial stimuli (including capsaicin). However, at this stage the sensory terminals still contain large amounts of transmitters but the sensory fibres are no longer able to effect sensory or efferent functions (Maggi *et al.*, 1987a). After recurrent prolonged stimulation of sensory C-fibres neuropeptide depletion is more likely to occur both centrally and peripherally. In addition to its ability to release neuropeptides it has been suggested that capsaicin could act via multiple mechanisms to inhibit repletion of these nerves including a blockade of axonal flow of transmitters and blockade of the transport of trophic factors particularly nerve growth factor (NGF; Otten *et al.*, 1980). It is important to note that additional binding sites for capsaicin have been localised at the axonal level and it is thought that these are responsible for this blockade (Otten *et al.*, 1980).

1.6.2 Mechanism of action of capsaicin; figure 1.3

The following sequence of events have been shown to occur following application of capsaicin to sensory C-fibres.



CGRP
 NKA/NKB
 substance P
 somatostatin
 VIP
 galanin
 corticotrophin-releasing factor
 bombesin-like peptides

Figure 1.3
Activation of Sensory C-fibres

1. A primary increase in Na^+ and Ca^{2+} conductance (influx). This action suggests the opening of an ion channel, followed by membrane depolarisation and block of C spike conduction. The involvement of Na^+ was indicated by a reduction in membrane depolarisation in its absence. However, capsaicin did not activate fast Na^+ channels because the depolarising action of capsaicin, on sensory nerves was tetrodotoxin resistant (Maggi *et al.*, 1986).
2. Influx of Ca^{2+} leading to a secondary increase in K^+ efflux (and possibly Cl^- influx) frequently resulted in a secondary hyperpolarisation (Marsh *et al.*, 1987; Wood *et al.*, 1989). This action was followed using radiotracer flux experiments (Wood *et al.*, 1988).
3. Neuropeptide release. Capsaicin has been reported to release neuropeptides directly from peripheral sensory fibres via a Ca^{2+} -dependent but tetrodotoxin-independent mechanism. This mechanism involves membrane depolarisation but the neuropeptides are not released as a result of a propagated impulse and therefore does not rely on an axon reflex response (Gamse *et al.*, 1979). This is in contrast to ortho- or antidromic activation of sensory C-fibres which often results in an axon reflex response and the release of sensory neuropeptides from the peripheral and the central nervous system. This release involves impulse propagation and is Ca^{2+} , local anaesthetic and tetrodotoxin sensitive (Gamse *et al.*, 1979).

There is a general understanding that Ca^{2+} is very important in capsaicin-mediated events. Perhaps the most convincing piece of evidence to suggest a role for Ca^{2+} is that exposure to Ca^{2+} -free medium protected sensory

fibres from capsaicin desensitisation (Santiciola *et al.*, 1987). However, it was concluded that failure of capsaicin to induce a response in Ca^{2+} -free medium may depend on many factors as detailed below.

- * Presence of Ca^{2+} may be necessary for the interaction of capsaicin with its 'receptor'.
- * Protection may involve the proposed Ca^{2+} dependence of the sensory receptor potential.
- * Ca^{2+} entry is required for capsaicin-induced neuropeptide release.
- * The actions of the neuropeptides, once released, may be Ca^{2+} dependent.

More recently novel agents like ruthenium red (see section 1.8.1) have further indicated the importance of Ca^{2+} in capsaicin-mediated events.

1.6.3 Neurotoxic effects of capsaicin; figure 1.4

It has been suggested that there is a role for Ca^{2+} in capsaicin-mediated nerve degeneration leading to toxicity and eventually cell death. Degeneration is thought to involve excessive Ca^{2+} entry at many levels resulting in the irreversible destruction of a large percentage of cells in sensory ganglia (Jancso *et al.*, 1977). However, the neurotoxic actions usually require high concentrations and persistent application of capsaicin. In addition to Ca^{2+} influx into the cell, which in itself could be damaging (Maggi & Meli, 1988), Maggi and Meli (1988) reported that a characteristic feature of capsaicin-poisoned nerves was swollen mitochondria; possibly indicating that Ca^{2+} could be taken up by the

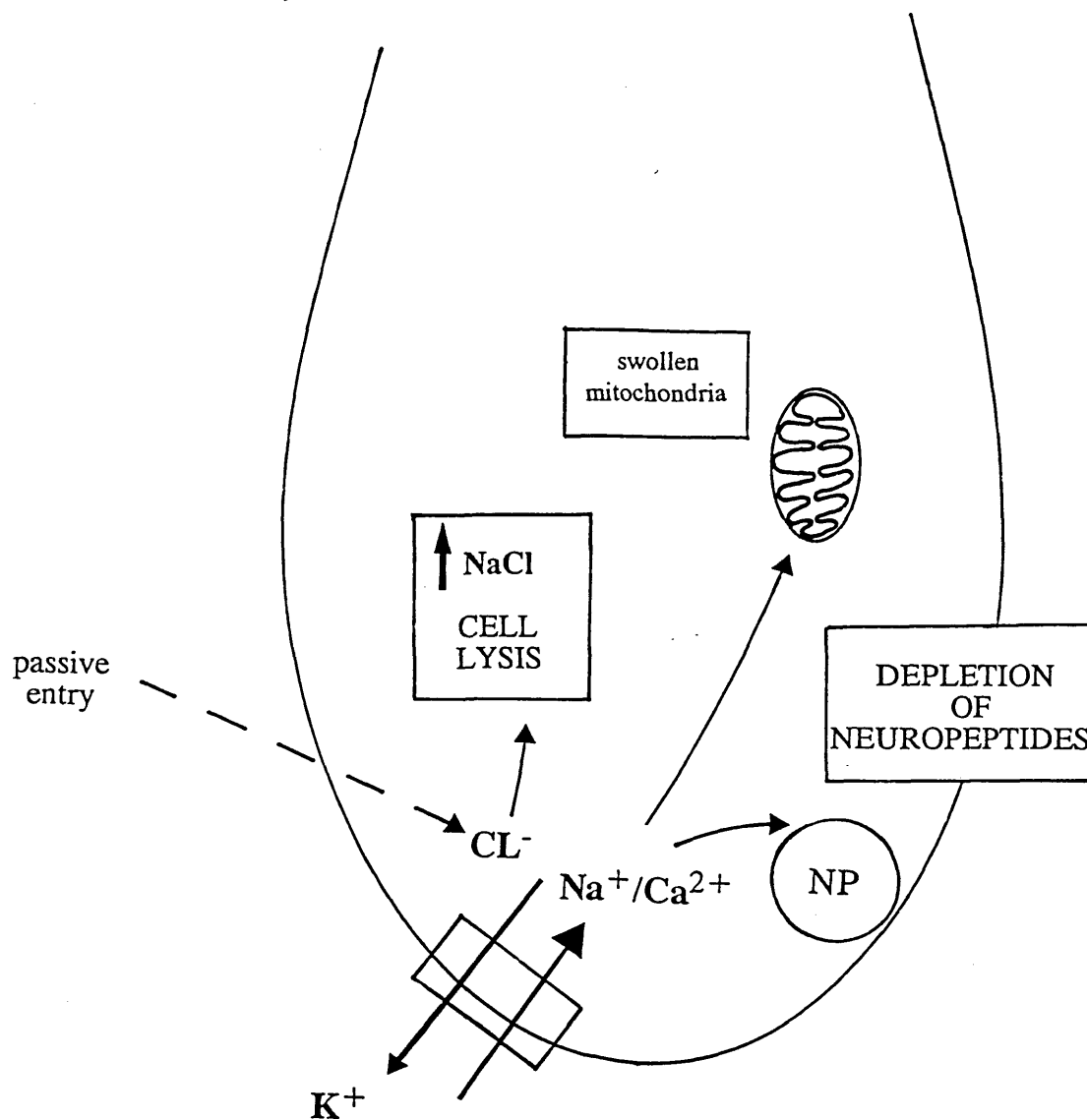


Figure 1.4
Neurotoxic effects of Capsaicin on Sensory C-fibres

mitochondria. This mitochondrial damage is closely coincident with changes in membrane conductance. Further evidence to implicate Ca^{2+} in the deleterious effects of capsaicin was that the disruption of microtubular and neurofilament organisation in C-fibres was similar to the damage induced in the peripheral nerve by the calcium ionophore A23187 (Marsh *et al.*, 1987). A second damaging mechanism might involve the influx of Na^+ through capsaicin-activated channels together with the passive movement of Cl^- into the cell. The net effect could be the uptake of NaCl followed by swelling of the cell and osmotic lysis.

1.6.4 Capsaicin and nerve growth factor

The protein nerve growth factor (NGF) is a naturally occurring trophic substance (Otten *et al.*, 1980). NGF is thought to be synthesised near the peripheral terminal of primary afferent neurons and gains access to the cell bodies of sensory neurons by undergoing retrograde axoplasmic transport after receptor mediated pinocytosis (Stockel *et al.*, 1975). It is thought that NGF is required for the maintenance of neonatal neurochemistry of sensory C-fibres (Baron Van Evercooren *et al.*, 1982), including axoplasmic transport of neuropeptides and their levels in the nerve terminals. There is convincing evidence to show that capsaicin damages sensory neurons and this neurotoxin could destroy the perikarya of sensory neurons by interfering with the actions of NGF (Otten *et al.*, 1983). It was found that doses of capsaicin which inhibited retrograde transport of NGF (Miller *et al.*, 1982) also depleted substance P and CGRP from the nerve terminals (Lindsay *et al.*, 1989). Otten *et al.* (1983) reported that in newborn rats concomitant administration of NGF partially antagonised the deleterious effects of capsaicin on substance P containing neurons. To further support the hypothesis that capsaicin interferes with NGF

transport, anti-NGF antibodies, applied systemically in rats, mimicked some of the actions of capsaicin (Taylor *et al.*, 1985). Therefore the protective action of NGF on sensory neurons could be impaired by capsaicin.

1.6.5 Ca^{2+} channels on neurons

There are voltage-sensitive calcium channels (VSCC) in the central and peripheral nervous system (Miller *et al.*, 1988). Some of these are dihydropyridine (DHP)-sensitive whereas others are insensitive. Three main categories of VSCC have been characterised; T-, N- and L-types.

It is possible to distinguish between different types by the use of pharmacological agonists and antagonists or by comparing the activation profiles of these channels using electrophysiological techniques. Omega conotoxin has been shown to be a good antagonist of N-type channels (Maggi *et al.*, 1988c) whereas DHP antagonists are thought to interact preferentially with L-type channels (Miller *et al.*, 1988).

Miller *et al.* (1988) used substance P-LI release from dorsal root ganglia (DRG) neurons, *in vitro*, as an indication of C-fibre activation. They concluded that capsaicin produced a large influx of Ca^{2+} into DRG cells by bypassing normal VSCC or other pathways. In contrast, when cells were depolarised by other stimuli such as K^{+} or by application of bradykinin, substance P-LI release appeared to be dependent on L-type channels (Miller *et al.*, 1988). Peterson and co-workers showed that the action of capsaicin on the total Ca^{2+} current in DRG neurons was blocked by $50\mu\text{M}$ Ni; L-type channels remain active in the presence of this cation (Peterson *et al.*, 1989), therefore L-type channels were not implicated in the capsaicin response. However these studies could not exclude

activation of VSCC of the N- or T-type. More recently this same group has reported that following Ca^{2+} entry into the nerve, capsaicin caused an increase in intracellular Ca^{2+} , which triggered inactivation of VSCC for around 30 minutes. The general opinion, at this present time, is that it is not known what 'site' on neural tissue is activated by capsaicin however a direct receptor-channel linkage seems probable (Rang *et al.*, 1987).

1.6.6 Secondary messengers and capsaicin

In recent years, evidence has accumulated for a regulatory role for intracellular second messengers such as cyclic nucleotides (cAMP, cGMP), inositol polyphosphates and diacylglycerol in controlling ion fluxes. The second messenger events (if any occur) initiated by capsaicin in sensory neurons are therefore of interest and may help to unravel which channels could be important in capsaicin-mediated events.

Wood *et al* (1989) were the first to demonstrate an increase in cGMP after capsaicin pretreatment of DRG neurons but they reported that the cAMP response seen was probably an indirect action of capsaicin releasing neuropeptides which subsequently acted on other capsaicin-insensitive cells. The increase in cGMP was totally dependent on extracellular Ca^{2+} which suggests that Ca^{2+} influx was a prerequisite for the cGMP response to capsaicin. It was also observed that DHP channel blockers inhibited this increase in cGMP although having no effect on ^{45}Ca uptake in this system (Wood *et al.*, 1988) or Ca^{2+} induced cellular damage (Marsh *et al.*, 1987). Wood *et al* (1989) failed to inhibit the capsaicin response with pertussis toxin which inactivates G protein subunits, G_i and G_o . This, however does not rule out a role for other, as yet unknown G proteins in the capsaicin-induced cGMP response. These

observations suggested that Ca^{2+} entry through a capsaicin-specific cation channel led to the depolarisation of the cell which in some way could be functionally apposed to guanylate cyclase. However, Bevan et al (1989) reported that they had no evidence for the involvement of any second messenger systems in the transduction process induced by capsaicin on DRG neurons. All the studies to date would suggest that second messengers do not play a significant role in the control of capsaicin-evoked ion fluxes. Probably the best explanation for the reported increase in cGMP is that Ca^{2+} stimulated Ca^{2+} -sensitive enzymes that induced a secondary rise in the levels of intracellular mediators such as cGMP.

1.6.7 Resiniferatoxin (RTX)

RTX is a diterpine derivative obtained from the plant family Euphorbiaceae. It was isolated on the basis of extraordinary activity in the mouse ear reddening assay (Hergenhahn *et al.*, 1974), in which it exhibited three orders of magnitude greater potency than the most potent of the typical phorbol esters. RTX has a similar structure to phorbol esters. However, RTX appeared not to be a tumour promoter, was a poor activator of brain protein kinase C and did not compete with phorbol esters for protein kinase C binding sites (Driedger & Blumberg, 1980). In addition, the response to RTX was not affected by the protein kinase C inhibitor staurosporine (Dray *et al.*, 1990b). The differences in responses to RTX and phorbol esters can probably be explained by the fact that at position R (see figure 1.2) the phorbol ester has a free hydroxy group which is critical for tumour promotion. RTX is esterified at this position with 4-hydroxy-3-methoxy phenylacetate; this substituent has been shown to be essential for its unusual irritant activity (Schmidt & Evans, 1979).

Figure 1.2 also shows that RTX bears a similar homovanillyl ester group that is included in the capsaicin molecule. Recently RTX has been described as a potent capsaicin analogue (Szallasi & Blumberg, 1989). Both capsaicin and RTX induced hypothermia, neurogenic plasma extravasation, and pain in the rat (de Vries & Blumberg, 1989; Szallasi & Blumberg, 1989). It has been postulated that RTX and capsaicin bind to the same binding site on sensory nerve terminals (Dray *et al.*, 1990b).

1.7 Modulation of capsaicin-sensitive nerves

The number of substances that are known to modulate the function of capsaicin-sensitive nerves is rapidly increasing. This is almost certainly due to increasing evidence for the involvement of neurogenic mediators in many inflammatory responses.

Neural blockade by tetrodotoxin and local anaesthetics has already been described. Anaesthesia is probably not a useful therapeutic option since it also inhibits other afferent fibres, however, recently other agents have been described (eg ruthenium red) that may have more specific inhibitory actions. It has been postulated that prejunctional inhibition of neuropeptide release from sensory nerve endings may be a more useful approach in controlling neurogenic inflammation (Barnes *et al.*, 1990).

1.8 Blockade of sensory neuron activation

1.8.1. Ruthenium red

Ruthenium red (ammoniated ruthenium oxychloride) is a cationic dye. It has been shown to block the depolarisation-induced Ca^{2+} entry in synaptosomes (Tapia *et al.*, 1985). Anatomical studies have shown that the intracellular penetration of ruthenium red was very poor but that it did bind to certain mucopolysaccharidic acid residues on the cell membrane (Luft, 1971). However other workers have reported that significant amounts of ruthenium red penetrate into nerve fibres. Ruthenium red positive material has been established in the axoplasm, Schwann cell cytoplasm, extracellular matrix and the axo-glial contacts of the axon-myelin Schwann cell complex (Dolapchieva *et al.*, 1986). The

interactions between capsaicin, Ca^{2+} fluxes and neuropeptides release from sensory C-fibres has already been discussed. In light of these important relationships, many workers have been using ruthenium red as an investigative tool to examine the mechanism of action of capsaicin on neural tissues.

In vitro studies

Maggi et al (1988) demonstrated that ruthenium red (30nM - 10 μ M) blocked contractions in the rat isolated urinary bladder induced by capsaicin. A similar antagonism of the actions of capsaicin was observed in other experiments; contraction of the isolated guinea-pig ileum (Takaki *et al.*, 1989; Chahl, 1989) or isolated guinea-pig bladder (Maggi *et al.*, 1988a). In the latter preparation, it was also established the ruthenium red (30nM - 3 μ M) reduced the release from muscle strips of substance P-LI. Maggi and co-workers clearly demonstrated that ruthenium red also protected against capsaicin desensitisation of sensory C-fibres (Maggi *et al.*, 1988a; Maggi *et al.*, 1988b). In addition they reported that ruthenium red (10 μ M) inhibited the capsaicin-induced contraction of guinea-pig isolated bronchus (and associated tachykinin release) and the positive inotropic response in the atria (with the associated CGRP release), but responses to electrical field stimulation of these preparations were left unaffected by this dose of ruthenium red (Maggi *et al.*, 1989a). However higher doses of ruthenium red (30 μ M) did partially block electrical field stimulated and KCl evoked contraction of the bronchus and the inotropic effects on the atria. Therefore it was postulated that, at higher concentrations, ruthenium red might block N-type Ca^{2+} channels. However it is unlikely that this mechanism accounts for the blockade by ruthenium red of capsaicin-induced responses because omega conotoxin, an N-type receptor antagonist did not significantly modify capsaicin induced depolarisation (Maggi *et al.*, 1988c).

In vivo studies

There have not been many studies investigating the actions of ruthenium red in vivo but there is considerable interest in this area.

Maggi et al (1989) demonstrated that topical administration of ruthenium red (10-100 μ M) to the serosal surface of the urinary bladder in urethane-anaesthetised rats prevented the motor response of the bladder to topical administration of capsaicin. At 100 μ M topical ruthenium red prevented the blood pressure rise induced by capsaicin but not that induced by sudden bladder distention in rats. After intrathecal administration ruthenium red (800ng/rat) did not however effect the blood pressure rise produced by topical administration of capsaicin onto the bladder.

Amann and Lembeck (1989a) investigated the effect of ruthenium red on capsaicin-induced stimulation of polymodal nociceptors in the isolated perfused rabbit ear with an intact neuronal connection. Although the capsaicin response, a depressor reflex in systemic arterial blood pressure and release of substance P-LI, was abolished by ruthenium red (20 μ M) the response to bradykinin and acetylcholine remained. Interestingly, when the ear was perfused with a Ca^{2+} -free medium the capsaicin-evoked depressor reflex was enhanced (Amann *et al.*, 1989a); this enhanced response was inhibited by ruthenium red (0.6-20 μ M). However, perfusion of the ear with Ca^{2+} -free medium did inhibit the release of substance P-LI. The fact that capsaicin-induced excitation of primary afferents was antagonised in the absence of extracellular Ca^{2+} suggests that this inhibitory action of ruthenium red was not only mediated by inhibition of transmembrane Ca^{2+} fluxes. Recently Amann et al (1990), have perfused the rabbit ear with 50°C perfusate. This stimulus not only induced a depressor response but evoked

substance P-LI release. Both of these parameters were at least partially inhibited by ruthenium red (20 μ M). In addition, the heat-induced depressor reflex was inhibited by ruthenium red in the absence of extracellular Ca²⁺.

1.8.2 Other agents

Capsaicin itself can induce blockade; high doses of capsaicin can induce desensitisation, deplete stored neuropeptides and in some circumstances the nerves can degenerate. Due to the interest in the antinociceptive potential of capsaicin-induced neuropeptide depletion, more specific analogues of this neurotoxin have been developed. One of these is olvanil (NE 19550); this drug may have anti-inflammatory, as well as antinociceptive properties (Dray *et al.*, 1990a).

Disodium cromoglycate (DSCG), and nedocromil are used therapeutically in asthma treatment. Both of these drugs have been shown to inhibit activation of sensory fibres (Dixon *et al.*, 1980). In addition, frusemide, has inhibitory actions on sensory C-fibres (Barnes *et al.*, 1990). This drug is a loop diuretic but its mechanism of action on sensory fibres is not fully understood.

1.9 Prejunctional inhibition of neuropeptide release

1.9.1 Opioids

Morphine inhibited plasma extravasation in the rat paw induced by antidromic stimulation of the saphenous nerve; this response was reversed by the opioid receptor antagonist naloxone (Bartho & Szolcsanyi, 1981). This inhibitory effect was mediated by μ -opioid receptors (Fossard & Barnes, 1987). Further

evidence to suggest that opioids could modulate sensory nerves was presented by Rogers and Barnes (1989); they showed that morphine inhibited the secretory response induced by capsaicin. One of the problems in defining an action of opioids is that there are central and peripheral opioid receptors. To try and overcome this problem, BW443C was developed, which is an opioid that cannot penetrate the brain. It was discovered that this drug still had peripheral activity and more importantly it inhibited bronchial hyperactivity in rats (Adcock *et al.*, 1988; Adcock & Beesley, 1989).

So far only inhibitory actions of opioids have been discussed. Recently, it was suggested that opioids can, in some instances, promote sensory neuropeptide release via μ -,delta-and kappa-opioid receptors (Crain & Shen, 1990). It was postulated that this dual (inhibitory and excitatory) modulatory mechanism may help to account for the observations of Knox and Dickenson (1987). They demonstrated that in single unit recordings from dorsal horn neurons, the application of low concentrations of opioids facilitated nociceptive responses, whereas, higher concentrations resulted in the inhibition of a painful response.

1.9.2 Other agents

There are numerous other agonists that are reported to inhibit the release of neuropeptides. Amongst these is corticotrophin-releasing factor, which has been co-localised with substance P and somatostatin in peripheral nerves (Skofitsch *et al.*, 1984). Corticotrophin-releasing factor inhibited neurogenic plasma leakage in rats (Wei & Kiang, 1989). However, somatostatin itself can inhibit the release of substance P-LI in response to nerve stimulation (Lembeck *et al.*, 1982). Another putative sensory neuropeptide, galanin, inhibited non-adrenergic non-cholinergic neural bronchoconstriction in guinea-pig. This

same response was also inhibited by R-alpha-methyl-histamine, an H₃ receptor agonist. H₃ receptors have recently been localised in central and peripheral nerves (Ichinose & Barnes, 1990). Somatostatin, however, had no effect on this non-adrenergic non-cholinergic response (Giuliani *et al.*, 1989), suggesting that prejunctional receptors on sensory nerves may vary at different sites (Barnes *et al.*, 1990). Another peptide, neuropeptide Y, which is a co-transmitter in adrenergic fibres, inhibited the contractile response produced by activation of capsaicin-sensitive primary afferents in the guinea-pig (Lundberg & Saria, 1987). This is not an exhaustive list of putative modulators of sensory neuropeptide release. At present, research is being directed into discovering their mechanisms of action on the presynaptic membrane and evaluating their therapeutic potential.

2.1 In vivo measurements of inflammatory activity using the rabbit skin bioassay

Rabbit skin was chosen to measure inflammatory activity in vivo for the following reasons:

- 1 Rabbits are easily housed, maintained and are not expensive especially when considering the data output possible from one experiment.
- 2 Rabbits have a large dorsal skin area and the dorsal skin is fairly uniform. These characteristics allow the inflammatory activity in a large number of test sites to be determined in one experiment.
- 3 Rabbits have accessible ear veins and arteries which are useful in many procedures including: introducing drugs and test agents (eg. anaesthetics), measuring systemic blood pressure and obtaining frequent blood samples (eg. for histology).
- 4 Redness is a cardinal sign of inflammation. Rabbit skin has a relatively low basal blood flow, therefore a larger window exists to measure increases in local blood flow.

There are many different aspects of inflammatory reactions that can be monitored in rabbit skin. In this thesis neutrophil accumulation, oedema formation (plasma protein exudation) and local blood flow were determined. Many of these procedures are radioisotopic but new procedures are being

introduced which do not necessitate the use of isotopes. A good example of this new technology is the laser Doppler flow meter, which measures local blood flow using a non-invasive probe.

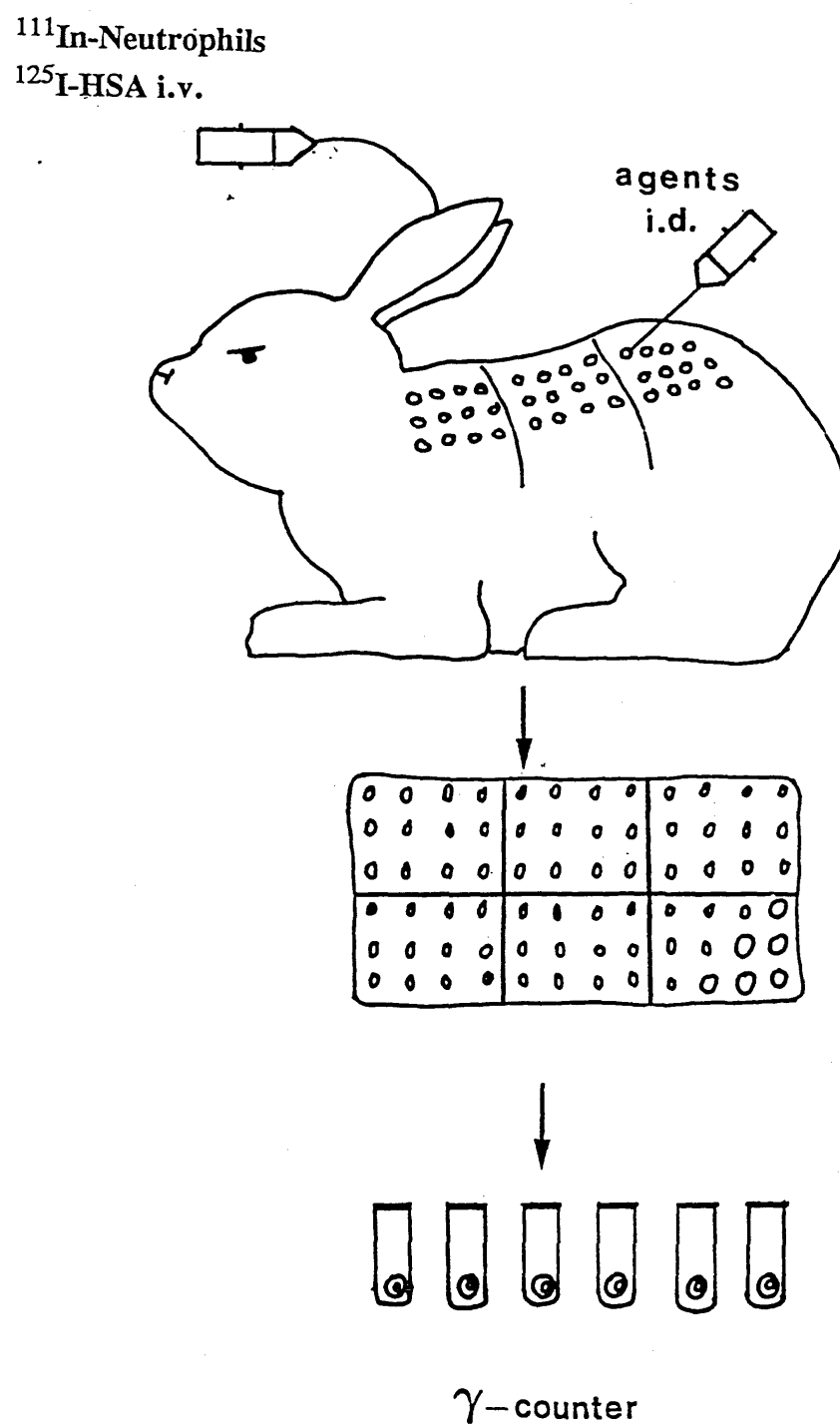


Figure 2.1
 Measurement of Oedema Formation and ^{111}In -Neutrophil Accumulation

2.2 In vivo Measurement of oedema formation in rabbit skin (figure 2.1)

Male New Zealand White rabbits (2.0-3.5kg) were anaesthetised with sodium pentobarbitone (Sagatal, 30mg/kg, i.v. with maintenance doses as necessary). The rabbit dorsal skin was then shaved and ^{125}I -human serum albumin (^{125}I -HSA; 0.2MBq/rabbit) was injected intravenously. To allow a visual record of the inflammatory reaction 1ml of a 2.5% Evans Blue dye solution was also i.v. injected. The agents under investigation were injected intradermally in 0.1ml volumes, each treatment having six replicates with injections given according to a balanced site plan. At the end of a test period a 10ml cardiac blood sample was collected into heparin (10Uml^{-1}). The animal was then killed by an overdose of sodium pentobarbitone, the dorsal skin was removed and the injection sites were punched out (17mm diameter punch). The skin sites, together with plasma samples, were counted in a manual multiwell gamma counter (1260 multigamma 2; LKB Wallac Ltd). All counts were automatically corrected for background counts and detectors were normalised for the radioisotope used. In experiments where ^{125}I was the sole radioisotope used, the samples were counted for 30 seconds over the energy range 10KeV-500KeV. In all experiments the mean ^{125}I counts per minute in 1ml plasma were determined from the cardiac samples collected at the end of each experiment. From this value the ^{125}I count per μl plasma was calculated thus allowing μl plasma per test site to be determined.

2.3 Preparation of ^{111}In -neutrophils

Male, New Zealand White rabbits were anaesthetised with sodium pentobarbitone (30mg/kg i.v. with an additional 15mg/kg i.v. before surgery). A carotid artery was exposed and cannulated with a 16 gauge Abocath catheter

(Venisystems). A total of 72ml blood was collected equally into eight 50ml tubes. Each tube contained 1ml of acid citrate dextrose (160mM disodium hydrogen citrate + 0.28M glucose in sterile water). A further 13ml of blood was collected into tri-sodium citrate (3.5% in sterile water). Autologous citrated plasma was produced after the citrated blood was centrifuged twice (3000g for 7 minutes at 20°C). To each tube 6mls of Hespan (3% final concentration) was added; this step removed the majority of red blood cells and the rest were separated by centrifuge facilitated sedimentation (25g for 15 minutes at 20°C). The leucocyte-rich supernatant was removed and centrifuged (300g for 7 minutes at 20°C). This step produced a leucocyte pellet and a platelet-rich plasma supernatant. The supernatant was removed and centrifuged twice (2000g for 10 minutes; 2500g for 10 minutes, both at 20°C) to produce autologous platelet poor plasma (PPP). The leucocyte pellets were carefully resuspended in approximately 5mls of citrated plasma.

Neutrophils were separated from mononuclear cells and remaining erythrocytes using a two layer discontinuous Percoll-plasma gradient (50%/70%; each layer was 3ml). The cell suspension was layered using a 19 gauge needle onto two gradients and centrifuged (400g for 17 minutes at 20°C). This spin resulted in a mononuclear cell band, on top of the 50% Percoll layer, a neutrophil band in the interface between the 50% and 70% layers and an erythrocyte pellet. The neutrophils (and mononuclear cells when required) were harvested using a pipette in 1.0-1.4ml volumes.

A 10 μ l sample of cell suspension recovered from the Percoll/plasma gradient was diluted 2000-fold in azide free balanced electrolyte solution (Isoton 2). The total number of red and white blood cells was measured in a Coulter counter (Model M2, Coulter electronics Ltd). Erythrocytes were then lysed using

20 μ l Zaponin (potassium cyanide in Isoton 2) and the number of leucocytes measured. From this value the total number of leucocytes recovered from the gradient was determined.

10-20 μ ls of ^{111}In dium chloride (5-20MBq) in 0.04M hydrochloric acid was chelated to 2-mercaptopyridine-N-oxide (40 μ g in 100 μ l phosphate buffered saline; pH7-5), and incubated with $2.5-4.5 \times 10^7$ neutrophils for 15 minutes at 20°C. 10mls of PPP was used to suspend the cells. A 10 μ l sample was taken from the suspension (sample 1) before it was centrifuged (400g for 7 minutes 20°C). This procedure was repeated twice (samples 2 and 3). Finally the washed ^{111}In -labelled neutrophils were resuspended in 3ml of PPP and i.v. injected into the ear vein of a recipient rabbit via a 19 gauge canular followed by an equal volume of sterile saline. A 10 μ l sample of the final cell suspension was also taken (sample 4).

Sample 4 was diluted 100-fold and the radioactivity in counts per minute (CPM) determined. From this the CPM per ^{111}In -neutrophil was calculated as:

$$\text{CPM/neutrophil} = \frac{\text{CPM injected into recipient}}{\text{No of neutrophils injected}}$$

where CPM injected into recipient = CPM in sample 4 x 300

Samples 1, 2 and 3 were also diluted 100-fold and counted in a gamma counter. From these the total CPM used during the labelling procedure was calculated as:

$$\text{Total CPM} = \text{CPM injected} + \text{CPM in samples 1} + 2 + 3.$$

The percentage binding of ^{111}In to the neutrophils was then calculated as;

$$\% \text{ binding} = \frac{\text{cell-associated CPM in sample } 3 \times 100}{\text{total CPM}}$$

The ^{111}In counts per neutrophil was determined and used to express neutrophil accumulation in each skin site in terms of number of labelled leukocytes.

In experiments where ^{111}In -neutrophil accumulation was determined, oedema formation (^{125}I -HSA accumulation) was also monitored (figure 2.1). To allow detection of each isotope, they were counted separately in limited energy ranges (^{125}I 10-100KeV; ^{111}In 100-500 KeV) for 60 seconds. The counts were automatically corrected for spill up (0.03 %) and spill down (25.03 %) in a manual multiwell gamma counter (1200 multigamma 2; LKB-Wallac Ltd).

2.4 Experimental protocols using ^{125}I -HSA and ^{111}In -neutrophils

^{125}I -HSA and ^{111}In -neutrophils were intravenously injected 5 minutes before the intradermal injection of some or all of the test agents. The measurement period of an inflammatory reaction was taken from when the radioisotopes were injected i.v. until the end of the experiment. At this time the rabbit was killed with an overdose of sodium pentobarbitone (Expiral 200mg/kg i.v.).

In the standard protocol plasma protein leakage and, where appropriate, neutrophil accumulation, were measured over a 30 minute period. This protocol was adapted according to experimental requirements as follows.

Protocol A

In order to measure the time course of PGE_2 and CGRP, these vasodilators were intradermally pre-injected at defined times before a second superimposed injection of bradykinin (10^{-10} mol/site) or saline (see figures 5.4 and 6.3a). ^{125}I -HSA was intravenously injected 5 minutes before bradykinin and oedema formation was measured for 30 minutes. This protocol allowed the duration of the ability of a given vasodilator to potentiate oedema formation induced by bradykinin. The biological half life ($t^{1/2}$) of the vasodilator activity was calculated as the time taken for the vasodilator to lose half its potentiating activity.

Protocol B

IL-1 induced protein synthesis dependent neutrophil accumulation in rabbit skin (Rampart & Williams, 1988). An experimental protocol was therefore designed to examine the inflammatory profile of IL-1 (see figure 6.1). IL-1 (1.4×10^{-14} mol/site) or saline were pre-injected 0, 1, 2 or 3 hours before a second super-imposed injection of either a vasodilator or saline. In these experiments the radioisotopes (^{125}I -HSA and ^{111}In -neutrophils) were injected 5 minutes before the vasodilator and responses were measured over a 1 hour period. This protocol allowed inflammatory responses to be monitored 0-1, 1-2, 2-3 and 3-4 after the injection of IL-1. IL-1, in all further experiments, was injected intradermally 1 hour before a vasodilator or saline

Protocol C

In order to determine the long lasting effects of CGRP, the potentiation of oedema formation and neutrophil accumulation induced by direct-acting (bradykinin and platelet activating factor) and neutrophil-dependent (FMLP,

C5a des Arg and LTB₄) mediators by CGRP (10^{-11} mol/site) was measured over defined periods (see figures 5.7 and 5.8). Inflammatory responses over 30 minutes were investigated by injecting the mediators five minutes after the injection of ¹²⁵I-HSA and ¹¹¹In-neutrophils. When investigating the long lasting potentiating action of CGRP (see figure 5.7) the protocol was as above except that a second set of test agents was injected 30 minutes after the first set. The measurement period in these experiments, which was 60 minutes, began when the first set of mediators was injected. This allowed oedema formation and neutrophil accumulation to be measured for 30 and 60 minutes. Accumulation for the 30-60 minute period was determined by subtraction.

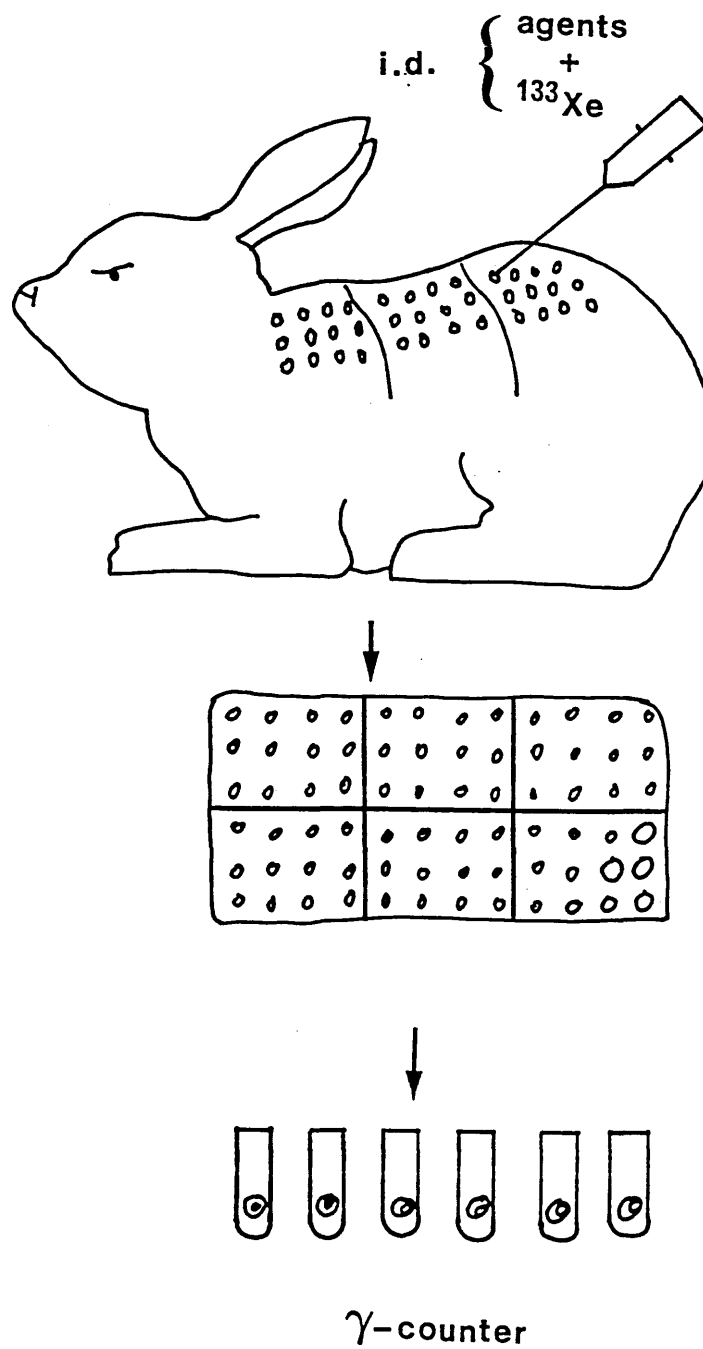
In a separate set of experiments, the first set of test agents was injected 30 minutes before intravenous ¹²⁵I and a second set was injected five minutes after the radioisotope. The measurement period of 30 minutes began when the second set of test agents were intradermally injected. This protocol allowed the direct measurement of 0-30 and 30-60 minute periods of a given inflammatory response (see figure 5.8).

Protocol D

A "two stage" protocol was used to test the effectiveness of intravenous test agents (see figure 4.4). In these experiments, the first set of short acting mediators were intradermally injected 30 minutes before a second set of identical mediators into different sites. The rabbit was killed after a further 30 minutes. ¹²⁵I was intravenously injected 5 minutes before the first set of mediators whereas, anti-CGRP Fab (2ml/kg) was intravenously injected 5 minutes before the second set of mediators. This protocol allowed the responses induced by intradermal mediators to be measured over 30 minutes in the presence and absence of systemic antiserum.

Figure 2.2

Measurement of Changes in Blood Flow



2.5 Multiple site ¹³³Xenon clearance method of measuring local blood flow (figure 2.2)

A rabbit was anaesthetised and prepared as described in section 2.2 above. Test agents (0.1ml at 4°C), were mixed with ¹³³xenon (1MBq per test agent) and intradermally injected into the dorsal skin according to a balanced site plan. After a 15 minute clearance period the rabbit was killed with an overdose of sodium pentobarbitone. The punched out injection sites were counted in a multiwell gamma counter (1260 multigamma 2; LKB Wallac Ltd). The samples were counted for 30 seconds over the energy range 10KeV-115 KeV.

Blood flow changes produced by intradermal injections of agents were calculated as a percentage increase (or decrease) of ¹³³Xe washout over that in the saline-injected control sites. Results were calculated from the following formula.

$$\text{blood flow change} = \frac{(\ln X_{e_s} - \ln X_{e_a}) \times 100\%}{(\ln X_{e_i} - \ln X_{e_s})}$$

where: X_{e_s} = counts/min saline-injected skin
 X_{e_a} = counts/min test agent-injected skin
 X_{e_i} = count/min in 0.1ml volume of injection fluid

2.6 Non-invasive measurement of local blood flow using the laser Doppler flow meter.

A rabbit was anaesthetised with sodium pentobarbitone (30mg/kg iv) and shaved. In addition, the surface hair of these rabbits was removed using a depilator (Immac). Immac was liberally applied to the dorsal skin of the rabbit for 5-10 minutes before being gently removed. Excess Immac was cleaned away using tap water to reveal smooth skin. The prepared rabbit was left covered at room temperature for 30 minutes.

Changes in blood flow were recorded using a Perimed IIb laser Doppler flow meter. The laser probe was secured in a holder 3-4mm away from an injection site on the dorsal skin. After a steady baseline had been established, at each site, the gain level was recorded and readings were taken at sites that had received intradermal vasodilators, saline and at an uninjected site. Readings were taken three times for 20 seconds at 0, 5, 10, 15, 30, 45, 60, 75, 90 and 120 minutes after each injection.

Results were recorded as mean % flux value (number of red blood cells moving in the volume measured by the path of the laser beam). The baseline value at each site was subtracted from each reading before results were expressed as % flux above basal flow. The time taken for each vasodilator to lose half its biological activity ($t^{1/2}$), measured using the laser Doppler flow meter, was calculated.

2.7 Raising and purification of sheep anti-rabbit neutrophil antiserum (ANS)

Anti-rabbit neutrophil antiserum was raised by Dr P Hellewell in sheep using a method adapted from Knicker and Cochrane (1965).

Glycogen-induced rabbit peritoneal neutrophils (1.5×10^8 cells) were emulsified with an equal volume of Freund's complete adjuvant (FCA) and injected subcutaneously into the dorsal skin of a sheep. The animal was boosted weekly for 7 weeks with rabbit neutrophils emulsified in Freund's incomplete adjuvant (FCA). After the 8th week blood was removed via the carotid artery. The antiserum was absorbed against red blood cells (5×10^9 cells per 20ml of antiserum) for 30 minutes at room temperature.

The IgG antibody of the antiserum was prepared according to the method of Steinbuch and Audran (1969). For every 10ml of antiserum, 20ml of sodium acetate (0.06M; pH4.0) was added followed by 0.747ml of n-octanoic acid. The solution was incubated at room temperature for 30 minutes before centrifugation (3500g, 20 minutes at 20°C). 4ml of 1.0M sodium phosphate pH7.4 was added to the supernatant followed by saturated cold ammonium sulphate in a volume equal to that of the total solution. The solution was mixed for 30 minutes and then placed on ice (2°C) for a further 60 minutes. Following centrifugation (3500g, 30 minutes at 2°C), the supernatant was discarded and the pellet resuspended in a volume of sterile saline equal to the original volume. The IgG preparation was then dialysed 3 times against 10 volumes of saline at 4°C and passed through a 0.22 μ M filter. The preparation was stored at -20°C. Before use, *in vivo*, the specificity of the antibody was improved by absorbing the antibody against unwanted cell types. Monocyte-rich, platelet-rich and erythrocyte rich solutions were harvested as described in section 2.3 above. Firstly a

monocyte-rich solution (2ml) was added to the antiserum (30ml). The solution, which was incubated at 20°C for 15 minutes, was inverted every 5 minutes. The solution was then centrifuged (400g, 5 minutes at 20°C) and the pellet was discarded. This procedure was followed for platelet-rich and then erythrocyte-rich solutions. The sheep anti-rabbit neutrophil antiserum (ANS) was then aliquoted and stored at 20°C.

2.7.1 The effect of ANS on circulating leucocyte counts

A 1ml blood sample was collected into a heparinised tube, from an anaesthetised rabbit, via the ear vein. A 10 μ l sample was taken to determine the whole leucocyte count as described in section 2.3 and smears were also taken and stained enabling a differential count (neutrophil: monocytes) to be obtained.

ANS (1ml) was intravenously injected into a second ear cannula. Blood samples were then taken for analysis at 15, 30, 45, 60, 90 and 120 minutes after the first ANS injection. A booster injection of ANS (1ml) was administered 60 minutes after the first injection.

2.7.2 Staining procedure for leucocytes

Blood smears were air dried for at least 1 hour and then fixed in methanol (5 minutes). The slides were directly transferred to 50% May Grunwald stain and then 10% Giemsa, both for 5 minutes. After washing in tap water the slide was ready for differential cell counting. From the coulter count analysis (whole leucocyte count) and the differential count between neutrophil/monocyte, the number of neutrophils and monocytes per ml of whole blood was calculated.

2.7.3 Neutrophil Depletion

In further experiments ANS (1ml), was intravenously injected at the same time as intradermal injections of IL-1 or other mediators. Booster injections of ANS (1ml) were given 45 and 90 minutes afterwards. Oedema formation induced by IL-1 and potentiated by CGRP, given 1 hour after the IL-1, was measured for 120 minutes. Blood samples were taken 5 minutes before and 120 minutes after the injection of IL-1. These samples were analysed as described in sections 2.3 and 2.7.2 above.

2.8 Raising of goat anti-human alpha CGRP antiserum

Anti-human alpha CGRP antiserum was raised with the help of Dr PJ Jose

Two methods for cross-linking CGRP (Bachem, 76.3% pure) were used. Firstly, CGRP was cross-linked to itself (CGRP-CL); 10 μ l of glutaraldehyde (1%) was added to human alpha CGRP (1mg in 0.2ml PBS). Secondly, CGRP was cross-linked in the presence of ovalbumin (45mgml⁻¹ in PBS, CGRP-OA); 10 μ l of glutaraldehyde (1%) was added to human alpha CGRP (1mg in 0.3ml ovalbumin). Both solutions were mixed rapidly and incubated in the dark at room temperature for 90 minutes. A further 10 μ l of glutaraldehyde (1%) was added to the solutions and incubated for 12 hours under the same conditions. After being dialysed twice against 20 volumes of PBS at room temperature the immunogens (CGRP-CL and CGRP-OA) were diluted in PBS and frozen (-20°C).

For immunisations the goat was sedated with diazepam (2mg/kg intramuscular). On day 0 and day 21 the goat received a total of 140 μ g of CGRP-CL (in FCA) which consisted of 20 intramuscular injections of CGRP-CL (0.1ml) and 4 subcutaneous injections of CGRP-CL (0.25ml). On day 42 330 μ g of CGRP-CL (in FIA) was administered using a similar dosing regimen. On days 63 and 84, 330 μ g of CGRP-OA (in FIA) were administered using the same dosing regimen. Test samples of blood (20-100ml) were bled from the goat via the jugular vein on days 50 and 72. The goat was finally bled out on day 91.

The anti-CGRP titre of each of the bleeds was tested by competitive binding radioimmunoassay. The dilution of anti-serum needed to displace 45% of bound ¹²⁵I-CGRP was: day 50, 1/1,700, day 72 1/10,000, day 91 1/4600. It was interesting to note the increase in titre after immunisation with CGRP-OA. Due to the volume of serum harvested on day 91, this batch was used in all bioassay experiments.

2.8.1 Preparation of anti-CGRP IgG

The IgG antibody fraction of the antiserum was prepared according to the method of Steinbuch and Audran (1969) and as described in section 2.7 above. The volume of the IgG preparation was adjusted (using sterile saline) to equal the original volume.

2.8.2 Preparation of anti-CGRP Fab

Anti-CGRP IgG was thawed and centrifuged (2500g for 5 minutes at 20°C). To enable the cleavage of IgG antibody to the smaller Fab fragments, by mercuripapain, cysteine and EDTA (pH7.4) were added so that their final

concentrations were 10mM and 2mM respectively. After mixing mercuripapain (1% w/w of IgG where 15mls of goat serum contains 300mg IgG) was added and the solution was incubated (37°C for 16 hours). After incubation, iodoacetamide (1mg/ml) was added and the Fab preparation was dialysed 3 times against 10 volumes of 10mM TRIS HCL buffer (pH 7.8).

2.8.3 Anion-exchange HPLC of anti-CGRP Fab

The Fab preparation was centrifuged (4000g for 17 minutes at 20°C) and filtered (Millipore, 0.22 μ M). The preparation was applied to a DEAE column (TSK 545), equilibrated in 10mM TRIS HCL (pH 7.8) at a flow rate of 1.2ml/min. The column was rinsed with 10ml of column buffer and the buffer solution that passed straight over the column (the breakthrough) was collected in a sterile glass beaker. A 60 minute linear gradient from 1-0.5M sodium chloride in column buffer was run at a flow rate of 1.0ml/min and sequential 1 minute fractions were collected. The breakthrough and the fractions were collected and tested for their ability to bind human alpha CGRP under competitive binding assay conditions. Two precipitation methods were used; protein A binding agarose (PABA; 5%) was used as a precipitant and donkey anti-goat serum (DoAG; 1/10) was used as a second antibody. These two methods allowed differentiation between Fab and IgG fractions; DoAG detected Fab and IgG but PABA detected IgG alone. The collected fractions had anti-CGRP activity using either method, suggesting that IgG had been retained on the column, but the breakthrough solution lacked activity when PABA was used as a precipitant. In all further purifications the breakthrough, which contained the Fab fraction, was collected alone.

The Fab preparation was freeze-dried and dialysed three times against 10 volumes of saline at 4°C. The volume of the Fab preparation was then adjusted (using sterile saline) to equal the original volume of antiserum.

The % recovery of the anti-CGRP activity (Fab and IgG) compared to whole anti-CGRP antiserum (equivalent to 50% displacement of ^{125}I -CGRP) was: anti-CGRP IgG 45%; anti-CGRP Fab (before HPLC) 20%; anti-CGRP Fab (final) 10-15%.

2.8.4 Purification of normal goat serum (control)

Serum was collected from a non-immunised goat. Control IgG and Fab were prepared using the same procedures as described in section 2.8 above.

2.9 Purification of spinal cord vasodilator activity

New Zealand white rabbits (3-5kg) were anaesthetised and killed by an overdose of sodium pentobarbitone (60mg/kg). The spinal cord was then dissected from the spinal vertebrae and frozen at -20°C. At a later date the spinal cord was then thawed and homogenised with a hand held glass homogeniser, in a cold NaHCO_3 solution (1.4%; 4°C) containing indomethacin (10^{-6} mol/ml). The concentration of spinal cord was 200mg/ml. The spinal cord extract was centrifuged (2500g, 5 minutes at 4°C) and the supernatant was kept at 4°C. The extract was assayed for inflammatory activity in rabbit skin using the standard protocol.

2.10 Extraction of spinal cord vasodilator activity using affinity chromatography

CNBr-activated sepharose 4B gel was prepared by incubating it with 50ml HCL (1mM) for 15 minutes to give approximately 7ml of wet gel. 5mls of anti-CGRP IgG (which contains approximately 100mg protein) was diluted in coupling buffer (NaHCO₃ buffer; 0.1M, pH8.3 containing NaCl 0.5M). Anti-CGRP IgG solution was then added to wet gel and mixed end over end for 2h at room temperature. The gel was then transferred to a buffer containing the blocking agent glycine (0.2M) and then washed with coupling buffer, acetate buffer (0.1M, pH4.0 containing NaCl 0.5M) and finally washed in coupling buffer again. After filtering the gel was stored at 4°C.

2.10.1 The effect of affinity chromatography on responses induced by spinal cord exudate

The volume of exudate was then split into two portions. One portion was passed over the affinity gel conjugated with anti-CGRP IgG. Both portions of exudate (spinal cord, sc; extracted spinal cord, sc*) were injected into rabbit skin. Responses were measured over 30 minutes.

2.11 Preparation of capsaicin

A fresh stock solution of capsaicin was prepared daily using absolute alcohol, tween 80 and saline in the following ratio.

alcohol: tween 80: saline (20:10:70)

The capsaicin was further diluted in sterile saline and intradermally injected alone or in combination with other mediators.

2.12 Preparation of C5a des Arg

Zymosan activated plasma was used as a source of C5a des Arg. New Zealand white rabbits were anaesthetised with sodium pentobarbitone, cannulated via the carotid artery and bled into heparinised tubes (10Uml⁻¹). Rabbit plasma was incubated (30 minutes at 37°C) with zymosan (1mgml⁻¹). Zymosan was removed by centrifugation (2500g for 2 x 10 minutes). Before use the zymosan activated plasma was desalted. This was completed using either a Sephadex G-25M (PD-10) column or by being dialysed three times against 10 volumes of saline. Activated plasma was then stored in aliquots of 1ml at -20°C. The C5a des Arg content of activated plasma (3×10^{-11} mol 0.1ml⁻¹) was measured by radioimmunoassay (Jose *et al.*, 1983).

2.13 Statistical analysis of results

All values for means are quoted together with the standard error of the mean (sem). Where appropriate two way analysis of variance (Two way ANOVA) was used to evaluate the statistical significance of differences between means. Two levels of significance were used at the 1% and 5% levels ($p < 0.01$ and $p < 0.05$).

2.14 Materials

Rabbits were from Froxfield farm, Froxfield or Hacking and Churchill, Huntingdon. Sagatal and Expiral (sodium pentobarbitone) were from May and Baker Ltd, Dagenham. $^{111}\text{InCl}_3$, ^{125}I human serum albumin and $^{133}\text{Xenon}$ were from Amersham International plc, Little Chalfont. Hydroxyethyl starch (Hespan) was from Dupont Ltd, Stevenage. Percoll and Sephadex G-25M (PD-10) columns were from Pharmacia Ltd, Milton Keynes. Saline (sterile, pyrogen free) was from the Boots Company, Nottingham. Bradykinin, formyl-methionyl-leucine-phenylalanine (FMLP), mercaptopyridine N-oxide, prostaglandin E_2 (PGE_2), zymosan A, actinomycin D, ruthenium red, capsaicin, substance P and neurokinin A and B were from Sigma chemical company, Poole. Donkey anti-goat IgG was from Nordic Immunological Laboratories, Maidenhead. Heparin sodium BP (0.3% Creasol as preservative) was from Paines and Bryne Ltd, Greenford. Platelet activating factor was from Bachem, Bubendorf, Switzerland. Evans blue dye, histamine acid phosphate, di-sodium ethylenediaminetetraacetic acid (EDTA) were from British Drug houses Ltd, Poole. Mepyramine maleate was from May and Baker Ltd, Dagenham. Resiniferatoxin was from Fluka Ltd. Endothelin was purchased from Peninsula Laboratories, St Helens. Immac was purchased from Anne French.

The following were generous gifts: WEB2086 from Dr H Heuer, Boehringer Ingleheim, West Germany. Human alpha and beta CGRP from Dr U Ney, Celltech Ltd, Slough. Recombinant IL-1 alpha and beta from Dr D Westmacott, Roche Products Ltd, Welwyn Garden City. Prostacyclin (PGI_2) from Dr B Whittle, Wellcome, Beckenham. LTB_4 from Miles Laboratories, Stokes Poges.

1. To investigate the inflammatory actions of capsaicin injected intradermally in rabbit skin by measuring oedema formation, changes in blood flow and neutrophil accumulation in vivo.
2. To try and identify inflammatory mediators released by capsaicin in rabbit skin and to attempt to modulate the release of neuropeptides from sensory C-fibres in vivo.
3. To raise and purify a selective anti-CGRP antiserum (IgG and Fab fragments) to determine the presence of CGRP in rabbit skin and spinal cord. Furthermore, the anti-CGRP antiserum will be used to investigate the possible role for CGRP in capsaicin-evoked responses in rabbit skin.
4. To further examine the vasodilator activity of CGRP in rabbit skin and to compare the actions of this long-lasting vasodilator with those of the prostanoid vasodilators PGE₂ and PGI₂. In addition to evaluate three different methods of measuring vasodilator activity in rabbit skin; potentiation of oedema formation, ¹³³xenon clearance and the non-invasive laser Doppler probe
5. To determine the importance of CGRP in inflammatory responses mediated by neutrophil-dependent (eg C5a des Arg and IL-1) and direct-acting (eg bradykinin and histamine) mediators of increased microvascular permeability.

Pro-inflammatory actions of capsaicin**3.1 Introduction**

Capsaicin, which selectively activates sensory C-fibres, has been reported to have many inflammatory activities (Maggi & Meli, 1988). A large percentage of work in this area has been completed in vitro, although in recent years more in vivo techniques have been used to demonstrate the specific actions of this hot pepper extract. In this study, oedema formation, blood flow changes and neutrophil accumulation have been measured in vivo, in the rabbit skin, to investigate inflammatory responses induced by capsaicin. These tests allow a relatively large number of agents to be measured in vivo for inflammatory activity. Specific antagonists and antiserum have been employed to try and implicate or eliminate the involvement of selected inflammatory mediators in this inflammatory response.

3.1.1 Pro-inflammatory profile of capsaicin

Capsaicin induced dose-dependent oedema formation in the rabbit skin over 30 min, as shown in figure 3.1. In addition oedema formation induced by many inflammatory mediators was potentiated by capsaicin (figure 3.5). Oedema formation can be a synergistic response between a mediator of increased microvascular permeability and a vasodilator ('Two mediator hypothesis,'(Williams, 1977a)). At lower doses capsaicin (10^{-6} mol/site or less), when injected alone, appeared to potentiate oedema formation induced by other mediators without inducing marked plasma leakage, suggesting that it can

increase blood flow but not microvascular permeability at these doses. Indeed, when local blood flow was measured directly in the rabbit skin, using a multiple site ^{133}Xe clearance technique, it was found that low doses of capsaicin increased blood flow in a dose dependent manner (figure 3.3). At a dose of 10^{-6}mol/site capsaicin potentiated oedema formation induced by histamine in a time-dependent manner (figure 3.2). Initially capsaicin markedly potentiated plasma protein leakage, however, a second phase of vasodilatory activity appeared when capsaicin was pre-injected for 1h before the i.v. injection of ^{125}I .

At a higher dose capsaicin ($3 \times 10^{-6}\text{mol/site}$) induced oedema formation when injected alone (figures 3.1 and 3.4). When CGRP (10^{-11}mol/site) was co-injected with capsaicin ($3 \times 10^{-6}\text{mol/site}$) no further potentiation of oedema formation was observed. Results : capsaicin 44.4 ± 7.2 , capsaicin + CGRP 45.4 ± 5.6 and CGRP alone $14.4 \pm 1.7\mu\text{l/site}$, mean $\pm$ SEM, $n=6$ replicate sites. However, figure 3.5 demonstrates that a synergistic interaction still occurred between a mediator of increased microvascular permeability and capsaicin at a dose of $3 \times 10^{-6}\text{mol/site}$. These results could suggest that an increase in blood flow was the predominant response to capsaicin at all doses, but that a mediator of increased microvascular permeability was released when higher doses of capsaicin were injected. Interestingly, when oedema formation induced by capsaicin was measured over a longer period of time (120 vs 30 min) an enhanced response was recorded (figure 3.4).

Capsaicin, even at higher doses did not induce neutrophil accumulation in the rabbit skin over 30 min, but capsaicin markedly enhanced cell accumulation induced by the neutrophil-dependent mediator FMLP (figure 3.5), presumably as

a consequence of vasodilator release from sensory fibres. However, it is interesting to note that FMLP-induced neutrophil accumulation was enhanced to a greater degree than oedema formation by capsaicin at a dose of 10^{-6} mol/site.

Neutrophil-dependent inflammatory processes have been shown to be slower in onset than responses induced by direct-acting mediators (Wedmore & Williams, 1981). In the light of this finding and the fact that the 120 min oedema response induced by capsaicin was considerably greater than the 30 min oedema response (figure 3.4), the possibility of neutrophil accumulation occurring in response to capsaicin was tested over 120 min. The response induced by capsaicin was compared to responses induced by C5a des Arg, a chemotactic complement fragment and the non-chemotactic mediator bradykinin. The results of this experiment over 120 minutes were : capsaicin (3×10^{-6} mol/site) 6197 ± 1408 , bradykinin (10^{-10} mol/site) + PGE_2 (3×10^{-10} mol/site) 6921 ± 771 , C5a des Arg (3×10^{-11} mol/site) + PGE_2 $48,066 \pm 2289$, mean $\pm$ SEM, $n=6$ replicate sites. These data suggest that oedema formation induced by capsaicin was not dependent on the presence of neutrophils.

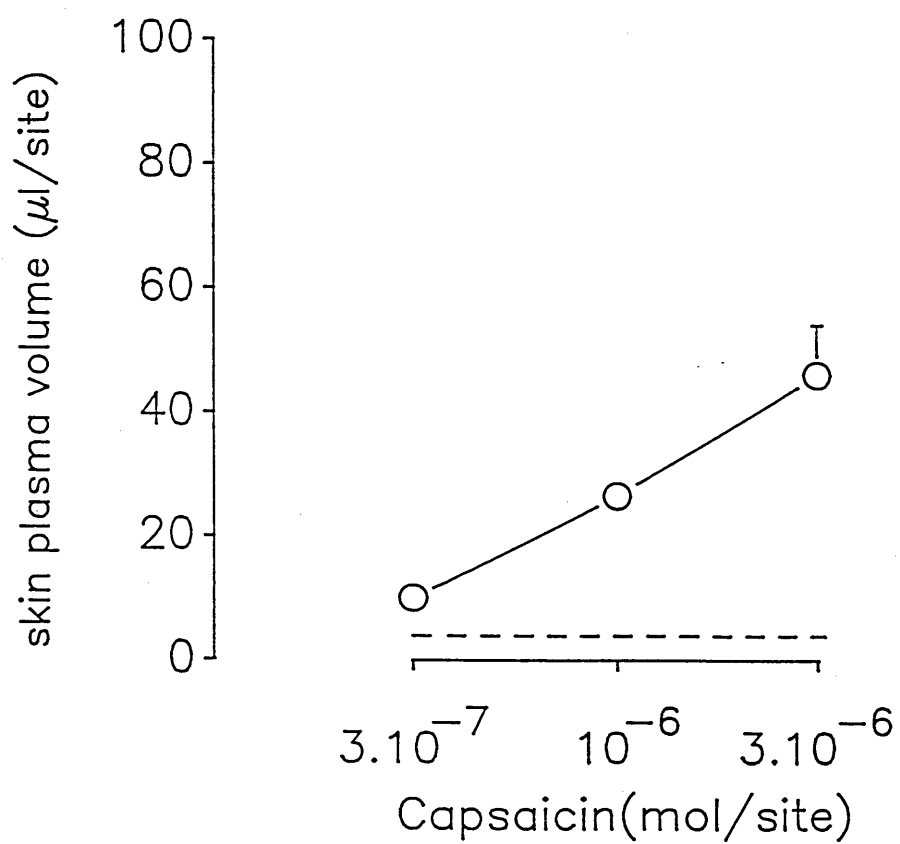


Figure 3.1

Oedema formation (μl/site) induced by capsaicin over 30 min in rabbit skin. Responses to capsaicin (○) and saline (dashed line) are indicated. Results are expressed as mean $\pm$ SEM, n=6 replicate sites.

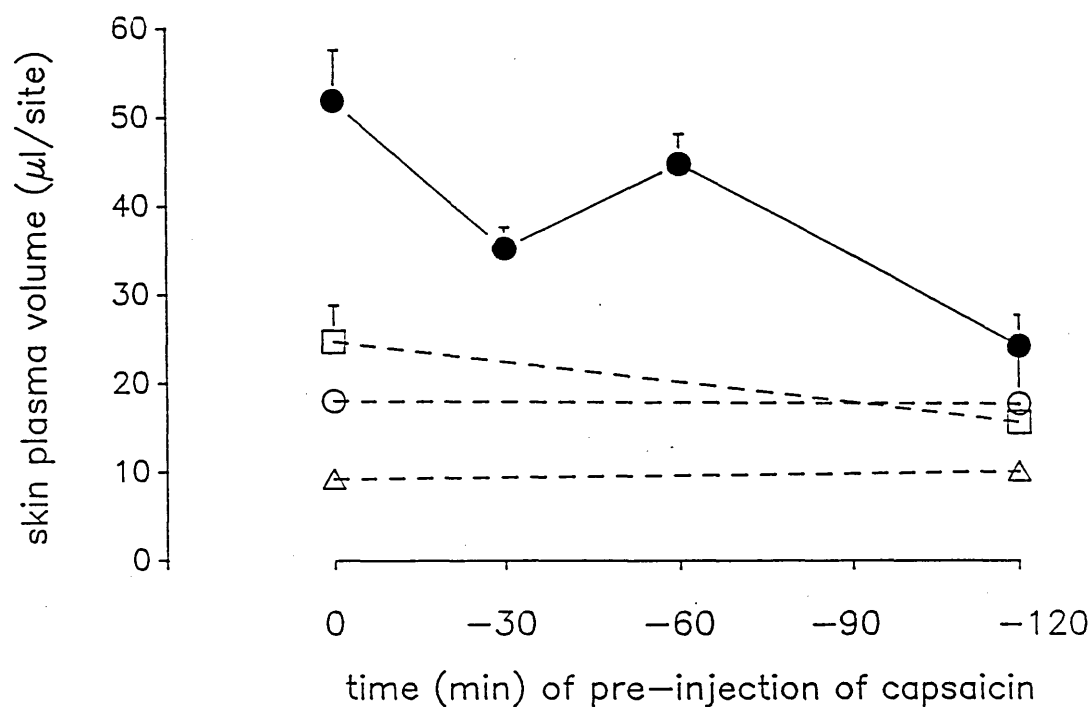


Figure 3.2

The effect of different pre-treatment times of capsaicin on histamine-induced oedema formation ($\mu\text{l}/\text{site}$). Saline or capsaicin ($10^{-6}\text{mol}/\text{site}$) were i.d. pre-injected 0, 30, 60 or 120 min before a second injection of saline or histamine ($3 \times 10^{-9}\text{mol}/\text{site}$). Oedema formation was then measured over 30 min in the rabbit skin. Symbols represent responses to: Capsaicin ($\square$), histamine ($\circ$), histamine + capsaicin ($\bullet$) and saline ($\triangle$). Results are expressed as mean $\pm$ SEM $n=6$ replicate sites.

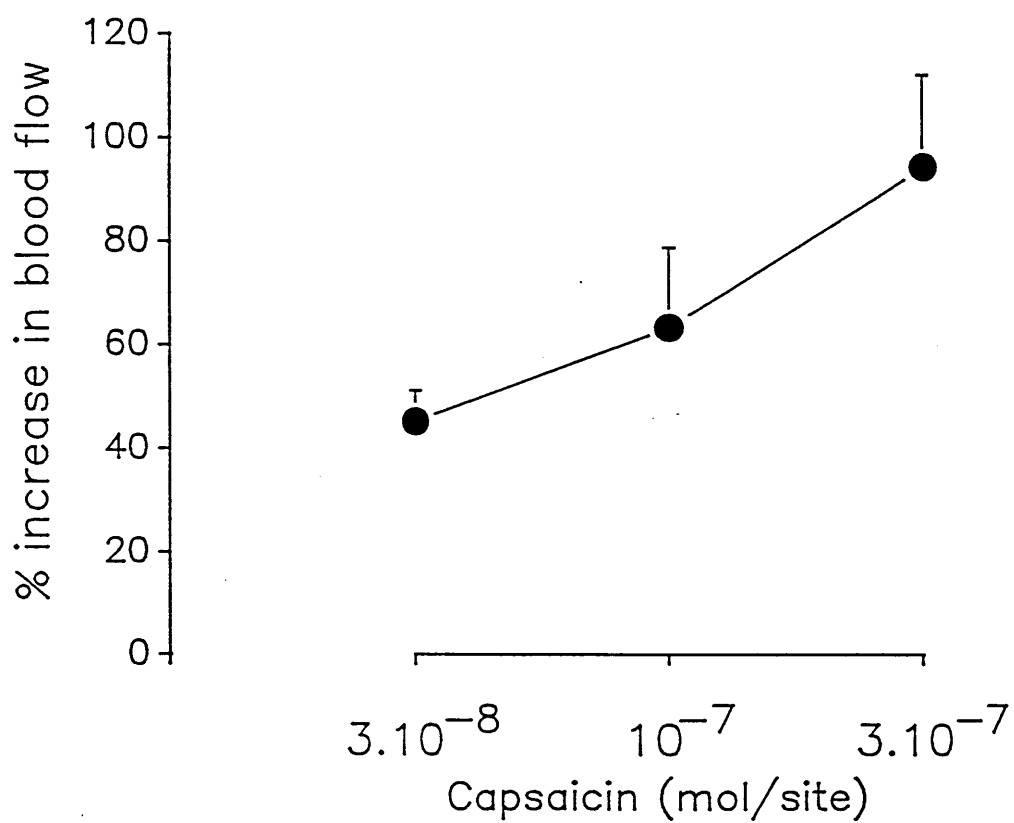


Figure 3.3

The effect of capsaicin on local blood flow in the rabbit skin. A dose-dependent increase in blood flow (%) induced by capsaicin (●) was measured over 15 min. Results are expressed as mean $\pm$ SEM, n=4-5 rabbits.

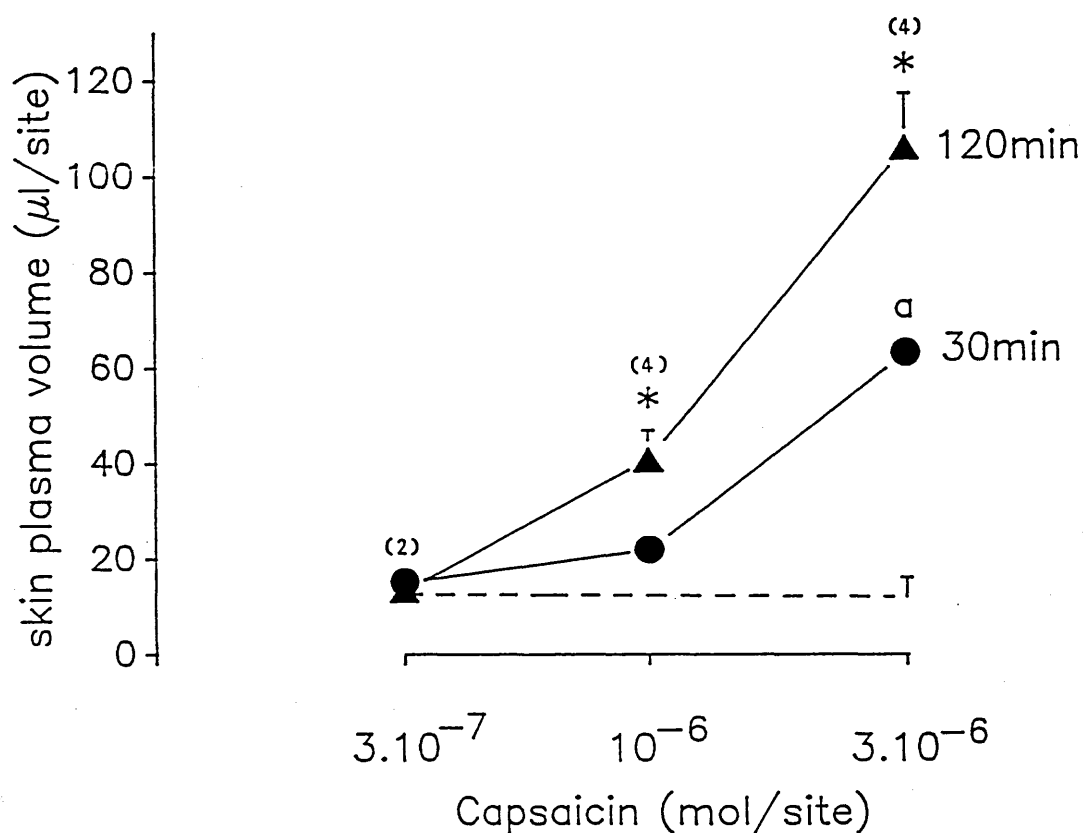


Figure 3.4

The effect of capsaicin on oedema formation (μl/site), in rabbit skin. Capsaicin was either injected 30 min (●) or 120 min (▲) before the rabbit was killed. Responses to saline are indicated by a dashed line. Results are expressed as mean $\pm$ SEM, n=2-4 rabbits. Significant differences between the 30 min and 120 min responses to capsaicin ($1 - 3 \times 10^{-6}$ mol/site) are indicated by * ($p < 0.05$). Significant differences between capsaicin (30min response) and saline are indicated by a □ ($p < 0.05$; two way ANOVA).

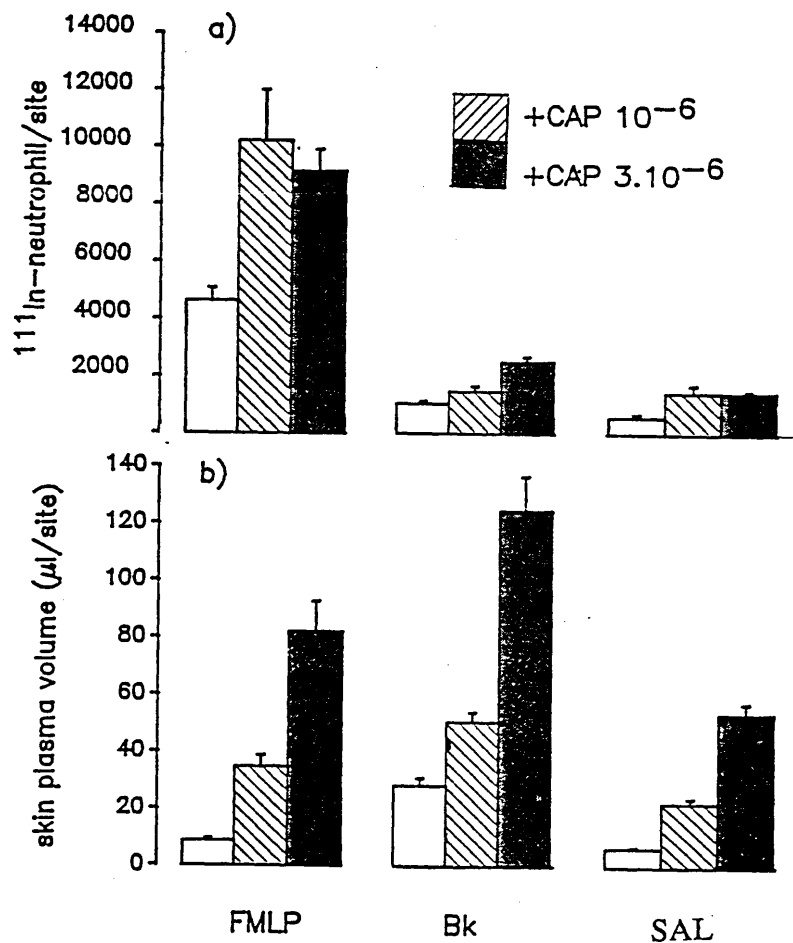


Figure 3.5

The effect of capsaicin on neutrophil accumulation, in rabbit skin, over 30 min. a) neutrophil accumulation (^{111}In -neutrophil/site) and b) oedema formation ($\mu\text{l}/\text{site}$) were induced by FMLP ($5 \times 10^{-11}\text{mol}/\text{site}$), bradykinin (Bk; $10^{-10}\text{mol}/\text{site}$) or saline, alone (open columns) or in the presence of capsaicin ($10^{-6}\text{mol}/\text{site}$; hatched columns; $3 \times 10^{-6}\text{mol}/\text{site}$; filled columns). Results are expressed as mean $\pm$ SEM, $n=6$ replicate sites.

3.2 Effect of pharmacological antagonists on capsaicin-induced responses.

It has been reported that capsaicin can promote the synthesis and release of prostaglandins in some species (Juan *et al.*, 1980). However, prostaglandins are probably not involved in capsaicin-evoked responses in the rabbit skin because indomethacin, which blocks prostaglandin generation (eg PGE₂, PGI₂) by inhibiting the cyclooxygenase pathway, had no marked effect on oedema formation induced by capsaicin although responses to arachidonic acid were inhibited (table 3.1).

Results from the previous section indicated that responses induced by capsaicin were not dependent on the presence of neutrophils in the rabbit skin. Therefore, antagonists of direct-acting mediators of increased microvascular permeability (eg histamine and PAF) were tested against inflammation induced by capsaicin. WEB 2086 (10^{-7} mol/site) has proved to be a very effective PAF antagonist but this pharmacological agent had no effect on oedema induced by capsaicin (table 3.2). Mepyramine (10^{-9} mol/site) and cimetidine (3×10^{-8} mol/site) H₁ and H₂ antagonists respectively, had no significant effect on capsaicin-induced oedema formation. Likewise, the combination of indomethacin and cimetidine (table 3.2) failed to inhibit the capsaicin response.

It was also important to establish whether the responses described in this chapter were dependent on an intact neural network. Lignocaine (0.5 - 5mg/ml), which as a local anaesthetic inhibits action potential propagation in neurons, failed to block capsaicin-induced oedema formation in rabbit skin (figure 3.6). This result suggests that the local response mediated by capsaicin in the rabbit skin was induced by the direct release of biologically active substances from the activated neuron rather than via an axon reflex response.

Table 3.1a

TEST AGENT	SKIN PLASMA VOLUME ($\mu\text{l}/\text{site}$)	
		+INDO (10^{-8})
Capsaicin (3×10^{-6})	57.9 ± 7.3	48.5 ± 4.9
Bk (10^{-10}) + Capsaicin (10^{-6})	67.1 ± 9.0	69.3 ± 10.0
Bk (10^{-10}) + AA (10^{-9})	70.6 ± 7.1	37.0 ± 7.5
Bk (10^{-10})	32.5 ± 8.0	ND
NaHCO ₃	5.3 ± 0.9	6.1 ± 0.8

Table 3.1b

TEST AGENT	INCREASED BLOOD FLOW (%)	
		+INDO (10^{-8})
Capsaicin (3×10^{-7})	69.6 ± 24.4	76.5 ± 17.4
NaHCO ₃	ND	5.8 ± 12.7

Table 3.1

The effect of indomethacin on a) oedema formation ($\mu\text{l}/\text{site}$), measured over 30 min and b) increased blood flow (%), measured over 15 min, induced by capsaicin. The doses of mediators, which were diluted in 1.4% NaHCO₃, are expressed as (mol/site). Results are expressed as mean $\pm$ SEM, n=6 replicate sites (ND = not determined).

Table 3.2

The effect of a) WEB2086, b) mepyramine and c) cimetidine on oedema formation induced and/or potentiated by capsaicin. Oedema formation ($\mu\text{l}/\text{site}$) was measured in rabbit skin over 30 min. Doses of mediator are expressed as (mol/site). Results are expressed as mean $\pm$ SEM n=6 replicate sites.

TEST AGENT		SKIN PLASMA VOLUME (μ l/site)	
a)	PAF antagonist		+WEB (10^{-7})
	Capsaicin (3×10^{-6})	70.6 ± 6.0	64.7 ± 5.7
	PAF (10^{-9}) + CGRP (10^{-11})	44.8 ± 4.5	22.6 ± 2.6
	PAF (10^{-9})	8.0 ± 0.9	ND
	saline	8.2 ± 0.4	8.0 ± 0.9
b)	H ₁ antagonist		+Mepyramine (10^{-9})
	Capsaicin (3×10^{-6})	65.7 ± 7.0	58.5 ± 4.8
	HA (3×10^{-9}) + CGRP (10^{-11})	50.0 ± 7.6	20.7 ± 4.2
	HA (3×10^{-9})	11.5 ± 2.2	ND
	saline	6.4 ± 0.7	11.3 ± 2.0
c)	H ₂ antagonist		+Cimetidine (3×10^{-8})
	Bk (10^{-10}) + Capsaicin(10^{-6})	67.1 ± 9.0	57.9 ± 11.0
	HA (10^{-8})	29.7 ± 5.8	21.7 ± 2.8
	Bk (10^{-10})	32.5 ± 8.0	ND
	Bk + capsaicin (10^{-6})+Indo (10^{-8})	69.3 ± 10.0	73.7 ± 6.8
	saline	5.3 ± 0.9	4.7 ± 6.8

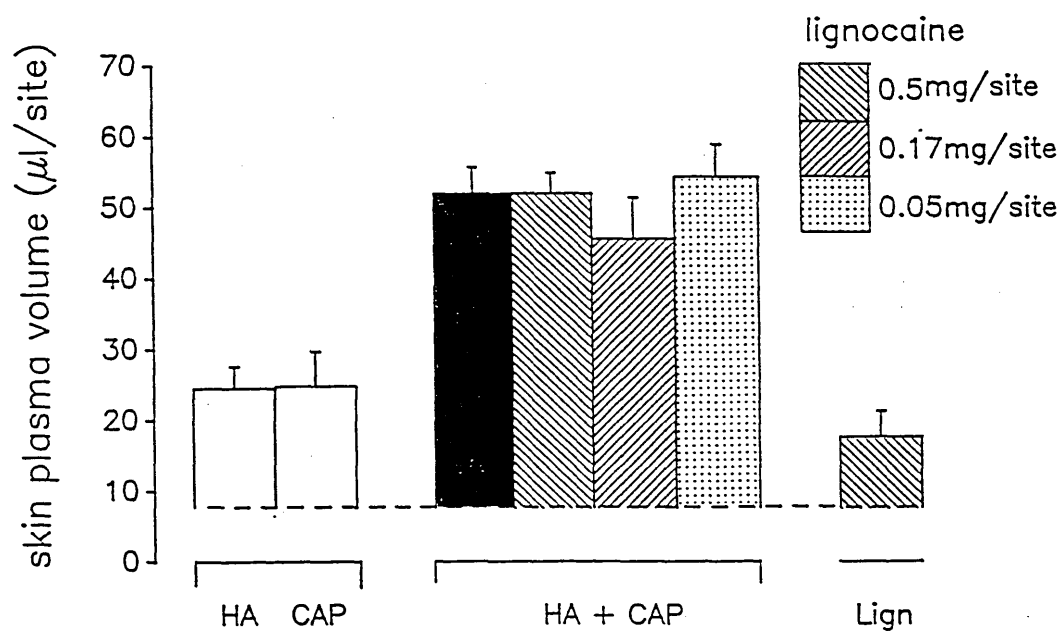


Figure 3.6

The effect of lignocaine (lign; 0.5 - 5mg/ml) on oedema enhanced by capsaicin (10^{-6} mol/site). Oedema formation (μ l/site) was induced by histamine (HA, 3×10^{-9} mol/site;) and measured over 30min. The dashed line represents responses at saline injected sites. Results are expressed as mean $\pm$ SEM, n = 6 replicate sites.

3.3 The effect of ruthenium red on capsaicin-induced responses.

Maggi and co-workers (Maggi *et al.*, 1988b; Maggi *et al.*, 1988a), demonstrated that capsaicin induced contractions in the rat isolated urinary bladder. Furthermore, they demonstrated that this response could be inhibited by ruthenium red, thus prompting the use of ruthenium red in the rabbit skin assays.

Ruthenium red dose-dependently inhibited plasma leakage induced by capsaicin (3×10^{-6} mol/site) over 30 min but had no activity alone (figure 3.7). It was demonstrated that ruthenium red (10^{-8} mol/site) did not affect responses induced by bradykinin and CGRP, but ruthenium red significantly inhibited oedema formation induced by capsaicin (figure 3.8). To examine the selectivity of ruthenium red further, responses to FMLP, histamine and PAF were tested; oedema induced by these other mediators was not affected by ruthenium red (figure 3.9). Capsaicin alone induced significantly more plasma protein leakage over 120 min than over 30 min (figure 3.4). Ruthenium red (10^{-8} mol/site) also inhibited the longer 120 min response: capsaicin (3×10^{-6} mol/site) 113.7 ± 7.7 ; capsaicin + ruthenium red 51.9 ± 13.3 ; ruthenium red 21.2 ± 5.4 ; saline 14.3 ± 1.2 , mean $\pm$ SEM $n=3$ rabbits. The responses induced by capsaicin alone and in the presence of ruthenium red were significantly different; $p < 0.05$ (two way ANOVA).

It was decided to explore the inhibitory effect of ruthenium red on capsaicin further using the ^{133}Xe clearance technique to measure increased blood flow. Figure 3.10 demonstrates the dose-dependent increase in blood flow induced by capsaicin. This increase was significantly inhibited by ruthenium red (10^{-8} mol/site), however it was noted that inhibition was not complete;

aproximately 50-60% of this response remained. CGRP induced a substantial increase in blood flow but this response and the small response induced by bradykinin were both not affected by ruthenium red.

Resiniferatoxin (RTX) has been reported to induce neurogenic inflammation in the rat (Szallasi & Blumberg, 1989). In this model RTX was 100-1000 times more potent than capsaicin. Using ^{133}Xe clearance, the ability of RTX to stimulate blood flow in the rabbit skin was tested. Unlike capsaicin, RTX was weak in increasing blood flow (figures 3.11 and 3.12). Surprisingly increased blood flow induced by a vasodilator dose of CGRP ($3 \times 10^{-12}\text{mol/site}$) was dose-dependently inhibited by RTX (figure 3.11). To compare the activities of capsaicin and RTX further, the small increase in blood flow induced by RTX was monitored in the presence of ruthenium red. Ruthenium red inhibited oedema formation and vasodilatation induced by capsaicin, but in contrast ruthenium red significantly enhanced increased blood flow induced by higher doses of RTX ($1-3 \times 10^{-9}\text{mol/site}$; figure 3.12). This result suggests that RTX could release vasoconstrictor and vasodilator substances in the rabbit microvasculature but the vasodilator activity was only apparent when ruthenium red inhibited the vasoconstrictor response. The identities of these endogenous bioactive substances are unknown.

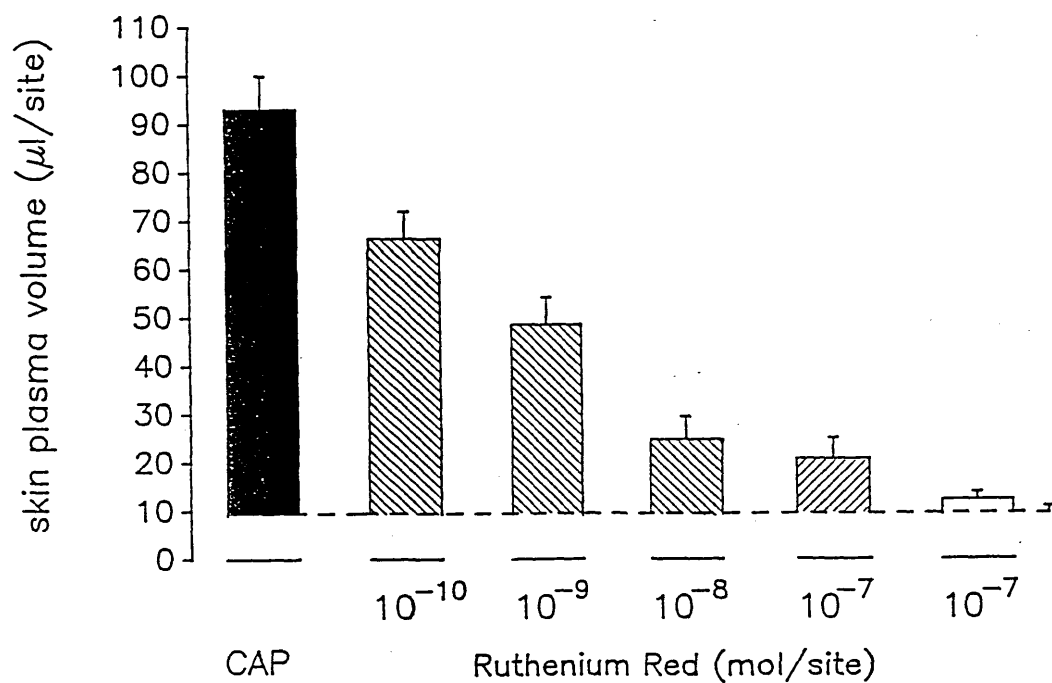


Figure 3.7

The dose-dependent inhibition of capsaicin-induced oedema formation (µl/site) by ruthenium red, over 30 min. Ruthenium red (10^{-10} - 10^{-7} mol/site) or saline were pre-injected 5 min before capsaicin (3×10^{-6} mol/site). Responses are represented by : capsaicin (filled columns), capsaicin + ruthenium red (hatched columns), ruthenium red (open column) and saline (dashed line). Results are expressed as mean $\pm$ SEM, n=6 replicate sites.

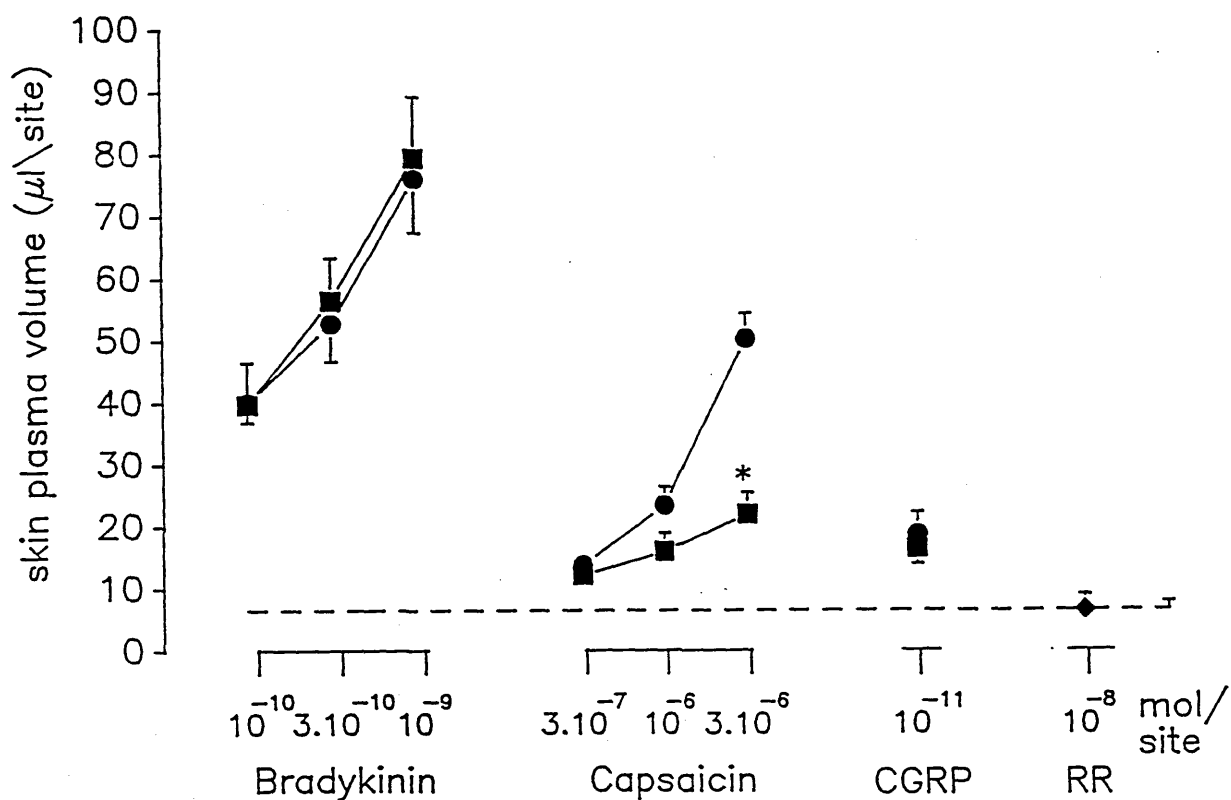


Figure 3.8

The effect of ruthenium red (RR; 10⁻⁸mol/site) on oedema formation (μl/site) induced by capsaicin (3 x 10⁻⁷ - 3 x 10⁻⁶mol/site), bradykinin (Bk, 10⁻¹⁰ - 10⁻⁹mol/site) and CGRP (10⁻¹¹mol/site), over 30 min. Ruthenium red or saline was pre-injected 5 min before capsaicin, bradykinin or CGRP. Oedema formation (μl/site) induced by mediators alone (●) and in the presence of ruthenium red (■) or ruthenium red alone (◆) were measured. Responses to saline are indicated by a dashed line. Results are expressed as mean ± SEM, n=4 rabbits. Asterisk (*) represents a significant difference (p<0.01, two-way ANOVA) between capsaicin (3 x 10⁻⁶mol/site) alone and in the presence of ruthenium red.

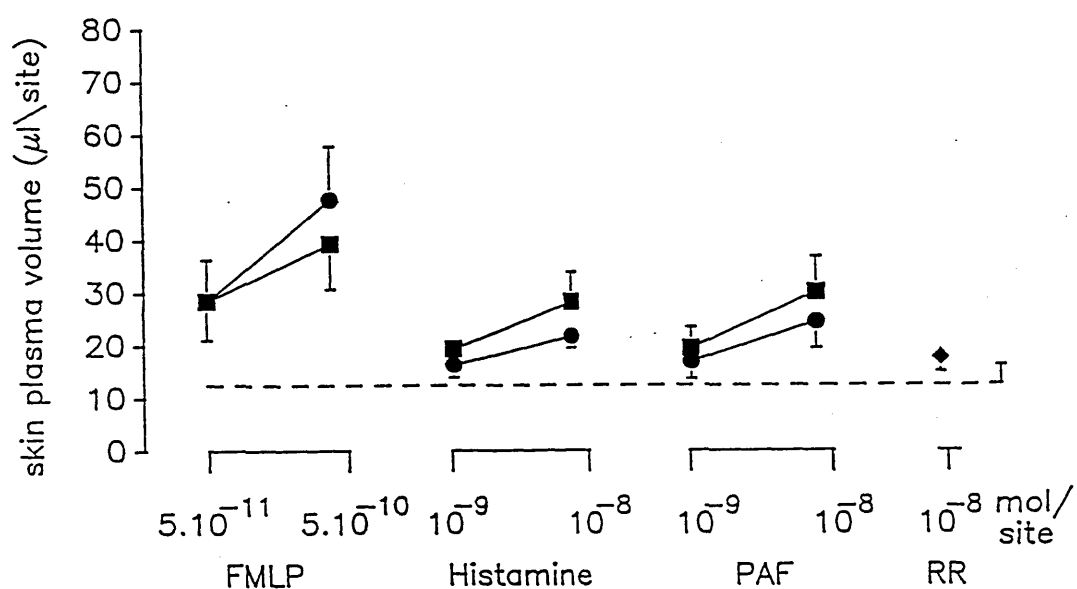


Figure 3.9

The effect of ruthenium red (RR; 10^{-8} mol/site) on oedema formation induced by FMLP (5×10^{-12} - 5×10^{-11} mol/site), histamine (10^{-8} - 10^{-7} mol/site) and platelet activating factor (PAF, 10^{-9} - 10^{-8} mol/site), over 30 min. Ruthenium red or saline were pre-injected 5 min before FMLP, histamine and PAF. Oedema formation (μ l/site) induced by mediators alone (●) and in the presence of ruthenium red (■) or ruthenium red alone (◆) were measured. Responses to saline are indicated by a dashed line. Results are expressed as mean $\pm$ SEM, n=4-5 rabbits.

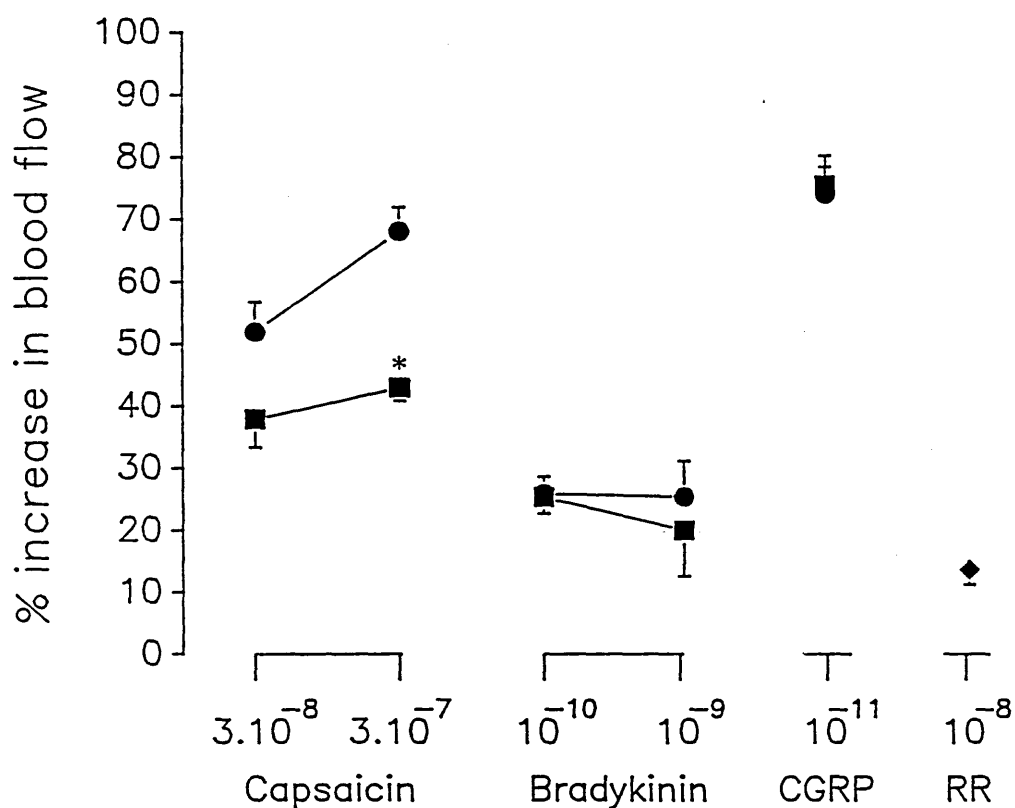


Figure 3.10

The effect of ruthenium red on increased blood flow induced by capsaicin (3×10^{-8} - 3×10^{-7} mol/site), bradykinin (10^{-10} - 10^{-9} mol/site) and CGRP (3×10^{-12} mol/site) over 15 min. Ruthenium red (10^{-8} mol/site) or saline were pre-injected 5 min before capsaicin, bradykinin and CGRP. Increased blood flow (%) induced by mediators alone (●) and in the presence of ruthenium red (■) or ruthenium red alone (◆) were determined. Results are expressed as mean $\pm$ SEM, $n=5-6$ rabbits. Asterisk (*) indicates a significant difference ($p < 0.05$, two-way ANOVA) between capsaicin (3×10^{-7} mol/site) alone and in the presence of ruthenium red.

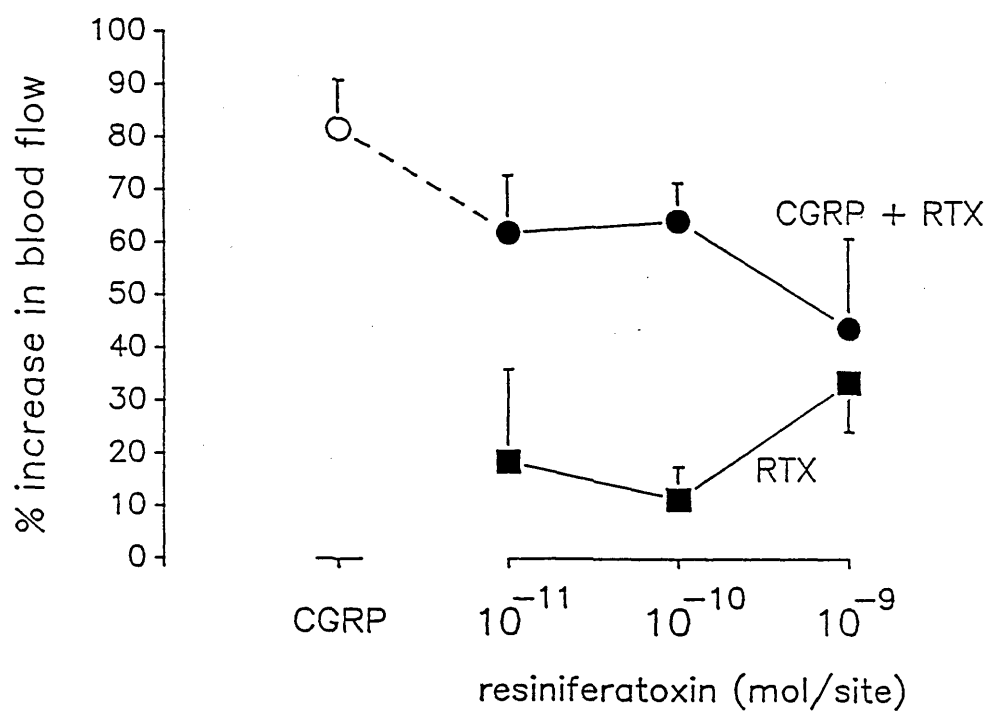


Figure 3.11

The effect of resiniferatoxin (RTX) on increased blood flow induced by CGRP. RTX and CGRP (3×10^{-12} mol/site) were injected i.d. in rabbit skin and increased blood flow was measured over 15 min. Symbols represent response to: RTX (■), CGRP (○) and CGRP + RTX (●). Results are expressed as mean $\pm$ SEM n=6 replicate sites.

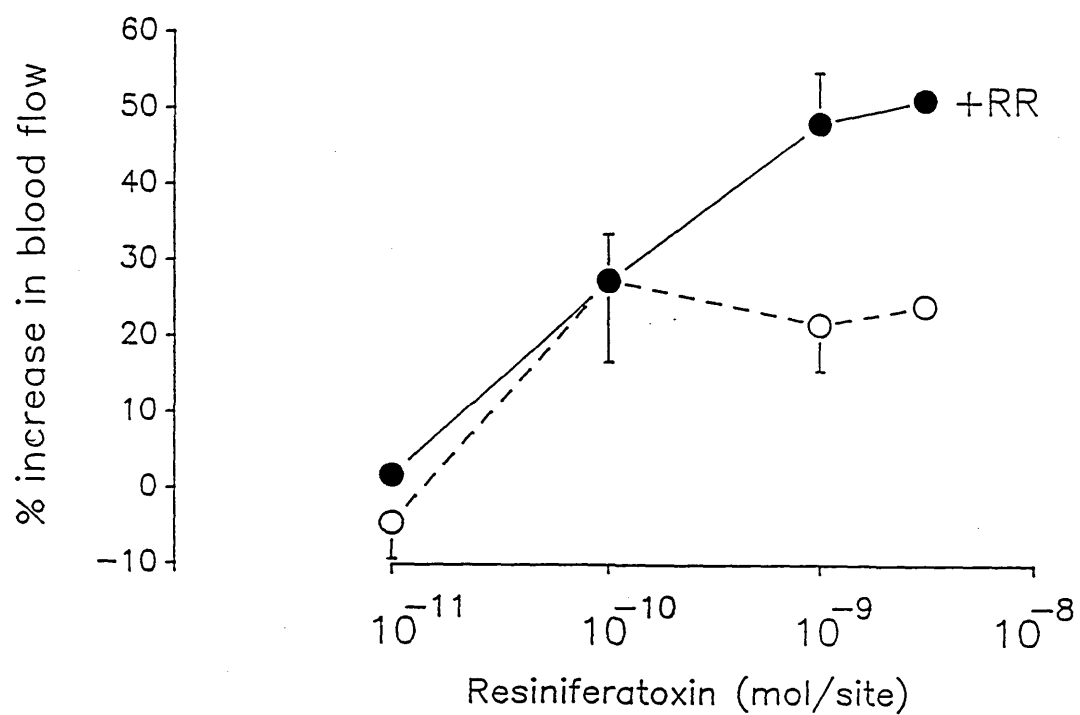


Figure 3.12

The effect of ruthenium red (10^{-8} mol/site) on responses induced by resiniferatoxin (RTX). Saline or ruthenium red were pre-injected 5 min before RTX. Increased blood flow induced by RTX alone (○) and in the presence of ruthenium red (●) was measured over 15 min. Results are expressed as mean $\pm$ SEM $n=2-3$ rabbits.

Chapter 4 Goat anti-human alpha CGRP antibody

4.1 Introduction

CGRP, a potent vasodilator, has been implicated in inflammatory processes in many species. To investigate the role of the neuropeptide CGRP, *in vivo*, a goat anti-human alpha CGRP was raised and purified to give IgG and the papain-digested fragment Fab. However, before the anti-CGRP antibody could be used as an investigative tool, the ability of this antibody to neutralise the vasodilator activity of exogenous CGRP and the selectivity of the antibody had to be established in rabbit skin. Therefore experiments were designed to investigate the ability of the anti-CGRP antibody to inhibit vasodilatation induced by CGRP and other mediators. In some experiments blood flow was measured by xenon clearance but in other experiments the ability of a given mediator to potentiate oedema formation, as a result of its vasodilator activity, was determined. Experiments were also designed to examine a vasodilator activity extracted from rabbit spinal cord. The anti-CGRP antibody was tested against this activity to try and establish whether the antibody recognised and neutralised an endogenous rabbit vasodilator. The concentration of anti-CGRP antibody used in these experiments was equal to the concentration in the original goat serum (100%), unless otherwise stated.

4.2 Neutralisation of the vasodilator activity of exogenous CGRP in rabbit skin

IgG and Fab fragments of anti-CGRP antibody, when pre-injected 5 min before mediators, significantly inhibited oedema formation potentiated by CGRP (10^{-11} mol/site) in rabbit skin (figure 4.1). Neither IgG nor Fab had any

inflammatory activity when injected i.d. alone in rabbit skin (figure 4.1). These experiments demonstrated that the relatively high titre of this antibody allowed both IgG and Fab to be used as neutralising antibodies in rabbit skin.

Using the same pre-treatment time (5 min), it was observed that anti-CGRP IgG had a profound inhibitory effect on oedema responses potentiated with rat alpha CGRP and human alpha CGRP. However the ability of VIP to potentiate bradykinin-induced oedema formation was not affected by the anti-CGRP IgG (figure 4.2). To emphasise the specificity of the anti-CGRP antibody cross-reactivity experiments, completed under radio-immunoassay conditions, demonstrated that anti-CGRP cross-reacted with human alpha CGRP, human beta CGRP and rat alpha CGRP but not with amylin amide, VIP, human calcitonin, eel calcitonin, PGE_2 , and $\text{PGF}_{2\alpha}$ (table 4.1). The specificity of the anti-CGRP antibody is further shown in figure 4.3. The vasodilators CGRP and PGI_2 potentiated neutrophil accumulation and oedema formation induced by C5a in rabbit skin (figure 4.3). Responses which were dose-dependently enhanced by CGRP were virtually abolished by the anti-CGRP IgG (figure 4.3). However responses potentiated by PGI_2 were unaffected by anti-CGRP IgG.

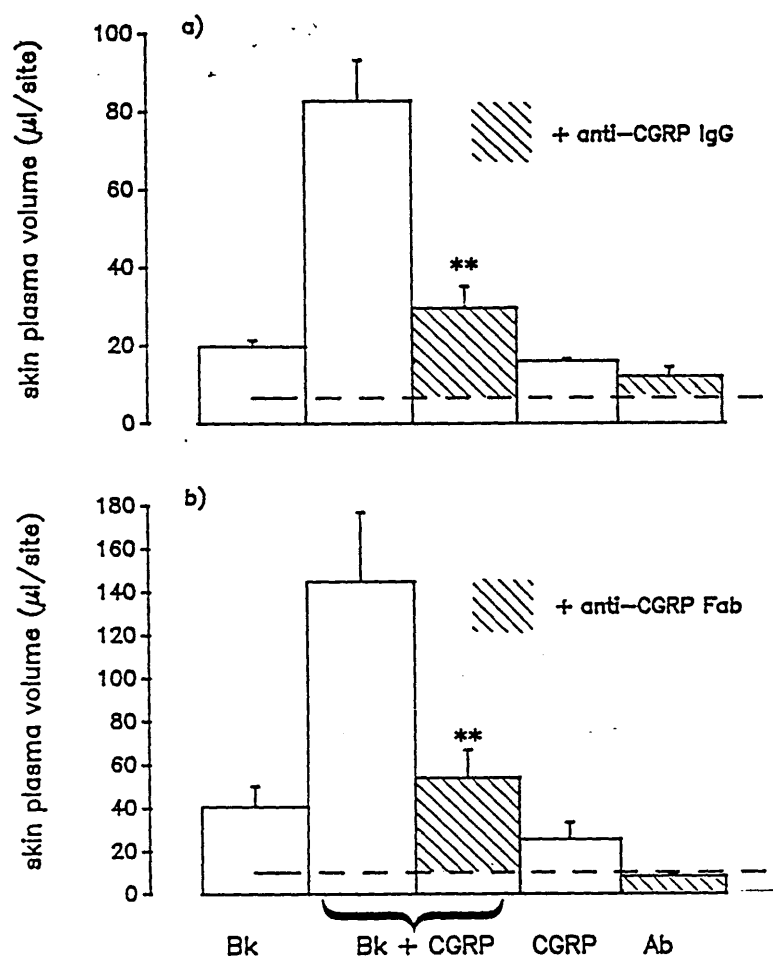


Figure 4.1

The neutralisation of the potentiating activity of CGRP (10^{-11} mol/site) using a) IgG and b) Fab fragments of a goat anti-human CGRP antibody, in rabbit skin. Saline, for control, or anti-CGRP (Ab) were pre-injected 5 min before mediators. Oedema formation (μ l/site) induced by bradykinin (Bk; 10^{-10} mol/site) + CGRP alone (open columns) and in the presence of anti-CGRP (hatched columns) was measured over 30 min. The dashed line represents the responses at saline-injected sites. Results are expressed as mean $\pm$ SEM, $n=3$ rabbits. Significant differences between the response to Bk + CGRP and Bk + CGRP + anti-CGRP antibody are indicated by ** $p < 0.01$ (two way ANOVA).

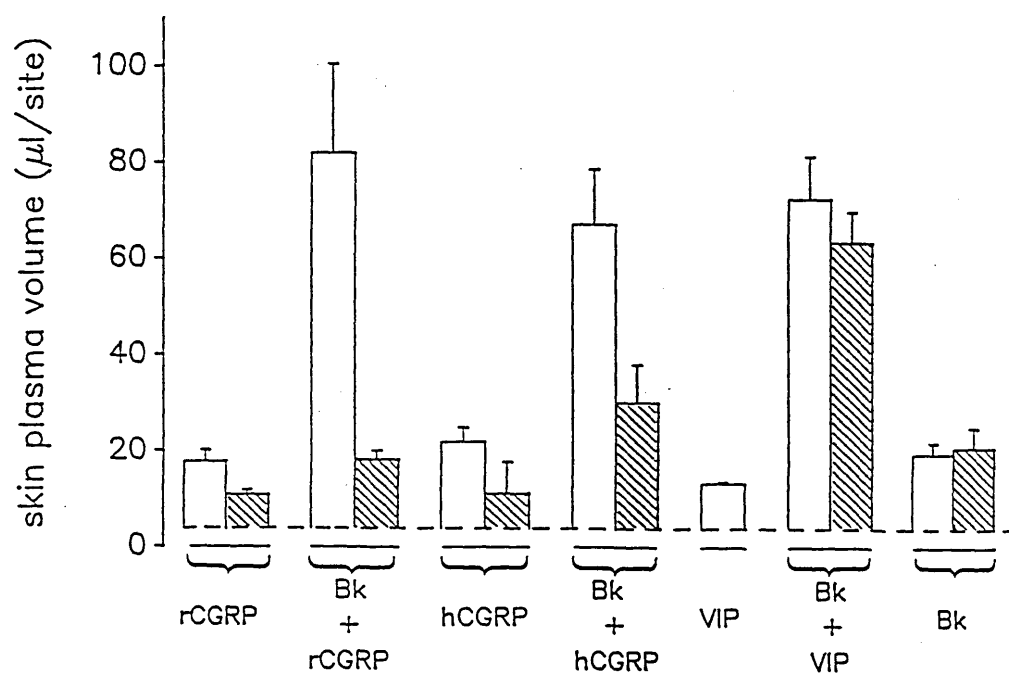


Figure 4.2

The effect of anti-CGRP IgG on responses induced by bradykinin (Bk; 10^{-10} mol/site) and potentiated by rat alpha CGRP (rCGRP; 10^{-11} mol/site), human alpha CGRP (hCGRP; 10^{-11} mol/site) and vasoactive intestinal peptide (VIP; 10^{-10} mol/site). Saline or IgG were pre-injected 5 min before mediators. Oedema formation (μ l/site) induced by mediators alone (open columns) and in the presence of IgG (hatched columns) was measured over 30 min, in the rabbit skin. Dashed line represents responses at saline-injected sites. Results are expressed as mean $\pm$ SEM $n=6$ replicate sites in 1 rabbit.

¹ Cross-reaction (%)	
human alpha CGRP	100
human beta CGRP	68
rat alpha CGRP	15
VIP	<<0.1
amylin amide	<<0.1
human calcitonin	<<0.1
eel calcitonin	<<0.1
PGE ₂	<<0.1
PGF ₂ alpha	<<0.1

Table 4.1

Cross reactions, determined by radio-immunoassay of various mediators, with the anti-human alpha CGRP serum relative to human alpha CGRP.

¹Calculated from the mass of human alpha CGRP required to displace 50% of the ¹²⁵I human alpha CGRP from the antiserum.

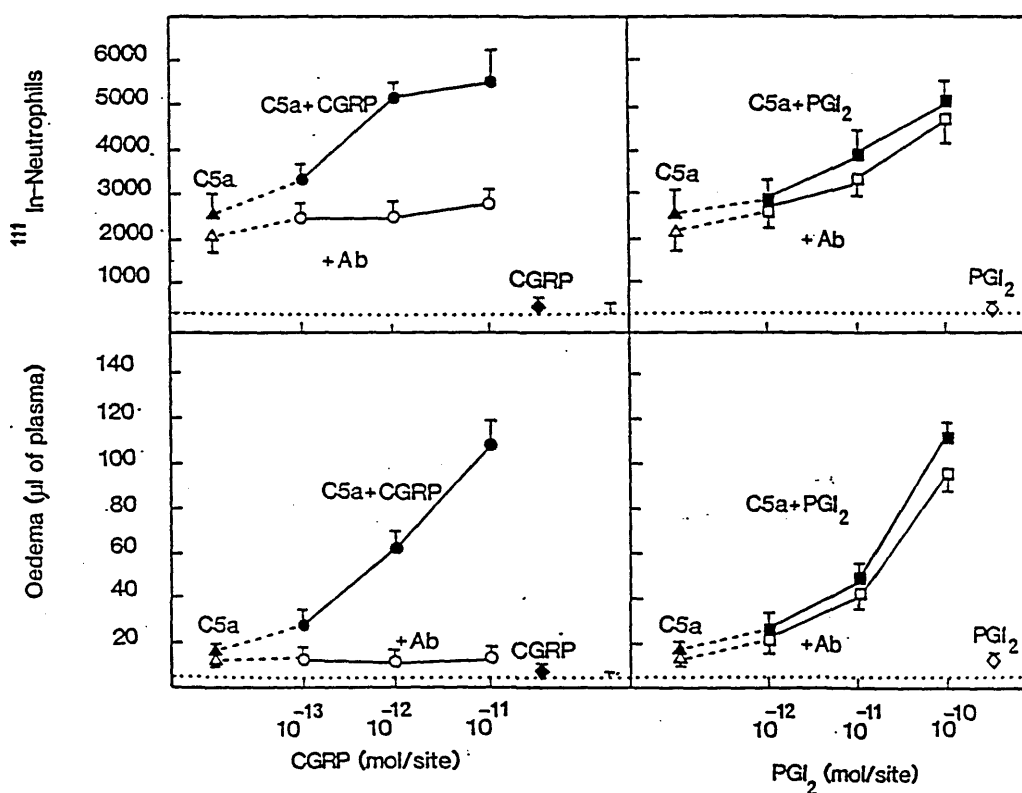


Figure 4.3

The effect of anti-CGRP IgG on neutrophil accumulation (^{111}In -neutrophil/site) and oedema formation (μl /site) in rabbit skin. Saline or IgG were pre-injected 5 min before mediators. Responses were induced by C5a ($5 \times 10^{-11}\text{mol/site}$) and potentiated by CGRP and PGI_2 . Symbols represents responses to: C5a ($\blacktriangle$), C5a + IgG ($\triangle$), C5a + CGRP ($\bullet$), C5a + CGRP + IgG ($\circ$), C5a + PGI_2 ($\blacksquare$), C5a + PGI_2 + IgG ($\square$), CGRP ($\blacklozenge$) and PGI_2 ($\blacklozenge$). Dotted line represents responses at saline-injected sites. Results are expressed as mean $\pm$ SEM $n = 6$ replicate sites.

4.3 The role of CGRP in capsaicin-evoked responses : the use of goat anti-CGRP antibody.

Capsaicin can release neuropeptides from sensory C-fibres (Gamse *et al.*, 1981; Maggi & Meli, 1988). In the present study anti-CGRP antibody was used to examine whether the neuropeptide CGRP was released from sensory fibres in rabbit skin in response to capsaicin. Furthermore, experiments were designed to try and elucidate the mechanism of neuropeptide release.

There have been reports that an anti-CGRP antibody, given i.v., neutralises responses induced by capsaicin and topical mustard oil in the rat (Louis *et al.*, 1988; Louis *et al.*, 1989). In my study anti-CGRP Fab, which had a similar antibody titre to the anti-CGRP IgG used by Louis and co-workers, administered i.v. (2ml/kg) did not neutralise oedema formation induced by histamine + CGRP (10^{-11} mol/site). Similarly, responses to histamine + capsaicin (10^{-6} mol/site) were also unaffected (figure 4.4).

As already described, local anti-CGRP IgG and Fab significantly inhibited responses induced by exogenous CGRP. However, anti-CGRP Fab when pre-treated for 5 min did not markedly affect inflammatory responses induced by capsaicin, therefore a longer pre-treatment time was tested (table 4.2a and 4.2b). Longer (40 min) or shorter (5 min) pre-treatment times of anti-CGRP Fab equally inhibited responses induced by CGRP (table 4.2a). Figures 4.5 and 4.6 further demonstrate that using a pre-treatment time of 40 min, anti-CGRP IgG and Fab equally and dose-dependently neutralised the vasodilator activity of exogenous CGRP. Furthermore, when anti-CGRP Fab was i.d. pre-injected 40 min before capsaicin a significant and dose-dependent inhibition of oedema formation potentiated by capsaicin was observed (figure 4.7 and table 4.2a).

However, anti-CGRP IgG also pre-treated for 40 min, failed to inhibit this response significantly (figure 4.7). These results show that the intact IgG was much less effective than anti-CGRP Fab at neutralising oedema formation induced by capsaicin (figure 4.7). This finding could be explained by the difference in size, and therefore the diffusing ability, between intact IgG and the Fab fragments; IgG 150-160KDa, Fab 40-45KDa.

133 xenon clearance is a sensitive method for measuring increased blood flow. CGRP (3×10^{-12} mol/site), PGE₂ (10^{-10} mol/site) and VIP (10^{-11} mol/site) induced an increase in local blood flow (figure 4.8). In these experiments a longer pre-treatment time of the antibody was used (40 min). The vasodilator response induced by CGRP was inhibited by anti-CGRP Fab, however responses to VIP and PGE₂ were unaffected (figure 4.8). The neutralisation by anti-CGRP fab of increased local blood flow induced by capsaicin was dose-dependent and this inhibition was significant at sites that had received a high vasodilator dose of capsaicin (3×10^{-7} mol/site; figures 4.9 and 4.10).

To ascertain whether the protein concentration of the anti-CGRP Fab affected clearance of 133 xenon, control Fab, raised in a non-immunised goat was prepared using a similar protocol to that used for anti-CGRP antibody. Once the protein concentrations of anti-CGRP and control Fab were determined by spectrophotometric analysis, the protein concentration of control Fab was adjusted to equal the concentration of anti-CGRP Fab. However, control Fab (100%) had no effect on oedema formation potentiated by CGRP or capsaicin (table 4.3).

These results strongly suggest that the anti-CGRP antibody was acting specifically and selectively to inhibit capsaicin-induced CGRP release. However, the maximal neutralisation of capsaicin-induced responses by the anti-CGRP Fab (given at optimal concentration and pre-treatment time) was approximately 40-50%. It was possible that the residual vasodilator activity was sensitive to ruthenium red. However the pre-injection of anti-CGRP (40 min) followed by the co-injection of capsaicin + ruthenium red resulted in the same level of inhibition in the presence of ruthenium red as effected by anti-CGRP Fab alone. Results: Capsaicin (3×10^{-7} mol/site) 75.3 ± 10.2 , capsaicin + anti-CGRP Fab (100%) 56.9 ± 6.3 , capsaicin $\pm$ ruthenium red (10^{-8} mol/site) 47.9 ± 7.3 , capsaicin + anti-CGRP Fab + ruthenium red $52.8 \pm 7.1\%$ increase in blood flow, n=6 replicate sites.

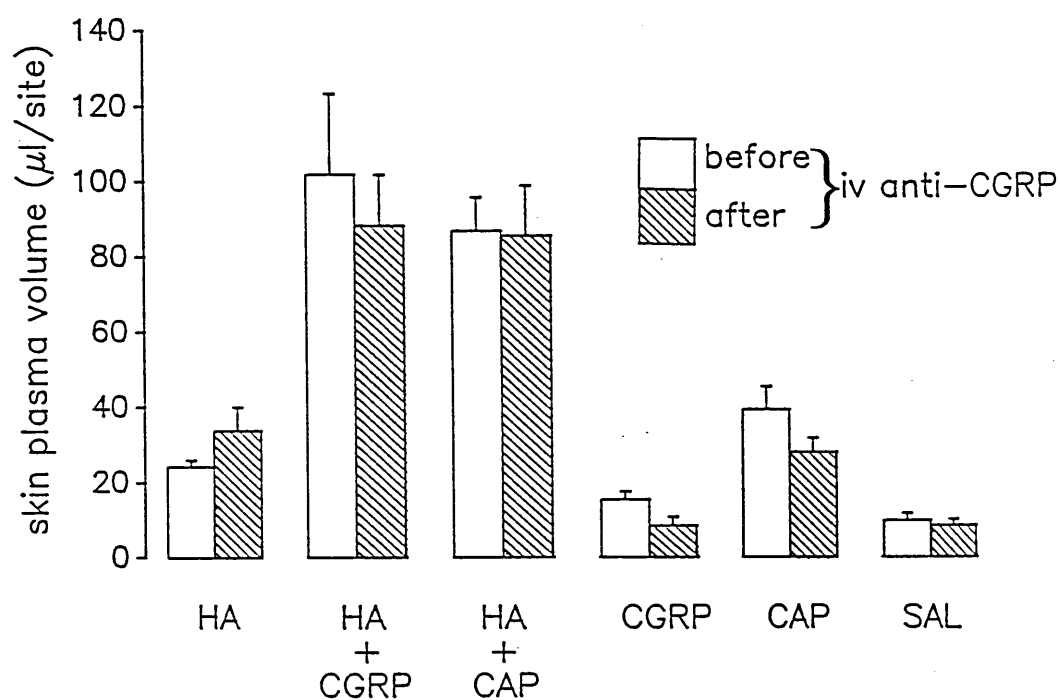


Figure 4.4

The effect of intravenous (i.v.) anti-CGRP Fab (2ml/kg) on oedema formation potentiated by intradermal (i.d.) capsaicin (10^{-6} mol/site). Oedema formation (μ l/site) induced by histamine (HA, 3×10^{-9} mol/site) was potentiated by either CGRP (10^{-11} mol/site) or capsaicin. The responses, over 30 min, before (open columns) and 5 min after i.v. anti-CGRP Fab (hatched column) were measured. Results are expressed as mean $\pm$ SEM, n=6 replicate sites.

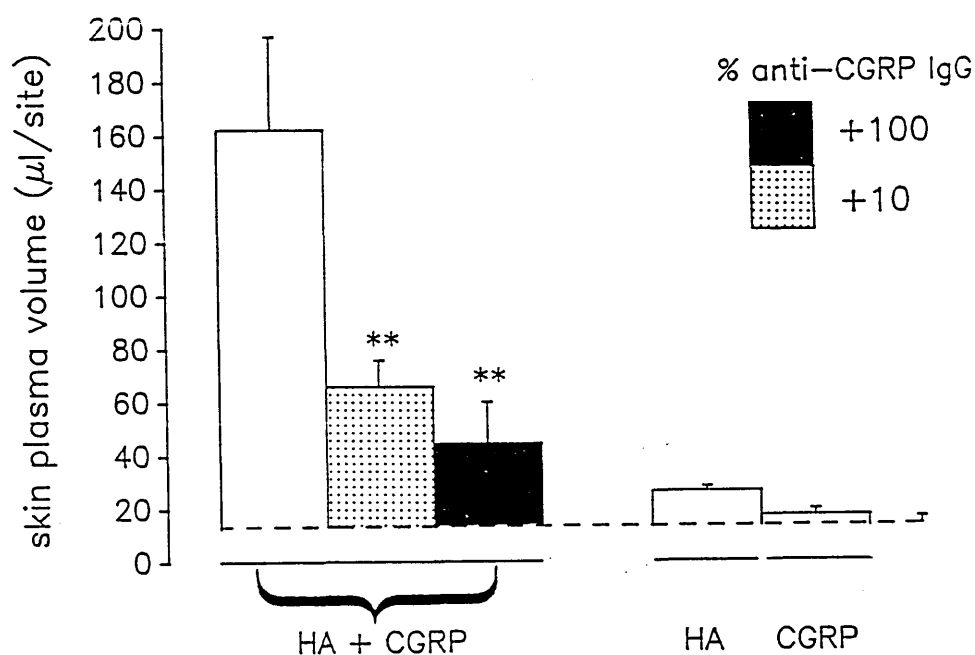


Figure 4.5

The dose-dependent neutralisation, using anti-CGRP IgG, of the ability of CGRP to potentiate oedema formation (μl/site) in rabbit skin. Saline or anti-CGRP IgG (100% or 10% of concentration in original serum) were pre-injected 40 min before mediators. Oedema formation induced by histamine (HA; 3×10^{-9} mol/site) and potentiated by CGRP (10^{-11} mol/site) was measured over 30 min. Dashed line represents responses at saline-injected sites. Results are expressed as mean $\pm$ SEM $n=3$ rabbits. Significant differences between responses induced by histamine + CGRP and HA + CGRP + IgG are indicated by ** $p < 0.01$ (two way ANOVA).

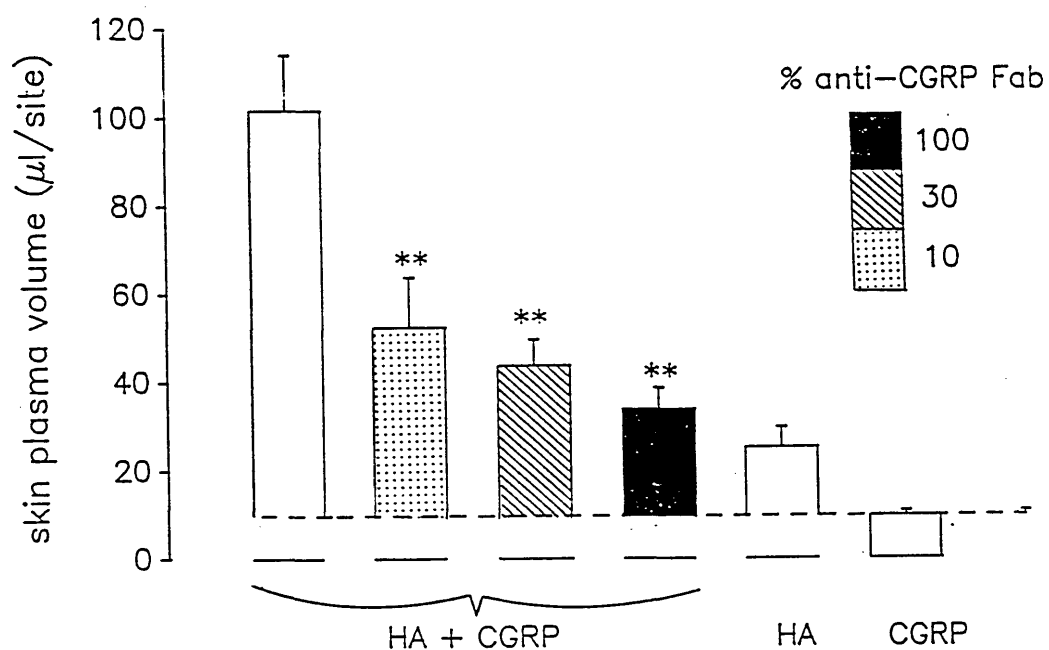


Figure 4.6

The dose-dependent neutralisation, using anti-CGRP Fab, of the ability of CGRP to potentiate oedema formation in rabbit skin. Saline or anti-CGRP Fab (100%, 30% or 10% of concentration in original serum) were pre-injected 40 min before mediators. Oedema formation ($\mu\text{l}/\text{site}$) induced by histamine ($3 \times 10^{-9} \text{mol}/\text{site}$) and potentiated by CGRP ($10^{-11} \text{mol}/\text{site}$) was measured over 30 min. Dashed line represents responses at saline-injected sites. Results are expressed as mean $\pm$ SEM $n=6$ rabbits. Significant differences between histamine + CGRP and histamine + CGRP + anti-CGRP Fab are indicated by ** $p < 0.01$ (two way ANOVA).

Table 4.2

The effect of pre-treatment time (min) of anti-CGRP Fab or saline for control on a) oedema formation ($\mu\text{l}/\text{site}$) and b) increased blood flow (%) induced by capsaicin. Pre-treatments were given at the times indicated before a second injection of the inflammatory mediators. Oedema formation was measured over 30 min whereas increased blood flow was measured over 15 min in the rabbit skin. Doses of mediators are expressed as (mol/site). Results are expressed as mean $\pm$ SEM, n=6 replicate sites.

Table 4.2a

Oedema formation ($\mu\text{l}/\text{site}$)

Pretreatment time (min)	HA(3×10^{-9}) + CGRP (10^{-11})		% inhib
	+ sal	+ Fab	
5	103 $\pm$ 20.9	45.5 $\pm$ 7.2	67.2%
40	136.2 $\pm$ 10.9	41.9 $\pm$ 6.5	79.3%
	HA(3×10^{-9}) + capsaicin(10^{-6})		
5	41.4 $\pm$ 4.4	40.0 $\pm$ 3.9	9.4%
40	61.9 $\pm$ 5.6	28.9 $\pm$ 2.8	53.3%

Table 4.2b

Increased blood flow (%)

Pretreatment time (min)	capsaicin ($3 \cdot 10^{-7}$)		% inhib
	+ sal	+ Fab	
5	119.9 $\pm$ 29.4	87.1 $\pm$ 10.0	26.6%
40	178.0 $\pm$ 24.6	104.2 $\pm$ 16.5	41.5%

Agent	skin plasma volume(μ l/site)		
	+ saline	+ control Fab	+ anti-CGRP Fab
a)			
HA + CGRP	110 $\pm$ 17.3	99.8 $\pm$ 12.7	36.2 $\pm$ 0.7
saline	10.1 $\pm$ 1.4	9.1 $\pm$ 2.3	16.9 $\pm$ 2.6
b)			
HA + Capsaicin	75.8 $\pm$ 6.5	74.6 $\pm$ 4.2	37.3 $\pm$ 3.4
saline	8.4 $\pm$ 2.1	7.7 $\pm$ 1.6	6.4 $\pm$ 0.5

Table 4.3

The effect of control Fab on oedema formation induced by histamine (3×10^{-9} mol/site) and potentiated by CGRP (10^{-11} mol/site) and capsaicin (10^{-6} mol/site). Anti-CGRP Fab (100%) and control Fab (100%) were pre-injected 40 min before other mediators and oedema formation was measured over 30 min. Results are expressed as mean $\pm$ SEM for a) n=3 rabbits and b) n=6 replicate sites.

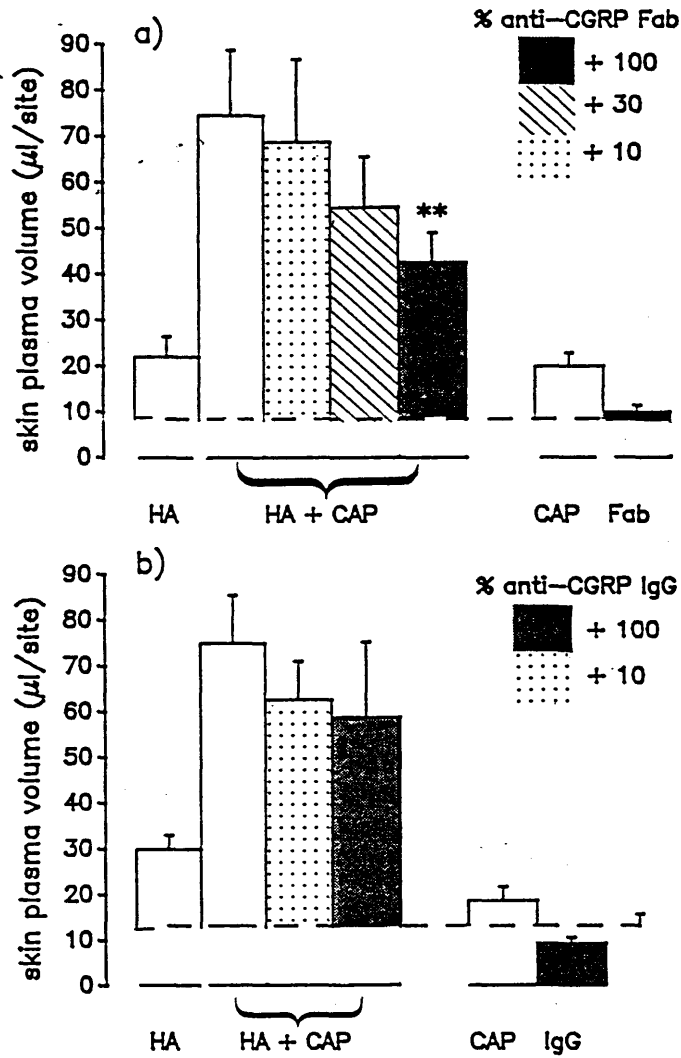


Figure 4.7

The effect of i.d. injections of either a) Fab or b) IgG anti-CGRP antibody on oedema formation potentiated by i.d. capsaicin (10^{-6} mol/site). Oedema formation (μ l/site) induced by histamine (HA; 3×10^{-9} mol/site) and capsaicin alone or in combination (open columns) was measured over 30 min. The effect of dilutions of anti-CGRP (100%, 30%, 10%) on these responses was determined. Antibodies (Fab or IgG), of equal protein concentration, or saline were pre-injected 40 min before mediators. Responses induced by saline are indicated by a dashed line. Results are expressed as mean $\pm$ SEM, $n=4-6$ rabbits. Asterisks (** $p < 0.01$) indicate significant differences ($p < 0.01$, two way ANOVA) between responses induced by histamine + capsaicin alone and in the presence of anti-CGRP antibody.

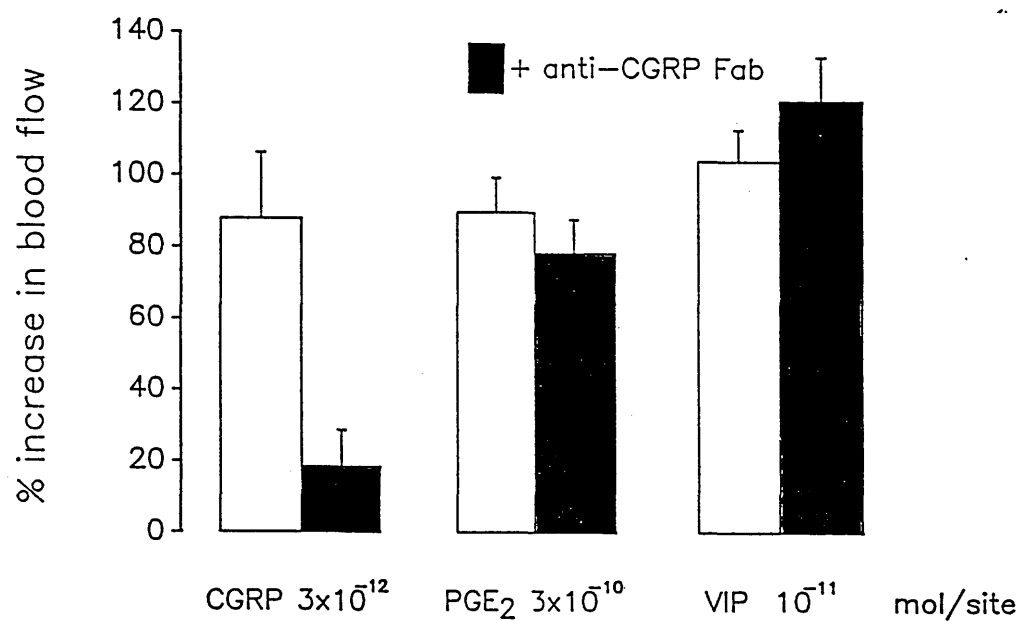


Figure 4.8

The effect of anti-CGRP Fab on increased blood flow (%) in rabbit skin. Saline or anti-CGRP Fab (100%) were pre-injected 40 min before vasodilators. Responses were induced by CGRP (3×10^{-12} mol/site) VIP (10^{-11} mol/site) and PGE₂ (10^{-10} mol/site) and measured over 15 min. Results are expressed as mean $\pm$ SEM n=6 replicate sites.

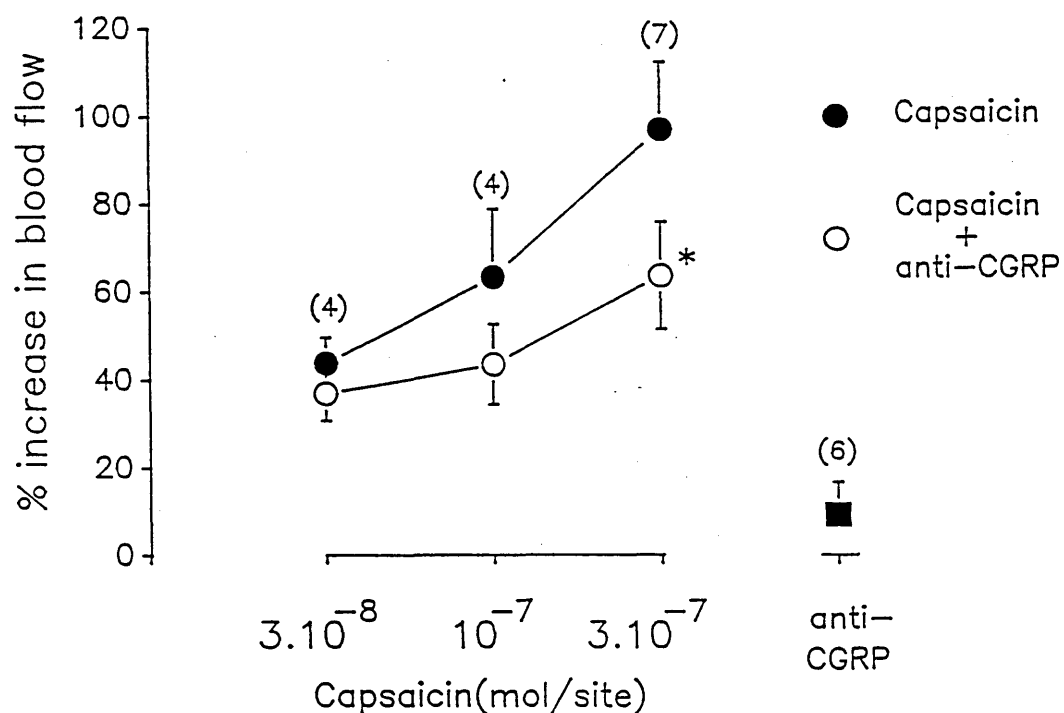


Figure 4.9

The effect of anti-CGRP Fab on increased blood flow induced by 3 doses of capsaicin in the rabbit skin. Anti-CGRP Fab or saline were pre-injected 40 min before capsaicin and the increased blood flow (%) induced by capsaicin was measured over 15 min. Symbols indicate responses to ; capsaicin (●), capsaicin + anti-CGRP (○) and anti-CGRP alone (■). Results are expressed as mean $\pm$ SEM, n=4-7 rabbits. Asterisk (*) represents significant differences ($p < 0.05$, two way ANOVA) between capsaicin alone and in the presence of anti-CGRP Fab.

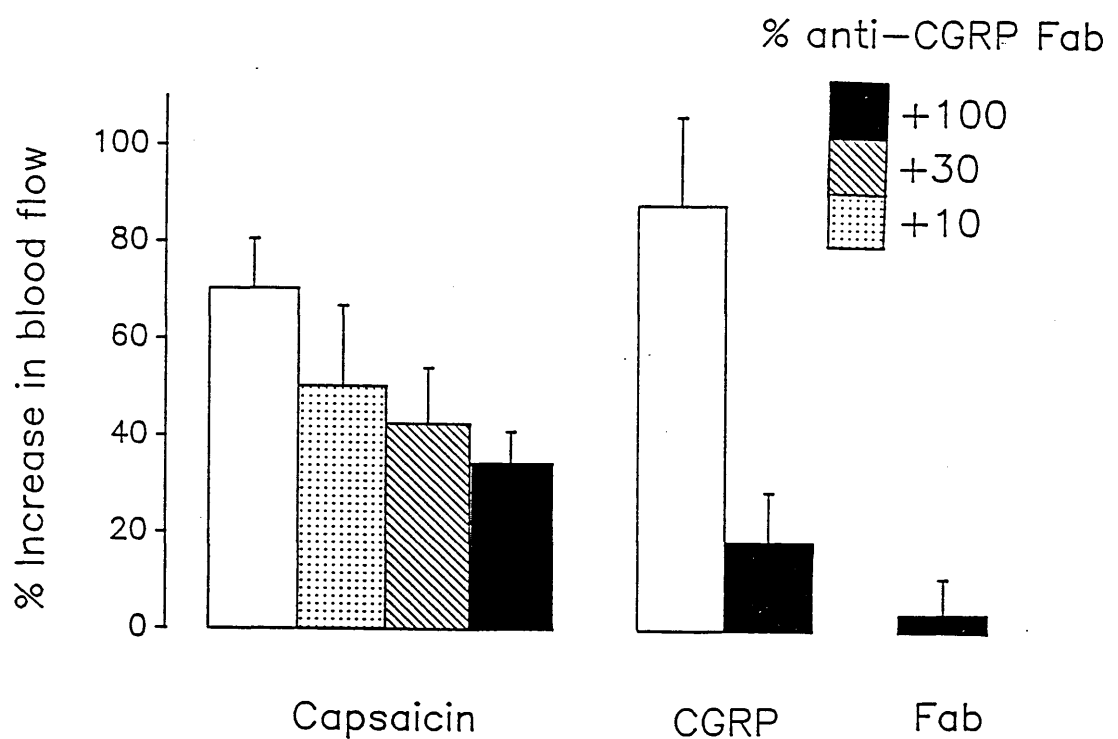


Figure 4.10

The effect of anti-CGRP Fab on increased blood flow (%) induced by capsaicin (3×10^{-7} mol/site) and CGRP (3×10^{-12} mol/site). Anti-CGRP Fab or saline were pre-injected 40 min before mediators. Increased blood flow induced by i.d. capsaicin and CGRP alone (open columns) or in the presence of dilutions of anti-CGRP Fab (100%, 30%, 10%) was determined. Results are expressed as mean $\pm$ SEM $n=6$ replicate sites in 1 rabbit.

4.4 Vasodilator activity extracted from rabbit spinal cord

In the last chapter evidence to suggest that capsaicin releases a vasodilator activity in rabbit skin was presented. Furthermore this activity was recognised and neutralised by anti-CGRP Fab. Further experiments were carried out to investigate if a vasodilator activity with the characteristics of CGRP could be extracted from rabbit spinal cord. In preliminary experiments, spinal cord extract in a cold (4°C) NaHCO₃ solution (1.4%), containing indomethacin (10⁻⁷mol/site) to block the conversion of arachidonic acid into prostanoid vasodilators, was injected i.d. into rabbit skin in an oedema assay. Exogenous vasodilators (CGRP and PGE₂) failed to enhance the relatively small amount of plasma protein leakage induced by the spinal cord extract. However spinal cord extract enhanced oedema formation induced by mediators of increased microvascular permeability (eg histamine and bradykinin; figure 4.11). Thus it appeared that this extract either contained a vasodilator substance or was able to generate the release of a vasodilator.

4.4.1 Neutralisation of rabbit endogenous vasodilator activity

The ability of the extract to induce vasodilatation was investigated in the presence of anti-CGRP IgG by the ability of spinal cord extract to potentiate histamine-induced oedema formation. Anti-CGRP IgG significantly inhibited the small response induced by spinal cord extract alone and also the ability of this extract to enhance oedema formation induced by histamine (figure 4.12). These results suggest that rabbit CGRP was present. In another experiment, spinal cord extract was passed over an affinity column (CNBr-activated sepharose 4B) conjugated with anti-CGRP IgG. Spinal cord extract, after this procedure did not markedly enhance oedema formation induced by histamine, suggesting that the

vast majority of the vasodilator activity was removed by the specific affinity gel. The small residual vasodilator activity of treated spinal cord extract was further inhibited in the presence of i.d. anti-CGRP IgG (figure 4.13).

It is therefore likely that the vasodilator activity which was extracted from rabbit spinal cord and recognised by the anti-human CGRP antibody, was a rabbit form of CGRP.

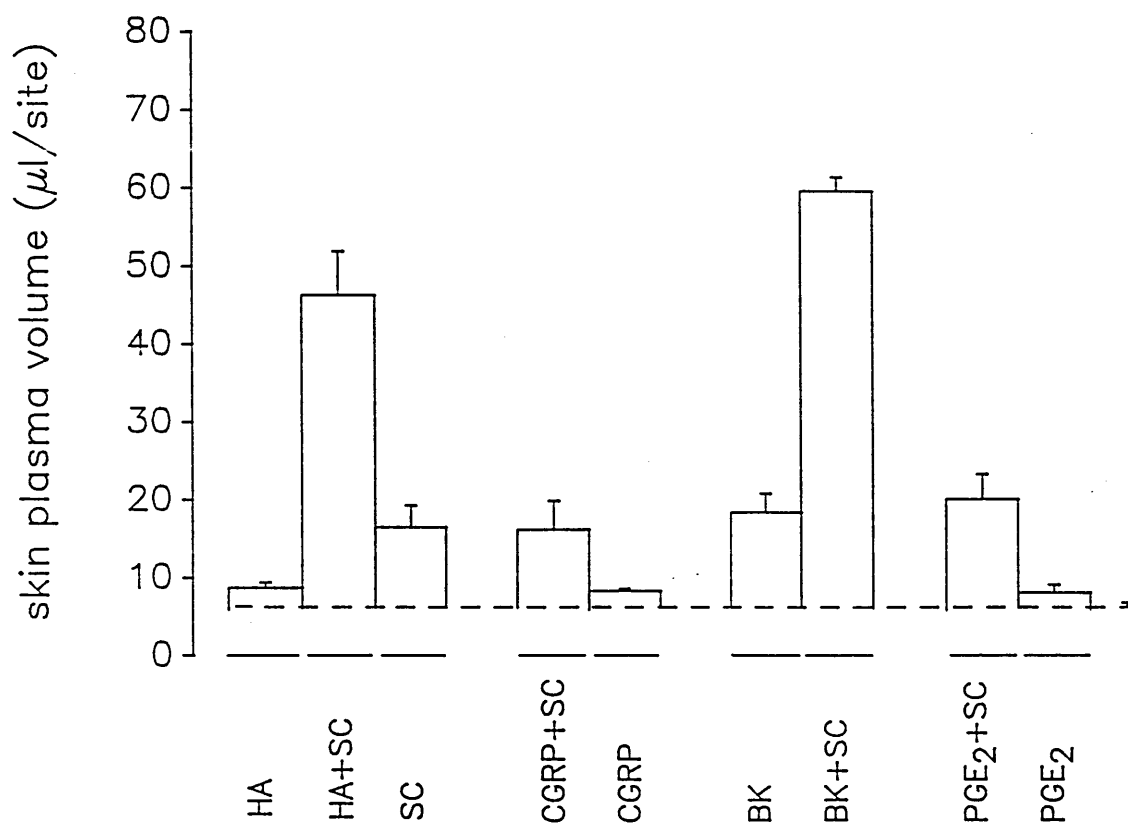


Figure 4.11

The effect of spinal cord extract on oedema formation ($\mu\text{l}/\text{site}$) in rabbit skin. Responses induced by rabbit spinal cord extract alone (SC; equivalent to 20mg of spinal cord/site in a NaHCO_3 solution, 1.4% at 4°C , containing indomethacin, $10^{-5}\text{mol}/\text{site}$) were measured alone or co-injected with histamine (HA; $3 \times 10^{-9}\text{mol}/\text{site}$), bradykinin (Bk; $10^{-10}\text{mol}/\text{site}$), CGRP ($10^{-11}\text{mol}/\text{site}$) or PGE_2 ($3 \times 10^{-10}\text{mol}/\text{site}$), over 30 min, in the rabbit skin. Dashed line represents responses at NaHCO_3 (1.4%)-injected sites. Results are expressed as mean $\pm$ SEM $n=6$ replicate sites.

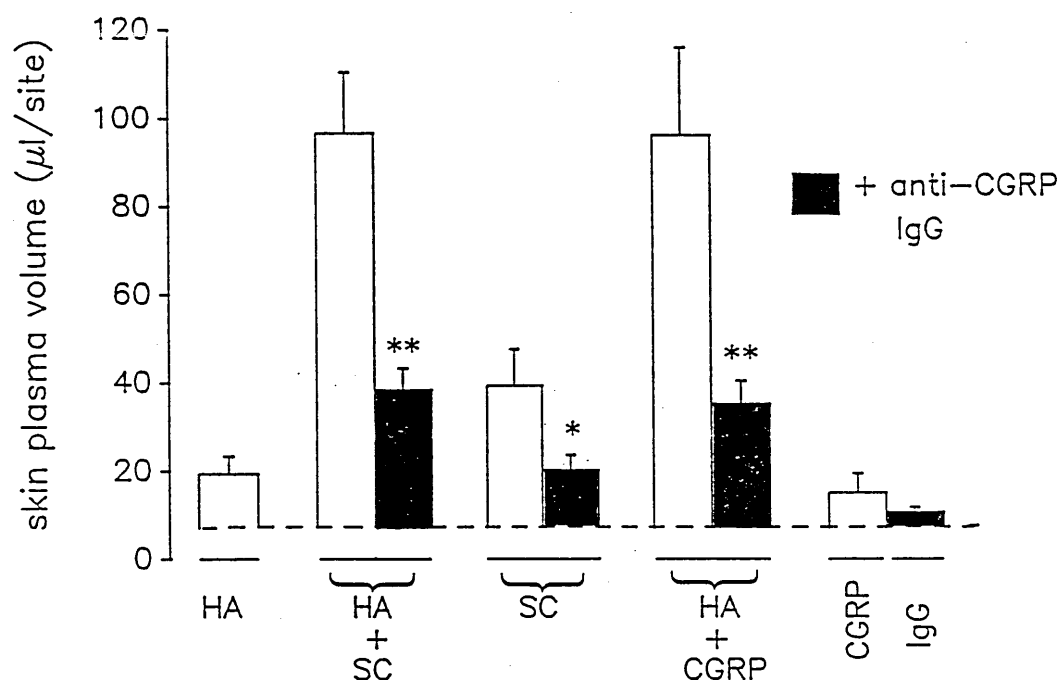


Figure 4.12

The effect of anti-CGRP IgG on responses induced by rabbit spinal cord extract in rabbit skin. NaHCO₃ (1.4%) or anti-CGRP IgG were pre-injected i.d. 5 min before mediators. Oedema formation (μl/site) induced by histamine (HA; 3×10^{-9} mol/site) was potentiated by CGRP (10^{-11} mol/site) or spinal cord extract solution (SC; equivalent to 20mg of spinal cord/site in a NaHCO₃ solution, 1.4% at 4°C, containing indomethacin 10^{-5} mol/site). The response to spinal cord extract alone was also determined. All responses were measured over 30 min. Dashed line represents responses at NaHCO₃ (1.4%)-injected sites. Results are expressed as mean $\pm$ SEM n=4 rabbits. Significant differences between responses in the presence and absence of anti-CGRP IgG are indicated by * $p < 0.05$ ** $p < 0.01$ (two way ANOVA).

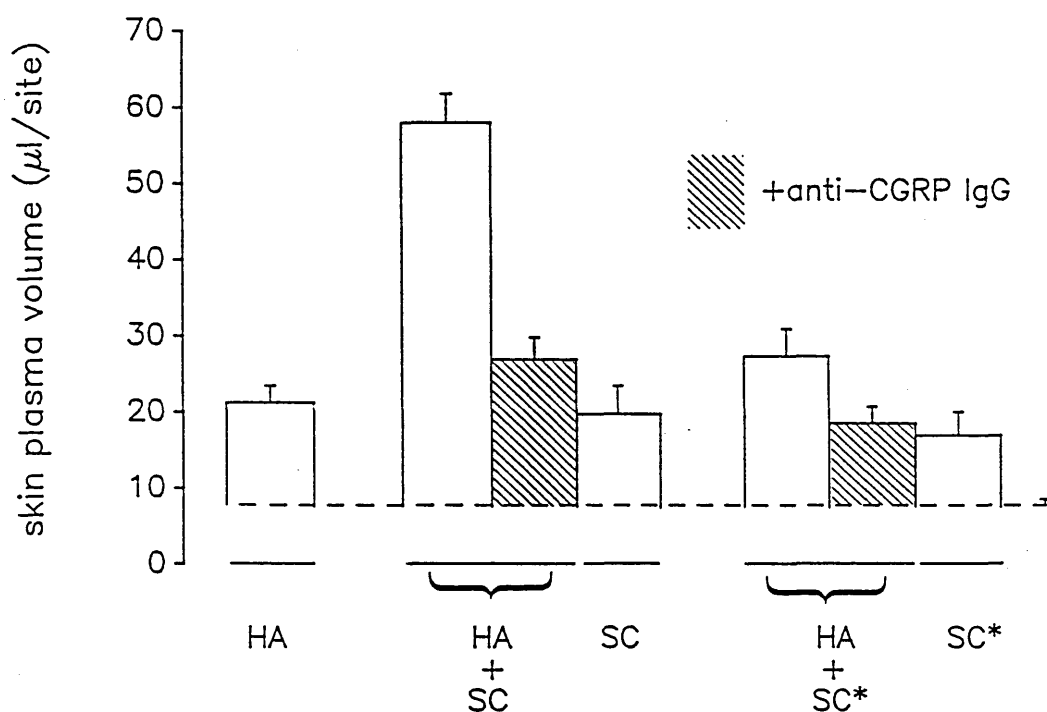


Figure 4.13

The effect of passing spinal cord extract solution over a CGRP affinity column on its ability to enhance oedema formation in rabbit skin. Spinal cord extract solution (SC; equivalent to 20mg spinal cord/site in a NaHCO_3 solution, 1.4% at 4°C , containing indomethacin, 10^{-7}mol/site) was split into two equal portions; half of the extract was passed over a CGRP affinity column, at 4°C (SC^*). Oedema formation ($\mu\text{l/site}$) induced by histamine (HA; $3 \times 10^{-9}\text{mol/site}$) and co-injected with SC or SC^* was measured for 30 min. Some sites were pre-treated (i.d., -5 min) with saline or local anti-CGRP IgG. Dashed line represents responses at NaHCO_3 (1.4%)-injected sites. Results are expressed as mean $\pm$ SEM $n=6$ replicate sites.

4.5 Other sensory neuropeptides involved in neurogenic inflammation

This study has presented evidence for the involvement of the vasodilator neuropeptide CGRP in capsaicin-evoked responses. However, the possibility remains that other neuropeptides could be released from sensory C-fibres in the rabbit skin. Neurogenic responses induced by capsaicin were partially, but not completely, neutralised by anti-CGRP Fab. In further studies the tachykinins and VIP were injected i.d. into rabbit skin for comparison with CGRP.

4.5.1 Tachykinins

NKA, NKB and substance P induce inflammatory responses in rat skin (Brain & Williams, 1989). However, none of these mediators induced oedema formation in rabbit skin even in the presence of a vasodilator (CGRP) or mediators of increased microvascular permeability (histamine and bradykinin; table 4.4). In addition substance P failed to increase local blood flow measured using ^{133}Xe clearance (Results: substance P, 10^{-11}mol/site $6.6 \pm 5.7\%$ increase in blood flow, $n=6$ replicate sites.)

4.5.2 Vasoactive Intestinal Peptide (VIP)

VIP has been reported to be a vasodilator in many vascular beds. When VIP (10^{-11}mol/site) was i.d. injected into rabbit skin an increase in blood flow was observed (VIP $89.1 \pm 8.2\%$ increase in blood flow, $n=6$ replicate sites). VIP (10^{-10}mol/site) also significantly potentiated oedema formation induced by bradykinin in the rabbit skin (figure 4.14). This response was similar to the enhancement of oedema formation by CGRP (10^{-11}mol/site). Furthermore, VIP alone did not induce any plasma protein leakage. Therefore, unlike the

tachykinins, VIP could be responsible for some neurogenic responses in the rabbit skin. Indeed from results presented in this thesis it is impossible to exclude the possibility that the residual vasodilatation induced by capsaicin and observed in the presence of anti-CGRP Fab, was due to the release of the vasodilator VIP.

Agents		Oedema Formation ($\mu\text{l}/\text{site}$)	
		+CGRP (10^{-11})	+Bk (10^{-10})
saline	8.5 ± 0.6	11.7 ± 1.1	29.9 ± 1.7
NKA	10.2 ± 1.3	11.8 ± 1.1	31.5 ± 1.9
NKB	8.3 ± 0.3	13.4 ± 1.1	27.1 ± 1.9
		+CGRP (10^{-11})	+histamine ($3 \cdot 10^{-9}$)
saline	9.2 ± 1.0	12.5 ± 1.5	18.0 ± 1.3
SP	9.9 ± 1.5	12.8 ± 1.1	17.4 ± 0.6

Table 4.4

The effect of NKA, NKB and substance P, all at 10^{-10} mol/site, at inducing or potentiating oedema formation in rabbit skin over 30 min. The doses of mediators are expressed as (mol/site). Results are expressed as mean $\pm$ SEM, n=6 replicate sites.

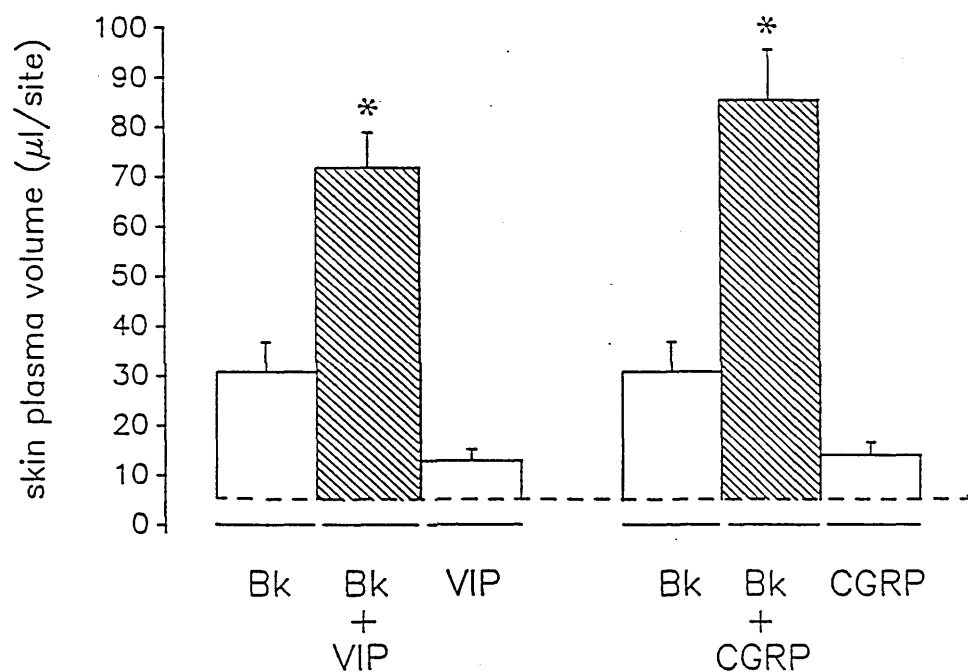


Figure 4.14

The effect of VIP (10^{-10} mol/site) and CGRP (10^{-11} mol/site) on oedema formation (μ l/site) induced by bradykinin (10^{-10} mol/site). The dashed line represents responses at saline-injected sites. Results are expressed as mean $\pm$ SEM, $n=3$ rabbits. Significant differences between bradykinin alone and in the presence of VIP or CGRP are indicated by * $p < 0.05$ (two way ANOVA).

Chapter 5 Biological activity of CGRP in rabbit skin

5.1 Introduction

Previously, CGRP has been shown to be a potent long lasting vasodilator (Brain & Williams, 1985; Brain *et al.*, 1985). Using a ^{133}Xe clearance technique, it was established that CGRP induced a dose-dependent increase in blood flow (Brain *et al.*, 1985). In this study, the actions of CGRP have been investigated further using various in vivo models in the rabbit skin.

The modulation of inflammatory responses by CGRP was investigated by measuring oedema formation and neutrophil accumulation. Using these techniques interactions between mediators of increased microvascular permeability (direct-acting and neutrophil-dependent) and sensory neuropeptides were determined. In addition, increased blood flow was measured using the laser Doppler blood flow meter. The introduction of the laser Doppler blood flow meter has allowed the direct non-invasive measurement of changes in local blood flow in many systems including skin. The possible interaction of CGRP with the novel endothelial cell product, endothelin, which has been shown to be a potent vasoconstrictor in many vascular beds, in the rabbit skin was also examined.

5.2 Modulation of oedema formation and neutrophil accumulation by CGRP

CGRP (10^{-11}mol/site) dose-dependently potentiated oedema formation induced by C5a des Arg and bradykinin (figure 5.1). These results support previous studies which demonstrated that CGRP, as a consequence of its vasodilator activity, potentiated inflammatory oedema formation induced by mediators of increased microvascular permeability (Brain & Williams, 1985;

Brain *et al.*, 1986b). C5a des Arg, FMLP and LTB₄ induce neutrophil-dependent oedema formation (Wedmore & Williams, 1981). It was observed that CGRP dose-dependently enhanced neutrophil accumulation and oedema formation induced by these chemoattractants in the rabbit skin (figures 5.1 and 5.2). The injection of CGRP (10^{-11} mol/site) alone did not induce oedema formation (figure 5.1) and at doses up to 10^{-10} mol/site, CGRP did not induce neutrophil accumulation either. (Results are as follows ¹¹¹In-neutrophil/site: CGRP (10^{-10} mol/site) 350 ± 25 , saline 304 ± 14 , mean $\pm$ SEM n=6 replicate sites.

5.3 Modulation of the vasodilator activity of CGRP by endothelin

As already described, CGRP potentiated oedema formation induced by mediators of increased microvascular permeability. The addition of the vasoconstrictor endothelin-1 caused a dose-dependent inhibition of oedema formation induced by bradykinin and FMLP (figure 5.3). Neutrophil accumulation induced by FMLP in the presence of CGRP was also inhibited by endothelin-1. Results: FMLP (5×10^{-11} mol/site) 897 ± 62 , CGRP (10^{-11} mol/site) 663 ± 62 , FMLP + CGRP 1197 ± 35 , FMLP + CGRP + endothelin (10^{-12} mol/site) 997 ± 96 , FMLP + CGRP + endothelin (10^{-11} mol/site) 739 ± 77 , saline 458 ± 80 ¹¹¹In-neutrophil/site mean $\pm$ SEM n=6 replicate sites. It would appear that the modulation of oedema formation by endothelin results from its potent vasoconstrictor activity in the microvascular bed in rabbit skin (Brain *et al.*, 1988)

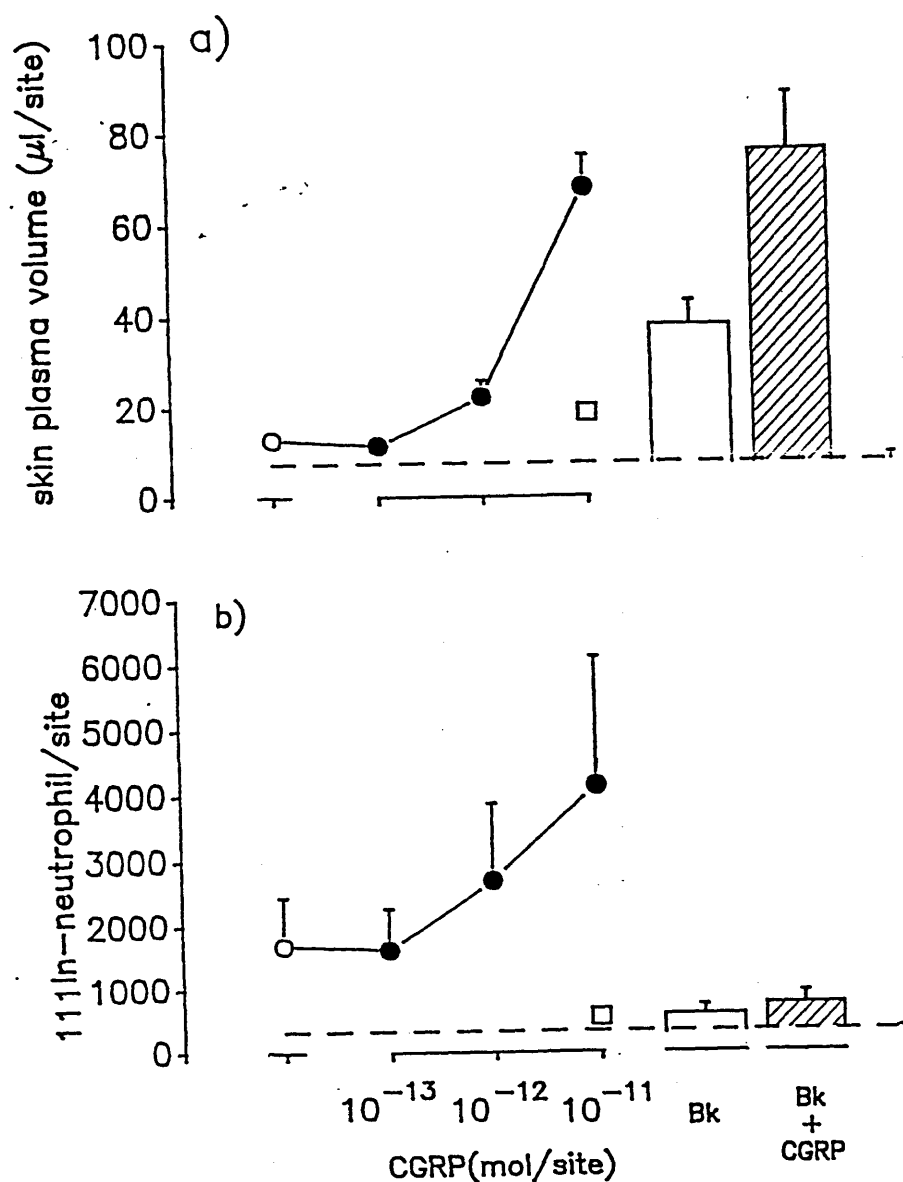


Figure 5.1

Potentiation by CGRP of a) oedema formation ($\mu\text{l}/\text{site}$) and b) neutrophil accumulation (^{111}In -neutrophil/site) induced by C5a des Arg ($3 \times 10^{-11} \text{mol}/\text{site}$) and bradykinin ($10^{-10} \text{mol}/\text{site}$) over 30min. The symbols represent responses to C5a des Arg ($\circ$), C5a des Arg + CGRP ($\bullet$), CGRP ($\square$). Responses to bradykinin are shown by columns (Bk, open column) and Bk + CGRP (hatched column). The dashed line indicates responses at saline-injected sites. Results are expressed as mean $\pm$ SEM, $n=4$ rabbits.

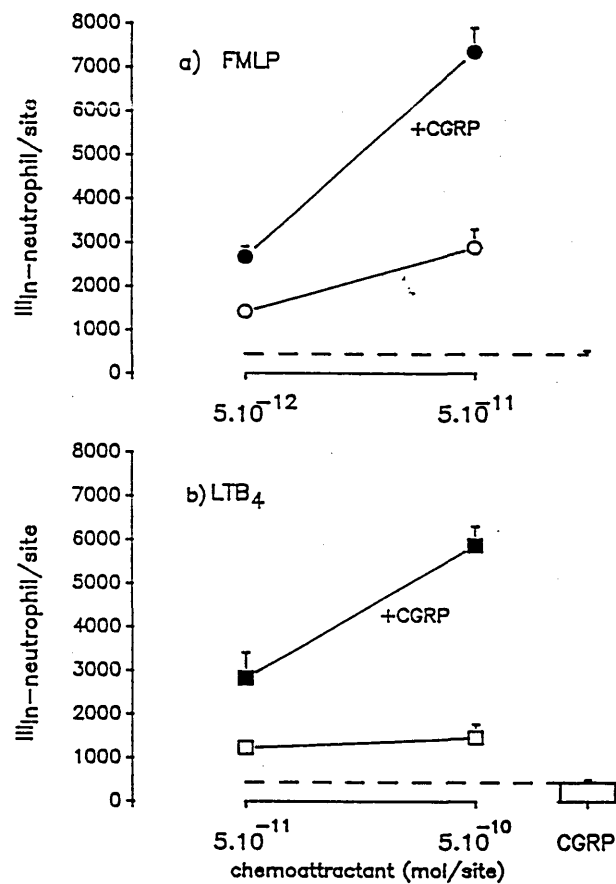


Figure 5.2

Potentiation by CGRP (10^{-11} mol/site) of neutrophil accumulation (^{111}In -neutrophil/site) induced by LTB_4 and FMLP over 30min. The symbols represent responses to LTB_4 (□) and FMLP (○) alone and in the presence of CGRP (■ and ● respectively). The dashed line indicates responses at saline-injected sites. Results are expressed as mean $\pm$ SEM, $n=6$ replicate sites.

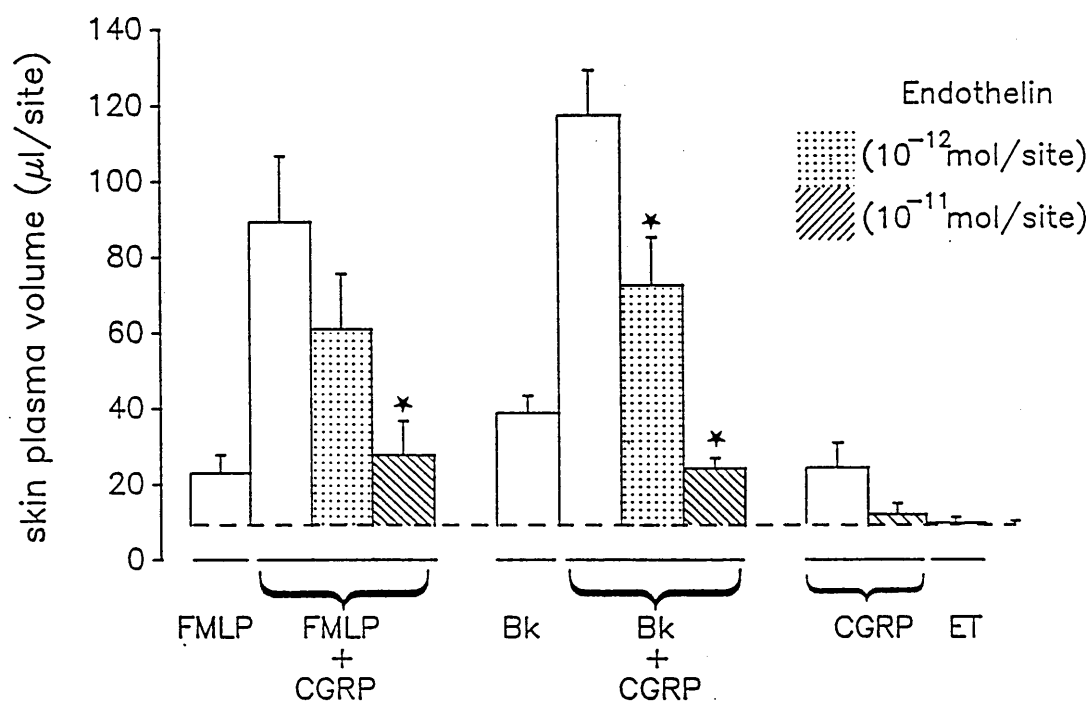


Figure 5.3

The effect of endothelin on oedema formation enhanced with CGRP (10^{-11}mol/site). Oedema formation induced by FMLP ($5 \times 10^{-11}\text{mol/site}$) and bradykinin (10^{-10}mol/site) was measured over 30 min. Endothelin was co-injected with other mediators. Dashed line represents responses at saline-injected sites. Results are expressed as mean $\pm$ SEM $n=4$ rabbits. Asterisk (*) represents significant differences ($p < 0.05$) between FMLP + CGRP in the presence and absence of endothelin.

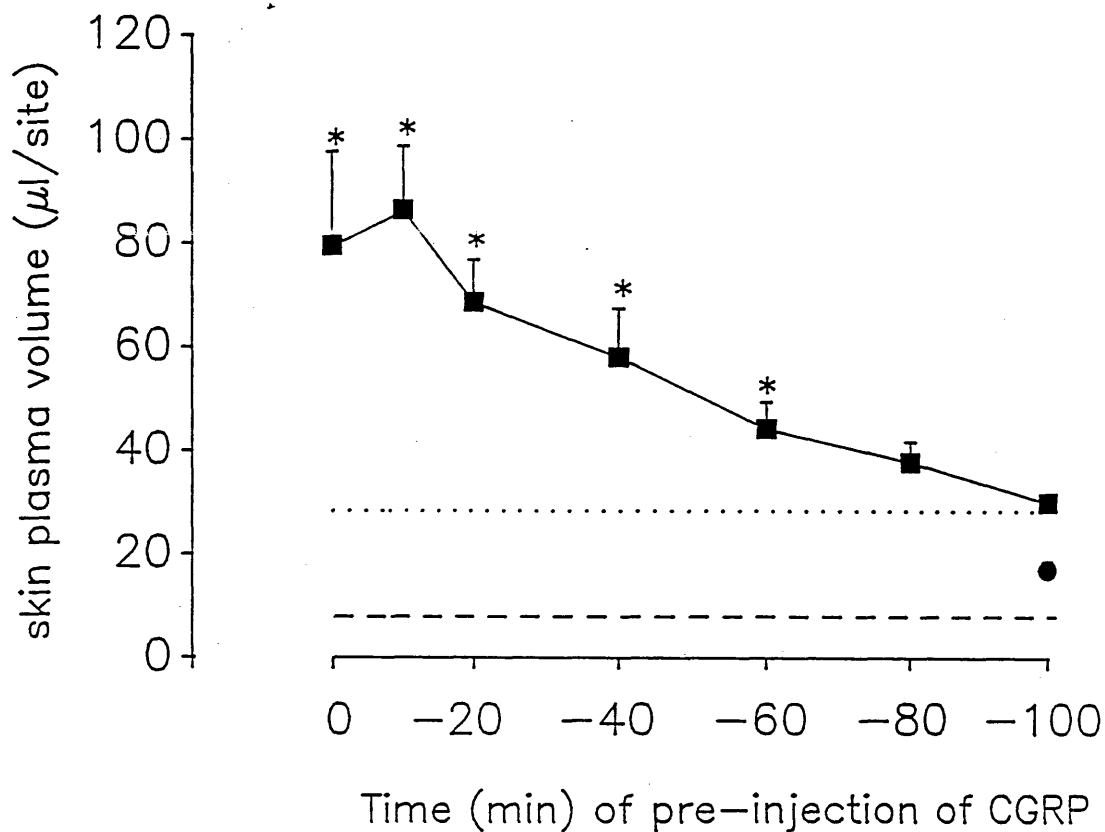


Figure 5.4

The duration of action of CGRP as a potentiator of bradykinin-induced oedema formation. CGRP at a dose of 10^{-11} mol/site (■) was injected into each rabbit 100, 80, 60, 40, 20 and 0 min before a superimposed injection of bradykinin (10^{-10} mol/site). CGRP alone (●) was also injected 100 min before a superimposed injection of saline. Oedema formation (μl/site) was measured for 30 min after bradykinin or saline injection. The dotted line indicates the response to bradykinin alone. The dashed line represents the response to saline. Results are expressed as mean $\pm$ SEM, n=4 rabbits. Significant differences between oedema responses induced by bradykinin alone and in the presence of CGRP are indicated by * $p < 0.05$ (two way ANOVA).

5.4 Comparison of CGRP with the prostanoid vasodilator PGI₂

The duration of vasodilator activity was indirectly measured by monitoring the ability of CGRP and PGE₂ to potentiate oedema formation induced by bradykinin. CGRP was determined to have a relatively long duration of vasodilator activity in rabbit skin (figure 5.4). The biological half life ($t^{1/2}$) of the vasodilator activity of CGRP (10^{-11} mol/site) was 39.3 ± 7.7 min. This result compares favourably with previous results from our laboratory (40.1 ± 7.5 min; (Brain & Williams, 1985) . However, PGI₂ (3×10^{-10} mol/site) was shown to have a much shorter duration of vasodilator activity ($t^{1/2}$ 12 ± 3 min; Peck and Williams, 1978). The introduction of the laser Doppler blood flow meter has allowed the duration of increased blood flow to be directly monitored. From these experiments the $t^{1/2}$ for increased blood flow induced by CGRP and PGI₂ was 68.4 ± 7.4 min and 26.2 ± 4.2 min respectively (figure 5.6). Using this technique, it appeared that the activities of both these vasodilators were more protracted, however, CGRP (10^{-11} mol/site) still showed an increased duration of vasodilator action when compared to PGI₂ (3×10^{-10} mol/site). In addition to having a more protracted vasodilatory profile, CGRP was also more potent than PGI₂ when considering its ability to enhance inflammatory responses induced by C5a des Arg (figure 5.6).

Further experiments were designed to investigate whether the differences in potency and duration of action of CGRP and PGI₂ affected the ability of these vasodilators to modulate inflammatory responses. Both vasodilators potentiated C5a des Arg-induced oedema formation and neutrophil accumulation to a similar extent over a 30 min period. Over the longer 30-60 min period, which was determined indirectly by subtraction of the 0-30 min response from the 0-60 min response, PGI₂ failed to potentiate the responses to C5a des Arg. However

CGRP significantly potentiated oedema formation equally over the 0-30 min and 30-60 min periods (figure 5.7; typical experiment). The results from this experiment were supported by statistical analysis of grouped data. CGRP (10^{-11} mol/site) significantly potentiated neutrophil accumulation ($p < 0.05$, $n = 4$ rabbits) and oedema formation ($p < 0.05$, $n = 4$ rabbits) induced by C5a des Arg over both the 0-30 and 30-60 min periods.

CGRP potentiated oedema formation induced by a range of mediators of increased microvascular permeability (figure 5.8). These experiments were designed to measure directly the ability of CGRP (10^{-11} mol/site) to potentiate oedema formation over the 0-30 and 30-60 min periods. CGRP potentiated oedema formation induced by all the mediators over the 0-30 min period. However during the 30-60 min period only oedema formation induced by the neutrophil-dependent mediators FMLP and C5a des Arg was significantly enhanced by CGRP.

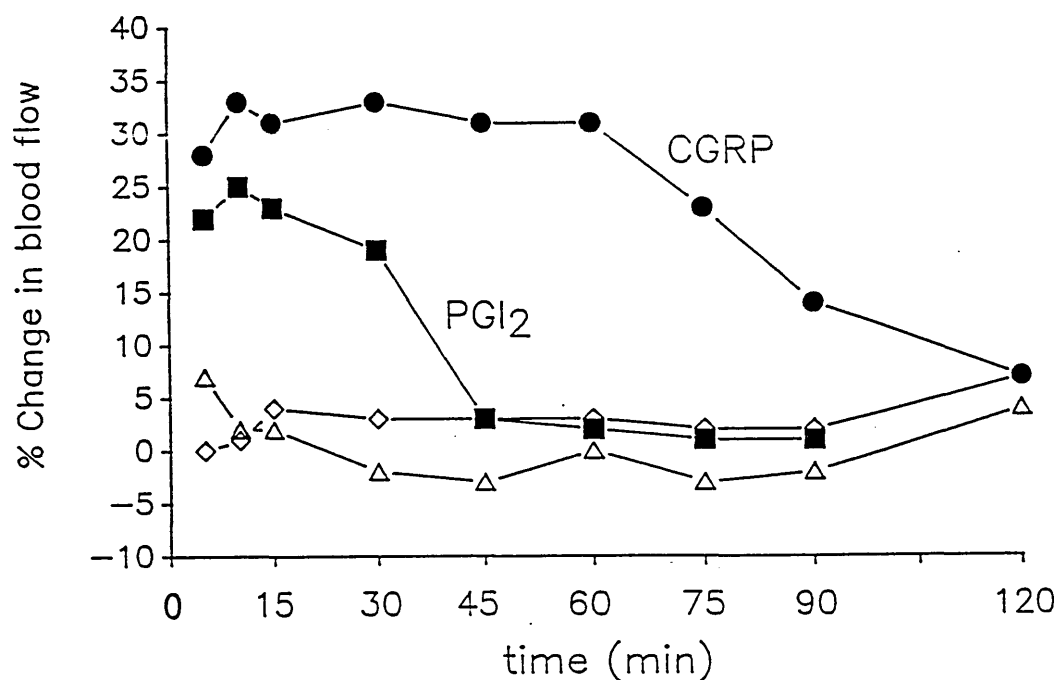


Figure 5.5

The duration of vasodilator activity, determined using the laser Doppler flow meter, of CGRP (10^{-11} mol/site) and PGI₂ (3×10^{-10} mol/site) in rabbit skin. Blood flow, calculated as % above basal blood flow for each mediator, was measured at sites i.d. injected with CGRP, PGI₂ and saline between 0 and 120 min after the injection. For comparison changes in blood flow at an untreated site was also measured. Symbols represent responses induced by CGRP (●), PGI₂ (■), saline (◇) and measurements at uninjected sites (△). Results are expressed as the mean of triplicate measurements taken at any one site in 1 rabbit. For group data (n=3 rabbits), the biological half life ($t^{1/2}$) for the duration of vasodilator activity induced by CGRP was significantly greater than that for PGI₂ ($t^{1/2}$ CGRP 68.2 ± 7.4 min ; $t^{1/2}$ PGI₂ 26.4 ± 4.2 min : $p < 0.05$ two way ANOVA).

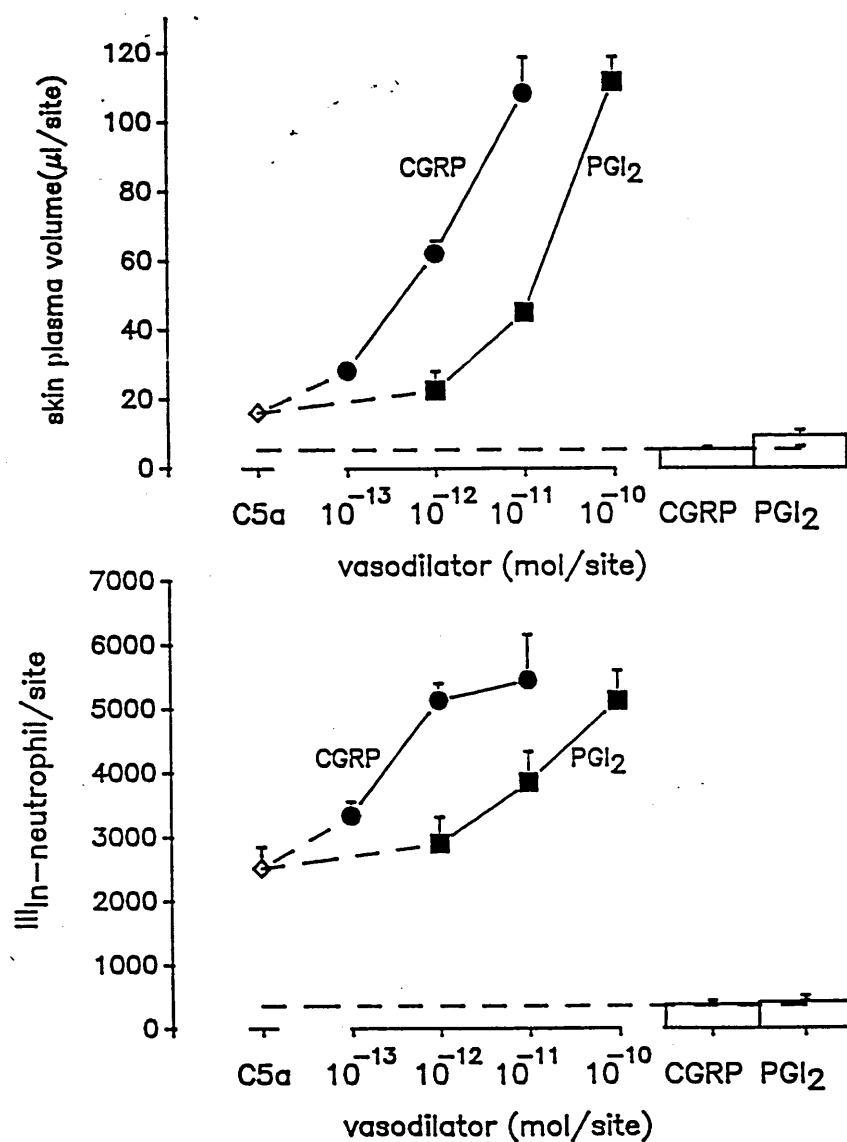
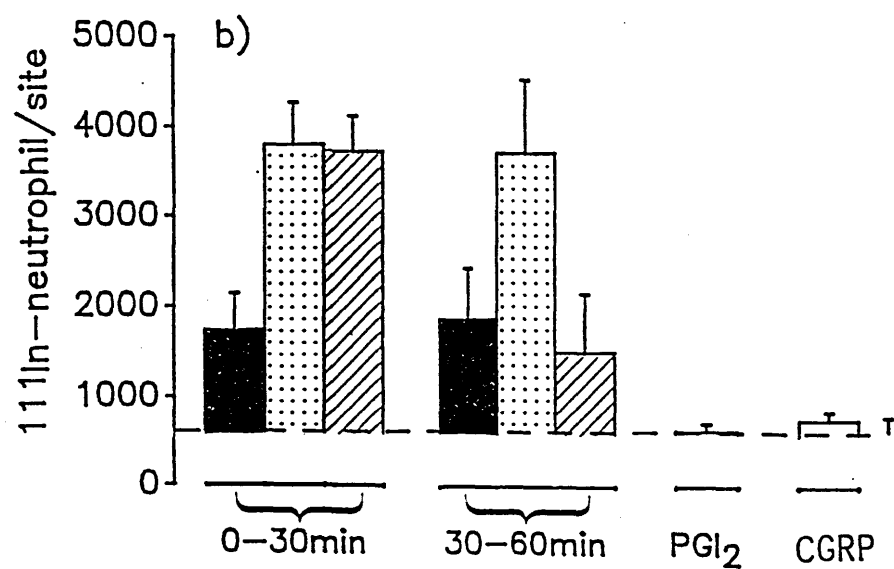
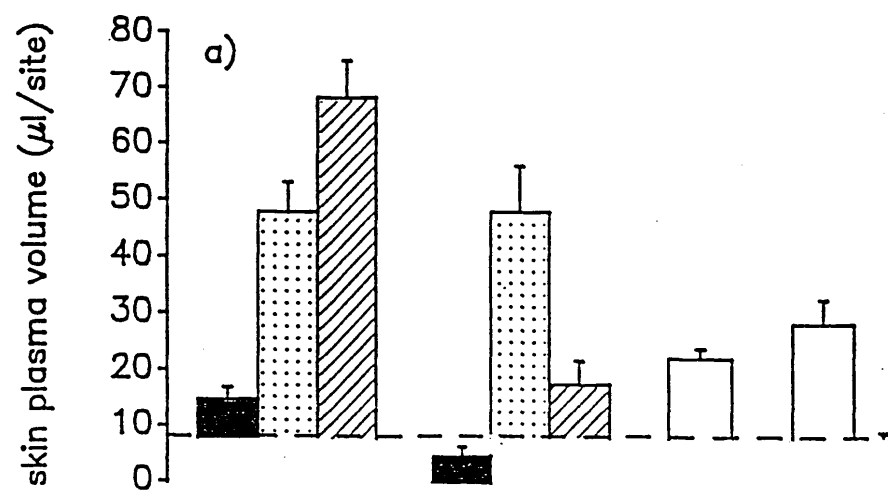


Figure 5.6

The effect of CGRP and PGI₂ on a) oedema formation (μl/site) and b) neutrophil accumulation (¹¹¹In-neutrophil/site) on responses induced by C5a des Arg. Responses in the rabbit skin were measured over 30min. The symbols represent responses to C5a des Arg (◊), C5a des Arg + CGRP (●) and C5a des Arg + PGI₂ (■). The dashed line indicates responses at saline-injected sites. Results are expressed as mean ± SEM, n=6 replicate sites.

Figure 5.7

The effect of CGRP (10^{-11} mol/site) and PGI₂ (3×10^{-10} mol/site) on a) oedema formation (μ l/site) and b) neutrophil accumulation (111 In-neutrophil/site) induced by C5a des Arg (3×10^{-11} mol/site) over 0-30 min and 30-60 min. Results for 30-60 min were determined by subtraction. Responses to C5a des Arg are shown by solid columns, C5a des Arg + CGRP stippled columns, and C5a des Arg + PGI₂ hatched columns. Responses to CGRP and PGI₂ were measured for 0-60 min. The dashed line indicates responses at saline-injected sites. Results are from 1 experiment expressed as mean $\pm$ SEM, n=6 replicate sites. For group data (n=4 rabbits) CGRP significantly ($p < 0.05$) potentiated all responses over 0-30 and 30-60 min. However PGI₂ only significantly ($p < 0.05$) potentiated responses measured over 0-30 min.



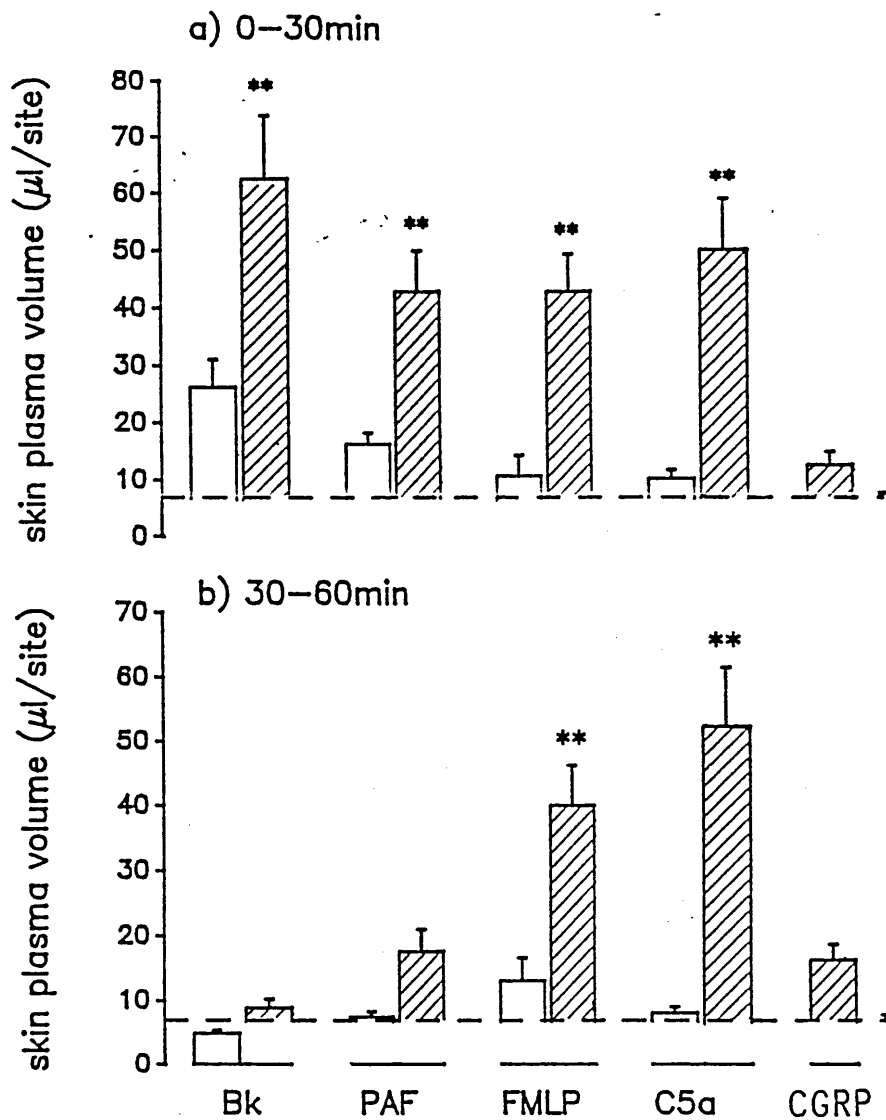


Figure 5.8

Potentiation by CGRP of oedema induced by mediators of increased microvascular permeability. Oedema induced by mediators (bradykinin; 10^{-10} mol/site, PAF; 10^{-9} mol/site, C5a des Arg; 3×10^{-11} mol/site and FMLP; 5×10^{-11} mol/site) alone (open columns) was compared to oedema induced by the mediators in the presence of CGRP (10^{-11} mol/site, hatched columns) over a) 0-30 min and b) 30-60 min. In these experiments oedema formation was measured directly over the 0-30 min and 30-60 min periods after i.d. test agents. The dashed line indicates responses at saline-injected sites. Results are expressed as mean $\pm$ SEM, $n=6$ rabbits. Significant differences between oedema induced by the mediator of increased microvascular permeability alone and in the presence of CGRP are indicated by ** $p < 0.01$ (two way ANOVA).

Chapter 6 The effect of CGRP on responses induced by IL-1

6.1 Introduction

Neutrophil accumulation induced by chemoattractants (C5a des Arg, LTB₄ and FMLP) is associated with plasma protein leakage (Wedmore & Williams, 1981). These inflammatory responses were synergistically potentiated by various vasodilators. There have been many reports describing neutrophil accumulation induced by IL-1 in the rabbit skin and synovial cavity. However, this cell accumulation was not accompanied by marked oedema formation even in the presence of the prostanoid vasodilator, PGE₂ (Rampart & Williams, 1988; Pettipher *et al.*, 1986). This discrepancy between inflammatory responses induced by C5a des Arg and IL-1 in the rabbit skin was intriguing. Furthermore, oedema formation was observed in the rat skin and rabbit eye in response to IL-1 through the generation of histamine and PAF respectively.

In this study the possibility of a synergistic relationship between IL-1 and the long lasting vasodilator, CGRP, was investigated in rabbit skin.

6.2 Synergistic interaction between IL-1 and the neuropeptide CGRP

Many inflammatory responses induced by IL-1 have been shown to be time-dependent as its actions are dependent on de novo protein synthesis. Experiments were therefore designed so that IL-1 alpha (1.4×10^{-14} mol/site) was pre-injected at several time points (0-3h) before a second injection of either a vasodilator dose of CGRP (10^{-11} mol/site) or saline alone. IL-1 alpha induced ¹¹¹In-neutrophil accumulation that was time-dependent. Maximal cell accumulation was observed when IL-1 was pre-injected 1h before accumulation

of ^{111}In -neutrophils was measured; this response was significantly potentiated by a subsequent injection of CGRP given i.d. at the beginning of the measurement period (figure 6.1b). Although little oedema formation was apparent at sites which received IL-1 alpha or CGRP alone, highly significant oedema formation was measured at sites pre-treated with IL-1 alpha 1h before CGRP (figure 6.1a).

It has been widely reported that IL-1 can induce the generation of prostanoid vasodilators in vivo. Therefore experiments were designed to test whether IL-1 alone was able to increase local blood flow, in the rabbit skin. When IL-1 (1.4×10^{-14} mol/site) was i.d. pre-injected either 1 or 2h before saline, no increase in blood flow was observed over 15 min. (Results: IL-1 (-1h) $5.8 \pm 1.1\%$, IL-1 (-2h) $10.6 \pm 5.52\%$ increase in blood flow, $n=6$ replicate sites.) Therefore the generation of vasodilators by IL-1 alone was not detected in the rabbit skin. These results demonstrate that, in this model neutrophil accumulation induced by IL-1 was accompanied by oedema formation in the presence of the neuropeptide vasodilator CGRP.

In preliminary studies CGRP (10^{-11} mol/site) and the prostanoid vasodilators PGE_2 (3×10^{-10} mol/site) were found to potentiate equally oedema formation. Both vasodilators enhanced neutrophil-dependent oedema formation induced by C5a des Arg (figure 6.2). However, the ability of these doses of vasodilators to potentiate oedema formation induced by IL-1 alpha differed. The neuropeptide CGRP significantly potentiated oedema formation induced by IL-1 alpha. In contrast to its effect on responses induced by C5a des Arg, PGE_2 failed to enhance markedly plasma extravasation induced by IL-1 alpha. The weak ability of PGE_2 to potentiate oedema formation induced by IL-1 alpha has been previously reported (Watson *et al.*, 1989; Pettipher *et al.*, 1986). Experiments

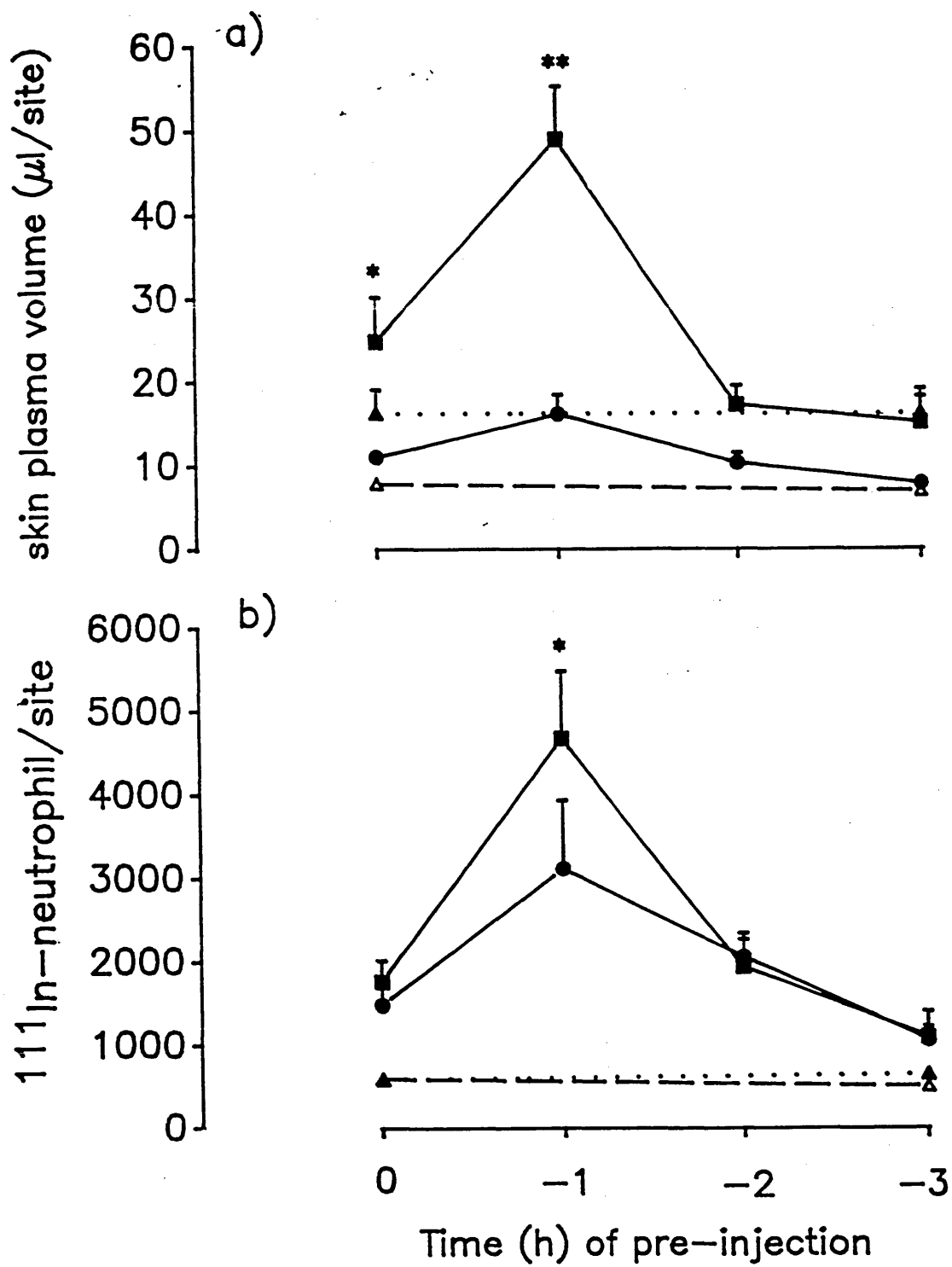
were then designed to determine why there was such a striking difference between the abilities of CGRP and PGE₂ to enhance oedema formation induced by IL-1.

Low doses of CGRP (10⁻¹¹mol/site) stimulated vasodilator responses in rabbit skin of a much longer duration than vasodilator responses to PGE₂ (3 x 10⁻¹⁰mol/site; figure 5.4 ; Brain and Williams, 1985). When the duration of vasodilator activity of different doses of PGE₂ was investigated, it was found that a higher dose of PGE₂ (3 x 10⁻⁹mol/site) had protracted vasodilator activity in rabbit skin. The time taken for the higher dose of PGE₂ to lose half its ability (t¹/₂) to potentiate oedema formation induced by bradykinin was 44.9 ± 4.0 min, similar to results obtained for CGRP (10⁻¹¹mol/site; figure 5.4, t¹/₂ 39.3 ± 7.7 min). In addition, the highest dose of PGE₂ (3x10⁻⁹mol/site) was found to enhance oedema formation induced by IL-1 alpha (figure 6.3b). Therefore, in the presence of a sustained increase in microvascular blood flow, induced by two chemically distinct vasodilators, the potent ability of IL-1 alpha to increase microvascular permeability was observed.

Capsaicin induces the release of a vasodilator activity. It was decided therefore to test whether endogenous release of putative rabbit CGRP could enhance oedema formation induced by IL-1. IL-1-induced oedema formation was significantly enhanced by CGRP (10⁻¹¹mol/site) however, only an additive effect was observed when IL-1 was injected 1h before capsaicin (10⁻⁶mol/site; figure 6.4).

Figure 6.1

Effect of IL-1 alpha on oedema formation and ^{111}In -neutrophil accumulation in rabbit skin. IL-1 alpha (1.4×10^{-14} mol/site) or saline were injected intradermally 3h, 2h, 1h and 0h before a second injection of either CGRP or saline. Oedema formation (fig 1a) or neutrophil accumulation (fig 1b) were measured by the iv injection of ^{125}I -albumin or ^{111}In -neutrophils for a 1h period after the second injection. The symbols represent responses to: IL-1 alpha ($\bullet$), IL-1 alpha + CGRP ($\blacksquare$), CGRP ($\blacktriangle$), saline ($\triangle$). Results are expressed as mean $\pm$ sem n=4 rabbits. Significant differences in a) oedema responses to IL-1 alpha + CGRP compared with CGRP and b) neutrophil accumulation induced by IL-1 alpha + CGRP compared to IL-1 alpha are indicated by ** $p < 0.01$, * $p < 0.05$ (two way ANOVA).



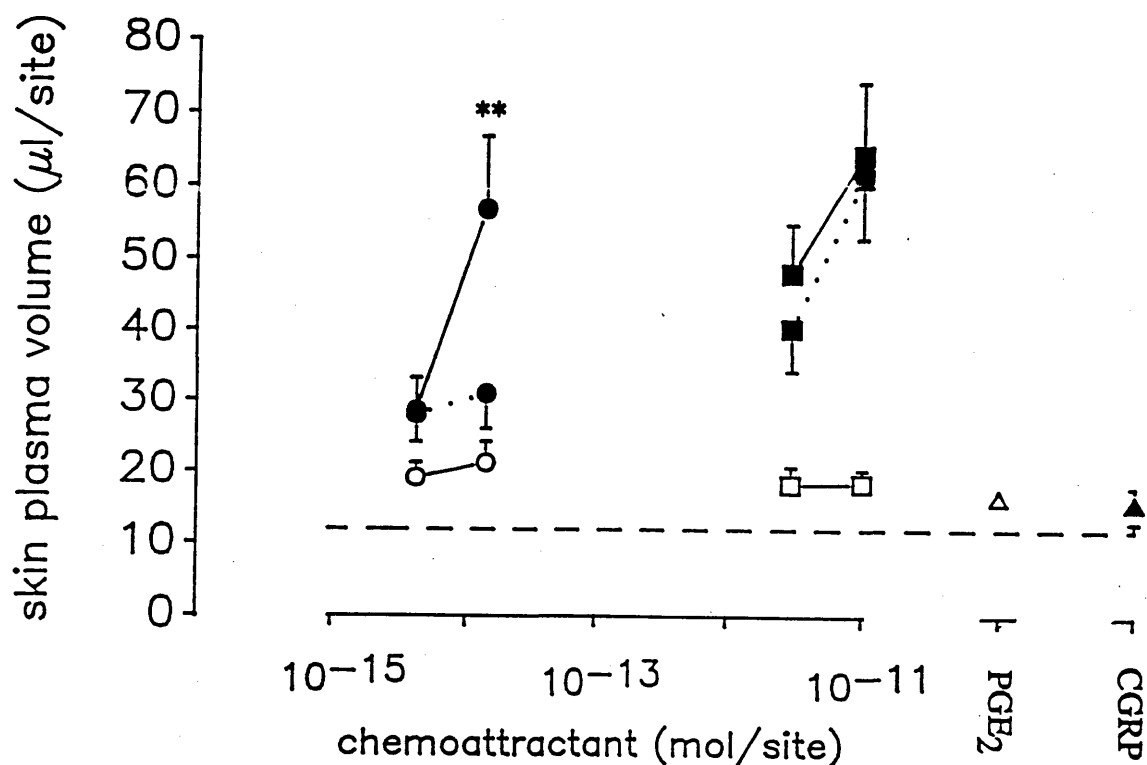


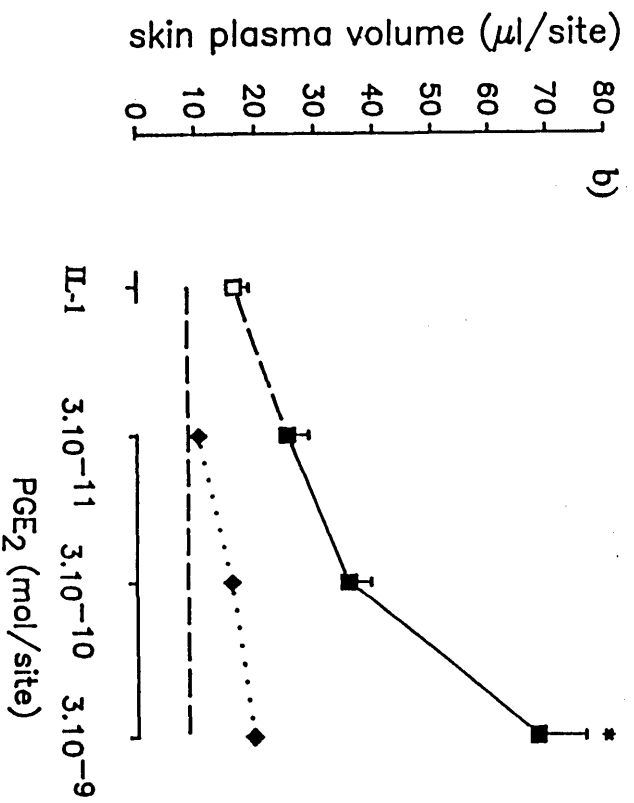
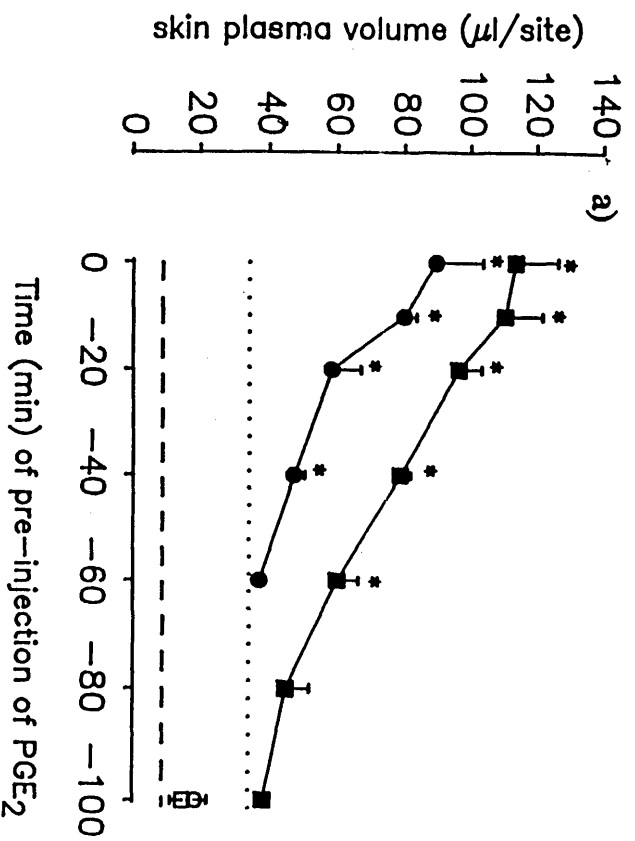
Figure 6.2

Effect of the vasodilators CGRP and PGE_2 on oedema induced by IL-1. IL-1 was injected 1h before C5a des Arg, CGRP (10^{-11} mol/site) and PGE_2 (3×10^{-10} mol/site). Oedema was then measured for a further 1h period. Responses at saline-injected sites are represented by a dashed line. The symbols represent responses to: IL-1 (○), IL-1 + CGRP (●—●), IL-1 + PGE_2 (●--●), C5a (), C5a + CGRP (■—■), C5a + PGE_2 (■--■), CGRP (▲), PGE_2 (Δ). Results are expressed as mean $\pm$ SEM, $n=6$ rabbits. Significant differences between responses induced by IL-1 + CGRP compared with IL-1 are indicated by ** $p < 0.01$ (two way ANOVA).

Figure 6.3

a) The duration of vasodilator activity of PGE_2 as a potentiator of bradykinin-induced oedema formation. PGE_2 at 3×10^{-10} mol/site (■) and 3×10^{-9} mol/site (●), was injected into each rabbit 100, 80, 60, 40, 20, and 0 min before a superimposed injection of bradykinin (10^{-10} mol/site). Oedema formation ($\mu\text{l}/\text{site}$) was measured for 30 mins after bradykinin injection. Other symbols represent: PGE_2 (3×10^{-10} mol/site) and PGE_2 (3×10^{-9} mol/site) injected alone. The dotted line indicates the response to bradykinin alone. The dashed line represents the response to saline. Results are expressed as mean $\pm$ SEM $n=4$ rabbits. Significant differences between oedema responses induced by bradykinin alone and in the presence of PGE_2 are indicated by * $p < 0.05$ (two way ANOVA).

b) The effect of increasing doses of PGE_2 on oedema induced by IL-1 (1.4×10^{-14} mol/site). IL-1 was i.d. injected 1h before PGE_2 . The symbols represent responses to: IL-1 (□), IL-1 + PGE_2 (■), PGE_2 (◆). Saline injected sites are represented by a dashed line. Results are expressed as mean $\pm$ SEM $n=6$ rabbits. Significant differences between IL-1 + PGE_2 and IL-1 alone are indicated by * $p < 0.05$ (two way ANOVA).



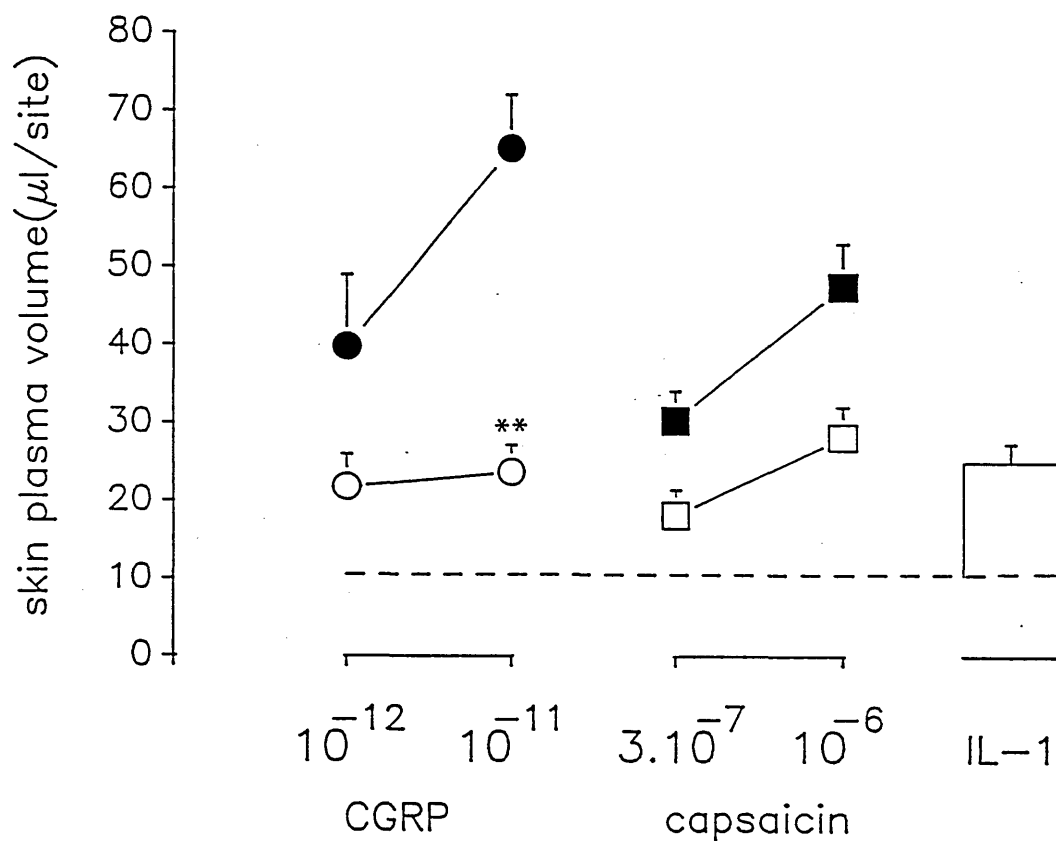


Figure 6.4

The effect of capsaicin on oedema formation induced by IL-1. Oedema formation ($\mu\text{l}/\text{site}$) induced by IL-1 ($1.4 \times 10^{-14} \text{ mol}/\text{site}$) was measured in the presence of CGRP (●) or capsaicin (■). This response was compared to responses induced by IL-1 alone (open column). Responses at saline-injected sites are indicated by a dashed line. Results are expressed as mean $\pm$ SEM, $n=5-7$ rabbits. Asterisks (**) represent a significant difference ($p < 0.01$, two way ANOVA) between IL-1 alone and in the presence of CGRP ($10^{-11} \text{ mol}/\text{site}$).

6.3 The effect of actinomycin D and anti-neutrophil antiserum on oedema formation induced by IL-1 + CGRP

Actinomycin D was used to test whether oedema formation induced by IL-1 + CGRP was dependent on de novo protein synthesis. In the presence of local actinomycin D (5×10^{-9} mol/site), administered at the same time as IL-1, oedema formation induced by IL-1 alpha was abolished (figure 6.5).

IL-1 induced neutrophil accumulation in the rabbit skin (figure 6.1b), it was decided therefore to examine whether plasma protein leakage induced by IL-1 was neutrophil-dependent. Sheep anti-rabbit neutrophil antiserum (ANS) was used to deplete circulating neutrophils. In preliminary studies ANS was i.v. administered at 0 and 60 min. It was observed that circulating neutrophils were initially greatly diminished (figure 6.5). However, the neutrophil counts started to recover at around 60 min after the first i.v. injection. Total white blood cell counts were slightly affected after the first injection of ANS but by 60 min total circulating leukocytes had returned to normal. To allow maximal depletion of neutrophils in further experiments ANS or saline was administered at 0, 45 and 90 min. In these experiments, after i.v. ANS neutrophils were depleted by $92.8 \pm 9\%$ whereas total leukocytes were only inhibited by $28 \pm 7.8\%$ (Results: before i.v. ANS 1.62 ± 0.53 , 120 min after i.v. ANS 0.13 ± 0.06 neutrophil/ml $\times 10^6$). Surprisingly, the control saline-treated rabbits were found to be neutrophilic 120 min after the i.d. injection of IL-1 (1.4×10^{-14} mol/site into 30 sites). These rabbits had approximately 5 times their basal level of neutrophils (Results: before i.v. saline 0.77 ± 0.36 , 120 min after i.v. saline 2.97 ± 0.9 neutrophil/ml $\times 10^6$). IL-1 can induce neutrophilia (Cybulsky *et al.*, 1986), therefore, it is possible that i.d. IL-1 releases neutrophils into the general circulation.

When oedema responses were determined responses induced by C5a des Arg + CGRP and IL-1 alpha + CGRP were significantly inhibited in ANS-treated rabbits. However, responses induced by bradykinin which increased microvascular permeability through a direct interaction with microvascular endothelial cells were not affected by ANS (figure 6.7).

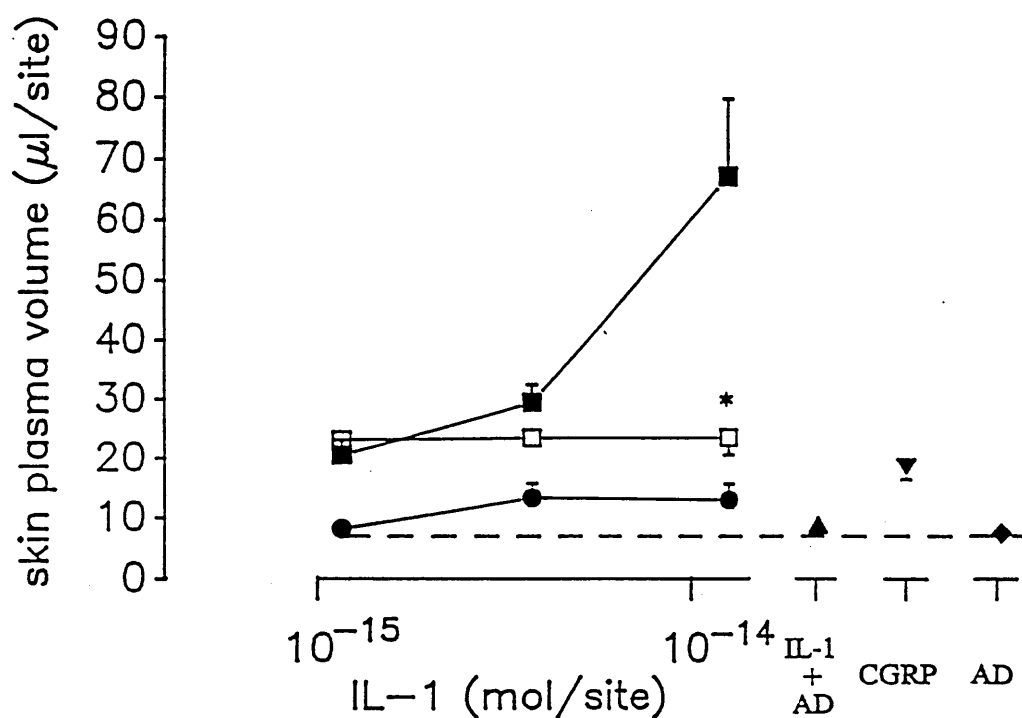


Figure 6.5

The effect of actinomycin D (AD; 5×10^{-9} mol/site) on oedema responses ($\mu\text{l}/\text{site}$) induced by IL-1 in the presence of CGRP (10^{-11} mol/site). The symbols represent responses to: IL-1 (●) IL-1 + CGRP (■), IL-1 + CGRP + AD (□), CGRP (▼), AD (◆) and IL-1 + Act D (▲). The response at saline-injected sites is represented by a dashed line. Results are expressed as mean $\pm$ SEM $n=4$ rabbits. Significant differences between IL-1 + CGRP compared with IL-1 + CGRP + actinomycin D are indicated by * $p < 0.05$ (two way ANOVA).

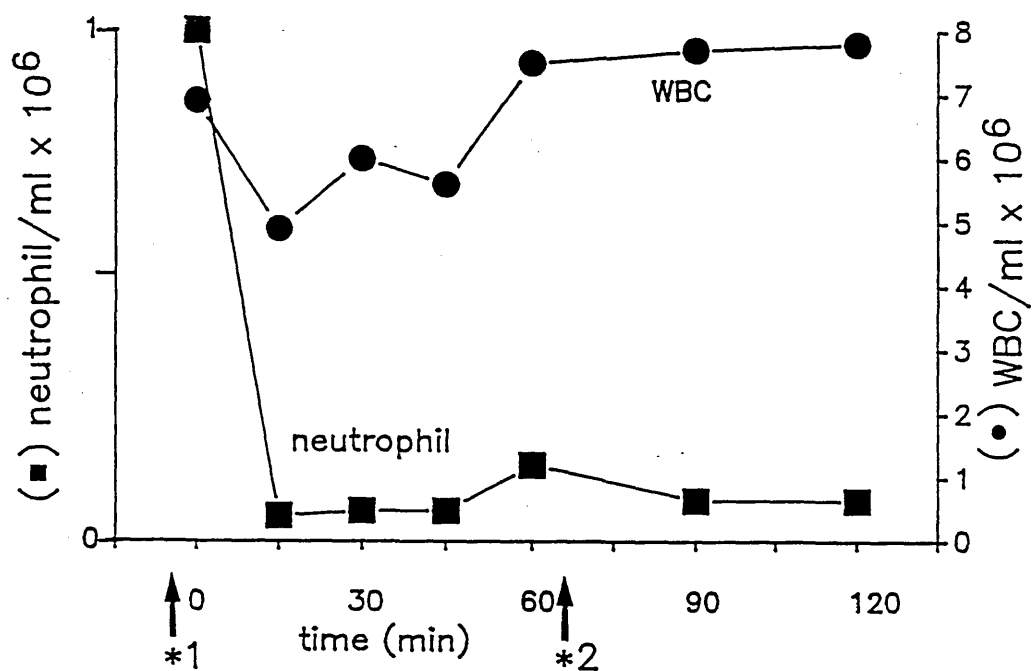
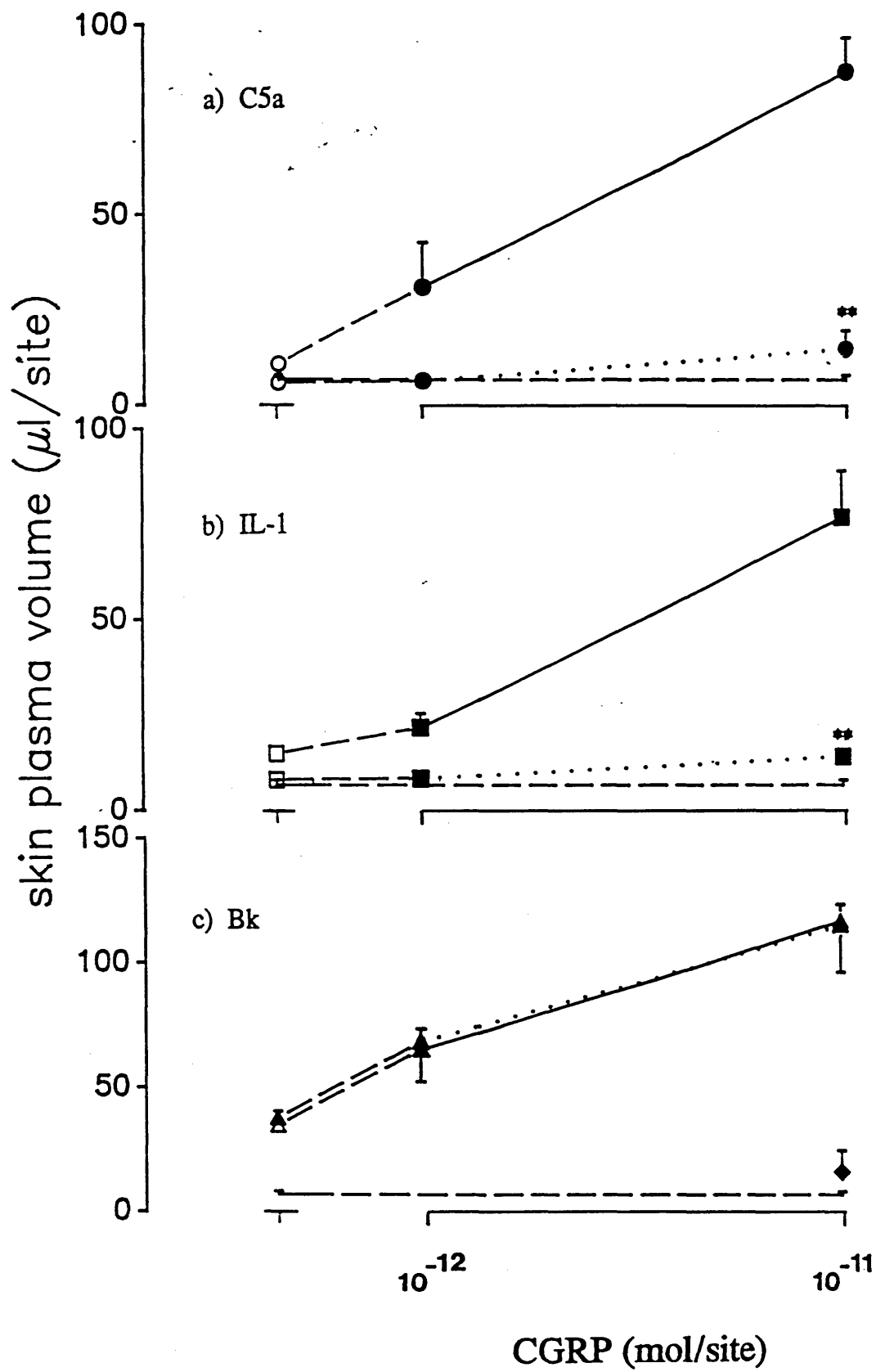


Figure 6.6

The effect of sheep anti-rabbit neutrophil antiserum (ANS) on circulating leukocyte counts in the rabbit. ANS was injected i.v. at 0 (*1) and 60min (*2). Total white blood cell counts (WBC; ●) and by differential, neutrophil counts (■) were determined at 0, 15, 30, 45, 60, 90 and 120 min. Results are expressed as cell number/ml of blood $\times 10^6$ for 1 rabbit.

Figure 6.7

The effect of neutrophil depletion on oedema formation ($\mu\text{l}/\text{site}$) induced by a) C5a des Arg ($3 \times 10^{-11}\text{mol}/\text{site}$), b) IL-1 alpha ($1.4 \times 10^{-14}\text{mol}/\text{site}$) and c) bradykinin (Bk; $10^{-10}\text{mol}/\text{site}$). Each agent was i.d. injected with CGRP and oedema formation measured over 1h. Solid lines show the response in saline-treated animals whilst the response in rabbits treated with a sheep anti-rabbit neutrophil antiserum (i.v.) is shown by the dashed line. The symbols represent: C5a des Arg ($\odot$), C5a des Arg + CGRP ($\bullet$), IL-1 ($\square$), IL-1 + CGRP ($\blacksquare$), bradykinin ($\triangle$), bradykinin + CGRP ($\blacktriangle$) and CGRP ($\blacklozenge$). Saline injected sites are represented by a dashed line. Significant differences between saline treated and neutrophil-depleted rabbits are indicated by ** $p < 0.01$ (two way ANOVA). Results are expressed as mean $\pm$ sem, $n=6$ paired rabbits.



6.4 The effect of a PAF antagonist WEB 2086 (10^{-7} mol/site) and a histamine H_1 antagonist on oedema induced by IL-1 + CGRP

The possibility that oedema induced by IL-1 was due to the release of endogenous PAF or histamine was examined using specific antagonists injected i.d. into rabbit skin. The PAF antagonist, WEB 2086, and the histamine (H_1) antagonist were co-administered with the vasodilator or saline. WEB 2086 (10^{-7} mol/site) inhibited oedema induced by PAF in rabbit skin as previously reported (Hellewell & Williams, 1989), but had no effect on oedema induced by IL-1 alpha and beta, in the presence of CGRP (table 6.1). The H_1 histamine antagonist chlorpheniramine (3×10^{-9} mol/site) abolished histamine-induced oedema formation in rabbit skin, but had no effect on responses induced by IL-1 alpha.

Table 6.1

The effect of a) a PAF antagonist and b) an H₁ antagonist on oedema induced by IL-1 + CGRP in rabbit skin. The antagonists were co-injected with CGRP. Doses of mediators are expressed as (mol/site). Results are expressed as mean $\pm$ SEM n=1 or 4 rabbits as indicated (ND = not determined). Significant differences between responses with and without antagonists are indicated by ** P<0.01 (two way ANOVA).

TEST AGENTS	OEDEMA(μ l/site)		n
a) PAF antagonist	+WEB2086 (10^{-7})		
IL-1 alpha + CGRP	55.1 $\pm$ 4.9	60.5 $\pm$ 4.8	4
PAF + CGRP	48.6 $\pm$ 8.5	21.6 $\pm$ 0.2	4**
CGRP	20.0 $\pm$ 1.3	ND	4
IL-1 beta + CGRP	74.9 $\pm$ 5.6	69.3 $\pm$ 3.9	1
PAF + CGRP	66.1 $\pm$ 6.2	35.0 $\pm$ 4.2	1
CGRP	34.7 $\pm$ 5.4	ND	1
b) H ₁ antagonist	Chlorpheniramine (10^{-9})		
IL-1 alpha + CGRP	58.5 $\pm$ 5.2	57.8 $\pm$ 5.7	4
HA + CGRP	53.0 $\pm$ 7.0	26.5 $\pm$ 3.4	4**
CGRP	22.3 $\pm$ 2.0	ND	4

7.1 Capsaicin

Capsaicin induced an increase in local blood flow and as a consequence of this action it potentiated oedema formation and neutrophil accumulation induced by inflammatory mediators. The threshold for vasodilator activity in rabbit skin was below 10^{-8} mol/site. The vasodilator response induced by capsaicin appeared to be biphasic (figure 3.2). The initial phase was fast in onset but the potentiating ability of capsaicin had declined 30 minutes after injection. A second phase, longer but less intense than the first, was evident at around 60 minutes after injection of the radioisotopes. These observations could be explained by the release of neuropeptides from different stores in the sensory terminals. Hakanson et al (1987) described the release of CGRP and tachykinins from sensory nerves in the rabbit iris. In their model capsaicin evoked a long-lasting but biphasic contraction. Their observations were consistent with an initial release from a rapid-release pool in addition to release from a slow-release pool of neuropeptides over a longer period of time. They also demonstrated that the rapid-release pool was refilled from the slow-release pool. In our rabbit model, the time difference between the first and second peaks of vasodilator activity could reflect the time needed to replenish a rapid-release pool from a slow-release pool. In addition to its ability to release neuropeptides from sensory terminals, capsaicin has also been shown to inhibit the axonal transport of neuropeptides from the cell bodies to the sensory terminals. This process could result in not only the total depletion of stored neuropeptides but also a blockade of the neurons ability to replenish stocks.

Recently a lot of attention has been focussed on nerve growth factor (NGF). This trophic factor promotes anterograde neuropeptide transport in sensory fibres at 1mm/hour (Lindsay *et al.*, 1989; Kashihara *et al.*, 1989). It has been reported that NGF antagonises the neurotoxic actions of capsaicin on sensory neurons (Taylor *et al.*, 1985). However, capsaicin has been shown to inhibit the retrograde transport of NGF (Miller *et al.*, 1982) therefore removing protection from the neurons.

Results from this thesis suggest that capsaicin responses, especially at higher doses ($> 10^{-6}$ mol/site), are prolonged. One could interpret from these findings that adequate supplies of neuropeptides (eg. CGRP) are maintained and not easily depleted. It is probable that a balance exists between endogenous NGF and exogenous capsaicin in the rabbit skin models. The balance probably depends partly on the length and intensity of the capsaicin stimulus. In addition there could well be a difference between responses induced in healthy neurons and those that occurred in diseased neurons or at least neurons that lacked certain essential factors (eg. NGF). It appears therefore, that at all doses of capsaicin a vasodilator response occurred and in our experiments neuropeptides (particularly CGRP) from whatever source were not limited to any great extent, over the time scale investigated in this thesis.

Further experiments were designed to identify the vasodilator activity released by capsaicin in rabbit skin. Juan *et al* (1980) reported that capsaicin stimulated prostaglandin biosynthesis from extraneuronal tissue. Furthermore, PGE₂ released by intraarterial injection of capsaicin into the isolated perfused rabbit ear, caused a dose-dependent reflex fall in blood pressure by stimulation of chemosensitive nociceptors. PGE₂ and PGI₂ are vasodilators in rabbit skin (Williams, 1979). However, indomethacin, a cyclooxygenase inhibitor, did not

affect the increase in local blood flow or potentiation of oedema formation induced by capsaicin. Therefore, in my hands, capsaicin induced vasodilatation that was not dependent on prostaglandin release.

Human, rat and chicken sequences for CGRP have been reported (figure 1.1). It is very interesting that they are so highly conserved. In this thesis anti-human CGRP Fab inhibited a vasodilator activity present in the homogenate of rabbit spinal cord (figure 4.12). In addition, an anti-CGRP affinity column bound this vasodilator activity (figure 4.13). In light of the very specific nature of anti-CGRP antiserum, raised and purified in our laboratory, and the homology between known CGRP structures, it is highly probable that a rabbit form of CGRP was released by capsaicin from rabbit skin. In the future it is planned that the putative rabbit CGRP will be extracted, using an anti-CGRP affinity column, eluted and purified. This will allow the full amino acid sequence for rabbit CGRP to be elucidated. The advantages of knowing this structure are two-fold.

- 1) The homology between human and rabbit CGRP could be compared.
- 2) Anti-rabbit CGRP antiserum could be raised which may prove to be a more efficient tool in investigating many inflammatory reactions of known or unknown neurogenic origin in rabbit skin (eg. delayed-type hypersensitivity and passive cutaneous anaphylaxis reactions).

Neuropeptides (CGRP and the tachykinins) have been localised in human skin (Gibbins *et al.*, 1987; Wallengren *et al.*, 1987b; Dalsgaard *et al.*, 1989). Immunohistochemical techniques have located CGRP-like immunoreactivity (CGRP-LI) in sensory nerves that are in close association with arterioles in the cutaneous microvasculature. Use of a specific anti-CGRP antiserum has

indicated a role for rabbit CGRP in capsaicin-induced responses in rabbit skin. Anti-CGRP Fab fragments significantly and dose-dependently inhibited capsaicin-induced increases in blood flow (Figure 4.9) and potentiation of oedema formation (figure 4.7). In these studies I found that there was a difference in the ability of intact anti-CGRP IgG and the digested anti-CGRP Fab fragments to neutralise capsaicin-induced responses; anti-CGRP IgG was much less effective (figure 4.7). However, the anti-CGRP IgG and Fab were equally able to neutralise responses induced by exogenous human CGRP. It has been reported that sensory nerve terminals are in close apposition to blood vessels (Gibbins *et al.*, 1987). Therefore released neuropeptides have a very short distance to diffuse to the target cell and effect a vasodilatory response. This close spatial arrangement could prove prohibitive to the entry of large molecules. Fab fragments are approximately 4 times smaller than IgG (40-45kDa and 150-160kDa respectively). Therefore the size of the anti-CGRP IgG could hinder its ability to neutralise endogenous rabbit CGRP, whereas anti-CGRP Fab has a potentially greater chance of competing in the synaptic cleft. Louis *et al* (1989) reported that anti-CGRP IgG, given systemically, inhibited the neurogenic responses mediated by topical mustard oil in rats. However, in my hands anti-CGRP IgG, given systemically at an equivalent concentration, had no effect on oedema responses potentiated by CGRP or capsaicin in rabbit skin. This discrepancy cannot easily be explained, but it is possible that differences in species or antisera could be responsible for varying observations.

Anti-CGRP Fab significantly inhibited capsaicin-induced increase in blood flow but this inhibition was only partial; approximately 50% of the response remained (figure 4.9). There are many possible theories to explain the residual component.

- 1) The size of the anti-CGRP Fab molecule, although smaller than the IgG, could still hinder the ability of this antiserum to enter the synaptic cleft and neutralise endogenous rabbit CGRP.
- 2) Anti-human CGRP antibodies could have a lower affinity for rabbit CGRP than human CGRP. Although this option remains a possibility, rabbit spinal cord extract (20mg/site) and exogenous human CGRP (10^{-11} mol/site) induced equal potentiation of oedema formation and were both completely inhibited by anti-CGRP IgG (figure 4.12).
- 3) The residual increase in blood flow could be induced by another vasodilator (known or novel). A list of putative candidates for this remaining activity must include vasoactive intestinal peptide (VIP).

Anand et al (1983) reported that topical capsaicin pretreatment inhibited axon reflex vasodilatation induced by VIP in human skin. Recently more direct evidence has been presented by Maggi et al (1990). They demonstrated that the inhibitory motor effect of capsaicin on muscle strips from human intestine was blocked by an anti-VIP antiserum. They postulated that VIP is probably localised in sensory nerves of the gut.

In rabbit skin VIP (10^{-10} mol/site) potentiated oedema formation (figure 4.14; Williams, 1982). Therefore VIP is certainly capable of mimicking neurogenic vasodilatation. Experimental tools that could be employed to confirm or eliminate a role for VIP in capsaicin-induced vasodilatation include; a VIP radioimmunoassay, anti-VIP neutralising antiserum and novel VIP antagonists.

At higher doses ($>10^{-6}$ mol/site) capsaicin not only potentiated oedema formation but induced oedema alone (figure 3.4). Presumably in these experiments capsaicin released a mediator that was capable of increasing microvascular permeability. There are two classes of mediators of increased microvascular permeability, direct-acting and neutrophil dependent (Wedmore & Williams, 1981). Capsaicin ($>10^{-6}$ mol/site) did not induce neutrophil accumulation in rabbit skin but as a consequence of its vasodilator activity, capsaicin potentiated neutrophil accumulation induced by other chemotactic mediators (eg. C5a des Arg). Therefore it is highly unlikely that the mediator of increased microvascular permeability released by capsaicin is neutrophil-dependent. However, specific antagonists of known direct-acting mediators, platelet-activating factor and histamine also had no effect on oedema formation induced by capsaicin (table 3.2).

In this study we were unable to identify the mediator of increased microvascular permeability released by capsaicin, but the profile of this inflammatory activity was examined. When oedema induced by capsaicin was measured over 120min an enhanced oedema response, when compared to the 30min oedema response, was measured. This demonstrates that the prolonged vasodilatation induced by this neurotoxin was matched by the release of a mediator of increased microvascular permeability that has either a very long duration of action in rabbit skin or is continuously released. Interestingly, the amount of vasodilator released by capsaicin (3×10^{-6} mol/site) was adequate to enhance oedema formation induced by capsaicin to a maximal level as a superimposed injection of CGRP (10^{-11} mol/site) had no effect.

7.1.1 Sensory nerve blockade

Significant progress has been made in recent years in identifying neuropeptides released by capsaicin and other mediators that can activate sensory fibres (eg. bradykinin). However, blocking the initial activation of sensory fibres could be of greater therapeutic benefit than using neuropeptide antagonists which are not yet specific or widely available. This approach could not only inhibit the release of neuropeptides but, in addition block orthodromic stimulation which would normally result in the perception of tactile sensations and pain.

Local anaesthetics inhibit impulse propagation in nerve fibres. They block the axon reflex response and inhibit neurogenic flare and plasma extravasation in skin (Jancso *et al.*, 1967; Foreman *et al.*, 1983a). However, lignocaine failed to inhibit inflammatory responses induced by capsaicin in rabbit skin (figure 3.6). Therefore in rabbit skin, it appears that capsaicin directly activated sensory C-fibres resulting in neuropeptide release that was not dependent on the conduction of action potentials.

The search for an agent that would block sensory neuropeptide release led to a cationic dye, ruthenium red. It is shown here that ruthenium red (10^{-8} mol/site) significantly inhibited oedema formation and increased blood flow induced by capsaicin in vivo in rabbit skin (figures 3.8 and 3.10). Responses induced by CGRP and other mediators of increased microvascular permeability however, were not affected by this cationic dye.

In vitro studies have previously demonstrated that ruthenium red inhibits transmembrane Ca^{2+} fluxes in nerves (Tapia *et al.*, 1985; Marsh *et al.*, 1987). In view of the importance of calcium entry for neuropeptide release (Gamse *et al.*, 1979), the effect of ruthenium red on capsaicin-evoked neuropeptide release was investigated; responses from a variety of isolated tissues were selectively inhibited by ruthenium red (Takaki *et al.*, 1989; Amann *et al.*, 1989b). It was therefore postulated that ruthenium red was interfering with Ca^{2+} entry either at the plasma membrane level or uptake into the mitochondria (Moore, 1971; Swanson *et al.*, 1974) and that this was the basis for its effects on capsaicin-mediated events.

Wood et al (1989) investigated the mechanism of action of ruthenium red further using in vitro patch clamping techniques. Measuring capsaicin-induced opening of selective ion channels in DRG cells, they demonstrated that excitation of peripheral nociceptors by capsaicin occurred in the absence of extracellular calcium. They also reported that the effect of ruthenium red on capsaicin-activated ion channel activity did not resemble ion-channel blockade. They concluded that ruthenium red may be acting in a manner more like a pharmacological antagonist, either interfering with the binding of capsaicin to the membrane site or preventing channel opening after capsaicin had bound. Recently in vivo evidence has been presented to suggest that ruthenium red not only affected calcium entry but that the site of action is more likely to be a capsaicin-activated cation channel. In this study by Amann et al (1989b) the inhibitory effect of ruthenium red against a capsaicin-induced depressor reflex in an isolated rabbit ear preparation with an intact neuronal connection was seen in a Ca^{2+} free perfusate. However, in these experiments neuropeptide release was completely abolished by the addition of EGTA (a Ca^{2+} chelator). The possibility

was therefore raised that ruthenium red interacted with more than one site on the sensory terminal (ie. Ca^{2+} channel and a specific binding site for capsaicin) and these sites may or may not be coupled.

My results support the interaction of ruthenium red with a specific binding site for capsaicin on sensory fibres as responses induced by bradykinin (an activator of sensory fibres) were not affected by this dye. Furthermore, Maggi et al (1988c) demonstrated that neuropeptide release evoked by antidromic electrical stimulation of sensory nerve fibres, was not inhibited by ruthenium red. In conclusion, if ruthenium red was acting solely to inhibit Ca^{2+} fluxes then a more universal inhibition of neuropeptide release would be expected. Evidence therefore supports the hypothesis that ruthenium red can affect Ca^{2+} fluxes but in addition can modulate neuropeptide release by a more specific mechanism of action.

Capsaicin-induced increase in blood flow was significantly but only partially inhibited by ruthenium red (figure 3.10). Neutralisation by anti-CGRP Fab also inhibited this response to the same degree (figure 4.9). Moreover, concomittant injection of ruthenium red and anti-CGRP Fab failed to inhibit the capsaicin-induced increase in blood flow further than either agent alone. The obvious conclusion from these findings is that ruthenium red and anti-CGRP Fab, by differing mechanisms, inhibit capsaicin-evoked vasodilatation mediated by the release of CGRP from capsaicin-sensitive neurons. The vasodilator responsible for the residual component may be a neuropeptide. As already discussed a possible candidate is the neuropeptide vasodilator VIP. However, an alternative possibility is that a novel neuropeptide vasodilator is released from capsaicin-sensitive neurons. Future experiments with ruthenium red will involve the identification of the vasodilator responsible for the residual response.

Resiniferatoxin (RTX), a non-tumour promoting phorbol ester with structural similarity to capsaicin (figure 1.2), was found to have some interesting biological properties. Extensive comparisons of the activities of RTX with those of capsaicin indicated that RTX acts as a superpotent capsaicin analogue (Szallasi & Blumberg, 1989). Both cause hypothermia, nociceptor stimulation, neurogenic inflammation, pain and subsequent desensitisation to these responses in rats (de Vries & Blumberg, 1989; Szallasi & Blumberg, 1989; Dray *et al.*, 1990b). For many of these responses RTX was 1,000-10,000 fold more potent than capsaicin. The connection between RTX and capsaicin was strengthened by an initial report that described the binding of RTX to a "capsaicin receptor" (Szallasi & Blumberg, 1989).

I investigated the inflammatory actions of RTX in rabbit skin and compared the findings with capsaicin-mediated responses. RTX did not markedly increase blood flow as measured using a ^{133}Xe clearance technique, even at a relatively high dose of 3×10^{-9} mol/site (figure 3.12). In fact when RTX was co-injected with CGRP (3×10^{-12} mol/site) a dose-dependent inhibition of increased blood flow was observed (figure 3.11). This result suggested that a vasoconstrictor was released by RTX in rabbit skin. However, it is likely that a vasodilator was also released as RTX alone did not induce a vasoconstrictor response in our model. In support of this possibility, Franco-Cereceda *et al* (1990) demonstrated that RTX evoked the release of CGRP-LI and induced bronchoconstriction in the guinea-pig lung. Furthermore, in their study both responses were inhibited by ruthenium red.

When the actions of RTX were investigated further it was found that ruthenium red did not attenuate but significantly enhanced RTX induced responses, revealing a ruthenium red resistant increase in blood flow (figure 3.12). It is possible that ruthenium red inhibited the release of a vasoconstrictor that normally would have masked the underlying vasodilator activity. I would therefore like to suggest that RTX induced the release of multiple vasoactive substances from capsaicin-sensitive neurons in rabbit skin.

Capsaicin-induced increase in blood flow was only partially inhibited by ruthenium red; a residual ruthenium red-resistant component was observed. One interesting possibility is that activation of a "capsaicin receptor" by RTX or capsaicin could result in the ruthenium red-resistant release of the same, as yet unidentified, neuropeptide vasodilator. In the future experiments are planned to investigate the mechanism of action of RTX further and to try and identify the vasoactive substances released by this toxin in rabbit skin.

7.1.2 Prejunctional modulation of capsaicin-sensitive neurons

Another route by which neuropeptide release can be modulated is via pre-synaptic receptors on the sensory nerve terminal. It is thought that receptors for opioids, somatostatin, NPY, galanin, GABA, histamine and corticotrophin-releasing factor exist on the sensory nerve terminal (Barnes *et al.*, 1990).

The inhibitory prejunctional effects of opioids on both central and peripheral sensory nerves have been widely studied. Both morphine and a number of synthetic enkephalin analogues inhibited neurogenic plasma extravasation in the rat paw; these responses were reversed by naloxone (Bartho

& Szolcsanyi, 1981). In addition an inhibitory effect of opioids was also observed on non-cholinergic bronchoconstriction induced by substance P in the guinea pig in vivo (Belvisi et al., 1988) and on nerve stimulation induced release of substance P in the cat knee (Yaksh, 1988).

Furthermore, opioids reduced the spontaneous firing of small afferents innervating inflamed synovial joints (Russell *et al.*, 1987). Human studies have also demonstrated that inhibitory opioid receptors are also present in the airways and morphine inhibited the secretory response normally evoked by capsaicin (Rogers & Barnes, 1989). Therefore there is a wealth of evidence supporting the modulatory role of opioids on sensory fibres.

Another neuropeptide, somatostatin that is often co-stored with CGRP in human skin has inhibitory actions on sensory neurons. Somatostatin inhibited the vasodilatation and plasma extravasation induced by saphenous nerve stimulation (Lembeck *et al.*, 1982). In light of the co-localisation of somatostatin and CGRP it is possible that somatostatin could modulate the release of the vasodilator neuropeptide. It would therefore be interesting to investigate the possible modulatory effect of somatostatin on CGRP- and capsaicin-evoked responses in rabbit skin.

Recently galanin-immunoreactive nerves have been identified and capsaicin pre-treatments resulted in a marked reduction of this neuropeptide in sensory fibres (Papka & Traurig, 1989). Guiliani et al (1989) reported that galanin inhibited the non-cholinergic contraction produced by field stimulation of the isolated guinea-pig bronchus. Their studies indicated a prejunctional neuromodulatory action of galanin on capsaicin-sensitive nerves.

In this thesis the prejunctional modulation of capsaicin-sensitive nerves was not investigated, however it would be very interesting to compare the effect of these agents with the actions of capsaicin and neuropeptide antagonists.

7.1.3 Endogenous activators of putative "capsaicin receptors"

Groups involved in sensory neuron pharmacology have been proposing the presence of a specific "capsaicin" receptor on sensory neurons for many years (Jancso *et al.*, 1967; Szolcsanyi & Bartho, 1978; Maggi & Meli, 1988). The use of ruthenium red would certainly support this proposal. However, there is a problem with the concept of a "capsaicin receptor" and that is capsaicin is not an endogenous substance in animals. Why would man (and other animal species) possess specific receptors for capsaicin? This question will probably be answered by the identification of an endogenous activator of "capsaicin receptors". The characteristics of the putative "capsaicin receptor" are described in the Introduction.

Ruthenium red has already proved to be a useful tool in probing capsaicin/RTX-induced responses but recently the effect of this dye on more physiological activators of sensory neurons has been investigated. Noxious heat (52°C) has been used to induce a depressor response and local release of neuropeptide in a perfused rabbit ear preparation with an intact neuronal connection (Amann *et al.*, 1990). Ruthenium red blocked capsaicin and heat-induced responses in this preparation and the two modalities would appear to activate sensory neurons in a similar way. Amann *et al* (1990) pointed out that their data did not exclude the possibility that heat indirectly activated nociceptors by releasing endogenous compounds. Dray *et al* (1990b) however reported that noxious (48°C) heat-induced depolarisation of a rat tail spinal cord preparation

was not affected by ruthenium red whereas capsaicin-evoked responses were completely inhibited by this dye. Therefore they would not agree with the conclusion that capsaicin and heat activate sensory fibres by a similar ruthenium red-sensitive mechanism. Bevan et al (1989) proposed that the endogenous activator of sensory neurons could be protons. They demonstrated that low pH solutions ($\text{pH} < 6.4$) caused an increase in cation permeability in DRG cells and furthermore in cell cultures, exposed to high doses of capsaicin (thus killing capsaicin-sensitive neurons), low pH solutions failed to activate cation channels. They concluded that neurons that responded to low pH solutions were restricted to the capsaicin-sensitive neurons. In light of these preliminary findings it would be very interesting to see whether ruthenium red interfered with proton-mediated responses induced in rabbit skin.

The actions of RTX on sensory neurons and its possible interaction with a "capsaicin receptor" have already been discussed. Other molecules which also bear structural similarity to capsaicin have been investigated to ascertain whether they activate sensory fibres in a similar manner. Piperine, a congener of capsaicin and extracted from black pepper mimicked the effects of capsaicin on the isolated guinea-pig ileum. In addition, cross-tachyphylaxis between piperine and capsaicin was observed (Takai *et al.*, 1990). The hypothesis that piperine interacted with a "capsaicin receptor" was further supported by the finding that ruthenium red inhibited piperine-mediated responses.

Patacchini et al (1990), compared a whole range of natural pungent substances on the rat bladder; capsaicin, piperine, mustard oil, eugeneol and curcumin. All agents tested dose-dependently contracted the rat bladder and produced desensitisation towards capsaicin. Development of cross-tachyphylaxis

among the natural pungent substances and inhibition of their respective responses by ruthenium red (Maggi *et al.*, 1989a), suggests a common site of action for these substances on sensory neurons.

In conclusion, all indirect evidence points towards the existence of a specific capsaicin receptor on sensory nerve fibres. Direct evidence to support this hypothesis has been elusive because of the relatively low affinity and the lipophilic nature of the available ligands. In response to these problems, James *et al* (1988) developed long lasting photoaffinity probes. These aryl azide probes are analogues of capsaicin and produced long lasting capsaicin-like Ca^{2+} uptake into cultured sensory neurons. The future use of these probes will aid the identification and characterisation of molecules from sensory neurons that bind these probes specifically. Moreover, it is hoped that these probes will allow the clarification of physiological functions for capsaicin binding sites.

7.2 CGRP

CGRP potentiated oedema formation induced by direct-acting and neutrophil-dependent mediators of increased microvascular permeability (figure 5.1a). This activity is not unique to CGRP; all mediators that induce vasodilatation potentiate oedema formation; see Introduction, "two mediator hypothesis". However, the threshold dose for vasodilator activity induced by CGRP was approximately 100fmol in rabbit skin, which is at least 10 fold lower than the threshold for vasodilator activity induced by the prostanoid vasodilator, PGI_2 (figure 5.6).

CGRP did not induce neutrophil accumulation in rabbit skin but as a consequence of its vasodilator activity, this neuropeptide dose-dependently enhanced neutrophil accumulation induced by chemoattractants (figure 5.1b and 5.2). Presumably an increase in blood flow allowed a greater number of neutrophils to pass through a given section of microvasculature, therefore a larger pool of potentially adherent neutrophils was available. In addition, a vasodilated bed would allow greater delivery of humoral chemoattractants to a site of inflammation. CGRP also enhanced plasma protein leakage associated with neutrophil accumulation (figure 5.6). However, it is interesting to note that a greater enhancement of C5a-induced neutrophil accumulation relative to oedema formation was observed when lower doses of CGRP were injected (figure 5.6). One explanation of this finding is that neutrophil accumulation is more sensitive than oedema formation to changes in intravascular hydrostatic pressure within the venule. In contrast, PGI₂ potentiated oedema formation and neutrophil accumulation in a similar manner. In light of these findings, it is possible that CGRP could promote neutrophil adherence induced by chemoattractants independently of its vasodilator activity. Enhancement of neutrophil accumulation by capsaicin also appeared more sensitive than plasma protein leakage to lower doses of capsaicin (figure 3.5).

Brain and Williams (1985) reported that the biological half life ($t^{1/2}$) of the ability of CGRP (10^{-11} mol/site) to potentiate oedema formation in rabbit skin was 39.3 ± 7.7 min. Identical studies in this thesis compare favourably with this result, (figure 5.4; $t^{1/2}$: CGRP 10^{-11} mol/site 40.1 ± 7.5 min) therefore confirming that CGRP is capable of inducing protracted vasodilatation especially when compared with PGI₂ (3×10^{-10} mol/site; $t^{1/2}$: 12 ± 3 min) and PGE₂

(3×10^{-10} mol/site; $t^{1/2}$: 18.6 ± 4.4 min, figure 6.3a). However, monitoring the ability of a vasodilator to potentiate oedema formation is an indirect way of determining the $t^{1/2}$ of that vasodilator.

Two methods available to measure more directly changes in blood flow are 133 xenon clearance and the use of a laser Doppler flow probe. The 133 xenon clearance method allows multiple mediators to be tested for their ability to increase blood flow in one skin bioassay. However, due to technical problems, this method has proved not to be ideal for examining the time course of a vasodilator response. In addition it is an invasive technique utilising radioisotopes. The laser Doppler flow probe uses non-invasive and non-isotopic technology and allows continuous monitoring of an increase in blood flow.

Using the laser probe CGRP induced a protracted vasodilator response when compared to PGI_2 (figure 5.5). However, the $t^{1/2}$ of vasodilator activities measured using the laser probe differed from previous findings (CGRP 10^{-11} mol/site 68 ± 7.4 min; PGI_2 3×10^{-10} mol/site 26.2 ± 4.2 min). These results demonstrate that CGRP increased blood flow for considerably longer than was estimated using its ability to potentiate oedema formation (figure 5.4). The obvious conclusion is that a small increase in blood flow is sufficient to be detected by the laser probe but not enough to markedly enhance venular plasma protein leakage. However, the laser probe measures blood flow at a more superficial level than the 133 xenon clearance technique and the differences in $t^{1/2}$ could reflect the measurement of blood flow in different cutaneous microvascular beds.

However, other possibilities exist to explain the apparent discrepancy between direct and indirect methods of measuring vasodilatation. Plasma protein extravasation leads to an oedematous response in a vasodilated bed: this response must be controlled. A reduction in the duration of potentiating activity induced by CGRP could be explained by an increase in the metabolism or clearance of this neuropeptide in oedematous surroundings. Brain and co-workers have investigated mechanisms by which CGRP is metabolised in vivo. It is stable to incubation in plasma and blood (Brain *et al.*, 1985). However, the protracted vasodilator response of CGRP was attenuated when substance P and CGRP were co-injected into human and rat skin. Further studies have shown that this breakdown of CGRP was effected by mast cell proteases such as tryptase and chymase which are released by substance P (Brain & Williams, 1988). The tachykinins were not active in rabbit skin (table 4.4) but a similar mechanism could be important for regulating the vasodilator activity of CGRP. However an increase in metabolism of CGRP was probably not responsible for the shorter duration of potentiating activity in rabbit skin because the duration of the chemically unrelated vasodilator PGI₂ was also reduced.

Another way to inhibit a vasodilator response is by the introduction of a vasoconstrictor. The endothelial product, endothelin is a potent vasoconstrictor (Yanagisawa *et al.*, 1988). Endothelin dose-dependently reduced oedema formation and neutrophil accumulation (figure 5.3) presumably by physiological antagonism. An alternative possibility to explain the difference between the potentiating ability of CGRP and its ability to increase blood flow is that the volume of extravascular fluid accumulating in response to a mediator of increased microvascular permeability could exert physical pressure on the microvasculature. This pressure could partially block blood flow ultimately leading to a reduction in the duration (and the maximal response) of an

inflammatory response. However Brain and Williams (1989) demonstrated that the laser Doppler flow meter is probably able to measure changes in blood flow at oedematous sites.

The results demonstrate that although CGRP potentiated oedema formation induced by direct acting mediators over short periods of time, a long lasting synergistic interaction exists between neutrophil-dependent mediators of increased microvascular permeability (C5a des Arg and FMLP) and CGRP in rabbit skin (figure 5.8). The shorter acting vasodilator PGI₂ did not cause a long lasting potentiation of oedema formation or neutrophil accumulation. This suggests that the protracted potentiating effect of CGRP is due to its sustained vasodilator activity in rabbit skin.

Our laboratory has previously shown, by measuring the time taken for each mediator to lose half its activity in rabbit skin ($t^{1/2}$), that C5a des Arg ($t^{1/2}$ 100 minutes) and FMLP ($t^{1/2}$ 50 minutes) are both long lasting mediators (Jose *et al.*, 1981; Wedmore & Williams, 1981). Histamine and bradykinin are known to be substantially shorter acting ($t^{1/2}$ 3-6 minutes; Wedmore and Williams, 1981) and their effects were not potentiated by CGRP over the 30-60 minutes period (figure 5.8). Therefore a sustained potentiation of oedema formation is only observed when vasodilators and mediators of increased microvascular permeability both possess long durations of action.

IL-1 is an important inflammatory mediator but unlike C5a des Arg, this cytokine is not directly chemotactic and stimulates neutrophil accumulation via a protein synthesis-dependent mechanism (Rampart & Williams, 1988). Studies on the effect of IL-1 as a mediator of oedema formation have elicited conflicting results. The injection of recombinant IL-1 into rabbit skin and synovial joints

caused pronounced neutrophil accumulation with little oedema formation (Pettipher *et al.*, 1986; Foster *et al.*, 1989; Watson *et al.*, 1989). This differed from studies in human skin (Dowd *et al.*, 1988) rabbit eye (Rubin & Rosenbaum, 1988) and rat skin (Martin *et al.*, 1988) where plasma protein leakage was observed. The combination of IL-1 and CGRP induced significant neutrophil accumulation and surprisingly oedema formation was also observed in rabbit skin (figure 6.1). These results revealed that the protracted cutaneous vasodilatation induced by CGRP was necessary in order to observe oedema formation induced by IL-1 in rabbit skin. In contrast, a dose of the vasodilator PGE₂ (10^{-10} mol/site) that maximally potentiated oedema induced by C5a des Arg failed to markedly enhance oedema formation induced by IL-1 (figure 6.2). However, a higher dose of PGE₂ (3×10^{-9} mol/site) which has a similar duration of vasodilator activity to CGRP (10^{-11} mol/site) produced a similar potentiation of oedema formation (figure 6.3). Thus it appears that when a microvascular bed is dilated by a long lasting vasodilator, oedema induced by IL-1 can be observed. Results from these studies also demonstrated that on a molar basis IL-1 was considerably more potent than the neutrophil-dependent inflammatory mediator C5a des Arg (figure 6.2).

Maximal plasma extravasation was observed over a 1 hour accumulation period when CGRP was injected 1 hour after intradermal IL-1. One explanation for the time-dependence of the oedema-inducing activity of IL-1 points to the need for de novo protein synthesis and expression of adhesive molecules on endothelial cells in culture (Bevilacqua *et al.*, 1985; Pober *et al.*, 1986; Dustin *et al.*, 1986; Bevilacqua *et al.*, 1989). This may occur on venular endothelial cells in vivo. In addition, IL-1 has been shown to stimulate the release of tumour necrosis factor (TNF) from monocytes and neutrophil chemotactic factor (IL-8) from endothelial cells in vitro (Philip & Epstein, 1986; Strieter *et al.*, 1988).

Recently Dr Paul Collins and co-workers from our laboratory have demonstrated the *in vivo* generation of IL-8 in the rabbit peritoneal cavity (Collins *et al.*, 1991). Therefore, these or other chemoattractant molecules may be acting as secondary mediators in the rabbit skin model. In support of this possibility it has recently been shown that both TNF and IL-8 induced neutrophil accumulation with accompanying oedema formation in rabbit skin (Foster *et al.*, 1989; Rampart *et al.*, 1989; Rampart *et al.*, 1989; Colditz *et al.*, 1989).

IL-1 has been reported to stimulate the release of the vasodilator PGE₂ from cultured endothelial cells, dermal fibroblasts, synovial cells and other cell types. Results from this thesis have demonstrated that vasodilatation is essential to observe oedema formation induced by IL-1. Therefore, the possibility that IL-1 induced the endogenous release of vasodilator prostaglandins in rabbit skin was examined. However, increased blood flow was not detected in response to IL-1 using a ¹³³xenon clearance method.

Unlike IL-1, capsaicin did induce the release of an endogenous vasodilator in rabbit skin; CGRP. In light of the marked oedema induced by IL-1 in the presence of CGRP it was decided to examine the effect of capsaicin on inflammatory responses induced by this cytokine. A potentiation of oedema induced by IL-1 was expected by capsaicin but only an additive response was observed (figure 6.4). It is possible that, in these experiments, the amount of CGRP released by capsaicin in rabbit skin was not sufficient to induce the profound vasodilatation needed to potentiate IL-1-induced oedema formation. However, the lack of synergistic interaction between IL-1 and capsaicin could be explained by more complex events. Macrophage-derived IL-1 has been shown to be the predominant agent responsible for the sustained increased synthesis of NGF after nerve lesion (Lindholm *et al.*, 1987). As discussed previously, NGF

protects neurons from the deleterious effects of capsaicin. Therefore it is possible that in our experiments the injection of IL-1 1 hour before capsaicin allowed the synthesis and anterograde transport of NGF in sensory neurons thus preventing neuropeptide release and potentiation of oedema formation.

7.2.1 CGRP Receptors

The existence of CGRP receptor(s) has already been discussed (see Introduction). Recently fragments of the CGRP molecule have been tested with some success for antagonistic activity on putative CGRP receptor subtypes.

Denis et al (1990) demonstrated that human CGRP₈₋₃₇ displayed relatively potent competitive antagonistic properties towards the action of native human alpha CGRP in the guinea-pig atrial and ileal preparations. In contrast, in the rat vas deferens, the antagonistic potency of human CGRP₈₋₃₇ was much weaker. This work led to the proposal of two types of receptor CGRP₁, and CGRP₂ which were human CGRP₈₋₃₇-sensitive and insensitive respectively. Further structure-activity studies by this group led to the discovery that [Cys(ACM^{2,7})]human CGRP was a selective agonist on CGRP₂ receptors (Denis *et al.*, 1990).

Human CGRP₈₋₃₇ has also proved to be a useful tool for determining whether the actions of CGRP are mediated via specific CGRP receptors or calcitonin receptors. It has been reported that in tissues that had predominantly CGRP receptors (rat liver plasma membrane) human CGRP₈₋₃₇ inhibited the binding of ¹²⁵I-[Tyr] rat CGRP and adenylate cyclase activation induced by human CGRP or human calcitonin (Yamaguchi *et al.*, 1988). However, the human CGRP₈₋₃₇ fragment alone had no activity. In tissues that had

predominantly calcitonin receptors (kidney LLC-PK cells), human CGRP₈₋₃₇ binding alone induced adenylate cyclase activation leading to cAMP production. In these preparations, human calcitonin and human CGRP induced increases in adenylate cyclase activity that was not affected by human CGRP₈₋₃₇. From binding characteristics of human CGRP₈₋₃₇ it can be presumed that receptors on rat liver plasma membrane are CGRP₁ receptors.

Han et al (1990) demonstrated that human CGRP₈₋₃₇ antagonised CGRP and nerve stimulation-induced vasodilatation in the mesenteric arterial bed. Their results suggested that human CGRP₈₋₃₇ could be a competitive antagonist of vascular CGRP receptors. Smaller fragments of the CGRP molecule have also been tested for antagonistic properties. CGRP₁₂₋₃₇ and [Tyr]-rat CGRP₂₈₋₃₇ antagonised CGRP-induced responses on the guinea-pig atria and opossum internal anal sphincter smooth muscle.

In light of all these in vitro studies it would be very interesting to use human CGRP₈₋₃₇ and other CGRP antagonists when they become available, in rabbit skin in vivo. These studies would help determine which receptor sub-type is responsible for the CGRP-induced vasodilatation reported in this thesis. Moreover, a specific vascular CGRP antagonist could determine whether all or part of the vasodilatory response induced by capsaicin was due to endogenously released CGRP. In the event that a partial inhibition is observed (mimicking the effects of the anti-CGRP antiserum), the hypothesis that another neuropeptide vasodilator is present in sensory neurons would be strengthened. Generally, a specific CGRP antagonist is needed to identify a role for CGRP in different inflammatory reactions.

Once CGRP has interacted with its receptor a vasodilation response is observed. The mechanism by which this neuropeptide dilates arteries and arterioles is largely unknown. However, recently the activation of K^+ channels has been implicated in this response (Standen *et al.*, 1989). Arterial dilations were partially reversed by blockers of the ATP-sensitive K^+ channel (glibenclamide and barium). In addition, CGRP hyperpolarised arterial smooth muscle and blockers of K^+ channels reversed this hyperpolarisation. Further evidence was provided by patch clamping studies on isolated arterial smooth muscle cells; CGRP opened single K^+ channels. These studies led to the conclusion that a substantial part of the relaxation produced by CGRP was induced by K^+ channel activation. The mechanism of the K^+ channel-independent vasodilator component remains to be determined but may involve Ca^{2+} channel inhibition or increased Ca^{2+} extrusion or sequestration.

7.3 Modulation of the release of neuropeptides from capsaicin-sensitive neurons in disease; therapeutic potential

The research in this thesis has demonstrated how a sensory neuron reacts to different stimuli under physiological conditions, eg. the mechanisms involved in the release of stored neuropeptides and the biological functions of these peptides in the microvasculature. This information is vital before we can begin to investigate and identify a role for capsaicin-sensitive neurons in pathological situations (eg. rheumatoid arthritis, asthma etc).

In the final section of this thesis different reactions and disease processes are discussed; one common denominator between them is the involvement of the sensory neuron. This basic similarity has allowed research into sensory

pharmacology of the skin (which is highly accessible) to benefit the understanding of a whole host of disease states in the joint, lung and other less accessible organs.

7.3.1 Cutaneous Hypersensitivity

There is evidence that neuropeptides localised in capsaicin-sensitive neurons may be involved in the modulation of immune functions. Opioid peptides, substance P, VIP, somatostatin, NKA and CGRP have been included among the peptides with immunoregulatory potential (Payan *et al.*, 1984; Foreman, 1987). The contribution of peptidergic neurons to the expression of type I-IV hypersensitivity reactions has been studied.

7.3.1.1 Delayed type hypersensitivity (type IV)

Girolomoni and Tigelaar (1990) reported that early events in a murine delayed type reaction were attenuated by pre-treatment with capsaicin suggesting neurogenic inflammation is a major component of the early irritant reaction to haptens. However, in later responses capsaicin pre-treated mice exhibited enhanced contact sensitivity reactions (Nilsson & Ahlstedt, 1989). Clinical observations by Wallengren and Moller (1986) agreed with this animal data; topical application of capsaicin-enhanced the tuberculin reaction and allergic contact dermatitis in humans.

These results indicate that peptidergic neurons regulate the expression of cell mediated, delayed in time cutaneous inflammatory reactions. The net effect of these neurons seemed to be suppressive because their impairment resulted in augmented reactions. Among the possible candidates present in these neurons

that may exert suppressive activities are peptides such as VIP, which is known to inhibit lymphocyte traffic from peripheral nodes (Moore, 1984), somatostatin which can inhibit lymphocyte proliferation and CGRP which was recently shown to inhibit macrophage activation in response to interferon-gamma (Nong *et al.*, 1989).

7.3.1.2 Cutaneous Anaphylaxis Reactions (type 1)

There are conflicting views over the role of capsaicin-sensitive neurons in IgE-mediated responses. In human atopsics, IgE-mediated responses consist of an itching sensation, a flare and a wheal reaction. Lundblad *et al* (1985) reported that after pre-treatment with capsaicin the flare and itching components of the response were suppressed. It is thought that capsaicin-sensitive neurons are activated by mediators, such as histamine, which are released upon mast cell degranulation by allergen exposure. However, the wheal response was unchanged after capsaicin pre-treatment. McCusker *et al* (1989) whilst agreeing that the wheal response was not affected by capsaicin pre-treatment did not observe a consistent effect on IgE-mediated acute flare responses either. The role of capsaicin-sensitive neurons is therefore not clear in IgE-mediated cutaneous responses in man. Due to the difficulties and ethics of performing mechanistic studies in man, a return to animal models of hypersensitivity is probably inevitable.

Ongoing experiments in our laboratory are investigating the role of capsaicin-sensitive neurons in hypersensitivity reactions in rabbit skin (Arthus, passive cutaneous anaphylaxis and delayed-type hypersensitivity reactions). Capsaicin pre-treatment experiments, which deplete all neuropeptides are probably not the best way to investigate these complex reactions although

initially, modulation of responses by capsaicin is a good indication that sensory neurons are involved in a particular reaction. Therefore anti-CGRP antiserum and specific neuropeptide antagonists are being used to investigate the role of CGRP and other neuropeptides in cutaneous responses.

7.3.2 Pain

Studies using capsaicin and capsaicin analogues NE-19550 (olvanil) and NE-21610, have shown them to be anti-nociceptive (Dray *et al.*, 1990a). When applied to peripheral fibres in a neonatal tail/spinal cord preparation in vitro, capsaicin induced activation, selective desensitisation and reduced responses to other noxious stimuli (heat, bradykinin). However olvanil and NE-21610 were considerably less potent than capsaicin on peripheral fibres but they did induce cross desensitisation to capsaicin. Therefore the analogues have the advantage of being anti-nociceptive without initially inducing a painful response.

It was suggested that anti-nociception induced by systemic capsaicin (and capsaicin analogues) may be due to effects on sensory mechanisms in the spinal cord rather than in the periphery. Studies by Kuraishi and co-workers demonstrated that a neuropeptide, released by capsaicin, was involved in pain transmission in the spinal dorsal horn (Kuraishi *et al.*, 1988). They demonstrated that an intrathecal injection of anti-CGRP antiserum inhibited either adjuvant or carrageenin-induced hyperalgesia in the rat paw. These studies suggested that endogenous CGRP promoted nociceptive transmission in the spinal dorsal horn. In addition they suggested that the capsaicin analogues effect their anti-nociceptive actions by modulating neuropeptide release in the spinal dorsal horn.

In contrast, a peripheral anti-nociceptive action for capsaicin in the treatment of pain associated with Guillain-Barre Syndrome, has been reported. Repeated topical applications of capsaicin decreased pain sensitivity in the area of topical application on human skin (Morgenlander *et al.*, 1990). It would be interesting to compare the effects of peripheral and systemic administration of capsaicin analogues in treating painful responses, when these agents become available clinically.

7.3.3 Wound Healing

Skin flap survival is a major problem in plastic surgery especially after burn injuries and skin grafts as well as cosmetic surgery. Much research has been devoted to increasing the nutritional blood flow in skin and nerve aiding the survival of skin flaps.

Kjartansson *et al* (1987a) investigated the role of capsaicin-sensitive neurons on the survival of experimental critical flaps in rats. They observed that pre-treatment with capsaicin caused a dramatic decrease in flap survival area compared to normal animals. Moreover, transcutaneous electrical nerve stimulation (TENS) increased cutaneous blood flow in the musculocutaneous flap (Kjartansson *et al.*, 1988). The mechanisms by which TENS caused increased blood flow and subsequent increased flap survival are not known however, two possible ways have been suggested. TENS treatment of reserpine pre-treated rats resulted in an increased blood flow, therefore an inhibition of sympathetic vasoconstrictory fibres or the release of vasodilator neuropeptides could result in an increased flap survival.

CGRP, as a potent vasodilator neuropeptide was an obvious candidate for the mediator of increased flap survival. Indeed CGRP was shown to increase the survival area of ischaemic tissue from 45% to about 90% (Kjartansson & Dalsgaard, 1987a). However, the concentration used was 2×10^{-5} times lower than those known to cause an increase in skin blood flow under normal conditions (Brain *et al.*, 1985). It is therefore possible that the mechanism by which CGRP increased survival of ischaemic tissue may be different from vasodilatation or that the sensitivity to this neuropeptide was altered during ischaemic conditions.

The non-invasive nature of TENS-induced increase in blood flow has recently been used with promising results on human patients with skin grafts to the breast and face. Although, the use of this therapy is in its infancy, the results from these operations will encourage the use of TENS in the treatment of other disease processes where an adequate blood supply is a major problem.

7.3.4 Rheumatoid Arthritis

Rheumatoid arthritis is characterised by pain, swelling, cartilage degradation and leukocyte accumulation. Another observation is that this disease often displays anatomical symmetry. Many studies have tried to implicate the sensory neuron in the pathogenesis of this degenerative disease. Gronblad *et al* (1988) observed that the human synovium is richly innervated by sensory nerve fibres. Levine and others suggested that the symmetrical innervation of joints by sensory and autonomic nerves, the way in which such nerves are controlled bilaterally by the nervous system, and differences in the density of innervation of different joints could explain why arthritic inflammation of joints tends to occur with bilateral symmetry (Kidd *et al.*, 1989; Levine *et al.*, 1990). In addition, several studies carried out in animals showed that pre-treatment with capsaicin

leads to a partial inhibition of the symptoms of adjuvant arthritis (Colpaert *et al.*, 1983; Inman *et al.*, 1989). Substance P has previously been suggested to be responsible for the pro-inflammatory actions seen in arthritis as an increased incidence of substance P containing nerves has been detected in connection with the arthritic joint in the rat (Levine *et al.*, 1984). Furthermore, the severity of arthritis was increased by substance P and antagonised by substance P antagonists (Levine *et al.*, 1984). However, recent evidence suggests that CGRP could be an important mediator. CGRP has been detected in small diameter nerves supplying the knee joint (Gronblad *et al.*, 1988). Moreover, capsaicin-induced release of CGRP from dorsal horn slices was increased in adjuvant arthritic rats (Nanayama *et al.*, 1989). This suggests that CGRP levels were elevated in a capsaicin-sensitive pool of nerve terminals from primary afferents. More importantly, in man CGRP has been shown to be present in synovial fluid as well as perivascular nerves, ideally suited for a role in the regulation of blood flow in the synovium (Larsson *et al.*, 1989; Kidd *et al.*, 1989).

Substance P has been shown to stimulate the release of inflammatory cytokines from cultured monocytes (Lotz *et al.*, 1988). As substance P and CGRP are co-localised in sensory nerves it is possible that the stimulation of sensory neurons at inflammatory sites could lead to substance P-induced production of IL-1 whose effects can then be enhanced by vasodilator neuropeptides.

Results in this thesis demonstrate that in the rabbit microvasculature, the use of CGRP has enabled the activity of IL-1 as a mediator of increased microvascular permeability to be investigated. Furthermore, CGRP and IL-1 could be concomitantly released at inflammatory sites such as the rheumatoid joint. In these circumstances, controlling neuropeptide release from sensory neurons could offer some relief to patients suffering from rheumatoid arthritis.

Whilst in the future capsaicin antagonists could be useful in suppressing CGRP release, the release of other potentially beneficial neuropeptides would also be inhibited. Recently, Louis et al (1990) demonstrated that circulating antibodies to CGRP (auto antibodies) reduced the number of secondary lesions normally observed in adjuvant-arthritic rats. In addition, α_1 glycoprotein levels in plasma were reduced in these animals. These results suggested that auto-antibodies are indeed able to ameliorate the severity of the adjuvant arthritis syndrome and that CGRP contributes to the inflammatory response seen in the later stages of the disease. If this preliminary research continues to prove encouraging then the future treatment of rheumatoid arthritis could involve the use of specific CGRP antagonists which would control blood flow and subsequently reduce plasma protein leakage.

7.3.5 Asthma and bronchial hyperreactivity

Sensory fibres are widely distributed in the lung and in particular around bronchial and pulmonary blood vessels and the alveolar wall (Cadieux *et al.*, 1986; Mak & Barnes, 1988). The presence of capsaicin-sensitive nerves in human airways that are capable of reacting to irritants like capsaicin, was demonstrated by Collier and Fuller (1984). In addition pre-treatment with capsaicin has indicated the involvement of sensory fibres in the pathogenesis of asthma. Furthermore, neuropeptides stored in and released from capsaicin-sensitive neurons produce many of the pathological features of asthma including contraction of airway smooth muscle, oedema formation, leukocyte accumulation, mucous hypersecretion and increased mediator release (Gamse *et al.*, 1980; Lembeck & Holzer, 1979; Lundberg & Saria, 1983; Barnes, 1987). In addition many of the features of asthma can be exacerbated by increased blood flow (Matran *et al.*, 1989). CGRP is stored in sensory fibres in the lung and has

binding sites that closely correlate with the sensory innervation (Mak & Barnes, 1988; Umeda & Arisawa, 1989). Therefore this vasodilator is ideally situated to induce profound vasodilatation leading to the exacerbation of many of the symptoms of an asthmatic response.

The initial stimulus for asthma is not fully understood. However, damage to airway epithelium, possibly as a result of eosinophil products (eg. major basic protein) causes the exposure of afferent nerve endings. In turn activation of sensory fibres leads to the release of neuropeptides and possibly the activation of an axon reflex. Histamine activates sensory fibres following allergen challenge in man and other animal studies (Alving *et al.*, 1990). The axon reflex may explain how patchy epithelial damage leads to widespread pathophysiological changes (Barnes, 1987).

Drug studies have given further evidence that capsaicin-sensitive neurons are important in asthma. The anti-asthmatic drug sodium cromoglycate was shown to suppress the activation of sensory C-fibres by capsaicin in the dog lung (Dixon *et al.*, 1980). Use of specific neuropeptide antagonists, ruthenium red and capsaicin antagonists may help to unravel some of the mechanisms involved in asthma.

The discussion so far has concentrated on the therapeutic potential of capsaicin antagonists and neuropeptide antagonists, particularly CGRP antagonists. It must be stressed however that there are several other approaches to manipulating neurogenic inflammation. Opioids modulate sensory neuropeptide release. One problem with this class of mediator is that it could have unwanted central as well as peripheral actions. This problem was overcome with the drug BW443C which exhibits potent anti-nociceptive effects but has

poor penetration of the blood brain barrier. The investigations into the other prejunctional modulators (galanin somatostatin etc) are in the early stages but it is hoped that these neuropeptides may have clinical benefit in the future. Another interesting approach concerns the prejunctional terminal and the finding that prejunctional receptors may have a common mode of action. The multiple prejunctional receptors may open a common K^+ channel. This proposal is supported by the finding that cromakalim which activates K^+ channels is effective in inhibiting neural bronchoconstriction in guinea-pig airways (Ichinose & Barnes, 1990).

In conclusion neurogenic inflammation may be an important component of diseases such as asthma and rheumatoid arthritis. Detailed investigations of the mechanisms involved in neurogenic inflammation in animal models will provide the basis for future therapeutic intervention in disease using novel compounds.

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