In the name of God

The beneficent The merciful

The actions of sensory neuropeptides on the synovial vasculature of normal and inflamed joints

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by

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Dedicated

to

my family

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Declaration and list of publication

I hereby declare that this thesis comprises my own original studies and does not include work forming part of a thesis presented for another degree in this or any other university. I performed every experiment described in this thesis and I am responsible for all data analysis.

Part of the work contained in this thesis has been or will published as follows:

Abstract:

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Karimian S.M., McDougall J.J., Ferrell W.R. (1995). Neuropeptidergic and autonomic control of vasculature of the rat knee joint revealed by

laser Doppler perfusion imaging. *Experimental Physiology* 80, pp 344- 348

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SUMMARY

Inflammation of the joint, arthritis, is a major problem in human society, affecting large numbers of individuals, including a large number of those aged 60 and over. A principle event in development of inflammation is an increase in blood flow. Different factors are involved with neurocirculatory control of which the neurokinins and calcitonin gene-related peptide (CGRP) may have an important role and the work described in this thesis is directed at investigating this.

There are few reports about the role of neurokinins in joint inflammation and how neurokinin receptors are affected and thus animal models of inflammation were used for these investigations, based on the rat knee joint.

Two techniques were used to measure joint inflammation:

a) microturbidimetry was used to examine knee joint perfusate to evaluate its protein content due to the extravasation effect of CGRP.

b) laser Doppler imaging (LDI) was employed to give an estimate of blood flow changes in response to application of neurokinins

In normal knee joints:

1) Intra articular injection of 10^{-6} mol of CGRP produced protein extravasation which was sustained throughout the period of infusion. This was a specific effect of CGRP and not merely a consequence of its potent vasodilator effect.

2) SP on its own induced a dose dependent and transient vasodilatation, this being mediated via NK1 and NK2 receptors.

3) NKA at higher doses induced vasodilatation and this vasodilator effect was chiefly mediated via NK2 receptors and a smaller part mediated via NK1 receptors.

4) NKB produced a potent and significant vasodilatation. This effect was mediated chiefly via NK1 and NK2 receptors and it is unlikely that NK3 receptors are present.

5) The neurokinin receptors in the rat knee joints are of the B type of NK2 and a possible subtype of NK1 receptors.

6) SP and perhaps other neurokinins are normally released from sensory nerves mediating and participating in regulation of vascular tone and basal blood flow.

B) In inflamed knee joints:

1) In the carrageenan-induced inflammation, there is a hyper-sensitivity to the neurokinin response with a significant increase in SP-induced vasodilatation.

2) In the acute capsaicin treated knee, an acute vasoconstriction occurred at different doses of capsaicin which in lower doses was followed by a vasodilatation which could be due to release of neurokinins from sensory nerve endings. Vasoconstriction by 2% capsaicin is irreversible over the time course of the experiments.

3) With acute and chronic inflammation induced by capsaicin, there is an alteration in the response to neurokinins and there is an attenuated response to the SP vasodilator effect on blood flow.

4) In adjuvant-induced arthritis, one week post induction the effect of SP was completely abolished with no response to SP at any dose. This attenuation changes to a vasoconstriction effect at week 3.

5) There is a contralateral effect of adjuvant-induced arthritis, the response to SP being significantly attenuated in the contralateral knee. This response revealed a neurogenic effect of inflammation in one knee being exerted on the contralateral joint.

6) In all three different models of inflammation, the response to the neurokinins was altered but there was not any change to the 5HT and adrenaline response in adjuvant-induced inflammation.

7) In all of these three models of inflammation neuropeptides have a key role in initiating of the signs of inflammation. The further joints changes will depend on the nature of the inflammatory agent and individual treatments would be required to reduce inflammation and joint damage.

CHAPTER ONE

GENERAL INTRODUCTION

AND

LITERATURE REVIEW

1.1.1 INTRODUCTION

Inflammation is an important component of disease in many clinical conditions, and it is generally associated with disordered blood flow. Arthritis, inflammation of the joints is a specific example in which changes in blood flow are an important factor in disease development. In the healthy state joints permit a controlled and almost frictionless movement, and this depends upon integrity of the joint and nutrition of synovial tissue. Four elements of synovial tissues need consideration: the compact surface zone, the surface zone layer, packing tissue and the vasculature (Edwards 1988). Synovial nutrition critically depends on the circulation and microcirculation (Mc Kibbin & Maroudas 1979). All vascular beds in the body leak a small amount of water to the surrounding tissue, which enters the lymphatic drainage system. Synovial tissue is unusual in that some of the water passes in and out of the cavity (and hyaline cartilage) before becoming lymph (Edwards 1988). Since any change in the circulation could have serious effects on joint movement, increased vascular permeability in inflamed synovial tissue could lead to an imbalance in Starling forces with resulting oedema and stiffness of movement (Edwards 1988).

The control of microcirculatory flow is complex, but recently neurokinins and calcitonin gene related peptide (CGRP) have been found in sensory nerve endings. These peptides have significant effects on the vasculature bed and their permeability (Maggi *et al* 1993a; Otsuka & Youhioka 1993; Poyner 1992) and so may have role in the pathogenesis of inflammation.

To examine microcirculatory changes, many methods of measuring blood flow have been developed (Liew & Dick 1981). Laser Doppler Imaging (LDI) is the most recent technique which is a development of single point laser Doppler flowmetery, allowing large areas to be scanned in a relatively short time.

1.1.2 AIMS OF THE STUDY:

Neurokinins and CGRP are newly discovered peptides but many physiological and pathological aspects of these peptides remain unclear especially regarding joints and the effect of these peptides in inflammation. CGRP is a potent vasodilator peptide which potentiates other neurokinin vasodilator effects (Cambridge & Brain 1992), but little is known of its effect on protein extravasation, and so the first objective is to examine this.

The second objective is characterize the neurokinin receptors mediating the effect of neurokinins on blood flow in the normal knee joint and to find out which neurokinins are involved in the normal regulation of articular blood flow.

The effect of neurokinins in different models of inflammation and arthritis is unclear, and so a third objective is to study the effect of neurokinins on the inflamed knee microcirculation and also evaluate the contralateral effect of inflammation. In the second and third parts of this study LDI was used and found to be an effective and user-friendly tool for the measurement of blood flow.

LITERATURE REVIEW

1.2 JOINTS

All bones are interconnected by articulations or joints to form the skeleton. These joint are composed of wide variety of connective tissue structures that permit varying degree of movement between adjacent bones. Three groups of joints are recognised and these are a) Synathrotic, b) Amphiarthrotic, c) Diarthrotic. There are also other types of classification based on the form of connective tissue which can be used. (Gray 1973).

1.2.1 Synathrosis: These joints are generally found in the skull and the contributing bony plates that compose them are held firmly together by fibrous or cartilagous elements (Mankin & Radin 1989). These joints are generally immovable or have a very limited movement, and can be further differentiated into three subclasses by the type of tissue involved in the union (Junqueira & Carneiro 1992). These joints, also called fibrous joints,, are temporary joints because the fibrous tissues become ossified and synostosis will develop over time.

1.2.2 Amphiarthrosis: These joints are characterised by the presence of broad, flattened discs of fibrocartilage connecting the articulating surfaces. The bony portion of the joints is usually covered by hyaline cartilage and the entire structure is invested with a fibrous capsule. Examples of such joints are those between the vertebra, the distal tibiofibular articulation and the pubic symphysis. The hyaline cartilage joints become ossified in adulthood and so have no movement, but fibrocartilaginous ones retain limited movement.

1.2.3 Diarthrosis: These joints include most of the body joints and have great mobility, such as the elbow and knee joints which have diarthrosis ligaments and a capsule of connective tissue to maintain the contact at the end of the bone (fig. 1.1). The capsule encloses a sealed articulating cavity that contains synovial fluid, a colourless transparent viscous fluid like egg white which is dialysed blood plasma with a high concentration of hyaluronic acid, a glycosaminoglycan of high molecular weight produced by B cells of the synovial layer (McCarty 1989). These joints are also called synovial joints, the capsule is composed of two layers, one external (fibrous layer) and one internal (synovial layer). The internal surface of the synovial membrane is usually lined by a layer of squamous or cubical cells. Underneath this cellular layer is loose or dense connective tissue with areas of adipose tissue. These layers are separated from each other by a small amount of connective tissue (fig. 1.2).

It has been shown that there are two type of cells lining the synovial membrane: **a)** A cells, similar to mononuclear phagocytes with a large golgi complex, endoplasmic reticulum, but only small number of lysosomes.

b) B cells which are similar to a fibroblast (fig. 1.3) (Junqueira & Carneiro 1992).

This thesis is particularly concerned with the knee joint, one of the largest of the diarthrodial joints, and one which is commonly affected in joint diseases.

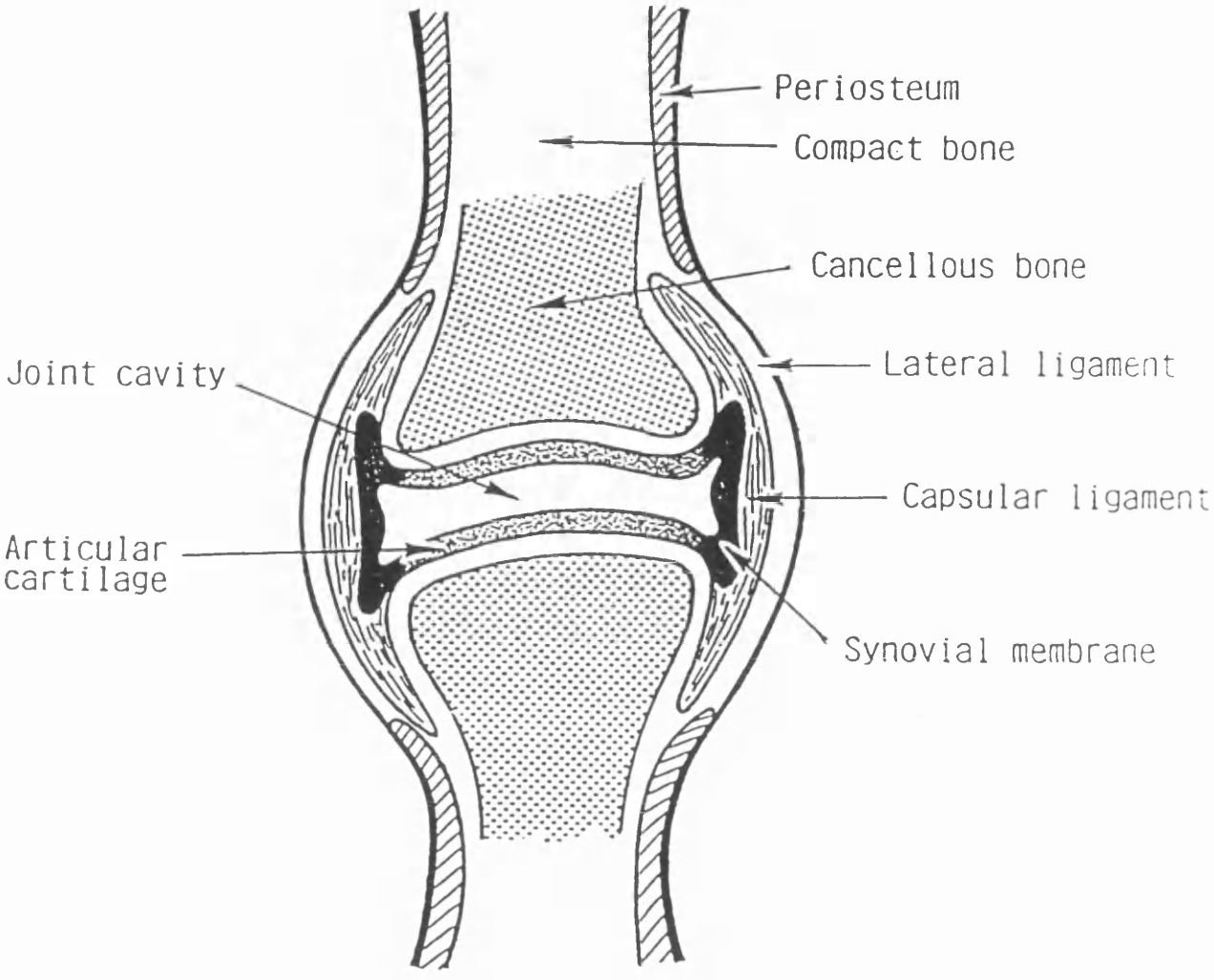


Fig 1.1: The structural view of a diarthroidal joint, showing synovial cavity and membrane

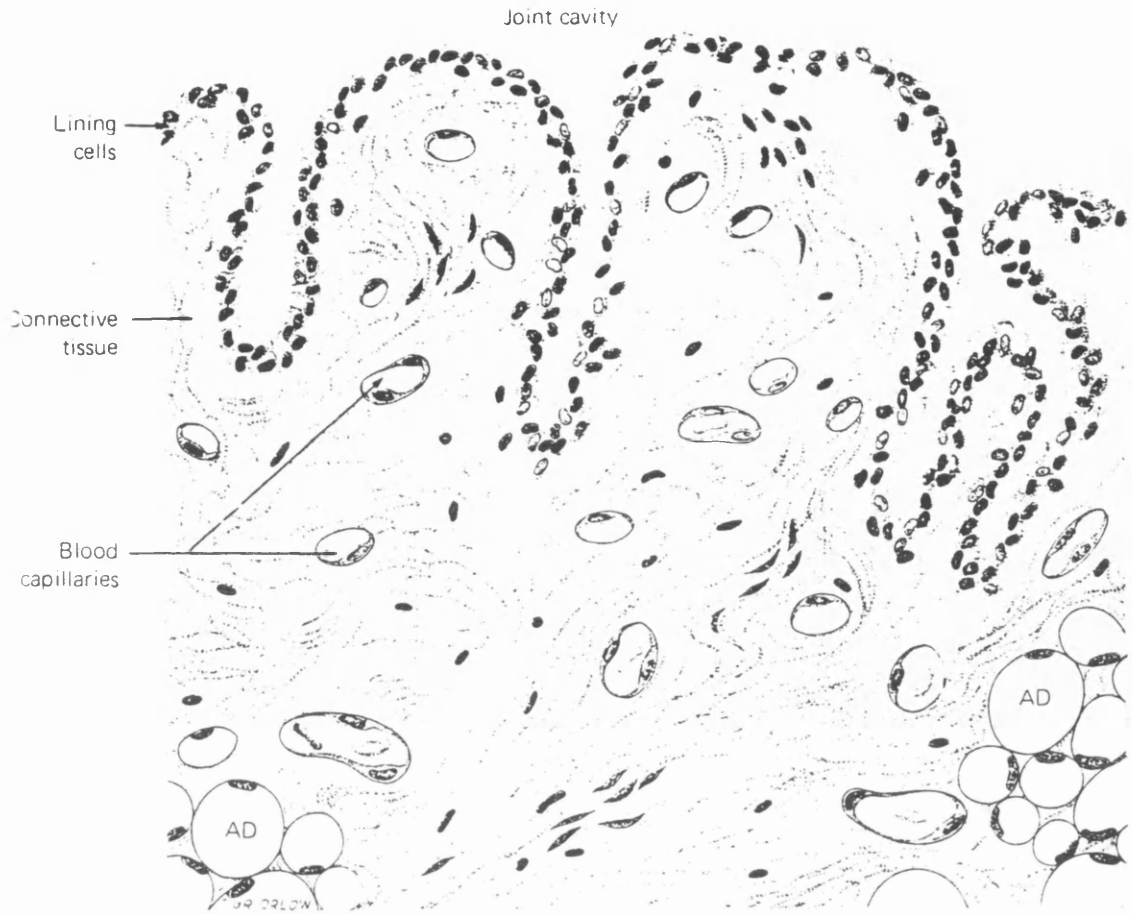


Fig 1.2: Histologic structure of the synovial membrane, with its lining connective cells in epithelioid arrangement. There is no basal lamina between the lining cells and the underlying connective tissue.(From Junquera 1992, in Basic histology)

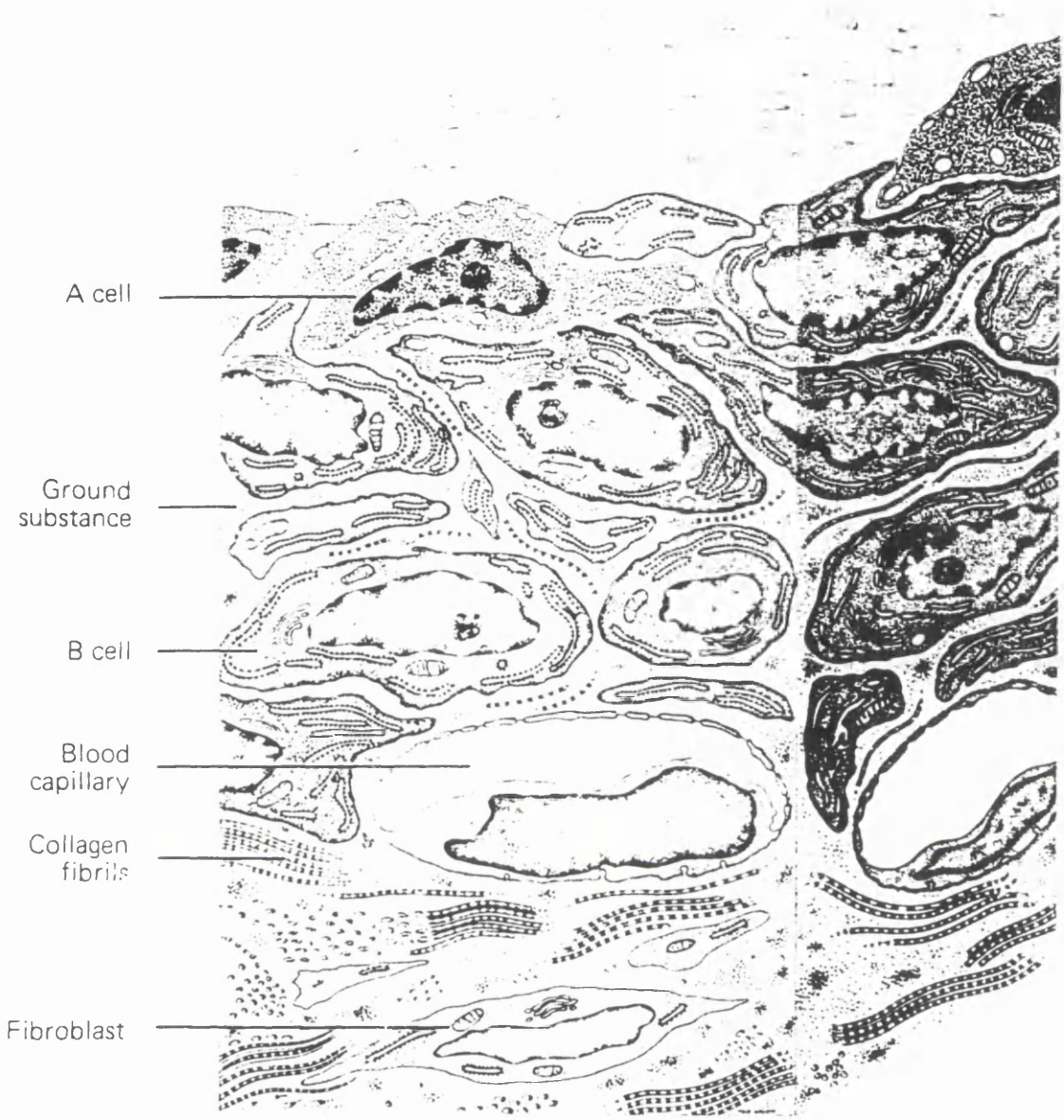


Fig 1.3: Schematic representation of the ultra-structure of the synovial membrane. A and B cell types are separated by a small amount of connective tissue ground substance. (From Junquera 1992, in Basic histology)

1.3 GROSS ANATOMY OF THE RAT KNEE JOINT:

On the flexor surface of the distal end of the femur just proximal to the condyles are two small but well defined articular surfaces for the sesamoid bones, the medial and lateral fabella, which develop in connection with the tendons originating at the head of triceps surae. The semilunar cartilages of the knee joint are also ossified in the rat (Chace 1955)

1.3.1 Arterial supply:

The femoral artery (fig. 1. 4) is a continuation of the external iliac which travels in the medial aspect of the thigh. It extends from the inguinal ligament to the point where the vessel passes between adductor brevis and caudo femoralis to the popliteal fossa where is it known as the popliteal artery. It has six branches, the superficial circumflex iliac, the highest genicular, the sapheneous (great saphenous), the profunda femoralis and perforating, the muscular, and the superficial branches.

The highest genicular artery (genu suprema) arises from the deep surface of femoral artery. In the majority of cases its origin is above the muscular branch to the adductor and superficial epigastric, but in some it may leave the femoral artery just above the knee. In the rat, this artery represents the combined muscular and articular branches of the highest genicular, and occasionally the muscular branches spring directly from the femoral artery, and in such cases the articular branch may arise with the superficial circumflex iliac branch of femoral or the superficial epigastric (fig. 1.4) (Chace 1955).

1.3.2 Venous drainage:

Joint drainage, from the medial inferior genicular, lateral inferior genicular, and middle genicular veins and tributaris vein is achieved by all of these veins. Ventral drainage begins with the junction of the anterior and posterior tibial vein at the upper margin of the popliteus muscle, and ends with drainage into the femoral vein. The femoral vein comes to a superficial position on the medial surface of the thigh and runs upward with the femoral arteries and leaves the tight by passing under the inguinal ligament and becoming the external iliac vein (Chace 1955)(fig. 1.4).

1.3.3 Nerve supply:

The femoral nerve, from the third and fourth lumbar nerves, appears from between psoas minor and iliacus and runs under the inguinal ligament in the company of the external iliac vessels before entering the thigh. It is divided into the posterior and anterior, the anterior comprising muscular branches. After giving various branches to the quadriceps femoris, it continues to the saphenous nerve (long saphonous) and runs superficially down the medial surface of the thigh and lower leg, accompanied by the saphenous artery and large saphenous nerve which takes the place of the medial sural branch of tibial nerve. (Chace 1955)(fig.1.5).

Joints are supplied by articular nerves the branches descending from main nerve trunks. Both the anterior and posterior articular nerves contains myelinated and unmyelinated afferent fibres and unmyelinated sympathetic post ganglionic fibres. The total number of nerve fibres has been determined by electron microscopy and, for

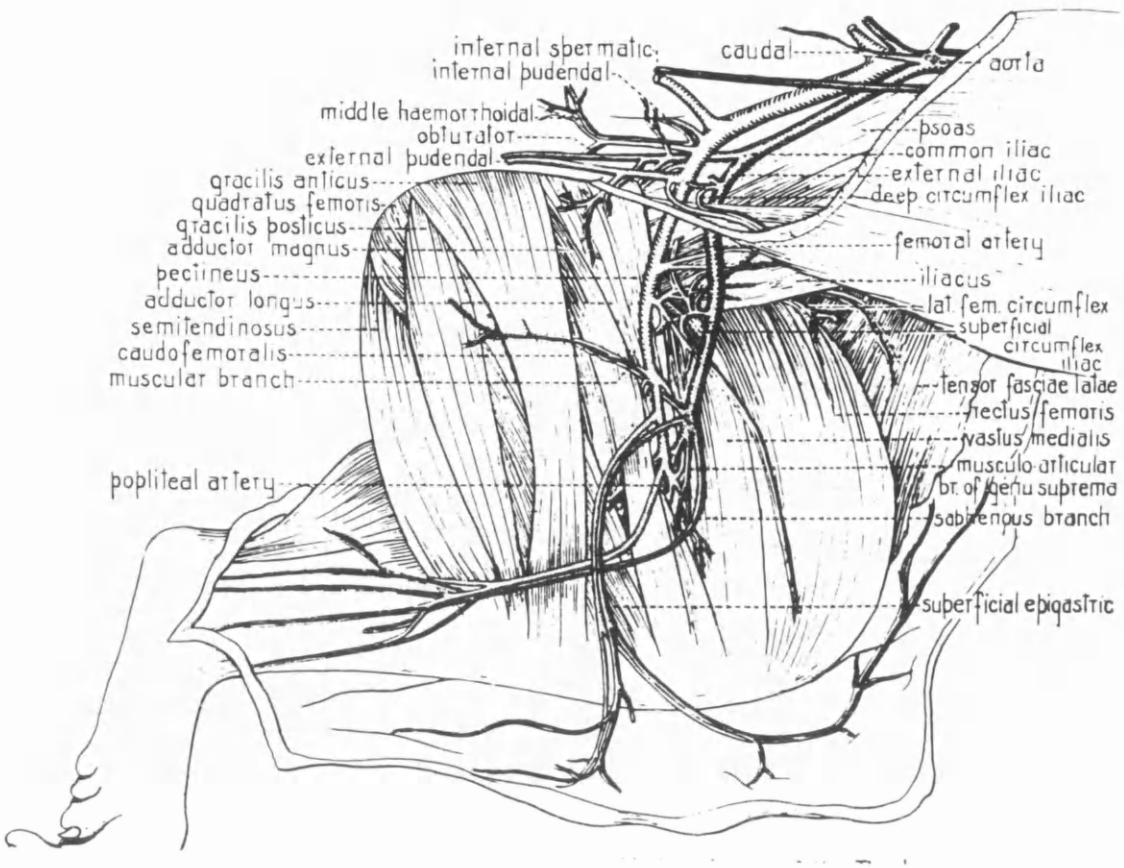


Fig 1.4: Superficial blood vessels of the medial aspect of the thigh. The femoral artery and vein. (From Chace, Green Eunic 1955, in Rat anatomy)

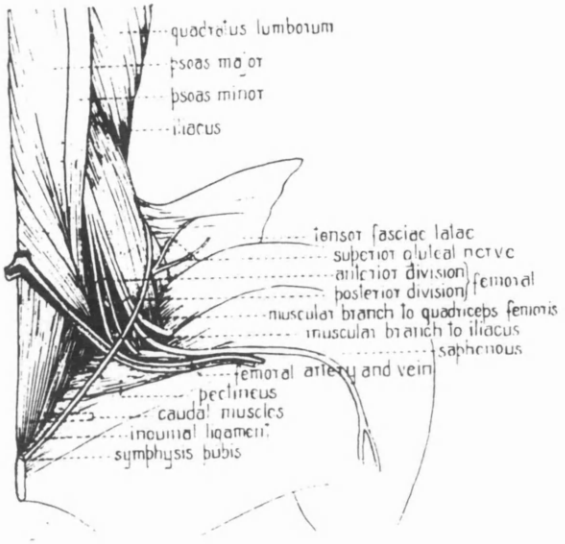


Fig 1.5: Divisions of left femoral artery (From Chace, Green Eunic 1955, in Rat anatomy)

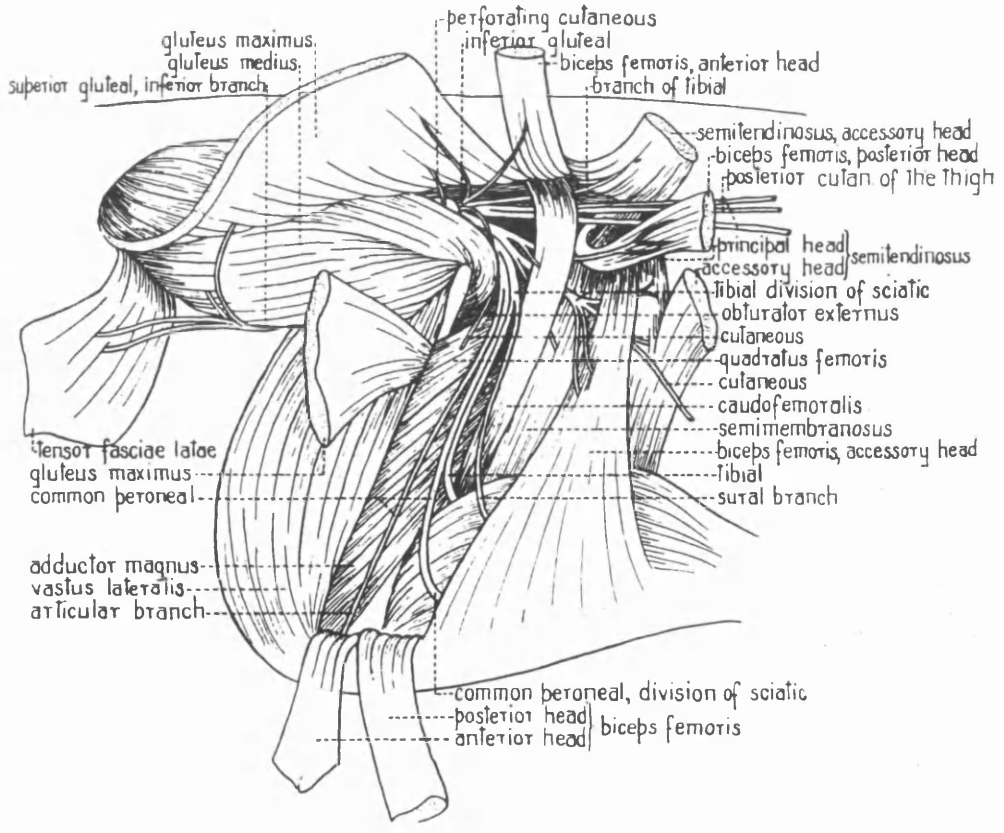


Fig 1.5: Divisions of the sciatic nerve

example the rat posterior atricular nerve (PAN) has 400 axons of which 20% are myelinated and 80% are unmyelinated and about half of the unmyelinated fibres are sympathetic efferents (Schaible & Grubb 1993).

1.4 CONTROL OF BLOOD FLOW

The peripheral circulation is essentially controlled by: neural (sympathetic, peptidergic), hormonal (cathecholamines etc.) and local factors at the tissue level.

The vessels that regulate blood flow throughout the body are called resistance vessels (arterioles) and these are very important in the central control of blood pressure regulation and blood flow (Berne & Levy 1991). The central nervous system regulates and controls systemic blood pressure.

The regulation of blood flow through each organs depends upon its moment to moment function and metabolic needs and local mechanisms override neural control in some situations, although the precise mechanisms are unknown. Four major hypotheses have been but forward to explain the local control of blood flow; 1) The myogenic hypothesis, which is related to degree of stretch of the smooth muscle which is the actuator in producing alternations in vascular resistance. 2) The metabolic hypothesis, in which it is believed that under conditions of consumption of oxygen, vasodilator metabolites are being produced at a constant rate, a transient increase in flow would wash out the metabolites at a more rapid rate and reduce their concentration thereby increasing vascular resistance. Conversely if perfusion pressure dropped the metabolites would be washed out at a

reduced rate and locally increase their concentration and thus decrease vascular resistance(West 1990). 3) tissue pressure hypothesis and 4) the flow dependent dilatation which acts in the certain tissues like kidney and arcade arterioles (Berne & levy 1991; West 1990). The local circulation is also affected by active and reactive hyperaemia, response to injury which activates various tissues components, low PO_2, high PCO_2, lactic acid, adenosine, bradykinin, histamine, hydrogen ion, and as recently reported sensory neuropeptides (Vander *et al* 1990). Most of the vasodilator theories assume that the vasodilator substance is released from the tissue mainly in response to oxygen deficiency (Guyton 1991). It is likely that a combination of all these determinants regulate local flow.

There is now good reason to believe that chemical mediators -neuropeptide hormones- are likely to be a dominant factor in regulation and under special conditions are produced at a constant rate. In general the joint blood vessels behave like those of the skin (Cobbold & Lewis 1956).

1.5 REGULATION OF JOINT BLOOD FLOW:

Blood flow through a microcirculatory bed varies from tissue to tissue depending on metabolic need. There is a large variation in capillary density for example, in muscle it is 2000 capillaries per mm^2 while in the skin it is about 50 capillaries per mm^2 (Stern *et al.* 1977). All the factors which are involved in the control of local blood flow regulation, including central and local factors, are also involved in the regulation of joint blood flow. In peripheral areas local control of joint

blood flow is likely to be more important than central control because nutritional needs can alter so quickly.

Apart from all these factors, there are other important factors which affect joint flow and which are important for nutrition. These are:

a) Changes in intra articular pressure. Phelps *et al* (1989) showed that the synovial microcirculation is reduced by moderate increases in hydrostatic pressure and that small changes in intra-articular pressure (9-12 mm/Hg) can cause an appreciable decrease in blood flow as measured by ^{133}Xe clearance. The clearance doubled if only 10 ml of effusate material was aspirated (Liew & Dick 1981). In patient with rheumatoid arthritis (RA) intra-articular pressure is related to local hemodynamics of the knee and to regional blood perfusion. Elevation of the intra-articular pressure may affect the hemodynamics of the knee joint (Bunger *et al* 1986).

b) Heat: When temperature increases either locally or centrally, blood flow is increased and this also applies to the knee (Liew & Dick 1981).

c) Exercise: blood flow through the knee segment increases during exercise, and it has been shown that there is an increase in blood flow in the calf muscles during resistive rhythmic contraction (Liew & Dick 1981). St'onge *et al* (1971) indicated that in the knee, there is a reduction in the clearance rate of Na^+ during the exercise, but the exact mechanism is unclear.

d) At rest, there is a generalised reduction in blood flow to the resting part both in the number and calibre of the blood vessels and this is probably the beneficial mechanism behind bed rest in acute rheumatoid arthritis (Liew & Dick 1981).

e) During inflammation: Inflammation induces marked increase in articular blood flow and this can be measured by techniques such as ^{133}Xe clearance `(Liew & Dick 1981) .

1.6 CELL MEMBRANE RECEPTORS

In recent years a large number of neuropeptide hormones have been identified including sensory neuropeptides and neurotransmitters (Utosoka & Masanavi 1993; Mussap *et al* 1993) and each peptide is recognised by a specific and distinct cell surface receptor which translates hormone recognition into a biological response (Nuki *et al* 1994). Each hormone receptor is a unique molecular species, and there are certain features which are similar among peptide receptors:

A) a mono binding region exposed on the external cell surface

B) a hydrophobic region which anchors them within the lipid bilayer of cell membrane

C) an activation site which interacts either with other membrane component or with the interior of the cell wall (West 1990).

Peptide hormone receptors are glycosilated and often contain more than one subunit and require phospholipid or a membrane

environment for optimum biological activity. Two general structures for cell surface hormone receptors have been identified:

1) first structure consists of seven hydrophobic membrane spanning helical surface loops between membrane segment from the ligand binding domain

II) The second structure consist of a single membrane spanning domain which separates the extracellular domain from intracellular domain (Kubo *et al* 1986).

There are approximately 10^4-10^5 receptors on a target cell and they have a continuous turnover and a potential half life and participation in the recycling period. Also, they are dynamic molecules and extensively regulated by the cell; for example long term exposure to a hormone causes decrease in receptor concentration (down regulation) while a low concentration of hormone causes an increase in receptor number and hypersensitivity (up regulation). (West 1990). Functionally there are four kind of receptors:

a) receptors for fast neurotransmitters, which directly couple to an ion channel.

b) receptors for many hormones and slow transmitters which couple to a effector system.

c) receptors which directly link to tyrosine kinase.

d) and finally steroid receptors.(Fig. 1.6)(Rang & Dale 1991).

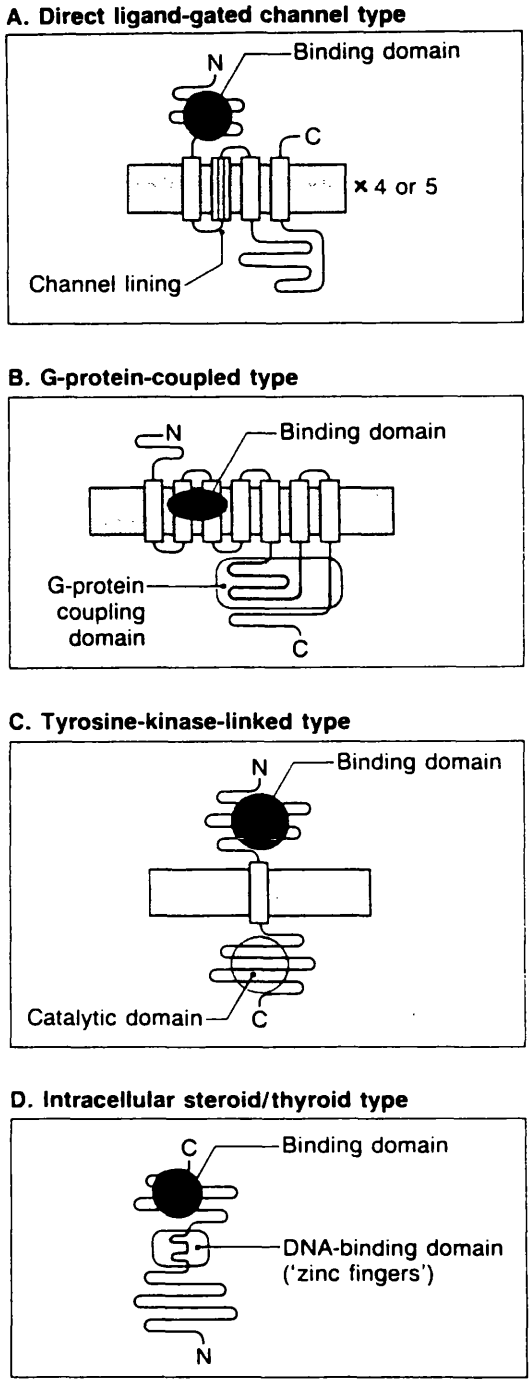


Fig 1.6: General structure of four receptor families (Rang and Dale 1991, in Pharmacology

The next step after association between hormone and receptor is signal generation, and here the mechanism is different depending on the coupling mechanism:

I) Activation of cyclic adenosine monophosphate, which has three subunits: a stimulating regulatory component, a catalytic component, and an inhibiting regulatory component

In the case of the activation of the stimulatory component, a catalytic unit will activate and this increases the formation rate of cyclic AMP , Mg AMP & GTP and production of ATP initiating a chain reaction to start up (Baldwin 1994).

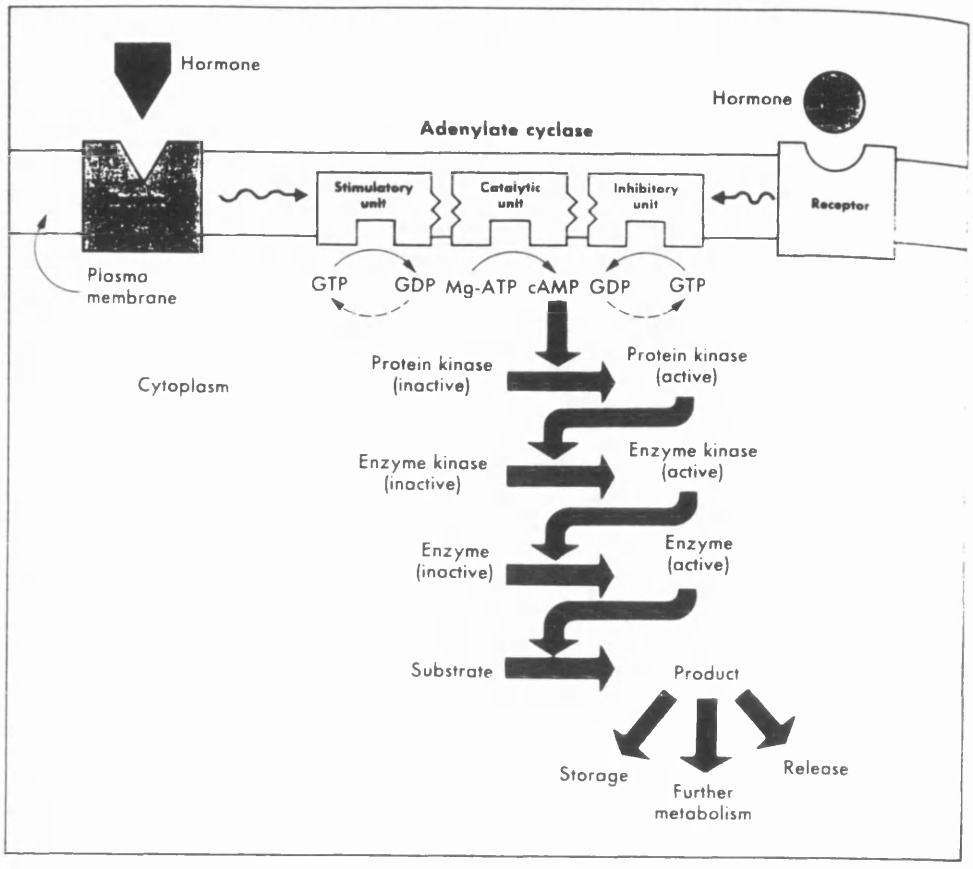
In other cases activation of the inhibitory component leads to deactivation and decreases of formation cAMP and ATP (fig. 1.7).

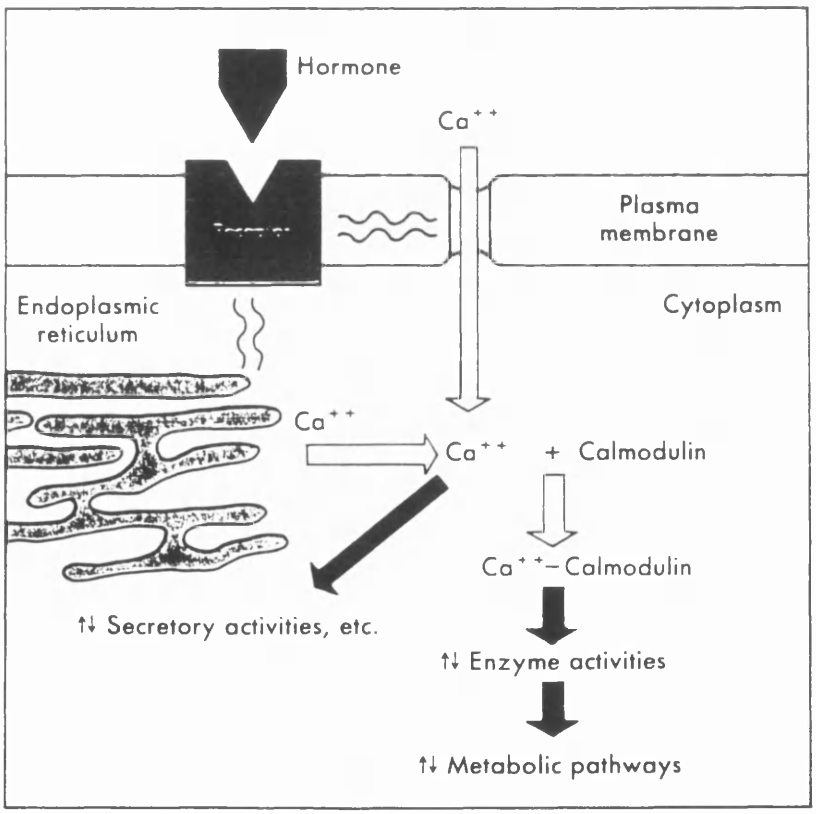
II) Calcium calmodulin: the activated receptor can open calcium channels and mobilise calcium into the cell, binding to calmodulin which is a free protein. This complex is a powerful regulator of the activity of many enzymes (Fig. 1.8).

III) Phospholipid products: About 10% of the lipid portion of membrane contains phosphatidyl inositol, a hormone receptor component which activates phospholipase, and consequently phosphatidyl inositole 4,5 phosphate splits into the two component: diacylglycerol and inositol tri-phosphate (IP3) which, with calcium ions, can activate protein kinase C and consequently activate the metabolic pathway.

Fig 1.7: Activation of cyclic AMP and consequent chain reactions. From Vander *et al.* (1990). in Human physiology

Fig 1.8: Mechanism of hormones action via calcium as second messenger. From Vander *et al.* (1990). in Human physiology


Hormone
Hormone
Adenylate cyclase
Stimulatory unit
Catalytic unit
Inhibitory unit
Receptor
Plasma membrane
GTP
GDP
Mg-ATP
cAMP
GDP
GTP
Cytoplasm
Protein kinase (inactive)
Protein kinase (active)
Enzyme kinase (inactive)
Enzyme kinase (active)
Enzyme (inactive)
Enzyme (active)
Substrate
Product
Storage
Release
Further metabolism


Hormone
Ca++
Plasma membrane
Endoplasmic reticulum
Cytoplasm
Ca++
Ca++ + Calmodulin
Ca++–Calmodulin
↑↓ Secretory activities, etc.
↑↓ Enzyme activities
↑↓ Metabolic pathways

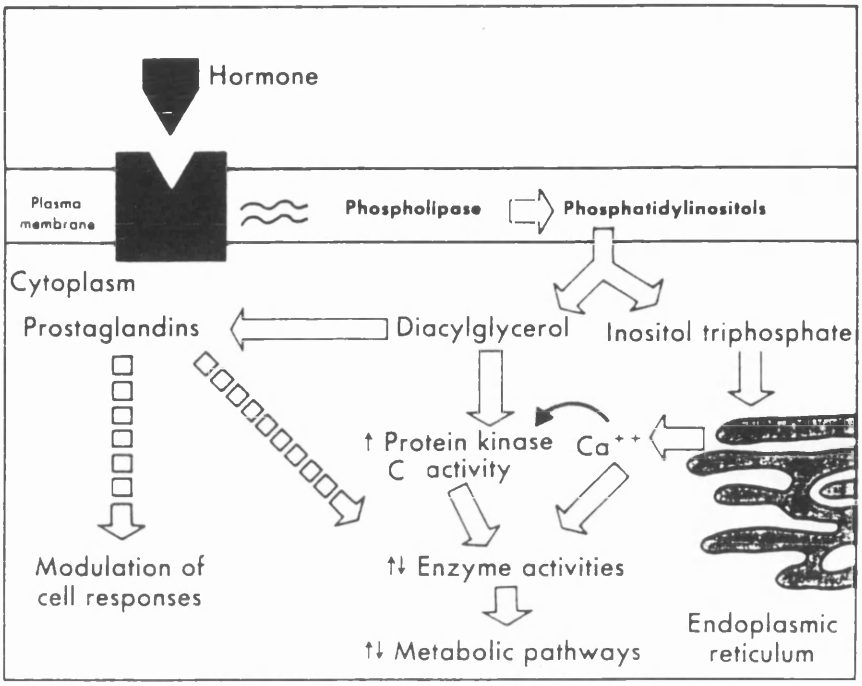
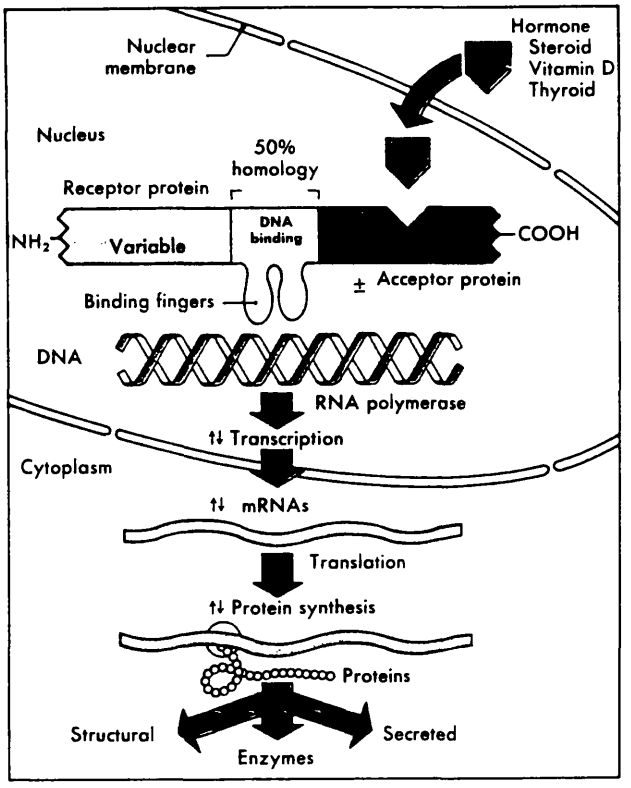
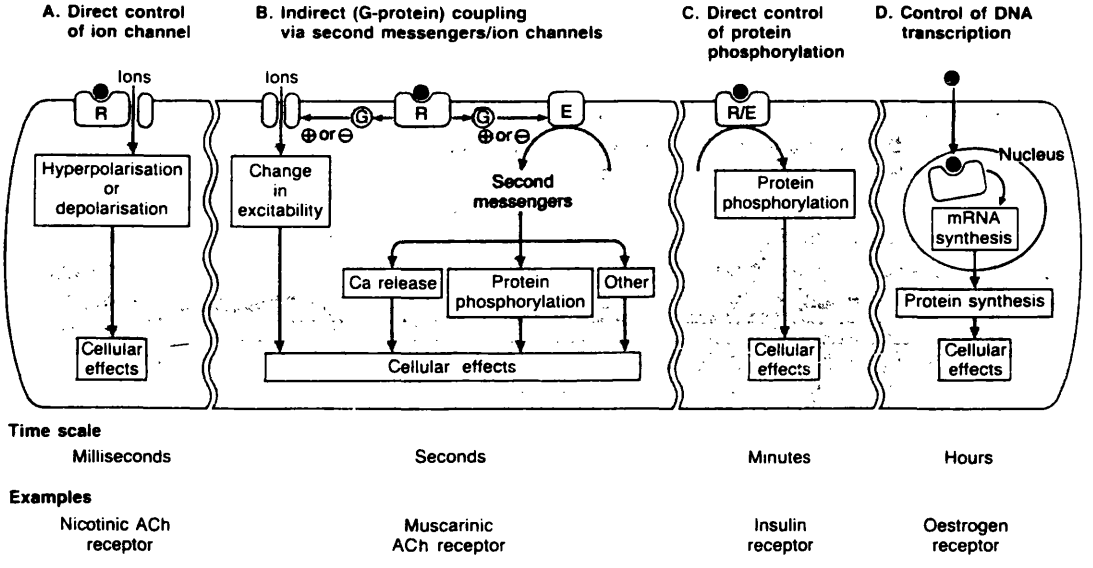


Fig 1.9: Mechanism of hormone action via membrane phospholipids. From Vander *et al.* (1990). in Human physiology

Fig 1.10 a: Mechanism of action of vitamin D, steroid and thyroid hormones. The hormone combine with a nuclear protein receptor. From Vander *et al.* (1990). in Human physiology

Fig 1.10 b: Types of receptor-effector linkage. (R= receptor: G= G protein: E= enzyme) (Rang and Dale 1991, in Pharmacology


Nuclear membrane
Hormone
Steroid
Vitamin D
Thyroid
Nucleus
50% homology
Receptor protein
NH2
Variable
DNA binding
COOH
± Acceptor protein
Binding fingers
DNA
RNA polymerase
↑↓ Transcription
Cytoplasm
↑↓ mRNAs
Translation
↑↓ Protein synthesis
Proteins
Structural
Enzymes
Secreted


A. Direct control of ion channel
B. Indirect (G-protein) coupling via second messengers/ion channels
C. Direct control of protein phosphorylation
D. Control of DNA transcription
Ions
R
Hyperpolarisation or depolarisation
Cellular effects
Ions
R
G
⊕or⊖
E
Change in excitability
Second messengers
Ca release
Protein phosphorylation
Other
Cellular effects
R/E
Protein phosphorylation
Cellular effects
Nucleus
mRNA synthesis
Protein synthesis
Cellular effects
Time scale
Milliseconds
Seconds
Minutes
Hours
Examples
Nicotinic ACh receptor
Muscarinic ACh receptor
Insulin receptor
Oestrogen receptor

IV) In some cases the receptor itself activates after binding to a hormone and after phosphorylation it can activate a cascade of metabolic pathways. (Fig. 1.9)

V) Some hormones can freely enter a cell and activate a transformation process in the nucleus by coupling with a cytoplasmic receptor through transformation and activation of DNA production, starting a cascade of production (Fig. 1.10A)(Berne & Levy 1990). All these mechanism are summarised in fig. 1.10B).

1.7. TACHYKININS

The first report of the discovery of tachykinins was given by Von Euler and Gaddum (Von Euler & Gaddum 1931). Substance P was the first neuropeptide discovered and was described as "a substance which lowered arterial blood pressure of an atropinised rabbit by peripheral vasodilatation". Later, in 1964, Gaddum named it substance P (SP), in which the "P" stands for the powder obtained after the extraction procedure. The chemical formula of SP remained unknown until 1970 when Leeman *et al.* reported the amino acid sequence of SP (Pernow 1983). In this period some other peptides with effects similar to SP were extracted from amphibian tissues. Erspamer and colleagues observed that SP and non-mammalian newly discovered peptides, (like Eledosin from octopus salivary glands), have similar biological activity (Erspamer 1981). Following this, other homologues of SP have been discovered, and all these peptides have a common c terminal with a phe-xaa-gly-leu-met,NH2 sequence. In 1985 two other peptides of this family were discovered, these are now

known as Neurokinin A (NKA) and Neurokinin B (NKB)(Erspamer 1981 ; Pernow 1983).

1.7.1 nomenclature:

After the discovery of each peptide, a specific name relating to its action was given to it, and so there was a different name for each peptide. Finally Erspamer and co-workers suggested the term of "Tachykinins" for these peptides, because their effect on the guinea pig ileum contraction was much quicker than of other peptides such as bradykinin (Bertaccini 1976). Later, the term "Neurokinin" came to be used for these peptide because all these peptides are derived from neurones. Presently the term 'tachykinins' is used for non-mammalian neuropeptides and 'neuropeptides' for mammalian neuropeptides (Regoli *et al* 1987a). At the Montreal IUPS satelite symposium, "Substance P and neurokinin" (1986) the following names were adopted for neuropeptides: 'SP' for Substance P, 'NKA' for Neurokinin A (also known as Neurokinin Alpha or neuromedin L or substance K), 'NKB' for Neurokinin B (also known as Neurokinin beta or neuromedin K) and 'TK' for tachykinins as a group defined by the C terminal (Henry 1987). Some newer neuropeptides with a common C terminal sequence have been isolated since and these are called "NPY" for neuropeptide Y and "NPK" for neuropeptide K (Leeman 1987).

1.7.2 Synthesis:

Two genes which encode the peptide sequence of neurokinins in mammals have been identified. The preprotachykininI (PPT1, also known as PPTa) encodes the sequence of SP and Neurokinin A, and preprotachykinin II (PPT2 or PPTb) encodes Neurokinin B (Nawa *et al*

1983; Bonner *et al* 1987; Nakanishi 1991). In translation different mRNA are produced by genes and these different mRNAs produce three different precursors, before finally, different neurokinins can be produced (Helke *et al* 1990). Neurones are the major source of tachykinin both in the CNS and in the peripheral nerves, but the major sources for tachykinins are in the peripheral endings of capsaicin-sensitive primary afferent neurones. TK synthesis occurs in the cell bodies of the capsaicin-sensitive primary afferent neurones which are located in the dorsal root ganglia (DRG) by the expression of PPTI. There is not enough evidence for the presence of PPT II in DRG (Mussab *et al* 1993; Maggi *et al* 1993a). Tachykinin-like immmunoreactivity has been detected in other tissues and cells, for example in the enteric neurone system (Deacon & Colon 1987), the heart (Hoover 1987), the primary afferents of the knee joint (Hanesch *et al* 1991), the upper and lower part of airway (Maggi 1990), the carotid artery (Kusakabe 1994) and in urinary bladder (Maggi *et al* 1993a; Ekstrom *et al* 1994;)(Fig. 1.11a & b).

1.7.3 Storage: Tachykinins are stored in large granules in synaptic vesicles of diameter up to 100 nm in diameter (Otsuka & Youhioka 1993). Substance P is found only in small or intermediate size vesicles (less than 50 nm) which indicates that it can be found in the unmyelinated fibres (Hanesch *et al* 1991).

1.7.4 Release: The mechanism of NK release is not fully understood, but an active calcium dependent release, high frequency dependent release, low K^+ ion concentration dependent release and finally capsaicin induced release have all been demonstrated (Maggi

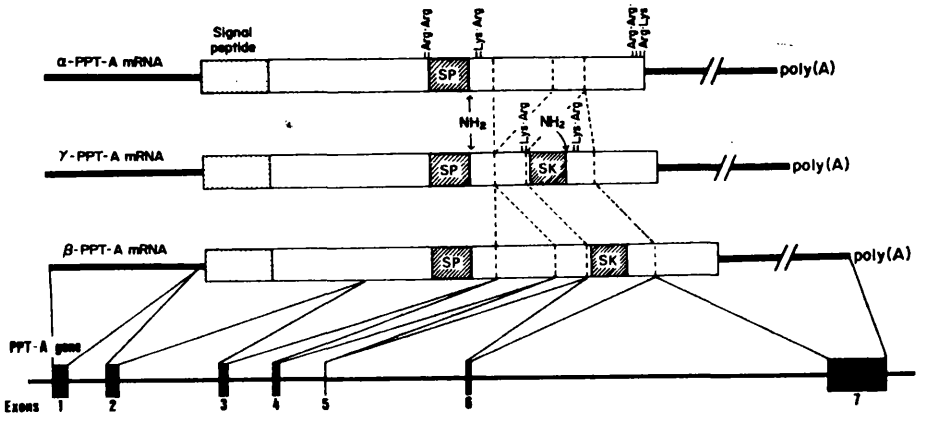


Fig 1.11A: Structure relationship between three preprotachykinin (PPT). A mRNAs and their coding gene. From Nakanishi 1991

SP	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
NKA	H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
NKB	H-Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2
Kassinin	H-Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2
Eledoisin	Pyr-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
Physalaemin	Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2
Neuropeptide K	H-Asp-Ala-Asp-Ser-Ser-Ile-Glu-Lys-Gln-Val-Ala-Leu-Leu-Lys- Ala-Leu-Tyr-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
Neuropeptide γ	H-Asp-Ala-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2

Fig 1.11B: Amino acid sequences of natural Tachykinins. The C-terminal sequence common to the peptides of tachykinin family is underlined. From Maggi 1993a

1993a). There is no report of physiological release of neurokinins except in the gut (Otsaka & Youhioka 1993; Maggi *et al* 1993a)

1.7.5 Degradation:

These peptides are highly susceptible to hydrolysis by tissues and cytosolic and membrane peptidases. Neuronal uptake has a minor role in terminating of the effect of tachykinins (Mussap *et al* 1993), therefore many of body organs are capable of the breaking down the tachykinins. The kidneys are the most effective organs and the spleen and liver are also effective organs in eliminating SP (Pernow 1983). Neutral endopeptidase (NEP) is widespread in brain, respiratory tract and gut (Maggi *et al* 1993a), and NEP breaks downs SP at three different hydrolysed bonds, 6, 7 and 9, these bonds are in amino acid group of hydrophobic residuaes (Matsas *et al* 1984). It has been shown that Neurokinin A and B are a good substrate for endopeptidases (Turner & Hooper 1987). Angiotensin converting enzyme can also hydrolyse neuropeptides in the C terminal region and certain other areas (Matsas 1984; Turner & Hooper 1987).

1.8.1 TACHYKININ RECEPTORS:

After the discovery of neurokinins a vast effort was begun to identify the Neurokinin receptors. Erspamer reported the first receptor for SP in 1981 (Erspamer 1981) and further research lead to the discovery of NKA and NKB receptors. There are now known to be multiple receptor and binding sites for tachykinins (Iverson *et al* 1987; Lee *et al* 1987). The nomenclature for these receptors is based on three tachykinins; NK1 is for receptors with highest affinity to substance P rather than NKA and B, NK2 has a higher affinity to NKA and NK3

has a higher affinity to NKB rather than the others (Maggi *et al* 1993a)(fig. 1.12a). These three neurokinins are therefore full agonists for these three receptors but with different affinities (Regoli *et al* 1987b).

NK1 which has 407 amino acid sequences has the highest affinity for SP and has been isolated from different tissues. There is a heterogenicity in neurokinin receptors which is based on; a) the affinity of the agonist for the receptor, b) metabolic breakdown and c) whether the glycosilated or non-glycosilated form of NK1 receptor is present (Lew *et al* 1990; Liu & Quirion 1991). This heterogenicity led to the development of very specific antagonists and the discovery of NK1 subtypes (Hall *et al* 1994; Meini *et al* 1994). NK1 receptor subtypes have been found in visceral smooth muscle and glands, respiratory, and urogenital system, blood vessel muscle, epithelium, exocrine cells and lymph nodules (Mussab *et al* 1993; Maggi *et al* 1993a)(fig. 1.12b).

Compared to other receptors, NK2 is the most responsive receptor for NKA. The first NK2 receptor was found in the bovine stomach with 384 amino acids and other forms of NK2 receptor have been found, for example in the rat stomach (390 amino acid sequence), human trachea (398 amino acid), human jejunum (398 amino acid) and in mouse genome (384 amino acid sequence) (Maggi *et al* 1993a). Different NK2 subtypes have been found, in hamster pulmonary artery (Maggi *et al* 1989), in human bronchus (Astofi *et al* 1994), and rat spinal cord. In general two subtypes of NK2 receptor have been described, NK2a and NK2b (Mussab *et al* 1993).

	NK-1	NK-2	NK-3
Agonists order of potency	SP>NKA=NKB	NKA>NKB≫SP	NKB>NKA≫SP
Selective agonists	SP methylester [Sar^9] SP sulphone GR 73,632	[βAla^8] NKA (4–10) GR 64,349	Senktide [MePhe^7] NKB
Selective antagonists	L 668,169 GR 82,334 Spantide II FR 113,680 CP 96,345 RP 67,580	L 659,877 MEN 10,207 MEN 10,376 R 396 MDL 29,913 Actinomycin D	?
Monoreceptorial bioassays for TK receptors	Dog carotid artery Guinea-pig vas deferens Rabbit jugular vein Rabbit vena cava Mouse bronchus Guinea-pig urethra	Rabbit pulmonary artery Rat vas deferens Hamster trachea Human bronchus Human colon Human urinary bladder	Rat portal vein

Fig 1.12A: The pharmacology of tachykinin NK1, NK2, NK3 tachykinin receptors. From Maggi 1993a

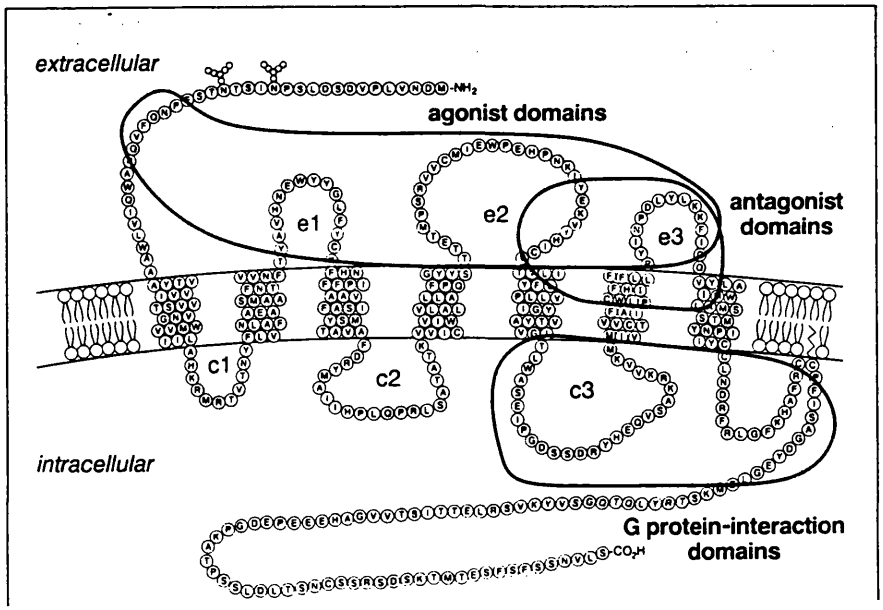


Fig 1.12B: Schematic model of structure of human NK1 tachykinin receptor showing various functional domain. **e**= extra-cellular loop: **c**= cytoplasmic loop. From Marso *et al.* 1989 in TIPS.

NK3 which has a 452 amino acid sequence is the most responsive receptor for NKB (Helke *et al* 1990). Recent findings about the human human NK3 receptor have revealed that 68% of its amino acid sequence is similar with NK1 and NK2 receptors (Buel *et al* 1992). Like other receptors, there is heterogenicity in NK3 receptors (Murray *et al* 1987). The NK3 receptor only has been found in the myoenteric plexus of guinea pig and the rat portal vein (Maggi *et al* 1993a) and in the gastro intestinal tract, oesophagus and urinary system (Mussab *et al* 1992; Maggi *et al* 1993a) but the numbers of receptors are much lower than other receptors.

1.8.2 Mechanism: the mechanism of action of neurokinin receptors are based on activation of the phosphoinositol pathway. Neurokinins activate this pathway via G proteins in the membrane and consequently there is increase in intracellular calcium (Ca^{2+}) (Hall 1987; Dam 1987). Neurokinins evoke membrane depolarisation, due to decrease of K^{+} ion conductance and inhibit the Ca^{2+} activated potassium channel (Otsuka 1993).

1.8.3 Agonists:

The natural agonist, SP has the highest affinity for the NK1 receptors and the most responsive agonist for the NK2 receptors is NKA, and NKB having the highest affinity for NK3 receptors. There is cross reactivity between these receptors and they respond to all of these peptides, but there are different levels of response (Drapeau *et al* 1987; Ireland *et al* 1991) . A great deal of effort went into the development of specific agonists and Watson (1983) developed the first SP agonist (Watling & Guard1992). Later different agonists for

tachykinin receptors were found, for example; GR73632 was developed as NK1 selective agonist which is a potent NK1 agonist either in vivo or in vitro. GR64349 was developed and confirmed as a potent and selective NK2 agonist. Senktide [(succinyl 1-asp^{6} , mephe^{8}) SP] was developed as a NK3 agonist and this is very potent and selective for the NK3 receptor; it is 10^{4} times more potent on NK3 receptors than NK1 and NK2 receptors (Buell *et al* 1992; Watling & Guard 1992) .

1.8.4 Antagonists:

The first attempt at developing a special neurokinin antagonist was by Leander (Leander *et al* 1981) by inserting a D amino acid in the backbone of the natural peptide. At this time different NK1 antagonists had been developed but most of them had low affinity for the receptor, and so attempts to find more specific antagonists led to a second generation of antagonists. The first antagonist of this generation was L659877 (L659874) which had a linear structure (Dion *et al.* 1990), and then MEN10207 was developed by Maggi *et al.* (Maggi *et al* 1993a; Quartara *et al* 1992). Recently the Fujisawa group developed a selective tripeptide NK1 receptor antagonist, FR113680 (whose amino acid sequence is AC THR-DTRP (CHO) PH-NMEB21) (Maggi *et al* 1993a). this compound originated from the octapeptide [d-pro^{4}D-TRP$^{7\text{-}9\text{-}10}$,Phe^{11}]SP 4-11. FR 113680 acts as a selective and competitive NK1 receptor antagonist. Later Fujisawa developed FK888 or 2-(N_ME) indolil-CO-HYP-NAL-NMEBZ1, from FR113680 which is an extremely potent and selective NK1 antagonist (Watling & Guard 1992; Fugi *et al* 1992)(fig. 1.,13). The Fujisawa group developed FK224 from a strain of *streptomyces*

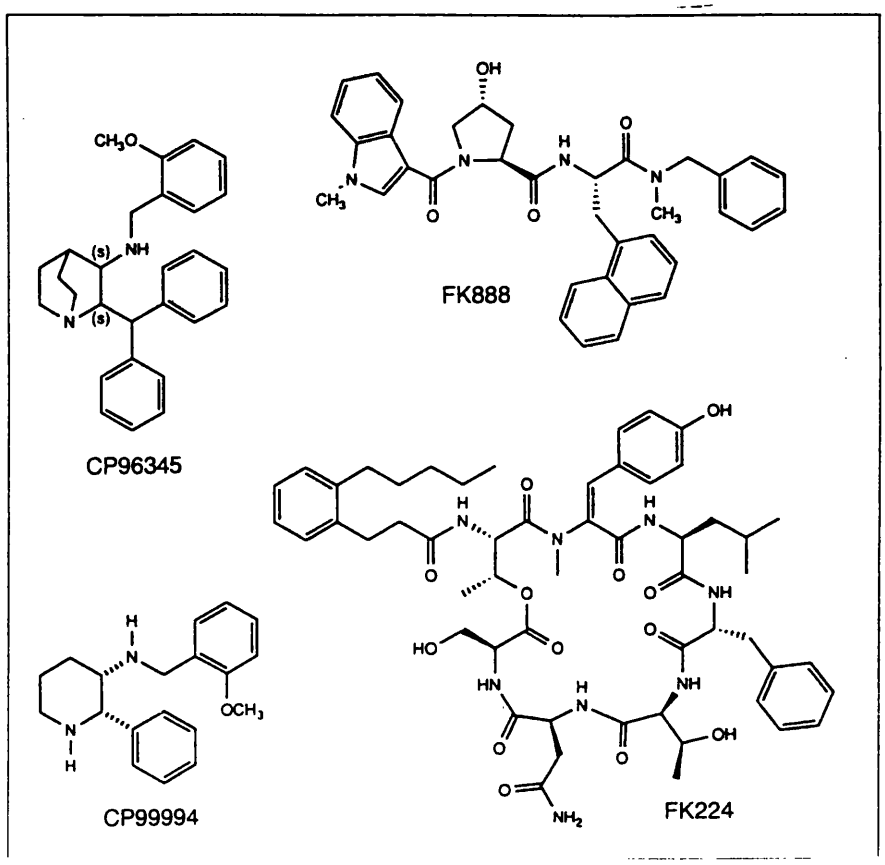


Fig 1.13A: NK1 tachykinin receptor antagonist

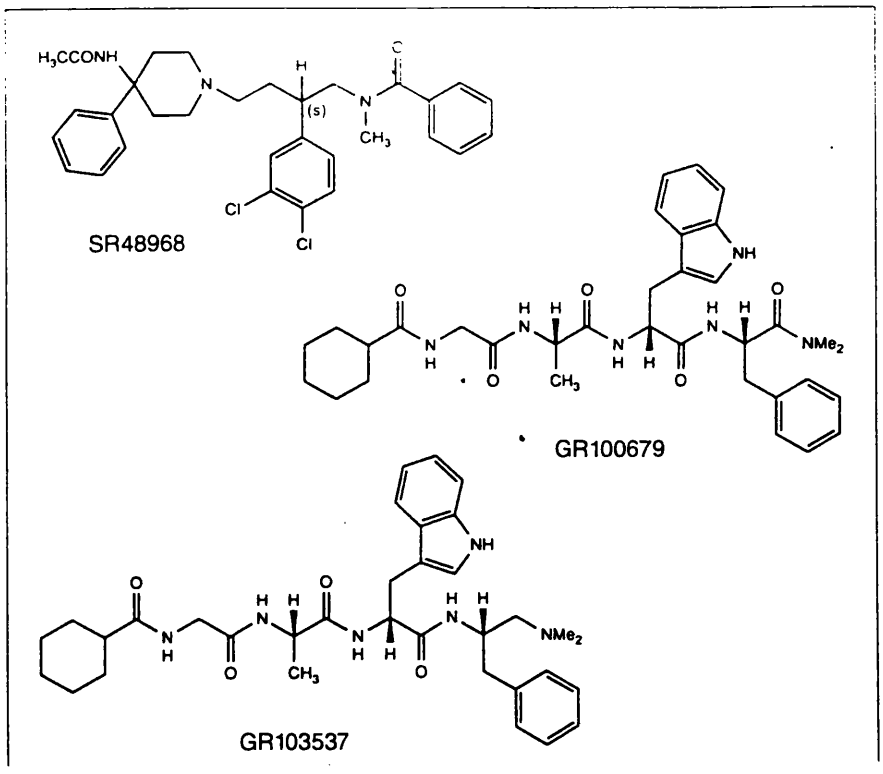


Fig 1.13B: NK2 tachykinin receptor antagonist

violaceo niger. This is a natural macrocyclic peptide, which is a NK1 and NK2 antagonist and inhibits the effect of both SP and NKA in vivo and in vitro (Morimoto *et al* 1992; Murrai *et al* 1992). These second generation antagonists generally were potent antagonists and were suitable for identifying neurokinin receptors. The third generation were defined as a nonpeptide antagonists, the advantage of these over the peptide antagonists being their resistance to degradation by tissue peptidases (Mussab *et al* 1993). Non peptide antagonists commenced with CP 96345 which is a potent and selective NK1 receptor antagonist (Maggi *et al* 1993a). A non peptide NK2 receptor antagonist (S) - N - Methyl-N [4-4 Acetyl Amino-4-Pheoxyl piperidinol - 2 - (3-4)] dichloro-phenyl benzamide or SR48968 was developed by Emonds (Emonds *et al* 1992; Advenier *et al* 1992) and is a very highly potent and specific NK2 receptor antagonist. This antagonist is a competitive antagonist with 100 fold higher affinity for NK2 receptors than other receptors (fig.1.13)(Watling & Guard 1992).

There has been little progress in developing NK3 antagonists and there are few reports in the literature (Petitet *et al* 1993). Recently [D-Pro^{2}, D - Trp^{6} ,^{8} , Nle^{10}] Neurokinin B has been reported as a NK3 antagonist, but a potent and selective NK3 antagonist has yet to be developed (Petitet *et al* 1993).

1.9 BIOLOGICAL ACTION

Neurokinin receptors are widespread and distributed in all body organs and they show different biological actions depending on the position of the receptor and the type of organ (Pernow 1983; Maggi *et al* 1993a; Otsuka & Youhioka 1993). SP acts as neurotransmitter in

peripheral afferent neurones and spinal cord. Release of SP is mediated by high concentrations of calcium and magnesium or by application of capsaicin or by noxious stimulation. SP elicits EPSP's (excitatory post synaptic potentials) and slow EPSP's in the second order neurone in the spinal cord and also in the mesenteric ganglionic cell. It is widely distributed in the central nervous system (CNS) and acts as stimulatory mediator in different parts of the CNS. There is a widespread presence of SP in salivary glands with a potent stimulatory action and in the GI tract where it affects movement via depolarisation of smooth muscle (Holzer *et al* 1980; Holzer *et al* 1981). SP also exists in nerves around the GI tract blood vessels and it can cause neurogenic inflammation in the GI tract. There is presence of SP in other parts of the GI tract, such as the liver, gall bladder, and pancreas (Otsuka & Youhioka 1993). NK1 receptors and SP are present in the respiratory system and have a role in vascular permeability control, sneezing, mucosal secretion and mucociliary activities (Maggi *et al* 1993b) NK1 agonists and SP are the most potent bronchoconstrictor agents known (Ireland *et al* 1991). Numerous SP immunoreactive nerve fibre have been found in the heart, and SP has a role in cardiovascular pain transmission, and in the carotid body, where it mediates the hypoxic response (Otsuka & Youkioka 1993). SP containing fibres have been observed in the adventitia and between the adventitia and medial border and it elicit a potent endothelium dependent vasodilatation which can lead to protein extravasation (Saito *et al* 1990). SP immunoreactive nerves are present in blood vessels around the renal cortex. SP is one of the most potent vasodilators and intra renal infusion of SP produce an increase in renal blood flow, urine volume, sodium excretion, increase in glomerular filtration rate and suppresses renin release. SP immunoreactive

neurones are also present in the pyelouretral region and with noxious mechanical or chemical stimuli, release of SP not only induces inflammatory vasodilatation but also enhances motility of the pyelouretral musculature and facilitates removal of the irritant (Maggi *et al* 1992; Otsuka & Youhioka 1993). Neurokinins are present in the male and female reproductive system, in the umbilical and placental fluid, and it seems that SP also has a regulatory role in foetal blood flow during development. The role of other neurokinins are as yet undiscovered (Di Tommoso *et al* 1987; Papka & Trauring 1987).

Neurokinins are present in the skin, muscles, and joints and have a role in controlling blood flow. In response to irritation and stimulation, they can be one of the majors factor mediating inflammation in the skin and in the joint by their action at nerve endings (Yaksh 1988).

1.10 NEUROKININS AND NEUROGENIC INFLAMMATION

Arthritis is a disease of unknown aetiology that manifests itself as a chronic inflammatory disorder of the synovial joints (Ferrell & Lam 1995). This inflammation can be generalised and spreads to multiple joints. Rheumatoid arthritis is an auto-immune disease which has characteristics other than joint inflammation, and the inflammation which results from peripheral release of neurokinins is termed neurogenic inflammation (Mapp *et al* 1993). It has been suggested that neurokinins could contribute to joint inflammation (Scott *et al.* 1994)

There is a vast network of myelinated and unmyelinated neurones in the articular capsule with free complex or encapsulated nerve endings. In normal joints small nerves are found and these are immunoreactive for neuropeptides. Calcitonin gene related peptide (CGRP), SP and NKA (Deizguerra *et al.* 1988) exist in the perivascular area. Twenty percent of these neurones, which are sensitive to capsaicin, are myelinated and the remainder are unmyelinated. It is generally thought that unmyelinated fibres are capsaicin sensitive (Kidd *et al* 1990). These unmyelinated neurones play an important role in inflammation and release of SP can be activated by electrical stimulation, noxious stimulation, heat and chemical irritation (White & White 1985; Otsuka & Youkioka 1993). The extravasation effect of neurokinins has been reported previously by Lam & Ferrell (1991a). Electrical stimulation of C fibres in posterior articular nerve (PAN), evokes plasma extravasation which is reduced by application of a SP antagonist (Ferrell & Russell 1985). Later, the involvement of neuropeptide and peptide containing neurones in inflammation and inflammatory disease became clearer, and the existence of post ganglionic nerve terminal substances as pro-inflammatory agents has been suggested (Basbaum & Levine 1991). SP has its effects partly via a direct effect on blood vessels and partly by an effect on mast cells (Basbaum & Levine 1991). Further reports have indicated that SP intensifies experimental synovitis suggesting that SP is a potent and specific stimulus for many of the features of RA in vitro and possibly in vivo SP can contribute to the development of several of the pathological findings in RA (Lotz *et al* 1987). Lam & Ferrell (1990) reported that SP can directly induce inflammation and that this effect is mediated by NK1 receptors (Lam & Ferrell 1993a). Inflammation is, in general, accompanied by protein

extravasation and it has been shown that neurokinins are involved as they can induce and enhance protein extravasation in joints (Lam & Ferrell 1991a) and both NK1 and NK2 receptors can mediate this protein extravasation in joints (Scott *et al.* 1994). In the bronchial tree, NKA has been shown to induce a dose dependent leakage of I^{125} labelled plasma protein and fibrinogen extravasation which is completely inhibited by the non peptide NK2 antagonist SR48968. This shows the powerful role of NK2 receptors in mediating plasma extravasation, and this effect is a direct one on NK2 receptors and is not by activation of histamine release. In the guinea pig dura, NK1 receptors induce plasma protein extravasation (O'Shaughnessy & Conneer 1993). These effects can be accompanied by vasodilatation and increasing blood flow both in the skin (Brain & Williams 1989) and knee joint (Lam & Ferrell 1993b). This effect is exaggerated in inflammation and this suggested the presence of multiple neurokinin receptors in the knee joint which could mediate the inflammatory response (Scott *et al.* 1992; Tousingnant *et al* 1994). There are some reports of the bilateral effects of neurogenic inflammation of neuropeptides, which can contribute to the bilateral inflammation process (Bileviciute *et al* 1993; Mapp *et al* 1993; Kidd *et al* 1989). This symmetrical effect suggests that this is mediated by a neuronal pathway with a precise topographic projection (Kidd *et al* 1989). Mono arthritis most commonly leads to the induction of bilateral arthritis which implies the involvement of the nervous system and sensory neuropeptides in this condition (Mapp *et al* 1993). In arthritis and inflammation the distribution and response of receptors to neurokinins will change. In acute inflammation there is an increase in response to neurokinins but in chronic arthritis the response to

neuropeptide is attenuated (Scott *et al.* 1992; Lam & Ferrell 1993b; Mc Dougall *et al.* 1994).

1.11 CALCITONIN GENE RELATED PEPTIDE (CGRP)

The first indication of the existence of CGRP, in the rat, was obtained in 1983 (Rosenfield 1983). Later, a similar peptide was discovered in humans (Morris *et al* 1984) and after this other reports were published about CGRP with a slightly different structural sequence.

The discovery of CGRP was one of the first examples of a biologically active agent to be discovered by application of recombinant DNA technology in the absence of prior biological information (Holzer *et al* 1994). CGRP is a 37 amino acid polypeptide and the amino acid sequence varies in different species of mammals and birds. In some species for example the rat and man, two different types of CGRP have been isolated (fig. 1.14).

In man, the CGRP gene is transcribed from a larger precursor RNA with 6 Exons which subsequently split into α- CGRP and β-CGRP. The primary expression is the same in neural tissues; splicing to each CGRP is fully and precisely regulated by the CGRP producing tissues, and translations from RNA are in different pathways depending on tissue specificity and tissue factors (Bennett & Amara 1992). CGRP can be found in body fluids and tissues, but the molecular weights differs in each (Fig. 1.15) (Wimalawansa 1993).

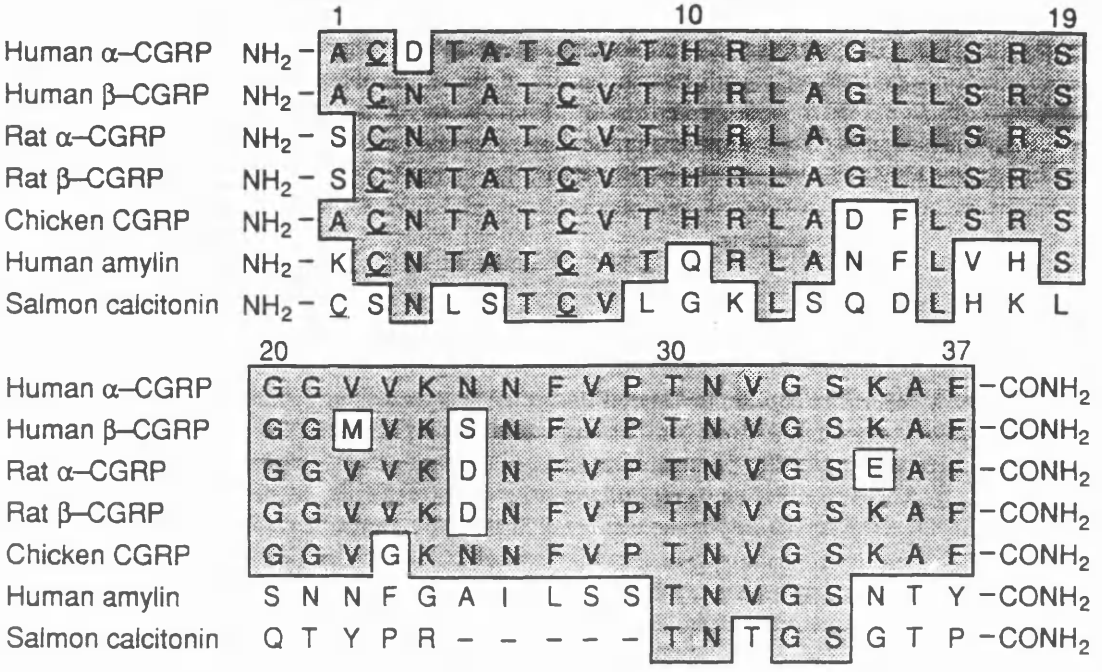


Fig 1.14: Structure of human and rat α and β CGRP, chicken CGRP, human amylin and salmon calcitonin. Area of sequence identity are shaded From Paynor 1992)

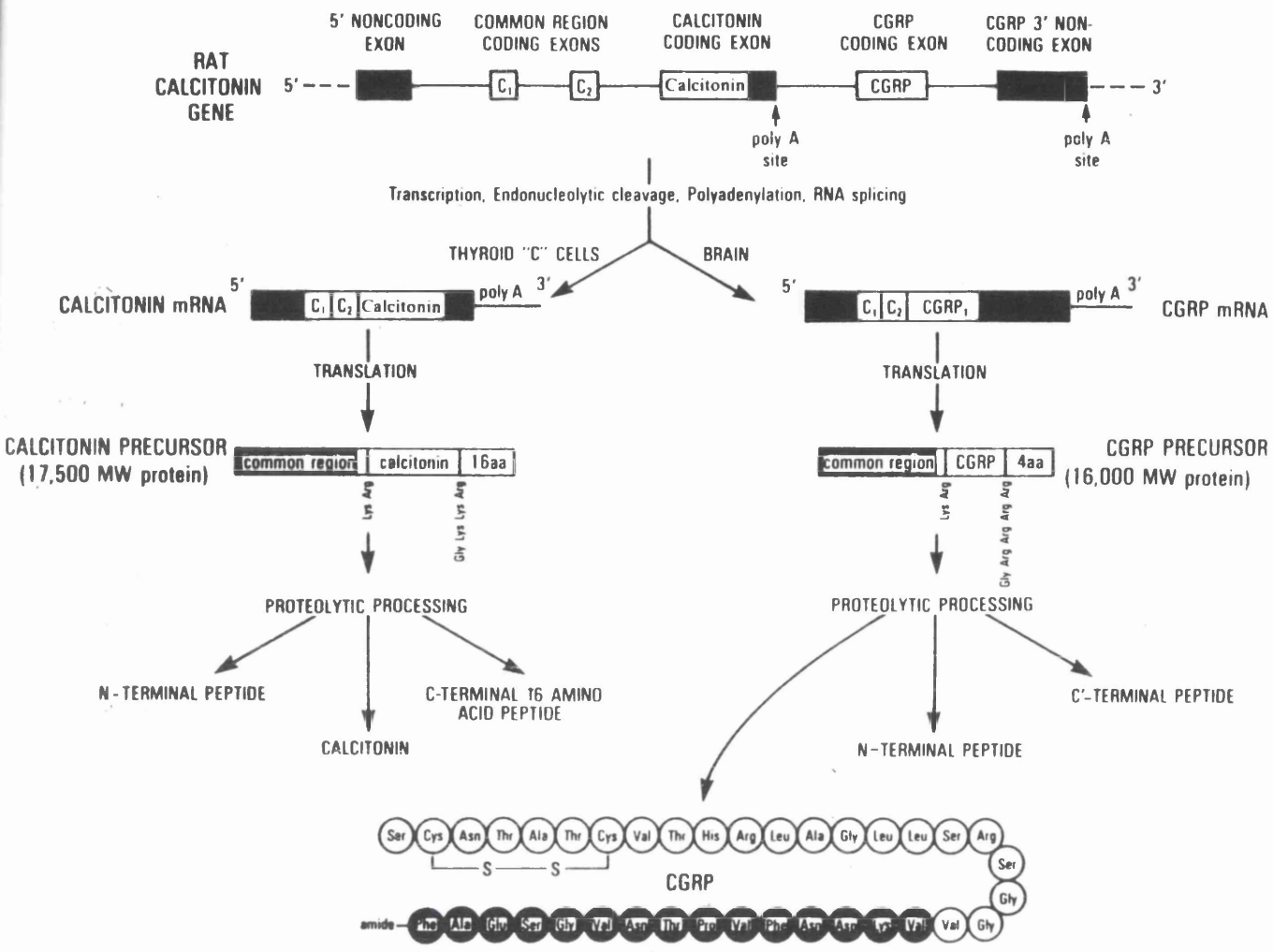


Fig 1.15: Alternative RNA processing pathways in expression of the calcitonin gene, which predict the synthesis of a novel neuropeptide in the brain. From Rosenfield 1983 in Nature

1.11.1 CGRP receptors:

CGRP receptors have been isolated from different organs. The molecular weight of the CGRP receptor is 66000 D and it consists of one subunit. The suggestion that there may be of different types of CGRP receptor, because of different responses in different organs, has not yet been proved but it is possible that there is more than one class of receptor for CGRP (Wimalawansa 1993). Quirrion *et al* (1992) suggested the existence of at least two classes of CGRP receptor which he has named $CGRP_1$ and $CGRP_2$. $CGRP_1$ receptors appear to be sensitive to antagonistic activity of extended C terminals, and $CGRP_2$ receptors possesses high affinity to a linear analogue of [cys (ACM)2,7] αCGRP and a third subtype now can be recognised by its low affinity to both of the others (Quirrion *et al* 1992). Poyner (1992) classified four subtypes of CGRP receptors:

a) CGRP1a, which is antagonised by CGRP(8-37). Rat α CGRP is equipotent with human α CGRP at this receptor

b) CGRP1b, which is sensitive to CGRP (8-37), and is 10 fold more potent than human α CGRP

c) CGRP2, with a low sensitivity to CGRP (8-37)

d) CGRP3, which is CGRP and Calcitonin equipotent and detected only in binding studies (Poynor 1992).

1.11.2 Distribution and mechanism of action

Different mechanisms of action have been suggested for the effect of CGRP. Quirrion *et al* (1992) believes that it acts by coupling

with G proteins, and by activating other mechanism in the cell. Anderson & Clasent (1989) reported that CGRP in the soleus muscle stimulates active Na^+- K^+ transport and causes a decrease of intra-cellular Na^+ and a minor increase in K^+ and a hyperpolarization which can be blocked by ouabain. Kageyama *et al* (1993) reported that, in the porcine coronary artery, CGRP acts via a cAMP dependent mechanism and Momse *et al* (1993) reported a similar mechanism in the gubernaculum of the urinary tract.

1.11.3 Agonist and antagonist: In addition to natural CGRP, CGRP of the [cys (Acm)2,7] type are selective agonists, and only CGRP(8-37) and (12-37)CGRP, which act on different CGRP receptor subtypes have been shown to be antagonists (Poyner 1992; Hayes 1993) .

CGRP is co-localised and co-released with Calcitonin in parafollicular C cells of the thyroid gland, and with SP in sensory nerves (Quirrion *et al* 1992), and also with acetylcholine in motoneurones (Csillik *et al* 1992). This assumes a regulation of the number and function of actylecholine receptor molecules. CGRP is present in variety of central and peripheral neurones and also seems to accumulate at the motor end plate (Kashihara *et al* 1989) and in a very wide range of body organs, such as gut and in mesentric resistance vessels, and it also regulates further CGRP release via a negative feed back mechanism (Nuki *et al* 1994). It also inhibits pancreatic enzyme secretion via the vagal nerve (Ying *et al* 1993), and has a regulatory function in the respiratory system (Tung cheng *et al* 1993), circulatory system, muscle, joints and in the brain (Sarina *et al* 1992).

CGRP shows diversity in its effects. It is likely to have role as a neurotransmitter, neuromedulator, local hormone, and as a trophic factor which causes rapid changes in neuronal activity, and relaxation of many types of smooth muscle. It also has a role in metabolism, and changes in gene expression (Royner 1992). CGRP is a potent vasodilator, acting on blood vessel smooth muscle and inducing hypotension in conscious animals (Abdehrahman *et al* 1992). The vasodilatation and hypotentsive effect of CGRP acts via endothelium derived nitric oxide (NO), and not via opening K^+ ATP channels and so can be attenuated by L NAME (Abdehrahman *et al* 1992). In muscle CGRP has a potent effect on glycogen synthetase activity and muscle glucose metabolism and has a physiologic role in skeletal muscle glucose metabolism (Rosseti *et al* 1993) . In the bloodstream there is a large concentration of CGRP with an unknown source and it may have an important role in the regulation of both systemic and local blood flow, since it is stored in the peripheral terminals of sensory nerves. It is possible that in ischaemia it will be released and regulate blood flow (Goto *et al* 1992). Recently CGRP has been implicated as one of the major factor in menopausal flushes (Tung Cheng *et al* 1993). CGRP is the most potent vasodilator known (Brain *et al.* 1985) and this effect is a direct action of CGRP and does not involve any other vasodilator mediator. This vasodilator effect of CGRP in some vessel in rat aorta is dependent on endothelium (Brain *et al.* 1985) and in other vessels is independent of endothelium (rat coronary artery)(Hiraty *et al.* 1988).

In endothelium independent relaxation, CGRP acts directly on vascular smooth muscle receptors and it has been demonstrated that it

can stimulate adenyl cyclase in these tissues and provides a biochemical pathway leading to relaxation of smooth muscle (Hiraty *et al.* 1988), or open the ATP sensitive K^+ channel which leeds to hyperpolarization and relaxation of the muscle (Nelson *et al.*1990)

In the endothelium dependent vasodilator effect of CGRP, activation of CGRP receptors in certain vascular beds releases Nitric oxide (NO), which dilates vessels by stimulation of the guanylate cyclase (Holzer 1994).

1.11.4 CGRP in inflammation:

It is now well established that CGRP containing neurones exist in the knee joint (Hanesch *et al* 1992). There is an increase in the release of CGRP during inflammation (Donnerer *et al* 1992), compared with normal conditions. CGRP on its own can participate in the formation of intra-articular oedema (Cruwys *et al* 1992), and even enhances the vasodilator action of other peptides by positive feed back (Lam and Ferrell 1993b). In conclusion CGRP can be an effective mediator of inflammation.

1.12. MEASUREMENT OF BLOOD FLOW

Measurement of blood flow in different organs became a necessity after the discovery of the circulation but measurement of joint blood flow with its special anatomical compartment has proven to be much more complicated. A variety of methods have so far been tried and the first to try to estimate joint blood flow was Horwath & Hallander (1949) who used a calorimetry method involving

intraarticular temperature. Skin temperature depends on the balance between two type of processes, those by which heat is dissipated from skin surface to the atmosphere (conduction, convection, evaporation and radiation) and those which deliver heat from the body to the skin surface by tissue conduction and flow transport. The heat coming to surface of skin comes from the deeper tissues which are warmer due to the reactions of cellular metabolism, and the flow rate of the heat depends upon tissue heat generation and thermal conductivity which varies in different situations. The advantage of this method was its simplicity and ease of measurement but its disadvantage was the large variation in the skin temperature and the poor correlation between temperature and blood flow.

Venous occlusion plethysmography has been used to measure blood flow, the non invasive nature of this method makes it mostly applicable in man. This is a classical method which was first studied by Brudie and Russell (1905) for measuring regional limb blood flow. The basic principle is that changes in regional volume of a limb or organ are directly related to changes in blood flow. The usual protocol is to perform venous occlusion with a proximal cuff inflated to 40-60 mmHg so that induced changes in volume are directly related to arterial inflow only. The limitation of technique is obvious, the usage being limited to those whole areas which can be occluded measurement is not continuous and also it is not possible to tell from which tissue the flow is mainly derived (Bonny *et al* 1952). Cobbold & Liewis . (1952) measured knee joint blood flow by the bubble method, first introduced by Bronner *et al* (1949). They studied the effect of

heating and cooling and of adrenaline on blood flow, and found a correspondence between the blood flow and these stimuli.

Transcutaneous PO_2, originally designed for neonatal monitoring of arterial PO_2, measures only flow to the skin and flourescein angiography (Oberge *et al* 1982) in which sodium flourescein is injected intravenously to study skin illuminated with UV light, is similarly limited (Cobbold & Leiwis 1956).

Radioisotopic methods for example radioactive xenon (^{133}Xe) and technetium (^{99}Tc) have been used for local skin blood flow measurement and their washout rates are proportional to flow. The radioactive ^{133}Xe technique has also been used to measure blood flow through many organs (Dick *et al* 1970a) including joints (Liewick & Dick 1981) and ^{99}Tc has been used in many organs in order to measure blood flow which is based on uptake of the isotope ^{99}Tc (Dick *et al* 1970b).

Recently a radiolabeled microsphere technique has been used to measure of joint blood flow in animals. By this method the number of microsphere flowing into an area provided an estimate of blood flow (Ferrell *et al.* 1990).

In the past 10 years Laser Doppler Flowmetry (LDF) has been used to monitor blood flow in various tissue (Obeid *et al.* 1990; Lam & Ferrell 1991b;Oberg *et al* 1982; Shepherd 1987). Since this technique is used in most experiment in this thesis, considerable space will be devoted to description of its operation and use. LDF relies on the Doppler shift effect, a shift in frequency which photons undergo when they collide with a moving object, in this case moving red blood

cells. The Doppler shift is illustrated in practice by the change which can be heard in the sound of the siren of a moving train as it approaches and recedes.

1.12.1 History of Doppler : John Christian Doppler (1803-1850) delivered his famous theory in 1842, and in that theory, the correct elementary formula is derived from the motion between the source and the observer along a line between them. The extension of this theory to the motion of both source and observer at the same time appeared in 1846, in which he mentioned the application of the theory both in acoustic and optics (Woodruff 1971). This theory was later verified by Ballot (Woodruff 1971).

1.12.2 Discovery of Lasers: Townes (1951) knew that as the wavelength of microwave radiation grew shorter its interaction with molecules became stronger and made it a more powerful spectroscopic tool. Making a device to generate the required wavelength became possible in 1953, which was called a MASER (Microwave amplification by stimulated emission of radiation) but Townes realised that shorter wavelengths beyond the microwave, infrared and visible light, probably offered even more powerful tools for spectroscopy (Karles 1988). In 1957 Townes discussed this matter with Schawland and the possibility of making this device, and after eight months they made their first LASER (Light amplification by stimulated emission of radiation) device. In 1960 a more practical Laser, the Ruby Laser was built by Majman. The most commonly used Laser now a days is a Helium-Neon Laser first operated by Javan in 1961. Many years elapsed between the marriage of the Doppler theory and the discovery of the Laser.

Attempts using this technique as a velocimeter started early in 1964. The high intensity and narrow line width of the Laser quickly converted the light beating phenomenon into a standard laboratory technique and the final step in the development Laser Doppler Velocimeter (LDV) was combination of the laser beam with the Doppler effect. Light scattered by particles or impurities in a moving fluid will be Doppler shifted by an amount proportional to the local fluid velocity and by mixing the scattered light with unscattered light Yeh & Cummins (1964) succeeded in measuring reactive change in blood flow and Laser Doppler Flowmetry (LDF) was born with this experiment (Shepherd 1990).

1.12.3 Principle of operation: The principle of LDF is based on the fact that a light beam from a low power laser source is directed via an optical fibre to the tissue, and the light scattered back from the tissue is collected by one or more other optical fibres and analysed. All the fibres are arranged in parallel within a single probe. Light which is scattered from moving blood cells undergoes Doppler shifting, the change in frequency depending upon the velocity of the cells. The red blood cell moves with a velocity of about 0.4-0.8 mm/second in capillaries (Fronken *et al.* 1977). Since the microvascular bed constitutes an intricate network of interlinking small blood vessels, the angle between the red blood cell velocity vectors and the beam propagation vector of the diffused scattered light can be regarded as randomised (Bonner & Nossal 1990). In cells which are moving away from the light source at velocity of 1mm/sec, the frequency falls about 4KHz and in the cells moving at the same rate in the opposite direction the frequency is increased the same amount (Kageyama *et al.* 1993).

From the alteration of the photon current, an output signal that is related to the flux of red cells in a specific tissue volume is generated. Fig. 1.16 presents a simples schematic diagram of the process. Special processing must be used to attenuate noises from the system and different adjustments must be made for the measurement of different ranges of blood flow (Obeid *et al.* 1990).

1.12.4 Depth of penetration: In human skin the red laser beam (wavelength 633nm) can penetrate and detect flow in vessels up to 1-2 mm below the skin surface. In the gut, it has been shown that the beam can detect blood flow in vessels up to 6 mm below the gut wall surface. In the pulp of intact human teeth, with the combined thickness of the enamel and dentine, the depth of penetration is about 2-3.5 mm. There will not be a critical depth below which flow cannot be detected but the sensitivity of the detector will decrease progressively as the thickness of the overlying tissue increases (Wårdell 1991).

1.12.5 Performance of the Laser Doppler Flowmeter :

Both the performance of the LDF in comparison with other methods for tissue blood flow evaluation and its value in clinical and research applications have been assessed in number of investigations. The LDF technique has been compared with xenon wash out in human skin and the correlation coefficient was found to be about 0.9 (Stern *et al.* 1977), and also it was compared with heat clearance technique were the advantage of LDF was proved (Enkema *et al.* 1981). Comparison between LDF and the microsphere technique was performed by Win *et al.* (1994) and they found that LDF technique is a powerful and appropriate technique for the measurement of blood

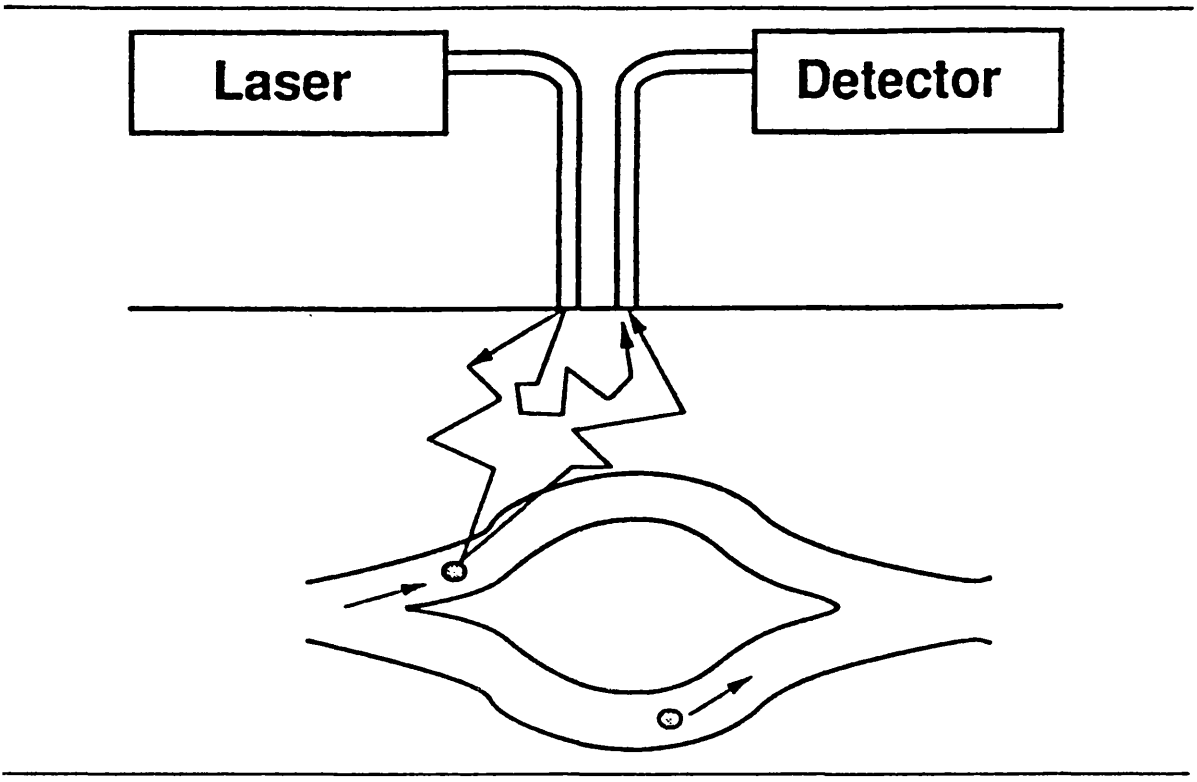


Fig 1.16: Laser- Doppler measurement of tissue perfusion. Laser light is typically delivered to tissue and returned to a detector by fibre optic light guides. Light in tissue is diffusely scattered by stationary tissue. Such light reaches the detector without being Doppler-shifted. Photons that encounter moving red blood cells experience a Doppler shift. From Shepherd 1990, in laser Doppler flowmetry

flow. Comparison between LDF and other techniques was performed to evaluate blood flow in different organs (VongSavon *et al.* 1993). LDF has also used for measurement of knee blood flow (Khoshbaten & Ferrell 1990; Najafipour & Ferrell 1993a; Najafipour & Ferrell 1993b).

1.12.6 Advantages and limitation of LDF

Advantages of this technique are that it is easily learned, non invasive and repeatable, unlike methods such as isotope clearance and radiolabelled microspheres (Hales *et al.* 1978) Linearity in the relation between the flux and red cell velocity, along with long term stability and fast response time make flowmeters ideal for monitoring rapid blood flow changes.

Quantitative absolute measurement of microvascular flow is not easy to perform with any available method. LDF gives an output value depending on the flux of red blood cells which is in arbitrary units The meaning of this value as a true representation of blood flow is problematic since exact penetration depth, vessel diameter and the exact angle between the laser beam axis and the flow axis is uncertain at the given point in time.

Red blood cells are transported close to each other possibly in Rouleaux formation, which can act as a larger particle when meeting laser light causing greater forward scatter and consequently underestimating the flux of these cells in the larger vessel. And finally another limitation of this technique is the extreme sensitivity to movement artefacts (Bonner *et al* 1990).

Despite these limitations, LDF demonstrates a good performance for the measurement of blood flow has been established as a good technique for this purpose and is being used in an ever increasing number of applications.

1.13.1 LASER DOPPLER PERFUSION IMAGING (LDI):

This is a system which allows multiple points on tissue to be scanned in a short space of time so that a larger area of tissue perfusion can be mapped. LDI is a new improved apparatus which can capture, analyse, process and display colour-coded images of tissue perfusion. A conventional scanner has two mirrors, the positions of which are controlled by two stepping motors, which guide a low power laser beam to the tissue surface (fig. 1.17) The mirrors move the laser beam sequentially over tissue, step by step, through a maximum of (64 X 64) pixels, making a total number of 4046 pixels. This area can covered in 4.5 minutes and represents 12X12 cm^2. At each point the beam penetrates tissues, depending on the power of the laser beam and the tissue structure, and light is scattered back and detected by a photo detector positioned in the scanner head. The detector transforms scattered light to equivalent electrical signals and each of these transformed values is stored in the computer memory for further signal processing and data analysis. After termination of the scanning procedure over the area, a colour-coded perfusion image can be displayed on a monitor and then the software generates perfusion values and allows statistical and data analysis (Wårdell 1991). The system also comprises a personal computer equipped with an analogue

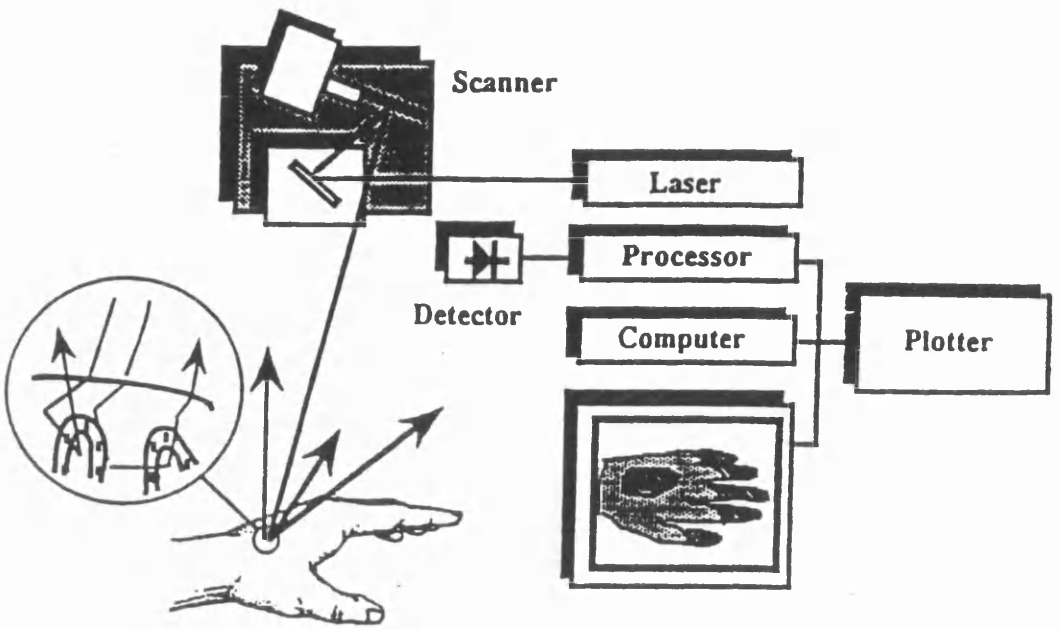


Fig 1.17: Block diagram of laser Doppler imager. From Wårdell 1993

to digital converter board (DT2811 data translation Inc USA), a signal processing unit installed in the computer for control of the stepping motors and also a detector amplifier in the scanner head. The scanner head is fixed to a laser beam generator tube. This machine can be situated on a tripod for ease of adjustment over tissues. A colour plotter is connected to the computer for a hard copy of images. Because ambient light can interfere with the measurement lights must be switched off or tissues covered by black fabric when in operation.

1.13.2 Mode of action: At the end of a scan the following steps can be carried out:

A) Signal processing: After eliminating signal noises, the processed signal scales linearly with tissue perfusion and a correction factor is added, derived from the angle between the detector and measurement area(fig. 1.18) (Wårdell 1991).

B) Image generation: The signal consist of two separate parts:

1) Total light intensity (TLI) which can be used to separate the background from the tissue

2) Perfusion image data which now can be displayed on a monitor. The image can be presented either in absolute colour code or relative colour code mode.

In relative colour code mode the highest value will be set to 100% and other values will be scaled to that. In absolute colour code mode the operator can select the highest and lowest value. The selected upper value sets to 100% and lower value sets to 0 % and the resulting image will appear in six different colours and each point will

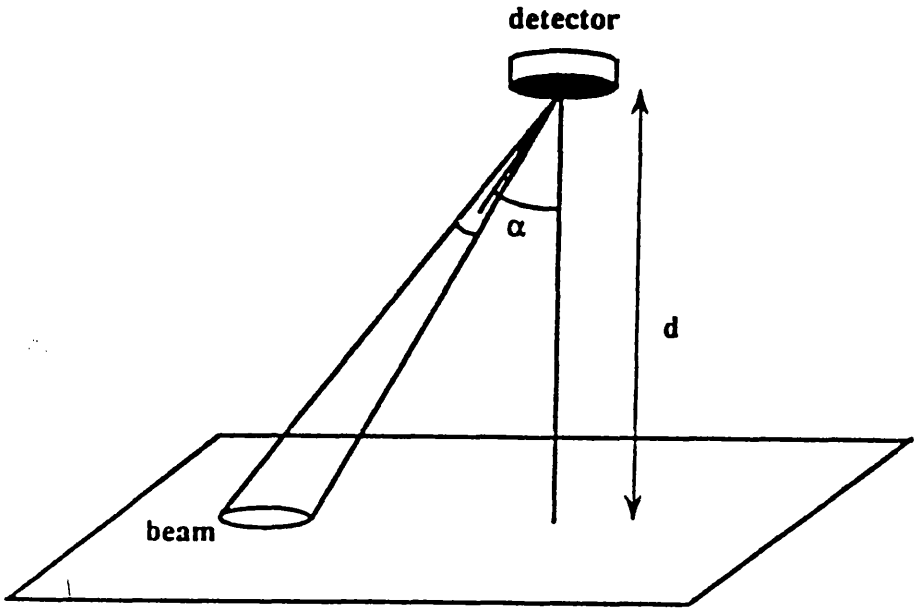


Fig 1.18: The position of the detector in relation to the light spot on the tissue surface. From Wårdell 1991

have its own specific value. Values between 80-100% are coded brown, 64-80% red, 48-64% yellow, 32-48% green, 16-32% blue and 0-16% navy blue (fig. 1.19). Other additional information is also given on the final image.

Advantages of this technique are generally similar to LDF which was described earlier. Absence of continuous measurement of blood flow is however a limiting factor. Movement artefact can still be a problem but less so than with LDF.

1.13.3 Data analysis: A specially written software package can be used and different functions are available in this programme which can convert physiological perfusion data to the numerical data. In the statistics option, the area of the image can be selected and within the selected area, the total number of pixels can be measured and a plot (histogram) of colour-coded pixels can be shown and the mean, standard deviation and number in the sample are calculated and displayed. Further statistical analysis can be performed on this numerical data.

LDI is well correlated with perfusion, the sensitivity of LDI has been compared with other known techniques and in all comparisons there has been a close correspondence between LDI and those other techniques (Wårdell *et al* 1993a) and correlation co-efficients have been greater than 0.97 (Wardell *et al* 1993b). The use of this technique is now increasing and blood flow in different organs is now being assessed with this technique. It has been used in the oral cavity and GI (Troilious *et al.* 1991) and also in animal research (Lam & Ferrell 1993a; Lam *et al* 1993D; McDougall *et al* 1994).

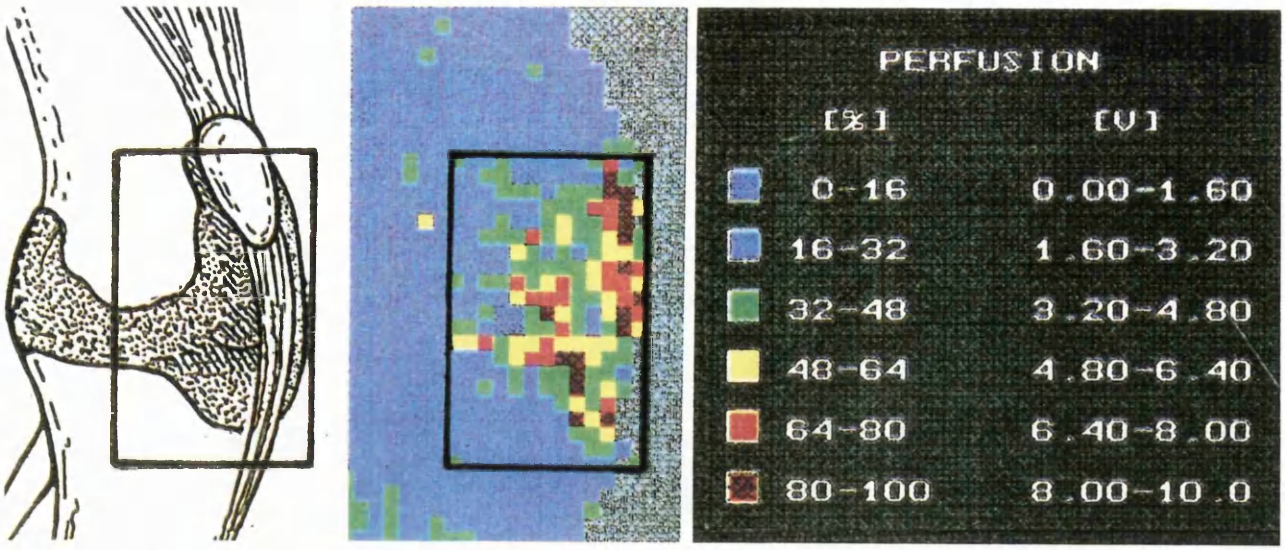


Fig 1.19: Laser Doppler perfusion image of medial aspect of the normal rat knee joint A) Diagramatic representation of one of the rat knee joint area scanned by the LDI. B) Colour coded perfusion image of the scanned area. For all measurements, the area of interest was outlined (black rectangle). Background values (grey areas) were ignored in these calculation. C) Colour coded for relative difference in perfusion, ranging from dark blue (lowest) to dark red (highest) and the corresponding voltage of the LDI signal (0-10 V). The same range of values were used in all scans including those illustrated in this thesis.

CHAPTER TWO

MATERIALS

AND

METHODS

MATERIALS

2.1.1) Instruments: The following instruments were used during the experiments:

Dissecting microscope M650, Wild Heerbrugg (Switzerland)

Dissecting table, Palmer (England)

Clock, Pye (England)

Balance Metler AE50, (Europe Instrument, Oxford)

Water bath, Grant instrument LTD (England)

Cordless cautery, Warecrest, C28(England)

Neurolog system, pressure amplifier, AC/DC, amplifier, Filter (England)

Laser Doppler Perfusion Imager, Lisca Developments (Sweden)

Multi-trace polygraph, Lectromed (England)

Shaker, Heidolph (Germany)

Polythene cannula size 2 and 3 (England)

Syringes size 1-20 ml, Plastipack (Ireland)

Surgical instruments and operating table accessories (from different sources)

Infusion pump, Gilson Minipuls (France)

Rack drive (departmental design)

Micropipet, Labsystem (Finland)

Digital thermometer, model THGH, Harvard, USA

Digital electronic micrometer , Mitomoto RS (Japan)

UV visible spectrophotometer model 4050, LKB (England)

Pressure transducer (Elcomatic EM 751)

2.1.2)Drugs: In addition the following drugs were used:

Freund's complete Adjuvant (Sigma)

Alcohol (70% and 100%)

Benzethenium chloride (Sigma)

Captopril (Sigma)

Lambda Carrageenan (Sigma)

EDTA (Sigma)

FK 224 (Fujisawa Pharmaceutical Co, Japan)

Heparinised saline

Neurokinin A (Novabiochem)

Neurokinin B (ICI)

Substance P (Cambridge research biochemical)

Calcitonin Gene-Related Peptide (Celtech; Bachem inc)

Cremophore (Sigma)

Normal (0.9%) Saline

Phenoxybenzamine Hydrochloride (Smithkline and French)

Capsaicin, (Fluka, Switzerland)

Hypnorm, (fentanyl fluanisone, Janssen)

Urethane , (ethyl carbamate, Sigma)

Valium (diazepam, Roche)

FK 888 (Fujisawa pharmaceutical Co, Japan)

SR 48968 (Sanofi research, France)

Isoproterenol (Sigma)

Euthetal - Pentobarbitone (120mg / ml)(May and Baker)

METHODS

2.2: ANIMAL PREPARATION: All experiments were performed on adult male Wistar rats (Albus ratus norvegicus), 300-500g weight. In total 480 rats were used for the work of this thesis.

2.3: ANAESTHESIA: Two methods of anaesthesia were used in the experiments, terminal and recovery anaesthesia. In terminal anaesthesia a solution of 25% urethane (Ethyl carbamate) (1-1.5 g / kg / IP) was injected into the peritoneal cavity, and in for recovery anaesthesia, Hypnorm 0.5 mg/kg /IM and diazepam 2mg/kg / IP were injected.

2.4: SURGICAL PROCEDURES

2.4.1: Carotid artery cannulation: In some experiments, the right common carotid artery was cannulated to monitor blood pressure during the experiment and for injection of drugs.

2.4.2: Anatomical aspect: The innominate artery, (brachio cephalic) derived from the aortic arch, extends anteriorly to the right as far as the level of the sternoclavicular joint where it is divided into the right common carotid and the right subclavian artery (Chess green 1955). The left common carotid arises directly from aortic arch. Both carotids after leaving the thorax, extend anteriorly in parallel with the trachea up to the level of the thyroid glands, where is a division into external and internal carotid.

In order to cannulate, the carotid (right, left or both) was freed from surrounding connective tissue and adjacent vagus, then the distal part (rostral) was ligated by a thread. Two other threads were used to

make two separate loops around the carotid and these were tied loosely, one proximal, the other distal. In order to stop blood flow a clamp could be used or it could be stopped by pulling up the free end of a third loop which had been placed previously. Then a small incision was made between these two ligatures, the heparinised cannula could then be inserted into the artery and secured in place by tightening the proximal thread. The cannula could be inserted deeper after removing clamp and then gently secured in its new place with the other pre-prepared threads.

The free end of the cannula was connected to a transducer which processed the signal which after amplification, was transferred to a chart recorder to give a continuous trace of blood pressure.

The pen recorder was calibrated between 0-200 mm Hg via a calibrating pressure pump.

2.4.3: Exposure of the knee joint: In all experiments where knee joint exposure was necessary, the anaesthetised rat was placed in dorsal recumbency and the fur over the knee joint shaved and the skin over the knee was excised in an oval shape, to expose the medial aspect of knee joint, and any connective tissues and fatty components over the knee joint were removed (fig. 2.1).

2.4.4: Denervation of the knee joint

In some experiments denervation of the knee joint was necessary. To achieve this, the anaesthetised rat was placed in dorsal recumbency and the fur over the thigh shaved and an incision made on the upper part of thigh. The skin was freed from underlying tissues

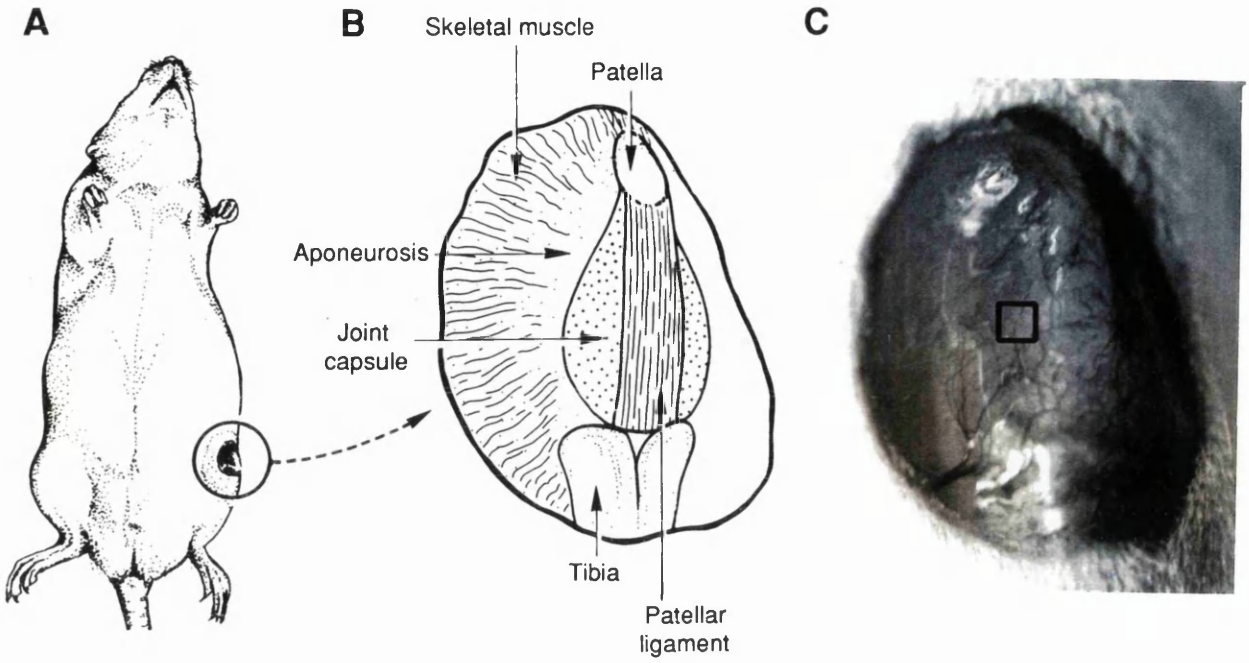


Fig 2.1: Photographic illustration of an exposed rat knee prepared for assessment of knee joint perfusion by laser Doppler imaging (LDI). (A) Drawing of rat indicating the exposed medial aspect of the knee joint which is photographically illustrated in (C). (B) Diagram shows the principal anatomical features of the knee joint (from lam & Ferrell 1993b)

by blunt dissection. The fat pad of the thigh was removed by electro-cautery and the saphenous nerve exposed. The nerve was freed from the adjacent artery and connective tissues and sectioned proximally. For rats in the recovery groups, the skin was sutured twice, once sub-cutaneously and then overlying skin to prevent wound reopening by the animal. In the terminal experiments the wound was closed by a Michell clips.

2.5: INTRA-ARTICULAR INFUSION

The anaesthetised rat was placed in dorsal recumbency, and the skin over the knee joints was shaved and carefully excised. Two 25 gauge needles, connected to an infusion pump were inserted into the joint, the inflow needle positioned so that it passed through the joint cavity (between the femoral condyles) to lie in the posterior region of joint, and the outflow needle was inserted so that its tip lay in the anterior region of the knee joint cavity through the patella tendon, or it could also be inserted in the small cavity under the patellar ligament (Fig. 2.2).

Saline (0.9%) was perfused into the knee joint at the rate of 0.1 ml /4 min. Joint aspirate was collected every 8 min with a collecting machine. A new sampling tube was placed every 8 min under the aspirate tube (fig. 2.3).

2.6: MICROTURBIDIMETRY:

This procedure is necessary to measure the amount of protein in the joint aspirate. After collecting samples, reagents were added to measure the protein content of each sample. The method is based on

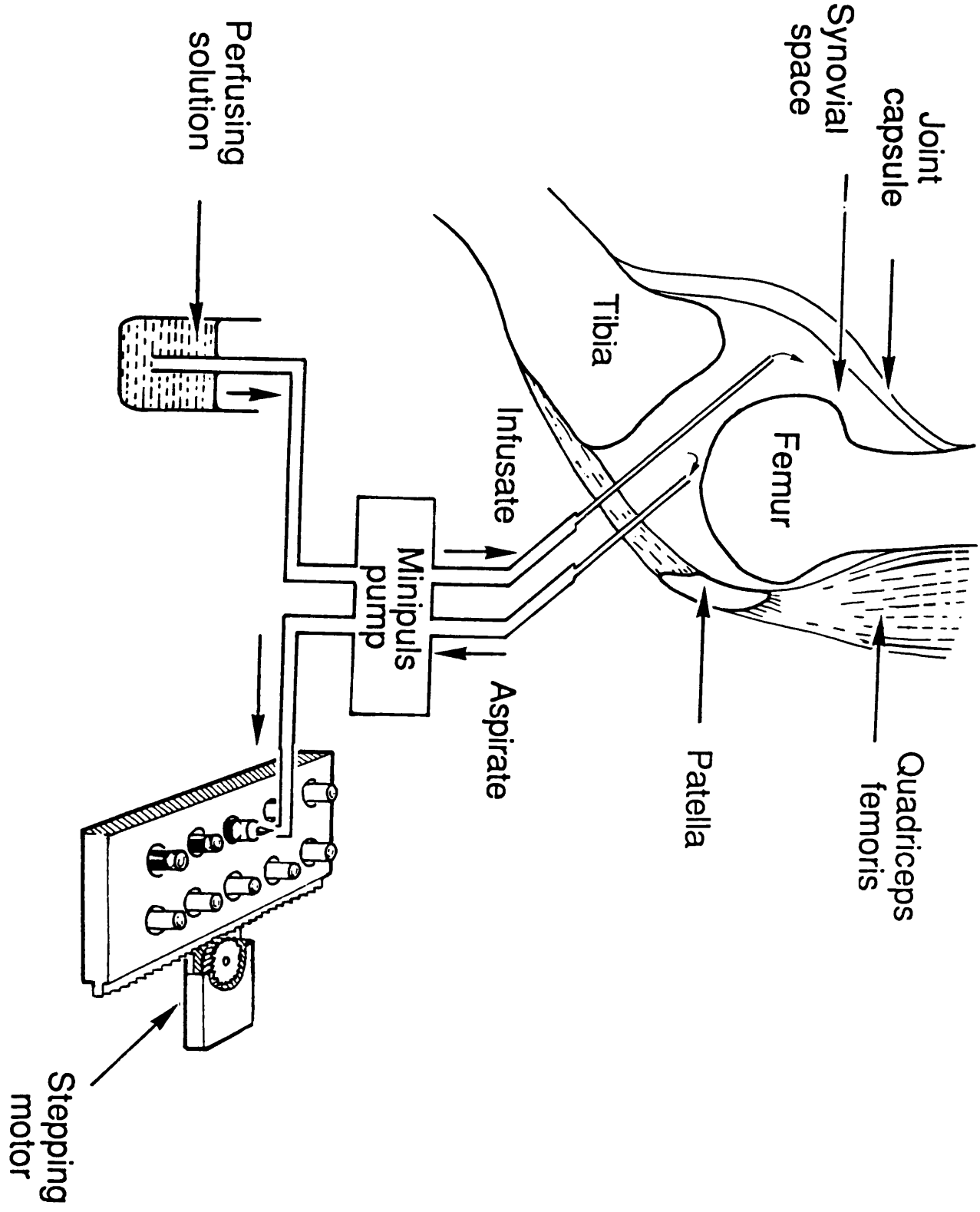


Fig 2.2: Schematic illustration of intra-articular infusion set-up and collection of aspirated samples.

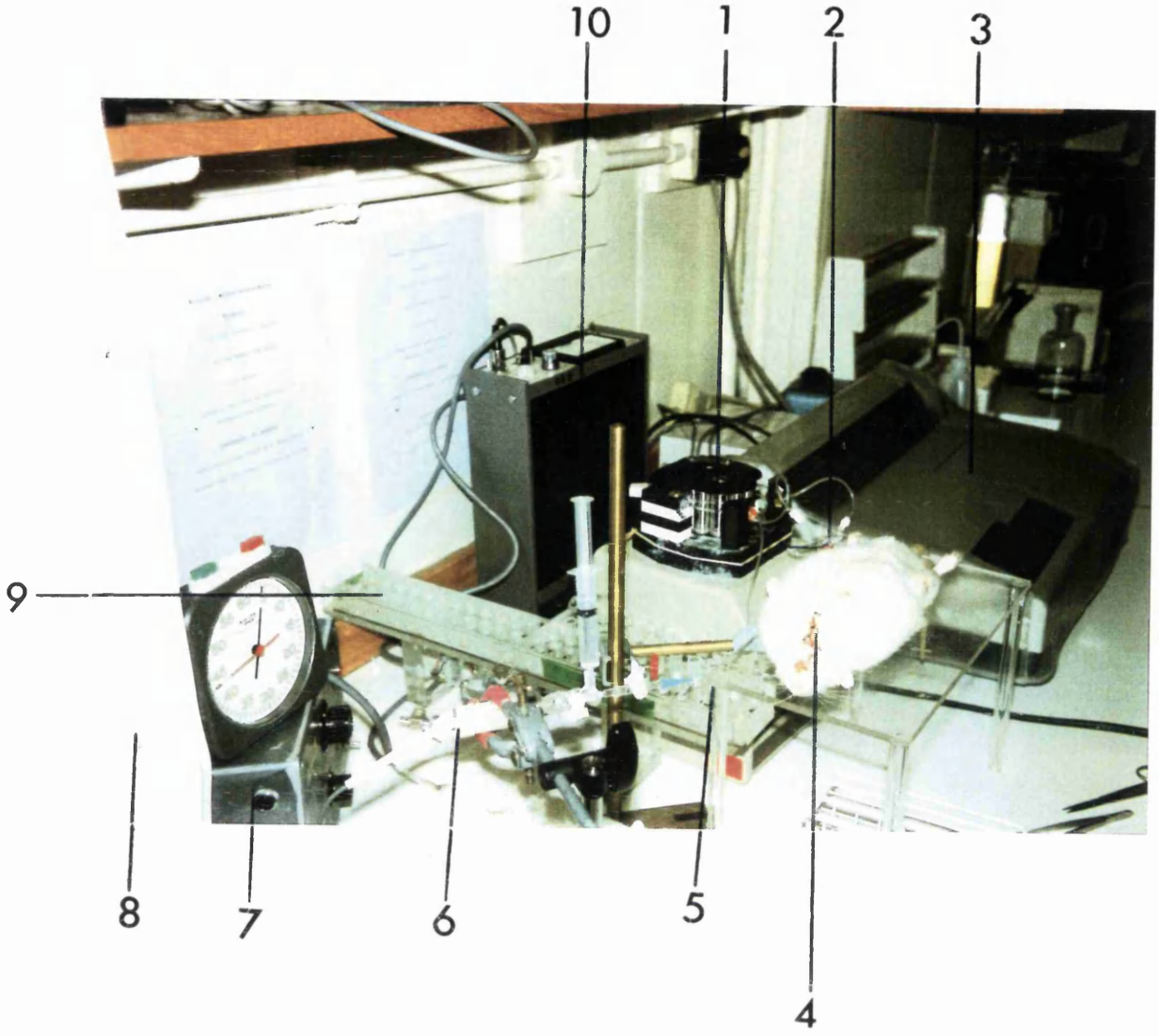


Fig 2.3: Photograph shows the experimental set-up and position of the animal at the end of cannulation and insertion of inflow and outflow needles. 1) minipuls pump 2) Infusate and aspirate needles 3) UV visible spectrophotometer 4) Carotid cannula 5) Aspirated samples 6) Blood pressure transducer 7) Stepping motor timer and controller 8) Pen recorder paper 9) Sample rack and driver 10) Blood pressure amplifier

the observation that, in alkaline solution, protein reacts with the quaternary ammonium salt, benzyl methyl {2 - [2 - (p -1 ,1, 3, 3 - tetra methyl buthyl phenoxy) ethoxy] ethyl} ammonium chloride (benzethonium chloride), to produce turbidity.

0.1 ml of the sample was mixed with 1 ml of 0.2% benzethonium chloride solution in distilled water plus 4 ml of 3.2% NaOH in 1.23% EDTA solution in distilled water. The solutions are vortexed and left for ten minutes for chemical processing and the amount of turbidimetry was measured by spectrophotometry at 360 nm. The reading of the machine was adjusted to zero in solvent plus saline before sample reading. Standard protein samples (0-500 mg/l) were prepared for each set of experiments to minimise variability . The amount of the protein in the sample was detected by comparing the reading of the sample with the reading of each protein calibration curve. This method was described by Iwata & Nishikasa (1979), gives a linear and sensitive readings between 50-500 mg /l but cannot detect very low protein concentrations.

This technique was then modified and changed the concentration of the solutions. In the modified version, 0.2 ml of perfusate was mixed with a final solution of 0.4 ml of 0.2% benzethonium chloride plus 1.6 ml of 3.2% NaOH in 1.23% EDTA and the mixture was vortexed and left for 15 min for chemical processing. After this, the procedure was the same as the Iawata & Nishikasa (1979) method. In the modified method the sensitivity and linearity of the reading was increased between 5-500 mg /l (fig. 2.4).

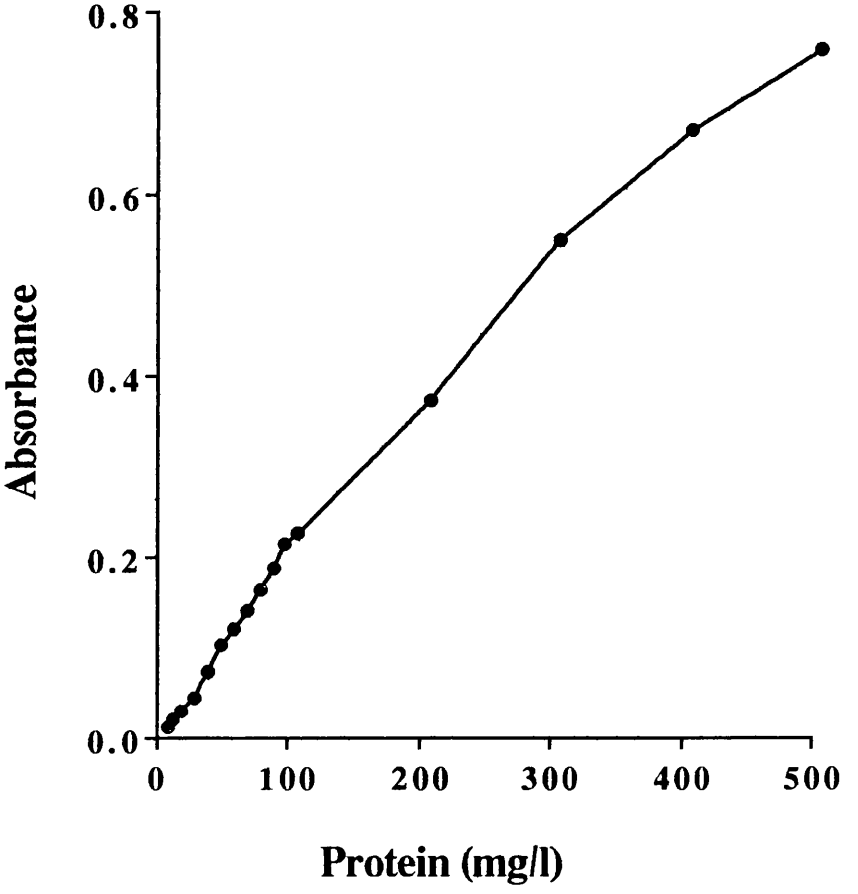


Fig 2.4: Modified protein calibration curve between 5-500 mg/ l

2.7: GENERAL METHOD FOR INDUCTION OF INFLAMMATION

Different models of inflammation were required in different sets of experiments. To induce inflammation, rats were anaesthetised and the fur over the knee joint was shaved and the skin disinfected. The sterile inflammatory agent used e.g. (Carrageenan, Capsaicin or Freund's Adjuvant) was prepared and 0.2 ml (of one of these,) was injected through a 25 G hypodermic needle. Passing through the patellar tendon into the joint cavity, 0.1 ml was injected into the posterior region and 0.1 ml injected to the anterior region of the joint cavity. After a period (depending on the nature of the inflammatory agent) inflammation will develop in the joint.

2.8: DRUG ADMINISTRATION: In all of these experiments, vasoactive drugs were applied topically. The drugs were prepared and warmed and then applied as a bolus on the knee joint with a Micropipet. Warmed saline applied to the joint surface was used during the experiment to prevent dehydration and a fall in the knee temperature.

2.9: MEASUREMENT OF KNEE BLOOD FLOW

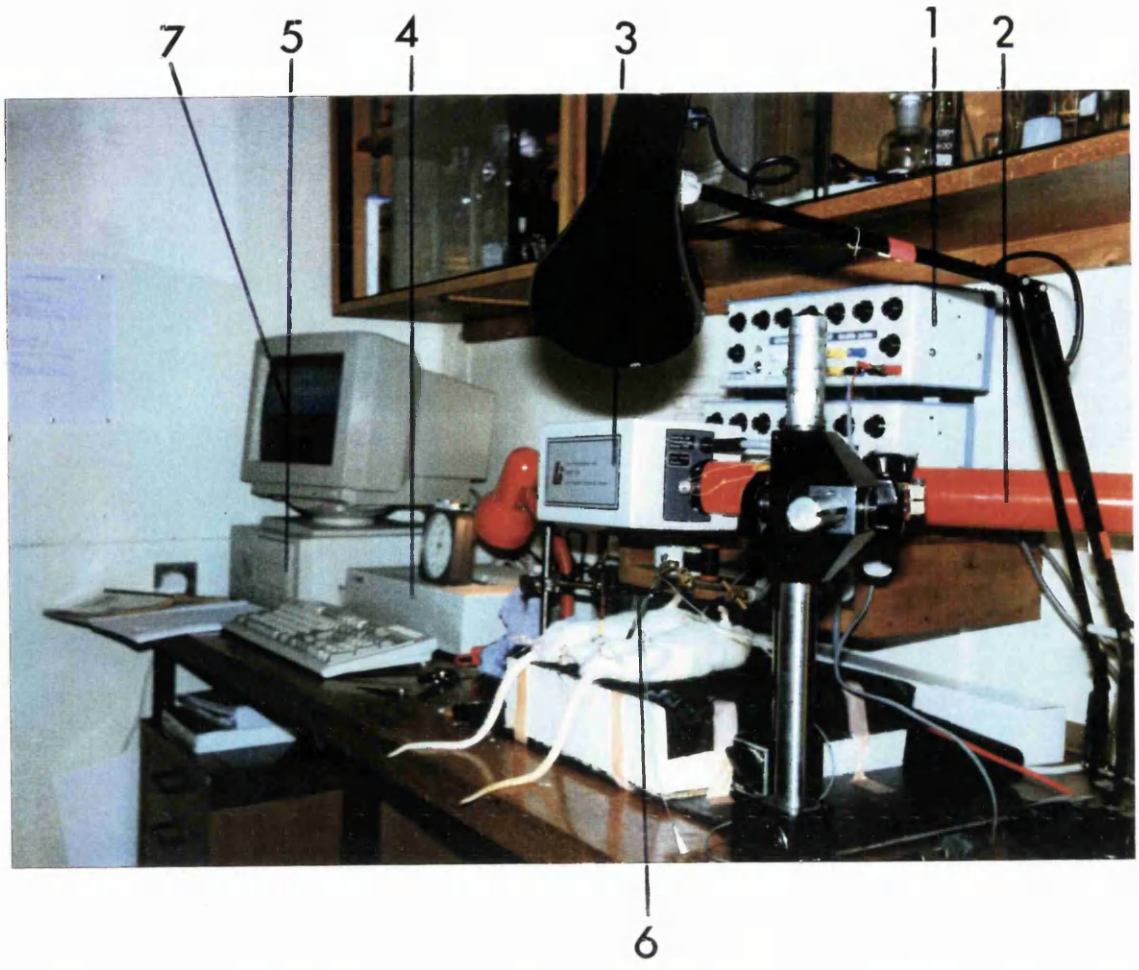
As mentioned in chapter one, knee joint and synovial circulation have been evaluated by different techniques such as, measurement of skin temperature, synovial fluid temperature, plethysmography, bubble flowmetry, isotope clearance, vital microscopy and the microsphere technique. There are disadvantages to most of these methods, for example in the temperature measurement technique variability is high

and there is a strong influence from environmental temperature (Greeenfield *et al.* 1951). In the ^{133}Xenon method, lipid solubility is one of the limiting factors in blood flow measurement (Dick *et al.* 1970a). and in some of these techniques (eg. isotope clearance) the long time of measurement is a limiting factor. Furthermore, almost all of these methods are not suitable for measuring rapid changes in blood flow. With the Laser Doppler flowmetery (LDF) method, blood flow can measured continuously, but only at single point and in a small volume of tissue, and slight changes in probe position may cause a very big change in measured blood flow because of variation in tissue architecture.

Laser Doppler Imaging (LDI) is newly developed technique. The principle of the technique has been described in chapter one and is based on collecting back scattered Doppler- shifted laser light, to create an image of blood flow distribution in an area over approximately 12 cm^2 in approximately 4.5 minute (fig. 2.5).

2.9.1: Experimental set-up: The LDI instrument was fixed on a tripod 10 inches above the operating table. LDI has a low power He-Ne laser source of a class two type which produces a 633 nm red beam. The laser beam is controlled by a software program (PIM, Lisca AB) which controls the stepping mirrors and guides the laser beam (800 μm) over the tissue. The scan starts from the top right of the selected area and terminates in the bottom left. The scan can be taken in high or low resolution mode which allows for zooming in on a specific area. The maximum size of scan area is 150 cm^2. In this format, the machine will scan 64 pixels in both X and Y axes making an area of 4064 scanned pixels with a scanning period of 240 sec. The

Fig 2.5: Photograph shows the experimental set-up and position of the animal at the end of the preparation 1) Stimulator 2) Laser tube 3)laser Doppler scanner head 4) Printer 5) Computer linked to the set-up 6) Stimulating electrodes 7) PIM programme on the monitor 8) Rats exposed knee joint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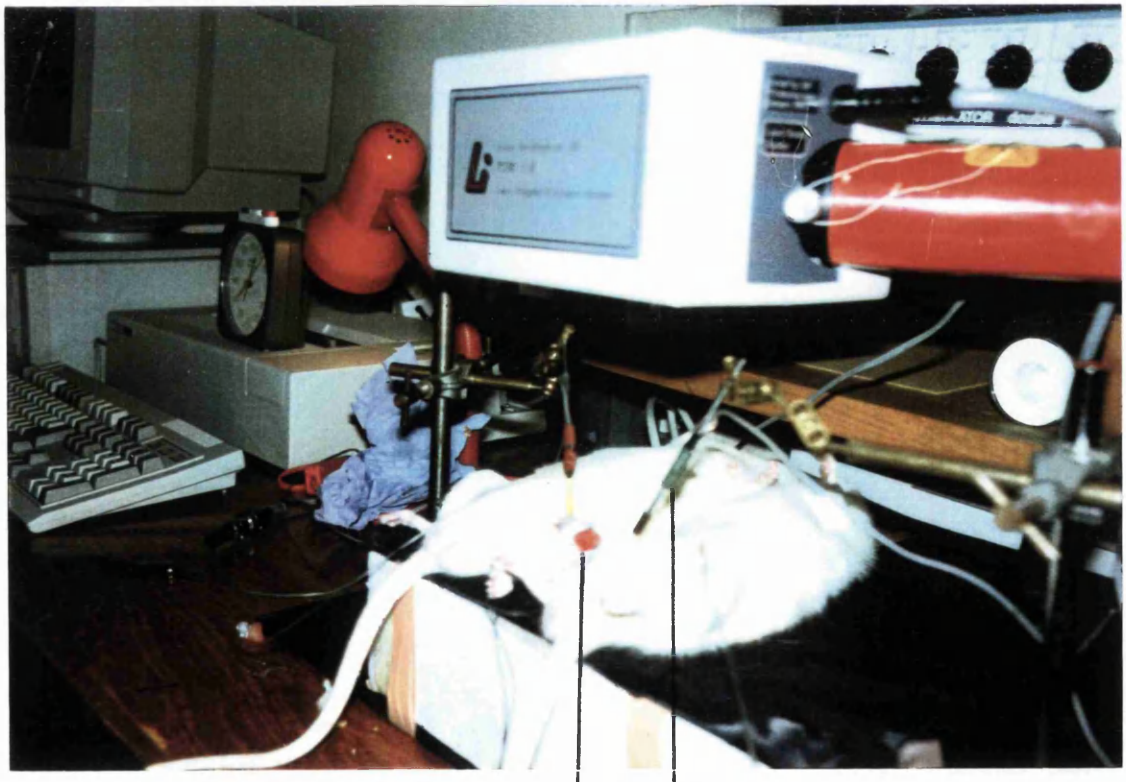
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6

size of scan area can be adjusted by the operator by selecting a smaller area to scan, and the required scan time becomes shorter. This allows simultaneous scanning of the opposite knee of two separate rats placed in close proximity. Background threshold is also adjustable and may be altered, depending on the type of experiment. In these experiments the normal back ground threshold was set to 5.81, and the scans were operated in high resolution mode.

In the presence of moving red blood cell in the capillaries, partially back-scattered Doppler-shifted light is detected by a photo detector in the scanner head. It converts instaneous light intensity into an electrical signal (volts) that is proportional tissue perfusion. A colour-coded perfusion image is then displayed on the monitor which can be output to a colour printer. For display purpose these images use six colours to encode perfusion (see chapter one), but the actual voltage values at each image are held in the memory or stored on disk and utilised for further statistical calculation of perfusion within a given area. Additional statistical analysis can be performed by the "VIEW" statistical programme which was exclusively written for image analysis.

2.9.2: Analysis: Statistical analysis of the scans was done using VIEW software (Moor Instruments). Statistical analysis of a selected rectangular area is possible in this programme and the data is shown in a bar chart form and numeric values ($\pm$ SD) as a percentage change in blood flow. The size of the rectangular area selected can be changed to include most of the articular tissue with minimal inclusion of surrounding muscle. In each set of experiments the scan was taken before administration of drug, as a control scan, and immediately after

administration of drugs to minimise variation and error. Further scans could be taken subsequently as a time dependent response. A residual signal is generated from tissue even when circulation is occluded and flow stopped completely (biological zero)(Holloway 1980; Tenland *et al.* 1983) and it has been recommended that this value should be subtracted from all observed values (Fargell 1990; Nilson 1990). Thus at the end of experiments a scan was taken of the joint in a dead animal as the biological zero (i e LDI signal when flow is absent). The value obtained for this biological zero was subtracted from the other values to allow calculation of pure blood flow changes (Nilson 1990). Values below the background threshold value are excluded from the calculation of voltage values.

2.9.3: Penetration depth: To evaluate depth of penetration of the red laser beam, a scan was taken prior an intra-articular injection of 10 PM adrenaline, (a potent vasoconstrictor) and another scan was taken after one minute and then ten minutes post injection. The scans showed blood flow changes in the articular and synovial vessels but the surrounding tissue was unaffected (fig. 2.6).

At each measurement site the beam penetrates the tissue to a depth of at least 250 μm (Wårdell 1991). It has been shown that 90% of capillaries in the joint capsule are located in the thin strip of synovium which lines the synovial cavity and many of these capillaries are within 20 microns of the joint space (Knight & Levick 1983), therefore the above result suggests that the red laser penetrates through the capsule to detect synovial blood flow, as underlying cartilage is avascular and laser penetration to the dense cortical bone is

Fig 2.6: Laser Doppler perfusion images of rat knee joints.

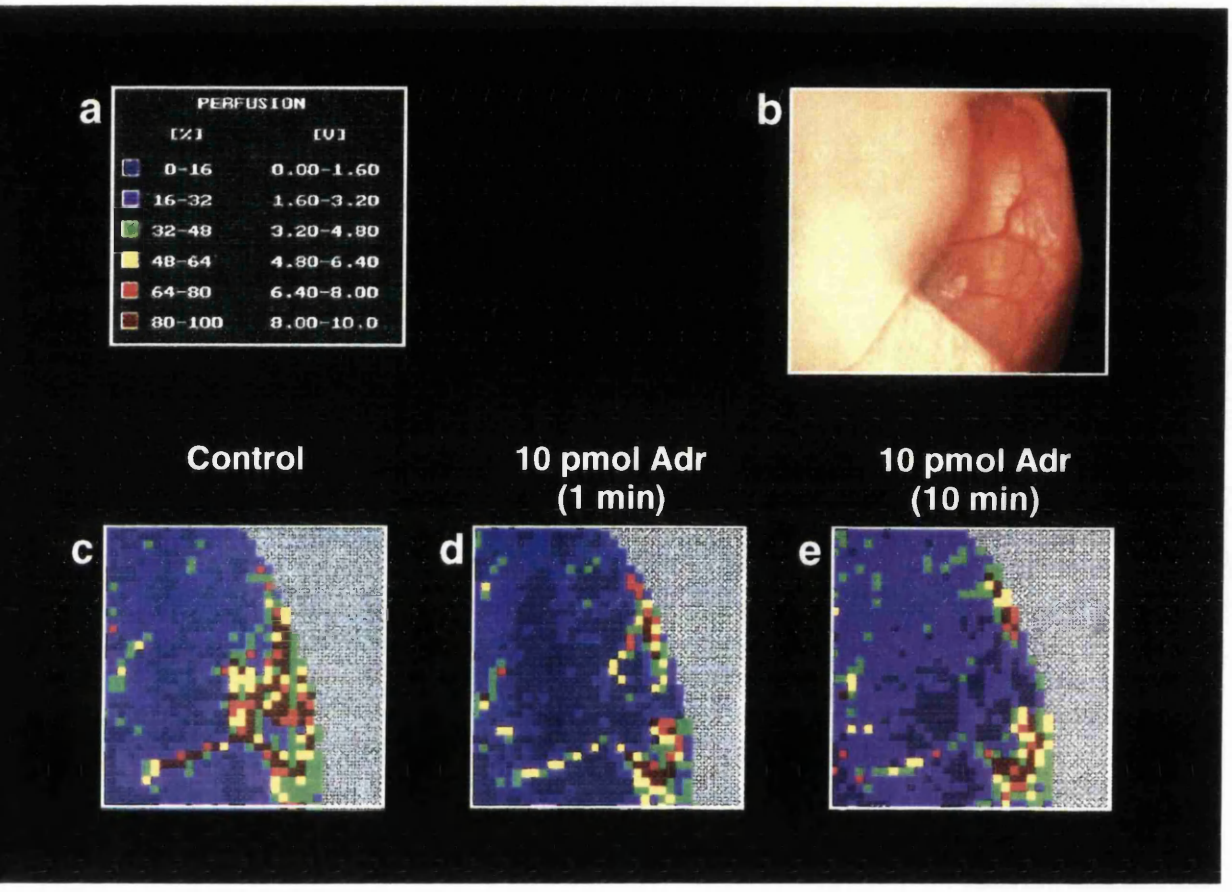
A) colour-coded system for showing perfusion values rang from dark blue (lowest) to dark red (highest), along with corresponding voltages (0-10V) of the LDI signal. The same range of values were used in all scans.

B) Photograph of the exposed medial aspect of a rat left knee joint, showing the high degree of vascularity of the region. The white area lateral to the large branching blood vessel corresponds roughly to the joint capsule.

C) Perfusion image of the medial aspect of the left knee joint of a normal animal prior to any experimental procedure.

D) Image from the same knee 1 minute after intra-articular injection of 10 pmol of adrenaline (Adr). A reduction in perfusion is present over the capsule region.

E) 10 minutes after injection of adrenaline, the underperfused area is somewhat larger, but surrounding regions are unaffected, suggesting that there has been no diffusion of adrenaline from the joint.


a
PERFUSION
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[V]
0-16
0.00-1.60
16-32
1.60-3.20
32-48
3.20-4.80
48-64
4.80-6.40
64-80
6.40-8.00
80-100
8.00-10.0
b
Control
10 pmol Adr
(1 min)
10 pmol Adr
(10 min)
c
d
e

negligible, any changes in blood flow detected by LDI exclusive refer to changes in synovial perfusion.

2.9.4: Stability of LDI measurement:

The flux reading in the first scan after initial preparation is relatively high because of the surgical procedures and tissue reaction, but the blood flow slowly comes down and after 15-20 min achieved a stable base line. This base line blood flow was stable during the experiments. The control scan reading after each drug application was the same as previous control scan. Statistical analyses were performed between controls of different sets of experiments and none of them were significantly different (one-way anova p=0637, n=30)(fig. 2.7).

2.9.5: Validity of the LDI technique: The LDI technique provides a convenient means of sampling a large volume of tissue in a relatively short time-frame thereby allowing the determination of the spatial distribution of tissue perfusion. This is particularly useful for analysis of joint capsule perfusion because of the complex geometry of joints, the highly anastomotic blood supply and the heterogeneous nature of the tissues which constitute the capsule. A significant advantage of LDI over laser Doppler flowmetery (LDF) is related to the heterogeneity of the responses observed. From the perfusion images (fig. 2.8) it is obvious that there is considerable variation in perfusion in adjacent areas, but closer inspection (fig. 2.8 b & c) shows that the response to SP is also heterogeneous, with perfusion at some points showing no change whilst other points show an increase in perfusion in excess of 300%. By taking measurements over an area of

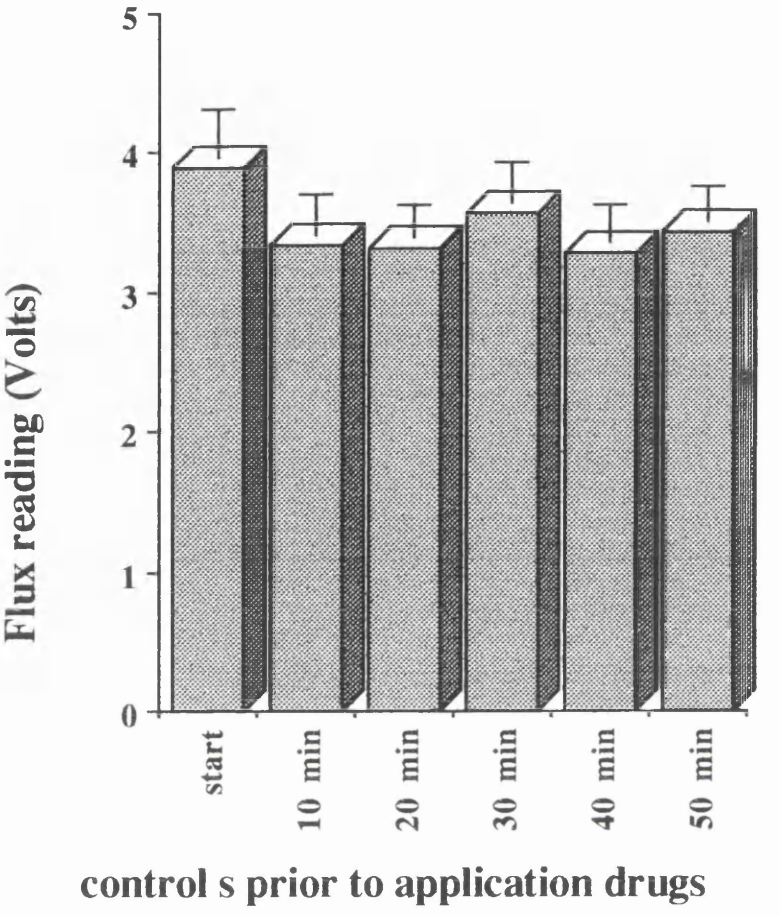


Fig 2.7: Flux reading (volts) of control scans prior to drug application (mean ±SEM, n=35) obtained during dose response curve. Differences between the readings are not significant (see text).

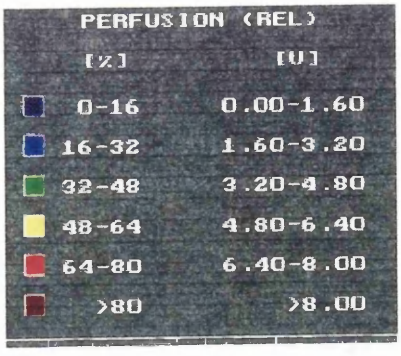
Fig 2.8: Laser Doppler perfusion images of rat knee joints.

A) colour-coded system for showing perfusion values

B) Perfusion image of medial aspect of 2 rat knee joints prior to any experiment.

C1) Image from same knees after topical administration of SP at 10^{-9} mol.

C2 &3) Image from other knees to the same dose of SP


PERFUSION (REL)
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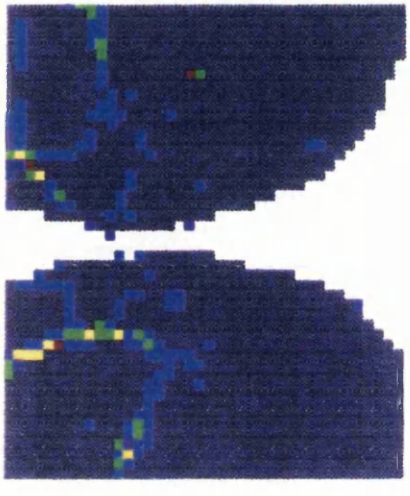
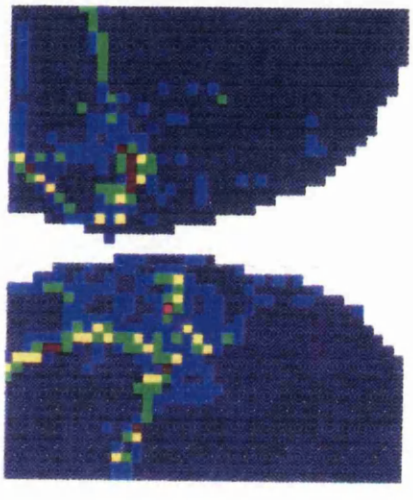
0-16
0.00-1.60
16-32
1.60-3.20
32-48
3.20-4.80
48-64
4.80-6.40
64-80
6.40-8.00
>80
>8.00

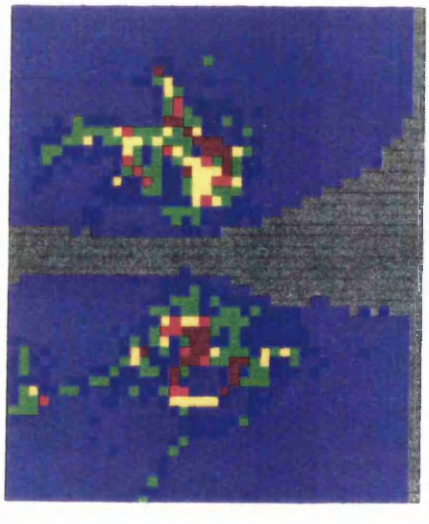
A



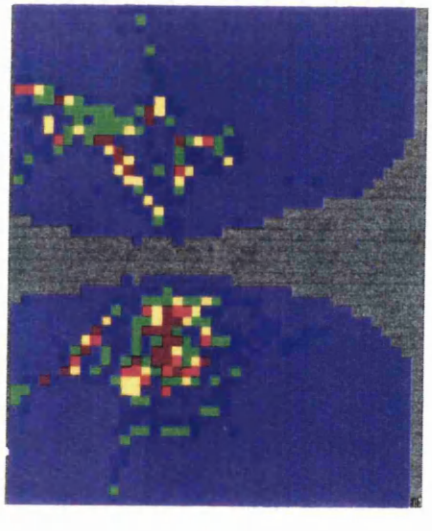
B



C_1



C_2



C_3

the joint capsule, LDI provides a clear view of the overall response of the tissue whereas LDF, as it only samples at a single point, is less likely to yield a representative sample. Past experience of LDF has shown that a great deal of sampling is required to find a site on the capsule which gives a similar response to a given drug between different animals (W. R. Ferrell personal communication). Without such standardisation, in a tissue such as the joint capsule, the values obtained with LDF would be very difficult to interpret. By contrast, LDI makes such standardisation unnecessary, and has the added advantage that any variation in the pattern of joint perfusion could be readily detected. This could prove to be useful in examination of the pattern of perfusion in acutely or chronically inflamed joints. One clear disadvantage of LDI compared to LDF is the poorer temporal resolution of the former. LDF provides a continuous measure of perfusion at a single point whereas LDI to some extent "integrates" the response over time, depending on the area of tissue scanned. This could be particular problem when examining transient events. In previous work using laser Doppler flowmetry to continuously monitor blood flow, it was observed that application of SP produced a transient dilatation, reaching a peak in about 40 sec and returning to baseline at about 110 sec (Lam and Ferrell 1993b). Thus, with a scan time of 100 sec, as typically used in the present study, it is possible that some of the response could be missed. To check this, images from rats were examined, placing small windows at the upper and lower borders of the knee joint and the vasodilator effect of SP examined. As scanning proceeded in a proximal to distal direction, if a significant temporal effect of SP were present, it would be expected that the percentage change in perfusion at the upper window would be greater than the

value for the lower window *i.e.* vasodilatation would be greater at the upper border of the knee as the response to SP would have waned by the time the scan reached the lower border. However, the mean percentage difference in perfusion between the two areas was only 2.8±3.3% and this difference was not significant (P=0.395; paired t-test; n=41 paired measurements). This suggests that the response to SP lasted long enough to be substantially captured during the scan. This may in fact be due to the fact that during the LDI scan time of ~100 sec, about the first 20 sec covers extra-articular tissues proximal the joint and about the last 20 sec also covers extra-articular tissues distal to the knee. Thus, the joint capsule, sandwiched in the middle, is reached at a near-optimal time.

2.10: TERMINATION OF THE EXPERIMENT:

At the end of the experiment the rat was painlessly killed by Euthetal (Pentobarbital 120 mg/ ml) via the IP route.

2.11: STATISTICAL ANALYSIS: Data analysis was performed on the values obtained from image analysis. "Minitab" software was used, and a modified version of the Shapiro Wilk test was used to test whether the data were normal distributed. Two statistical techniques were used, the Student t-test and ANOVA .

Two-tailed paired t test was used to compare the result of a special treatment of a group of image data with control image data in the same group of rats.

ANOVA was used to compare the data from two sets of experiments with different treatment and concentration of drugs. One

or two factor ANOVA (depending on experiments) was used and in both tests , the difference between two compared groups was considered significant when P values were less than or equal to 0.05.

CHAPTER THREE

PLASMA PROTEIN EXTRAVASATION

INTO THE RAT KNEE JOINT

INDUCED BY

CALCITONIN GENE-RELATED PEPTIDE

3.1 SUMMARY

1) Calcitonin Gene-Related Peptide (CGRP) is a 37 amino acid peptide which is the most potent vasodilator known, but there is no report of CGRP-induced protein extravasation, which could be due to the insensitive methods previously used to measure plasma protein extravasation.

2) Experiments were performed to investigate the protein extravasation effect of CGRP in the rat knee joint.

3) Intra-articular infusion of 10^{-6} M CGRP induced protein extravasation which was sustained throughout the period of infusion.

4) Both lower (10^{-5}) and higher (10^{-7}) concentrations of CGRP failed to produce extravasation. Failure at the highest concentration of CGRP was the likely consequence of a significant fall in arterial blood pressure which occurred with administration of CGRP at this concentration. In the presence of arterial hypotension (induced by the α_1, α_2, adenoreceptor antagonist, phenoxybenzamine) 10^{-6} M CGRP failed to induce plasma protein extravasation.

5) Plasma extravasation induced by CGRP was a specific effect of and not merely consequence of its potent vasodilator effect. A similar vasodilator response induced by a β adenoreceptor agonist, isoproterenol, failed to induce protein leakage.

6) These findings suggest that CGRP can also alter blood vessel permeability and therefore could contribute to neurally-mediated inflammatory responses.

3.2 INTRODUCTION

Calcitonin gene-related peptide (CGRP) is a 37 amino acid polypeptide which is encoded by the calcitonin gene. mRNA derived from this gene is processed so that it contains sequences coding either for calcitonin or CGRP, which is under the control of tissue dependent factors (Amara *et al.* 1982). Translation of the mRNA results in a 16000 Dalton (D) protein which is then cleaved to give a 3900 D α CGRP (Rosenfeld *et al.* 1983). The second form is β CGRP which is a product of a separate gene and only the peptide produced by this gene is biologically active. The mRNA encoding this peptide which is referred to as β CGRP appears to be the only mature transcript of β CGRP gene. Hybridisation histochemically reveals a similar distribution of α and β CGRP mRNA. This β CGRP is a new member of a family of related gene with a potential function in regulating the transduction of sensory and motor information (Amara *et al.* 1985)

Neuropeptides such as substance P (SP) are known to increase blood vessel permeability and thereby result in plasma protein extravasation into the extra vascular space. In synovial joints this process results in protein appearing in synovial fluid (Cruwys *et al.* 1992; Lam & Ferrell 1991a; Scott *et al* 1991) which is normally an ultra filtrate of plasma. Thus, using a marker which labels plasma proteins and estimating its concentration in synovial fluid, an indirect measure of synovial blood vessel permeability can be obtained.

CGRP is known to be a potent vasodilator of synovial blood vessels (Cambridge & Brain 1992; Lam & Ferrell 1993a; Lam & Ferrell 1993b) but previous experiments have failed to demonstrate

that CGRP can, of itself, elicit plasma extravasation (Cambridge & Brain 1991; Cruwys *et al.* 1992). However, this may have been the result of the relative insensitivity of the techniques employed where markers such as Evans blue (Cruwys *et al.* 1992, Lam & Ferrell 1991b) and ^{125}I labelled albumin (Cambridge & Brain 1992) were used. It has recently been shown that direct measurement of the protein content of synovial fluid by micro-turbidimetry is much more sensitive than plasma labelling methods (Scott, Lam & Ferrell 1991). Thus, the present study was performed, using the micro-turbidimetric method of protein assay, to re-evaluate whether CGRP can influence the permeability of synovial blood vessels.

3.3 MATERIAL AND METHOD

Experiments were performed in male Wistar rats (350-500g) deeply anaesthetised by intra peritoneal injection of urethane (1.13g/kg). The method for intra-articular injection was as described in chapter 2.4.1. In a sub-group of rats the carotid artery was canulated for monitoring blood pressure.

Normal saline (0.9%) was perfused into the synovial cavity (Gilson minipuls) at a rate of 25 µl / min and the effluent from the joint was collected every eight minutes. Saline perfusion was continued for up to one hour after which the perfusate was changed to one consisting of saline in which α-CGRP (Celltech) was dissolved in different concentrations, or another saline solution as a time control, and the second perfusion continued for up to one hour. In most animals both knees were used. After all the samples had been collected, reagents were added to measure the protein content of each sample.

The technique used in this study was described in chapter 2.4.2.

Samples of infusate containing only CGRP in concentrations up to 10^{-5} M were analysed with this method, but yielded no measurable protein. In a smaller series of animals, synovial effluent was collected whilst synovial perfusion was monitored using laser Doppler flowmetry (Lam & Ferrell 1993b) Assessment of changes in vascular calibre was by calculating changes in vascular resistance (mean arterial blood pressure divided by blood flow). Statistical analysis of the data was performed using MINITAB software. A modified version of the Shapiro-Wilk test showed that the data were normally distributed. Comparisons between mean values was by student's t-test or ANOVA where appropriate. Means ± standard error of the mean (SEM) are used throughout and all quoted P values are two-tailed.

3.4 RESULTS

Intra-articular perfusion of CGRP produced plasma protein extravasation into the synovial cavity of the rat knee, but only at a concentration of 10^{-6} M (fig. 3.1, n=24). Both lower (10^{-7} M) and higher (10^{5} M) concentrations failed to elicit extravasation (fig. 3.2, n=11 and 3.3, n=17) and therefore the concentration/response relationship was not investigated beyond this range. The responses at these two concentrations did not differ significantly from the response to saline infusion (fig. 3.4).

It was noticeable that protein extravasation gradually declined during the experiment at both of the concentrations of CGRP and the saline infusion. By contrast, 10^{-6} M CGRP produced a sustained response throughout the one hour infusion period. Although protein

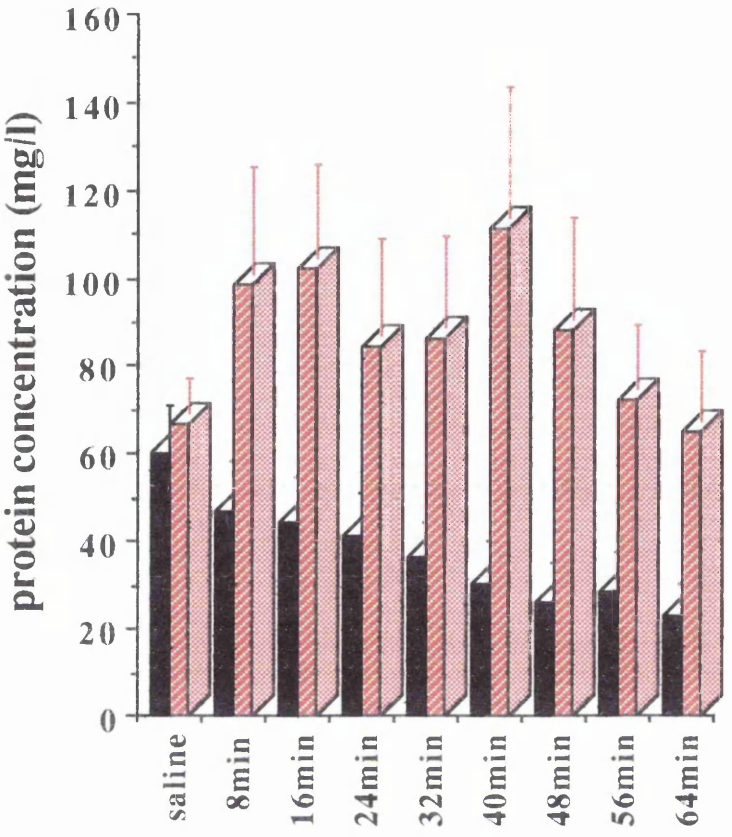


Fig 3.1: time course of plasma protein extravasation into the rat knee joint in response to intra-articular infusion of 0.9% saline, (black column, n=5) and CGRP at concentration of 10^{-6} M, (red column, n=24).

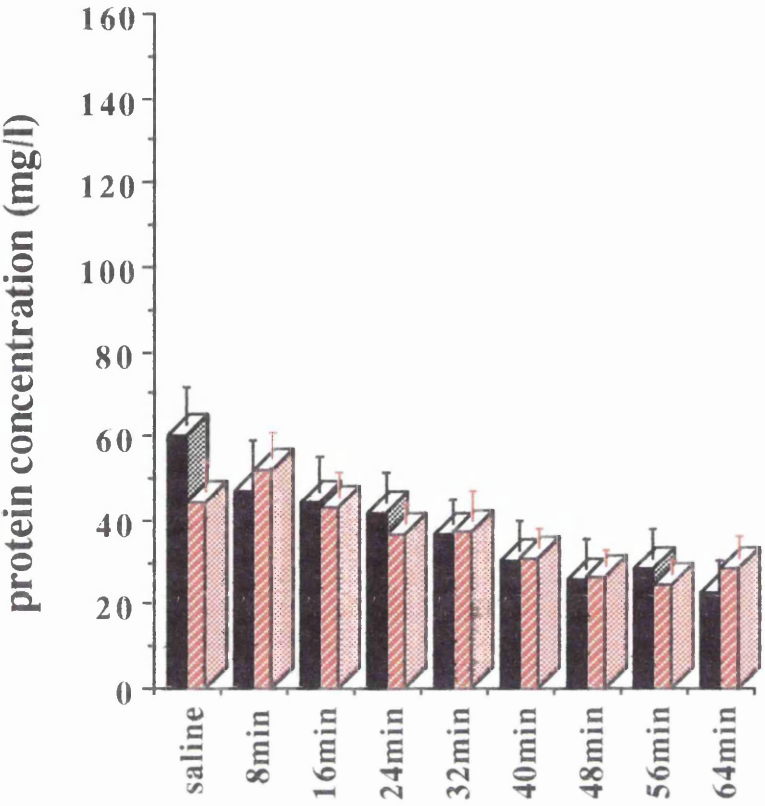


Fig 3.2: time course of plasma protein extravasation into the rat knee joint in response to intra-articular infusion of 0.9% saline, (black column, mean ±SEM, n=5) and CGRP at concentration of 10^{-7} M, (red column, mean ±SEM, n=17).

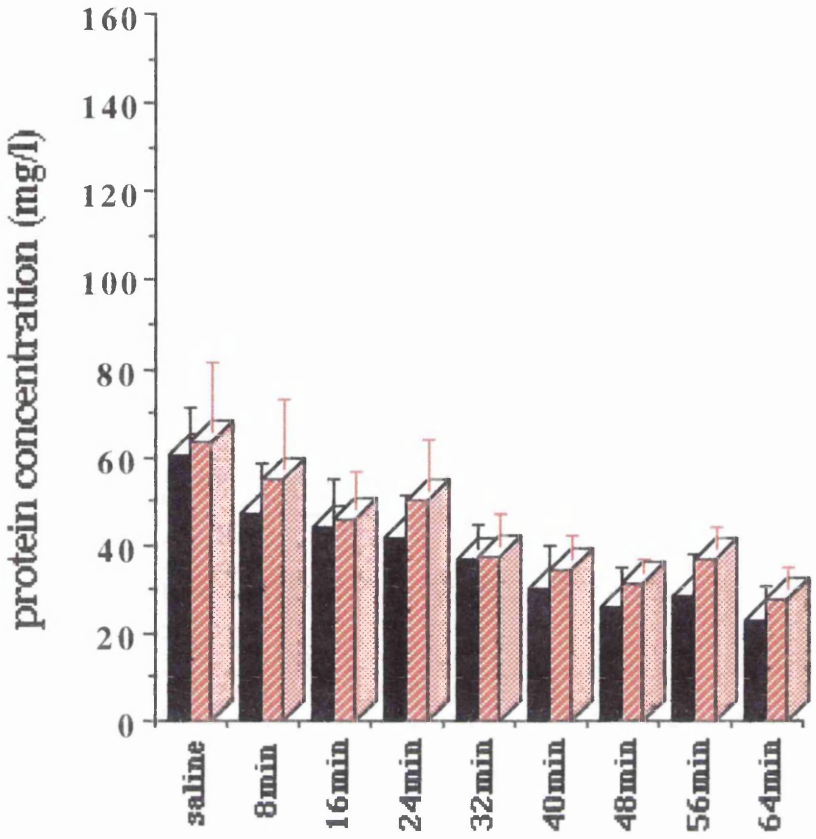


Fig 3.3: time course of plasma protein extravasation into the rat knee joint in response to intra-articular infusion of 0.9% saline, (black column, mean ±SEM, n=5) and CGRP at concentration of 10^{-5} M, (red column, mean ±SEM, n=11).

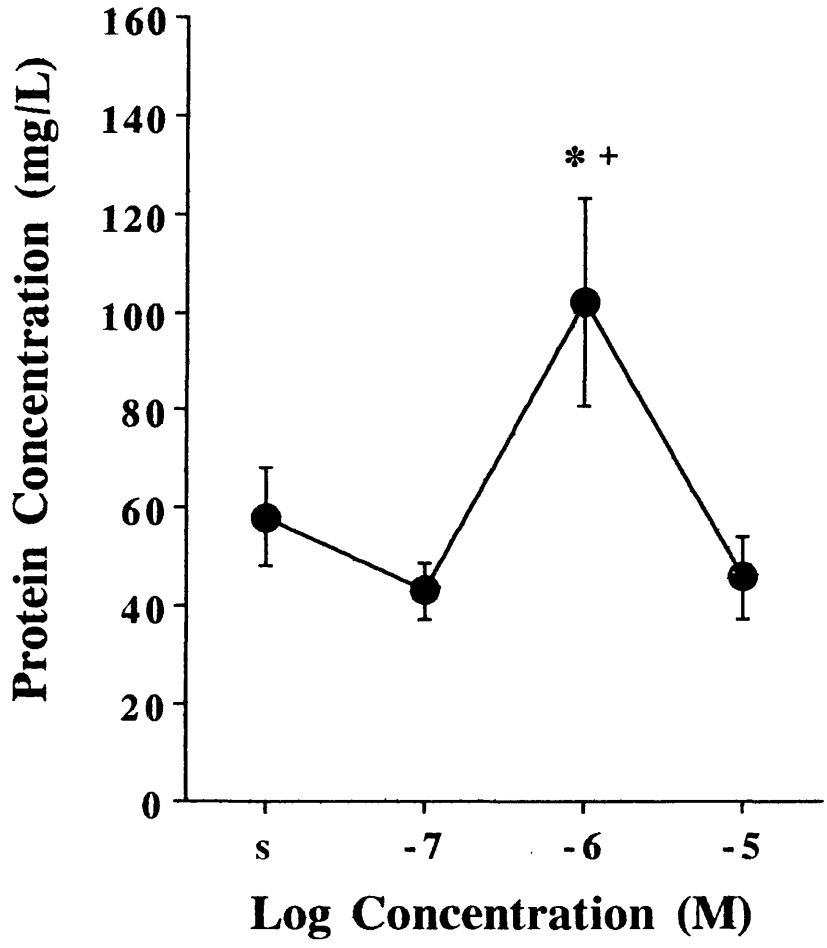


Fig 3.4 Protein concentration in synovial effusate during saline infusion (S) and 16 min after commencement of infusion of different concentrations of CGRP. * mean significantly different from saline infusion ($p<0.05$), + mean significantly different from lower and upper concentration ($p< 0.05$).

extravasation occurred at the 10^{-6} M concentration in each animal tested, the time course, particularly the time taken to reach the peak response, varied between animals, leading to large variations in protein measurements at the different times. Comparison of the time/response curves by ANOVA revealed a significant difference due to concentration ($P<0.0001$, $n=24$), with the 10^{-6} M infusion of CGRP differing significantly from the saline infusion, the 10^{-7} M and the 10^{-5} M CGRP infusions ($P<0.001$ in all cases).

The apparent lack of a dose-dependent effect of CGRP, as exemplified by the absence of protein extravasation by the 10^{-5} M infusion of CGRP, may have been due to the systemic vasodilator effects of CGRP. As shown in fig. 3.5 this dose produced a pronounced fall in mean arterial blood pressure and as synovial perfusion is directly related to the arterial perfusion pressure, it is likely that at this concentration of CGRP, the fall in synovial blood flow was sufficient to mask any protein extravasation.

Comparison of the time/response curves by ANOVA showed a significant difference due to treatment ($P<0.0065$). The fall in blood pressure induced by 10^{-5}M CGRP differed significantly from that occurring with saline ($P<0.05$) and 10^{-7} M CGRP ($P<0.05$). Although it appeared that the lower concentrations of CGRP might produce a modest fall in blood pressure with time, overall, the responses at both 10^{-6}M and 10^{-7}M CGRP did not differ significantly from that occurring with saline infusion. Plasma extravasation induced by 10^{-6} M CGRP (98.8± 24mg/l; n=9) could be virtually abolished (27±10 mg/ l; n=4) in the presence of hypotension induced by intravenous administration of the α_1, α_2-adrenoceptor antagonist

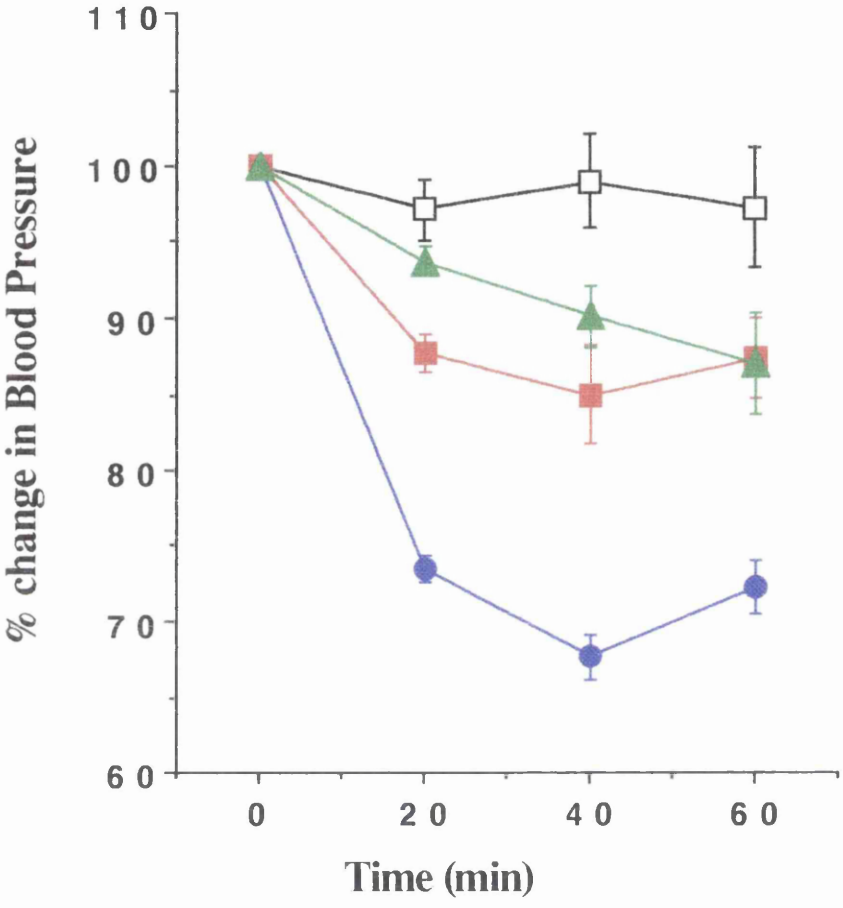


Fig 3.5 Percentage change in mean arterial blood pressure in response to intra-articular infusion of 0.9% saline (❑) and CGRP at concentrations of 10^{-7} M (▲),10^{-6} M (■), and 10^{-5} M(●). Mean ±SEM, n= 4-5.

phenoxybenzamine prior to intra-articular infusion of 10^{-6} M CGRP. Phenoxybenzamine, in producing a 27± 4% (n=4) fall in blood pressure which was similar to that occurring with 10^{-5} M CGRP (67.6±1.5% of control; n=4), did not itself produce significant protein extravasation (27± 10mg/l).

It was also observed that vasodilatation of articular blood vessels induced by the β_1, β_2-adrenoceptor agonist isoproterenol was not accompanied by significant protein extravasation. Infused into the joint at a concentration of 10^{-5} M, isoproterenol produced a 13.46± 2.9% (n=6) fall in vascular resistance, but this was not accompanied by significant protein extravasation (29.8± 11 mg/l). This vasodilatation was similar to that occurring with 10^{-6} M CGRP which produced a 11.9±2.1% (n=6) fall in vascular resistance but, as described earlier, yielded significant protein extravasation (111.1±30.2 mg/l). These findings argue that CGRP-induced extravasation is a specific effect, and not merely a consequence of its vasodilator action.

3.5 DISCUSSION

The results of this investigation have shown that contrary to previous studies (Cambridge & Brain 1992; Cruwys *et al.* 1992; Lam & Ferrell 1991a), CGRP can, in addition to its known vasodilator effects, also produce an increase in vascular permeability. It is unclear whether this is a direct effect on endothelial cells, but it is unlikely to be due to purely to its vasodilator effect as vasodilatation induced by the β-adrenoceptor agonist was not accompanied by plasma extravasation. Agents such as SP are known to have both a vasodilator effect (Lam & Ferrell 1993a; Lam & Ferrell 1993b) and to increase

synovial blood vessel permeability (Cruwys *et al.* 1992, Lam & Ferrell 1991a) In addition, at a concentration of 10^{-5}M, although CGRP would have produced the greatest degree of vasodilatation of synovial blood vessels, plasma extravasation was nevertheless absent. As explained earlier, the absence of protein extravasation at this concentration is probably related to reduced synovial perfusion, and this is strengthened by the observation that there is absence of protein extravasation during intra-articular infusion of 10^{-6}M CGRP in hypotensive animals. In addition, in a previous study it was shown that SP-induced plasma protein extravasation decreased at higher concentrations in acutely inflamed rat knee joints due to hypotension following systemic uptake of SP from the more permeable articular blood vessels (Scott, Lam & Ferrell 1992).

The progressive downward drift in protein concentration observed in the present study with saline, 10^{-5}M and 10^{-7}M CGRP probably reflects the gradual washout of protein from the knee joint cavity.

Another interesting feature to emerge from this study was the long-lasting nature of the extravasation induced by CGRP. It was shown that SP produced only transient increase in blood vessel permeability despite maintained intra-articular infusion (Scott, Lam & Ferrell 1991). In some respects this parallels the vasodilator effects of these two agents, with SP producing transient dilatation of synovial blood vessels whereas CGRP produces long-lasting dilatation (Lam & Ferrell 1993b). This difference is thought to reside in the rapid degradation of SP compared to CGRP (Brain & William 1989) but in the present experiments the intra-articular concentrations were

maintained by continuous infusion and therefore this mechanism cannot explain the findings of the present study. It is possible that some other mechanism may be present to account for these differences in plasma extravasation and this requires further investigation.

The findings of this study indicate that CGRP can by itself enhance vascular permeability, in addition to its ability to potentiate the effects of other inflammatory mediators which induce plasma extravasation (Brain & Williams 1989; Cambridge & Brain 1992; Cruwys *et al.* 1992). As CGRP is present in many peripheral nociceptive afferents, it could contribute to neurogenically-mediated inflammatory responses both by producing vasodilatation and by increasing vascular permeability.

CHAPTER FOUR

NEUROKININS

IN THE

RAT KNEE JOINT

SECTION ONE

SUBSTANCE P AND ITS RECEPTORS

4.1.1 SUMMARY

1) Experiments were performed to identify the type of Substance P (SP) receptors in the rat knee joint.

2) Laser Doppler Imaging (LDI) was used to investigate changes in blood flow.

3) FK888 (an NK1 antagonist), SR48968 (an NK2 non peptide antagonist), and FK224 (an NK1 and NK2 antagonist), in different doses were used in the experiments.

4) SP on its own induced a dose dependent transient (2 min) vasodilatation, (doses between 10^{-9} to 10^{-13} mol). Maximum vasodilatation was 40±7% when a dose of 1nmol was used. Higher doses failed to increase the vasodilator effect and the response decreased because of systemic vasodilatation and a fall in arterial blood pressure.

5) Both FK888 and SR48968 on their own failed to inhibit the vasodilator effect of SP in the rat knee joint.

6) FK224 dose dependently inhibited the vasodilator effect of SP in the rat knee joint. An equal combination of FK888 and SR48968 also inhibited the vasodilator effect of SP.

7) These data suggest that the vasodilator effect of SP is mediated via NK1 and NK2 receptors.

8) Captopril, an NEP inhibitor, reduced by 35% systemic blood pressure, and even in the presence of this low blood pressure SP still

produced a small dose dependent vasodilatation which lasted over 15 min

4.1.2 INTRODUCTION

SP is a peptide with an 11 amino acid sequence which was first discovered by Van Euler in 1931. This water soluble peptide is released from sensory nerve endings. SP containing sensory nerves are widely distributed in different organs, and participate in a variety of physiological and pathological events including inflammation (Lam & Ferrell 1990; Lam *et al.* 1993) and arthritis (Smiths 1989) and also activate neural pathways leading to rheumatoid synovitis (Lotz *et al* 1987). Recently different receptors for the neurokinins have been recognised (Maggi *et al* 1993a; Maggi *et al* 1994) this being made possible by the discovery of neurokinin agonists and antagonists.

The distribution of neurokinin receptors varies in different organs and in different species of animals. In the rat knee joint it is not clear which type of neurokinin receptor mediates the effects of SP on blood flow. These experiments were therefore performed to clarify the receptors mediating the effects of SP.

4.1.3 MATERIALS AND METHOD

Male Wistar rats (each approximately 350-500g, in-house colony) were deeply anaesthetised by intra-peritoneal injection of urethane (2g/kg). Subsequently, the skin over the knee was excised to expose the medial aspect of the knee joint. Relative changes in capsule blood flow (voltage difference of test minus control were monitored by Laser Doppler Imaging (LDI). As described in chapter 2, perfusion of

the exposed joint capsule was monitored during application of SP or a combination of SP with one of the antagonists. A scan was taken as a control immediately prior to application of any drugs. The normal time interval between successive scans was 5 minutes, which allows enough time for the recovery from the effect of the previous application of SP.

The vasodilator effect of the neurokinins is transient with the peak response occurring at 40±5.35 sec and return to baseline level in 111.43±8.7 (Lam & Ferrell 1993b). Details of the preparation and method are described in chapter 2.

Warmed SP (Cambridge Research Biochemicals), FK888 (Fujisawa), FK224 (Fujisawa), SR48986 (Sanofi) were administered as a bolus to the surface of the joint in a volume of 0.1 ml. SP was applied in 6 different doses from 10^{-13} to 10^{-8} mol, whilst three different doses (10^{-12} mol, 10^{-10} mol, 10^{-8} mol) of each antagonist were co-applied with SP. Warmed physiological saline (0.9% Cl Na) was regularly applied to the tissue surface to prevent dehydration.

Statistical analysis of the data was performed using "Minitab" software. A modified version of Shapiro Wilk test showed that the data were normally distributed . The comparisons between mean values was by ANOVA or "Student" t test where appropriate. Mean ± standard error of mean (SEM) was used and all quoted P values are two tail with P values equal or less than 0.05 being considered significant.

4.1.4 RESULTS

Basal perfusion: Imaging of blood flow to the medial aspect of the rat knee joint revealed that this region is highly perfused and is very vascular compared with surrounding tissue and skeletal muscle at regions adjacent to the joint capsule. Also in the joint tissue there is considerable variation in blood perfusion (fig. 4.1.1).

As the initial measurements involve comparison of basal blood flow between different treatments, rectangular areas chosen for calculation of the mean voltage were of the same size (23x15 measurement points). The rectangles selected comprise a total of 345 pixels on each image sufficient to include most of the articular tissue with minimal inclusion of the surrounding tissue.

The effect of SP showed a significant dose dependent increase in blood flow ($p<0.001$, n=35, Anova). At a dose of 10^{-13} mol there was a slight non-significant increase in blood flow, but there were a significant vasodilator responses with application of higher doses. The greatest vasodilator response occurred with SP 10^{-9} mol which increased of blood flow by 45.1 ± 8.6 %, n=35 (fig. 4.1.2,).

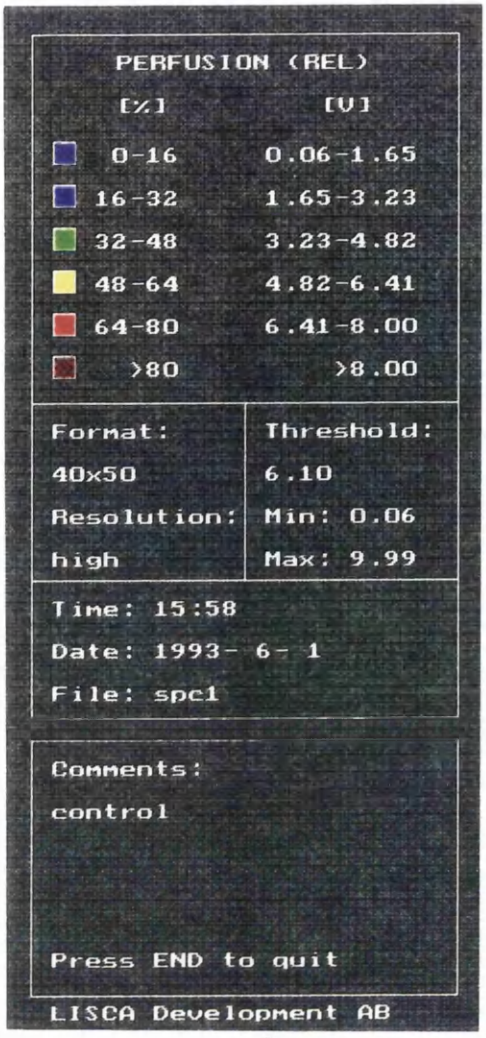
The effect of antagonists: three different antagonist at three different doses were used to see whether these can block the vasodilator effect of SP.

SR48986 (a NK2 non peptide antagonist) at three different doses was applied topically with SP to the knee joint (10^{-12} mol, 10^{-10} mol, 10^{-8} mol). SR48968 failed to reduce SP induced vasodilatation at all applied doses. Application of SR48968 at 10^{-12}

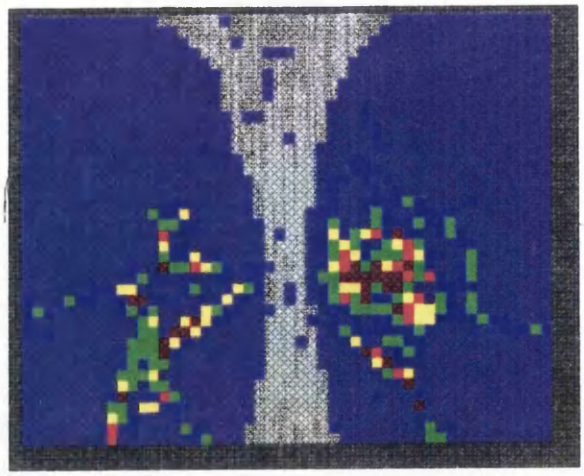
Fig 4.1.1: Laser Doppler perfusion images of rat knee joints.

A) colour-coded system for showing perfusion values

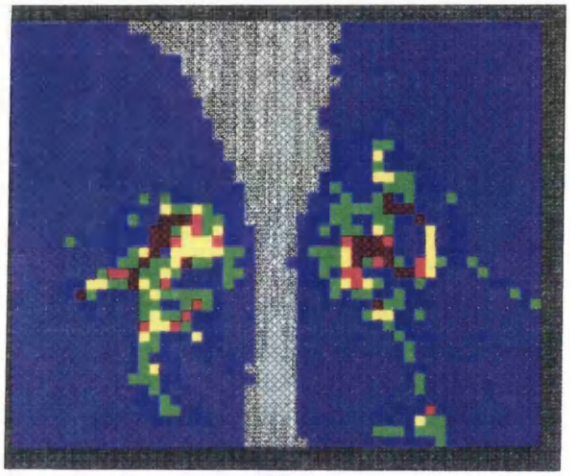
B) Perfusion images in different knees prior to any experiment


PERFUSION (REL)
[%] [V]
0-16 0.06-1.65
16-32 1.65-3.23
32-48 3.23-4.82
48-64 4.82-6.41
64-80 6.41-8.00
>80 >8.00
Format:
40x50
Resolution:
high
Threshold:
6.10
Min: 0.06
Max: 9.99
Time: 15:58
Date: 1993- 6- 1
File: spc1
Comments:
control
Press END to quit
LISCA Development AB

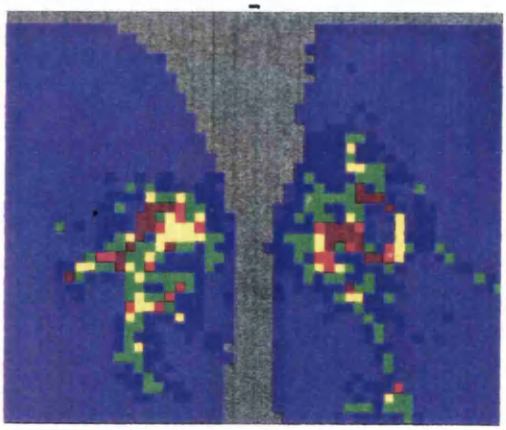
A



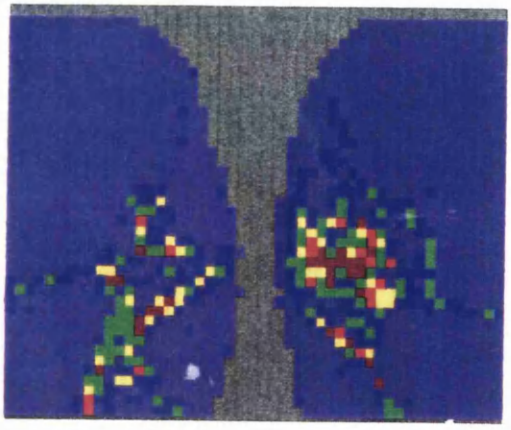
B



B



B



B

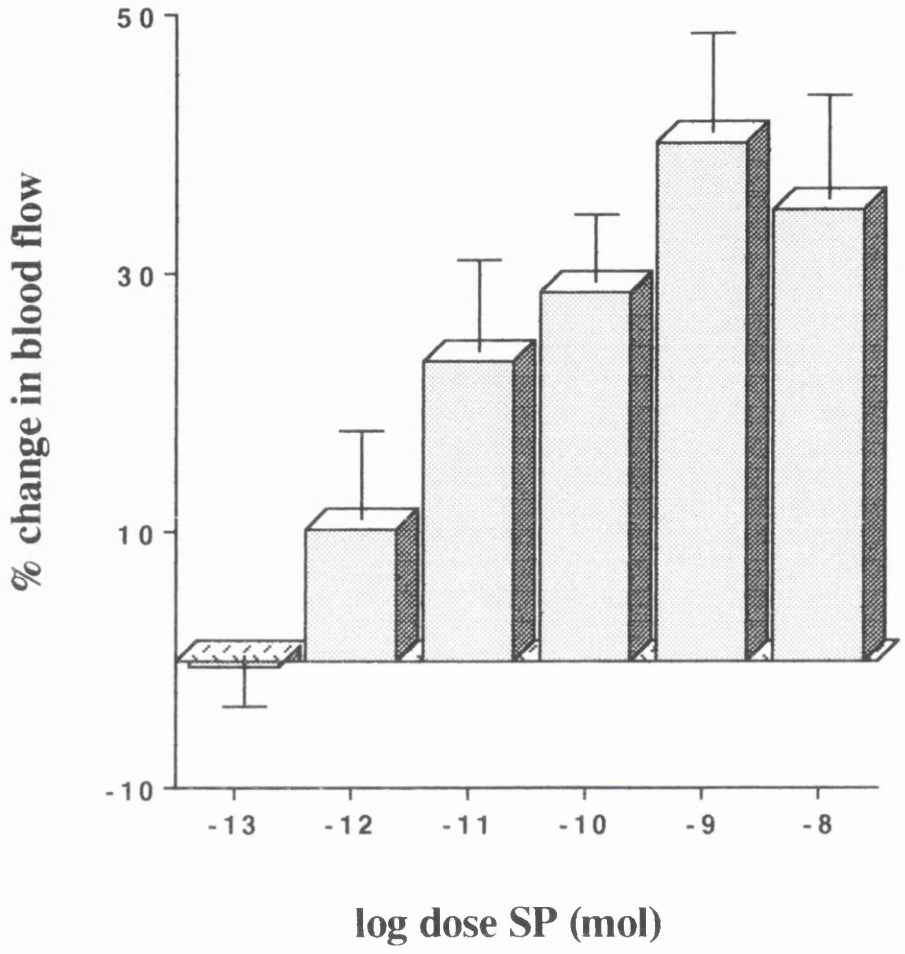


Fig 4.1.2. Changes in synovial blood flow during topical application of SP to the knee joint capsule (mean ±SEM, n=35)

mol slightly reduced the response to SP but it was not significantly different from control (P=0.064, n=24, Anova) (fig. 4.1.3a).

Application of SR48968 at doses of 10^{-10} mol and 10^{-8} mol did not reduce the vasodilator effect of SP, and seemed to slightly intensify it, but this effect was not significant (n = 12, p = 0.06 and n =8, p = 0.37 respectively) (fig. 4.1.3b, and c).

FK888 (an NK1 antagonist) was topically applied together with SP to the rat knee joint as described previously. Application of 10^{-12} mol FK888 with different doses of SP had no significant effect on vasodilatation. As seen in fig. 4.1.4a, 10^{-12} mol FK888 significantly reduced the vasodilator effect of 10^{-12} SP but failed at other doses of SP (p 0.513, n=12, Anova). This antagonistic effect of FK888 has been reported before (Lam *et al* 1993a).

Application of 10^{-10} mol FK888 with SP at different doses, except dose 10^{-10} mol, failed to reduce the vasodilator effect of SP (p= 0.2, n=12, Anova) (fig. 4.1.4b).

10^{-8} mol FK888 failed to reduce the vasodilator effect of SP on the rat knee joint (p = 0.61, n=16, two way Anova) (fig. 4.1.4c). In general FK888 reduced SP vasodilator effects at some doses but overall this reduction was non-significant

Three different doses of FK224 (an NK1 and NK2 antagonist) were used together with SP. FK224 at a dose of 10^{-12} mol failed to reduce the SP- induced vasodilator effect (p=0.23, n=8, Anova) (fig. 4.1.5a). This dose of FK224 inhibited the SP effect at some doses (10^{-12} mol), but overall the effect is unclear.

Fig 4.1.3. Changes in synovial blood flow during topical application of SP to the knee joint capsule alone, and with a non peptide NK2 antagonist, SR48968 at different doses. Values are mean ±SEM.

A) Application of SP alone (○, n=35) and together with SR48968 at 10^{-12} mol (▲, n=24)

B) Application of SP alone (○, n=35) and together with SR48968 at 10^{-10} mol (■, n=12)

C) Application of SP alone (○, n=35) and together with SR48968 at 10^{-8} mol (●, n=8)

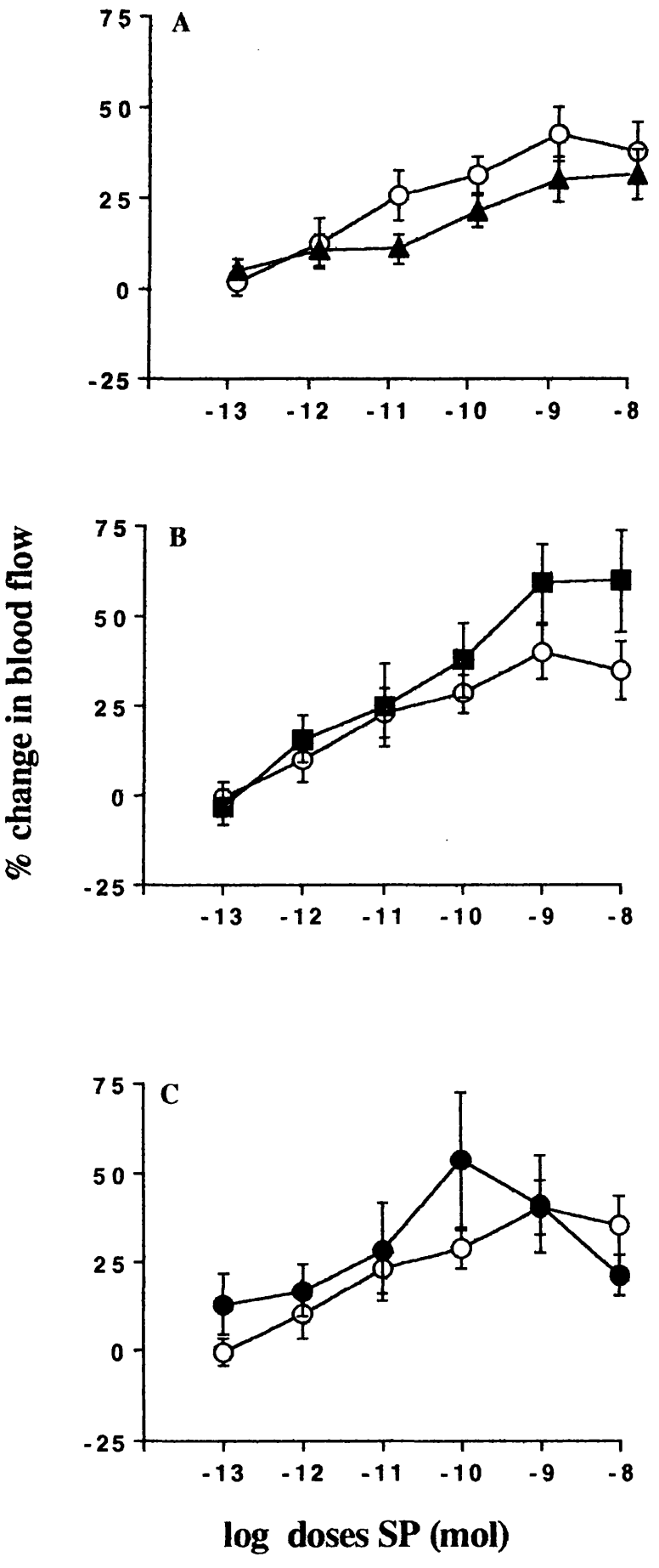
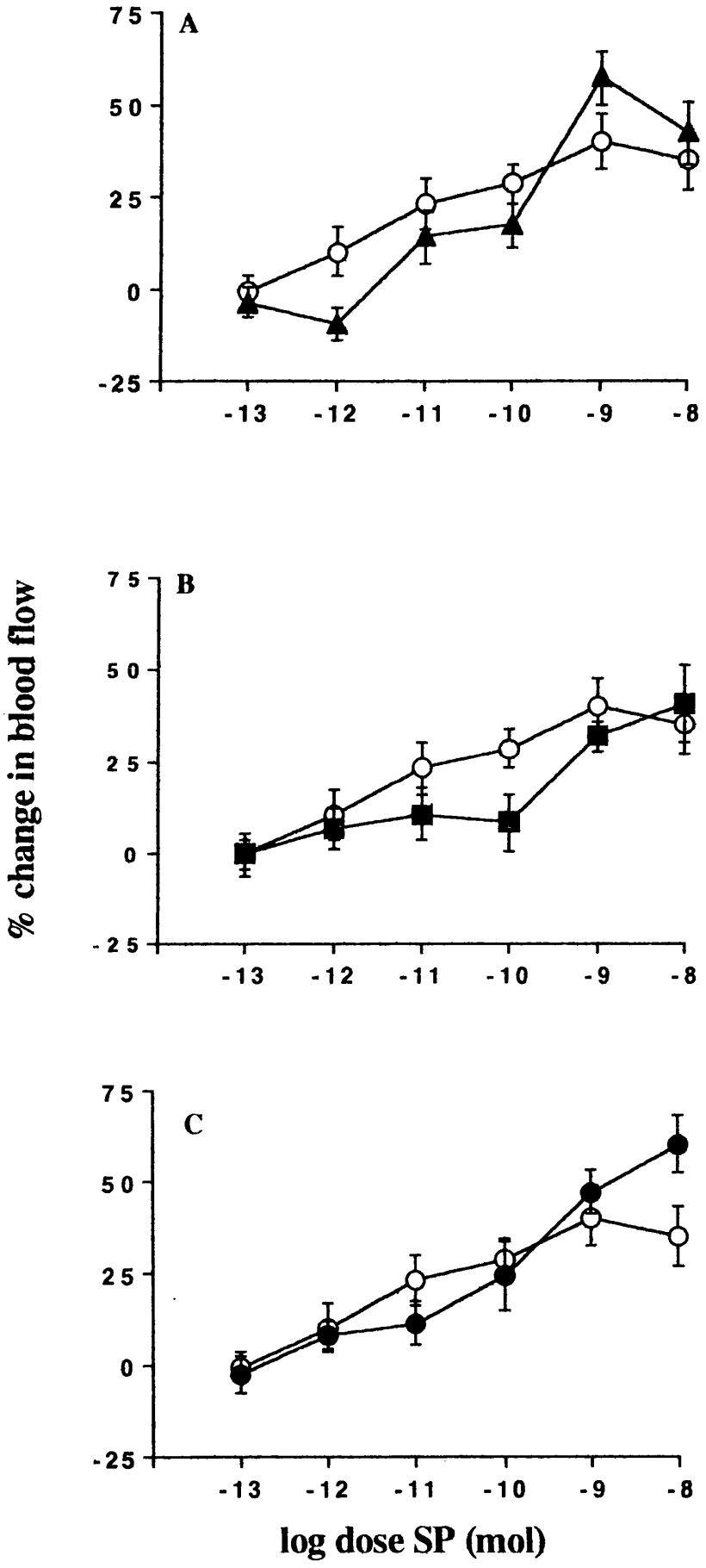

A
B
C
75
50
25
0
-25
-13
-12
-11
-10
-9
-8
% change in blood flow
log doses SP (mol)

Fig 4.1.4. Changes in synovial blood flow during topical application of SP to the knee joint capsule, alone and with an NK1 antagonist, FK888 at different doses. Values are mean ±SEM.

A) Application of SP alone (○, n=35) and together with FK888 at 10^{-12} mol (▲, n=12)

B) Application of SP alone (○, n=35) and together with FK888 at 10^{-10} mol (■, n=12)

C) Application of SP alone (○, n=35) and together with FK888 at 10^{-8} mol (●, n=16)


A
B
C
75
50
25
0
-25
-13
-12
-11
-10
-9
-8
% change in blood flow
log dose SP (mol)

FK224 at a dose of 10^{-10} mol reduced the vasodilator effect of SP significantly ($p=0.047$, $n=24$, two way Anova). This dose reduced SP effects on blood flow and shifted the dose response curve to right. At some points of the dose response curve the reduction is not significant but overall the reduction is significant (fig. 4.1.5b).

Co-application of FK224 (10^{-8} mol) with SP at various doses, fully inhibited SP's effect on blood flow ($P<0.0001$, $n=12$, Anova) and shifted the SP dose response curve to right (fig. 4.1.5c). At this doses of FK224 there is not only no response to SP from 10^{-13} to 10^{-10} mol but also there is a slight vasoconstrictor response. In general, FK224 dose dependently inhibited SP's effect on blood flow.

Co-application of both FK888 and SR48968 each at a dose of 10^{-8} mol with SP was performed and this significantly ($p=0.01$, $n=8$, Anova) reduced the vasodilator effect of SP at most doses, and shifted the dose response curve down (fig. 4.1.6).

4.1.5 DISCUSSION

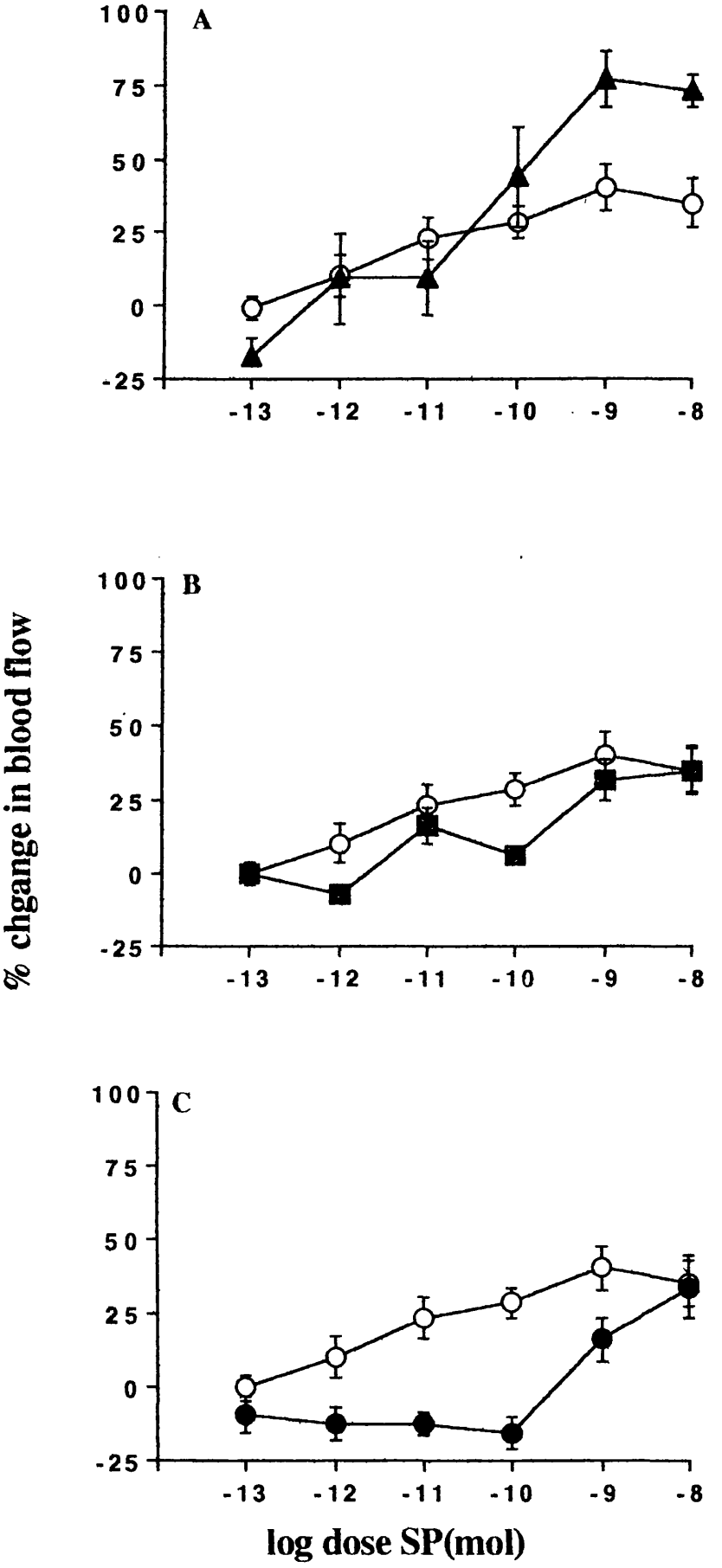
SP, an inflammatory mediator, is a potent vasodilator with a widespread effect all over the body. Local injection of SP can induce inflammation and oedema in the rat knee joint (Lam & Ferrell 1990). SP can also induce plasma protein extravasation in the rat knee joint (Scott *et al.* 1991). Inhibiting the effect of SP on blood flow and permeability by an appropriate and selective antagonist would have an important role in preventing pathological effects of SP. SP induced a dose-dependent vasodilatation in the rat knee joint, from 10^{-12} to 10^{-8} mol. At a dose of 10^{-8} mol, there is fall in blood flow compared to 10^{-9} mol, which could be due to the systemic absorption of SP.

Fig 4.1.5. Changes in synovial blood flow during topical application of SP to the knee joint capsule, alone and with an NK1 and NK2 antagonist, FK224 at different doses. Values are mean ±SEM.

A) Application of SP alone (○, n=35) and together with FK224 10^{-12} mol (▲, n=8)

B) Application of SP alone (○, n=35) and together with FK224 10^{-10} mol (■, n=24)

C) Application of SP alone (○, n=35) and together with FK224 10^{-8} mol (●, n=12)


A
B
C
100
75
50
25
0
-25
-13
-12
-11
-10
-9
-8
% chgange in blood flow
log dose SP(mol)

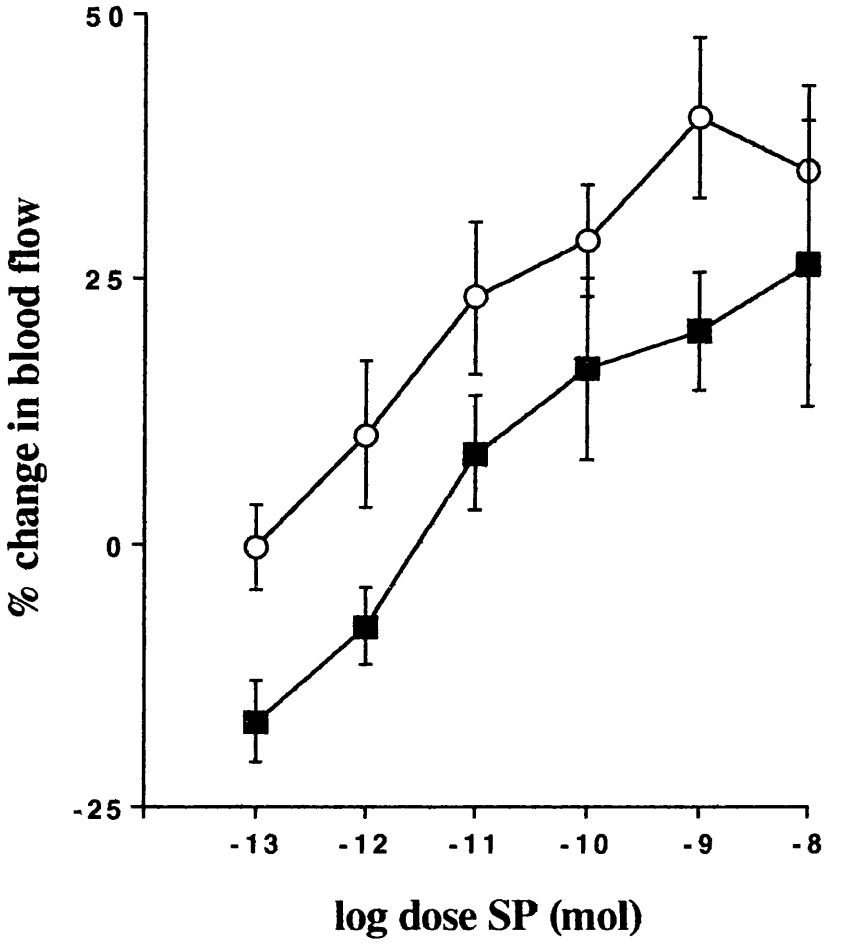


Fig 4.1.6. Changes in synovial blood flow during topical application of SP to the knee joint capsule, alone (○, mean ±SEM, n=35) and during co-application with SR48968 and FK888, both at a dose of 10^{-8} mol, (■ , mean ±SEM, n=8)

Monitoring blood pressure showed about a 20% fall in the blood pressure at 10^{-8} mol which is enough to reduce blood flow in the knee joint (fig. 4.1.7). This effect is transient, lasts less than 110 sec (Lam & Ferrell 1993b) and blood flow subsequently returns to normal. Thus, as previously mentioned, five minute intervals between each application of SP is enough for blood flow to return back to baseline. Fig. 2.7 indicates that control scan over 5 min are similar and there is no significant difference between these.

Three different neurokinin antagonists were applied in combination with SP to find out which neurokinin receptors mediate the vasodilator effects of SP.

SR48968 in general failed to inhibit the vasodilator effect of SP, although at one dose it could significantly reduce the SP vasodilator effect (SR.$^{-12}$ with SP $^{-11}$). At higher doses it even increased the SP vasodilator effect. As SR48968 is a potent and selective non peptide NK2 antagonist (Emonds *et al.* 1992), its failure to significantly reduce the vasodilator effect of SP at all doses suggests that these effects are not purely mediated by NK2 receptors.

Application of FK888 together with SP also failed to inhibit SP's effects on blood flow. In a previous study by Lam and Ferrell (1993c), FK888 was found to be an appropriate antagonist and was able to inhibit vasodilator effect of SP in the rat knee joint, though they only used a single dose of FK888. As shown in fig.4, at some doses it significantly reduced SP effects on blood flow (FK888 10^{-10} mol with SP 10^{-10} mol , $p<0.01$) but this was not consistent at other doses, and overall the effect was not significant. As FK888 is a selective and

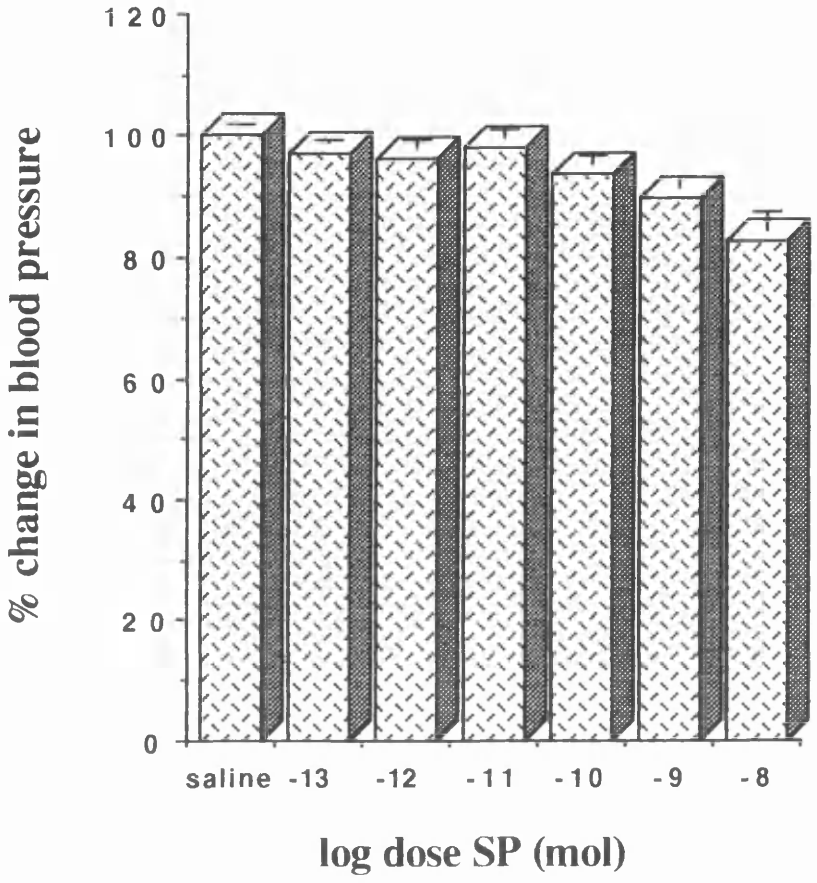


Fig 4.1.7. Percentage change in mean arterial blood pressure in response to topical application of SP to the joint capsule, (mean ±SEM, n=6)

potent NK1 antagonist (Murai *et al.* 1992), it's failure to completely inhibit the effect of SP, suggests that these are not fully mediated by NK1 receptors.

FK224 at a dose of 10^{-10} mol significantly ($p=0.047$) blocked SP effects and shifted the dose response curve to right and at dose of 10^{-8} mol it showed stronger inhibition of SP induced vasodilatation ($p<0.0001$). At doses of 10^{-13} to 10^{-10} mol, the response to SP was completely blocked by this antagonist, and a vasoconstriction response compared control was observed (fig. 4.1.5 b and c).

In general these data suggest that SP exerts its vasodilator effects via both NK1 and NK2 receptors which can be blocked by an NK1 and NK2 receptor antagonist such as FK224. The vasoconstriction which occurred in the presence of full blocking of NK1 and NK2 receptors, suggests that in the absence of a SP effect, other vasoconstrictor influences supervene resulting in a reduction in blood flow below the baseline control value. It could be concluded that SP may have a physiological role in regulating blood flow.

To verify whether the effect of SP is mediated by NK1 and NK2 receptors, a combination of a selective NK1 receptor antagonist (FK888) and selective NK2 receptor antagonist (SR48968) was used to try to block the vasodilator effect of SP. Application of both SR48968 and FK888 together with SP significantly inhibited the vasodilator effect on blood flow ($p=0.01$) and shifted the SP dose response curve downwards. This may be due to the vasoconstriction effect of FK888 and SR48968, as described in chapter 4.4

These data confirm previous findings with FK224, although the inhibition with FK888/ SR48968 combination at 10^{-8} mol was less than that obtained with FK224. This difference could be due to existence of atypical neurokinin receptors or neurokinin receptor subtypes. Lee *et al.* (1982) and Piercey *et al.* (1982) reported that there are several subpopulation of SP receptors. Also, the existence of atypical NK1 tachykinin receptors in the rabbit iris sphincter (Hall *et al.* 1994) supports the idea of atypical receptors perhaps being also present in the rat knee joint. Also Meini *et al.* (1994) have described the existence of NK1 receptor subtypes in the rat urinary bladder.

The inhibitory effects of these antagonist are likely to be purely due to their specific effect on neurokinin receptors. Although Zum yi wang *et al* (1994a) and Karlson *et al* (1994) have reported that these antagonists can have non-specific sympathetic effects which can cause slight vasodilatation, this appears at 10^{-4} mol which is more than a thousand fold higher than the maximum dose which was used in the present experiments. In addition, the time course of such effects is much longer than the transient SP time course on blood flow.

In conclusion these data suggests that SP induced vasodilatation in the rat knee joint is mediated by NK1 and NK2 receptors which may be atypical or a subtype of these receptors. A physiological effect of SP in controlling local blood flow may also be present. Further investigation is required to clarify this .

4.1. 6 SP INHIBITION BY NEUTRAL ENDOPEPTIDASE

Neutral endopeptidase (NEP) and angiotensin converting enzyme (ACE), are well known to convert and degrade SP (Couture *et al.* 1981). Several organs are capable of inactivating SP, and the liver has highest capability (Lembeck *et al* 1978).

Captopril (also known as SQ. 14225) was introduced as a NEP inhibitor (Couture *et al.* 1981), and it is also known as a hypotensive drug. Neurokinins will rapidly be degraded by NEP and eliminated The time course of vasodilatation by the neurokinins, with topical application, is less than 2 min. NEP inhibition in the tissues results in a long-lasting effect of neurokinins. In the experiments described below captopril was applied both topically and intravenously (IV) to evaluate changes in the neurokinin-induced vasodilatation in the rat knee joint.

4.1.6.1 Materials and methods: Six rats were cannulated to monitor blood pressure and drug administration. The procedures were as described in chapter 2. Captopril was applied topically or IV in a dose of 5.7 mg per kg (Shore *et al* 1992). SP was applied at different doses. The procedure and data analysis were as described in chapter 2 and 4.1,2

4.1.6.2 Results: IV application of captopril reduced systemic blood pressure up to 35% and this reduction was consistent and irreversible over 60 min (n=13). Topical application of captopril showed the same effect on blood pressure and reduced blood flow by 35% (n=5).

Topical application of SP on the knee joint in the captopril pre-treated rat revealed an attenuated response to SP. It was a weak response and also there was no significant difference between doses ($p< 0.05$). This attenuated response was consistent over 15 min.(fig. 4.1.8, n = 13).

4.6.3 Discussion: The response of the normal knee joint to SP is dominantly affected by the fall in blood pressure. SP at dose of 10^{-8} mol caused a <20% fall in the blood pressure and attenuated its local vasodilator effect on the knee joint (fig. 4.1.2, 4.1.7). A 35% reduction in systemic blood pressure should have a strong attenuating effect on SP mediated vasodilatation, but in fact SP showed a small but non dose dependent vasodilatation on the knee joint over 15 min. However in the intact knee the normal degrading period for SP is 1.5 min. Fig. 4.1.9 shows the time course of the effect of SP on blood flow after 0 , 5, 10 and 15 min post application of SP in the captopril pre-treated rat and compared with SP in the normal rat. SP in higher doses, even in captopril pre-treated rats, showed a further reduction in systemic blood pressure.

SP is a potent vasodilator peptide and it is synthetised and stored in a vast variety of tissues and body organs (Pernow 1983) This substance is immediately destroyed and inactivated by NEP and when this is inhibited an acute reduction in blood pressure can result from rapid vasodilatation. Therefore it could be concluded that the systemic hypotensive effect of captopril could be due to its inhibitory effect on NEP, thereby preventing the degradation of SP. SP has an essential role in controlling blood pressure and so slight changes in its metabolism can produce a vast changes in systemic blood pressure.

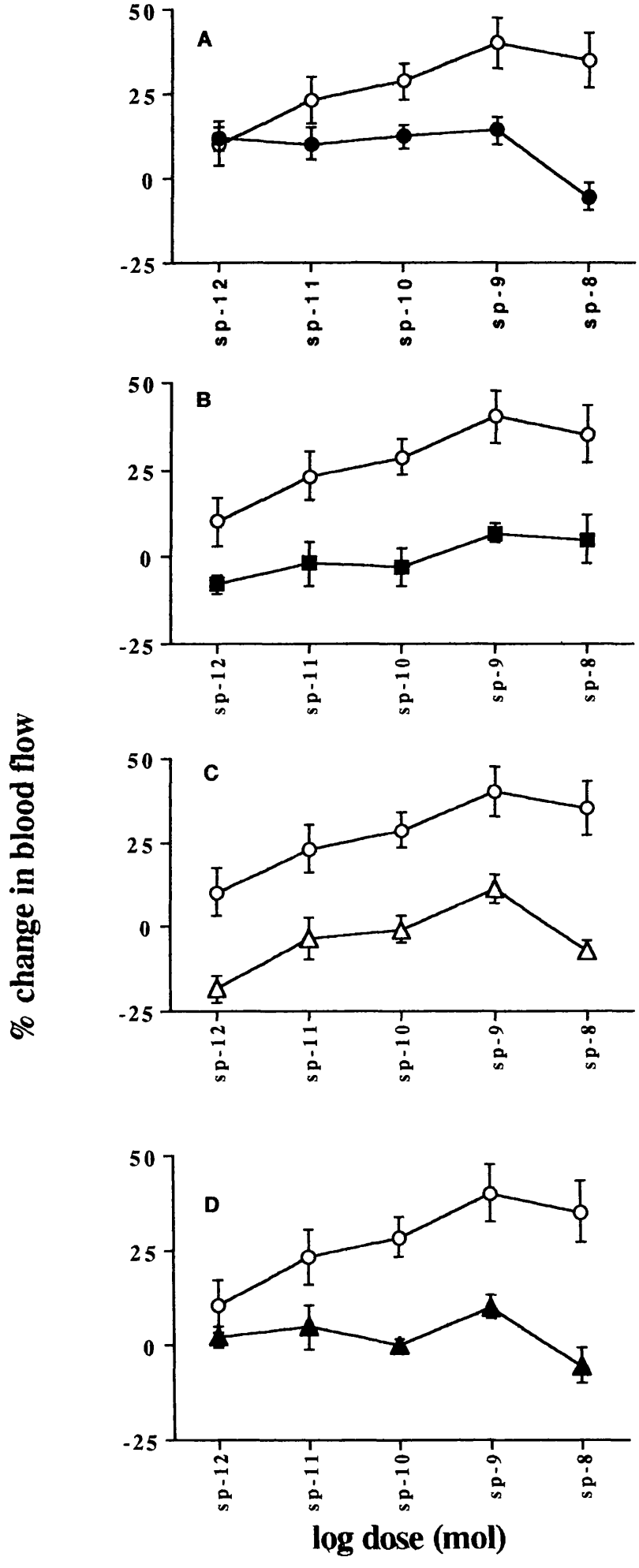
Fig 4.1.8. Changes in synovial blood flow during topical application of SP to the knee joint capsule in the intact knee and after IV. treatment with captopril (10^{-7} mol), at different times: Values are mean ±SEM.

A) Application of SP in intact knee (○, n=35) and immediately after treatment with captopril (●, n=10)

B) Application of SP in intact knee (○, n=35) and 5 min after treatment with captopril (■, n=10)

C) Application of SP in intact knee (○, n=35) and 10 min after treatment with captopril (Δ, n=10)

D) Application of SP in intact knee (○, n=35) and 15 min after treatment with captopril n=10 (▲, n=10)


A
B
C
D
50
25
0
-25
sp-12
sp-11
sp-10
sp-9
sp-8
% change in blood flow
log dose (mol)

Due to marked systemic effect of captopril on blood pressure it was not possible to do further experiments .

SECTION TWO

NEUROKININ A AND ITS RECEPTORS

4.2.1 SUMMARY

1) Experiments were performed to identify the action of NKA on neurokinin receptors in the rat knee joint.

2) Laser Doppler Imaging (LDI) was used to investigate changes in blood flow.

3) FK888 (an NK1 antagonist), SR48968 (an NK2 non peptide antagonist), and FK224 (an NK1 and NK2 antagonist), in different doses were used in the experiments.

4) NKA on its own at lower doses slightly decreased blood flow but at higher doses it dose dependently increased blood flow (doses between 10^{-10} to 10^{-13} mol). Maximum vasodilatation achieved was 59.25% ± 13.44 when a dose of 10^{-8} mol was used.

5) FK888 at higher doses inhibited the vasodilator effect of NKA in the rat knee joint.

6) FK224 at some doses inhibited the vasodilator effect of NKA but overall failed to reduce its effect on blood flow.

7) SR48968 at 10^{-8} mol partially, significantly and dose dependently reduced the vasodilator effect of NKA

8) These data suggest that the vasodilator effect of NKA is chiefly mediated via NK2 receptors which could be a subtype of these receptors, and a small part of its action is mediated via NK1 receptors.

4.2 2 INTRODUCTION

The existence of NKA first was reported in 1983 by several groups (Kangaw *et al.* 1983; Kimura *et al.* 1983; Manamino *et al.* 1984), and further investigation showed that this peptide is widely distributed in both central and peripheral nervous systems. NKA is released in response to noxious and other stimuli and has a variety of biological actions (Maggi *et al* 1993a) and is produced from a single preprotachykinin gene (SP/NKA gene: Nawa *et al* 1983). This peptide has a very wide distribution and synthesis occurs in different parts of the body (Pernow 1993, Deacon and Colon1987, Maggi 1993b) and gives rise to different physiological and pathological effects (Geppeti *et al* 1987; also see chapter 1). However there is only one reports about the effect of NKA on the rat knee joint vasculature (Lam & Ferrell 1993B). These experiments were performed to clarify the effect of NKA on rat knee joint blood flow and the receptors mediating this response.

4.2.3 MATERIALS AND METHODS

Experiments were performed on 53 Wistar rats, the experimental procedures and data analysis were as described previously (chapter 2).

NKA was utilised at six different doses (10^{-13}-10^{-8} mol) with and without three different antagonists, SR48968, FK888, FK224. Antagonists were applied over the same dose range and by the same route as described previously (see chapter 4.1.3). Statistical analysis was performed using minitab software, and the comparisons between mean values was by two way Anova or two tail Student t test (see chapter 4.1.3).

4.2.4 RESULTS

Basal perfusion and selected rectangular measurement area is as described in chapter 4.1.4.

The effect of NKA: NKA at lower doses had variable effect on blood flow. The 10^{-13} mol dose increased non-significantly blood flow and doses of 10^{-12} mol and 10^{-11} mol slightly decreased blood flow compared to basal blood flow values. From 10^{-10} mol blood flow increased dose dependently up to a maximum of 59.2±13.4%, n=14 (fig. 4.2.1).

The effect of antagonists: Three different antagonists at three different doses were used (10^{-12} mol, 10^{-10} mol, 10^{-8} mol).

SR48968, which is a non-peptide NK2 antagonist, was applied topically with different doses of NKA. Co-application of SR48968 at 10^{-12} mol with NKA failed to reduce the vasodilator effect of NKA and even intensified the vasodilator effect of NKA at 10^{-9} mol, but overall SR48968 failed to inhibit the effects of NKA ($p = 0.96$, n=8, Anova) (fig. 4.2.2a).

Application of SR48968 at 10^{-10} mol with NKA showed different responses, with NKA at 10^{-12} mol to NKA 10^{-10} mol increasing NKA's effect on blood flow but at other doses of NKA, decreased its effect on blood flow. In general, SR48968 at 10^{-10} mol failed to reduce the vasodilator effect of NKA ($p= 0.58$, n= 8, Anova) (fig. 4.2.2b) and the dose response curves were not significantly different from control.

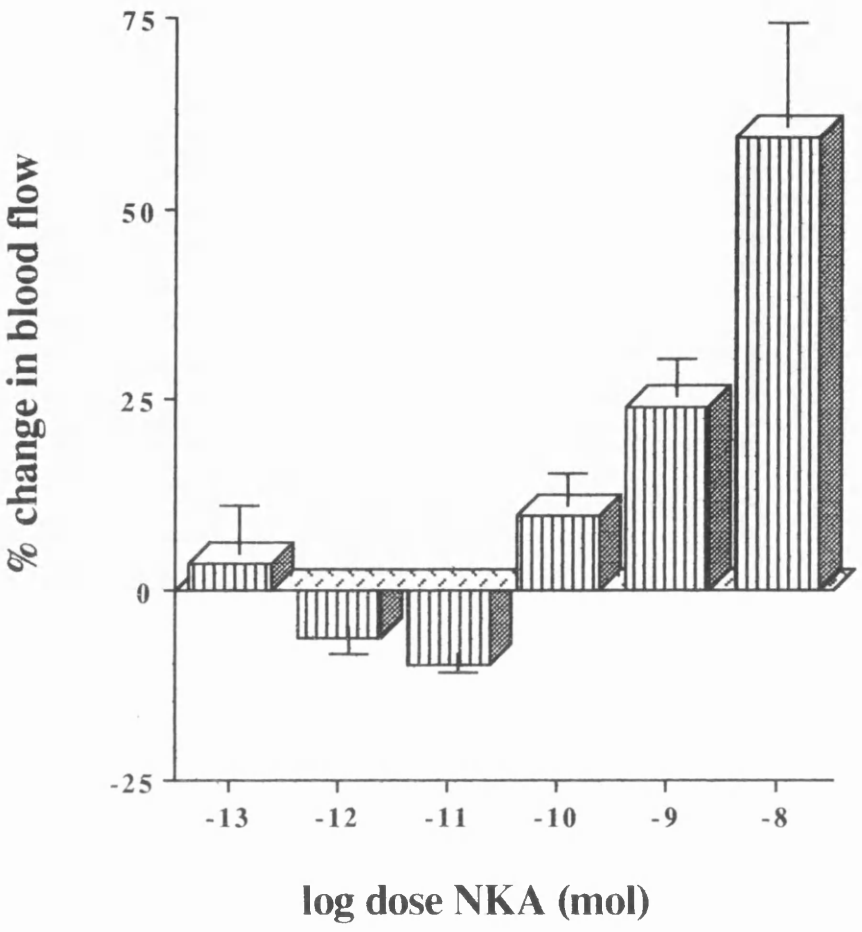


Fig 4.2.1 Changes in synovial blood flow during topical application of NKA to the knee joint capsule (mean ±SEM, n=14)

In contrast, SR48968 at 10^{-8} mol significantly (P=0.002, n=8, Anova) inhibited the effect of NKA (fig. 4.2.2c).

FK888 which is a specific NK1 receptor antagonist was applied at three different doses together with NKA. FK888 at 10^{-12} mol with different doses of NKA, not only failed to inhibit NKA but also increased significantly the NKA effect on blood flow ($p<0.001$, n=12, Anova)(fig. 4.2.3a).

FK888 at 10^{-10} mol applied with NKA also increased the vasodilator response of NKA and although this effect was less obvious than FK888 at 10^{-12} mol, and it too was significant (p=0.001, n=12. Anova) (fig. 4.2.3b).

Co-application of FK888 at 10^{-8} mol with NKA reduced the effect of NKA on blood flow at doses 10^{-13} to 10^{-9} mol but increased the vasodilator effect of NKA at a dose 10^{-8} mol (fig. 4.2.3c). In general, FK888 at 10^{-8} mol partially and significantly reduced the effect of NKA on blood flow (p=0.02, n=12, Anova).

FK224 is an NK1 and NK2 receptor antagonist. This antagonist was also used at three different doses. Application of FK224 at 10^{-12} mol together with NKA not only inhibited NKA-induced vasodilatation, but also significantly increased its vasodilator effect (p=0.007, n= 8, Anova) (fig. 4.2.4a).

FK224 at 10^{-10} mol intensified the effects of NKA on blood flow at all doses, which was significant compared with NKA on its own ($P<0.0001$, n=8, Anova) (fig. 4.2.4b)

FK224 at a dose of 10^{-8} mol co-administered with NKA showed a variable effect on blood flow, at some doses increasing and at others decreasing the vasodilator effect of NKA. Overall it failed to reduce the effect of NKA on blood flow ($p= 0.32$, n=8, Anova) (fig. 4.2.4c).

Application of both FK888 and SR48968 at a dose of 10^{-8} mol with NKA, inhibited the vasodilator effect of NKA except at a dose of 10^{-11} mol. This reduction of blood flow was significant ($p=0.017$. n=8, Anova) in compared to control (fig. 4.2.5).

4.2.5 DISCUSSION

NKA is a ten amino acid peptide which is produced by PPT1, the same gene which produces SP. NKA has a 4 amino acid sequence similar to SP (Helke *et al* 1990). This peptide exists with other neuropeptides in the sensory nerve endings of both central and peripheral nervous systems and participates in different biological responses (Maggi *et al* 1993a). NKA, beyond a dose of 10^{-10} mol, increases blood flow with a maximum effect at a dose 10^{-8} mol (59.2± 13.4 % increase in blood flow). To find out which receptors mediate this effect three different antagonists at three different doses were topically co-applied with NKA.

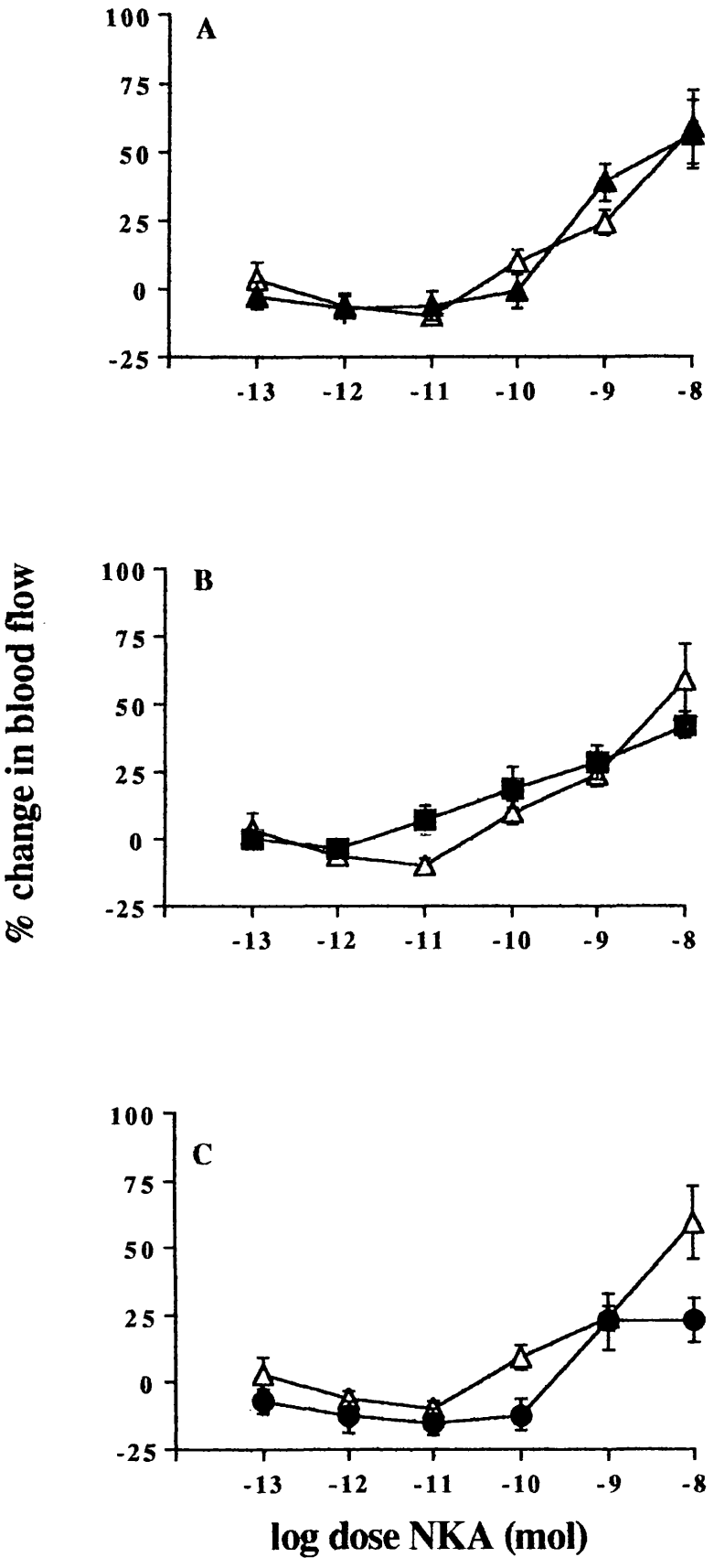
SR48968 at a dose of 10^{-12} mol failed to reduce NKA induced vasodilatation and 10^{-10} mol SR48968 produced variable effects, and except at a dose NKA 10^{-8} mol, it failed to reduce the vasodilator effect of NKA. SR48968 at 10^{-8} mol significantly and dose dependently reduced effects of NKA on blood flow (two way Anova, $p=0.002$). This reduction in the NKA effect on blood flow is not strong and the dose response curve is only slightly shifted to right which

Fig 4.2.2. Changes in synovial blood flow during topical application of NKA to the knee joint capsule alone, and with a non peptide NK2 antagonist, SR48968 at different doses: Values are mean ±SEM.

A) Application of NKA alone (Δ, n=14) and together with SR48968 at 10^{-12} mol (▲, n=8)

B) Application of NKA alone (Δ, n=14) and together with SR48968 at 10^{-10} mol (■, n=8)

C) Application of NKA alone (Δ, n=14) and together with SR48968 at 10^{-8} mol (●, n=8)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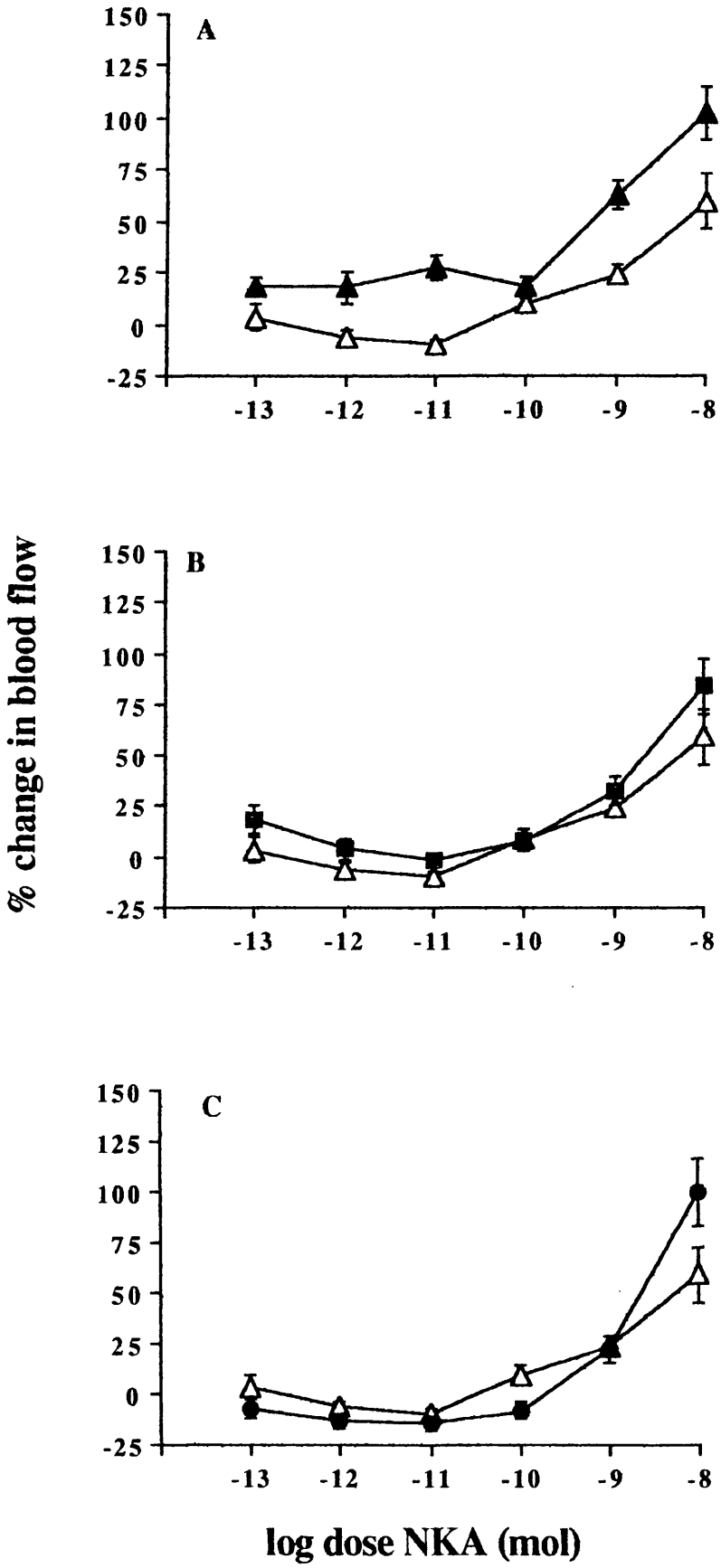
-11
-10
-9
-8
% change in blood flow
log dose NKA (mol)

Fig 4.2.3. Changes in synovial blood flow during topical application of NKA to the knee joint capsule alone, and with an NK1 antagonist, FK888 at different doses: Values are mean ±SEM.

A) Application of NKA alone (Δ, n=14) and together with FK888 at 10^{-12} mol (▲, n=12)

B) Application of NKA alone (Δ ,n=14) and together with FK888 at 10^{-10} mol (■, n=12)

C) Application of NKA alone (Δ ,n=14) and together with FK888 at 10^{-8} mol (●, n=12)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-25
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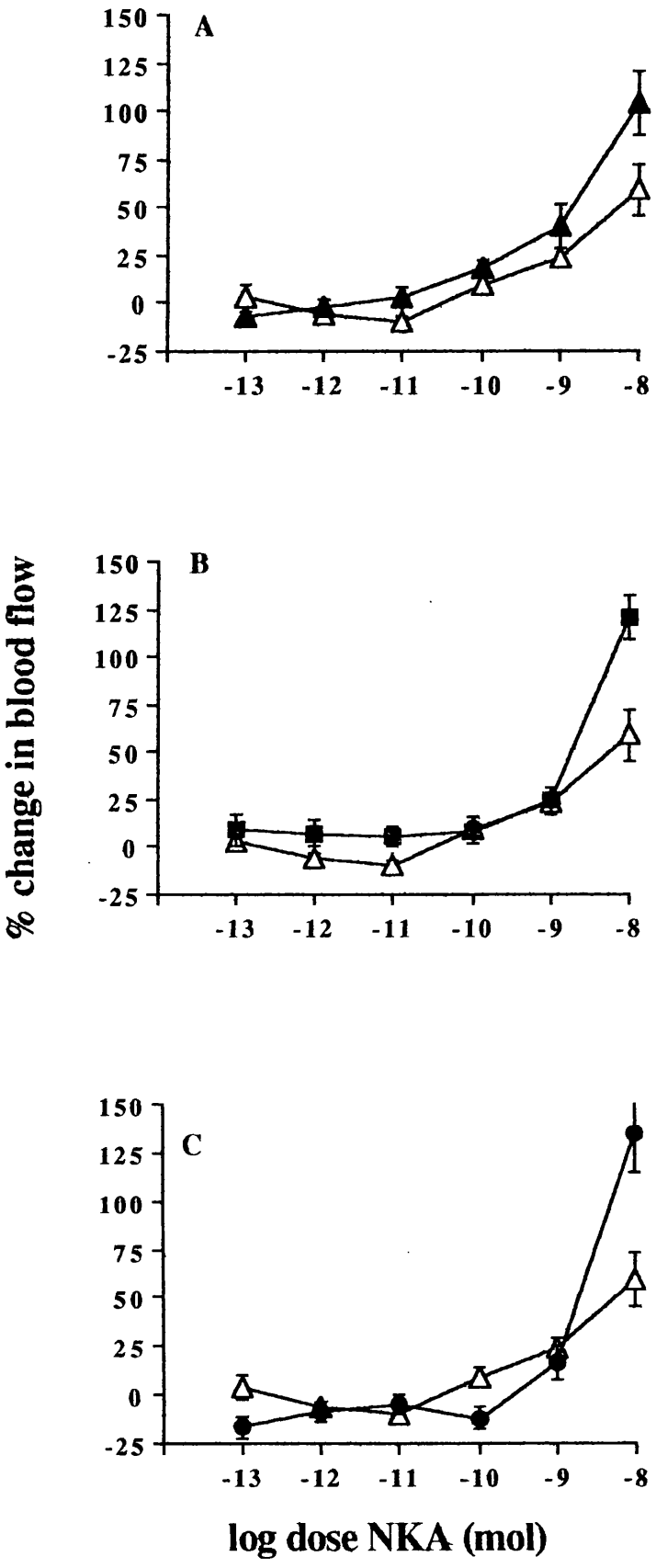
-9
-8
% change in blood flow
log dose NKA (mol)

Fig 4.2.4. Changes in synovial blood flow during topical application of NKA to the knee joint capsule alone, and with an NK1 and NK2 antagonist, FK224 at different doses: Values are mean ±SEM,

A) Application of NKA alone (Δ , n=14) and together with FK224 at 10^{-12} mol (▲, n= 8)

B) Application of NKA alone (Δ , n=14) and together with FK224 at 10^{-10} mol (■, n= 8)

C) Application of NKA alone (Δ , n=14) and together with FK224 at 10^{-8} mol (●, n= 8)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% change in blood flow
log dose NKA (mol)

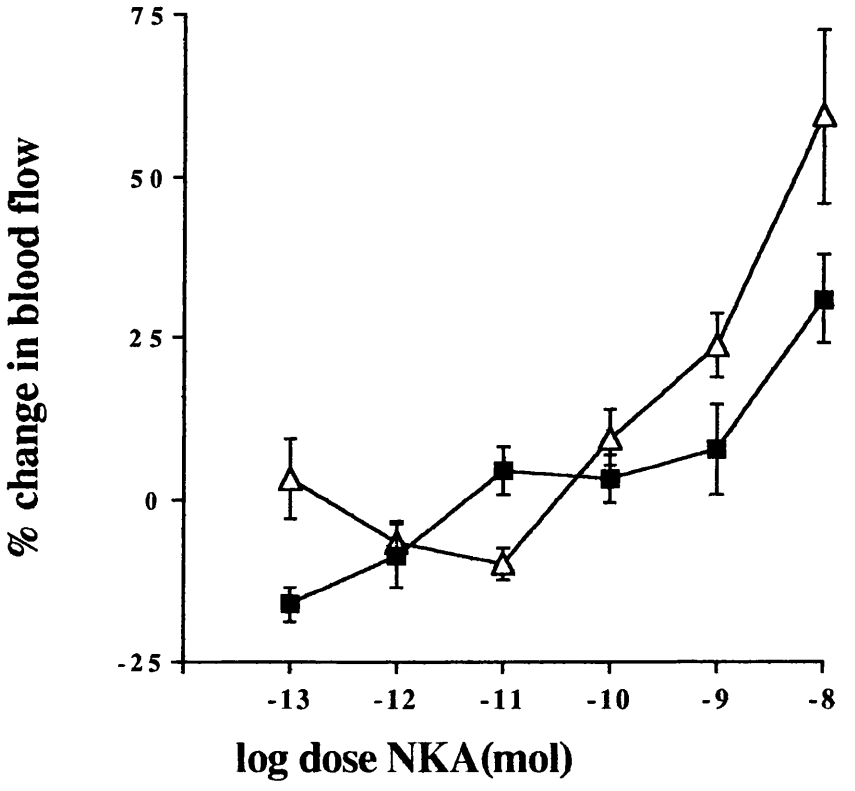


Fig 4.2.5. Changes in synovial blood flow during topical application of NKA to the knee joint capsule, alone (Δ, mean ±SEM, n=14) and co-application with SR48968 and FK888, both at dose of 10^{-8} mol, (■ , mean ±SEM, n=8)

suggests a partial inhibition of NKA. As SR48968 is known to be a specific NK2 antagonist, these data suggest that NK2 receptors mediate a part of the vasodilator effect of NKA on blood flow.

Co-application of both 10^{-12} and 10^{-10} mol FK888 with NKA, enhanced the vasodilator effect of NKA on blood flow, which was significant (P=0.01) for the 10^{-10} mol dose. This enhancing effect of antagonists at lower concentrations was observed also with the other antagonist SR48968. FK888 on its own does not have any effect on blood flow in doses of 10^{-12} and 10^{-8} and at 10^{-10} mol non significantly increased blood flow (see 4.4). Therefore the enhancing effect of NKA with co-application of FK888 could be due to a direct effect on blood vessels or activation and release of other vasodilator agents.

10^{-8} mol FK888 significantly reduced the vasodilator effect of NKA at lower doses and enhanced effects of NKA on blood flow at 10^{-8} mol. These data suggest that FK888 can partially inhibit the vasodilator effect of NKA on blood flow, which indicates that NKA can also activate NK1 receptors and mediates a part of its effect via these..

FK224 at dose of 10^{-12} and 10^{-10} mol was unable to reduce NKA-induced vasodilatation, and even significantly enhanced this effect. FK224 at 10^{-8} mol had a variable effect on NKA induced vasodilatation. at lower doses of NKA this effect is attenuated and at higher doses this effect is enhanced. At 10^{-10} mol this antagonist weakly inhibits NKA-induced vasodilatation but this was not significant.

Combination of FK888 and SR48968 both at 10^{-8} mol significantly (P=0.017) reduced the NKA effect on blood flow. This reduction, except at a dose of 10^{-11} mol, was dose dependent, and shifted the dose response curve to right. In conclusion, NKA has a higher affinity for NK2 receptors than NK1 receptors, and mediates its effect on blood flow generally via these receptors, although NKA can cross react on NK1 receptors. SR48968 showed a weak antagonistic response for NK2 type of receptors. Hallin *et al* (1994) suggested further classification for NK2 receptors in spinal cord, and Advenier *et al* (1992) described that SR48968 is more active on the NK2a receptor than the NK2b. As SR48968 did not show strong antagonistic effect in the rat knee joint, it seems that NK2b receptors mediate the vasodilator effect of NKA. There is cross reactivity with NK1 receptors and a small portion of the NKA effect on blood flow is mediated via these receptors. Previous work has shown that bronchoconstriction in the conscious guinea pig is mediated predominantly by NK2 receptors with only a minor involvement of NK1 receptors (Chan *et al* 1994).

SECTION THREE

NEUROKININ B AND ITS RECEPTORS

4.3.1 SUMMARY

1) Experiments were performed to identify the action of NKB on neurokinin receptors in the rat knee joint.

2) Laser Doppler Imaging (LDI) was used to investigate changes in blood flow.

3) FK888 (a NK1 antagonist), SR48968 (a NK2 non-peptide antagonist) and FK224 (a NK1 and NK2 antagonist), were used at different doses in these experiments.

4) NKB on its own produced a potent and significant dose dependent vasodilatation in the rat knee. The maximum vasodilatation achieved was 105% increase from base line with a dose of 10^{-8} mol NKB.

5) SR48968 at a dose of 10^{-8} mol significantly reduced the effect of NKB on blood flow.

6) FK888 in a dose of 10^{-8} mol slightly but non significantly reduced NKB-induced vasodilatation.

7) FK224 at 10^{-8} mol completely and significantly blocked the vasodilator effect of NKB.

8) Combination of FK888 and SR48968 in a dose of 10^{-8} mol. completely and significantly blocked the effects of NKB on blood flow.

These data suggest that NKB mediates its vasodilator effects via NK1 and NK2 receptors in the knee joint, and it is unlikely that NK3 receptors are present.

4.3.2 INTRODUCTION

NKB is a ten amino acid peptide, which belongs to the tachykinin family with a common C terminus. NKB is the only tachykinin derived from pre-pro-tachykinin II (PPTII) (Bonner *et al* 1987). In spite of expression of PPTI, in dorsal root ganglia (DRG), the PPTII gene is not expressed in DRG hence the absence of NKB in central or peripheral nerve terminals of capsaicin-sensitive primary afferents is expected (Maggi *et al* 1993a) and NKB is mainly found within the CNS. Some investigators have reported the existence of NKB in rat peripheral nerves (Tateishi *et al* 1990), but tissue levels are extremely low compared to SP or NKA and in many cases NKB is undetectable.

The release of this peptide is via an active calcium dependent mechanism (Bonner *et al* 1987), and it is thought to exert different physiological actions, but there are few reports about the specific effects of NKB in peripheral tissues.

Three different and distinct receptors, NK1, NK2 and NK3 mediate the biological effects of the neurokinins. NK1 and NK3 are G protein coupled receptors with pharmacologically distinct profiles (Gether *et al* 1993). However there is little known about the effects of NKB in rat knee joint blood vessels and the receptors mediating these effects, and these experiments were performed to investigate these issues.

4.3.3 MATERIALS AND METHOD

The experiments were performed on 42 male Wistar rats (~350-500g). Anaesthetic procedures and preparation for experiments, technical and statistical methods were as described in chapter 4.1.3.

NKB at six different doses (10^{-13} to 10^{-8} mol). was used in these experiments Three different antagonists, FK888, FK224 and SR48968 at three different doses (10^{-12}, 10^{-10}, 10^{-8} mol) were co-applied with NKB. The rest of the methods have been described previously (chapter 2). Statistical analysis in this study was by two way Anova and student t test as described previously (see 4.1.3).

4.3.4 RESULTS

Basal perfusion and selected rectangular measurement area is as described in 4.1.4.

The effect of NKB: NKB on its own produced a potent and significant increase in blood flow. This vasodilatation began at a dose of 10^{-12} mol and the maximum response was at 10^{-8} mol with 105.7± 13.6 %, n=8, increase in blood flow over basal values (fig. 4.3.1).

The effect of antagonists: SR48968 at dose of 10^{-12} mol co-applied with NKB, in some cases decreased and in some cases enhanced the vasodilator effect of the NKB, but overall these changes were not significant (p=0.24, n=8, Anova) (fig. 4.3.2a).

Application of 10^{-10} mol SR48968 with NKB not only failed to reduce NKB-induced vasodilatation but also significantly (p=0.01, n=8, Anova) increased its effect on blood flow at all doses (fig. 4.3.2b).

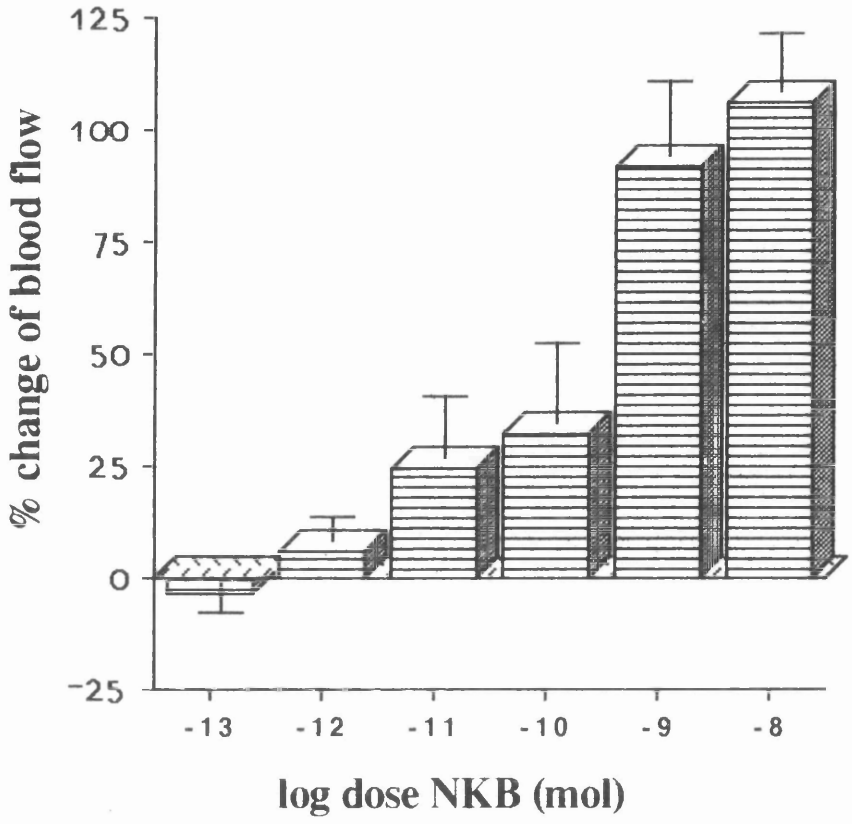


Fig 4.3.1 Changes in synovial blood flow during topical application of NKB to the knee joint capsule (mean ±SEM, n=8)

SR48968 at a dose of 10^{-8} mol co-applied with NKB weakly but significantly ($p=0.033$, $n=8$, Anova) reduced NKB-induced vasodilatation (fig. 4.3.2c).

FK888 at all three doses failed to inhibit NKB-induced vasodilatation. FK888 at 10^{-12} and 10^{-10} mol had no effect on NKB-induced vasodilatation and at 10^{-8} mol non-significantly reduced the effect of NKB ($p=0.8$, $n=8$. $p=0.5$, $n=8$ and $p=0.4$, $n=8$ respectively) (fig. 4.3.3a, b and c respectively).

FK224 at a dose of 10^{-12} mol co-applied with NKB, reduced its effect on blood flow, but this reduction was not significant ($p=0.1$, $n=8$, Anova) (fig. 4.3.4a).

FK224 at 10^{-10} mol significantly ($p=0.012$, $n=8$, Anova) inhibited NKB induced vasodilatation and slightly shifted the dose response curve to the right (fig. 4.3.4b).

The vasodilator effect of NKB was completely and significantly ($p<0.001$, $n=10$, Anova) blocked by FK224 at 10^{-8} mol, and except for the 10^{-8} mol dose which gave only 45% vasodilatation, the other doses did not evoke any response and blood flow remained at baseline values (fig. 4.3.4c).

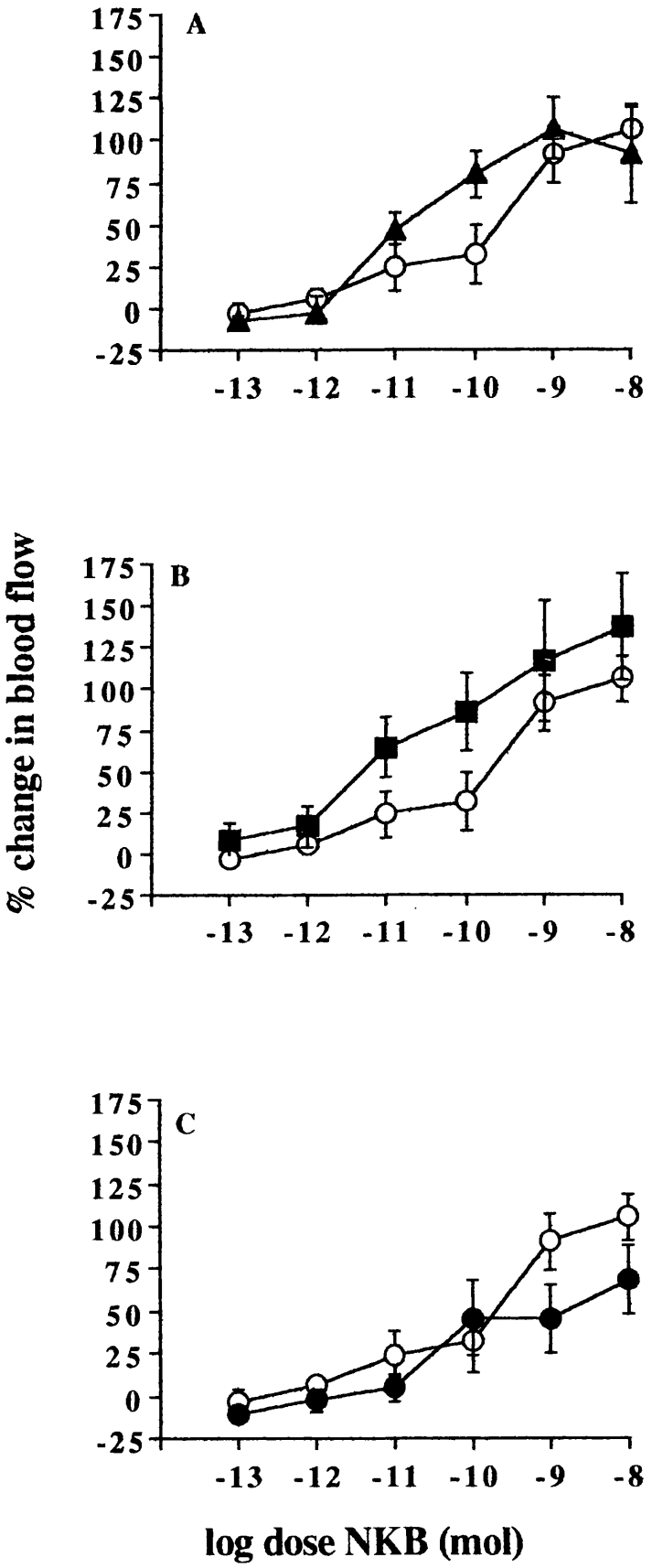
Co-application of FK888 and SR48968 both at 10^{-8} mol with NKB completely blocked the effect of NKB on blood flow at lower doses and at higher doses. Significant ($p<0.0001$, $n=8$, Anova) and dose dependent inhibition of NKB occurred, shifting the dose response curve to the right (fig 4.3.5).

Fig 4.3.2. Changes in synovial blood flow during topical application of NKB to the knee joint capsule alone, and with a non peptide NK2 antagonist, SR48968 at different doses: Values are mean ±SEM.

A) Application of NKB alone (○, n= 8) and together with SR48968 at 10^{-12} mol (▲, n=8)

B) Application of NKB alone (○, n= 8) and together with SR48968 at 10^{-10} mol (■, n= 8)

C) Application of NKB alone (○, n=8) and together with SR48968 at 10^{-8} mol (●, n=8)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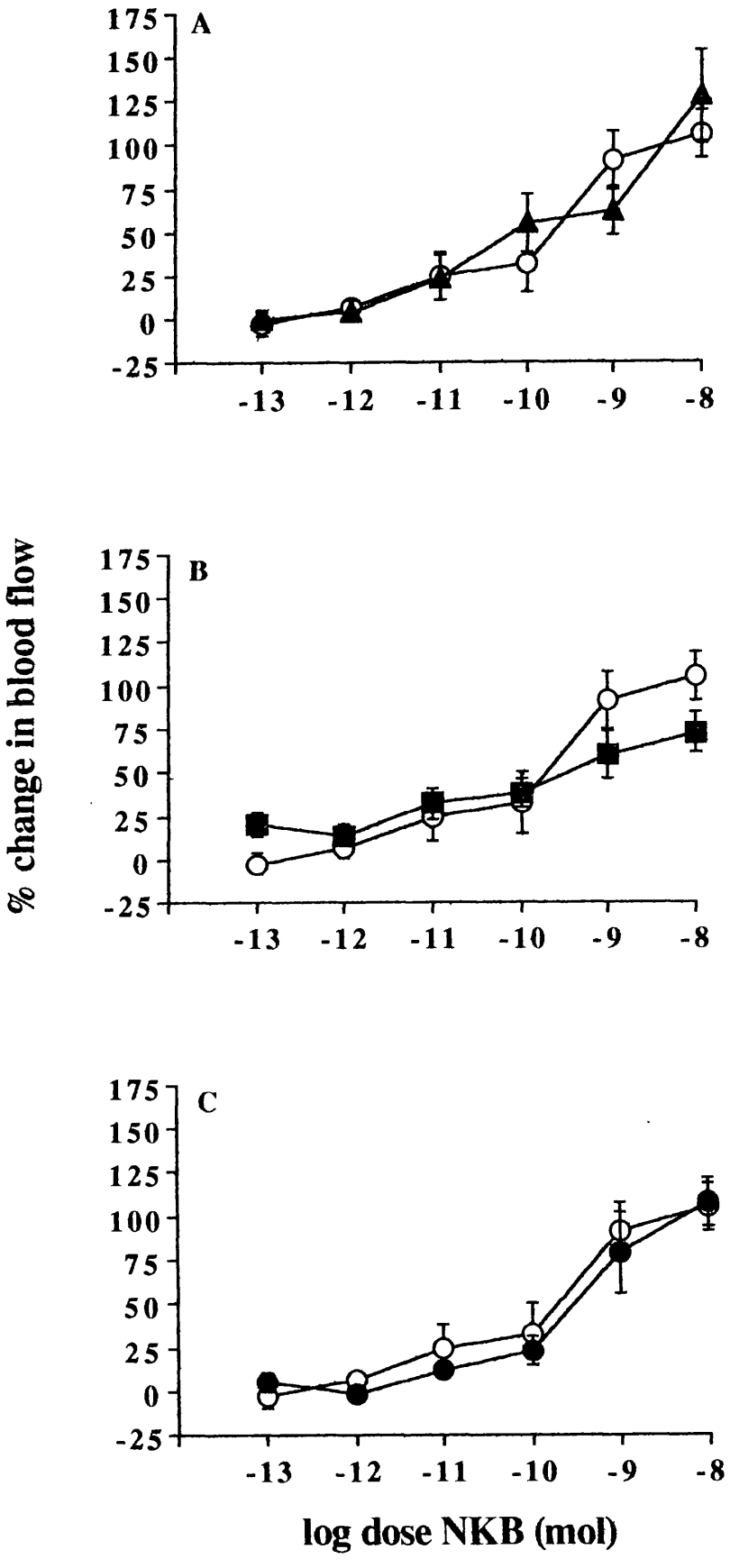
% change in blood flow
log dose NKB (mol)

Fig 4.3.3. Changes in synovial blood flow during topical application of NKB to the knee joint capsule alone, and with an NK1 antagonist, FK888 at different doses: Values are mean ±SEM.

A) Application of NKB alone (○, n = 8) and together with FK888 at 10^{-12} mol (▲, n= 8)

B) Application of NKB alone (○, n= 8) and together with FK888 at 10^{-10} mol (■, n= 8)

C) Application of NKB alone (○, n= 8) and together with FK888 at 10^{-8} mol (●, n=8)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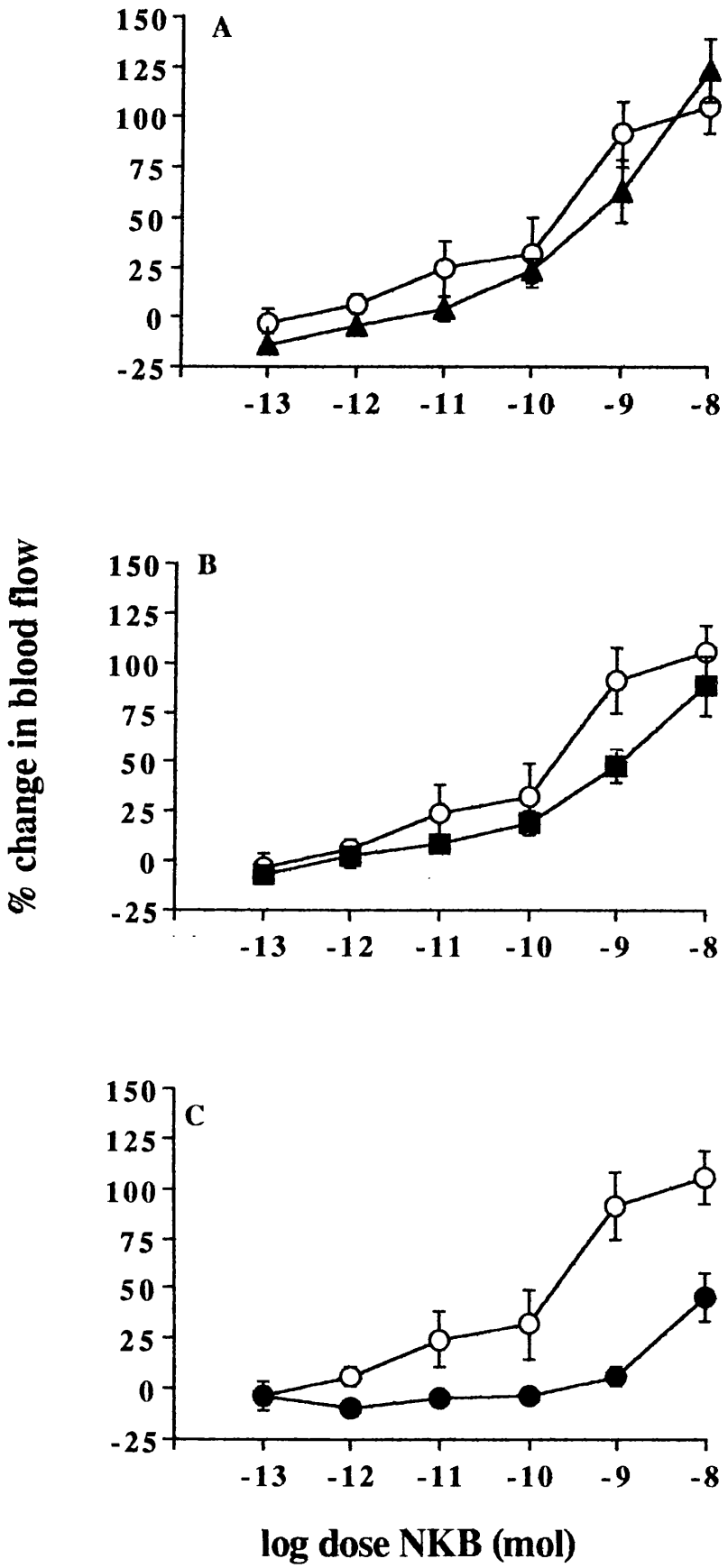
-8
% change in blood flow
log dose NKB (mol)

Fig 4.3.4. Changes in synovial blood flow during topical application of NKB to the knee joint capsule alone, and with an NK1 and NK2 antagonist, FK224 at different doses: Values are mean ±SEM.

A) Application of NKB alone (○, n= 8) and together with FK224 at 10^{-12} mol (▲, n=8)

B) Application of NKB alone (○, n= 8) and together with FK224 at 10^{-10} mol (■, n=8)

C) Application of NKB alone (○, n= 8) and together with FK224 at 10^{-8} mol (●, n=10)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-8
% change in blood flow
log dose NKB (mol)

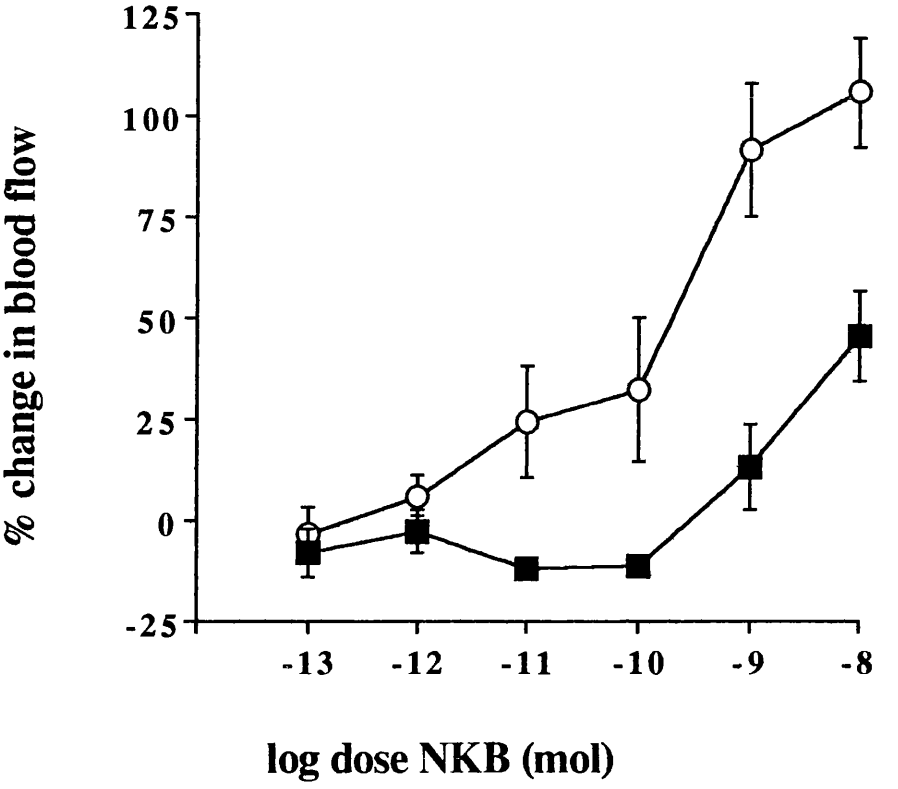


Fig 4.3.5. Changes in synovial blood flow during topical application of NKB to the knee joint capsule, alone (○, mean ±SEM, n=8) and co-application with SR48968 and FK888, both at dose of 10^{-8} mol, (■, mean ±SEM, n=8)

4.3.5 DISCUSSION

There is little data published about NKB and its effect on different organs. NKB in the rat the knee joint potently and dose dependently increased blood flow. An effect which was highly significant ($p<0.0001$ one way Anova). This vasodilatation is transient and like other neurokinins lasts only a maximum of two minutes. Maximum vasodilatation was 105.7±13.6 % increase from base line which showed the greatest effect of the three neurokinins.

SR48968 at doses of 10^{-12} and 10^{-10} mol enhanced the effect of NKB on blood flow, this becoming significant at 10^{-10} mol ($p= 0.01$, $n=8$). This enhancement could be due to a dual effect of the antagonist which will be discussed later. SR48968 at 10^{-8} mol slightly but significantly reduced the NKB effect on blood flow ($p=0.038$, two way Anova). This reduction indicates involvement of NK2 receptors in this response. FK888 failed completely to inhibit NKB at doses of 10^{-12} and 10^{-10} mol and slightly but non significantly reduced its effect at 10^{-8} mol. As FK888 is a specific NK1 antagonist, NKB appears to mediate its effects via other neurokinin receptors.

Application of FK224 at doses of 10^{-12} and 10^{-10} mol dose dependently inhibited NKB which at 10^{-10} mol was significant ($p=0.012$, $n=8$). At 10^{-8} mol FK224 strongly and significantly ($p<0.0001$, $n=8$) blocked NKB effects on blood flow and even at 10^{-9} mol NKB failed to dilate blood vessels. These data strongly suggest that NKB mediates its effects via both NK1 and NK2 receptors. In view of the finding with FK888, NK1 receptors in the rat knee joint may be a subtype of NK1 receptor and also the NK2 receptor could be a NK2b receptor.

These finding were verified by application of both FK888 and SR48968 mol, which significantly and dose dependently reduced the effect NKB on blood flow which supports the FK224 findings.

With regard to these data, it could be concluded that NKB may mediate its effects on blood flow via an atypical NK1 receptor and a subtype of NK2 receptor. Complete inhibition by FK224 indicates that there is no other neurokinin receptor in the rat knee joint and the significant reduction in blood flow by co- administration of FK888 and SR48968 indicate lack of NK3 receptors in the rat knee joint. In the literature the only report about the existence of peripheral NK3 receptors indicates that there are found in the rat portal vein (Mastrangelo *et al* 1987).

4.3.6 GENERAL DISCUSSION

All three neurokinins dose dependently and significantly induced vasodilatation in the rat knee joint, and these response were significantly different from each other. The rank order of potency was NKB > SP > NKA (fig. 4.3.6). There is no significant difference between SP and NKB at lower doses, but at higher doses the difference is highly significant and NKB overall demonstrates a potent effect on blood flow. It could be that SP has a hypotensive effect at the highest doses but NKB does not.

Three distinct neurokinin receptors exist for these three neurokinins, and subdivision of these receptors were also found. These three neurokinins are capable of activating these receptors, but with different affinities and potencies (Regoli *et al* 1987b). The mechanism

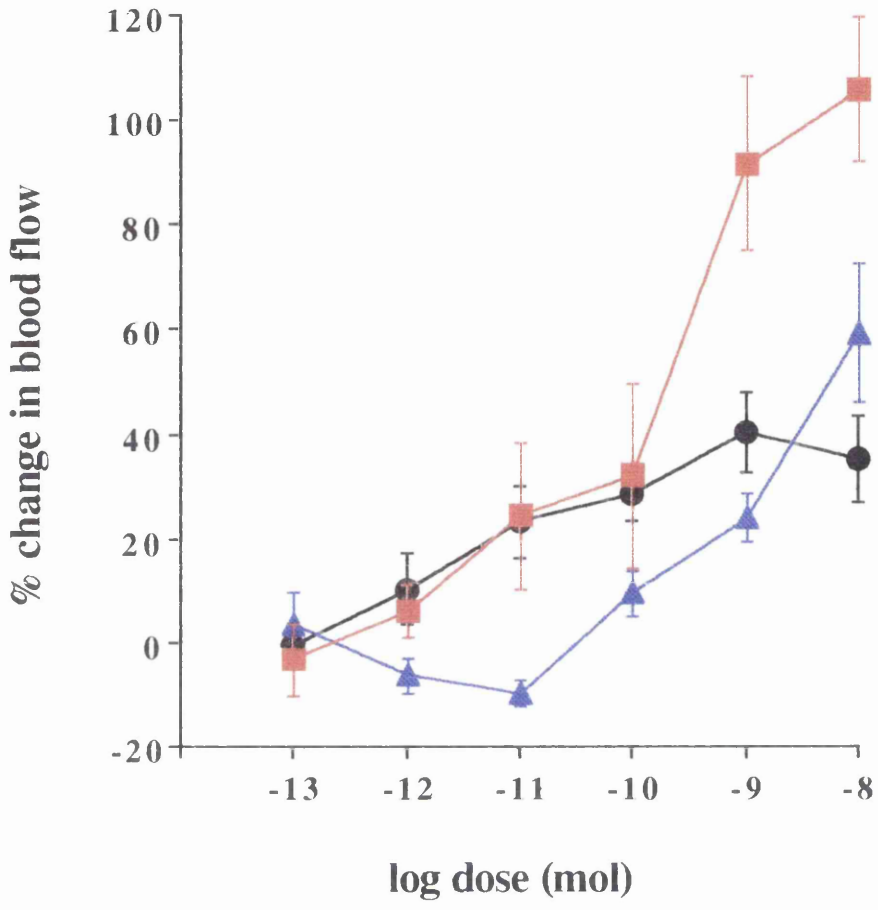


Fig 4.3.6 Changes in synovial blood flow during topical application of SP (●, mean ±SEM, n=35), NKA (▲, mean ±SEM, n=14), NKB (■, mean ±SEM, n=8), to the knee joint capsule

is via activation of a second messenger (Phosphatidyle inositol hydrolysis) with differing potency (Nakjima *et al* 1992).

Application of three different antagonists demonstrated different effects. SR48968 at 10^{-12} mol failed to inhibit the vasodilatation induced by three neurokinins, and even slightly increased the SP effect on blood flow.

SR48968 at 10^{-10} mol enhanced effects of neurokinins on blood flow instead of attenuating vasodilatation, which was significant with NKB. SR48968 at 10^{-8} mol significantly enhanced the SP vasodilator effect .

These data suggest that SR48968 has dual effect on rat knee joint blood flow: a specific effect on NK2 receptors which blocked the vasodilator effect mediated by NK2 receptors and an indirect effect which potentiated the neurokinin effect on blood flow. In the presence of NK2 receptors the inhibitory effect of SR48968 overcomes its indirect effects. The mechanism underlying the indirect effect is not clear. It may activate other mechanisms and release other vasodilator agents, or the receptors may have a regulating and interfering effect on each other, and blocking one receptor type may influence other receptors type and potentiates their responses. The effect of SR48968 which is not a specific antagonist for SP, shows clear potentiation of the vasodilator effect of SP.

For both NKA and NKB there appears to be both an antagonistic and a potentiating effect of SR48968, which by potentiation of neurokinins and competitive inhibition of their receptors, altogether shows a very weak antagonistic effect. SR48968 is a potent antagonist

for NK2a receptors but has also effects on NK2b receptors, (Advenier *et al* 1992a). The weak response to NKA in the rat knee joint suggests the existence of NK2b receptors in this area. These finding were confirmed by others in rat peripheral tissues and NK2b receptors have been reported in rat smooth muscle (Maggi *et al* 1993a).

FK888, which is a selective NK1 antagonist, like SR48968 increased neurokinin effects on blood flow, which could be due to an indirect effect as described before. FK888 showed a weak antagonistic effect on SP, which could partially be due to interaction with the indirect effect of FK888 and also due to a subtype of NK1 receptor which was confirmed with application FK224.

FK224, a NK1 and NK2 receptor antagonist, completely blocked the effect of SP on blood flow which reveals that SP also mediates its effects via NK2 receptors. Significant but not complete reduction of SP increased blood flow by combination of FK888 and SR48968 again verifies that a subtype of NK1 receptor and NK2b receptor which mediates SP induced vasodilatation.

The NKA data revealed that this peptide mediates its vasodilatation via NK2 receptors, but as the SP and NKA data suggest, this receptor may be of the NK2b receptor subtype.

Complete block of NKB induced vasodilatation by FK224 and a combination of FK888 and SR48968 suggests that there are no NK3 receptors in the rat knee joint and that NKB mediates its effects by acting at both NK1 and NK2 receptors. Thus it is possible that a subtype of NK1 receptor and the NK2b receptor mediate the effects of all the neurokinins on the rat knee joint.

These experiments revealed that SP, NKA and NKB exert their effects via both NK1 and NK2 receptors and activation of only one of those receptors by one of these neurokinins may be necessary to show its full effects on blood flow. Blocking of one neurokinin receptor type by a specific antagonist would not reduce that neurokinin's effect on blood flow because the other receptor type will remain active and thus be able to show the agonist's full effect. It is possible that to achieve a full response by the agonist does not require occupation of all the receptors (depend on the nature of the receptor and the agonist). Partial receptor occupation would be enough to achieve to the highest response, therefore blocking of one receptor type neurokinins would not be enough to reduce the neurokinin's effect on blood flow. Thus there would not be a significant change in blood flow in compared to the effect of the neurokinin on its own. To effectively block the neurokinin's effect on blood flow blockade of both neurokinin receptors is necessary, as observed in these experiments.

SECTION FOUR

PHYSIOLOGICAL ROLE OF NEUROKININS

IN THE REGULATION OF

SYNOVIAL BLOOD FLOW

4.4.1 SUMMARY

1) Experiments were performed on Male wistar rats to clarify physiological role of the tachykinins in regulating synovial blood flow.

2) Laser Doppler Imaging (LDI) was used to investigate changes in blood flow.

3) FK888 (an NK1 antagonist), SR48968 (an NK2 non-peptide antagonist), and FK224 (an NK1 and NK2 antagonist), at three different doses and also phenoxybenzamine (an α_1& α_2 adenoceptor antagonist) were used in these experiments.

4) Neither FK888 nor SR48968 on their own significantly influenced joint blood flow

5) Co-administration of FK888 and SR48968 significantly and dose dependently reduced basal blood flow, which showed ~20% reduction from control at 10^{-8} mol.

6)FK224 significantly, but less potently, reduced basal blood flow

7) Phenoxybenzamine administration resulted in ~30% increase in basal blood flow. This effect was reduced by 60% during co-administration with of antagonists FK888 and SR48968 at 10^{-8} mol.

8) In the denervated knee joint, co-administration of FK888, and SR48968 at 10^{-8} mol showed a 6% increased basal blood flow which did not significantly differ from vehicle treatment.

9) Phenoxybenzamine-mediated vasodilatation was not inhibited by co-administration with FK888 and SR48968 at 10^{-8} mol in the denervated knee.

10) These data suggest that SP and perhaps other neurokinins are normally released from sensory nerve ending and participate in regulating vascular tone and thus basal blood flow.

4.4.2 INTRODUCTION

Neurokinins are present in unmyelinated sensory fibres in the both central and peripheral nervous systems (chapter 1, and chapter 4, sec 4.1, 4.2, 4.3), and such fibres also innervate the rat knee joint (Konttinen *et al.* 1990).

Experiments described earlier (chapter 4, sec 4.1, 4.2, 4.3) showed the presence of neurokinin receptors as there is a potent vasodilator effect with exogenous application of neurokinins to the rat knee joint however whether SP and other neurokinins have a role in regulating synovial perfusion is unknown at present and it was investigated in this study.

4.4.3 MATERIAL AND METHOD

Experiments were performed on male Wistar rats (~350-500g). Two groups of rats were used, an intact group and a knee joint denervated group. The procedure of terminal anaesthesia and experimental procedures in the intact group were described earlier in section 4.1. In the denervated group, five rat were anaesthetised with hypnorm and diazepam and the medial aspect of knee joint denervated, as described in chapter two. After allowing one week to elapse for

degeneration and depletion of sensory nerve fibres, the same procedures used in the intact group, were repeated on this group of rats. Three different antagonists FK224, FK888, SR48968 at three different doses 10^{-12} mol, 10^{-10} mol, 10^{-8} mol and phenoxybenzamine an α 1 and $\alpha 2$ receptor antagonist at a dose of 10^{-6} mol were used in these experiments . Statistical analysis of the data was performed. The comparisons between mean values was by Student's t test (see 4.1.3)

4.4.4 RESULTS

Effect of antagonist: Saline and the vehicle for the neurokinin antagonists on their own did not have any significant effect on basal blood flow. (0.2±1.4%, n=44, and 0.7±2.2%, n=29 increase from base line perfusion, respectively) and also did not show any significant difference with each other (p=0.85, two tail t test) (fig. 4.4.1).

Application of SR48968 on its own at different doses produced variable and non-significant effects on blood flow. SR48968 at 10^{-12} mol slightly increased blood flow (0.64±3.1%, n=27, T test), and a 10^{-10} and 10^{-8} mol slightly decreased blood flow (-0.6±2.2%, n=22 and -1.3±2.8%, n=22, respectively), but none of these responses differed significantly from the vehicle effect (10^{-12} p=0.8, 10^{-10} p=0.8, 10^{-8} p= 0.9, respectively, paired two tail t test) (fig. 4.4.1)

Application of FK888 on its own at different doses like SR48968, produced variable but non-significant responses on blood flow. At 10^{-12} mol FK888 slightly and non significantly decreased blood flow (-2.7±2.9%, n=28) and at 10^{-10} and 10^{-8} mol did not produce any significant effect on basal blood flow (1.5 ± 3.1, n=36 and

-2.1±2.6%, n=40, respectively)(10^{-12} p=0.3, 10^{-10} p= 0.9, 10^{-8} p= 0.3, respectively, paired two tail t test) (fig. 4.4.2).

Application of FK224 on its own reduced blood flow and this reduction occurred at all doses. At 10^{-12} mol FK224 reduced blood flow (-5.3±4%, n=14) but this reduction of blood flow was not significant (p=0.3, n=14) compared to vehicle. At 10^{-10} mol FK224 significantly reduced blood flow (-8.3±2.2% p = 0.01, n=28, paired t test). FK224 at 10^{-8} mol also significantly reduced blood flow (-5.9±2.1 p=0.03, n=25, T test) (fig. 4.4.3).

Co-application of FK888 and SR48968 dose dependently reduced basal blood flow at all doses. The 10^{-12} mol dose non significantly reduced blood flow (-6.3±4.8%, p = 0.24, n=24, t test). At 10^{-10} mol this became significant (-12±3.3% p=0.002, n=24, t test) and more so at 10^{-8} mol (-19.9±2.7 p=0.0001, n=38, t test) (fig. 4.4.4a & b).

In the denervated rat knee co-application of FK888 and SR48968 (10-8 mol) failed to reduce basal blood flow and actually produced a small but non-significant increase in flow (6.8±4.5%, p=0.38, n=29)(fig. 4.4.5)

Application of phenoxybenzamine (10^{-6} mol) significantly increased basal blood flow (31.3±6.2%, p= 0.001, n=12) in the normal joint but when phenoxybenzamine was co-administered with FK888 and SR48968 the increase in blood flow was substantially and significantly (P=0.001, n=30, t test) attenuated. In the denervated joint the response to the same dose of phenoxybenzamine was reduced (16.8±11.7%, p= 0.33 n=8). but this reduction was not significant

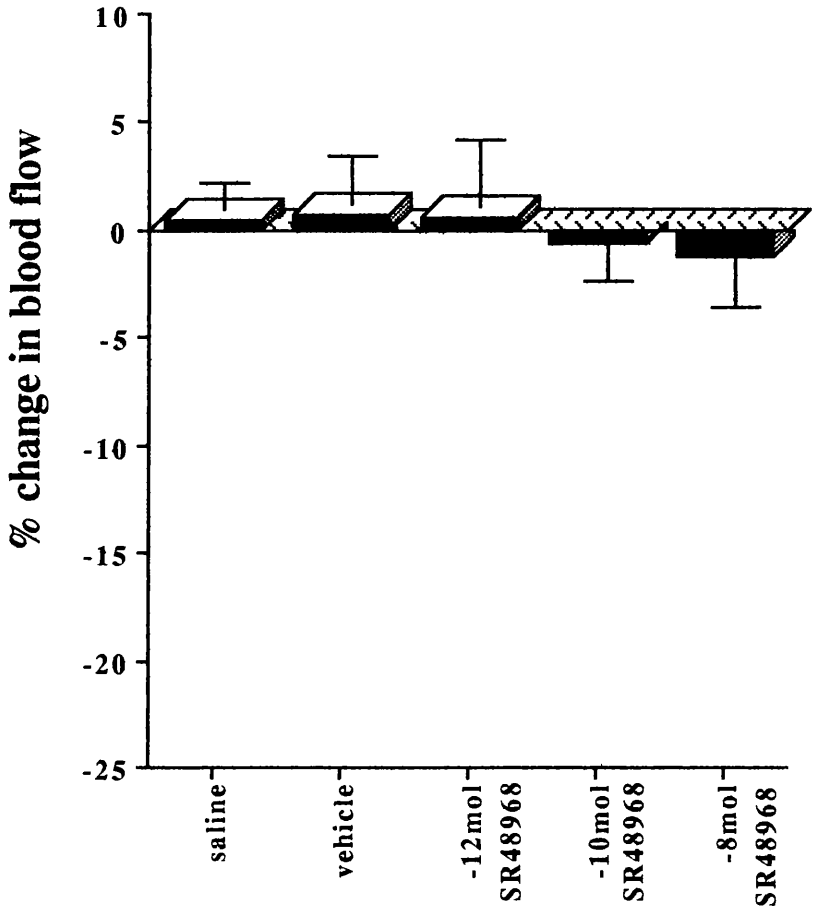


Fig 4.4.1. Changes in synovial blood flow during topical application of saline, vehicle for the neurokinin antagonist and SR48968 in different doses to the knee joint capsule. Values are mean ±SEM.

Saline (n=44), vehicle (n=29), SR48968 at 10^{-12} (n=27), SR48968 at 10^{-10} (n=22), SR48968 at 10^{-8} (n=22), there were not any significant difference between different treatments.

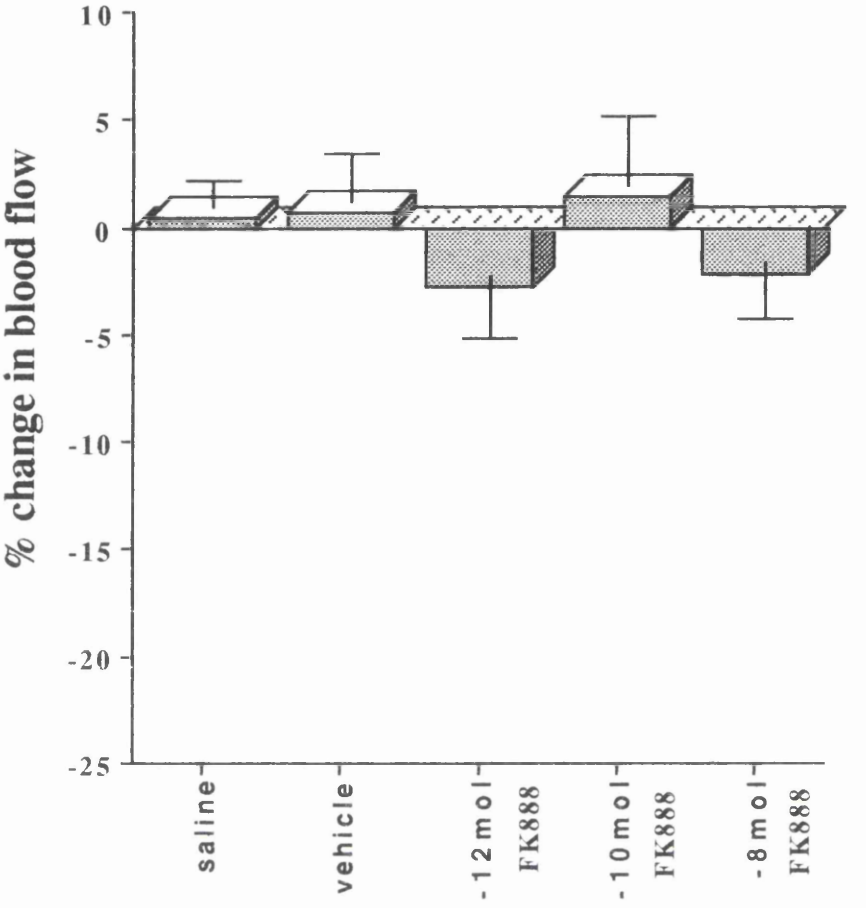


Fig 4.4.2. Changes in synovial blood flow during topical application of saline, vehicle for the neurokinin antagonist and FK888 in different doses to the knee joint capsule. Values are mean ±SEM.

Saline (n=44), vehicle (n=29), FK888 at 10^{-12} (n=28), FK888 at 10^{-10} (n=36), FK888 at 10^{-8} (n=40), there were not any significant difference between different treatments.

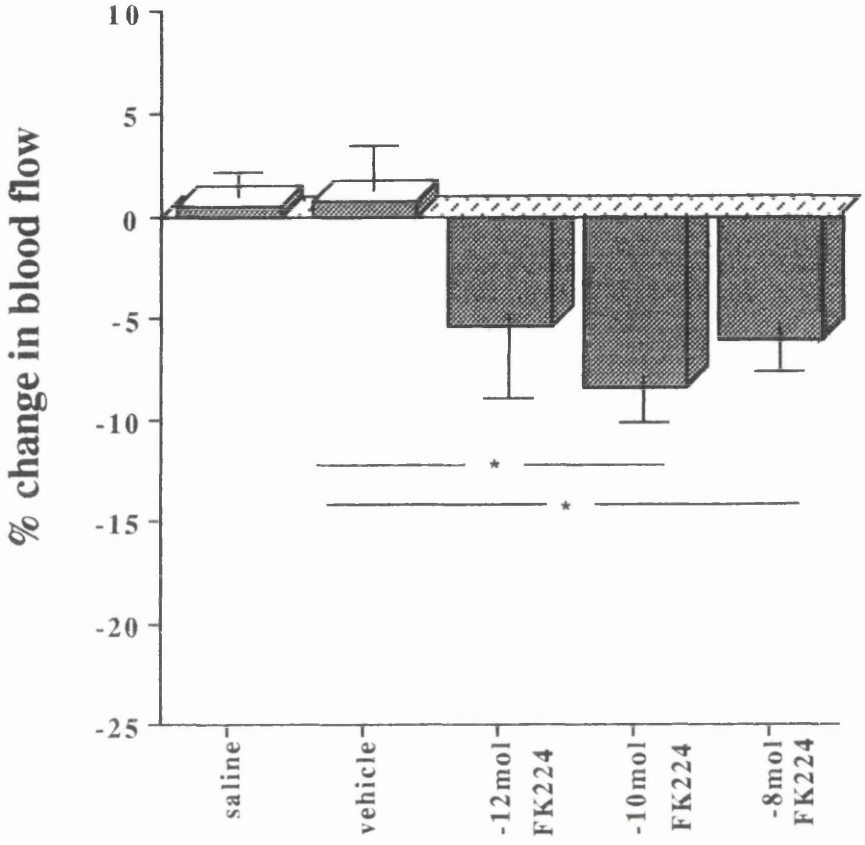


Fig 4.4.3. Changes in synovial blood flow during topical application of saline, vehicle for the neurokinin antagonist and FK224 in different doses to the knee joint capsule. Values are mean ±SEM.

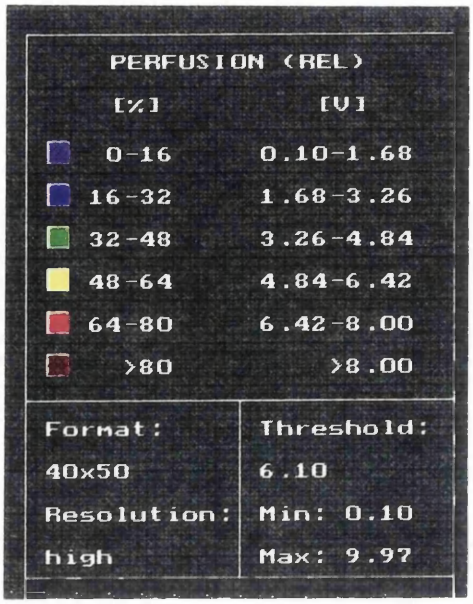
Saline (n=44), vehicle (n=29), FK224 at 10^{-12} (n=14, FK224 at 10^{-10} (n=28), FK224 at 10^{-8} (n=25). * means differs significantly, from vehicle. For significant value see text

Fig 4.4.4a: Laser Doppler perfusion images of rat knee joints.

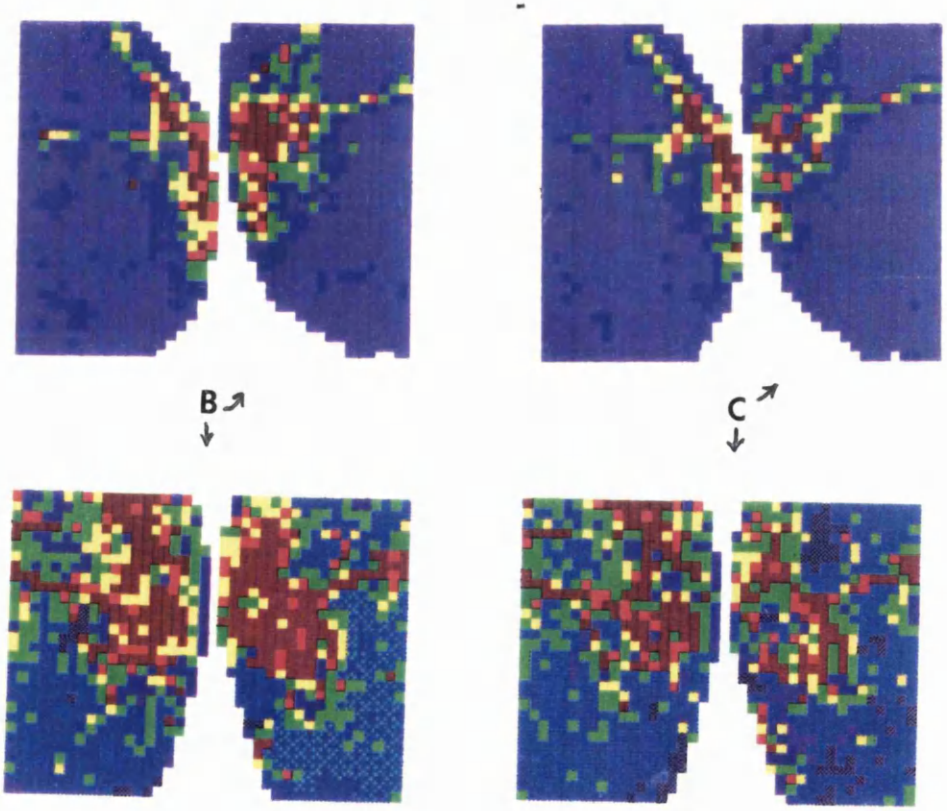
A) colour-coded system for showing perfusion values .

B) Control perfusion image of the knee joint.

C) Co-application of FK888 & SR48968 both at 10^{-8} mol. There is a significant reduction in basal blood flow.


PERFUSION (REL)
[%]
[V]
0-16
0.10-1.68
16-32
1.68-3.26
32-48
3.26-4.84
48-64
4.84-6.42
64-80
6.42-8.00
>80
>8.00
Format:
40x50
Resolution:
high
Threshold:
6.10
Min: 0.10
Max: 9.97

A


B
C

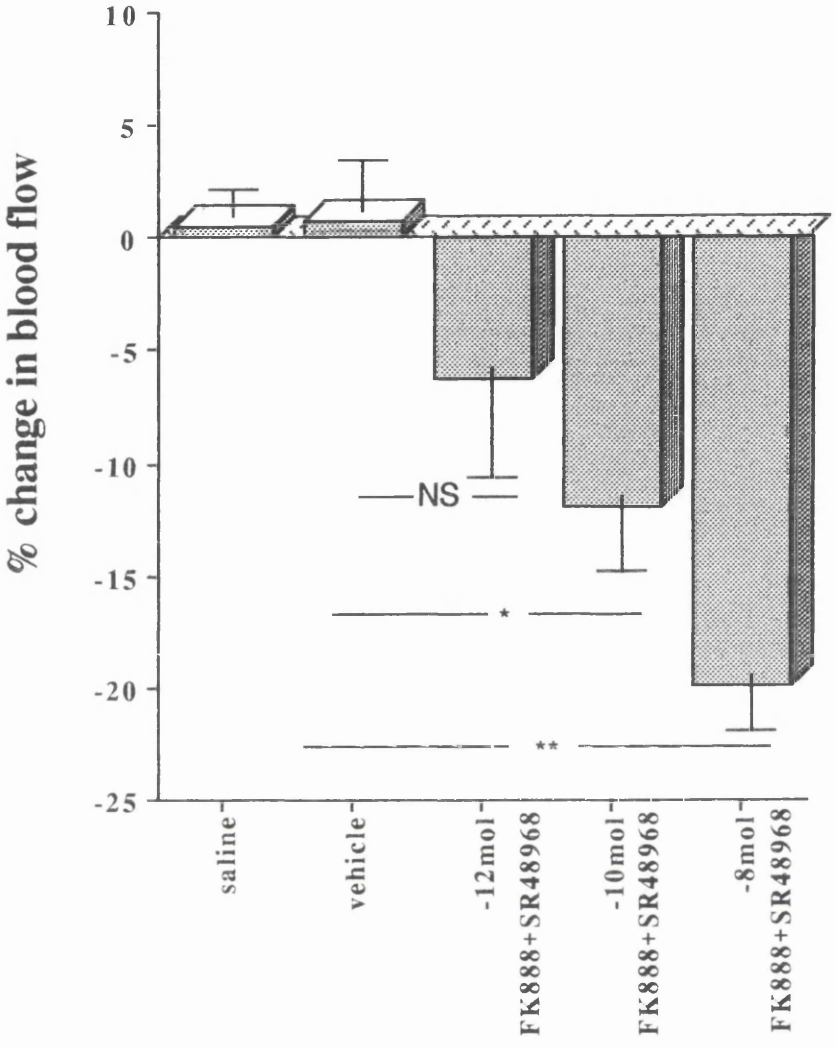


Fig 4.4.4b. Changes in synovial blood flow during topical application of saline, vehicle for the neurokinin antagonist and both SR48968 + FK888 in different doses to the knee joint capsule. Values are mean ±SEM.

Saline (n=44), vehicle (n=29), SR48968 + FK888 at 10^{-12} (n=24, SR48968 + FK888 at 10^{-10} (n=24), SR48968 + FK888 at 10^{-8} (n=38). * means differs significantly, from vehicle . For significant value see text

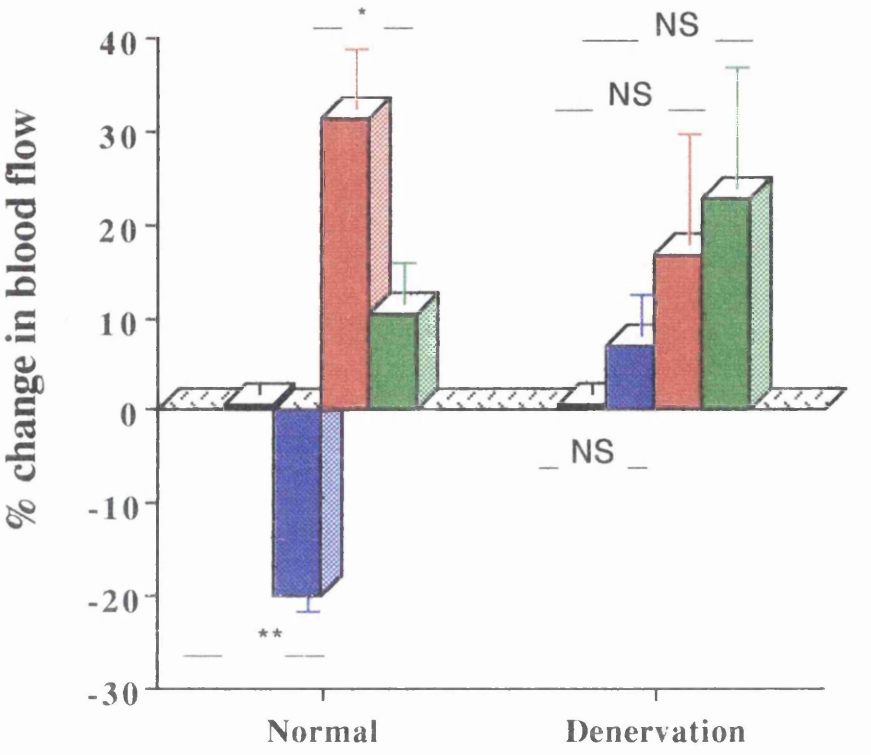


Fig 4.4.5. Changes in synovial blood flow during topical application of saline, and both SR48968 + FK888 both at 10^{-8} mol alone and with phenoxybenzamine (10^{-6} mol) to the normal and denervated knee joint capsule . Values are mean ±SEM.

Saline (■, n=44), SR48968 + FK888 (■, n=29), phenoxybenzamine (■, n=12), SR48968 + FK888 with phenoxybenzamine (■ , n=38). * means differs significantly from vehicle . For significant value see text

compared to vehicle (fig. 4.4.5). Contrary to the normal joint, co-administration of phenoxybenzamine and FK888 and SR48968 (10^{-8} mol) gave a response (22.78±12.86%) which did not differ significantly (p=0.14, n=5, t test) from phenoxybenzamine alone (fig. 4.4.5).

4.4.5 DISCUSSION

As the vehicle neurokinin for the antagonists contained alcohol, it could be argued that a part of the vascular response to the antagonists was due to alcohol, but application of vehicle alone to the knee joint did not show any effect on basal blood flow. The vehicle applied to the knee joint had the highest alcohol concentration which was used with the 10^{-8} mol dose of antagonist in these experiments, but even so, it had as little effect on blood flow as application of saline.

Previous experiments confirmed that SP acts at both NK1 and NK2 receptors, and as SP mediates its effect on blood vessels via these receptors three different antagonists, FK888, FK224 and SR48968 were used to antagonise its action on the vascular bed.

Neither FK888 nor SR48968 at the three doses used influenced synovial perfusion compared to vehicle, which suggests that in the previous experiments (section 4.1, 4.2, 4.3) the results were solely due to the effect of the antagonists on blood flow.

FK224 reduced basal blood flow at all three doses but this was significant only at the 10^{-10} and 10^{-8} mol doses (p=0.01, n=28 and 0.03, n=25).

Co-administration FK888 and SR48968 at the three doses potently and dose dependently reduced blood flow, the greatest reduction being 19.9±2.6%. Blood pressure was monitored in these experiments to confirm that these effects are not due to a systemic hypotensive effect of the antagonists. Application of both antagonists to the knee joint did not show any effect on blood pressure even at the highest doses.

These two series of data suggest that there is a basal release of SP in the normal knee joint which produces vasodilatation and participates in regulating blood vessel tone via NK1 and NK2 receptors, and thus blocking these receptors reduces basal blood flow.

To confirm these findings, phenoxybenzamine 10^{-6} mol applied to the knee joint produced a 31.3±6.2% increase in basal blood flow, but it did not show any significant effect on systemic blood pressure. Co-administration of FK888 and SR48968 (10^{-8} mol) with the same dose of phenoxybenzamine reduced the vasodilator effect of phenoxybenzamine on its own (by more than 60%) (10.4±0.5%) which was highly significant (fig. 4.4.5).

These findings suggest that there is normally sympathetic vaso-constrictor tone which is offset by the vasodilator action of neurokinins and basal blood flow shows a balance between the sympathetic vasoconstrictor and neuropeptidergic vasodilator influences.

As SP and other neuropeptides exist in sensory nerve endings, to check that this action was mediated by sensory neurokinins, in five rats

the medial aspect of joint was surgically denervated and one week later the above experiments repeated.

In the denervated knee, co-administration of FK888 & SR48968 (10^{-8} mol) produced a 6.8±4.5% increase in perfusion, which was not significantly different from the effect of the vehicle. The lack of effect of these antagonists is indicative of the absence of neurokinins in the denervated joints

In these rats phenoxybenzamine-induced vasodilatation did not change significantly with co-administration of antagonists. which also indicates the absence of neurokinins.

These finding all indicate that SP and perhaps other neurokinins are normally released from sensory nerve ending without apparent activation of these fibres and to some extent offset sympathetic vasoconstrictor "tone". This implies that these neurokinins have an important regulatory function in vascular function in rat knee joints and perhaps in other vasculature beds.

CHAPTER FIVE

EFFECTS OF INFLAMMATION AND NERVE DEPLETION ON SYNOVIAL BLOOD FLOW

5.1 SUMMARY

1) Experiments were performed to evaluate the effect of different models of inflammation on neurokinin-mediated vasodilatation

2) Laser Doppler imaging (LDI) was used to investigate changes in blood flow.

3) Carrageenan-induced acute inflammation increased basal blood flow and enhanced the vascular response to SP.

4) Capsaicin was utilised to induce chronic nerve depletion and was acutely applied to the knee joint. Chronic capsaicin treatment increased basal blood flow and also altered SP-induced vasodilatation.

5) Injection of different doses of capsaicin into the knee joint produced an acute vasoconstriction at all doses, followed by a dose dependent vasodilatation. This secondary effect could be due to the release of vasodilator mediators from sensory nerve endings.

6) Capsaicin in chronic application substantially attenuated SP-induced vasodilatation and with acute application dose dependently attenuated the SP effect on blood flow.

7) Adjuvant was applied to induce chronic inflammation. Adjuvant significantly reduced basal blood flow in the first week but this abated by the third week. Temperature and knee diameter increased in the first week after administration.

8) Adjuvant altered and attenuated the SP effect on blood flow, which was consistent across the three weeks.

9) A contralateral inflammatory effect of the adjuvant was revealed in these experiments. An increase in the temperature and diameter and alteration of the SP effect on blood flow was observed in the contralateral knee joint.

10) These data suggest that different agents can induce neurogenic inflammation and the response to neurokinins is completely altered in different models of inflammation. This alteration may contribute to the process of inflammation.

5.2 INFLAMMATION IN THE JOINTS

5.2.1 INTRODUCTION

Recent studies have identified an important contribution of the nervous system to inflammation and inflammatory disease. In particular, substances released from the peripheral terminals of small diameter primary afferent fibres and from sensory nerve endings have been implicated in several major components of inflammation and in inflammatory models in the rat (Basbaum *et al.* 1991). Inflammation in fact is a major component of the body's defence mechanism, but the initiating factors in chronic inflammatory disease such as arthritis in synovial joints remain unclear (Scott *et al.* 1994). The acute inflammatory process is characterised by three main stages :

1) vasodilatation and increased blood flow to an area.

2)Increased vascular permeability with leakage of plasma from the micro-circulation.

3)Migration of phagocytic leukocytes from the micro-circulation into the surrounding tissue.

Acute inflammation may also be an initiating factor in chronic inflammatory disease (Scott *et al.* 1994).

In the inflamed joint many events and changes appear in the affected area, and there is an alternative response to different neurokinins. For example in the acutely inflamed joint enhancement of SP induced protein extravasation has been reported (Scott *et al.* 1992).

However there is relatively little known about neurokinin receptors and their response in acute and chronic models of joint inflammation.

It would be expected a priori that the best screen test for evaluation of neurokinin receptors and their role in the different stage of inflammation would be based on experimental models of arthritis. In this study three different models of inflammation were induced in the rat knee joint. Acute inflammation can be induced by intra-articular injection of carrageenan and chronic inflammation by freunds adjuvant. Application of capsaicin into the knee joint caused depletion of sensory nerves. These models of inflammation were used to assess the responsiveness of blood vessels to neurokinins in these conditions.

5.3 SECTION ONE

ACUTE INFLAMMATION INDUCED BY:

CARRAGEENAN

5.3.1 Introduction: The *Algae chondrus crispus* is the main source of carrageenan. The term carrageenan was first used by Stanford in 1862 but a polysaccharide with simpler properties had been isolated and described previously by Schmids in 1844 (Rosa 1972). Later material with similar composition and physical properties has been isolated from other type of seaweeds, but carrageenan was restricted to polysaccharide extracted from *Algae chondrus* and *Gigatina stella.*

Carrageenan is a sulphated polysaccharide which has been fractionated with potassium chloride into two separate components: κ

carrageenan is the part which acts under influence of potassium ions and λ carrageenan is insensitive to the potassium ion. κ has 40% and λ has 60% of unfractioned carrageenan. κ carrageenan is composed of sulphated D galactose and 3, 6 anhydro- D -galactose with a branch component with a molecular weight between 1.8-3.2 $x10^5$ and λ carrageenan is composed almost entirely of sulphated D galactose with molecular weight between $4\text{-}7*10^5$. The ability of carrageenan to induce inflammation is related to the chemical structure of galactose, the configuration of the unit plays no apparent role, and the compound has a wide spectrum of interference with biological systems. The use of carrageenan as an irritant was first introduced in 1962 (Lam & Ferrell 1989a);. carrageenan induces acute oedema and a chronic granolumatous response which characterised by an influx of polymorphonuclear leukocyte in the synovial fluid and accompanied by the proliferation of synovial membrane and the carrageenan induced acute inflammatory response is mediated by several mediators released in the area (Willoughby & Dirosa 1971). The time course of Carrageenan induced oedema has been described in the presence of different phases of development of oedema (Vanarman *et al.* 1965; Vinegar *et al.* 1969). Overall, carrageenan is a well established agent to induce acute inflammation.

5.3.2 MATERIALS AND METHOD

Two groups of rats were selected: group 1 was used as a control and group 2 had an inflamed knee. 0.2 ml of 2% carrageenan was injected into the knee joint 24 hours prior to the experiments under a general anaesthetic (0.1 ml to the posterior and 0.1 ml to the anterior region) (see Chapter 2). SP was administered in a bolus at different

doses to the inflamed and intact knee joints. LDI imaging and data analysis were as described previously (chapters 1&2)

5.3.3 RESULTS AND DISCUSSION

Carrageenan alone induced acute inflammation and significantly ($p=0.01$, $n=20$, t test) increased basal blood flow which confirm the finding of Lam & Ferrell (1993a)(fig. 5.1a & b). Application of SP at different doses in the inflamed knee joint significantly and dose dependently ($P=0.001$, $n=20$, 2way anova) increased blood flow in comparison to the effect of SP in the normal rat knee (fig. 5.2). Lam and Ferrell (1989a) demonstrated that the inflammatory response to carrageenan is mediated by neurokinin receptors and blocking neurokinin receptors strongly attenuated its inflammatory response. Lam and Ferrell (1989a) also suggested a lack of sympathetic influence on articular blood vessels in carrageenan-induced inflammation. It has been shown that in carrageenan inflammation within the synovial capsule, the general metabolism of chondrocytes is temporarily depressed and it is suggested that the factors produced in the inflammatory response produce intracellular structural abnormalities and altered membrane function (Byres *et al.* 1985). Overall this enhanced response to the application of SP could result from reduced sympathetic tone, hyper-sensitivity of the inflamed joint to the exogenous applied SP or alteration of neurokinin receptors in carrageenan-induced acute inflammation.

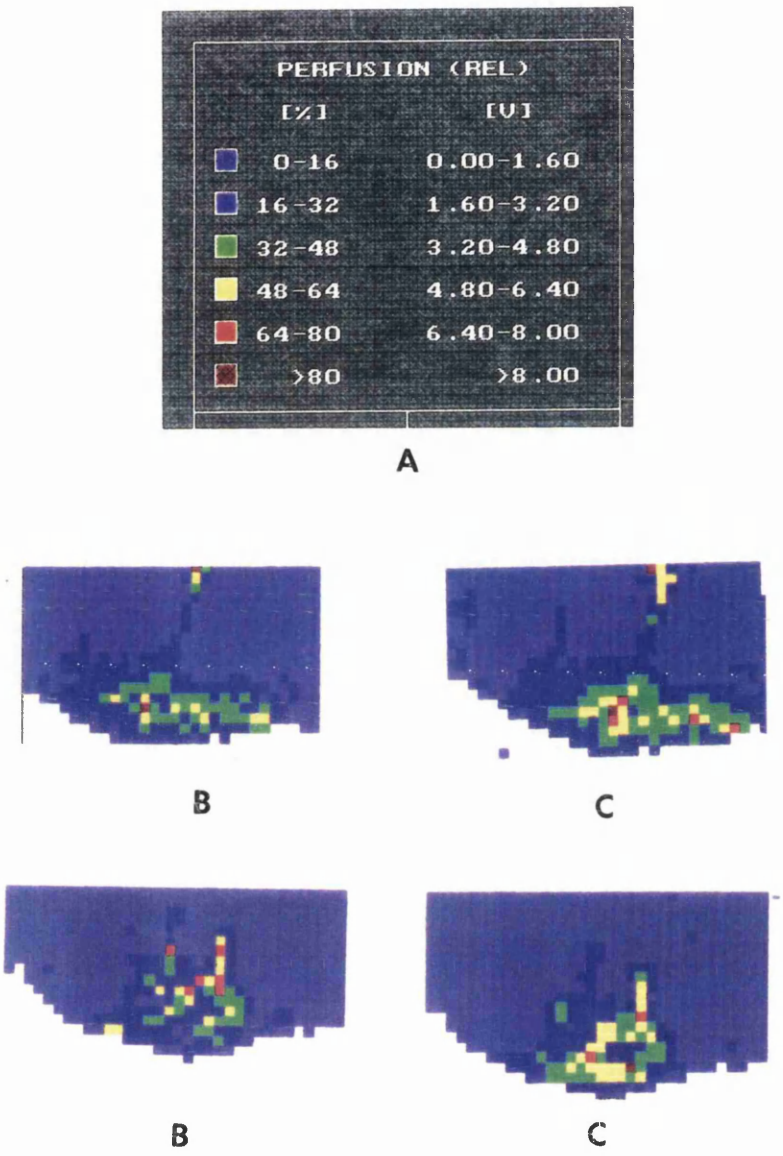


Fig 5.1 a: Laser Doppler perfusion images of rat knee joints.

A) colour-coded system for showing perfusion values

B) Basal perfusion images of the rat knee joints in control (intact) knee.

C) knees after 24 hours after carrageenan treatment, show a higher basal perfusion in compared to control knees.

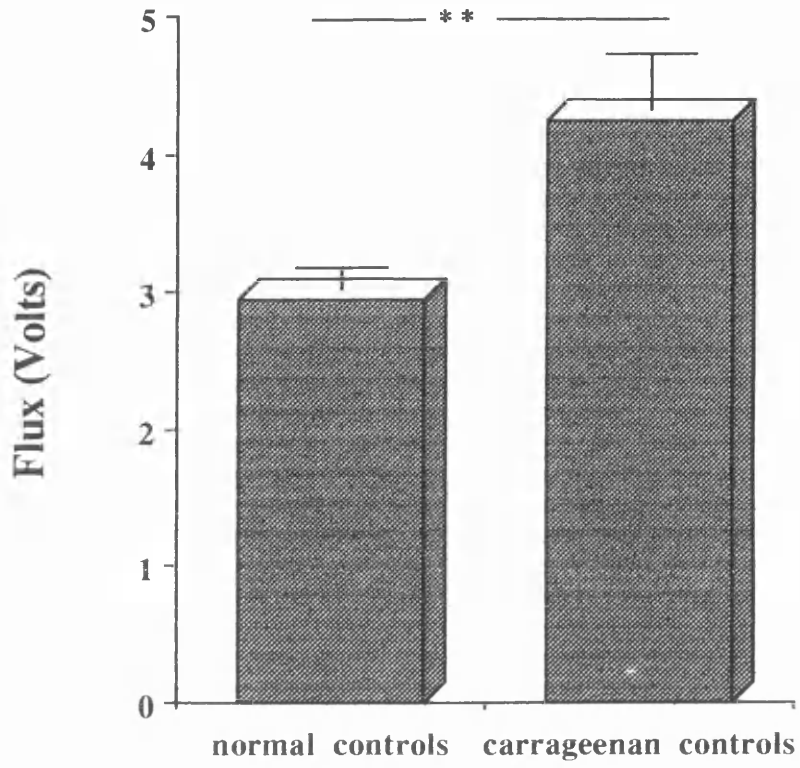


Fig 5.1b. Flux reading of (volts) first control scan in normal knees (n=35, P=0.01) and carrageenan-treated knee (n=20). * means carrageenan flux value differs significantly from normal flux value.

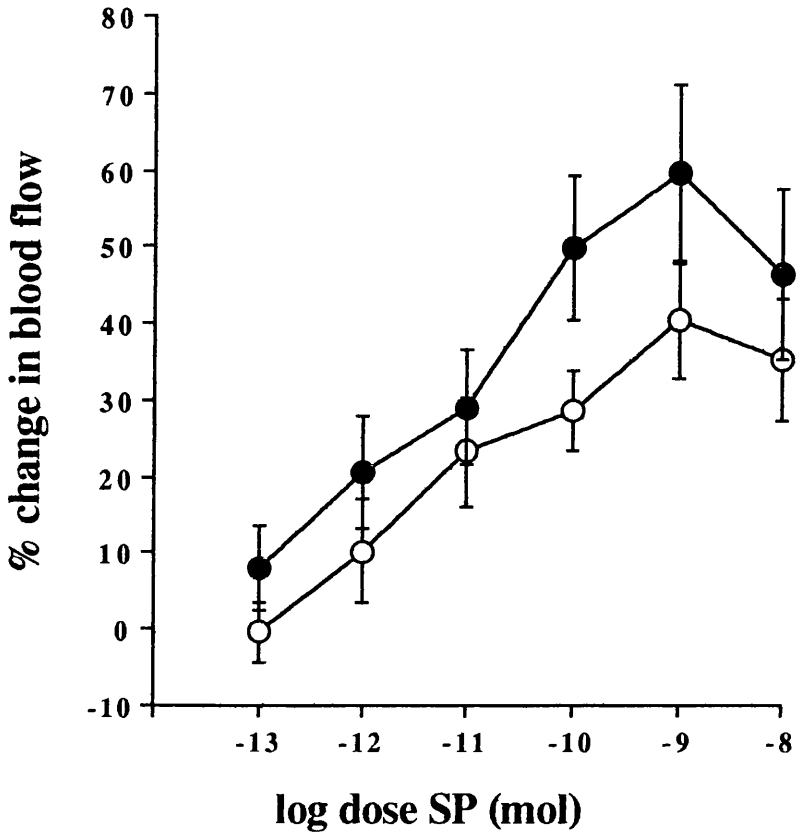


Fig 5.2. Changes in synovial blood flow during topical application of SP to the knee joint capsule, in the normal knee (○, mean ±SEM, n=35) and carrageenan pre-treated knee (●, mean ±SEM, n=20)

5.4 SECTION TWO: CHRONIC INFLAMMATION

ADJUVANT MONOARTHRITIS

5.4.1 Introduction:

The mechanisms underlying the formation and development of chronic inflammatory diseases such as rheumatoid arthritis are still uncertain. In order to study the pathogenesis of these disorders, a number of experimental models of arthritis have been designed (Gardner, 1960). Of these, injection of Freund's complete adjuvant into the joint capsule induces an unilateral monoarthritis (Grubb, *et al.* 1988). Ajuvant was described over 40 years ago (Stoerk *et al* 1954) as an agent for induction of arthritis. Adjuvant was made of a variety of dead mycobacteria, usually *mycobacterium tuberculosis* and M *butiricum* or M *phelei*, suspended in vegetable or mineral oil. *Corinea bacterium* and *nocardia asteroides* have also been used to induce arthritis (Billingham and Davies 1979). The concentration of mycobacteria in the vehicle suspension can influence the arthritis which develops and so it was necessary for the optimum concentration of mycobacteria to be determined. The time course of adjuvant-induced arthritis has been described by several investigators such as Newbold (1963) and Billingham and Davies (1979). It is clear from the literature that the inflammatory syndrome is essentially the same whatever the laboratory, and that rat strains have recovered from later phases of disease after 21 days. The time of onset of inflammation varies between 3-13 days in different reports. Baugartner *et al.* (1974) have described five phases in the course of adjuvant arthritis, starting with acute local inflammation within 1 to 4 days so by day 7 to 12

there is a remission of inflammation which later changes to a very extensive inflammation and osteogenesis by day 12 - 28. Finally at day 35, the last stage with associated permanent deformity with minimal inflammation occurs. Adjuvant inflammation is produced by several different factors and the presence of adjuvant is a trigger for activation of a multi-factorial process involving the lymphatic system, immunological mechanisms (fig. 5. 3), histological processes and lysosomal enzyme activation (Hanching 1984).

Freunds adjuvant-induced arthritis is being increasingly used as a model of chronic inflammation. Examination of synovium from adjuvant treated rats shows a reduction in synovial neuropeptidergic nerves when compared with normal samples (Konttinen *et al.* 1990) and similar findings have also been seen in patients with rheumatoid arthritis (Grönblad *et al.* 1988; Mapp *et al.* 1990). The tachykinin substance P (SP) is present in afferent nerve terminals (Hockfelt, *et al,* 1975) and is released in response to electrical antidromic stimulation of their fibres (Yaksh, 1988). The role of synovial nerves in the development of various forms of acute inflammation has previously been reported (Lam & Ferrell, 1991b) and it is thought that their effects may be mediated by local release of SP which is known to be an initiating factor for inflammation in this region. By using the technique of laser Doppler perfusion imaging (LDI), it has been possible to examine these neurogenic and neuropeptidergic effects on joint blood flow (Lam & Ferrell, 1993a). These experiments showed that topical application of SP to the exposed joint surface produced a potent vasodilatation although this particular effect was found to be transient. Intra-articular injection of carrageenan, an agent known to

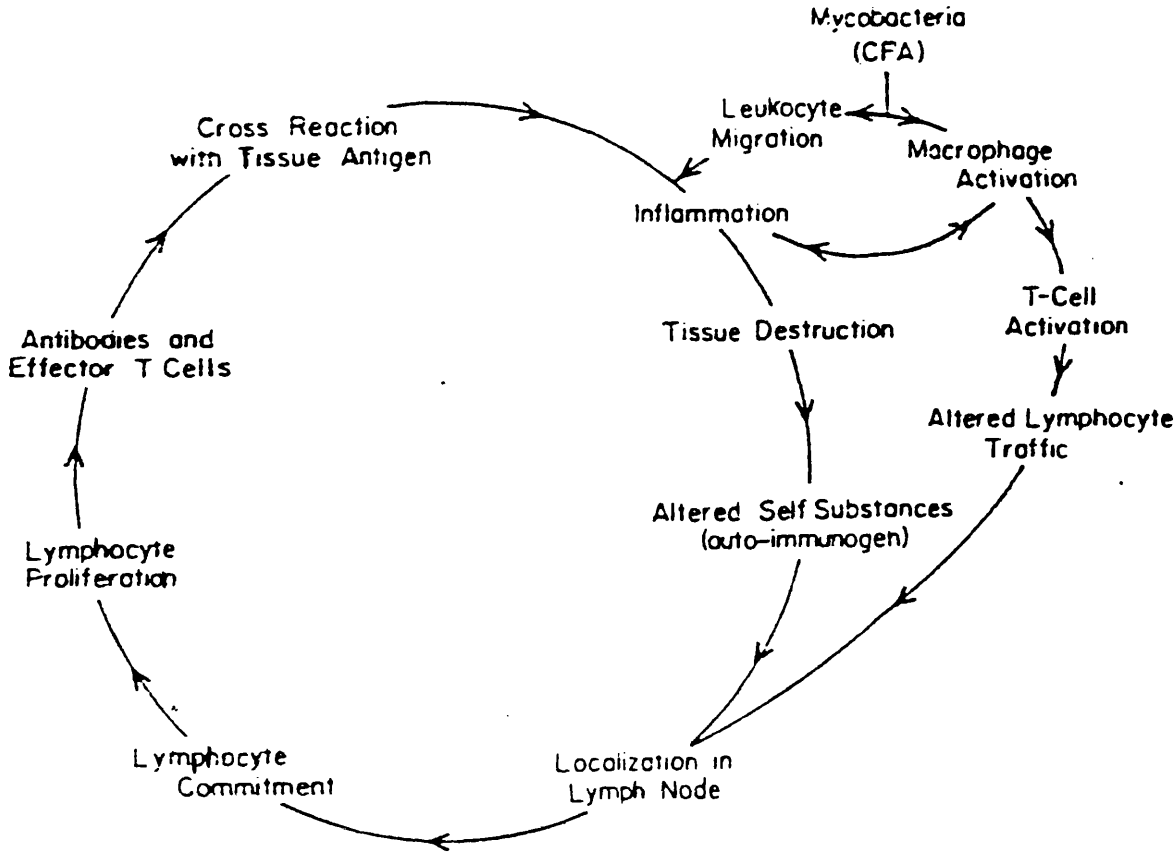


Fig 5.3: Immunopathogenesis of adjuvant arthritis,. From Chang Y. H. 1984

induce acute joint inflammation, attenuated the neurogenic vasoconstrictor response but enhanced the SP-mediated vasodilatation (Lam & Ferrell, 1993). In the experiments described below Freund's Adjuvant induced a monoarthritis which was followed for three weeks to follow up any eventual changes of the response to SP in both the ipsilateral and contralateral knees

5.4.2 MATERIAL AND METHODS

Thirty male Wistar rats from an in-house colony (aged 170 days- approx. 330 g in weight) were used in this study. The animals were deeply anaesthetised by Hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml, Janssen: 0.1 ml/300g i.m.) and diazepam (2.5 mg/kg, i.p). The width of the knee joint was measured by a digital micrometer (Motoyo instruments, Japan) and temperatures were measured by a digital thermometer (Harvard USA). The skin over the knee joint was shaved and 0.2 ml of Freund's complete adjuvant (Sigma) was injected into the synovial cavity of the right knee joint (0.1 ml into the posterior region and 0.1 into the anterior region). The animals were allowed to recover and the inflammatory response was assessed by measurement of knee diameter and temperature at one, two and three weeks post injection. Comparison between mean diameters and temperature was by a two-tail paired student t-test.

For the terminal blood flow experiments, the 30 animals were deeply anaesthetised by i.p. injection of urethane (2g/kg, Sigma). Here, 10 animals were examined at one and three weeks post injection and a further 10 normal rats were used as controls. An ellipse of skin overlying the joint was excised to expose the medial aspect of the

knee. SP (Cambridge Research Biochemicals) was warmed (37°C) prior to administration as a bolus applied to the exposed surface of the joint in a volume of 0.1 ml (doses ranged from 10^{-13} to 10^{-8} mol). Warmed (37°C) physiological saline (0.9% NaCl) was regularly applied to the knee joint surface to prevent tissue dehydration. Relative changes in synovial blood flow (voltage difference of test minus control) were monitored by a laser Doppler perfusion imager in both the normal group and the adjuvant treated group. Image analysis and statistics were as described in chapter 2.

5.4.3 RESULTS

5.4.3.1 Inflammatory response and basal joint perfusion

Injection of Freund's adjuvant into the rat knee caused a considerable increase in knee diameter at one week post-injection which abated by the third week (Fig. 5 4). At week 1, the adjuvant-treated knee width increased by 1.797±0.544 mm (mean ± S.E.M.) which compared to week 0 was significantly different (P=0.013, n=9, two-tailed paired t-test) from control knees which increased in size due to growth of the rats. By week 3, the inflamed knee had only increased by 1.195 ±0.35 mm from the week 0 level and this was found to be not significantly different (P=0.315; n=8 for adjuvant animals and n =10 for untreated animals, two tail t test) from a normal knee which had grown by 0.744 ± 0.11 mm over the three week period. Knee joint temperature also significantly increased at week 1 in injected compared to control knees and also at week 2 and week 3 the knee temperature in adjuvant treated was significantly higher than at the start (P=0.001, n=9, t test) (fig. 5.5). The contralateral knee did show

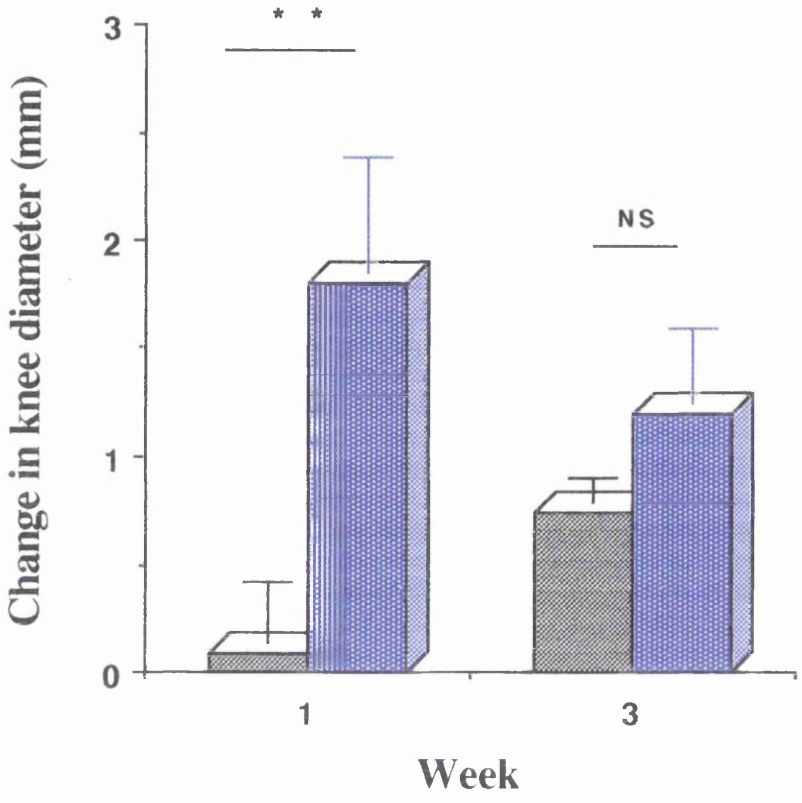


Fig 5.4, Changes in knee joint diameter at one and three weeks in response to intra-articular injection of Freund's adjuvant (blue column, n=9) compared to normal (untreated, black column, n=10). * * means differs significantly at week one. P=0.013

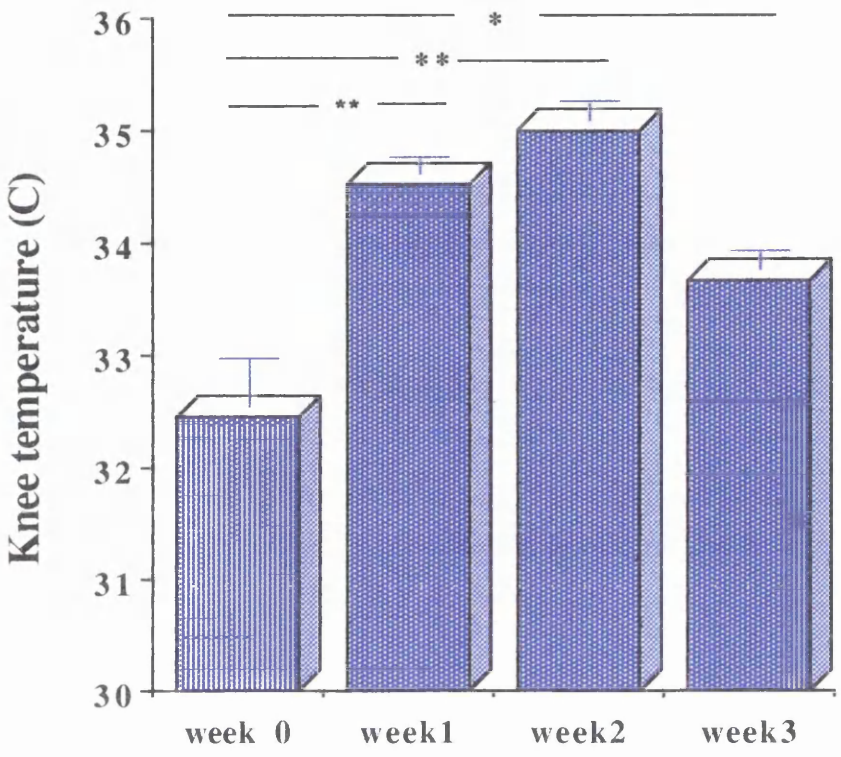


Fig 5.5. Changes in joint temperature at three weeks in response to intra-articular injection of Freund's adjuvant. Week 0 refers to the experimental starting day. (control and week 1, n=10, and week 2, n=9, week 3 n=9). * means week 1 differs significantly from week 0. For significant value see text

a significant (P=0.043, n=9) increase in diameter at week one and a significant ($p<0.01$, n-9, t test) increase in temperature.

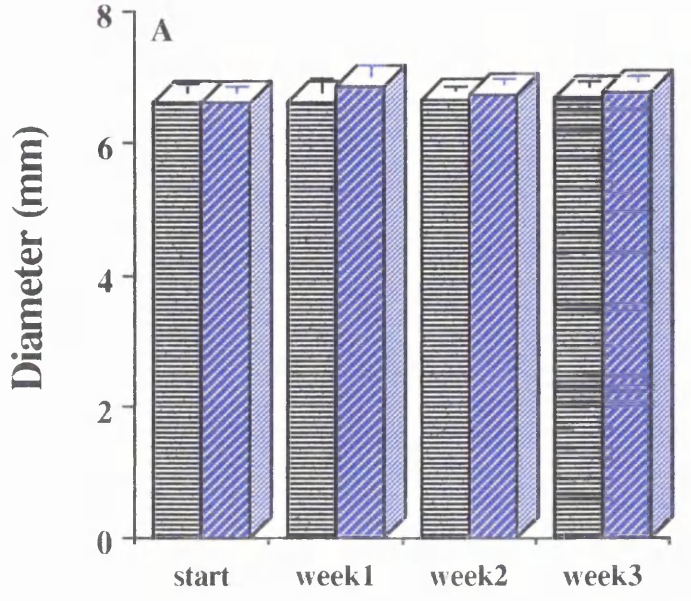
Freund's complete adjuvant was found to have no significant effect on ankle diameter and temperature over the 3 weeks ($P>0.05$; n =15 for each group tested, t test) thereby confirming an absence of a systemic effect of the adjuvant (fig. 5.6 a & b).

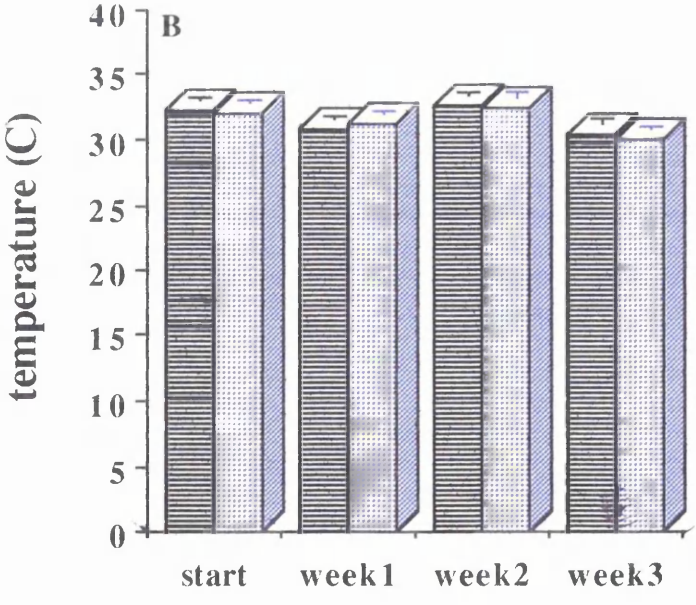
One week after induction of arthritis, a marked reduction in joint perfusion occurred as revealed by a fall in the LDI flux values measured at the beginning of each experiment. Perfusion then returned back towards control levels by the third week (Fig. 5.7)

5.4.3.2 Neurokinin-mediated vasodilatation

Substance P, when applied topically to the exposed surface of normal knee joints, showed a significant dose-dependent augmentation of joint blood flow ($P<0.001$; n =10, anova) culminating in a peak rise of 45.1±8.6% with the 10^{-9} mol dose. Figs. 5.3, 5.8 show that adjuvant treatment, however, completely abolished this hyperaemia and in some instances a constrictor response to SP could be observed. At week 1, SP had no significant effect on synovial blood flow ($P=0.511$; n=10, anova) but by the third week of inflammation vasoconstriction was recorded ($P<0.001$; n=9); both groups of animals were found to be significantly different from the control rats ($P<0.001$). A fall in articular perfusion of approximately 40% occurred when 10^{-8} and 10^{-12} mol doses of SP were administered to the joint but the intermediate doses produced less of an effect.

Fig 5.6: Changes in ipsilateral (blue column) ankle diameter (A) and temperature (B) at three weeks in response to knee joint intra-articular injection of Freund's adjuvant compared to the contralateral (black column) ankle. week 0 refers to the experiments starting day. (n=15). There is not any significant difference between the two ankle (ipsilateral and contralateral, adjuvant treated knee joint) both in diameter and temperature within the three week and also compared to week 0.


A
Diameter (mm)
8
6
4
2
0
start
week1
week2
week3


B
temperature (C)
40
35
30
25
20
15
10
5
0
start
week1
week2
week3

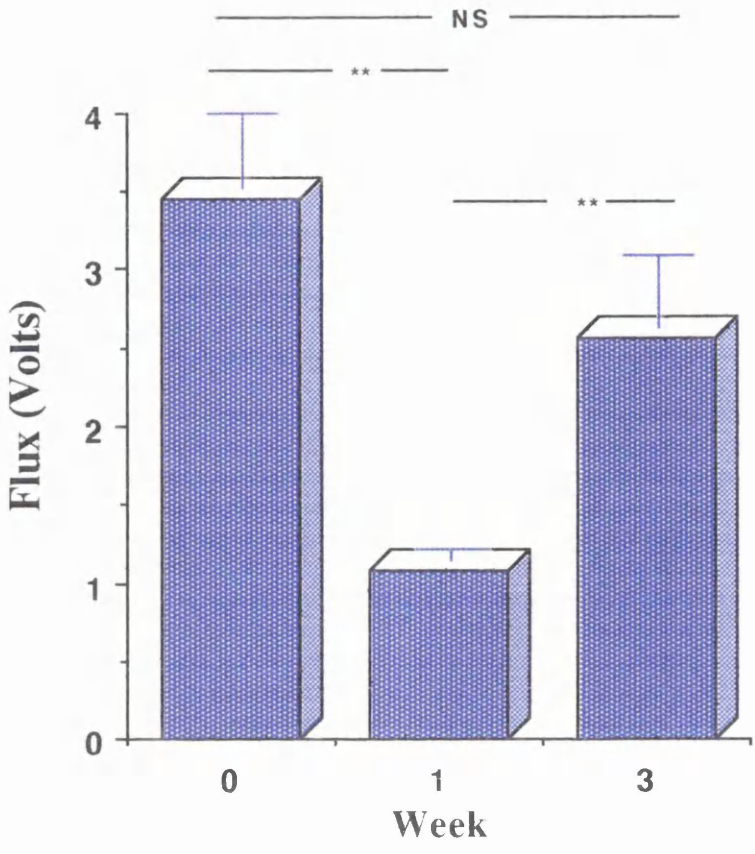


Fig 5.7. Alteration in basal blood flow at one and three weeks following intra-articular injection of Freund's adjuvant (mean ± SEM, n=10 for both control, week 0, and week 1 data and n=9 for week 3 group). ** refers to significant difference between week 0 and week 1, and week 3 and week 1. P=0.001

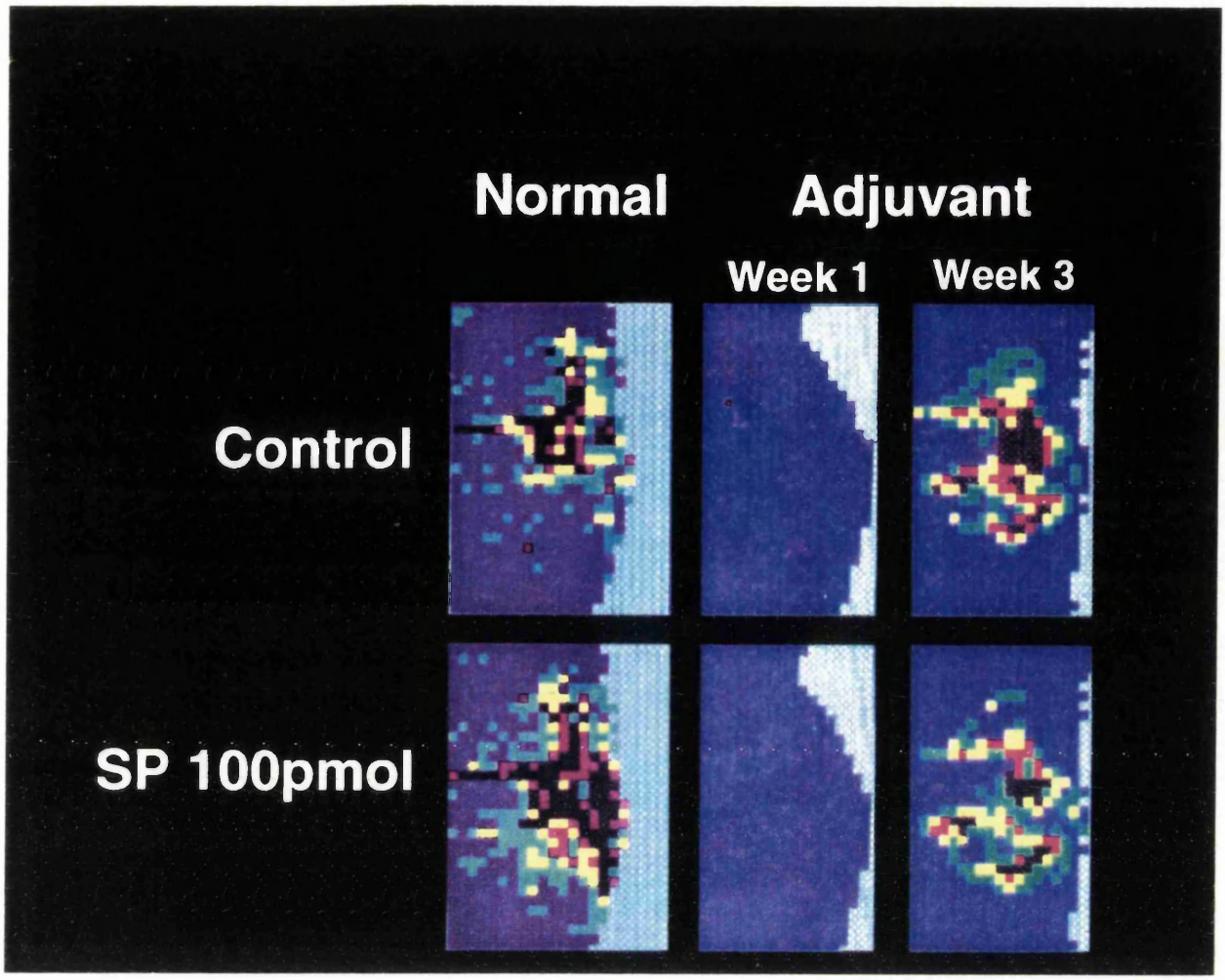


Fig 5.8a: Images showing changes in perfusion of normal and adjuvant-treated knees in response to application of substance P (SP) Perfusion is encoded using a six colour scale covering 0-10V. Control images indicate a substantial reduction of perfusion in adjuvant treated knees at week 1 which recovers by week 3. However, in adjuvant-treated knees there is no vasodilator response to SP at either weeks 1 or 3.

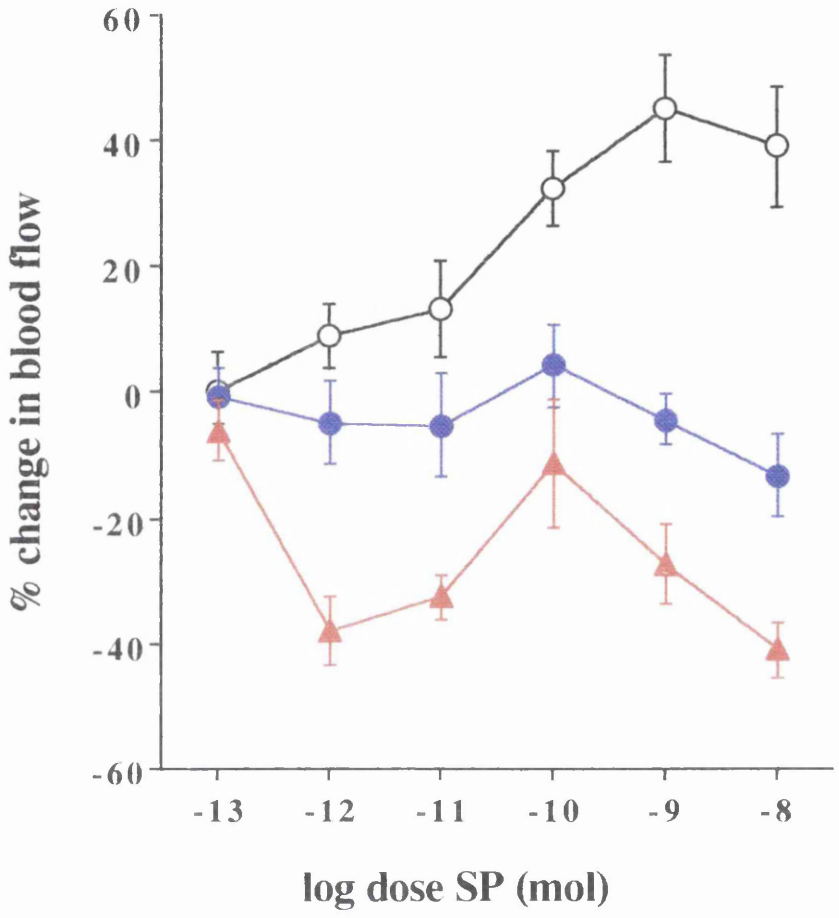


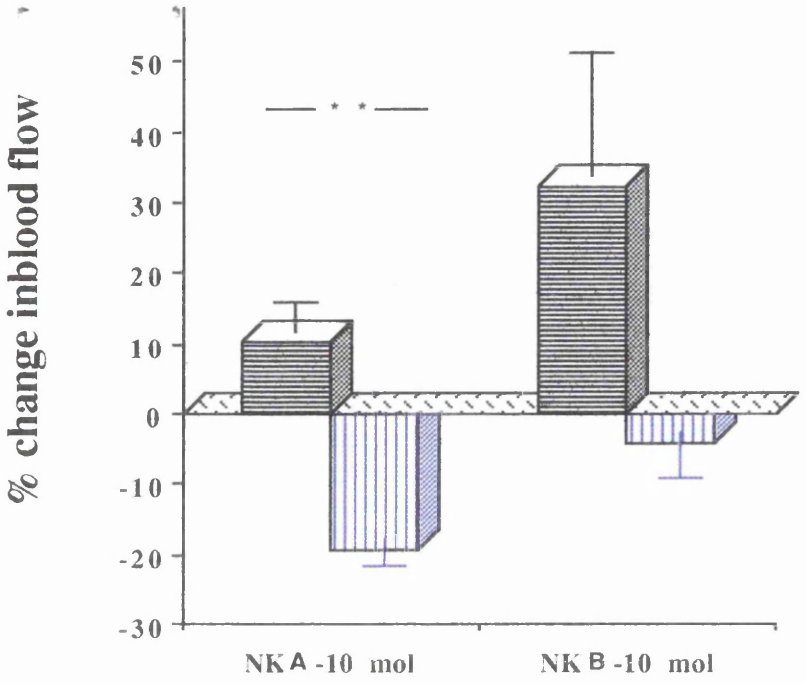
Fig 5.8b Changes in synovial blood flow during topical application of SP to the joint capsule in normal (○, mean ±SEM, n=10), in adjuvant induced chronically inflamed knees at week one (●, mean ±SEM, n=10), and week three (▲, mean ±SEM, n=8). The sequence of doses administered was randomised.

Application of NKA and NKB (both at 10^{-10} mol) to the surfaces of the exposed normal knee produced a 10.3±4.2% and 32.4±16.7% vasodilatation respectively. Adjuvant treatment at week three, however, completely abolished this hyperaemia and in some instances a vasoconstrictor response to these neurokinins was observed (Figs. 5.9 A) and this was significantly different from the control response ($p<0.0001$, n=7, t test). Application of 5 hydroxytryptamine (5HT 10^{-11} mol) to the exposed normal and adjuvant treated joint did not show any significant ($p> 0.05$, n=7, t test) effect on basal blood flow and application of 10^{-12} mol of adrenaline (epinephrine) to the normal and adjuvant treated joint showed an acute vasoconstriction effect which in adjuvant treated knee was slightly and significantly ($P<0.05$, n=7) greater than the normal knee (Fig. 5.9B).

5.4.4 DISCUSSION

Injection of Freund's complete adjuvant into rat knee joints caused a considerable increase in diameter and temperature of the joint and this was used as an index of the magnitude of the inflammatory response. Since no discernible change in ankle diameter occurred in any of the rats, it is clear that inflammation was localised to the knee and hence a polyarthritic model was avoided. Despite this observation, the contralateral knee could not be used as a control as it is known that an experimentally-induced monoarthritis causes bilateral changes in peptide content of synovial fluid (Bilevicuite *et al.*, 1993). This effect could result in a modification of basal blood flow in the contralateral knee thereby masking any possible neurogenic or peptidergic responses. Indeed, the contralateral knee did show a small but significant increase in diameter and a significant increase ($p<0.01$) in

Fig 5.9 A and B: Changes in synovial blood flow during topical application of NKA and NKB both doses at 10^{-10} mol, 5HT at a dose 10^{-11} mol and adrenaline at a dose 10^{-12} mol in the normal knee joint (black column, mean ±SEM, n=7) and at three weeks in the chronic adjuvant treated knee (blue column, mean ± SEM, n=7). * and ** refers to significant difference between the effect of NKA, NKB and adrenaline on blood flow in the normal knees with those in adjuvant treated knees. For significant value see text


% change inblood flow
50
40
30
20
10
0
-10
-20
-30
*
NK A -10 mol
NK B -10 mol

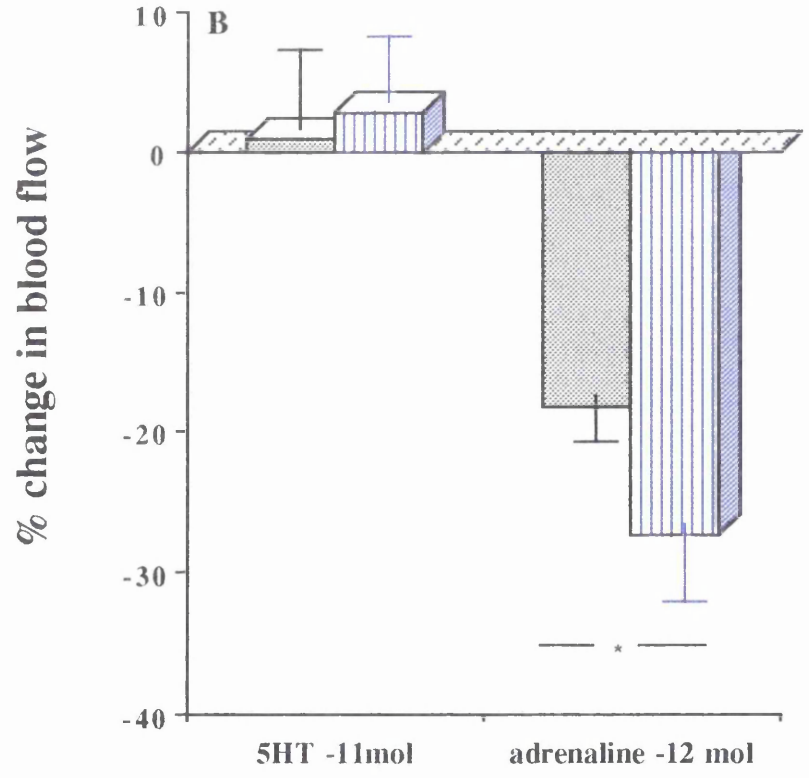
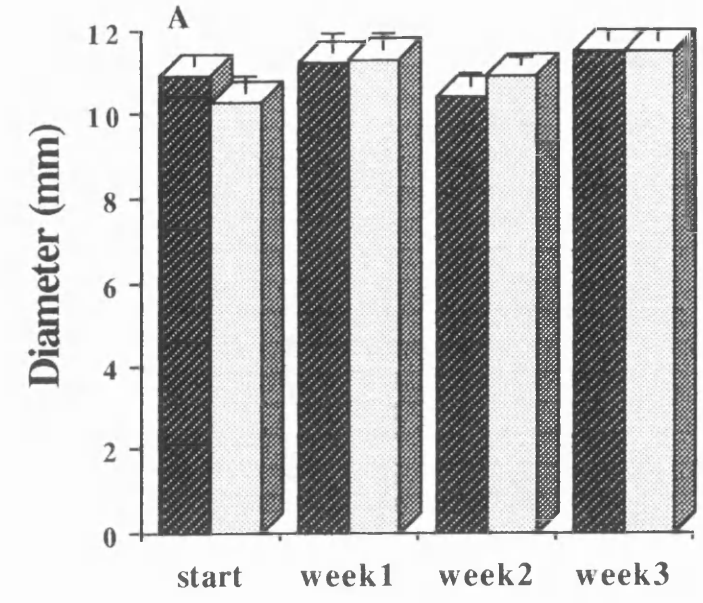
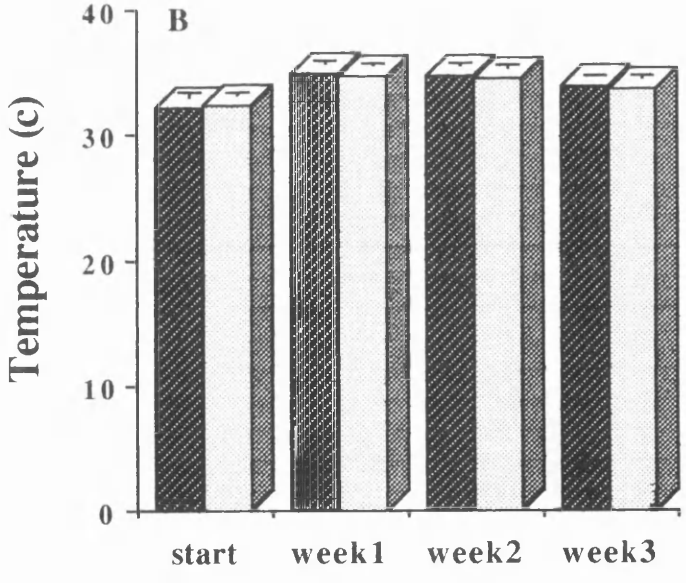

B
% change in blood flow
10
0
-10
-20
-30
-40
*
5HT -11mol
adrenaline -12 mol

Fig 5.10 A and B: Changes in knee joint diameter and temperature over three weeks in response to intra-articular injection of paraffin (vehicle of Freund's adjuvant , dark column) compared to contralateral knee (control, white doted column). week 0 refers to the experimental starting day. (n=5). There is not any significant difference between the two knees both in diameter and temperature within three weeks.


A
Diameter (mm)
12
10
8
6
4
2
0
start
week1
week2
week3


B
Temperature (c)
40
30
20
10
0
start
week1
week2
week3

temperature, suggesting that a mild, perhaps neurogenically-mediated, inflammatory response had occurred, confirming the unsuitability of the contralateral knee as an internal control. Injection of the paraffin alone (a necessary part of the control studies, since paraffin is the vehicle for Freund,s adjuvant) did not show any significant effect in knee diameter and temperature compared to un injected knee, verifying that the response to the to adjuvant is due to its active component ($p > 0.05$, $n=5$, t test)(fig. 5.10 a&b).

One week after adjuvant treatment, chronically inflamed knees exhibited a fall in basal blood flow which subsequently returned towards normal two weeks later. This initial fall in perfusion is probably real and not due to an inability of the laser to detect blood flow in the enlarged knee. The swelling of the joint is not due to a thickening of the capsule but may be ascribed to accumulation of tissue fluid which does not significantly affect the penetrative power of the laser beam. In addition, blood vessels on the surface of the adjuvant arthritic knee are still visible to the naked eye and therefore should be detectable by the LDI system. The monoarthritis model produces a localised lesion with little systemic disturbance and treated animals are found to be normotensive. Thus, the hypoperfused knee observed one week post adjuvant injection cannot be ascribed to circulatory disturbances which might occur in the more severe conventional adjuvant polyarthritic model. Microscopic inspection of the arthritic knees suggested that the hypo perfusion might to some extent be the result of localised areas of tissue necrosis and possible blood vessel ablation.

Differences between the arthritic and control rats could not be ascribed to differences in the reproducibility of measurements as basal flux values at week 1 in arthritic rats show lower variability than the measurements obtained in control rats or at week 3 (fig. 5.7). In addition, flux readings obtained prior to administration of SP were found to be stable throughout the experiments. The LDI technique is suitable for comparison of rats at different ages as the scan area can be increased to accommodate the larger knee joint in the older animals. Interestingly, comparison of flux values between animals of different ages revealed no significant differences in perfusion, suggesting that there is no alteration of vascularity of the joint as a function of age.

As articular cartilage is dependent for its nutritional requrements on synovial perfusion (McKibbin & Maroudas, 1979), then this initial reduction in joint blood flow could cause the integrity of the joint to deteriorate and lead to degenerative changes. It has previously been shown that adjuvant-induced inflammation in the rat knee joint abolishes sympathetic vasoconstriction and the neuropeptidergic vasodilator response to SP at one week (McDougall, *et al* 1994). The investigation outlined here has shown that these alterations are not transitory but persist three weeks post-injection of adjuvant, even though the inflammatory process appears to be abating by the third week.

Topical application of SP to the exposed surface of rat knees elicited a dose-dependent dilatation in normal rats which was maximal at 10^{-9} mol. Chronically inflamed joints were unresponsive to SP for the whole three weeks of the study and in some instances even showed vasoconstriction. This finding suggests that the SP receptors are either

radically transformed or possibly inactivated by the inflammatory process. Previous work has shown decreased binding of Bolton-Hunter labelled SP in chronically inflamed rat knee joints (Walsh, *et al*, 1993) supporting the view that a variation in either receptor expression or decreased efficacy of the receptor has occurred. In addition, it is possible that enzyme systems which degrade neuropeptides such as SP are altered by the inflammatory process. Adjuvant monoarthritis is known to cause an increased SP content of dorsal root ganglia (Smith, *et al*, 1992) and this increased amount of neuropeptide is transported toward the periphery in the axoplasmic flow (Donnerer, Schuligoi & Stein, 1992). It may be this accumulation of SP at the periphery which is contributing to the modification of the SP receptors either by toxic means or via a down regulatory mechanism. Experiments on another group of adjuvant treated knees revealed that the vasodilator response of the inflamed knee joint to NKA and NKB was like SP also reversed, whereas the responses to application of 5HT and adrenaline were not altered in the adjuvant treated knee compared to the normal joint and even showed a significantly stronger effect of adrenaline in the absence of natural neurokinin effects.In blood vessels the response to 5HT is variable, the overall effect varying according to the size of the vessels (Rang & Dale 1992). Using LDF it has been shown that 5HT at 10^{-11} mol produces vasodilatation in the rat knee joint (Lam & Ferrell, unpublished data). However in the present experiments this dose showed only a small effect on blood flow in the normal knee which was not changed significantly in the adjuvant treated knee, but the response was small and made it difficult to assess the effect of 5HT on its own. As 5HT has a variable effect on systemic blood pressure (Rang & Dale 1992) higher doses of 5HT could have caused blood

pressure changes and affect the results. In future experiments it would need a full dose response curve for 5HT to assess its effect on knee joint blood flow whilst monitoring blood pressure.

These results indicate that in the adjuvant treated knee all neurokinin receptors are modified and that this modification does not necessarily affect other receptor types in the joint.

Acute inflammatory responses induced by intra-articular injection of carrageenan show nerve-mediated vasoconstriction to be attenuated but the dilator effects of SP to be greatly enhanced (Lam & Ferrell, 1993a), whereas adjuvant induced inflammation altered both SP mediated vasodilatation and sympathetic vasoconstriction (McDougall *et al.* 1994). Combined with the findings of the present study, it would appear that the integrity of sympathetic transmission and neurokinin receptor activation in rat knee joints declines as inflammation becomes more chronic. Loss of these neurovascular controls could contribute to degenerative changes which commonly accompany chronic inflammatory joint diseases.

5.5 CONTRALATERAL EFFECT OF ADJUVANT MONOARTHRITIS

5.5.1 Introduction: The contralateral effect of neurogenic arthritis began to be recognised some years ago. Axotomy of the nerve to one muscle of the frog induced sprouting and synapse formation by the homologous intact nerve on the opposite side and this has since been observed in three different muscles, the cutaneous pectoralis

(Rotshenker 1979), the sartorious (Ring *et al.* 1983), and piriformis (Elizade *et al.* 1983), Similar events also occurred in mammals and it was found that in the intact muscle of the normal rat, spouting and synapse formation in an ongoing process which can be enhanced by contralateral axotomy (Rotshenker & Tai M. 1985). Bilevicuite *et al.* (1993) showed bilateral changes in synovial fluid neuropeptide content to monoarthritis.

Allnatt *et al.* (1990) reported that sapheneous nerve injury and degeneration in one rat leg suppressed the ability of the contralateral nerve to evoke plasma extravasation, which indicated that there is a neuronic contralateral effects in the body organs. Kidd *et al.* (1989) reported that synovial damage resulted in acute inflammation in the damaged joint and in a neurogenically mediated infiltrate of inflammatory cells in the contralateral joint.

Neuropeptides are known to have an important role in the inflammatory process (Marzou *et al.* 1989; Scott *et al.* 1994). They can share and interact with the neuropeptide's second messengers and so can connect certain immunological stimuli to the biosynthesis of inflammatory mediators (Marzou *et al.* 1989). In addition there has been a report describing the contralateral effect of monoarthritis on the spinal cord (Mapp *et al.* 1993), but to date little work has been done on the effect of ipsilateral arthritis on the vascular response of the contralateral joint to tachykinins.

In section 5.3 it was reported that administration of adjuvant caused significant increase in temperature of the contralateral knee and

to further investigate this matter, the experiments described in this section were carried out.

5.5.2 MATERIAL AND METHOD

Experiments were performed on two groups of rats, an intact group as control and an adjuvant treated group. Freund's adjuvant was injected into the knee joint to induce chronic monoarthritis and the temperature and width of the knee joint measured as an assessment of the inflammatory response at 1, 2 and 3. weeks. In addition, the response of the contralateral knee to SP at different doses was examined. In these experiments the contralateral effect of adjuvant was studied at the third week. The method of induction arthritis and measurement of temperature and width was the same as described in 5.3.2. and LDI imaging and data analysis performed, as described previously in chapter 2.

5.5.3 RESULTS

At week 1 the knee width significantly increased in the contra-lateral knee, compared to week 0 (control) ($p=0.043$ $N=9$), but there was no significant difference in diameter of the contralateral knee between week 2 or week 3 compared to week zero. The temperature in the contralateral knee significantly increased at weeks 1,2 and 3 compared to control (week 0) ($p<0.05$, $n=9$, t test) (Fig. 5.11).

Topical application of SP to the contralateral knee was performed at week 3. As shown in fig. 5.12 the vasodilator effect of SP on the blood flow was significantly attenuated and shifted the dose response curve to the right ($p=0.01$, $n=9$, anova).

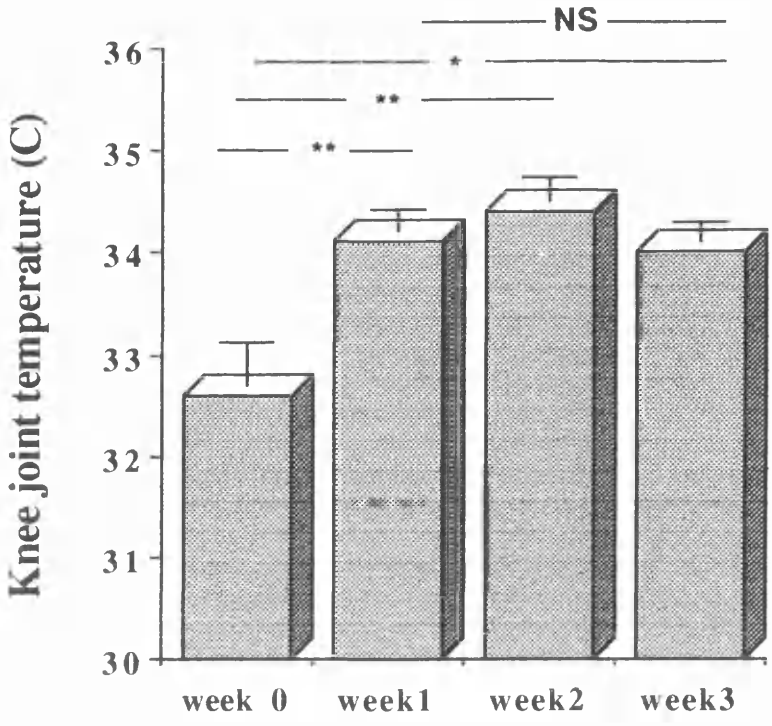


Fig. 5.11: Changes in contralateral knee joint temperature at three weeks in response to intra-articular injection of Freund's adjuvant compared to week 0. week 0 refers to the experimental starting day. (n=9). * refers to significant difference between week 1, 2 and 3 with week 0. For significant value see text

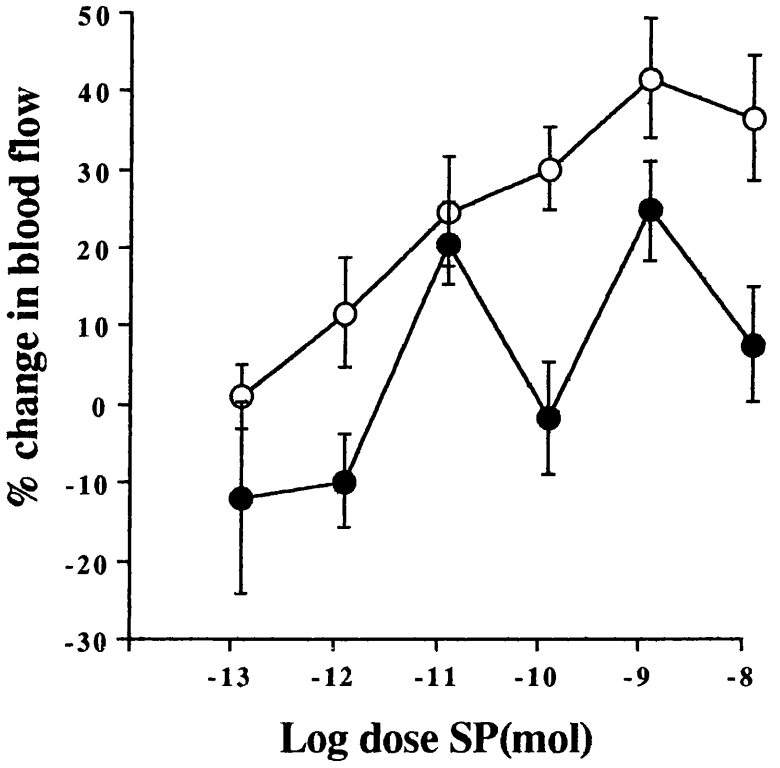


Fig 5.12: Changes in synovial blood flow during topical application of SP to the joint capsule in normal (○, mean ±SEM, n=10), and in contralateral knees at week three of the adjuvant treated animals (●, mean ±SEM, n=9),

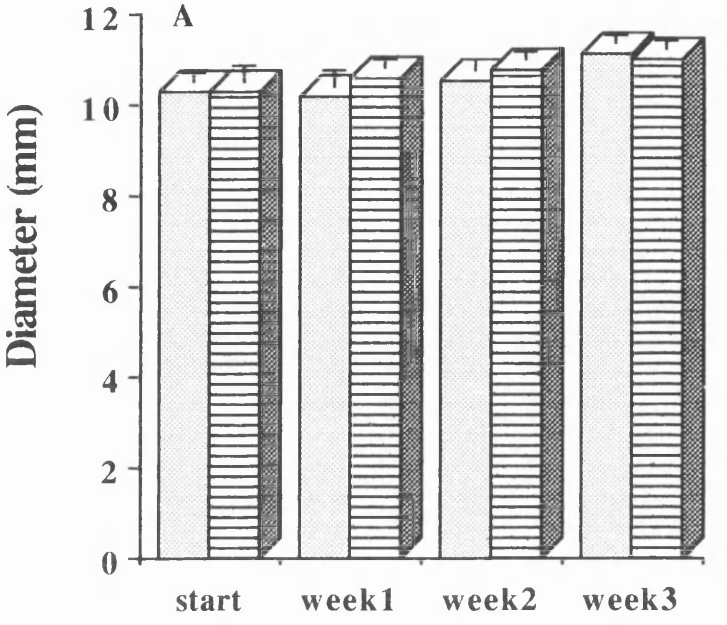
In the control group temperature and diameter changes over three weeks were not significant and increased knee diameter due to the natural growth at week three was not significant compared to week 0 ($p > 0.05$, $n=5$, t test)(fig. 5.13).

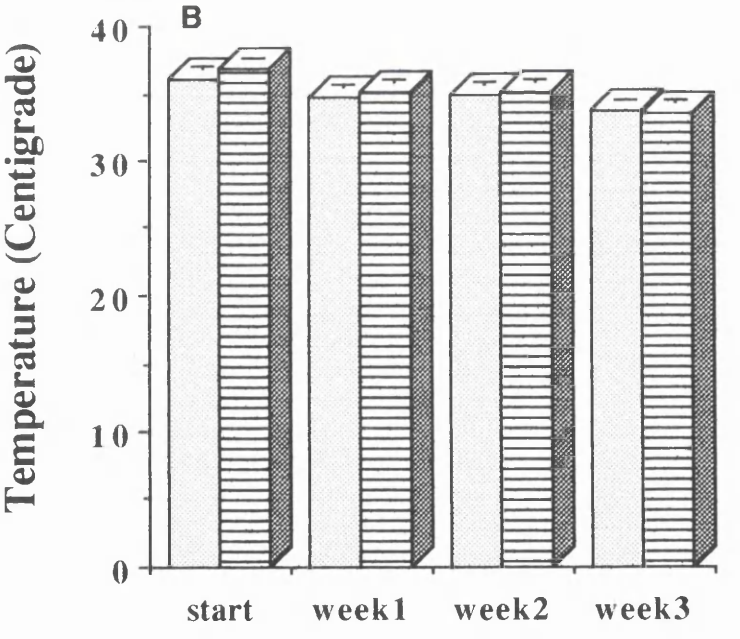
5.5.4 DISCUSSION

The temperature in the contralateral knee was significantly increased at all three weeks compared to control values, ($P<0.05$, $n=9$). Knee diameter at week 1 was significantly increased compared to week zero ($p=0.01$, $n=9$), but it abated by week 2 and week 3 and there was not any difference between values obtained at these weeks and the corresponding control values.

The attenuation of the effect of SP on blood flow by ipsilateral inflammation was not as strong on the contralateral side but it was significant compared to control. The changes in the temperature and diameter and attenuation of the SP effect on blood flow suggest a neurogenic symmetrical effect. In the control group no significant changes in temperature and diameter of the joint were seen over three weeks. Measurement of ankle diameter and temperature in the ipsilateral and contralateral sides over three weeks did not show any significant ($n=15$, $P > 0.05$) changes compared to week zero suggesting that the results cannot be explained by a purely systemic effect of the adjuvant . A functional relationship seems to exist between the sensory nerve fibres in the periphery and neuropeptides in the dorsal horn of the spinal cord. These peptides are therefore well placed to mediate a symmetrical response by a putative central reflex (Mapp 1993). The proposal is that after nociceptor activation secondary to joint damage,

Fig 5.13 A and B: Changes in knee joint diameter and temperature at three weeks compared to week 0. Both knees are intact (right knee, doted column left knee lined column). week 0 refers to the experimental starting day. (n=5). There is not any significant difference between two knee both in diameter and temperature within three weeks.


A
Diameter (mm)
12
10
8
6
4
2
0
start
week1
week2
week3


B
Temperature (Centigrade)
40
30
20
10
0
start
week1
week2
week3

preganglionic sympathetic neurones in the autonomic cell column of the spinal cord are selectively activated. These neurones project across the spinal cord to their counterparts on the other side and joint damage could therefore results on both sides (Kidd *et al.* 1989). Considering these finding and the existence of similar response to SP on the contralateral and at the ipsilateral knees, a neurogenic contralateral effect of adjuvant monoarthritis could be a possible explanation.

5.6 SECTION THREE

INFLAMMATION INDUCED BY

CAPSAICIN

5.6.1 Introduction: Capsaicin is the pungent ingredient which is found in a wide variety of red peppers of the genus *capsaicin.* Chemically it is a derivative of vanillyl amid, 8 methyl-n- vanillyl 6-no- nonamide (fig. 5.14) with a molecular weight of 305.42. Hot peppers have been known to humans since prehistoric times, but it has only recently been realised that capsaicin, a pure pepper substance, exerts a long term sensory receptor blocking action and capsaicin has now become well established as an important probe for sensory neurones (Holzer 1991).

Two effects of capsaicin can be differentiated: firstly an action which is grossly selective for thin afferent neurones of mammalians species, with short lasting stimulation followed by desensitisation, and secondly a transient depression with no long lasting consequences for the cell which is seen in most species (Holzer 1991). Capsaicin leads

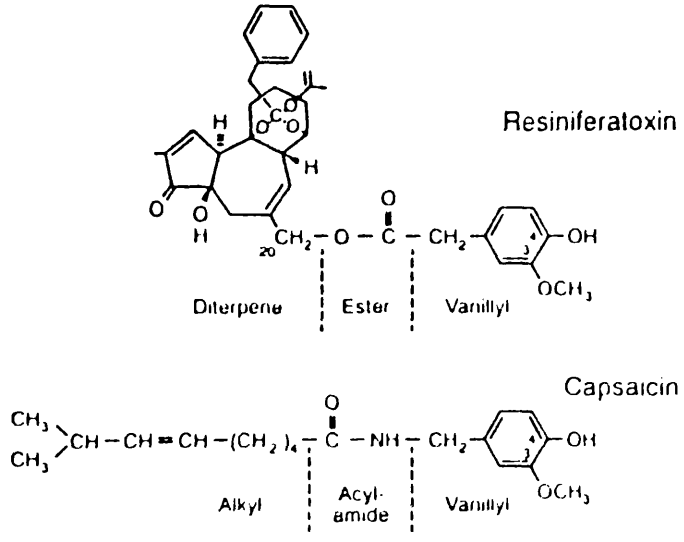


Fig 5.14: Chemical structures of capsaicin and resiniferatoxin

to a depolarisation of thin primary afferent neurones and the majority of SP containing afferents are depleted by capsaicin. CGRP and neurokinin containing neurones are also deplete by capsaicin (Holzer 1991), but the monoamine system is not damaged by capsaicin in the CNS and spinal cord and even slightly increases the amount of monoamine in these cells. In the rat, application of capsaicin on the sciatic nerve leads to a 34% reduction of cell bodies in the dorsal rout ganglia (DRG) and 32-40% reduction of unmyelinated fibres but capsaicin does not have any effect on myelinated fibres (Jansco and Lawson 1990; Ferrell *et al.* 1992).

Capsaicin's action on sensory neurones is mediated by a specific recognition site with the pharmacological properties of a receptor. These receptors activate Ca^{++} and Na^{+} ion channels and produce a stimulatory effect (Bevan and Szolcsany 1990)(fig. 5.15). This effect on ion channels is distinct from the voltage dependent cation channel in the cell membrane and taken together capsaicin stimulates sensory neurones which increases conductance and induces depolarisation. Calcium influx is induced by this mechanism and intracellular calcium concentration increases with consequent activation of intracellular enzymes and neuro-transmitter release (Holzer 1991).

Both excitation and desensitisation are dependent on extra cellular calcium concentration and the L type of voltage sensitive calcium channel (Maggi *et al.* 1989).

Capsaicin also has a direct action on metabolism and cellular proliferation. This effect is dose dependent, but the mechanisms by which it stimulates DNA synthesis is not yet clear (Mutacci *et al.*

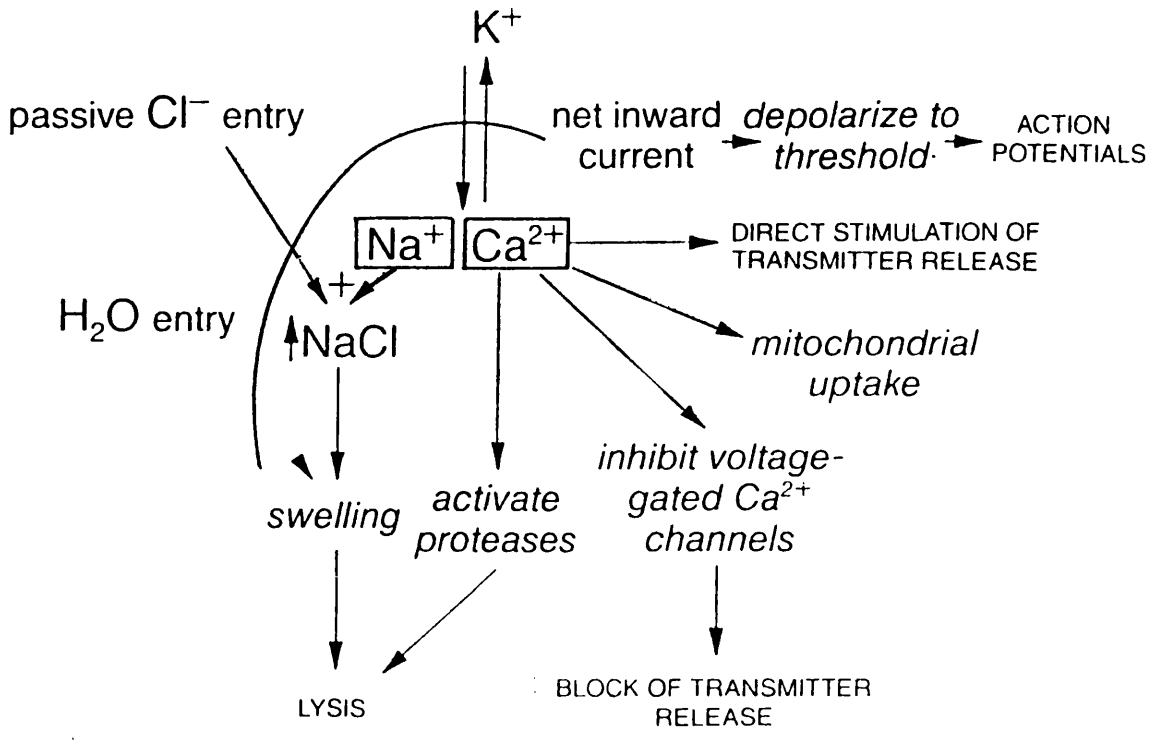


Fig 5.15: Scheme of action for capsaicin. From Bevan S. & Szolcsanyi 1990

1990). Subsequently Capsazepine was discovered and found to be competitive antagonist for capsaicin (Dray 1992)

In this study capsaicin was used to induce acute inflammation and chronically deplete nerve fibres. Chronic capsaicin treatment was used to deplete knee joint nerve endings to examine the tissue and vasculature response to SP. Acute capsaicin was used to release neurokinins and thereby induce neurogenic inflammation. The neuropeptidergic response was examined in both cases to elucidate the effect of neurokinins on the rat knee joint vasculature.

5.6.2 PART ONE

CHRONIC CAPSAICIN TREATMENT

Capsaicin, is known to cause depletion of neuropeptides such as SP from sensory nerve fibres and is neurotoxic when locally applied to nerves (Holzer, 1991). Unmyelinated but not myelinated nerve fibres supplying the rat knee joint are depleted after intra-articular injection of capsaicin (Ferrell, Lam and Montgomery, 1992). Thus capsaicin pretreatment of the joint could reveal the effect of SP, and also neurokinin receptor antagonists on knee joint vasculature.

5.6.2.1 MATERIALS AND METHODS:

Two groups of male Wistar rat were used in these experiments, an intact group and capsaicin-treated group. Degeneration of the nerves supplying the knee was induced by intra-articular injection of 0.2 ml of 2% capsaicin (Sigma) into the rat knee 5-7 days prior to the

start of experiments. Previous work has shown that this period is sufficient to obtain a significant reduction in unmyelinated fibres (Ferrell *et al.*, 1992).To control and compare with an intact group, 10 rats were injected with 0.2 ml of the capsaicin vehicle (ethanol, cremaphor EL in 0.9% saline). SP (Cambridge Research Biochemicals) was administered as a bolus applied to the surface of the joint in a volume of 0.1 ml. Phenoxybenzamine (10^{-6} mol) and FK888 and SR48968 (both 10^{-8} mol, an NK1 & NK2 antagonist respectively) also were used in these experiments. Warmed ($37^{o}C$) physiological saline solution (0.9% Na Cl) was regularly applied to the knee joint surface to prevent tissue dehydration. Arterial blood pressure was measured by cannulation of a carotid artery, connection to a pressure transducer (Elcomatic EM751) and monitoring the transducer output on a pen recorder (Lectromed Multitrace 4). Experimental set-up and image analysis was as described in chapter 2

5.6.2.2 RESULTS

Although the images suggest a higher basal blood flow in capsaicin-treated as opposed to vehicle-treated knees (fig. 5.16b), the mean voltage signals (±SEM) corresponding to each group (4.95±0.52 and 4.15±0.35 V respectively) did not differ significantly between the two groups (P=0.21, two sample t-test; n=12 and 15 knees respectively). The heterogeneity of perfusion in the knee joint capsule is clear from the images in figure 5.16 and analysis of two separate square areas (an array of 4 by 4 measurement points) in the same knee of control rats, one at the upper border and one at the lower border of the joint, showed significant (P=0.0014; n=47 paired measurements)

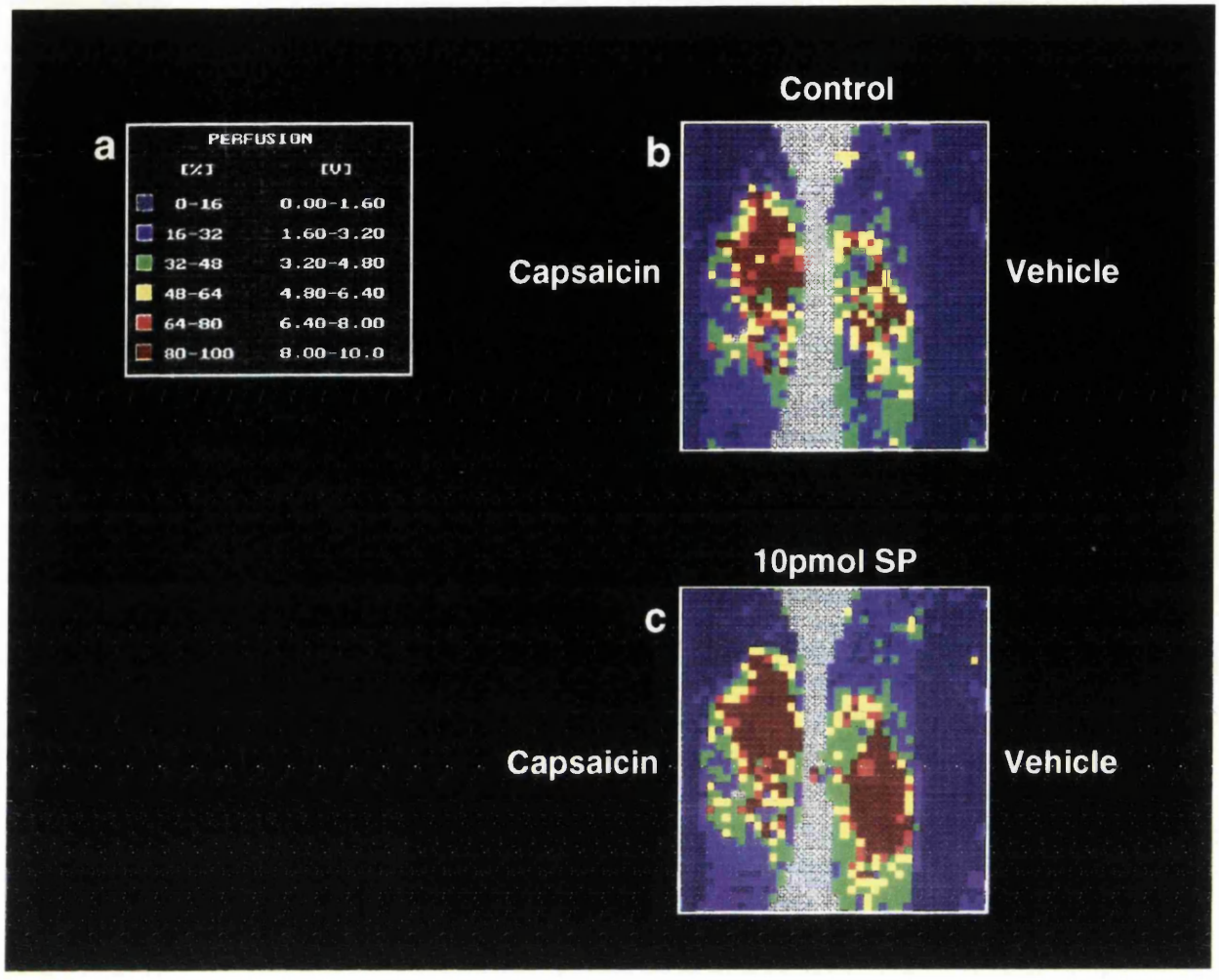


Fig 5.16: Laser Doppler perfusion images of rat knee joints.

A) colour-coded system for showing perfusion values

B) Images of knees from two rats, one pre-treated with capsaicin, the other with vehicle, prior to any intervention.

C) The same knees as in B immediately after topical application of 10 pmol of substance P (SP) to both knees. The vehicle-treated knee shows significant vasodilatation, but this is much less evident in the capsaicin-treated knee.

differences in the LDI signals of 2.2±0.11 V and 2.82±0.16 V respectively.

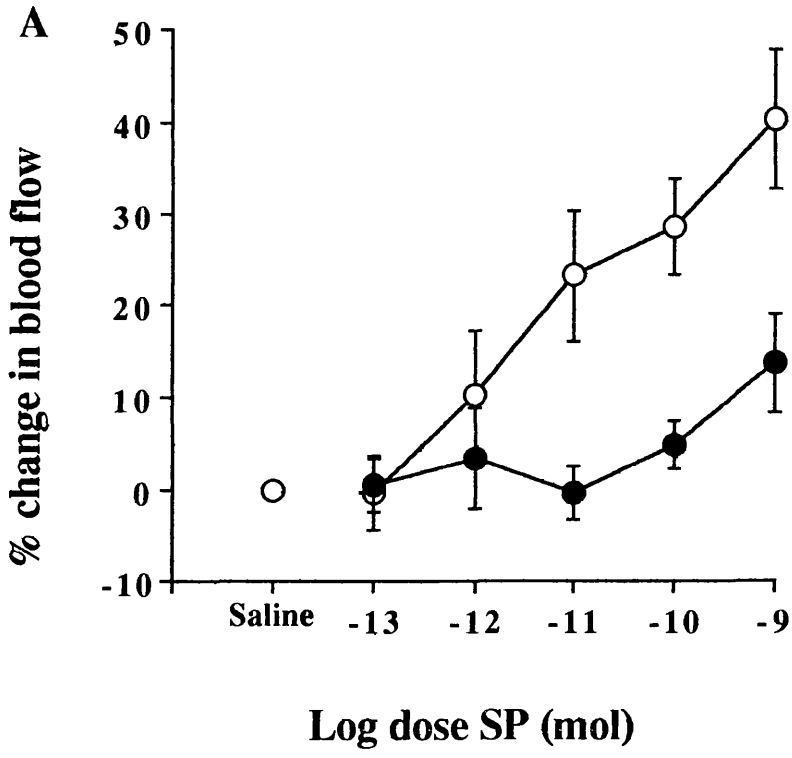
The responsiveness of articular blood vessels to neuropeptides was assessed by examination of the dose/response relationship to SP which was administered directly to the joint in a volume of 100µl. It was clear that SP produced significant vasodilatation in the both normal and vehicle-treated knee but had much less effect in capsaicin-treated knees (fig. 5.16.b& c). There was not any significant difference (P=0.27,vehicle n=10, intact n=35, two tail anova) between intact and vehicle treated rats in their response to SP.

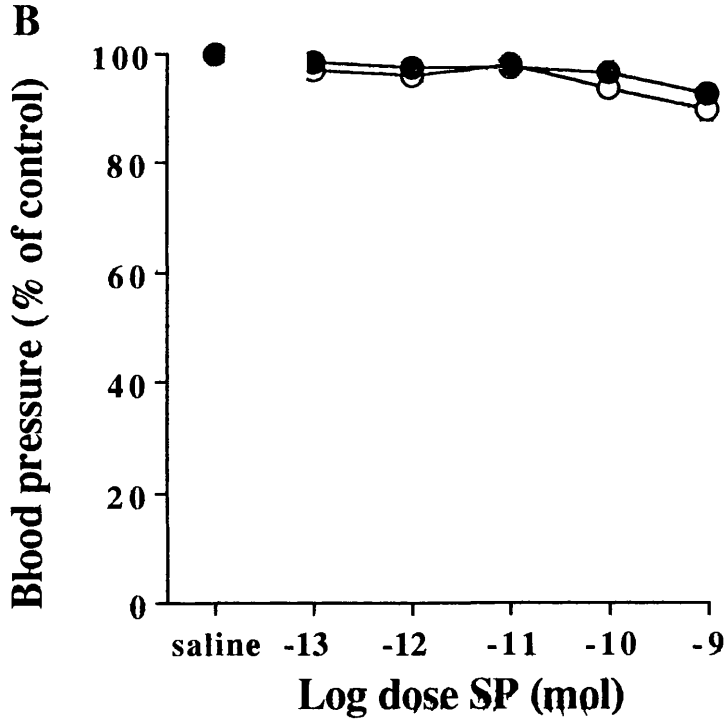
This difference in response was observed across a range of doses of SP (fig. 5.17A) and two-factor ANOVA revealed a significant dose-dependent effect (P=0.026) and a very significant difference between intact and capsaicin-treated knees (P<0.0001; n=35 & n=12 knees, respectively). Application of phenoxybenzamine (10^{-6} mol) in the 2% capsaicin treated knee slightly but non significantly reduced blood flow. Phenoxybenzamine in the intact knees, increased basal blood flow by 30% and the difference between these groups was highly significant (p <0.001,n=26, paired t test) (fig. 5.18).

Application of both FK888 and SR48968 (both 10^{-8} mol) in the 2% capsaicin pre-treated knee decreased basal blood flow but this did not differ significantly compared to the intact group. Co-application of phenoxybenzamine with FK888 and SR48968 in the 2% capsaicin pre-treated knee, slightly decreased basal blood flow as in the intact knees but the difference was not significant (p >0.05, n=17, t test) (fig. 5.18).

Fig 5.17A. Changes in synovial blood flow during topical application of SP to the knee joint capsule, in normal knees (○, mean ±SEM, n=35) and in 2% capsaicin pre-treated knees (●, mean ±SEM, n=12)

Fig 5.17B Percentage change in mean arterial blood flood in response to topical application of SP to the joint capsule in normal knee (mean ± SEM, ○, n=5) and in 2% capsaicin pre-treated knees (●, mean ±SEM, n=12). There is not any significant difference on blood pressure between different treatments.


A
% change in blood flow
50
40
30
20
10
0
-10
Saline
-13
-12
-11
-10
-9
Log dose SP (mol)


B
Blood pressure (% of control)
100
80
60
40
20
0
saline
-13
-12
-11
-10
-9
Log dose SP (mol)

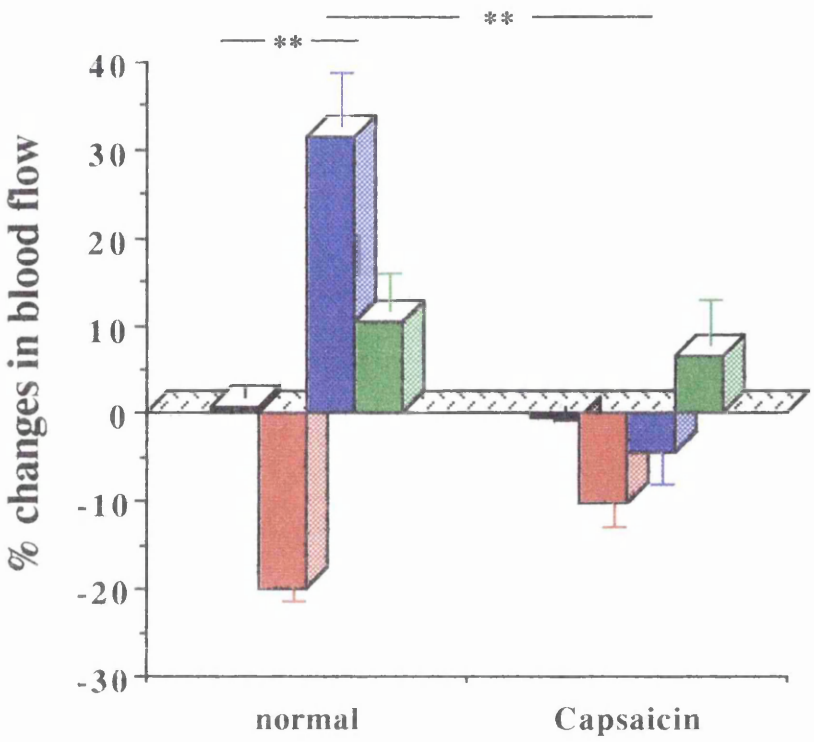


Fig 5.18. Changes in synovial blood flow during topical application of saline, and SR48968 + FK888 both at 10^{-8} mol alone and with phenoxybenzamine (10^{-6}) to the normal and 2% capsaicin pre-treated knee joint capsule . Values are mean ±SEM and for normal and capsaicin treated knees are:

Saline (■, n=44, and 57), SR48968 + FK888 (■, n=29 and 17), phenoxybenzamine (■, n=12 and 26), SR48968 + FK888 with phenoxybenzamine (■, n=38 and 9). * means differs significant.

As capsaicin could have induced a mild synovitis, in addition to its neurotoxic effects, it is possible that the attenuated vasodilator response to SP in capsaicin-treated knees could be attributed to more rapid uptake of SP in these joints. Rapid uptake of SP by synovial capillaries could then give rise to systemic vasodilatation and consequent transient hypotension, which has been observed at SP doses greater than 1 nmol (Lam and Ferrell, 1993b). This was checked in the present study by monitoring arterial blood pressure whilst applying different doses of SP to vehicle-treated and capsaicin-treated knees. As shown in fig. 5.17B, mean arterial blood pressure remained relatively steady across a range of doses and there was no difference in the blood pressure response to SP between vehicle and capsaicin-treated knees ($P=0.228$, one factor ANOVA, $n=5$).

In addition, repeated scans following SP administration showed that significant differences from pre-SP administration only occurred in the scan immediately following SP administration. This was true for capsaicin pre-treated rats, indicating that the time course of the dilator response to SP in this group was similar to that occurring in the vehicle group.

5.6.2.3 DISCUSSION

The present experiments have revealed that SP-mediated vasodilatation is substantially reduced in knees pre-treated with capsaicin.

The effect of topically applied SP on joint blood flow was of interest as capsaicin treatment shifted the SP dose/response curve to the right. This is in accordance with previous work where it was shown

that increased vascular permeability induced by SP was vastly attenuated by pre-treatment of the joint with capsaicin (Lam and Ferrell, 1989b). Application of phenoxybenzamine in capsaicin pre-treated knees slightly but non significantly reduced blood flow, while phenoxybenzamine in the intact knee induced an increase of more than 30% in blood flow, possibly due to the effect of peptidergic control of blood flow in the absence of sympathetic tone. This effect on blood flow was reduced to near zero in the capsaicin pre-treated knee because of elimination of peptidergic neurones due to capsaicin. Also application of neurokinin antagonists (FK888 & SR48968) in the capsaicin-treated knee slightly reduced blood flow compared with the intact knee which demonstrated the lack of efficacy of sensory neuropeptides. This observation also verifies previous findings which indicated that in the capsaicin treated knee sympathetic post ganglionic fibres are resistant to the neurotoxic effect of capsaicin (Karimian *et al.* 1995). Capsaicin inhibits the action of exogenous SP by a mechanism unrelated to the integrity of the afferent nerve fibres innervating the knee joint and peripheral nerve section has no effect on this inhibition (Lam & Ferrell 1989b). It has also shown that the inhibitory effect of SP is not primarily due to depletion of mediator cell such as mast cell (Lam & Ferrell 1989b). Such findings suggest that capsaicin is capable of modifying post-junctional receptor-mediated effects of SP, in addition to its known neurotoxic effects. The blood pressure recordings indicate that this is a true effect and not simply a consequence of more rapid uptake of SP by capsaicin-treated joints leading to arterial hypotension at lower doses of SP.

5.6.3 PART TWO

ACUTE CAPSAICIN TREATMENT

(NEUROGENIC INFLAMMATORY MODEL)

In the literature, capsaicin is described as inducing a variety of responses via different mechanisms, and therefore the development of responses to capsaicin may be altered by chronic or acute application of this agent. Acute capsaicin treatment excites primary afferent neurones and cause depletion of neurokinins from sensory nerve endings and consequently neurogenic inflammation develops. In these experiments the effect of acute capsaicin treatment in the rat knee joint was studied to investigate its effect on blood flow and the vascular responses to SP.

5.6.3.1 MATERIALS AND METHODS:

Two groups of rats were deeply anaesthetised (see chapter 2). Group one was the control and group two the capsaicin-treated rats. 0.1 ml of capsaicin at different doses was injected into the anterior capsule of the rat knee joint some minutes before the recordings SP was used in these experiments to evaluate if there is any change of response to SP in acute capsaicin treatment. Systemic blood pressure was monitored by carotid artery cannulation to assess the effect of the drugs on blood pressure. LDI imaging, data analysis was as described previously (chapter 2)

5.6.3.2 RESULTS

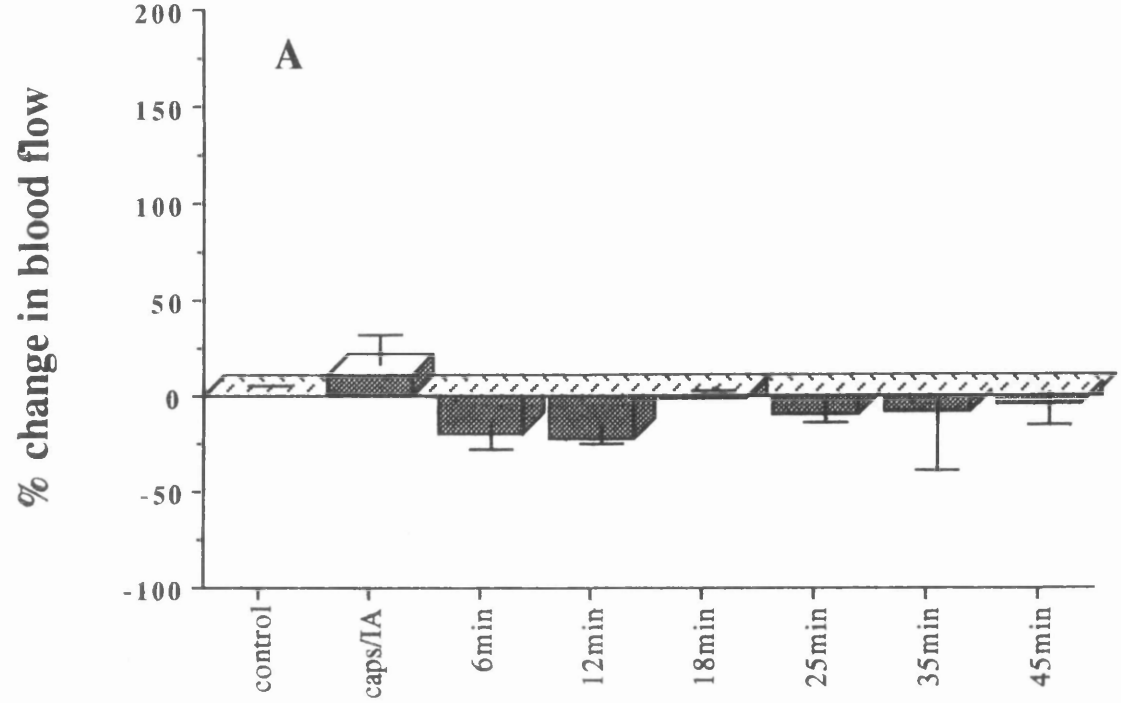
5.6.3.2.1 The effect of capsaicin on blood pressure: Injection of 0.1 ml saline into the anterior capsule produced a transient fall in blood pressure returning to normal in a few seconds. Injection of 0.1 ml capsaicin vehicle alone to the anterior capsule caused a 21±8% (n=5) fall in blood pressure which lasted a maximum of 30 sec and returned back to normal.

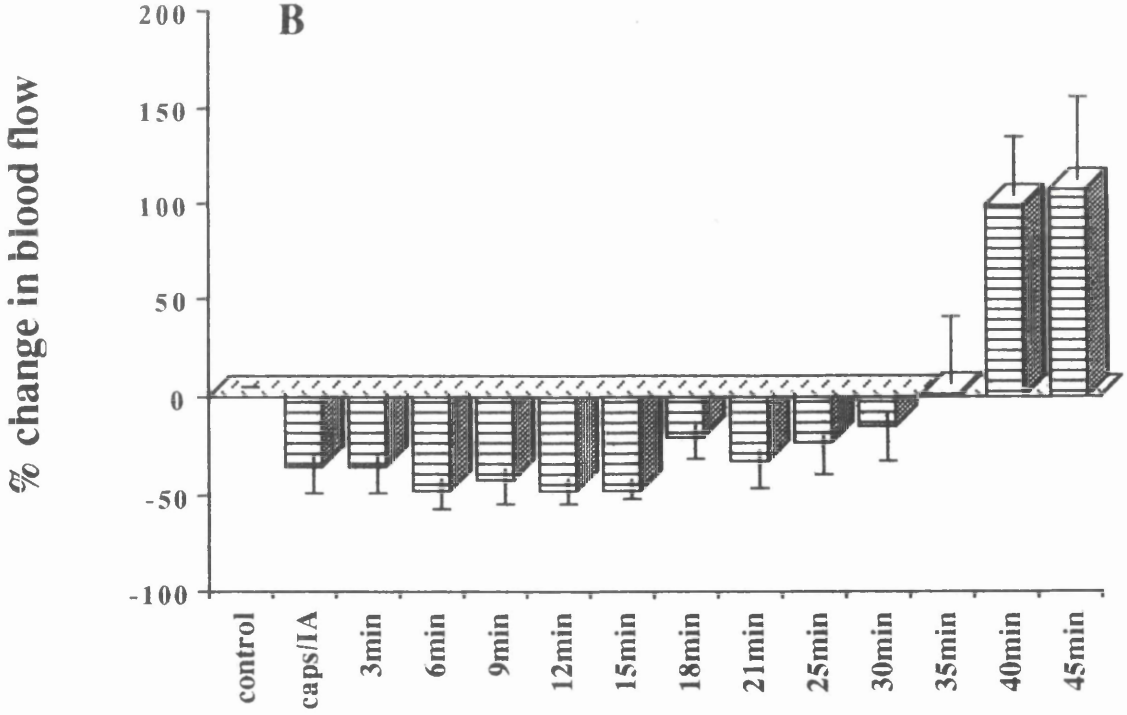
Infusion of 0.1 ml of 0.02% and 0.2% capsaicin into the rat knee joint did not have any significantly different effect on blood pressure compared to vehicle (n=5). Infusion of 2% capsaicin into the joint caused an 18±6% (n=5) increase in blood pressure which returned to normal within ten minutes .

5.6.3.2.2 The effect of capsaicin in the knee joint: Capsaicin induced a potent and dose dependent vasoconstriction which lasted a minimum of 40 minutes, whilst the vehicle did not show any significant effect ($p > 0.05$ n=5)(fig. 5.19A). Intra-articular injection of 0.02% capsaicin (0.1 ml) caused almost 40% (n=8) reduction in blood flow (fig. 5.19 B) which lasted 40 minutes and an increase in blood flow following this vasoconstriction. Intra-articular infusion of 0.2% capsaicin induced a 75% (n=8) reduction in knee blood flow which was stable for a period of 40 min followed by vasodilatation (fig. 5.20 A, n=8). Intra-articular application of 2% capsaicin induced a greater than 50% (n=5) reduction in blood flow which was stable, constant and irreversible (fig. 5.20 B).

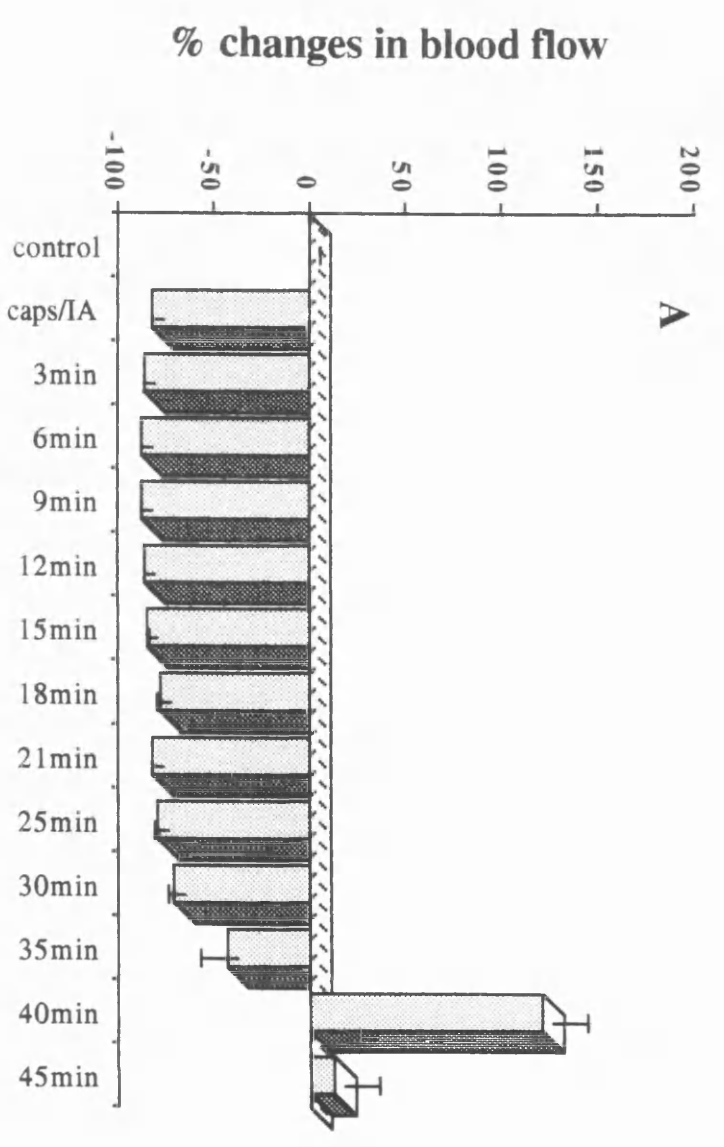
5.6.3.2.3 Application of SP: SP at different doses was applied in conjuction with capsaicin.. All different capsaicin doses attenuated

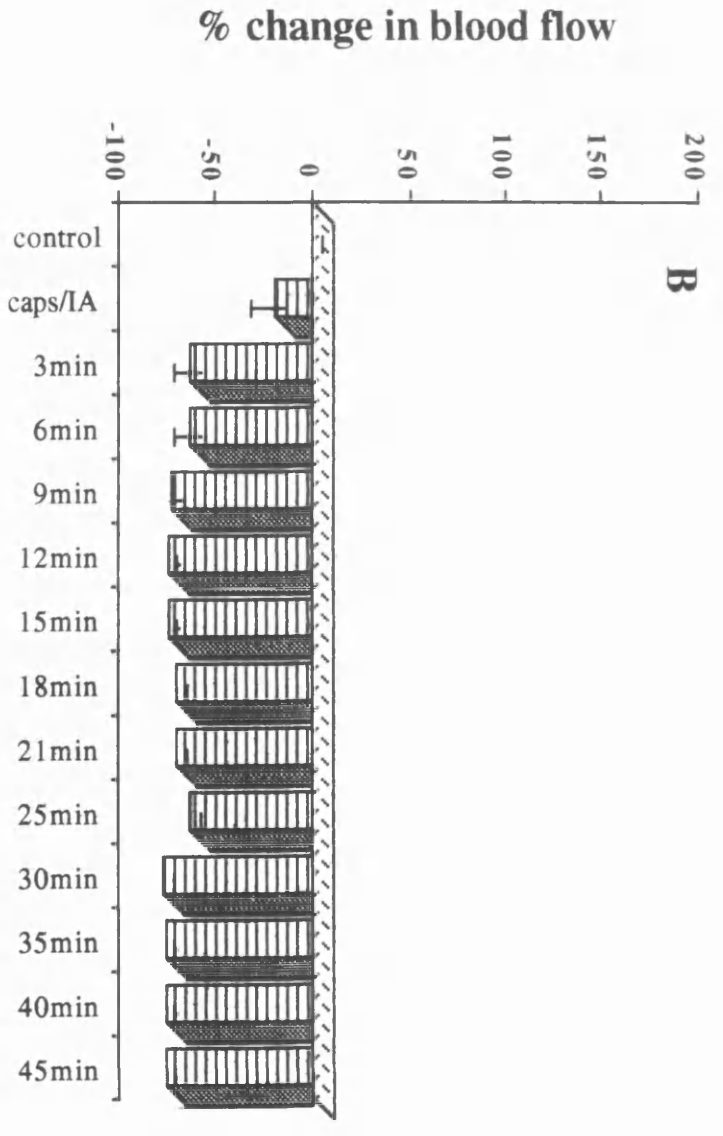
5.19 A and B: Time course and changes in synovial blood flow during intra-articular injection of capsaicin vehicle (fig A: n=5, $p>0.05$) and 0.02% acute capsaicin (fig B: n=8)


A
% change in blood flow
200
150
100
50
0
-50
-100
control
caps/IA
6min
12min
18min
25min
35min
45min


B
% change in blood flow
200
150
100
50
0
-50
-100
control
caps/IA
3min
6min
9min
12min
15min
18min
21min
25min
30min
35min
40min
45min

5.20 A and B: Time course and changes in synovial blood flow during intra-articular injection of 0.2% acute capsaicin (fig A: n=8) and 2% acute capsaicin (fig B: n=8)


% changes in blood flow
-100
-50
0
50
100
150
200
control
caps/IA
3min
6min
9min
12min
15min
18min
21min
25min
30min
35min
40min
45min
A


% change in blood flow
-100
-50
0
50
100
150
200
control
caps/IA
3min
6min
9min
12min
15min
18min
21min
25min
30min
35min
40min
45min
B

the effect of SP on blood flow. In the 0.02% capsaicin pre-treated group, SP at lower doses induced a slight but non- significant increase in baseline blood flow and with the highest concentration produced a sharp reduction of blood flow, but the difference between SP doses was not significant. The response to SP was significantly different ($P=0.004$, $n=8$, two way anova) from the control group dose response curve and this curve was shifted to the right (fig. 5.21 a).

The effect of SP on 0.2% capsaicin pre-treated knees was attenuated compared with the control group and the dose response curve was significantly shifted to the right ($P<0.001$, $n=8$, two way anova). This attenuation was more potent than in the 0.02% pre treated group but the difference between the two treatment was not significant (fig. 5.21 B). In the 2% capsaicin pre-treated group, the effect of SP doses was completely attenuated and SP did not show any effect on blood flow. The attenuation of the SP effect on blood flow was significant compare to the control group ($P< 0.001$, $n=10$, anova). The effect of different doses of SP on blood flow was not significantly different between the capsaicin treated groups and SP did not show a significant effect on blood flow (fig. 5.21c).

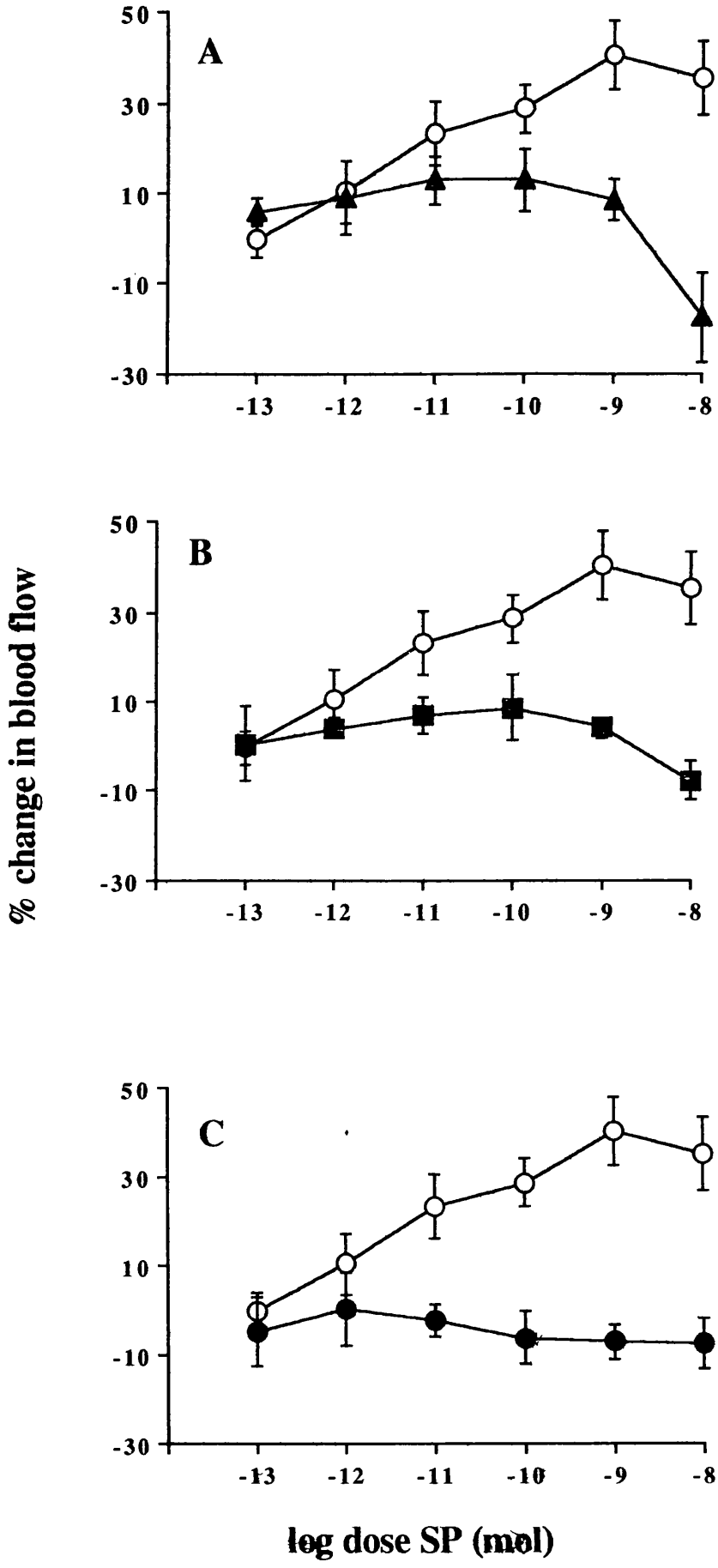
5.6.3.3 DISCUSSION

These experiments revealed the different effects of capsaicin. Intra-articular application of capsaicin induces a dose dependent vasoconstriction in which lasted a minimum of 40 minutes at all doses. Intra-articular injection of saline, 0.02% and 0.2% capsaicin, caused a transient effect on blood pressure which probably is due to the nociceptive effect of the needle and the capsule distention by the

Fig 5.21A. Changes in synovial blood flow during topical application of SP to the knee joint capsule, in normal knees (○ ,mean ±SEM, n=35 and at 0.02% acute capsaicin treated knees (▲ mean ±SEM, n=8). Difference is significant p=0.004 ANOVA

Fig 5.21b. Changes in synovial blood flow during topical application of SP to the knee joint capsule, in normal knees (○ ,mean ±SEM, n=35 and at 0.2% acute capsaicin treated knees (■ mean ±SEM, n=8). Difference is significant p<0.001 ANOVA

Fig 5.21c. Changes in synovial blood flow during topical application of SP to the knee joint capsule, in normal knees (○ ,mean ±SEM, n=35 and at 2% acute capsaicin treated knees (● mean ±SEM, n=10). Difference is significant p<0.001 ANOVA


A
B
C
50
30
10
-10
-30
-13
-12
-11
-10
-9
-8
% change in blood flow
log dose SP (mol)

infused fluid. Distension of the proprioceptive afferents, such as those in the joint capsule, are known to be able to reflexly influence blood pressure (W.R. Ferrell personal communication). Intra-articular injection of 2% capsaicin causes a transient increase in blood pressure which lasts for few minutes and returns backs to normal within 10 min. In general capsaicin did not have a significant long term effect on systemic blood pressure. Intra-articular injection of vehicle for the capsaicin did not show any significant effect on the vascular bed and over 40 min blood flow was constant and similar to control base line levels. Therefore vasoconstriction of the knee is the major and local effect of capsaicin on blood flow. This vasoconstrictor effect was followed by a marked vasodilatation which only occurred with the first two concentrations of capsaicin (0.02% and 0.2%).

The vasoconstrictor effect described above could be a hyperactivity of sympathetic tone due to influx of calcium ions through the calcium channels activated by capsaicin or could be due to depletion of neuropeptides which have an effective role in offsetting sympathetic sympatetic vasoconstrictor tone (see chapter 4.4). Vasoconstriction is irreversible in the 2% capsaicin-treated knee joint over the time course of experiment.

As articular cartilage is critically dependent on synovial fluid formation for its nutrition (McKibbin and Matoudas 1979). This long lasting vasoconstriction followed by a marked and dose dependent vasodilatation could be due to the effect of capsaicin on unmyelinated sensory neurones and the release of neurokinins (Holzer 1991) and calcitonin gene related peptide (CGRP) which is a potent vasodilator (Hughes *et al.* 1994)

In all cases following intra-articular application, capsaicin reacts with unmyelinated capsaicin-sensitive neurones and produces two separate reactions. In the first stage capsaicin desensitises and inactivates these neurones with an apparent vasoconstrictor effect possibly due to enhanced sympathetic tone. In the second stage release of neurokinins and CGRP from these neurones will have a vasodilator effect.

The capsaicin treated joint attenuated the SP vasodilator effect dose dependently. The lowest attenuation was in the 0.02% capsaicin pre-treated knee, which shifted the dose response curve to the right and significantly reduced the SP effect on blood flow. The highest attenuation due to SP was in the 2% capsaicin treated knee. Here the SP induced vasodilator effect was totally abolished and in fact there was no response to different doses of SP and the attenuating effect of 0.2% capsaicin was between these two.

These effects of SP in the acute capsaicin treated knee was similar to the chronic capsaicin treated group, and demonstrated a attenuated response to the exogenous neuropeptide, which could be due to the insensitivity of the neurokinin receptors. As SP is an inflammatory agent and capsaicin supresses its inflammatory effect (Lam & Ferrell 1989b), it could be used for treatment of arthritis and reduce its inflammation and pain by topical application of capsaicin cream (Deal *et al.* 1991) These data suggest that a long lasting acute vasoconstriction, changes in neuronal sensitivity and subsequent release of neurokinins are initiating factors for neurogenic inflammation. These finding support previous work (Jansco *et al.*

1967), but have been visualised, for the first time, using the LDI technique.

5.7. GENERAL DISCUSSION

Three different models of inflammation were studied in these experiments, Carrageenan, a galactose fraction, induces acute inflammation by activating immune cells and releasing different inflammatory mediators. Acute oedema develops in the injected area and there is also alteration of membrane function. Carrageenan induced inflammation and significantly enhanced the vasodilator effect of SP on blood flow. SP-containing neurones and SP receptors seem to have an important effect in development of inflammation as evidenced by the enhanced vasodilator response to SP (fig. 5.22).

Capsaicin which is selectively neurotoxic for sensory unmyelinated fibres, was used to induce inflammation. With chronic capsaicin treatment, which causes sensory nerve ablation, SP did not show any effect on blood flow, and capsaicin modified receptors which mediates the effect of SP. When capsaicin was acutely applied to the joint at different doses, a short period vasoconstriction appeared followed by a larger dose dependent vasodilatation at lower concentrations of capsaicin. This demonstrated the effect of capsaicin on sensory neurones and on the release of vasodilator mediators or loss of vasoconstrictor tone.

Adjuvant activates immunological, histological, lysosomal and lymphatic systems to induce chronic arthritis. This inflammation also

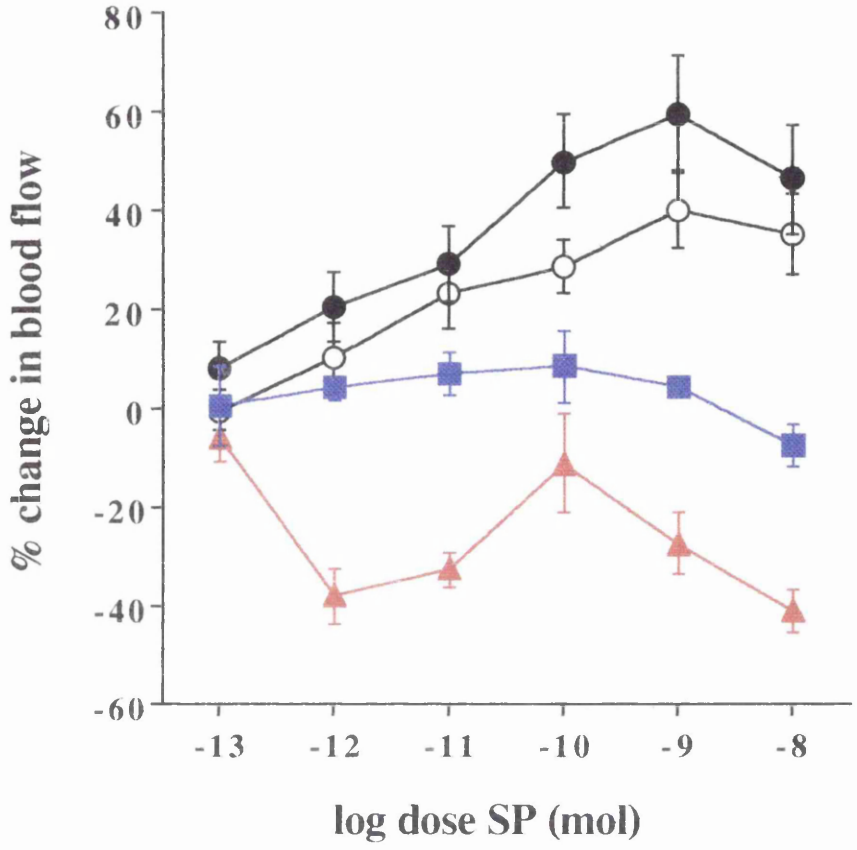


Fig 5.22: Changes in synovial blood flow during topical application of SP to the joint capsule in normal (○, mean ±SEM, n=10), carrageenan induced inflammation (●, mean ±SEM, n=20), 0.2% acute capsaicin (■, mean ±SEM, n=8), and in chronic adjuvant treatment week 3 (▲, mean ±SEM, n=9). The sequence of doses administered was randomised.

attenuated SP-induced vasodilatation and thus altered tissue response to SP. Adjuvant also demonstrated a contralateral inflammation accompanied by elevation of temperature. An increase in joint diameter and an alteration of the effect of SP on blood flow also occurred. Such marked differences in response to SP suggests fundamental differences in the mechanisms of inflammation in each model. It cannot be assumed that chronic inflammation is simply an extended acute inflammatory response. Although acute capsaicin administration induces acute inflammation in skin, in the joint marked vasoconstriction occurred, masking any inflammatory response. Thus, capsaicin cannot be used to investigation ipsilateral neurogenic inflammation, whereas in carrageenan-induced acute inflammation a marked vasodilatation occurs which demonstrates a different route to achieve acute inflammation. Chronic capsaicin treatment, causing nerve depletion, seems to mimic some of the adjuvant responses, certainly at week 1. This similarity may be due to the depletion of nerves in the synovium of adjuvant arthritic rats (Kontinen 1990), but these were only investigated at one week. It would be interesting to follow capsaicin-treated rats to 3 weeks post injection and examine the response of the synovial vasculature to SP (fig. 5.22).

The three agents produced a inflammation with altered neurokinin responses showing that they may play a role in the induction of these inflammatory responses. Neurokinins could provide clues to unlocking the mysteries of arthritis .

6 GENERAL CONCLUSION:

Joint nutrition is critically dependent on the synovial circulation and joint activity and blood flow regulation are closely related. The existence of calcitonin gene-related peptide (CGRP) and neurokinins has been found in sensory nerve endings and these have important actions in blood flow regulation.

In this thesis different aspects of the effect of these neuropeptides on blood flow has been studied. An interesting finding in these experiments was the protein extravasation effect of CGRP, which could be a contributory factor in joint inflammation.

The finding that NK1 and NK2 receptors mediate the effect of neurokinins on blood flow in the rat knee joint and that their actions are altered in the different models of arthritis considered here, suggests that these peptides may be involved in the inflammation. The enhanced SP-induced vasodilatation in carrageenan-induced inflammation contrasts with vasoconstrictor response to SP in adjuvant arthritis and suggests that chronic inflammation is not simply a continuation of an acute inflammatory response. and they have different pathways, though they can interact.

These experiments, for the first time revealed the contribution of neurokinins in the regulation of normal basal blood flow. It is possible that there is continual release of neurokinins from sensory nerve endings even in the absence of application of noxious stimuli.

Future experiments are indicated to continue work on different models of inflammation over longer periods to determine how long

neurokinin responses are altered and also establish the contribution of other agents, e.g. prostaglandins and nitric oxide and their interaction with neurokinins in the development of inflammation. Also of interest would be the extent to which neurokinins influence basic perfusion in acute and chronic inflammation. Recently discovered specific NK3 antagonists could give a clear clue in the investigation of the neurokinin effect on knee joint blood flow in the normal knee and inflamed knee.

In the normal knee joint over 60% of nociceptors are silent. However, activation of these receptors by different mechanisms (e.g.: chemical or physical factors) is followed by the release of neuropeptides (Otsuka & Yashioka 1993, Mc Carson 1994), which results in protein extravasation and vasodilatation in the area, and stimulates nociceptors to a greater discharge frequency such that a vicious circle then develops. Up to this level of inflammation the three models of inflammation have similar signs but beyond this, depending on the nature of the agent, will develop different effects and changes in the joint such as alteration in the response to SP. The involvement of these sensory nerve fibres in joint inflammation is suggested by observation that in both capsaicin and Adjuvant treated knees there is a reduced response to SP and in both cases there is known to be depletion of unmyelinated nerve fibres (Ferrell *et al* 1992, Konttinen *et al* 1990). However such similarities in signs, the future course of inflammation and the methods of therapy depend on the initiating factor and individual treatments are required, but in all of these inflammatory models neuropeptides play a major role .

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