THE CONTRIBUTION OF JOINT AFFERENT NERVES TO THE PATHOPHYSIOLOGY OF ARTHRITIS.

A thesis submitted to the University of Glasgow in candidature for the degree of Doctor of Philosophy in the Faculty of Medicine.

by

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Abstract

The contribution made by joint afferent nerves to the pathogenesis of arthritis can be summarised in relation to particular areas of the afferent pathway.

1. At the peripheral nerve terminals the release of neurotransmitters contributes to the inflammatory process by increasing vascular permeability and reducing vascular resistance. This process has been termed neurogenic inflammation.

2. When the action potentials reach the central terminals in the spinal cord, the overall afferent input to muscle efferent nerves is altered. This changes normal reflex patterns which may lead to joint deformity.

3. Relay of the signals to higher centres produces the sensation of pain and an altered awareness of limb position, both of which may lead to altered joint use and joint deformity.

The experimental work undertaken in the production of this thesis is concerned with the first two points above.

It was found that the neuropeptides substance P (SP) and neurokinin A (NKA) both produce a protein extravasation into the knee joint of the rat. Intra-articular perfusion of the joint

with substance P results in protein extravasation into the synovial cavity. The response is dose dependent from 10nM to 1 0μM. The protein content of the aspirated fluid from the synovial cavity rises for 12-16 minutes before falling sharply over an equivalent period, despite continuous perfusion with SP. The same transient response is observed with NKA. A preceding joint inflammation enhances the effect of substance P, and alters the response from a transient one to a persistent extravasation during SP perfusion. The inhibition of the SP response by the NK-1 receptor antagonist FK888 suggests an involvement of NK-1 receptors in protein extravasation. The inability of FK888 to reduce NKA induced extravasation, together with the inhibition of the NKA response with the NK-1 and NK-2 antagonist FK224, suggests the involvement of NK-2 receptors.

In the cat knee joint, the frequency response of low threshold joint afferents in the posterior articular nerve (PAN) to mechanical indentation of the joint capsule was shown to be independent of receptor type. Mechanical stimulation of these receptors reflexly excites α-motoneurones with a latency suggesting a di- or tri-synaptic pathway. This excitation can be reduced by conditioning electrical, or chemical, excitation of high threshold joint afferents. γ-motoneurones also display a period of excitation, of similar latency to the α-motoneorones, in response to electrical stimulation of low threshold joint afferents. Again, a preceding electrical stimulation of high threshold joint afferents inhibits the period of excitation induced in the γ-motoneurone by the low threshold joint afferents.

The interaction of the high threshold afferents with the low threshold afferents, without a direct effect on the motoneurones, suggests the possibility of a presynaptic influence on the low threshold joint afferents. It was shown that a conditioning electrical stimulation of the high threshold afferents in the PAN of the cat knee joint increases the intraspinal electrical threshold for antidromic stimulation of group II afferents in the PAN. This suggests a hyperpolarisation of the group II PAN terminals by the high threshold afferents, and this effect may account for the inhibition of the group II induced excitation of the motoneurones.

The protein extravasation induced by neuropeptides and the alteration of reflex responses by high threshold afferents may contribute to the pathology of the arthritic joint.

Dedication

This thesis is dedicated to my parents

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PUBLICATIONS

Papers

Ferrell, W.R., Baxendale, R.H. & Scott, D.T. (1994) Articular nociceptors inhibit the excitation of soleus motor units by low-threshold knee joint mechanoreceptors in the decerebrate cat. *Brain Research* (in press)

Lam, F.Y., Ferrell, W.R. & Scott, D.T. (1993) Substance P-induced inflammation in the rat knee joint is mediated by neurokinin 1 (NK_1) receptors. *Regulatory Peptides*, **46**, 198-201.

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Scott, D.T., Ferrell, W.R. & Baxendale, R.H. (1994) Excitation of soleus/gastrocnemius γ-motoneurones by group II knee-joint afferents is suppressed by group IV joint afferents in the decerebrate, spinalised cat. *Experimental Physiology* (in press)

Scott, D.T., Lam, F.Y. & Ferrell, W.R. (1991) Time course of substance P-induced protein extravasation in the rat knee joint measured by micro-turbidimetry. *Neuroscience Letters*, **129**, 74-76.

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Lam, F.Y., Ferrell, W.R. & Scott, D.T. (1992) Substance P-induced inflammation in the rat knee joint is mediated by neurokinin 1 (NK_1) receptors. *Regulatory Peptides*, **Supplement 1**: S96.

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Scott, D.T., Lam, F.Y. & Ferrell, W.R. (1991) Effect of acute joint inflammation on substance P-induced protein extravasation in the rat knee. **Abstract 105**, *Procedings of the 10th Iranian Congress of Physiology and Pharmacology, Ahwaz.*

FIGURES

Tables

INTRODUCTION

1.0 The joint

Joints are formed where two or more bones or cartilages are united by other tissues. They can be further classified on the basis of the tissue uniting the bones, into 1) fibrous joints 2) cartilaginous joints 3) synovial joints.

1) Fibrous joints: In these joints the bones or cartilage are united by fibrous tissue and hence have very limited, if any mobility e.g. joints uniting bones of the skull. The majority of these joints are only temporary, as the fibrous tissue joining the segments becomes ossified with a resulting synostosis.

2) Cartilaginous joints: In these joints the bones are united by hyalinecartilage (e.g. epiphyseal plates uniting the epiphysis and diaphysis of a long bone) or by fibrocartilage (e.g. pelvic symphysis). The hyalinecartilage joints become ossified in adulthood and so have no movement, while the fibrocartilaginous joints may have very limited movement.

3) Synovial joints: These joints are characterised by the presence of a joint cavity which is lined by synovial membrane (Fig. 1). The term "synovium", also used to describe the synovial membrane, is perhaps more applicable as the lining is not strictly a membrane (Levick, 1989a). The synovium is composed of loose or dense connective tissue lined by a loose array of macrophages (A-cells) and fibroblast derived cells (B-cells). There is no basal lamina separating the lining cells from the underlying connective tissue and the cells are separated from

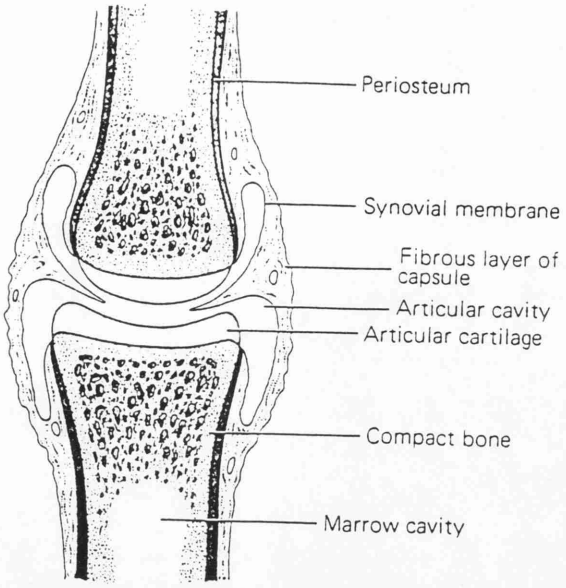


Figure 1: Schematic drawing of a synovial joint (Junqueira, Carneiro & Contopoulos, 1977)

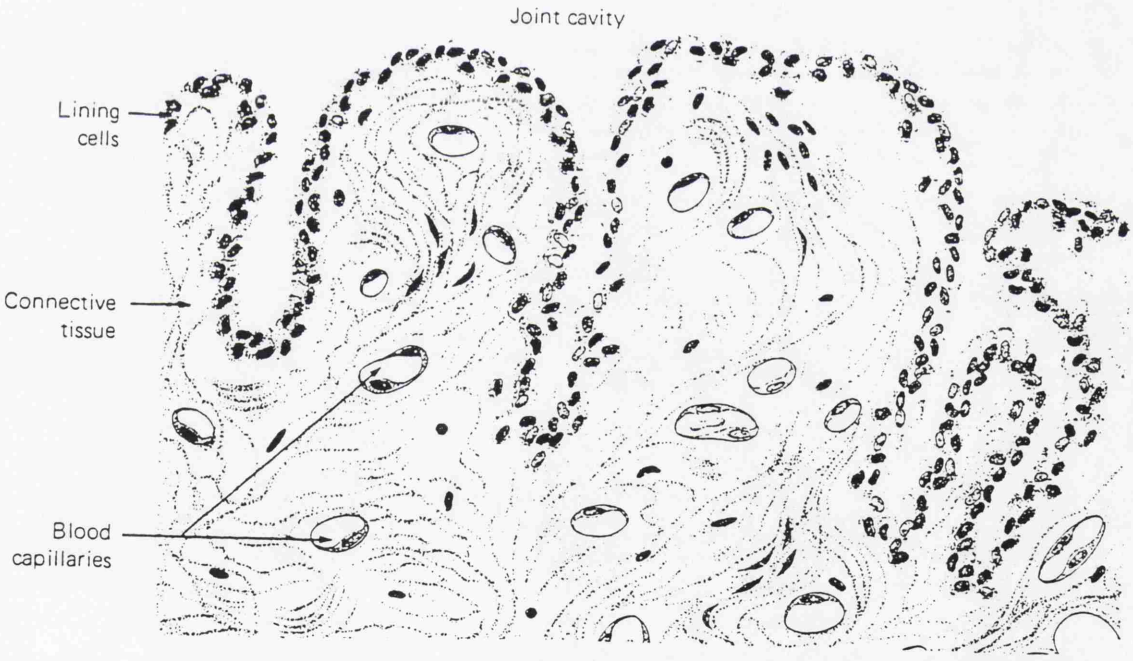


Figure 2: Histologic structure of the synovial membrane (Junqueira, Carneiro & Contopoulos, 1977)

each other by a small amount of connective tissue ground substance. The B cells are responsible for the secretion of interstitial matrix and lubricating molecules, while the A-cells clear debris from the joint cavity (Henderson & Edwards, 1987). The synovial layer has many folds which may penetrate deep into the joint cavity. It is also rich in capillaries and has variable amounts of adipose tissue (Fig. 2). The capillaries are concentrated in a well defined band approximately 10μM below the surface. The capillary density is high (500-700 mm^{-2}) and about half the capillary profiles bear fenestrations (Knight & Lēvick, 1983; 1984). External to the synovial membrane is a fibrous layer composed of dense connective tissue. This layer becomes more developed in some areas to form extracapsular ligaments, although these ligaments are also sometimes distinct from the capsule. The fibrous layer of the capsule varies considerably in thickness in different locations, occasionally disappearing and leaving only the synovial layer e.g. where a tendon passes over the capsule it may take the place of the fibrous layer. The fibrous layer attaches close to the margin of the articular surface, while the synovial layer and articular cartilage completely enclose the articular or joint cavity. Intracapsular ligaments, although inside the fibrous layer, are not in the joint cavity as the synovial membrane is reflected over them. The articular cartilage covers the articular surface of the bones. It is usually hyaline in type and is non-vascular, receiving nutrition from the synovial fluid. The fluid resembles a

Histological appearance	*Discharge pattern*	*Afferent fibre (dia)*	*Conduction velocity (m/s)*	*Afferent fibre classification*	*Location*	*Function*
Tendon organ	Slowly adapting	Myelinated (10–18 μm)	60–100	I (Aα)	Ligaments	Proprioceptor
Ruffini ending	Slowly adapting	Myelinated (5–12 μm)	20–70	II (Aβ)	Fibrous layer	Proprioceptor
Paciniform corpuscle	Rapidly adapting	Myelinated (5–12 μm)	20–70	II (Aβ)	Fibrous layer	Proprioceptor
Free nerve ending	Non-adapting	Myelinated (1–5 μm)	2.5–20	III (Aδ)	Fibrous layer & synovium	Nociceptor
Free nerve ending	Non-adapting	Unmyelinated (<1 μm)	<2.5	IV (C)	Fibrous layer & synovium	Nociceptor

Table 1: Classification of mammalian articular sensory innervation (Ferrell, 1992)

plasma ultrafiltrate in chemical composition (Levick, 1984; Knox, Levick & McDonald, 1988) except for its content of hyaluronate, the glycosaminoglycan responsible for the fluid's viscosity, which is actively synthesised by the synovial lining cells.

Synovial joints facilitate movement by allowing one bone to move in relation to another, while maintaining the joint surfaces in apposition. To achieve this the fibrous capsule and associated ligaments need to be strong enough to maintain apposition, and situated in such a manner as to allow movement. The ligaments are pliable but usually inelastic. The joint has also to provide as little resistance to movement as possible and protect itself against wear. The hyaline cartilage covering articular surfaces provides a durable surface which is continually being replaced. It also has a very smooth surface which, together with the lubrication provided by the synovia, reduces friction in the joint. Articular cartilage, together with articular disks and menisci (pieces of fibrocartilage inserted between articular surfaces) also reduce the effects of concussion.

The gross anatomy of the knee joint in man is similar to that of the cat and rat, the two animals most frequently studied, although there are minor anatomical differences (Hilderbrand *et al*, 1991).

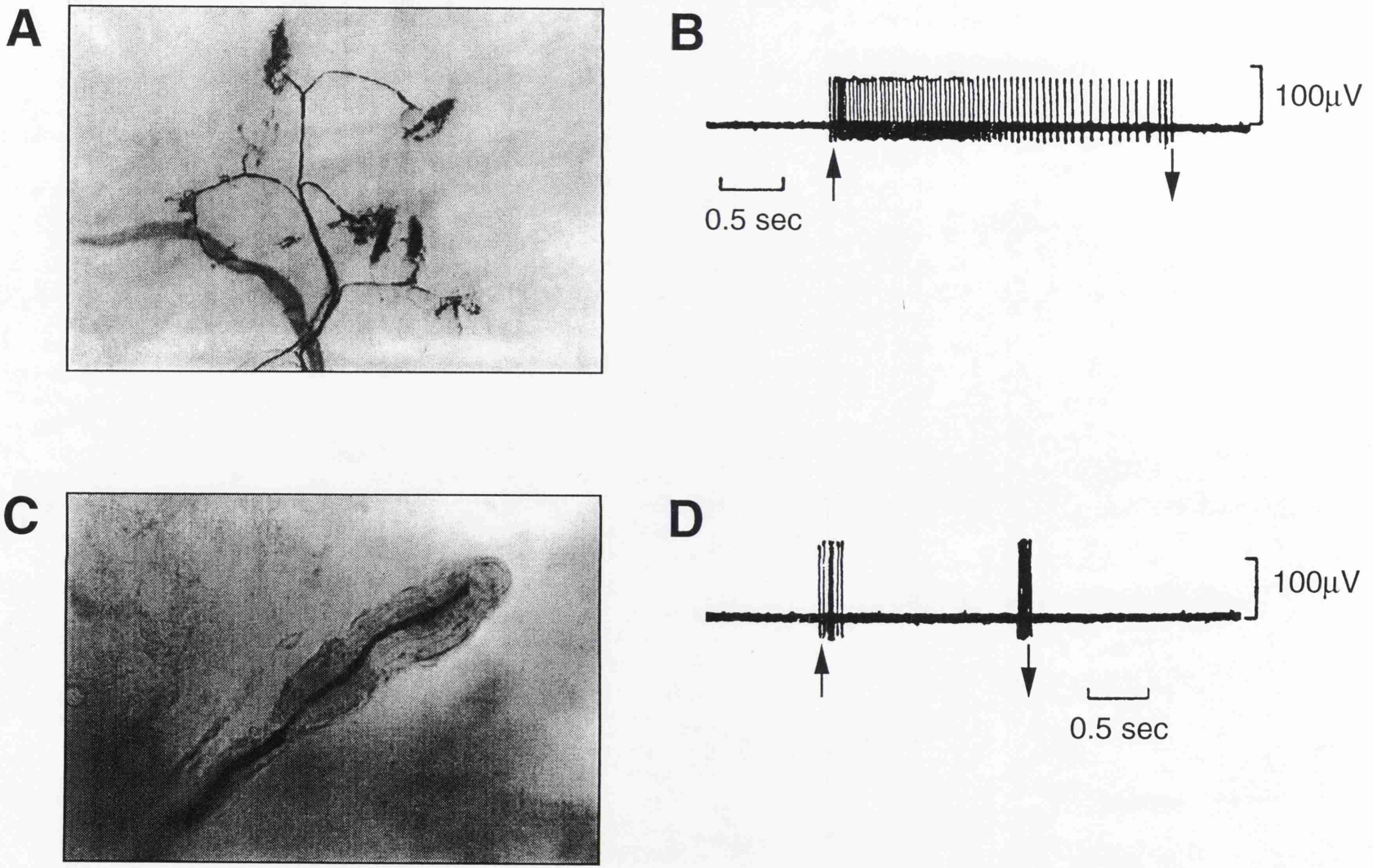
1.1 The joint receptors

Sensory nerve endings in the joint can be classified into four main morphologically distinct groups (Freeman and Wyke, 1967)(Table 1).

i) Ruffini type endings: These are encapsulated nerve endings, arranged in clusters of up to seven corpuscles emanating from one small diameter myelinated axon. Clusters are found almost exclusively in the fibrous layer of the capsule where they are the most numerous receptor to be found (Boyd, 1954; Freeman & Wyke, 1967). They can be found on both the flexion and extension sides of the joint (Strasmann, Halata & Loo, 1987). The corpuscle has a thin capsule, similar to the perineural sheath, which surrounds the nerve endings that form the core of the capsule. This terminal brushwork is intimately associated with collagen fibrils, that pass through the corpuscle to emerge at its poles and fuse with the joint capsule (Halata, 1977). The structure of the corpuscle makes it exquisitely sensitive to joint capsule stretching but relatively insensitive to compressive stress (Grigg & Hoffmann, 1982). These receptors produce a slowly adapting discharge in response to maintained stretch of the capsule (Boyd & Roberts, 1953; Boyd, 1954)(Fig. 3).

ii) Pacinian-type corpuscles: These receptor units consist of one to three elongated cylindrical bodies supplied by branches

Figure 3: Histological appearance and physiological response of the two types of organised nerve endings found in the cat knee joint capsule. The Ruffini ending (A) gives rise to a slowly adapting discharge in response to pressure applied to the joint capsule (B). Pressure is applied (↑) and held in a fixed position until removed (↓). The paciniform corpuscle (C) gives rise to a rapidly adapting response. Nerve endings are stained with the Gairns gold chloride method (Ferrell, 1992).


A
B
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C
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from a single axon (Boyd, 1954). They are found in the stratum fibrosum of the joint capsule (Boyd, 1954), periarticular loose connective tissue and muscle-tendon transition zones (Strasmann *et al*, 1987), and fibrous periosteum (Gardner, 1942). They are less frequently encountered than Ruffini-type endings and are in greatest preponderance towards the more lateral aspect of the joint (Boyd, 1954). The receptor consists of the terminal portion of the axon surrounded by longitudinally arranged concentric lamellae interposed by a fine granular layer (Halata, 1977). The structure of the corpuscle provides a rapidly adapting response to low threshold stimulation (Burgess & Clark, 1969a), and therefore requires a vibratory stimulus for continued activation (Fig. 3).

iii) Golgi-tendon organs: These are similar in structure and response to Ruffini-type endings but are only found in ligaments and tendons (Skoglund, 1956; Polacek, 1966). They are larger than other joint receptors and give rise to large myelinated axons.

iv) Free nerve endings: Unmyelinated and finely myelinated axons with unmyelinated terminals, which are not associated with specialised encapsulated structures at their peripheral ends, are termed free nerve endings. Afferent fibres with these endings provide the overwhelming majority of joint afferent innervation (Table 2)(Langford, 1983; Langford & Schmidt,

Nerve		Myelinated	Unmyelinated	Total
Tibial		7189	23,045	30,234
Med. Gastrocnemius				
Symp.	normal	801	2391	3192
	operated	860	1397	2257
VR+DRG	normal	893	1924	2817
	operated	0	804	804
Sural				
Symp.	normal	1076	3947	5033
	operated	1199	2397	3596
VR+DRG	normal	826	3214	4040
	operated	0	1052	1052
Med. Articular				
Symp.	normal	190	938	1129
	operated	192	438	628
Post. Articular				
Symp.	normal	270	929	1194
	operated	261	410	662

Table 2: The numbers of myelinated and unmyelinated axons in reprasentative cat hindlimb nerves. The normal side is used as a control for the contralateral operated side. Symp. = sympathectomy; VR+DRG = ventral rhizotomy and dorsal root ganglionectomy (Langford, 1983).

1983). Endings are found in the capsule and ligaments in both dense regular and irregular connective tissue. They occasionally accompany Ruffini-type corpuscles, while the majority occur near blood vessels (Langford, Schaible & Schmidt, 1984) and in the synovial membrane (Halata & Groth, 1976). After the loss of their myelin sheath, terminals remain enclosed within Schwann cell membranes until their final portion (Kruger, Perl & Sedivic, 1981). This sensory axon consists of a series of spindle-shaped "beads" connected by thinner segments. The beads and end bulb at the axon tip display the same ultrastructural features, all characteristic of receptive sites. The entire terminal tree therefore performs a sensory function through the multiple receptive sites formed by the beads (Heppelmann *et al*, 1990). The terminals contain numerous vesicles and mitochondria and are similar in this respect to synaptic boutons. These receptors respond to either innocuous or noxious mechanical stimulation, or both, in a non-adapting manner (Schaible & Schmidt, 1983a; b). Nerve terminals respon ding to application of algesic substances, as well as to mechanical stimulation, are classed as polymodal receptors.

The density of innervation at various sites remains uncertain, although ligaments may be well supplied (Marinozzi *et al*, 1991). Recent developments in immunohistochemical techniques using antisera against specific neuronal markers, combined with sensitive staining methods (Kidd *et al*, 1990a), have shown vastly increased small diameter nerve fibres in the joint

compared with previous studies using standard histological methods. Small diameter nerve fibres immunoreactive for protein gene product 9.5 were found in all sections of normal joint tissue and were scattered throughout the fibrous capsule, ligaments, tendons, and synovium. Nearly all these small diameter nerves in healthy synovium are immunoreactive for neuropeptides (Mapp *et al*, 1990; Grondblad *et al*, 1988). These included nerves containing immunoreactive substance P (SP) and calcitonin gene related peptide (CGRP), which are considered markers of sensory fibres, as well as nerves containing immunoreactive neuropeptide Y and its C flanking peptide, found in most peripheral noradrenergic neurones. Many of the SP and CGRP immunoreactive nerves were found in perivascular areas and numerous free fibres were also present, with some extending through the intima almost as far as the synovial surface.

Although most of the above information about receptors comes from the cat, human joints contain similar receptors (Schultz *et al*, 1984; Zimny, Schutte & Dabezies, 1986; Schutte *et al*, 1987; Zimny, 1988; Zimny, Albright & Dabezies, 1988; De Avila *et al*, 1989) of similar structure (Halata, Rettig & Schulze, 1985; Halata & Haus, 1989); as do those in the rat (Marinozzi *et al*, 1991).

1.2 Physiological characteristics of the receptors

The response of a receptor to a force is dependent on the structure of the receptor, as well as its relation to the tissue to which the force is applied and the nature of this tissue (Grigg, 1975). It has been shown that Ruffini-type endings are slowly adapting mechanoreceptors with low threshold to mechanical stress (Boyd, 1954; Eklund & Skoglund, 1960; Freeman & Wyke, 1967; Grigg & Hoffman, 1982), while Golgi tendon organs are slowly adapting but have a higher threshold (Boyd, 1954; Freeman & Wyke, 1967). Given the similar structure but different location of these receptors, the difference in threshold is probably due to the difference in nature of the surrounding tissue. Another receptor of similar structure to the above has been described (Grigg, Hoffman & Fogarty, 1982; Fuller, Grigg & Hoffman, 1991). The sensitivity of this Golgi-Mazzoni organ to compression, rather than stretch, of the capsule is again probably due to its relation to surrounding tissues. Pacinian corpuscles are rapidly adapting receptors with low threshold to mechanical stress (Freeman & Wyke, 1967; Burgess & Clark, 1969a). Free nerve endings, which have no specialised sensory endings, are non-adapting high threshold mechanoreceptors. These receptors have a large range of thresholds to mechanical stimulation (Schaible & Schmidt, 1983b).

It has been shown that, in the intact joint, the majority of slowly adapting receptors only respond to passive movement of

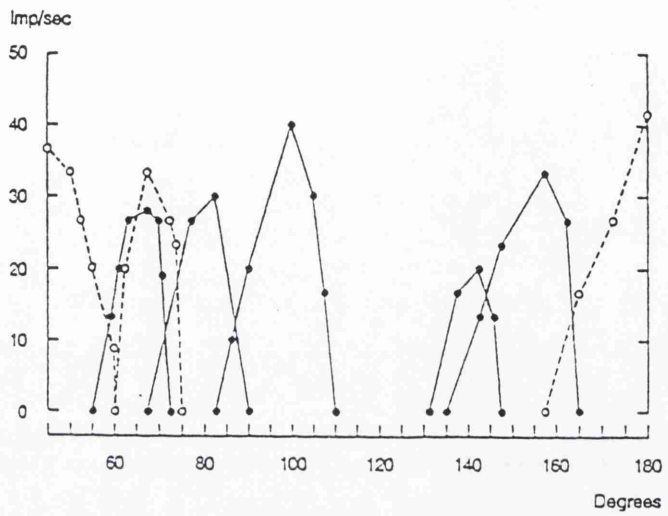


Figure 4: Graph of discharge rate of slowly adapting MAN afferents in relation to the angle of the knee joint (solid lines for one experiment and dashed lines for another). The figure is not representative of the total number of afferents recorded, the majority having maximal response at full extension or flexion (Skoglund, 1956).

the joint approaching full extension or flexion (Skoglund, 1956; Burgess & Clark, 1969a; Clark & Burgess, 1975; Millar, 1975)(Fig. 4). However, sufficient slowly adapting joint afferents responsive at intermediate joint positions exist, particularly in ligaments, to make signalling of joint position possible (Fig. 5)(Ferrell, 1980). In the awake cat, detectable joint receptors are not recruited in response to joint movement until after the earliest excitatory response of muscles acting across the knee (Marshall & Tatton, 1990). Presumably mechanical loading of the knee joint capsule is required for activation of the articular mechanoreceptors and is responsible for the tonic activity recorded in PAN and MAN in the midrange of joint movement in the awake cat. However, muscle contraction does not increase the proportion of joint receptors (18%) which are known to discharge spontaneously at intermediate angles in the absence of muscle tone (Ferrell, 1985).

Rapidly adapting receptors respond at various joint angles, but only at the initiation or termination of movement, and are therefore unsuited to monitoring joint angle (Boyd, 1954; Ferrell, 1980). Both rapidly and slowly adapting receptors respond to externally applied pressure and vibration (Grigg & Greenspan, 1977; Aloisi, Carli & Rossi, 1988), as well as to joint distension (Wood & Ferrell, 1984; Ferrell, 1987).

Mechanoreceptive units with fine afferent fibres respond to a variety of noxious and non-noxious movements of the joint. Measurement of the conduction velocity of these afferents

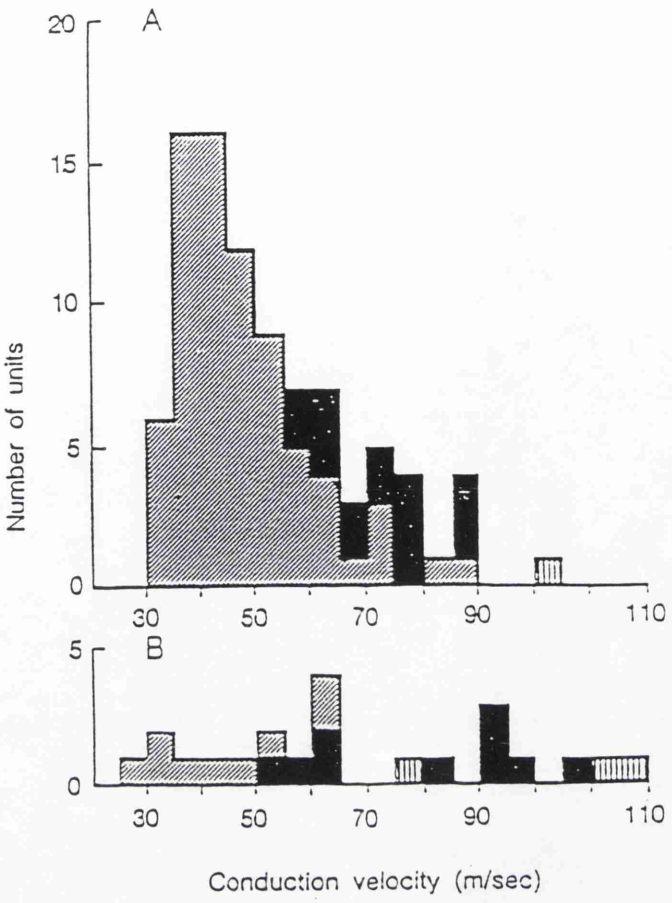


Figure 5: Conduction velocity spectra of slowly adapting knee joint receptors. (A) End-range units (B) Mid-range units. (▨) joint capsule units; (▪) ligament units; (▥) popliteal muscle units (Ferrell, 1980).

classes them indirectly as free nerve endings. Units responding to non-noxious movement can respond throughout the range of the joint with an irregular static discharge but no phasic response during the movement itself. The majority of fine afferents only respond to noxious movements of the normal joint and some do not respond to any movement (Schaible & Schmidt, 1983a). However, in the inflamed joint a higher proportion of units display resting discharges, the frequency of discharge is higher, the receptive fields are larger and many more units are activated with passive movement (Coggeshall *et al*, 1983; Gilbaud, Iggo & Tegner, 1985; Schaible & Schmidt, 1982; 1985). Significantly, as inflammation develops in a joint, afferents which could not previously be excited develop a sensitive spot in the joint capsule (Schaible & Schmidt, 1988a). The recruitment of a population of originally non-responsive afferent fibres will greatly enhance the total nociceptive inflow from the inflamed joint. These fine afferents are also excited and sensitised by close intra-arterial application of algesic substances or inflammatory mediators (Grubb *et al*, 1988; 1991; Schaible & Schmidt, 1988b; Neugebauer, Schaible & Schmidt, 1989).

1.3 The peripheral afferent pathway

The extrinsic innervation of the cat knee joint is composed of three distinct nerves; the posterior articular nerve (PAN), the medial articular nerve (MAN) and the lateral articular nerve (LAN)(Freeman & Wyke, 1967). The PAN is the largest nerve and innervates the posterior capsule as well as the posterior fat pads, the collateral ligaments, the posterior cruciate ligament and the posterior annular ligaments (Gardner, 1944; Skoglund, 1956; Freeman & Wyke, 1967). Occasionally, tendon organ afferents and muscle spindle primary and secondary afferents, all from the popliteus muscle, are found in the PAN (Lindstrom & Takata, 1972; Clark & Burgess, 1975; McIntyre, Proske & Tracey, 1978a; Gregory, McIntyre & Proske, 1989). The MAN innervates the medial and anterior capsule as well as the medial cruciate ligament, the infrapatellar fat pad and the medial meniscus. The LAN, when present, supplies the lateral capsule and the tibiofibular capsule (Gardner, 1944; Skoglund, 1956; Freeman & Wyke, 1967). The MAN branches from the saphenous nerve which is a branch of the femoral nerve, while PAN branches from the posterior tibial nerve, a branch of the sciatic and LAN branches from the common peroneal nerve (Figs. 6 & 7).

Joint afferents can be classified, on the basis of fibre diameter and conduction velocity, into groups I to IV (Fig. 8)(Boyd & Davey, 1968). On this basis tendon organs are associated with group I axons, paciniform and Ruffini endings with group II

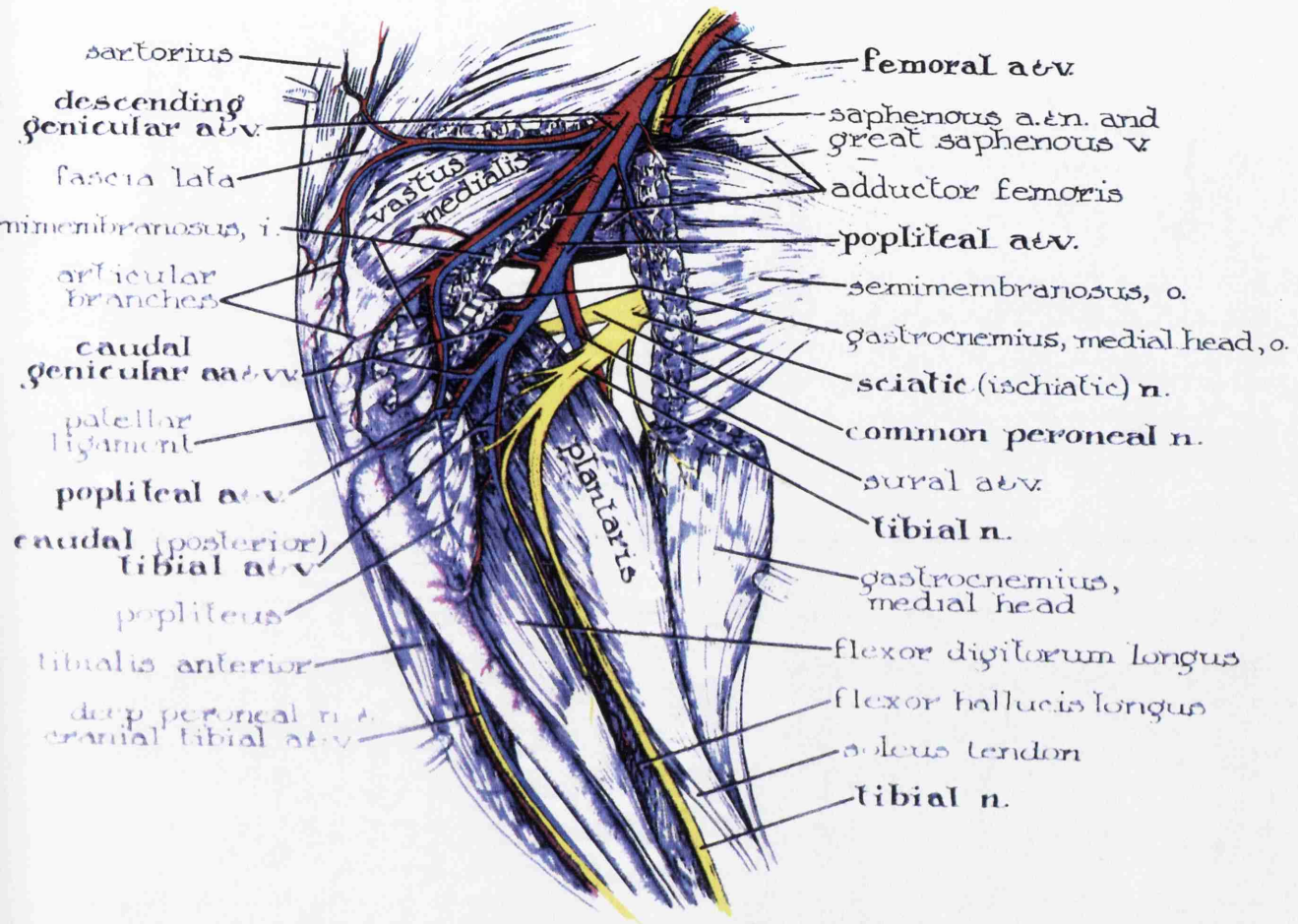


Figure 6: Medial aspect of the right stifle of the cat, illustrating nerves and blood vessels (Crouch, 1969).

axons, and free nerve endings with groups III and IV axons (Table 1)(Ferrell, 1992).

Early studies tended to over-emphasise the proportion of myelinated axons, as these are within the resolution of the light microscope. With the advent of the electron microscope it was discovered that both MAN and PAN are composed primarily of unmyelinated axons (Langford & Schmidt, 1983). Only twenty percent of the axons in MAN, and twenty two percent in PAN are myelinated. Of these axons, fifty percent of the unmyelinated and none of the myelinated are derived from the sympathetic chain. There are no ventral root axons in either nerve (Langford & Schmidt, 1983). The LAN is seldomly examined due to its inconsistent presence (Freeman & Wyke, 1967).

The innervation of the human knee is different from the cat's in nomenclature only (Gardner, 1948a; Kennedy, Alexander & Hayes, 1982).

The rat knee is supplied by a large PAN and small MAN, no LAN being discernible. As in the cat, approximately eighty percent of the axons in PAN are unmyelinated, although only about one third of these axons are afferent. All of the myelinated axons are afferent, and with a range of diameters from one to eight micrometers are smaller than in the cat. The MAN is more variable (Hilderbrand *et al*, 1991).

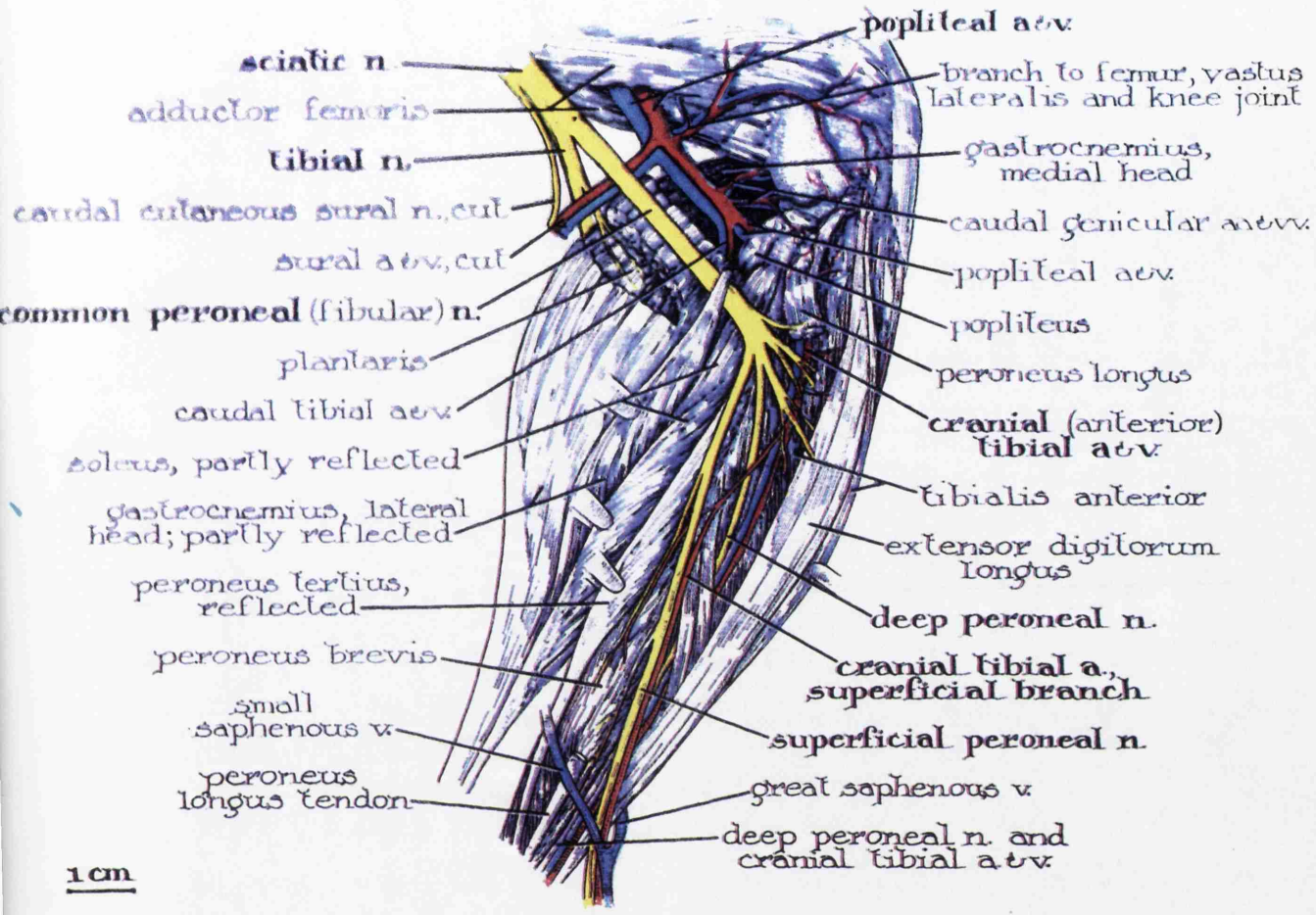


Figure 7: Lateral aspect of the right stifle of the cat, illustrating nerves and blood vessels (Crouch, 1969).

1.4 The central afferent pathway

Electrophysiological data has shown that PAN afferents enter the spinal cord via dorsal roots L6, L7 and occasionally S1, while MAN afferents enter via L5 and L6 (Gardner, 1948b; Skoglund, 1956; Clark & Burgess, 1975). Histological studies using horseradish peroxidase have confirmed these findings and also show that spinal cord terminations parallel the segments of entry. The terminations in the spinal grey matter are to lamina I and an area in the deep dorsal horn from lamina V to the dorsal part of lamina VII in the cat (Craig, Heppelmann & Schaible, 1987; 1988) and rat (Henderson & Saporta, 1987). Very few joint afferents ascend in the dorsal columns, the majority terminating, and activating interneurons in the lumbosacral and lower thoracic segments (Burgess & Clark, 1969b; Clark, 1972; Schaible, Schmidt & Willis, 1986). These interneurons have ascending axons (Schaible, Schmidt & Willis, 1987a) in the spinocervical (Harrison & Jankowska, 1984), spinoreticular (Fields, Clanton & Anderson, 1977), spinothalamic (Meyers & Snow, 1982) and spinocerebellar (Lindstrom & Takata, 1972) tracts. Primary afferent fibres extend as far rostrally as L1 and as far caudally as S2, mainly in Lissauer's tract and the dorsal column immediately adjacent to the dorsal horn (Craig, Heppelmann & Schaible, 1988). Higher order cells with joint inputs, such as in the dorsal column nuclei or cerebral cortex, also respond to

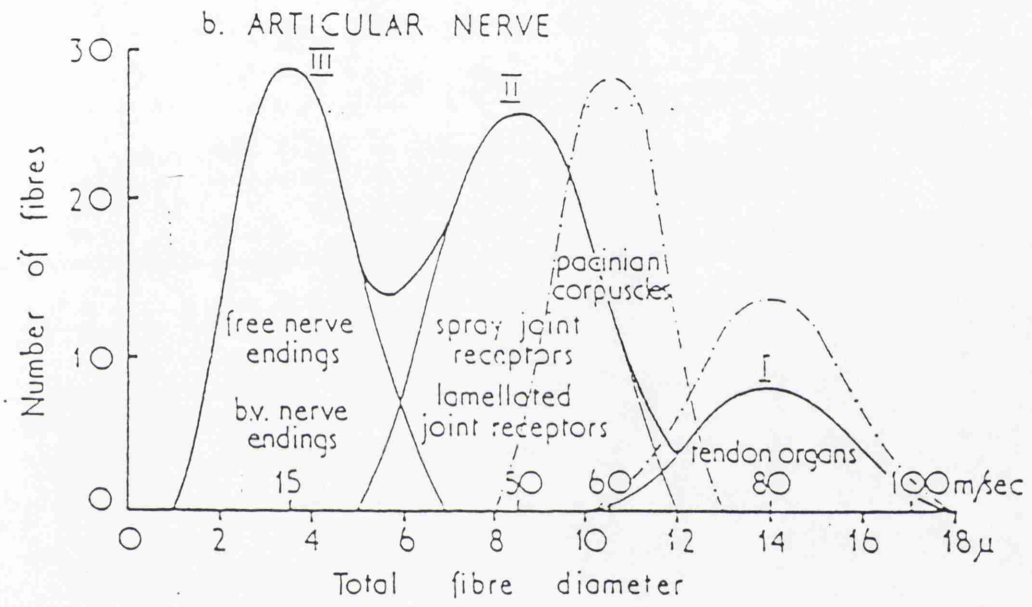


Figure 8: Fibre diameter, conduction velocity and distribution of articular receptor types (Boyd & Davey, 1968)

cutaneous and muscle inputs (Clark, Landgren & Silfvenius, 1973; Millar, 1979; Tracey, 1980).

1.5 Reflex effects from joint afferents

a) Effects on α-motoneurons

Joint afferents have been shown to influence α-motoneurones through various reflex pathways. When the excitability of flexion withdrawal and crossed extensor reflexes are examined in relation to joint position it is noted that withdrawal reflexes are more easily elicited when the knee is extended, while crossed extensor reflexes are more easily elicited with the knee flexed (Fig. 9)(Baxendale & Ferrell, 1981). That knee joint afferents are responsible for this action is demonstrated by abolishing the effect with knee joint anaesthesia, while tenotomising extensor and flexor muscles and maintaining their length does not affect the response. These effects are presumed to be mediated principally by group II afferents as non-noxious movements were used. Motoneurones represent only the final component in such reflex arcs and it is likely that joint afferents converge on neurones interposed in the arc rather than directly onto motoneurones (Lundberg, Malmgren & Schomburg, 1978). This could account for the relatively weak effects of joint afferents on motoneurone excitability compared to the stronger actions on transmission in reflex pathways. With both knee and ankle joints, externally applied pressure or passive joint movement may induce reflex effects on the muscles acting at those joints (Freeman & Wyke, 1967; Baxendale, Ferrell & Wood, 1987).

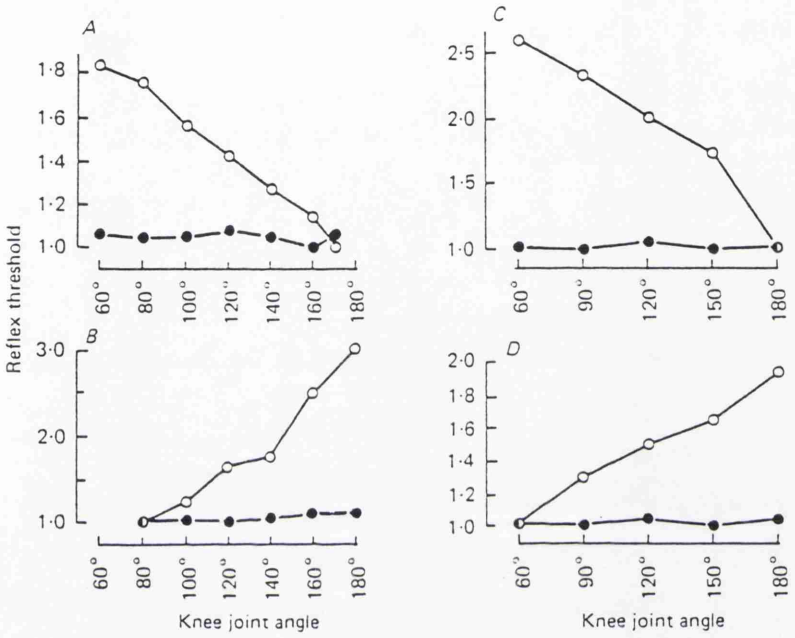


Figure 9: Modulation of the excitability of flexion reflex pathways to A biceps femoris, C tibialis anterior and crossed extensor reflex pathways to B vasti and D soleus by changing knee joint angle. Open circles = normal; Closed circles = knee joint anaesthesia. The excitability of the reflex pathway is expressed as the reflex threshold, which is the ratio of the stimulus intensity needed to elicit a reflex at each angle to the lowest intensity needed at any angle. Reflexes are elicited by electrical stimulation of the common peroneal nerve and measured as electromyograms of the particular muscle. Flexion withdrawal reflexes are most easily elicited when the knee is extended, while crossed extension reflexes are most easily elicited when the knee is flexed (Baxendale & Ferrell, 1981).

Although the effect from the joint afferents is weak, a clear excitation of extensor motoneurones is seen when other pathways are activated. Mechanical stimulation of low threshold knee joint afferents will provoke extensor muscle unit excitation, provided the muscle is under enough tension to provide adequate activity in muscle, and tendon organ, afferent pathways (Fig. 10)(Baxendale *et al*, 1987; Baxendale, Ferrell & Wood, 1988). Similarly, weak electrical stimulation of PAN (below twice the nerve threshold) will evoke post-synaptic potentials in α-motoneurones, but the effect is more potent if there is active input from Ib afferent fibres (Lundberg *et al*, 1978). Also, oligosynaptic projections of joint afferents to α-motoneurons of both flexor and extensor muscles acting at the joint have been shown to be mediated via Ia (Fedina & Hultborn, 1972) and Ib inhibitory neurons (Harrison & Jankowska, 1985). Rubral facilitation has also been used to disclose hitherto undetectable excitatory, as well as inhibitory potentials evoked via low-threshold joint afferents on both flexor and extensor α-motoneurons (Hongo, Jankowska & Lundberg, 1969).

Less is known about the reflex actions of group III and IV joint afferents on α-motoneurones. Intense electrical stimulation of PAN has been shown to produce excitation of α-motoneurones through the flexor reflex afferent system (Eccles & Lundberg, 1959; Holmquvist, 1961; Holmquvist & Lundberg 1961). Such electrical stimulation will excite all fibres in PAN, including group II fibres. However, it has been shown that experimental

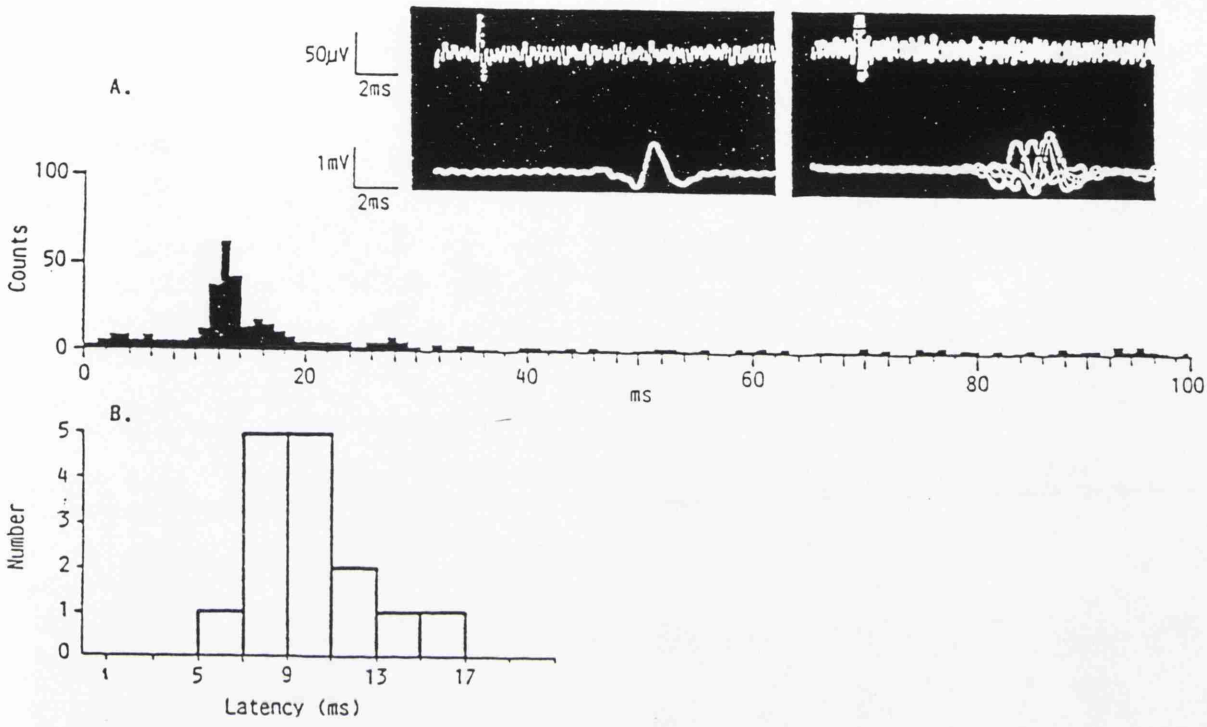


Figure 10: (A) Response of an individual motor unit (insets, lower trace; left=1 sweep, right = 5 sweeps) to repetitive mechanical stimulation of the posterior aspect of the joint capsule at an intensity sufficient to activate a single PAN receptor (insets, upper trace). The PSTH represents motor unit discharge over a one minute period of stimulation. (B) Reflex latencies from 15 motor units, as measured from the onset of the first PAN spike (Baxendale, Ferrell & Wood, 1988).

arthritis induced by intra-articular injection of carrageenan and kaolin results in selectively enhanced activity in group III and IV afferents (Coggeshall, *et al*, 1983). Using this method to selectively excite group III and IV axons, there is a significant increase in flexion reflex intensity as the inflammatory process develops, and the modulation of the flexion reflex intensity with altered joint angle is also abolished (Fig. 11)(Ferrell, Wood & Baxendale, 1988). The absence of modulation with joint angle could be due to either the excitatory input from the group III and IV fibres being much greater than from group II fibres, or to an inhibition of the group II pathways by the group III and IV pathways. Withdrawal reflexes in the rat are also enhanced with joint inflammation (Wall & Wolf, 1986) but, unlike the cat, there is no reversal with joint anaesthesia. Acute inflammation of the cat knee joint has also been shown to increase the resting discharge of flexor α-motoneurones, increase their response to local pressure applied to the knee, and increase their response to flexion and extension of the knee (He *et al*, 1988). Although these excitatory effects predominate, inhibitory effects are also present, including falls in resting discharge and in responses to movement.

Increased intra-articular pressure and joint distension excites predominantly joint mechanoreceptors (Wood & Ferrell, 1984), and evokes a reflex inhibition of α-motoneurons projecting to muscles around the joint, producing reduced quadriceps maximal voluntary contractions (deAndrade, Grant & Dixon, 1965;

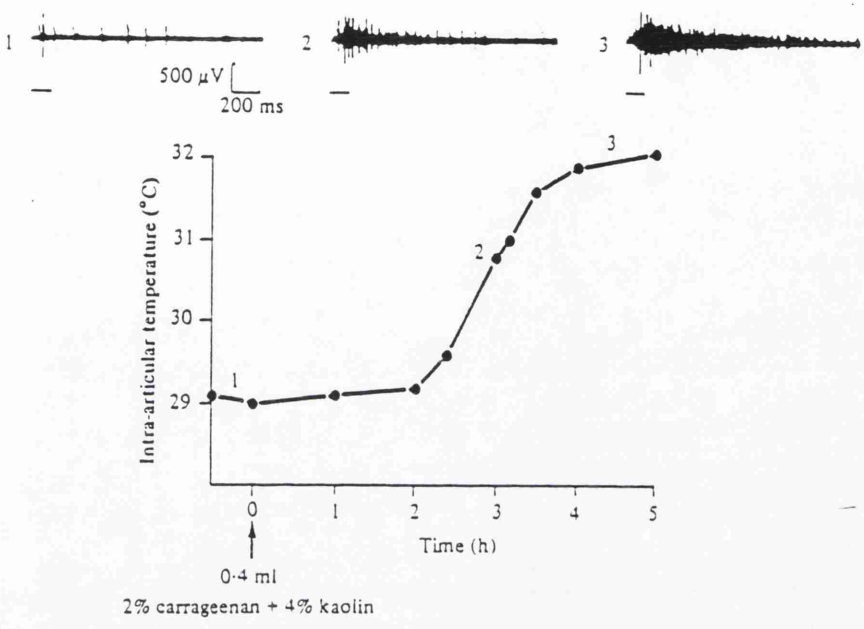


Figure 11: The effect of injection of carageenan and kaolin into the synovial cavity of the knee on intra-articular temperature and intensity of the flexion withdrawal reflex. The development of inflammation is estimated by monitoring the joint temperature. Electrical stimulation of the common peroneal nerve, sufficient to excite group III axons, elicits a flexion withdrawal response, measured as electromyographic recordings (EMG) in the simitendinosus muscle (1). Although the stimulus train is kept constant, reflex intensity increases as the inflammation develops (2,3). Reflex magnitude increases from the control EMG response by a factor of 4.85 (trace 2) and 8.22 (trace 3) (Ferrell, Baxendale & Wood, 1988).

Spencer, Hayes & Alexander, 1984; Wood, Ferrell & Baxendale, 1988).

b) Effects on γ-motoneurones

γ-motoneurones, the efferent nervous supply to muscle spindles (Fig. 12), receive synaptic inputs from widely ranging sources, including supraspinal nuclei, propriospinal neurones and primary afferents. Recordings made from single γ-motoneurones have shown reflex effects evoked by electrical stimulation of group I muscle afferents (Appelberg *et al*, 1983a), group II muscle afferents (Appelberg *et al*, 1983b), group III muscle afferents (Appelberg *et al*, 1983c) and cutaneous afferents (Johansson & Sojka, 1985); as well as from descending pathways (Johansson, 1988) and various parts of the brain (Granit & Kaada, 1952), including motor cortex (Gladden & McWilliam, 1977), cerebellum (Gilman & McDonald, 1967), thalamus (Yanagisawa, Narabayashi & Shimazu, 1963) and reticular formation (Shimazu, Hongo & Kubota, 1962).

High intensity electrical stimulation of the PAN evokes both excitation and inhibition of single γ-motoneurones (Voorhoeve & Van Kanten, 1962; Grillner, Hongo & Lund, 1969), and effects are observed in both extensor and flexor neurones (Johansson *et al*, 1986). Some cells are influenced only from high-threshold joint afferents, while some are only influenced from low-threshold joint afferents and some from both low- and high-threshold joint

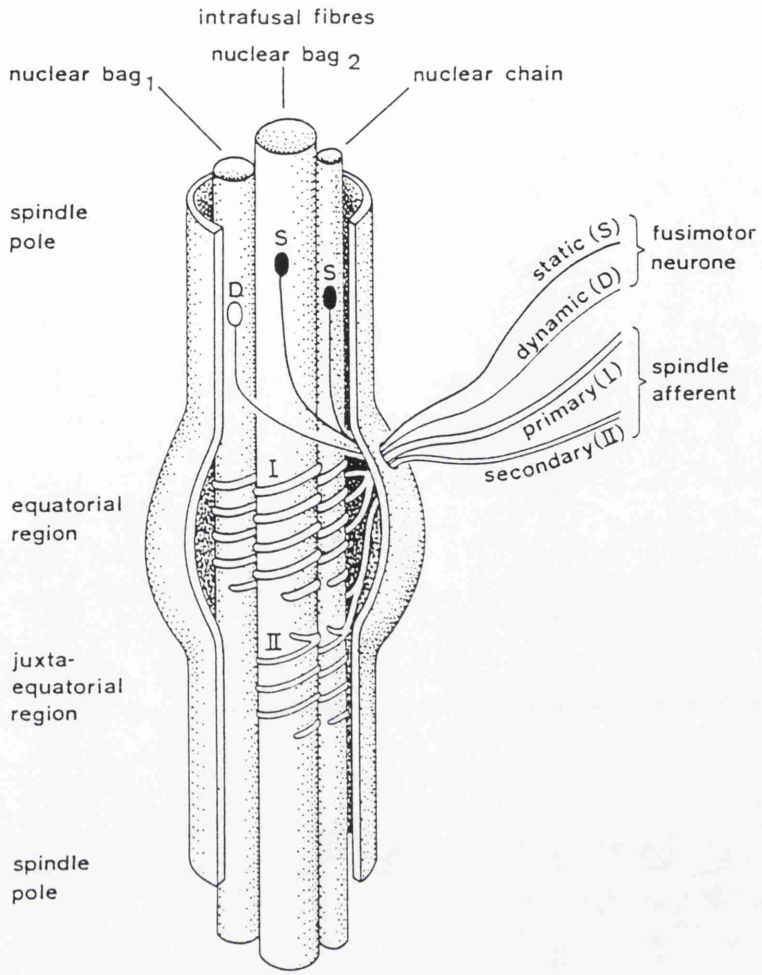


Figure 12: Simplified scheme of the mammalian spindle; each type of intrafusal muscle fibre being represented by a single fibre. The static and dynamic fusimotor neurones may be either exclusively fusimotor (γ-fibres) or mixed skeletofusimotor (β-fibres). Primary afferents occur on all three types of intrafusal fibre and are therefor excited by both static and dynamic fusimotor neurones. Secondary afferents are preferentially situated on bag_2 and chain fibres and are therefor activated by static fusimotor neurones only (Hulliger, 1984). (after Boyd et al, 1977)

afferents (Johansson *et al*, 1986). Among flexor γ-motoneurones excitatory effects are found to predominate, while for extensor γ-motoneurones excitation and inhibition are equally as common. Very frequent, and often potent, effects on nearly all (ninety-three percent) of the dynamic and static γ-motoneurones are found with electrical stimulation of the PAN (Johansson *et al*, 1986). Responses mediated by high threshold joint afferents are predominantly excitatory (McIntyre, Proske & Tracey, 1978b). Despite the PAN being a relatively small nerve, the responsiveness to stimulation is equal to, or greater than, the equivalent stimulation of muscle or cutaneous nerves.

Electrical stimulation of the PAN, limited to excitation of group II afferents only, evokes short latency excitation and/or inhibition of the background discharge of gastrocnemius/soleus γ-motoneurones (Fig. 13)(Baxendale *et al*, 1993).

Although electrical stimulation of afferent fibres implies more about the potential for control rather than its use, natural stimulation of muscle, skin and joint afferents also produce potent reflex effects on γ-motoneurones. Group I muscle afferents (Ellaway & Trott, 1978), group II muscle afferents (Appelberg *et al*, 1982), group III muscle afferents (Ellaway, Murphy & Tripathi, 1982) and cutaneous afferents (Johansson *et al*, 1989a; Davey & Ellaway, 1989) all produce reflex effects on γ-motoneurones. Natural excitation of both ipsilateral and contralateral joint afferents, by application of pressure to the capsule, can excite γ-motoneurones (Appelberg *et al*, 1979;

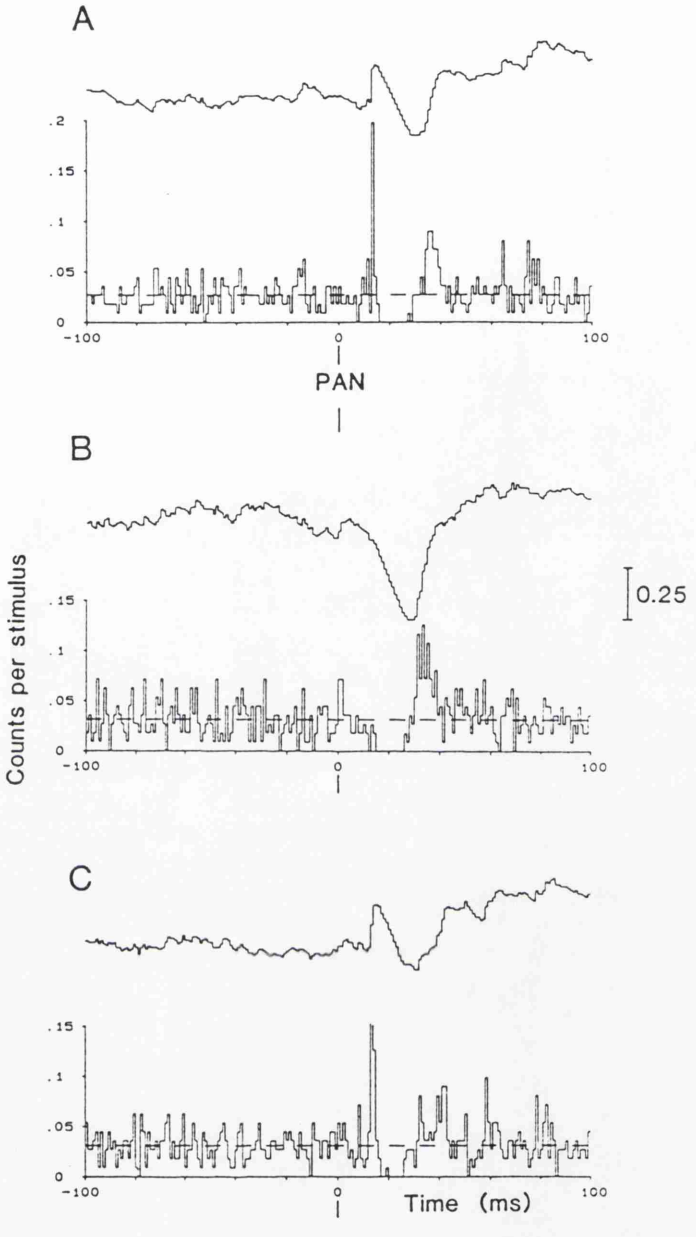


Figure 13: Patterns of response of three lateral gastrocnemius/soleus γ-motoneurones to electrical stimulation of the PAN. A, B, C: PSTH (below) and cusum (above) each constructed using 188 stimuli to PAN at 2.5T (A, B) and 3.6T (C). Horizontal dashed lines on each PSTH indicate mean expected counts in the absence of stimulation. Vertical calibration bar for the cusum gives the change from the expected probability of firing per stimulus (Baxendale *et al*, 1993).

Appelberg *et al*, 1981; Johansson, Sjolander & Sojka, 1988). Contralateral joint afferent excitation also contributes a considerable part of the response of the ipsilateral γ-muscle-spindle system to contralateral joint movement. Receptor afferents from the cruciate (Johansson *et al*, 1989b; Johansson, Sjolander & Sojka, 1990; Johansson *et al*, 1990) and collateral ligaments (Johansson *et al*, 1992; Sojka *et al*, 1992) of the knee have been shown to have potent reflex actions on the γ-muscle spindle systems of muscles acting at the knee. These are all low-threshold effects and many of them are sufficiently potent to be recorded at the level of the muscle spindle afferents. The spindle afferents are therefore not simple stretch receptors, as they are also influenced by γ-motoneurones, which themselves have polymodal properties (Fig. 14). The degree to which the various systems interact through interneurones supplying the γ-motoneurones is unknown, although reflex activation of muscle-spindle afferents has been shown to involve integration of cutaneous and proprioceptive afferents in man (Aniss *et al*, 1990), and joint and cutaneous afferents have been shown to interact in the regulation of γ-motoneurone discharge in the cat (Baxendale *et al*, 1990; 1993). Overall, γ-motoneurones are more responsive than α-motoneurones to low threshold stimulation, whether electrical (Johannson *et al*, 1986) or mechanical (He *et al*, 1988). This includes stimulation of the cruciate ligaments, mentioned above, which only required a tonically applied force of five to forty Newtons (sufficient to excite low threshold slowly

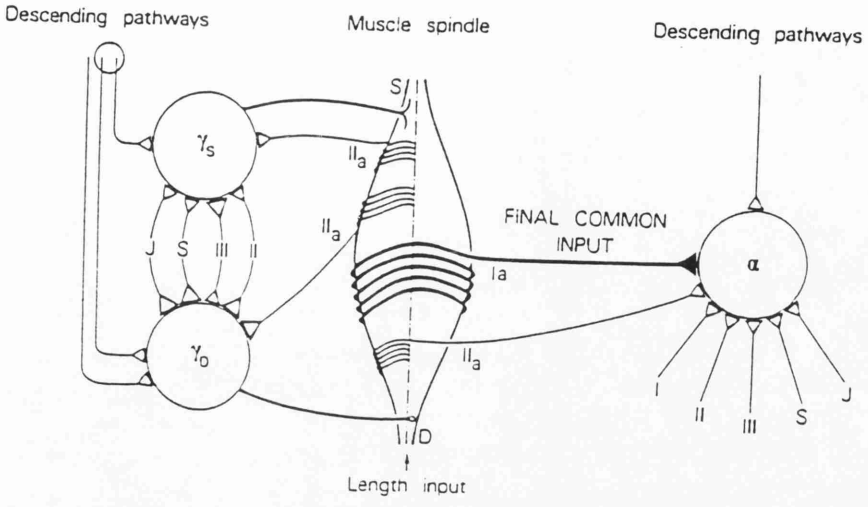


Figure 14: The scheme shows the multisensory convergence on static (γS) and dynamic (γD) γ-motoneurones from autogenetic (IIa) and heteronymous (II) muscle group II, from muscle group III (III) as well as from joint (J) and skin (S) afferents. The weak autogenetic actions from group I afferents onto γ-motoneurones are not shown.

At the level of the spindle, the polymodal signals from the γ-motoneurones interact with the mechanical input (muscle length). The primary (Ia) and secondary (IIa) spindle afferents then send a complex message to the central nervous system and α-motoneurone (Appelberg *et al*, 1983c).

adapting receptors) compared to around one hundred and thirty Newtons required to excite α-motoneurones (Solomonow *et al*, 1987).

Acute inflammation of the cat knee alters the reflex response of flexor γ-motoneurones to innocuous mechanical stimuli of the joint (He *et al*, 1988). The effects are similar, but more dramatic, than the effects on α-motoneurones. Some γ-motoneurones unresponsive to leg movement in control conditions, become responsive when inflammation of the knee has developed. Neurones showing control responses can be either inhibited or excited by the inflammation (Fig. 15). As the γ loop contributes to the power of maximal voluntary contractions in man (Hagbarth *et al*, 1986), it is possible that altered reflex activity of the γ-motoneurones in the inflamed joint leads to muscle weakness, joint stiffness and deformity.

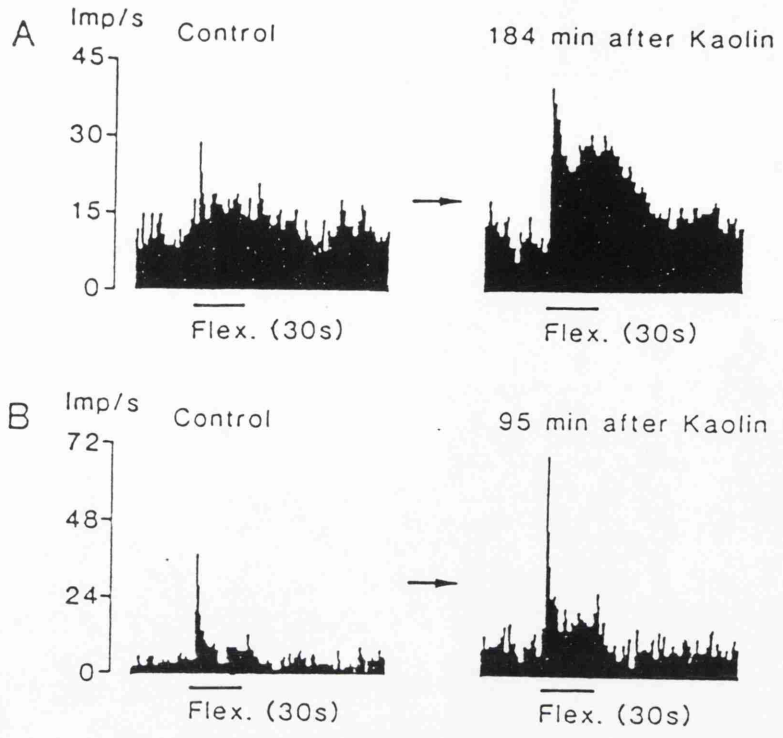
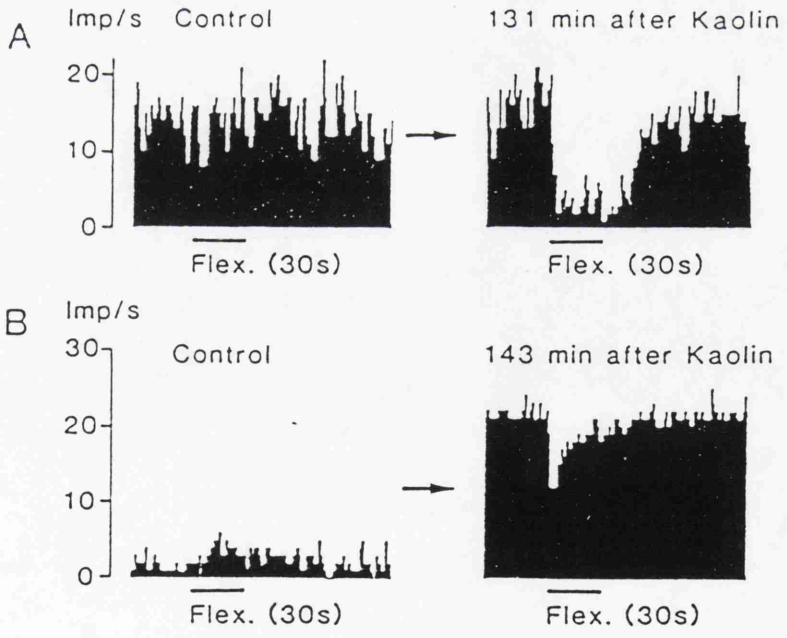


Figure 15: a) Peristimulus time histogram of the activity of two γ-motoneurones in nerve branches to posterior biceps and semitendinosus muscles, in response to limb flexion before and after the onset of acute inflammation. These neurones show an excitatory response to inflammation (He *et al*, 1988)



b) As in a) but these neurones show an inhibitory response to flexion after develpoment of inflammation.

1.6 Presynaptic control of joint afferents

The concept of presynaptic inhibition is important because it implies that afferent fibres are not passive conveyors of action potentials, but rather that conduction of impulses along the terminal arborisations, or transmitter release, is subject to control by other nerves. This allows selective control of the synaptic inputs to the target neurone. In contrast, postsynaptic inhibition would affect responses to all excitatory inputs acting on the soma and proximal dendrites. However, remote dendritic inhibition would also produce selective control (Green & Kellerth, 1966) and numerous attempts have been made to evaluate the relative contributions of these two inhibitory mechanisms. Depression of excitatory postsynaptic potentials (EPSPs) in the post synaptic neurone is indicative of inhibition, and work on motoneurones has shown that Ia EPSP depression occurs without other measurable postsynaptic changes. This, together with the maintenance of monosynaptic EPSPs evoked in the same motoneurone by different pathways, suggests presynaptic inhibition (Eide, Jurna & Lundberg, 1968; Rudomin, Nunez & Madrid, 1975). On the other hand, measurements of the passive properties of motoneurones indicated a consistent increase in the conductance of the distal dendrites, suggesting that postsynaptic inhibition is responsible for the depression of Ia EPSPs (Carlen, Werman & Yaari, 1980). However, it is not concluded that all of the inhibition is postsynaptic. The best evidence for the

elimination of a postsynaptic inhibition is the observation that EPSP depression occurs without changing the time course of the synaptic potential (Eccles, Schmidt & Willis, 1962b; Eide *et al*, 1968). If the EPSP on the distal dendrite is supressed by an increase in conductance of the dendrite, this should produce a faster falling phase of the EPSP (Eide *et al*, 1968; Rudomin *et al*, 1975).

Independent evidence for presynaptic inhibition is obtained by measuring the changes in membrane potential of the intraspinal arborisations of the afferent fibres themselves. Initially this was performed by recording the potential changes elicited in the central ends of the dorsal roots (Barron & Matthews, 1938; Eccles & Malcolm, 1946). These recordings showed a slow negative potential in the dorsal roots (DRP), which was attributed to a depolarisation of the central terminals of the afferent fibres (Barron & Matthews, 1938). Further evidence for this primary afferent depolarisation (PAD) is provided by showing that during the DRP the intraspinal terminals of the afferent fibres increase their excitability with a similar time course (Wall, 1958; Eccles, Magni & Willis, 1962a). In addition, recordings of the transmembrane potential changes of dorsal column afferent fibres show a depolarisation following stimulation of separate afferents (Eccles *et al*, 1962a; Eccles, Schmidt & Willis, 1963a).

Three hypothesis have been proposed to account for PAD (Davidoff & Hackman, 1984).

a) PAD is produced by electrical fields surrounding interneurones (Gasser & Graham, 1933).

There is evidence of electrical coupling between motoneurones and afferent fibres, as antidromic activation of motoneurones produces a depolarisation in afferent fibres (Grinnell, 1970). However, this is only of the order of several milliseconds, in contrast to the hundreds of milliseconds of PAD. Electrical interactions would not be expected to outlast the action potentials in the interneurones (Nelson, 1966).

b) PAD is produced by accumulation of extracellular potassium (Barron & Matthews, 1938).

The discovery of potassium-sensitive resins allowed the measurement of extracellular concentrations of potassium in the spinal cord. High frequency stimulation of nerves and dorsal roots increases potassium concentration and it is postulated that this is responsible for PAD (Krnjevik & Morris, 1972; 1974; 1975; Kriz, Sykova & Vyklicky, 1975). However, the changes are slower than simultaneously recorded changes in DRPs. Also the specificity of PAD argues against potassium accumulation as a mechanism. The intraspinal threshold of Ia fibres is reduced by conditioning stimulation of group I fibres from flexor muscles (Rudomin, Engberg & Jimenez, 1981). However, the same conditioning stimulation did not alter the threshold of vestibulospinal and rubrospinal fibres terminating close to the Ia fibres.

c) PAD is produced by axo-axonic contacts (Eccles, Schmidt & Willis, 1963b)

Axo-axonic contacts have been visualised in electromicrographs (Conradi, 1969; Rethelyi, 1970) and the presynaptic bouton of these synapses has been shown by immunocytochemistry (Barber *et al*, 1978, Maxwell *et al*, 1990) to contain glutamic acid decarboxylase, the enzyme that synthesises γ-aminobutyric acid (GABA), an amino acid strongly implicated as a neurotransmitter in presynaptic inhibitory pathways (Levy, 1977). Considerable pharmacological evidence exists for the role of the transmitter GABA in PAD. Muscle and cutaneous afferents are depolarised by iontophoretic application of GABA (Gmelin, 1976), semicarbazide-induced depletion of GABA reduces DRPs (Banna & Jabor, 1971), and inhibitors of GABA transaminase, a degradative enzyme for GABA, increases DRPs and excitability of afferent terminals (Davidoff, Grayson & Adair, 1973). GABA has been shown to produce depolarisation of neurones by increasing the conductance of chloride ions (Gallacher, Hagashi & Nishi, 1978). However, it may also block the inward calcium currents activated during the action potential, and it may be possible that this action rather than the depolarisation *per se* is responsible for the inhibition of transmitter release in PAD (Dunlap & Fischbach, 1981).

It has been proposed that suppression of transmitter release across the first sensory synapse is caused by a diminution in the presynaptic action potential which, in turn, is caused by

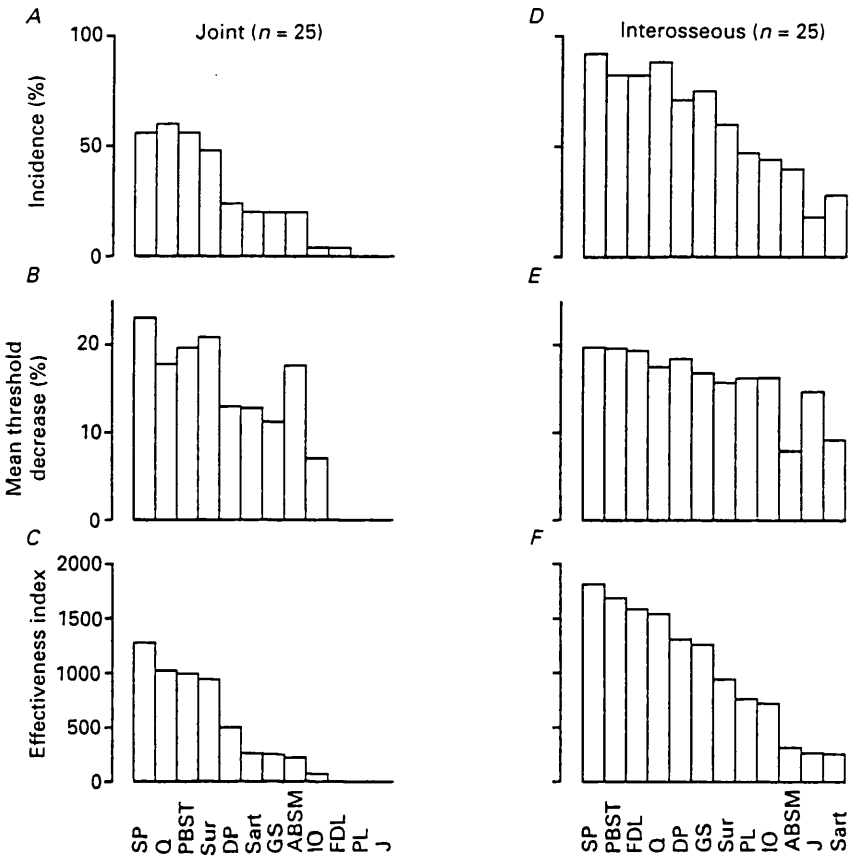


Figure 16: Comparison of the effectiveness of afferents in different nerves at inducing PAD in joint (A-C) and interosseal (D-E) fibres. The nerves from which PAD is evoked are indicated beneath the histograms. A and D, indicate the incidence of PAD evoked in the tested fibres with stimulation of each nerve indicated below the histograms. B and E indicate the mean percentage decrease in the amplitude of the threshold intraspinal stimuli required to elicite a response in 50% of trials. C and F represent the product of the incidence and percentage threshold decrease (Jankowska, Riddell & McCrea, 1993).

depolarisation (Eccles, 1964). The capacity of terminal depolarisation to decrease spike amplitude in the terminal and to reduce spike-induced transmitter release have been demonstrated (Hagiwara & Tasaki, 1958; Takeuchi & Takeuchi, 1962). The relationship between spike amplitude and postsynaptic response is steep (Katz & Miledi, 1967), suggesting that small changes in spike amplitude can markedly alter release.

With regard to sensory fibres of the joint, previous investigations have shown that these fibres can induce DRPs (Carpenter *et al*, 1963; Hongo, Jankowska & Lundberg, 1972; Quevedo *et al*, 1991) or depress presynaptic actions of group I afferents (Quevedo *et al*, 1991). Also PAD is induced, in fibres of the PAN, from group I (Ia and Ib) and group II muscle afferents and cutaneous afferents but not by conditioning stimulation of the PAN or interosseous nerve (Fig. 16)(Jankowska, Riddell & McCrae, 1993). Conditioning volleys used were not sufficient to excite group III or group IV afferents.

1.7) Neurogenic inflammation

1) The nervous system in inflammation

The traditional sensory role ascribed to the small diameter articular fibres has been shown to be only partially correct. Involvement of the nervous system in inflammatory responses has been recognised for almost a century and is based on the observation that antidromic electrical stimulation of dorsal roots or peripheral sensory nerves results in vasodilatation (Bayliss, 1901) and increased vascular permeability in the territory innervated (Jancso, Jancso-Gabor & Szolcsanyi, 1967). The release of substances from the peripheral terminals of nociceptive afferents was first implicated in the production of cutaneous wheal and flare responses (Lewis, 1927; 1936; 1941). Since then, evidence has accumulated in support of the notion that the "axon reflex" is mediated by unmyelinated afferent fibres. Neurogenic vasodilatation and plasma extravasation have been shown to occur during antidromic nerve stimulation at C fibre strength in both the skin (Jancso *et al*, 1967; Gasser, 1950; Kenins, 1981) and the joint (Ferrell & Russell, 1986; Ferrell & Cant, 1987); polymodal C fibres being responsible for the effect (Kenins, 1981).

Capsaicin (8-methyl-N-vanillyl-6-nonamide), the pungent ingredient in hot chilli peppers, exerts a unique excitatory action on a sub-population of afferent sensory neurones (Maggi & Melli,

1988). When applied to nerves it leads to release of neuropeptides and subsequent vasodilatation and plasma extravasation (Lundberg *et al*, 1983; Saria *et al*, 1983; Franco-Cereceda *et al*, 1987; Lynn & Shakhanbeh, 1988; Szolcsanyi, 1988; Manzini *et al*, 1989). Interaction of capsaicin with sensitive neurones results in an opening of cation selective ion channels (Wood *et al*, 1988b). This allows sodium and calcium ions to enter and potassium ions to leave the cell. The net effect is an inward current that depolarises and thus excites the neurones. However, not all sensory neurones are depolarised by capsaicin, the chemosensitivity being restricted to polymodal nociceptors, which are sensitive to a variety of noxious chemical, thermal and mechanical stimuli (Szolcsanyi, 1990; Holzer, 1991). The plasma extravasation induced by capsaicin is mediated indirectly through peptide released from primary afferent neurones acting at tachykinin NK-1 receptors (Eglezos *et al*, 1991). However, it is possible that capsaicin can also bind directly to NK-1 receptors as these receptors appear depleted when capsaicin is injected into the articular cavity (Lam & Ferrell, 1989b).

Systemic administration of capsaicin to adult or neonatal animals, or prolonged topical application to a peripheral nerve, is neurotoxic to the majority of unmyelinated sensory afferents (Jancso *et al*, 1967; Jancso & Kiraly, 1981). Intra-articular injection of capsaicin is also effective in selectively destroying unmyelinated afferents in the PAN of the rat knee joint (Ferrell, Lam & Montgomery, 1992). Systemic administration of capsaicin

can significantly attenuate adjuvant arthritis in rats (Colpaert, Donnerer & Lembeck, 1983), and persistence of capsaicin's anti-inflammatory effect for well over twenty days (Colpaert *et al*, 1983) is consistent with the depletion of substance P by capsaicin lasting weeks or months (Jessell, Iversen & Cuello, 1978); both effects outlasting capsaicin's bioavailability (Saria, Skoftisch & Lembeck, 1982).

Carageenan induced acute joint inflammation is also attenuated by prior treatment with capsaicin or surgical denervation (Fig. 17)(Lam & Ferrell, 1989a; 1991a), implying that both depend to some extent on the integrity of the sensory innervation of the joint, and thus have a neurogenically mediated component. Chemical sympathectomy had no effect on the induced inflammation, suggesting that the sympathetic system is not involved in carageenan induced inflammation.

There is also direct neurochemical evidence for BK-induced neuropeptide release from capsaicin-sensitive primary afferents (CSPA) in a number of different preparations (Hakanson *et al*, 1987; White & Zimmermann, 1988; Saria *et al*, 1988). Specific BK receptors are found on nociceptive primary afferents (Steranka *et al*, 1988; Manning & Snyder, 1989) and administration of BK not only generates sensory impulses but may also cause release of transmitters from peripheral sensory neurones. This is evidenced by the observation that BK-induced plasma protein extravasation in the skin and airways is reduced in animals pretreated with capsaicin (Arvier, Chahl & Ladd, 1977; Jancso,

Kirally & Jancso-Gabor, 1980; Lundberg & Saria, 1986). The anti-inflammatory effect of BK antagonists seems to be non-additive with that of cyclooxygenase inhibitors (Burch & De Haas, 1990), suggesting a facilitatory role of prostanoids in BK stimulation of primary afferents.

It should also be mentioned that neurogenic inflammation is not confined to skin and joint. It has been shown in a wide variety of internal structures, such as gall bladder, vagina, oesophagus, trachea, and ureters (Lundberg *et al*, 1984).

2) Neuropeptides in inflammation

It has become apparent that SP and related peptides contained in sensory C fibres (Hokfelt *et al*, 1975; Mapp *et al*, 1989; 1990) play an important part in what has come to be termed "neurogenic inflammation" for both the skin (Jancso *et al*, 1967; Lembeck & Holzer, 1979; Gamse, Holzer & Lembeck, 1980; Foreman, 1987) and the joint (Levine *et al*, 1984; Ferrell & Cant, 1987; Yaksh *et al*, 1988). Neuropeptides are contained in nerve fibres innervating the synovium (Mapp *et al*, 1989; 1990) as well as meniscus and bone (Hukkanen *et al*, 1992). Electrical stimulation of nerves supplying the joint induces release of SP from the articular fibres (Yaksh *et al*, 1988) and produces plasma protein extravasation into the synovial cavity (Ferrell & Russell, 1986). The neurogenic component of the joint inflammation induced by carrageenan is mediated via SP and

perhaps other neurokinins (Fig. 17)(Lam & Ferrell, 1989a). Neuropeptides have been found in synovial fluid aspirated from patients with rheumatoid arthritis (Lygren *et al*, 1986; Larsson *et al*, 1991; Marabini *et al*, 1991), although these studies are difficult to interpret as control samples from normal subjects cannot be acquired and opposing results are obtained, with one study showing an increase in SP (Marabini *et al*, 1991), whilst another showed an absence of SP (Larsson *et al*, 1991). Considering the difference in the rate of metabolism of SP and other peptides, an increased release of SP might not be detected as easily as for example calcitonin gene related peptide (CGRP). The absence of detectable amounts of SP in the arthritic joints could be due to a local interaction of SP and CGRP, as an excess of CGRP may lead to the depletion of SP locally (Brain & Williams, 1988). Significantly higher concentrations of neuropeptides are found in synovial fluid of gout, rheumatoid arthritis and psoriatic arthritis compared to osteoarthritis (Marabini *et al*, 1991; Hernanz *et al*, 1993)

Neuropeptides can be divided into a group of structurally related peptides termed tachykinins, and the peptide CGRP.

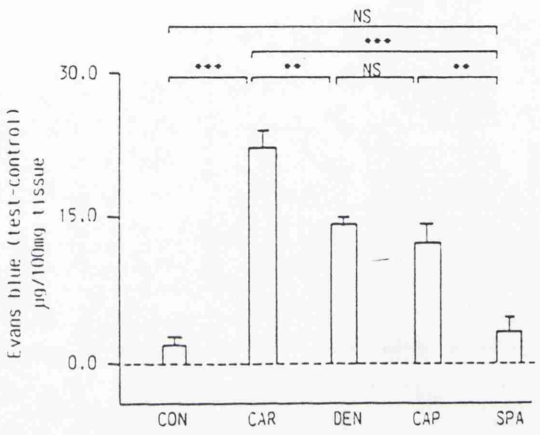


Figure 17: Histogram of Evans blue content of rat knee joint capsules (mean + SEM). Con = control response (bilateral injection of 0.9% saline); CAR = mean difference in dye content between the control knee and the other injected with 0.2 ml of 2% carrageenan; DEN = effect of 2% carrageenan injection in chronically denervated knees; CAP = response to carageenan injection in knees previously injected with 1% capsaicin; SPA = injection of 10ug of a substance P antagonist 15 mins before carageenan injection. **p< 0.01. ***p<0.001; n=5 (Lam & Ferrell, 1989a).

A) Tachykinins

i) Tachykinin synthesis and distribution

The term tachykinin (TK) is given to a family of peptides with the same COOH- terminal sequence of Phe-X-Gly-Leu-Met NH_2. As they are found principally in neurones, the term neurokinin is also used.

The undecapeptide substance P (SP) was the first tachykinin to be isolated (von Euler & Gaddum, 1931), purified and sequenced (Chang, Leeman & Niall, 1971). Subsequent studies of molecular biology and peptide biochemistry have expanded considerably the knowledge of various tachykinins in the mammalian nervous system. The tachykinins SP, neurokinin A (NKA; also called substance K), neurokinin A(3-10), neuropeptide K (NPK), and neuropeptide γ (NPγ) are produced from a single preprotachykinin gene as a result of differential RNA splicing and differential posttranslational processing (Fig. 18). The preprotachykinin I gene (PPT I) encodes the sequence of these tachykinins, while the PPT II gene encodes the sequence of neurokinin B (NKB) (Nawa *et al*, 1983; Nawa, Kotani & Nakanishi, 1984; Kotani, *et al*, 1986; Bonner *et al*, 1987; Krause *et al*, 1987). Three mRNAs from the PPT I gene transcription contain the sequences either to SP, NKA, NKA(3-10) and NPγ; to SP, NKA, NKA(3-10) and NPK; or to SP alone (Fig. 18)(Helke *et al*, 1990).

(a)

Gene	PPT mRNA	Peptide	Sequence
SP/NKA (PPT I) gene	α, β, γ	Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
	β, γ	Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
	β	Neuropeptide K β-PPT(72-107)NH_2	Asp-Ala-Asp-Ser-Ser-Ile-Glu-Lys-Gln-Val-Ala-Leu-Leu-Lys-Ala-Leu-Tyr-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
	γ	Neuropeptide γ γ-PPT(72-92)NH_2	Asp-Ala-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
	β, γ	Neurokinin A(3-10)	Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
NKB (PPT II) gene		Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2

(b)

Subtype	Ligand	Natural agonist potency	Relatively selective agonist
NK-1	[^{3}H]SP [^{125}I]BH-SP [^{125}I]Physalaemin [^{3}H]-[Pro^9]-SP	SP > NPγ ≥ NKA = NPK > NKB	[Sar^9, Met(O$_2$)^{11}]SP [β-Ala^8, Sar^9, Met(O$_2$)^{11}]SP(4-11) Ac-[Arg^6, Sar^9, Met(O$_2$)^{11}]SP(5-11) [pGlu^6, Pro^9]SP(6-11) (Septide) [Pro^9, Met(O$_2$)^{11}]SP [Cys$^{3-6}$, Tyr^4, Pro^9]SP
NK-2	[^{3}H]NKA [^{125}I]-[Iodohistidyl^1]NKA [^{125}I]BH-NKA [^{125}I]BH-Eledoisin [^{125}I]NPγ	NPK = NPγ ≥ NKA > NKB > SP	[β-Ala^8]NKA(4-10) [Nle^{10}]NKA(4-10) NKA(4-10)
NK-3	[^{3}H]-NKB [^{125}I]BH-NKA [^{125}I]BH-Eledoisin [^{3}H]Senktide	NKB > NKA > SP	[β-Asp^7, MePhe^8]NKB(4-10) [MePhe^8]NKB Succ-[Asp^7, MePhe^8]SP(6-11) (Senktide) [Cys$^{3-13}$]NKB [Pro^7]NKB

(c)

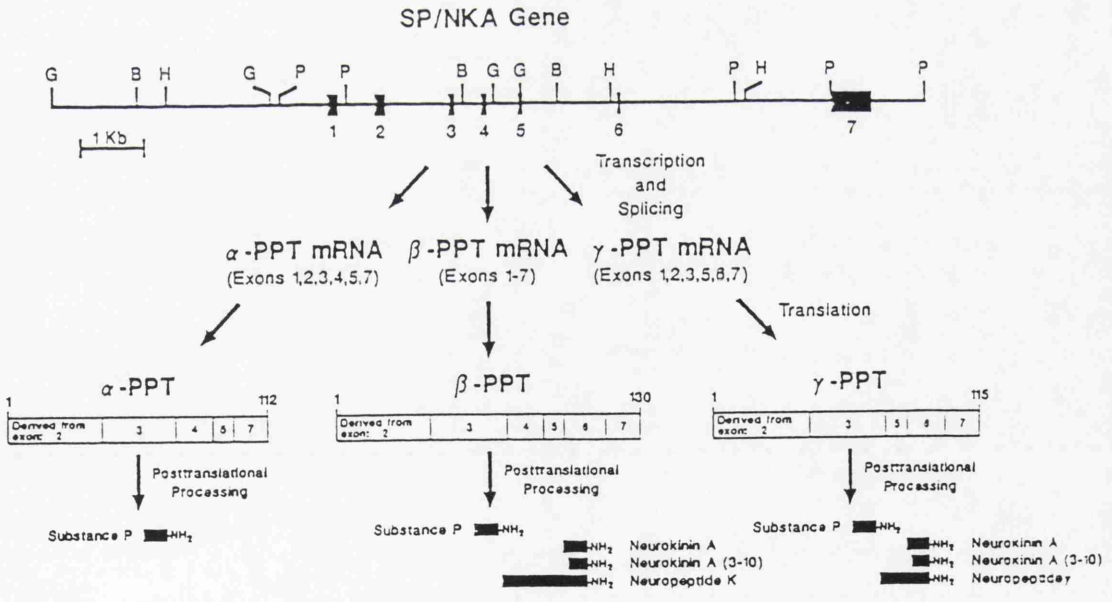


Figure 18: Mammalian tachykinin peptides (a) and receptors (b). Transcription of the PPT I gene, splicing of the primary transcripts and translation of the mRNAs (c) (Helke *et al*, 1990).

NPK and NPγ are N-terminally extended forms of NKA, while NKA(3-10) is NKA truncated to an octapeptide.

Neurones are the most common site for synthesis and storage of tachykinins, although TK-like immunoreactivity is found in other cell types, such as gut endocrine cells (Bartho & Holzer, 1985) and parenchymal cells of the carotid body (Cuello & McQueen, 1980). Although there is evidence for the release of tachykinins from non-neuronal cells (Ralevic *et al*, 1990), there is no evidence supporting synthesis in cells other than neurones.

Tachykinins are found at various sites in the CNS (Maggio, 1988), including the spinal cord sensory system. SP is found to be associated preferentially with spinal dorsal roots and with lamina I and II of the dorsal horn (Otsuka & Konishi, 1977). Approximately 50-60% of the SP content of the dorsal horn is in the primary afferent terminals, while the remainder is in spinal interneurones or bulbospinal neurones (Ljungdahl, Hokfelt & Nilsson, 1978). The function of substance P at these sites is thought to be as a sensory neurotransmitter involved in the mediation of peripheral pain sensation (Nicoll, Schenker & Leeman, 1980; Jessell, 1983; Pernow, 1983). As expected from the genetic origin of the tachykinins, SP and NKA co-localise throughout the mammalian CNS, while NKB is distributed independently (Kanasawa *et al*, 1984; Arai & Emson, 1986).

In the peripheral nervous system tachykinins are found in primary afferent neurones (Hokfelt *et al*, 1975), as well as in intrinsic gut neurones (Bartho & Holzer, 1985), intramural

bladder neurones (Crowe, Haven & Burnstock, 1986), certain sympathetic neurones (Kessler *et al*, 1981) and certain parasympathetic neurones (Sharkey & Templeton, 1984). Primary afferent neurones sensitive to capsaicin application are by far the most widespread source of neuronal tachykinins. This is indicated by the depletion of tachykinins following stimulation of chemosensitive or polymodal nerves with capsaicin (Maggi & Melli, 1988; Holzer, 1991). Synthesis of tachykinins occurs in the cell bodies of these neurones, which are located in the dorsal root ganglia (DRG). SP, NKA and other NKA-related peptides are produced in the DRG through expression of the PPT I gene (Too, Cordova & Maggio, 1989a; b). As the PPT II gene is not expressed in the DRG (Warden & Young, 1988), NKB is not found in the terminals of primary afferent neurones. However NKB is found in peripheral tissues (Tateishi *et al*, 1990) and although it is practically absent from both dorsal roots and DRG cells, it is contained within intrinsic spinal neurones (Ogawa, Kanazawa & Kimura, 1985). TKs synthesised in the dorsal root ganglia are transported (Keen *et al*, 1982), and released at nerve terminals both centrally and peripherally (Hokfelt *et al*, 1975). Release of SP and NKA at both central and peripheral nerve terminals takes place in response to depolarisation of the nerves, and is calcium dependent (White & Helm, 1982; Saria *et al*, 1984; Linderoth & Brodin, 1988; Yaksh *et al*, 1988). These tachykinins, therefore, can be classed as neurotransmitters. NKB also meets this criterion in the CNS (Lindefors *et al*, 1985a; b). SP-like

immunoreactivity is found in approximately seventeen percent of the MAN afferent fibres and sixteen percent of the PAN afferent fibres supplying the cat knee (Hanesch, Heppelmann & Schmidt, 1991), and in nerve fibres innervating the joint synovium (Mapp *et al*, 1989; Mapp *et al*, 1990). It is released by electrical stimulation of the nerves supplying the joint (Yaksh *et al*, 1988). Tachykinins are also released in response to physiological or pathophysiological stimulation. SP is released in the skin (Helme, Koschorke & Zimmermann, 1986) and spinal cord (Duggan *et al*, 1987), in response to noxious thermal stimulation of the skin and also in the spinal cord in response to innocuous stimulation of the peripheral field (Gov & Yaksh, 1987). Enhanced release of SP and CGRP occurs from terminals located in the lumbar dorsal horn of the spinal cord during carageenan-induced inflammation of the hindpaw (Garry & Hargreaves, 1992). This enhanced release does not require a sustained peripheral input as it is observed in vitro superfusion conditions. NKA is also released in the dorsal horn of the spinal cord following noxious cutaneous stimulation (Duggan *et al*, 1990). In animal models of arthritis, SP (Oku, Satoh & Takagi, 1987; Schaible *et al*, 1990) and NKA (Hope *et al*, 1990) are released in the spinal cord. A basal level of NKA is detected prior to any noxious stimulation, but on injection of kaolin and carageenan into a knee joint, release of NKA into the dorsal horn of the spinal cord is detected (Hope *et al*, 1990). Unilateral Freund's adjuvant-induced paw inflammation produces an

increase in SP and CGRP content of the sciatic supplying the paw (Donnerer, Schuligoi & Stein, 1992) as well as a marked increase in axonal transport. Levels in the paw are the same or reduced, suggesting reduced storage and increased release in inflamed tissues.

It should be noted that, because of the carboxyl-terminal homology among the tachykinins, most antisera raised against one peptide cross-reacts with other tachykinins (Milner *et al*, 1988). Immunochemical studies are therefore not as specific as chromatographic studies. Also, earlier studies did not know of the existence of the more recently discovered tachykinins, such as NPγ and NKA(4-10), and those studies involving radioimmunoassay and immunocytochemistry could not have accounted for the potential antibody cross-reactivities with these tachykinins.

On the other hand, metabolism of TKs by tissue peptidases may be so extensive as to influence the evaluation of the TK release. Neutral endopeptidase can cleave a variety of peptides including SP and NKA (Matsas, Kenny & Turner, 1984; Skidgel *et al*, 1984; Hooper, Kenny & Turner, 1985; Katayama *et al*, 1991). Hydrolysis of SP produces fragments (Conlon & Goke, 1984) which can crossreact with polyclonal and monoclonal antibodies (Cuello, Galfre & Milstein, 1979; Harmar & Keen, 1986). SP is normally released locally in the superficial dorsal horn of the cat spinal cord following electrical nerve stimulation (Duggan *et al*, 1988), but inhibition of peptidases allows the SP to spread more

widely (Duggan *et al*, 1992). Neurogenic plasma extravasation in the rat nasal mucosa is potentiated by peptidase inhibitors (Petersson *et al*, 1993)

ii) Tachykinin receptors

Three distinct neurokinin receptor types in various mammalian tissues have been confirmed; the neurokinin-1 (NK-1), neurokinin-2 (NK-2), and neurokinin-3 (NK-3) receptors, at which SP, NKA and NKB are the preferential endogenous agonists respectively (Beaujouan *et al*, 1984; Buck *et al*, 1984; Nawa *et al*, 1984; Regoli *et al*, 1985; 1987a; b; 1989; Lee *et al*, 1986; Maggi *et al*, 1987). Evidence for the existence of multiple TK receptors was first indicated by a greater potency than SP of non-mammalian TKs (eledoisin and kassinin), in certain mammalian tissues (Erspamer, 1981). Investigations, using bioassay, radioligand binding and in vivo physiological experiments followed. The existence of three types of receptors is confirmed by the isolation of three distinct genes encoding these receptors (Nakanishi, 1991). Although the natural TKs have different affinities for the different types of receptors, they are all capable of acting to some extent at all the receptors (Regoli *et al*, 1987a, Ingi *et al*, 1992) (Fig. 18). However, the concept of three distinct classes of receptors is reinforced by the discovery of agonists highly selective for one type of receptor (Dion *et al*, 1987; Drapeau *et al*, 1987; Regoli *et al*, 1988). As mentioned, the

criterion for distinguishing the three types of receptors is the degree of potency of the natural TKs. In the same way, the NK-1 and NK-2 receptors have been divided into different subtypes on the basis of differential binding of agonists and antagonists (Maggi *et al*, 1990). The study of individual receptor types is frequently undertaken using bioassay preparations, in which the biological response under study is mediated by only one of the three known receptor types. Heterogeneity of the NK-1 receptor has been suggested on the basis of differing affinities of agonists and antagonists to NK-1 receptors. Differing affinities are observed to NK-1 receptors expressed in different species (Garret *et al*, 1991; Gitter *et al*, 1991; Snider *et al*, 1991a; b; Fardin *et al*, 1992), receptors in different organs of the same species (Lew *et al*, 1990; Fardin & Garret, 1991), or receptors in the same preparation (Sakurada *et al*, 1991; Petitet *et al*, 1992). However, observed differences in the affinities of peptide ligands for NK-1 receptors could be due to differential metabolic breakdown by peptidases (Lew *et al*, 1990; Fardin & Garret, 1991), or to differences in receptor glycosylation (Burcher *et al*, 1991). Heterogeneity of NK-2 receptors has also been suggested in the same way. Differing affinities of agonists and antagonist to NK-2 receptors is observed in various different preparations (McKnight *et al*, 1988; Rovero *et al*, 1990; Ireland *et al*, 1991). Recently, the development of very selective agonists and antagonists for neurokinin receptors has allowed the identification of two possible types of NK-2 receptors,

provisionally termed NK-2A and NK-2B (Maggi *et al*, 1990; Van Giesbergen *et al*, 1991; Advenier, 1992b).

The main second messenger system coupled to activation of the three known receptor types is stimulation of phospholipase C leading to phosphoinositide breakdown and elevation of intracellular calcium (Guard & Watson, 1991). Adenylate cyclase stimulation and cAMP accumulation is also produced by SP acting on NK-1 receptors (Mitsuhashi *et al*, 1992), possibly through coupling with a different G-protein.

iii) Tachykinin receptor antagonists

Tachykinin receptor antagonists can be classified into peptides and nonpeptides.

The first peptide antagonists were based upon insertion of multiple D-amino acids into SP (Folkers *et al*, 1985) or were N-terminal truncated analogues of SP (Regoli, Escher & Mizrahi, 1984). These antagonists had poor selectivity (Rosell *et al*, 1983) and partial agonist activity, as well as other effects (Regoli, 1985). The selectivity and potency were subsequently improved by introducing constraints to the hexapeptide molecule and exchanging amino acids to produce various cyclic and linear analogues (Cascieri *et al*, 1986; Rovero *et al*, 1989; Dion *et al*, 1990; Iversen *et al*, 1990). There is now a vast array of tachykinin antagonists, the most relevant of which are discussed.

The more recent SP antagonists include the tripeptide FR 113680 [Ac-Thr-DTrp(CHO)-Phe-NMeBzl] which is a specific NK-1 receptor antagonist (Jukic *et al*, 1991; Morimoto *et al*, 1992a). It specifically antagonises [^{3}H]SP binding to guinea-pig lung membranes (Hagiwara *et al*, 1991) and inhibits substance P induced airway oedema and airway constriction in the guinea-pig (Murai *et al*, 1992a). A potent dipeptide derivative of FR113680 is FK888 (Fig. 19) [(2-(N-Me)indolil)-CO-Hyp-Nal-NMeBzl] which shows extremely high affinity for NK-1 receptors in the guinea pig ileum (Fujii *et al*, 1992). It inhibits the contraction of guinea-pig ileum induced by SP in the presence of atropine and indomethacin (NK-1 receptor bioassay) with a pA_2 value of 9.29. Inhibition of contraction of the rat vas deferens by NKA (NK-2 receptor bioassay) and of rat portal vein by NKB (NK-3 receptor bioassay) required FK888 concentrations of at least 10,000 times greater than was required to inhibit the NK-1 receptor bioassay. SP-induced airway oedema in the guinea-pig was inhibited after both intravenous and oral administration of FK888. This suggests that FK888 is a potent and selective NK-1 antagonist which is active both in vitro and in vivo (Fujii *et al*, 1992).

Another recently discovered antagonist with both NK-1 and NK-2 receptor selectivity is the cyclopeptide FK224 (Fig. 19). {N-[N^2-[N-[N-[N-[2,3-didehydro-N-methyl-N-[N-[3-(2-pentyl phyenyl)-propionyl]-L-threonyl]tyrosyl-L-leucynyl]-D-phenyl alanyl]-L-allo-threonyl]-L-asparaginyl]-L-serine-v-lactone}

(Morimoto *et al*, 1992b). FK 224 dose dependently inhibits [^{3}H] SP binding to rat cerebral cortical membranes (NK-1 receptor binding assay) and [^{3}H] NKA binding to rat duodenum smooth muscle membranes (NK-2 receptor binding assay) but does not affect [^{3}H] eledoisin binding to rat cerebral cortical membranes (NK-3 receptor binding assay). Also in bioassay experiments FK224 inhibits SP-induced contraction of guinea-pig ileum (NK-1) and NKA-induced contraction of vas deferens (NK-2) but does not affect NKB induced contraction of rat portal vein (NK-3) (Morimoto *et al*, 1992b). FK224 also inhibits the contraction of isolated guinea-pig trachea induced by SP and inhibits SP, NKA and capsaicin induced airway oedema (Murai *et al*, 1992b). These results suggest that FK224 is a selective NK-1 and NK-2 receptor antagonist which inhibits endogenous neurotransmitters released by capsaicin, as well as exogenous SP and NKA.

Non-peptide antagonists originate from the discovery of CP 96345 (Snider *et al*, 1991b)(Fig. 19), a compound which displays an exceedingly high potency and selectivity for NK-1 receptors. Although this antagonist also possesses significant affinity at Ca^{2+} channels (Guard & Watling, 1992), it has been useful in characterising the physiological contribution of NK-1 receptors in vivo. CP 96345 prevents excitation of dorsal horn neurons following noxious stimulation of the limb (Radhakrishnan & Henry, 1991) and blocks capsaicin-evoked plasma protein extravasation (Eglezos *et al*, 1991). It also reduces airway oedema induced by antidromic vagal stimulation, and plasma

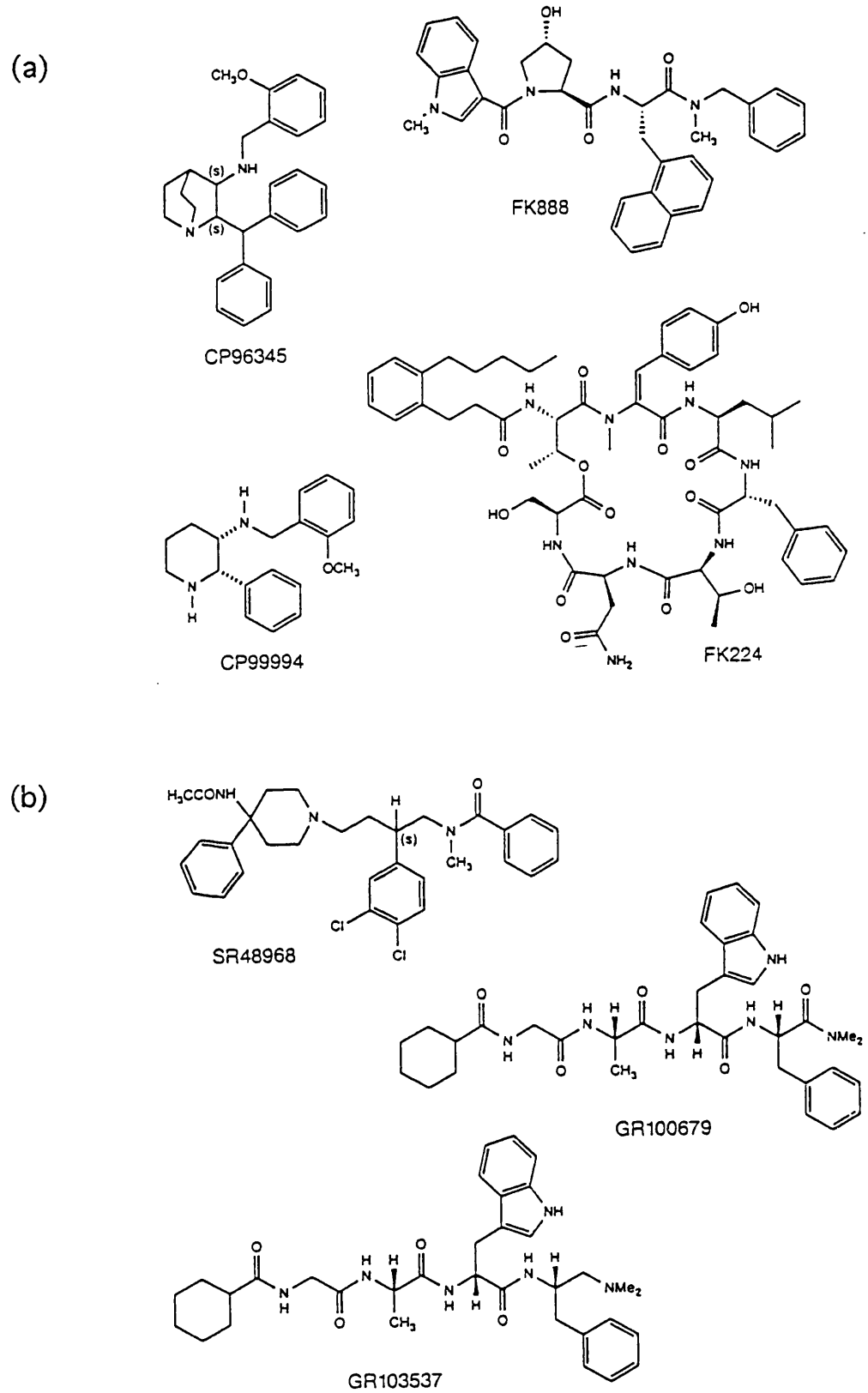


Figure 19: New NK-1 (a) and NK-2 (b) receptor tachykinin receptor antagonists (Watling & Krause, 1993)

protein extravasation in the rat paw induced by administration of SP, antidromic stimulation of the saphenous nerve or application of mustard oil (Lembeck *et al*, 1992). Intradermally injected CP 96345 inhibits the localised plasma protein extravasation of the rat hindpaw produced by stimulation of C fibres in the sciatic nerve (Xu *et al*, 1992), and SP or capsaicin induced increase in microvascular clearance in the hamster cheek pouch (Gao *et al*, 1993). Plasma extravasation in rat skin (Andrews, Helme & Thomas, 1989) and in the rat knee joint (Lam & Ferrell, 1991b; Lam, Ferrell & Scott, 1993) is mediated by the NK-1 receptor subtype. CP-96345, selectively inhibits SP-induced vasodilatation in the joint, whilst leaving the vasodilator responses to NKA and NKB unaltered (Lam & Ferrell, 1993b). This suggests that blood flow changes in the rat knee joint are mediated by multiple neurokinin receptor types, while plasma extravasation maybe limited to NK-1 receptors.

The non-peptide NK-1 receptor antagonist RP 67580, reduces plasma extravasation in rat skin induced by local application of capsaicin, or exogenous SP. In neonatal rats treated with capsaicin, SP-induced extravasation is still inhibited by RP 67580, suggesting a postsynaptic action of NK-1 antagonists (Moussaoui *et al*, 1993).

The first non-peptide NK-2 receptor antagonist was SR 48968 (Emonds-Alt *et al*, 1991)(Fig. 19). When tested in a variety of smooth muscle preparations it was found to have a very high potency at the NK-2 receptor (Advenier *et al*, 1992a). Differing

affinities in different species suggest that SR 48968 is more active at the NK-2A receptor subtype than on the NK-2B receptor subtype. The sensitivity of NK-2A receptors to SR 48968 is at least 1000 times higher than those of NK-1 and NK-3 receptors, and 100 times that of NK-2B receptors (Advenier *et al*, 1992a). In vivo SR 48968 antagonises NK-2 mediated bronchconstriction (Emonds-Alt *et al*, 1992) and specific NK-2 agonist induced contraction of isolated human bronchus, and NKA-induced rabbit pulmonary artery contraction (Advenier *et al*, 1992b).

iv) Tachykinins in inflammation

SP and related neuropeptides have been shown to exert potent and extensive proinflammatory actions such as vasodilatation (Ferrell & Cant, 1986; Lam & Ferrell, 1993a; b), increased capillary permeability (Ferrell & Russell, 1986), mast cell degranulation (Mazurek *et al*, 1981), secretion of PGE_2 and collagenase from synoviocytes (Lotz, Carson & Vaught, 1987), stimulation of secretion of IL-1-like activity from macrophages and astrocytes (Kimball, Perisco & Vaught, 1988; Martin *et al*, 1992) and cartilage and bone damage in adjuvant arthritis (Levine, Moskowitz & Basbaum, 1985). In addition, the neurokinins may play an important role in the activation of the immune system (Payan *et al*, 1987; Neveu & Le Moal, 1990)

The majority of the various biological actions exhibited by tachykinins (Pernow, 1983), are exerted by the common C-

terminal binding to tachykinin specific receptors. The N-terminal region also exerts biological actions and is thought to be important in determining the relative affinities of TKs for their receptors. Tachykinins have many biological actions, apart from their primary role of neurotransmission, including stimulation of secretions and smooth muscle contraction. Actions involved in inflammation are vasodilatation, plasma protein extravasation, stimulation of inflammatory cells, and mast cell degranulation.

a) Vasodilatation is measurable in vivo as a transitory fall in blood pressure, following administration of TKs (Maggi *et al*, 1985). In vitro, preparations of preconstricted blood vessels can also be made to dilate (D'Orleans-Juste *et al*, 1985). The effect is mediated by NK-1 receptors (Maggi *et al*, 1985; 1987; Couture *et al*, 1989) on endothelial cells (Stephenson, Burcher & Summers, 1986).

SP and related neuropeptides induce vasodilatation in the rat knee joint (Grice, Lam & Ferrell, 1990; Lam & Ferrell, 1993a; 1993b; Lam, Ferrell & Scott, 1993) (Fig. 20) and antidromic stimulation of C-fibre afferent nerves supplying the knee joint of the cat produces vasodilatation of joint capsule blood vessels (Ferrell & Cant, 1987). NK-2 receptors, as well as NK-1 receptors, may be involved in vasodilatation in the rat knee (Lam & Ferrell, 1993b). A laser Doppler perfusion imaging system (LDI) or the more conventional laser Doppler flowmeter (LDF) is used to assess changes in joint blood flow in these preparations. The LDF

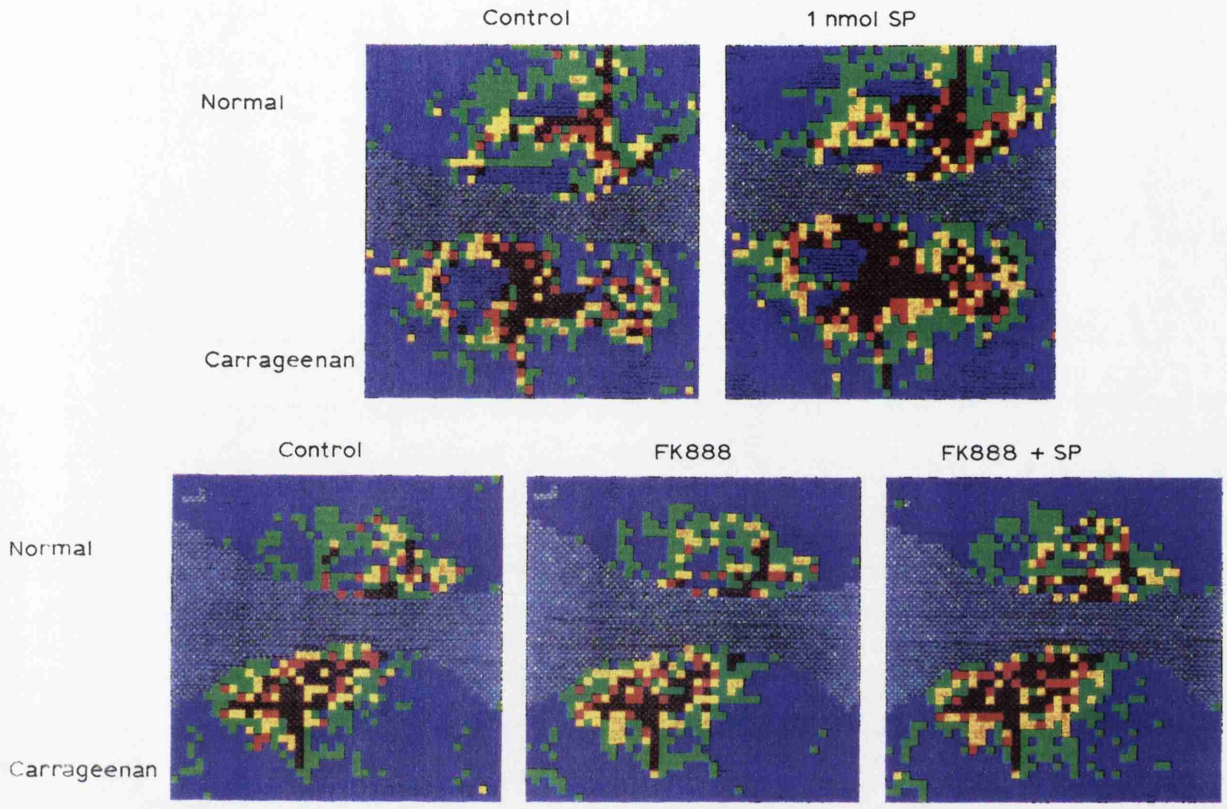


Figure 20: Perfusion images of the antero-medial aspect of the rat knee before (control) and after topical application of SP (1 nmol) in the absence and presence of FK888. The top panels are from normal animals whilst the bottom panels are from carageenan-treated animals. Colour codes are for relative differences in perfusion, ranging from dark blue (lowest) to dark red (highest). In the inflamed joint, vasodilatation to SP is enhanced compared to normal. Application of FK888 (10 nmol) alone produced no alteration of basal blood flow but substantially reduced SP-induced vasodilatation in both groups of animals (Lam, Ferrell & Scott, 1993).

provides measurements of tissue perfusion at a single location only, whereas the newly developed LDI system allows averaging and spatial mapping of blood flow changes for a selected tissue area up to 12 x 12cm wide. Alterations in blood perfusion of the knee joint as a consequence of vasoconstrictor or vasodilator effects could significantly influence the final manifestations of the inflammatory processes. Acute inflammation in the rat knee joint induced by prior carrageenan-injection resulted in an enhancement of the vasodilator responses to SP and CGRP as compared to normal joints (Lam & Ferrell, 1993a). The responsiveness of the rat knee joint microvasculature to sympathetic nerve stimulation was also substantially altered. Thus, electrical stimulation of the saphenous nerve produced small vasoconstrictor responses in carrageenan-treated knees, whereas in normal knees, potent vasoconstriction was observed (Lam & Ferrell, 1993a). Carrageenan is a model of acute inflammation similar to that occuring in human arthritic lesions. A single intra-articular injection of carrageenan results in an inflammatory response within the joint; this is characterised by an influx of polymorphonuclear leucocytes into the synovial fluid and proliferation of the synovial membrane (Cantazaro, Schwartz & Graham, 1971; Di Rosa, 1972; Santer, Sriratana & Lowther, 1983).

It is uncertain if a TK, in the physiological situation, reaches the receptor by direct release from nerve endings innervating the endothelial cells, or if circulating TK is responsible. Although

capsaicin sensitive nerves containing TKs are present in blood vessel walls (Wharton & Gulbenkian, 1989), it is uncertain if TKs released can gain access to the receptors on endothelial cells. In the porcine coronary arteries, vasorelaxation induced by capsaicin stimulation of nerve endings is unaffected by mechanical removal of the endothelium, but is inhibited by a CGRP antagonist (Franco-Cereceda, 1991). On the other hand, vasodilatation produced by nerve stimulation in the rabbit tenuissimus muscle is partially antagonised by an SP antagonist and is dependent on the endothelium (Persson *et al*, 1991). Although a unilaterally induced joint inflammation induces a bilateral release of SP, NKA, CGRP and neuropeptide Y in rat synovial fluid (Bileviciute *et al*, 1993), this is not associated with a rise in plasma levels It is possible that different vessels in different species have different permeabilities to the released TKs.

b) Plasma protein extravasation is also mediated through direct activation of NK-1 receptors on blood vessels (Abelli *et al*, 1989) as well as indirectly, by degranulation of mast cells and production of prostanoids (Jacques *et al*, 1989; Abelli *et al*, 1992). Electrical stimulation of the rat saphenous nerve produces extravasation of intravenous dyes from post capillary venules in the skin (Kowalski & Kaliner, 1987), although mast cells are not closely associated with permeable vessels or nerve endings displaying neuropeptide immunoreactivity (Baraniuk, Kowalski

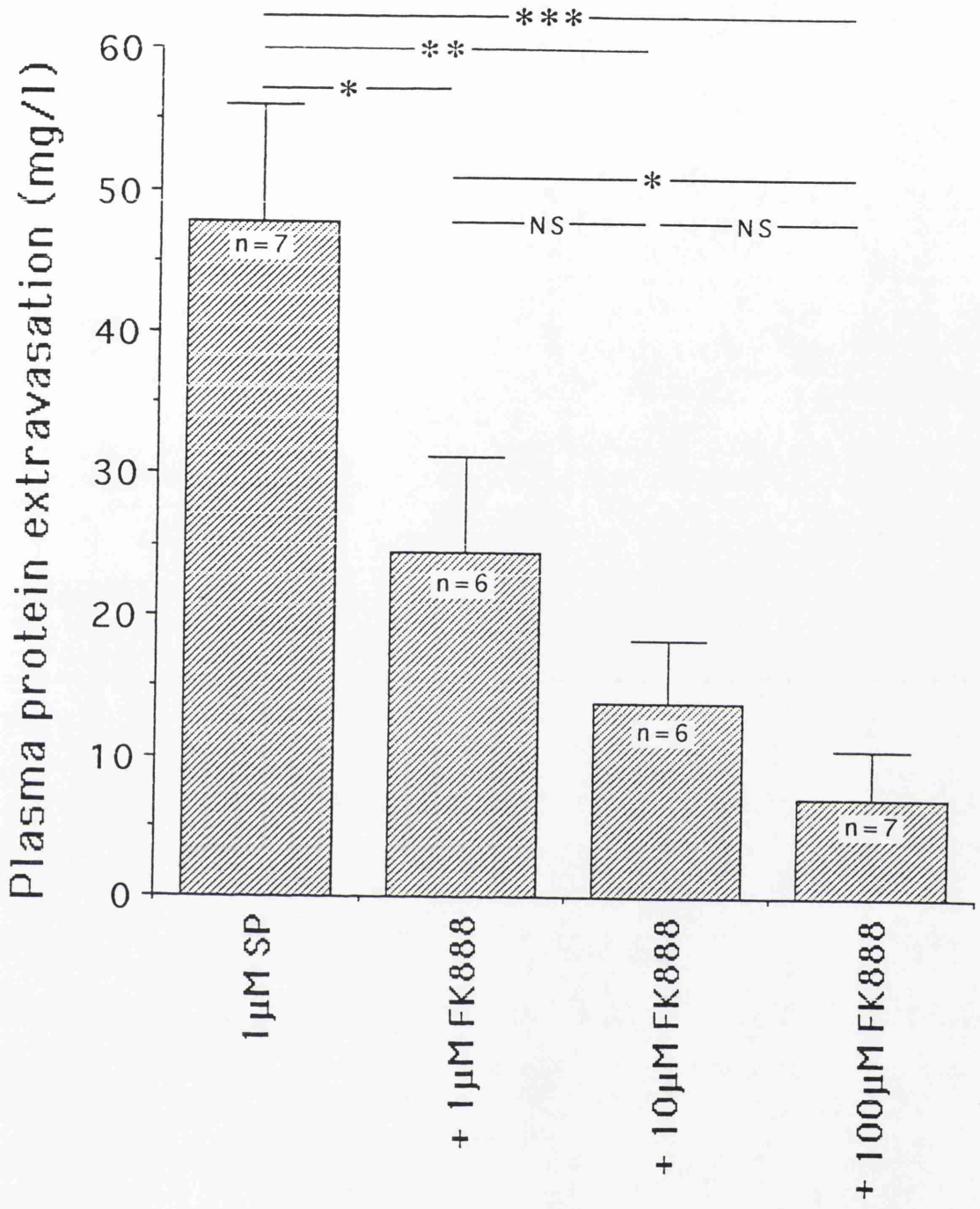


Figure 21: Effects of increasing concentrations of FK888 (1-100μM) on plasma protein extravasation in the rat knee induced by intra-articular perfusion of 1μM SP. Results are expressed as mean ($\pm$ S.E.M.) of the peak plasma protein extravasated by SP minus the preceding basal plasma protein levels. Significance difference (Mann-Whitney *U* test) **P*<0.05; ***P*<0.01; ****P*<0.001. Lam, Ferrell & Scott, 1993)

& Kaliner, 1990). Direct activation of the vascular bed by the neuropeptides is therefore more probable in this preparation. In the joint, antidromic stimulation of C fibre afferent nerves from the knee joint of the cat induces plasma protein extravasation into the joint capsule (Ferrell & Russell, 1986). This neurogenically induced plasma extravasation is completely inhibited by prior intra-articular administration of the SP antagonist D-Pro^{4}D-Try^{7,9,10}SP$_{4-11}$, which suggests that SP is the mediator of the response. SP injected into the joint cavity induces plasma protein extravasation (Lam & Ferrell, 1989a; b), and carageenan induced extravasation is inhibited by the SP antagonist D-Pro^{4}D-Try^{7,9,10}SP$_{4-11}$ (Lam & Ferrell, 1989a). Evidence to date, using the specific NK-1 agonist [Sar^{9}, Met(O$_2$)^{11}]-SP (Lam & Ferrell, 1991b), or the specific NK-1 antagonist FK888 (Lam *et al*, 1993) suggests that the NK-1 receptor is responsible for protein extravasation in the knee joint of the rat (Fig. 21).

c) Mast cell degranulation is produced, perhaps through direct G-protein activation, when the N-terminal of SP binds to the mast cell (Mousli *et al*, 1990). The C-terminal may also be involved, binding to NK-1 or NK-2 receptors which exist in certain mast cells (Krumins & Bloomfield, 1992). Mast cells are immunocompetent cells which have wide distribution within the body and are also present within the synovium. They are known to contribute to various inflammatory responses including those

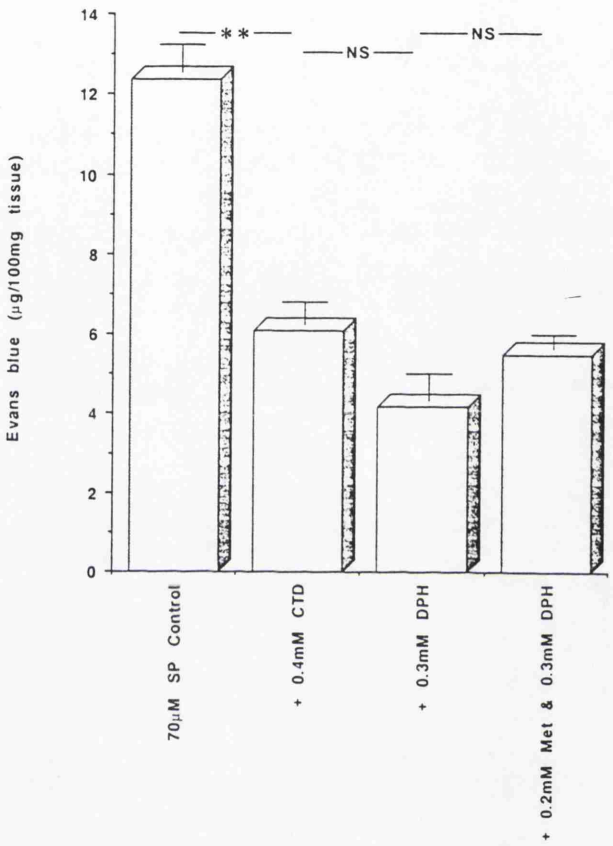


Figure 22: Control response to intra-articular injection (0.2ml) of 70μM substance P (SP) and the effect of pretreatment (15 mins. earlier) with cimetidine (CTD), diphenhydramine (DPH) or a combination of methysergide (Met) and DPH injected into the joint cavity. n=3-5. (Lam & Ferrell, 1990)

that occur in the skin (Kowalski & Kaliner, 1987) and the joint (Mican & Metcalf, 1990). Mast cell granules contain numerous preformed mediators, including histamine and serotonin, although histamine is the only amine found in human mast cells. In addition leukotrienes, prostaglandins and platelet activating factor can be produced (Metcalfe, Kaliner & Donlon, 1981; Tuominen *et al*, 1989; Wassaerman, 1984). There are increased mast cell numbers in rheumatoid synovial membrane (Crisp *et al*, 1984; Godfrey *et al*, 1984) and elevated histamine levels in rheumatoid synovial fluid (Frewin *et al*, 1986; Malone *et al*, 1986). When mast cells are activated during inflammatory responses (Kiernan, 1972; Foreman & Jordan, 1981) or acutely degranulated with compound 48/80 (Levi, Owen & Przeciakowski, 1982; Lam & Ferrell, 1990), plasma extravasation and vasodilatation are generated. Chronic depletion of mast cell contents, by pretreatment with compound 48/80, profoundly reduces inflammatory responses to injury (Kiernan, 1975; Lembeck & Donnerer, 1981) or substance P (SP) injection (Lam & Ferrell, 1990). Furthermore, intradermal and intra-articular injection of the rat mast cell constituents, histamine and serotonin, significantly increase plasma extravasation and vasodilatation (Bignold & Lykke, 1975; Majno & Palade, 1961; Northover & Northover, 1969; Lam & Ferrell, 1990). Drugs that inhibit degranulation of mast cells (e.g. disodium cromoglycate and flavonoids, such as quercetin) decrease oedema in the rat paw produced by various inflammatory agents (Gabor, 1979;

Goose & Blair, 1969). Histamine exerts its effects by interaction with cell-specific receptors designated H_1, H_2 and H_3 (Black *et al*, 1972; Arrang *et al*, 1987; Polk *et al*, 1988) Intra-articular injections of the H_1 blocker diphenhydramine, the H_2 blocker cimetidine, or the 5-HT antagonist methysergide, significantly attenuates SP-induced inflammation in the rat knee joint (Fig. 22)(Lam & Ferrell, 1990). Plasma extravasation and vasodilatation produced by antidromic peripheral nerve stimulation or injection of SP, are also inhibited by H_1-antagonists, or pretreatment with the mast cell degranulator, compound 48/80 (Arvier *et al*, 1977; Lembeck & Holzer, 1979). Mast cells are commonly found in close proximity to (Heine & Forster, 1975), or in direct contact with (Newson *et al*, 1983; Weisner-Menzel *et al*, 1981), peripheral nerve endings; specifically neuropeptide containing afferents (Skofitsch *et al*, 1985; Stead *et al*, 1987). Both tissue injury (Kiernan, 1972) and antidromic stimulation of peripheral nerves (Kiernan, 1971) can elicit mast cell degranulation, and injection of SP releases histamine from rat peritoneal mast cells (Kitada *et al*, 1980) and human cutaneous mast cells (Barnes *et al*, 1982; Hagermark *et al*, 1978).

d) When inflammation becomes more chronic in nature, a greater contribution is made by the inflammatory cells. These cells can be recruited or stimulated by TKs, as can other inflammatory mediators which themselves recruit the

inflammatory cells. TKs enhance lymphocyte proliferation (Payan *et al*, 1983), stimulate chemotaxis (Marasco *et al*, 1981) and stimulate monocytes (Wagner *et al*, 1987) and macrophages (Brunelleschi *et al*, 1990).

TKs are known to induce the production of cytokines (Kimball *et al*, 1988). These are a group of glycoproteins which modulate and control various aspects of body defence and repair, including inflammation. They include the interleukins, growth factors, colony-stimulating factors, and interferons. Interleukin-1 (IL-1) is the most extensively studied of these and will serve as an example of the function of this group. In vitro studies have shown that IL-1 activity can be produced by virtually all nucleated cell types (Oppenheim *et al*, 1986), including fibroblasts and osteoblasts (Martin & Resch, 1988). Many of the events associated with the inflammatory reaction can be mediated by IL-1 (Dinarello, 1984; Beck *et al*, 1986). It is thought to stimulate membrane events leading to increased phospholipase activity and the resultant release of arachidonic acid makes this substrate available for either prostaglandin or leukotriene synthesis, both of which contributes to acute inflammation. Synovial cells proliferate in response to IL-1 and secrete inflammatory mediators. Furthermore, IL-1 has been shown to be chemotactic for the major inflammatory mediators such as lymphocytes, mononuclear phagocytes and neutrophils. In macrophages, it induces secretion of tumour necrosis factor α, resulting in a higher cytotoxicity of these cells.

TKs can also induce secretion of eicosanoids from many cells, including synoviocytes (Lotz *et al*, 1987). These compounds include the prostaglandins, leukotrienes, thromboxanes, and lipoxins and are formed by the oxygenation of the polyunsaturated fatty acids arachidonic acid, dihomogamma linolenic acid and eicosapentaenoic acid. Membrane phospholipids are the main storage site for these fatty acids and oxygenation by either the cyclooxygenase or lipoxygenase pathways can take place in virtually any human cell type to produce the various prostaglandins and related compounds. Prostaglandins, products of the cyclooxygenase pathway, are produced on demand and participate in the development of the inflammatory reaction, principally by potentiating the effects of other mediators rather than by having a direct effect. They tend to have local actions, either on the cell of origin or nearby structures. For example, mast cells, often seen in large numbers in the synovium of early arthritis, produce prostaglandin which in turn induces histamine release from mast cells primed with anti-IgE antibody. Products of the lipoxygenase pathway are implicated as key mediators in inflammation. They have been shown to increase adherence of leukocytes to endothelial cells, facilitate enhanced vascular permeability, stimulate production of some interleukins and interferons, and to have an immunoregulatory function.

B) Calcitonin gene related peptide (CGRP)

CGRP is a thirty-seven amino acid peptide encoded by the calcitonin gene and generated via tissue specific RNA processing (Rosenfeld *et al,* 1983). It is found in various tissues, including the brain (Skofitsch & Jacobowitz, 1985) and the thyroid gland (Grunditz *et al,* 1986). In the periphery it occurs in DRG cell bodies (Gibbins, Furness & Costa, 1987; Gibson *et al,* 1984; Ju *et al,* 1987) and in primary sensory axons (Rodrigo *et al,* 1985; Terenghi *et al,* 1985), including nerve fibres supplying the knee joint of the cat (Hanesch *et al,* 1991). The co-localisation of CGRP with SP in sensory neurones (Gibbins *et al,* 1985; Wiesenfeld-Hallin *et al,* 1984), including nerves supplying the joint (Hanesch *et al,* 1991) suggests the possibility of a simultaneous release of these neuropeptides (Gulbenkian *et al,* 1986). CGRP and neuropeptides released simultaneously from nerve endings can have a synergistic effect. Intra-articular CGRP potentiates SP-induced plasma protein extravasation in the rat knee (Kidd *et al,* 1990b; Cambridge & Brain, 1992; Cruwys *et al,* 1992; Green, Basbaum & Levine, 1992), and potentiates tachykinin-induced increases in vascular permeability in the skin (Brain & Williams, 1985a; 1989) and in the airway (Gamse & Saria, 1985). CGRP microinjected into the grey matter of the spinal cord causes intraspinal spreading of immunoreactive SP released by nerve stimulation and noxious mechanical stimuli (Schaible *et al,* 1992).

CGRP produces a variety of biological effects including some which are pro-inflammatory. It is a potent vasodilator (Brain *et al*, 1985). In rabbit skin, oedema induced by histamine and bradykinin is potentiated by vasodilator doses of CGRP and capsaicin. Both these potentiating effects and capsaicin induced increases in blood flow are partially inhibited by prior treatment with selective CGRP antiserum (Buckley *et al*, 1992). In the rat knee joint, CGRP is a potent vasodilator and this effect is enhanced in the inflamed joint (Lam & Ferrell, 1993a). CGRP also modulates nerve-mediated vasoconstriction and this is attenuated by inflammation (Lam & Ferrell, 1992; 1993a). It has recently been found that SP attenuates the vasodilatory effect of CGRP (Lam & Ferrell, 1993b).

CGRP has also recently been found to induce plasma protein extravasation in the rat knee (Karimian & Ferrell, 1993) using the microturbidimetry technique, but not using Evans Blue (Cruwys *et al*, 1993)

The development of selective agonists and antagonists for CGRP receptors has led to the characterisation of two receptors, termed $CGRP_1$ and $CGRP_2$ (Dennis *et al*, 1989; 1990; 1991; Mimeault *et al*, 1991).

3) Interaction of the afferent system with the sympathetic system

The sympathetic nervous system may also contribute to neurogenic inflammation. Evidence supporting this idea arises from the finding that vascular permeability is increased after sympathetic nerve stimulation (Linde *et al*, 1974) and that the baseline plasma extravasation in the knee joint of cats is substantially reduced after lumbrosacral sympathectomy (Engel, 1941; 1978). Chemical sympathectomy with 6-hydroxydopamine (6-OHDA) (Helme & Andrew, 1985), reserpine (Gozsy & Kato, 1966), α-methyl-p-tyrosine or guanethidine (Green, 1974) has been shown to inhibit plasma extravasation evoked by intradermal injection of acetic acid, compound 48/80, polymixin B, histamine, 5-HT and thermal injury. Chemical sympathectomy reduces swelling and joint injury in rats with adjuvant arthritis (Levine *et al*, 1985). Adrenaline is thought to affect experimental arthritis by modulating the release of non-catecholaminergic factors from sympathetic post-ganglionic nerve (SPGN) terminals. This hypothesis is based on observations that rats treated with β2-adrenoceptor antagonists exhibit significantly reduced joint injury, whereas non-selective and β1-blockers have no effect (Levine *et al*, 1988). Thus, the action of low-dose adrenaline at presynaptic β2-adrenoceptors would enhance the release of factors such as PGE_2 (Basbaum & Levine, 1991), while high-dose adrenaline would act at presynaptic α2-adrenoceptors to inhibit

the release of these factors (Coderre *et al*, 1991). Also treatment with the β-blocker propranolol, and regional sympathetic block with guanethidine, reduces pain in patients with rheumatoid arthritis (Levine *et al*, 1986), while the arthritis-suppressing effect of high-dose adrenaline is antagonized by the selective α2-antagonist, yohimbine, and mimicked by the selective α2-agonist, clonidine (Coderre *et al*, 1991).

The afferent and sympathetic efferent systems may well interact to stimulate each other and amplify the inflammatory response. Nociceptors activated by the damaged joint will transmit information to the spinal cord and also release pro-inflammatory mediators via the axon reflex, which will in turn exacerbate the inflammation and further excite the C-fibres. At the same time SPGNs are activated either by C-fibre somatosympathetic reflexes or perhaps by excessive general autonomic function (Fitzgerald, 1989). (Rheumatoid arthritis, which normally strikes bilaterally, affects only the unaffected side of stroke victims and there is evidence of stress-induced flare-ups.) The sympathetic axons may then also release inflammatory mediators and further excite C-nociceptors, thus constituting a positive feedback mechanism that leads to severe pain and inflammation of the joint (Fig. 23).

Acute inflammation in the rat knee joint induced by prior carrageenan injection results in an enhancement of the vasodilator responses to SP and CGRP as compared to normal joints (Lam & Ferrell, 1993a). The responsiveness of the rat knee

joint microvasculature to sympathetic nerve stimulation is also substantially altered. Thus, electrical stimulation of the saphenous nerve produces small vasoconstrictor responses in carrageenan-treated knees, whereas in normal knees, potent vasoconstriction is observed (Lam & Ferrell, 1993a). The reduction of the vasoconstrictor response could represent decreased effectiveness of sympathetic neurotransmission in the inflamed joint, alteration of postsynaptic α-adrenoceptors (Gray & Ferrell, 1992), or perhaps liberation of vasodilator substances such as nitric oxide, neuropeptides or PGE_2, any of which could counteract sympathetic vasoconstrictor effects. In pathophysiological conditions, the microvasculature of the joint may be less influenced by its sympathetic innervation and this may result in a higher articular blood flow in the inflamed joint compared to normal.

Electrical stimulation of the saphenous nerve not only activates sympathetic efferent fibres, but also antidromically stimulates peripheral sensory nerve terminals, which results in the release of vasodilator neuropeptides (Yaksh *et al*, 1988). Normally, as mentioned previously, their release produces injury hyperaemia which facilitates the passage of mediators from systemic circulation for repair processes at the site of injury. As a consequence, the sympathetic vasoconstriction of blood vessels in the joint may be reduced. Thus, nerve-induced vasoconstriction is reduced in the presence of exogenously applied neuropeptides (Lam & Ferrell, 1993a). Previous findings

using LDF also confirm this observation for the normal joint (Lam & Ferrell, 1991a; Scott *et al*, 1992b). Of greater importance is that in acutely inflamed joints, sympathetic nerve stimulation in the presence of either SP or CGRP produces vasodilatation but not vasoconstriction of the articular blood vessels (Lam & Ferrell, 1993a). These findings are of significance as they imply that the presence of neuropeptides in inflammatory conditions could abolish sympathetic influences on the microvasculature.

There is no further evidence to substantiate sympathetic involvement in the carageenan model of acute joint inflammation and it has been shown that acute inflammation of rat knee joints induced by carrageenan is unaffected by reserpine pretreatment (Lam & Ferrell, 1991b). Thus participation of sympathetic neurones seems unlikely in this model of acute inflammation.

There is abundant evidence in support of the contribution of afferent nerves and, more specifically, of neuropeptides to inflammation. The involvement of the sympathetic innervation of the joint in the inflammatory process is uncertain. They are more likely to be of minor significance compared to contributions of peripheral sensory neurones in our particular model of acute joint inflammation induced by carrageenan. A bidirectional communication seems to exist between sensory nerves on one hand and inflammatory/immune cells on the other sustained by humoral factors which are released from both sides.

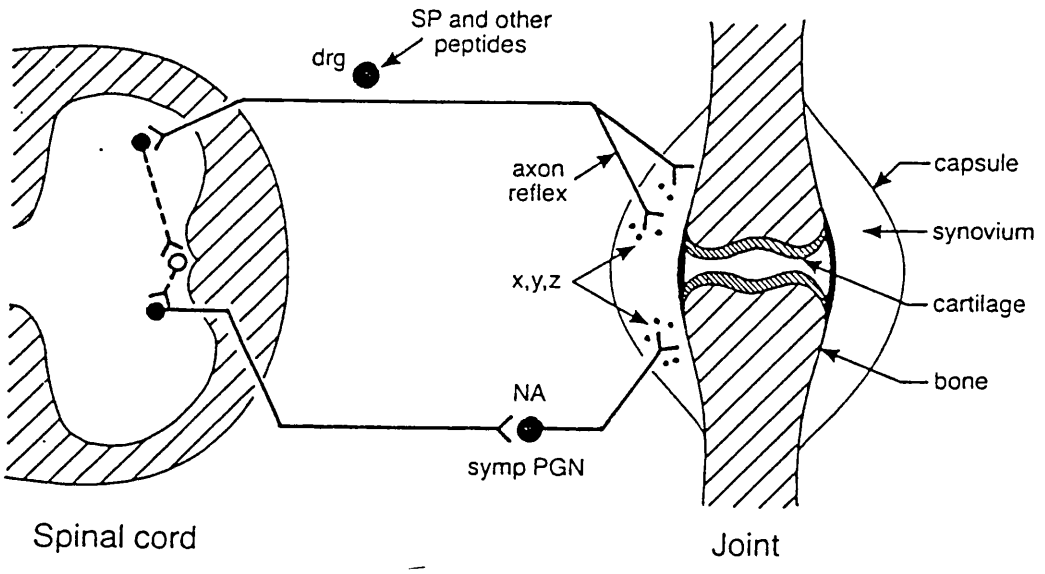


Figure 23: A model for the role of the peripheral branches of the sensory and sympathetic nervous systems on joint inflammation in arthritis.

SP= substance P; NA= noradrenaline; drg= dorsal root ganglion; symp PGN= sympathetic postganglionic neurone; x, y, z= proinflammatory agents released from sensory and sympathetic nerve terminals (Fitzgerald, 1989).

1.8 Joint afferent contribution to kinaesthesia and stataesthesia

Muscle, skin and joint afferents can all contribute to limb movement and position sense, as they all respond to various movements and positions of the limb and have the necessary central projections to convey information to higher centres. Experiments measuring the relevant importance of each group of afferents involve a) quantifying the response of each type of afferent to a range of movements and assessing the suitability of the response to contribute to a sensation of position or movement b) selectively removing the input of each group and reviewing the relative loss in kinaesthesia and position sense c) selectively enhancing the input from each group and reviewing any change in movement and position sense.

Original experiments suggested that the overwhelming majority of joint afferents were only active near the ends of the normal working range of the joints (Skoglund, 1956; Burgess & Clark, 1969a; Clark & Burgess, 1975; Millar, 1975) and the few mid-range afferents found were muscle afferents contaminating the joint nerve (Clark & Burgess, 1975; McIntyre *et al*, 1978). However, this was later contested with the discovery of a considerable number of mid-range units (Ferrell, 1980; Burke, Gandevia & Macefield, 1988). Although fewer joint afferents respond in the mid-range this does not exclude the role of the joint afferent in signalling joint position throughout the range of

movement of the joint. Muscle afferents undoubtedly have the necessary response characteristics to signal limb position (Matthews, 1977; McCloskey, 1978; Wei *et al*, 1984; Clark *et al*, 1985), while cutaneous afferents have limited capabilities (Matthews, 1982; Burgess *et al*, 1982; Ferrell & Milne, 1989). Although muscle afferents would seem capable of signalling limb position unaided, there is evidence to suggest that joint afferents make a significant contribution. Selective removal of joint afferent inputs by local intraarticular injection of anaesthetics or by partial joint deafferentation has resulted in a reduction of kinaesthetic and stataesthetic ability (Brown, Lee & Ring, 1954; Provins, 1958; Gandevia *et al*, 1983; Ferrell *et al*, 1985; Ferrell, Gandevia & McCloskey, 1987). Similarly, if muscle and cutaneous inputs are selectively removed there is a degree of kinaesthesia and stataesthesia maintained (Ferrell *et al*, 1987; Ferrell & Smith, 1988; Ferrell & Craske, 1992). Although removal of joint afferent input indicates a possible involvement in position sense, there may be compensation by the remaining classes of afferent such that the true contribution is masked. There is also considerable integration of the different afferent systems. Information conveyed by muscle spindle afferents is polymodal, as information is integrated from muscle, skin and joints (Johansson *et al*, 1987; Johansson *et al*, 1989). As indicated above capsular and ligamentous joint afferents clearly contribute to information transmitted by muscle spindle afferent via the γ-muscle-spindle system, and these afferents are clearly capable of providing the

CNS with information about the position and movement of the limbs (Hagbarth & Eklund, 1966; Eklund, 1972; Wei *et al*, 1986).

1.9 Joint pain and nociception

Severe chronic pain is a major clinical symptom experienced in rheumatoid arthritis, but despite this, the exact source of joint pain has not been clearly identified.

Specific receptors, responding to stimuli perceived as painful, were first observed in the skin (Blix, 1884). Partial nerve blocking techniques attributed the transmission of well localised pain sensations to Aδ-fibres, and more diffuse pain to C-fibres (Gasser & Erlang, 1929; Clark, Hughes & Gasser, 1935). In general, nociceptors only respond to stimuli capable of causing tissue damage, although in some cases they do respond to innocuous stimuli, which are then perceived as non-painful (Van Hees & Gybels, 1981; Beitel & Dubner, 1976). There is a good correlation between the discharge frequencies of nociceptors and the sensation of pain (Torebjork, 1979; Van Hees & Gybels, 1981), suggesting the involvement of temporal as well as spatial summation in the coding of nociceptive input from the periphery. Pain is a sensation which can only be reported by conscious humans, while in animals, the term nociception is used to describe the sensory sub-modality responding to those stimuli defined as capable of compromising the integrity of the organism (Sherrington, 1906). However, the concept of pain as an adjunct to protective reflexes is not always applicable, as alogens that do not produce tissue damage can elicit pain, and if released by

damaging stimuli, these alogens remain long after the damaging stimuli have been removed (McMahon & Koltzenburg, 1990).

In animals painful stimuli produce reflex responses such as flexion withdrawal of the limb, increased heart and respiratory rates, and pupilary dilatation. It has been shown that in rats, intradermal injection of adjuvant, a suspension of Mycobacterium butyricum, causes an inflammatory condition that resembles rheumatoid arthritis in humans (Pearson & Wood, 1959). Behavioural changes suggest that adjuvant arthritis in the rat is associated with chronic pain (De Castro Costa *et al*, 1981; Landis, Robinson & Levine, 1988). The arthritic rats lose weight, show reduced activity, have fragmented sleep patterns, hyperventilate, are irritable and hyperactive when handled and have increased sensitivity to paw pressure or flexion and extension of inflamed joints. Transection of appropriate ascending spinal tracts responsible for transmitting somatic sensory information in the central nervous system alleviates these symptoms to some extent (Dardick *et al,* 1986). As inflammation develops, the responses to somatic stimuli of spinal dorsal horn neurones (Menetrey & Besson, 1982), thalamic neurones (Gautron & Gilbaud, 1982) and cortical neurones (Lamour, Gilbaud & Willer, 1983) are dramatically changed. Nociceptive-specific spinal neurones with articular input develop increased levels of background activity and show increased sensitivity to light pressure on the inflamed skin, movement of the joint and pressure applied to the joint (Schaible, Schmidt &

Willis, 1987b; Schaible, Neugeauer & Schmidt, 1990). During the acute joint inflammation there are enhanced responses, not only to these stimuli applied to the inflamed limb, but also to stimulation of the normal contralateral limb. Furthermore, analgesics alleviate symptoms of pain (Kayser & Gilbaud, 1981; 1983) and arthritic rats select and develop a preference for solutions containing non-steroidal anti-inflammatory agents or low dose of opiate analgesics (Colpaert *et al*, 1980; Landis *et al*, 1988). Underlying these behavioural changes are neurophysiological and neurochemical changes in the peripheral nervous system and the central nervous system which may reflect long term alterations in central processing as well as changes in primary afferent responses.

Hyperalgesia and inflammatory pain are thought to result predominantly from the actions of various chemical mediators on nociceptive endings within the affected tissues (Lewis, Pickering & Rothschild, 1931; Deneau, Waud & Gowdey, 1953, Smith *et al*, 1966). Various inflammatory mediators excite or sensitize nociceptive endings in joints, including bradykinin (Kanaka, Schaible & Schmidt, 1985), prostaglandins (Schaible & Schmidt, 1988b), 5-HT (Grubb *et al*, 1988) and interleukins (Ferreira, 1989; Scott, 1990).

From the involvement of articular afferent neurones in nociception of the joint, it may be inferred that neuropeptides contained in these fibres are transmitters at the first central synapses of nociceptive pathways. Consistent with such a role are the findings

that electrical stimulation of C-fibre afferents (Yaksh *et al*, 1980) or noxious stimulation of the skin (Duggan *et al*, 1987; Kuraishi *et al*, 1985; Nance, Samynok & Nance, 1987; Oku *et al*, 1987) enhance the release of SP from the spinal cord. Also SP and NKA are both released into the spinal cord during development of arthritis in the cat knee (Schaible *et al*, 1990; Hope *et al*, 1990), while SP and NKA administered intrathecally enhance nociceptive reflexes (Laneuville, Dorais & Couture, 1988). It is very probable that neuropeptides such as neurokinin A (NKA) and calcitonin gene-related peptide (CGRP) co-existing with SP in afferent neurones (Dalsgaard *et al*, 1985; Hanesch *et al*, 1991) are co-released (Hua *et al*, 1986; Saria *et al*, 1988) and function as co-transmitters (Oku *et al*, 1987; Woolf & Wiesenfeld-Hallin, 1986).

METHODS and RESULTS

a) **Electrophysiology**

2.0 Anaesthesia and preparatory surgery

i) Anaesthesia

Inhalation anaesthesia employing a mixture of oxygen, nitrous oxide and halothane is used for both induction and maintenance. Induction involves a semi-closed method with the cat placed in a box ventilated with a mixture of eighty percent nitrous oxide and twenty percent oxygen/halothane mixture, halothane comprising three to four percent of the latter mixture. An adequate size of box, an imperfect seal on the box lid, and a flow rate of anaesthetic agents sufficient to remove exhaled gases reduces re-breathing of exhaled gases to a minimum. When the animal passes through stage II of anaesthesia (Campbell & Lawson, 1958) it is removed from the box and anaesthesia is continued with a face mask. Re-breathing is prevented by a flow rate greater than the animal's minute volume. When the animal has reached stage III, plane 2 of anaesthesia a tracheostomy is performed and the animal is ventilated via an endotracheal tube attached to an Ayers T-piece. Satisfactory anaesthesia is maintained with between one and two percent halothane. The expiratory limb, which has been occluded up to this point, is now opened and the fresh gas flow is set at twice the animal's minute volume to ensure that the exhaled gas is completely removed

from the expiratory limb during the expiratory pause. The volume of the expiratory limb is greater than the animal's tidal volume to prevent inhalation of atmospheric air.

The animal is maintained in stage III, plane 2 of anaesthesia (Campbell & Lawson, 1958). This stage is recognised by a regular automatic respiration of normal physiological character, muscular relaxation, constricted pupils, and absence of palpebral, corneal and pedal reflexes.

Gaseous anaesthesia is the anaesthetic of choice for both induction and maintenance of anaesthesia, due to its rapid elimination from the body and the subsequent return of normal reflex activity. Thus, in experiments requiring normal reflex function, the animal is anaesthetised until decerebration is accomplished, at which time anaesthesia can be discontinued and reflex activity returns. Decerebration is also required when muscle relaxants are in use. Muscle relaxants are used to prevent movement of the animal, especially during electrical stimulation of nerves. The muscle relaxant used here is pancuronium bromide (Pavulon, Organon Technica; 0.2mg/kg every 40 mins.), a non-depolarising, competitive relaxant which acts by competing for nicotinic receptors on the post synaptic membrane of the muscle. The action of approximately thirty minutes is terminated by build up of acetylcholine. During periods of muscle relaxation the animal is artificially ventilated with a respiratory pump.

In experiments not requiring reflex activity, induction is achieved by intra-peritoneal injection of sodium pentobarbitone (Sagatal; May & Baker) at a dose of 45mg/Kg. The animal is maintained at stage III, plane 2 by intravenous doses as required.

ii) Preparatory surgery

a) Tracheostomy

Once stable anaesthesia is achieved, the animal is placed in sternal recumbency and a pretracheal mid-line incision is made through the skin. All skin incisions are preceded by shaving of the skin. The skin is retracted, the superficial muscles covering the trachea separated by blunt dissection and the trachea exposed. The trachea is opened and a glass cannula inserted and tied in position. The glass cannula is connected to the Ayers T-piece by a small length of plastic tube, into which an eighteen gauge needle is inserted. This needle is connected to a pump and CO_2 analyser which enables the continual monitoring of end-tidal CO_2 values. If end-tidal CO_2 values stray below four percent or above six percent, the animal is paralysed (only if decerebrate) and artificially ventilated, the rate and depth of ventilation being adjusted to maintain an end-tidal CO_2 value of approximately five percent.

b) Cannulation

Both carotid arteries are exposed by blunt dissection, freed from surrounding connective tissue and the vagi, and ligated. The left carotid artery is cannulated proximal to the ligation to permit continual monitoring of the blood pressure. The skin incision in the neck is closed by Michel clips.

c) Decerebration

This is performed with the cat in sternal recumbency and its head clamped in a head holder (Roberts, 1951). A mid-line skin incision is made over the skull, the skin reflected and the right temporal muscle dissected free and reflected. A one centimetre hole is drilled in the skull with a trephine and the edges sealed with bone wax to prevent bleeding and the possibility of air embolism. The dura is incised and reflected and the vertebral arteries temporarily occluded by pressing on the wings of the Atlas. An inter-collicular cut is made by incising the brain just rostral to the bony tentorium. The forebrain rostral to this incision is removed and the cranium packed with cotton wool. On ensuring there is no bleeding from the brain stem, the vertebral arteries are released and the skin incision closed with Michele clips.

d) Temperature regulation

The animal's temperature is maintained at thirty eight degrees centigrade by means of a heated operating table thermostatically controlled by a thermistor inserted into the animal's rectum. When the animal is transferred to the experimental frame heating lamps provide the source of heat.

e) Posterior articular nerve dissection

With the animal in sternal recumbency, the left hind-leg extended backwards and the foot pronated, a skin incision is made from a point rostral to the popliteal fossa to a point just rostral to the point of the fibular tarsal bone. The popliteal fat pad is removed, using cautery to prevent bleeding, and the medial head of the gastrocnemius is removed from its insertions on the Achilles tendon and the caudal aspect of the femur. This exposes the caudal aspect of the joint capsule and the PAN, which is identified as a small nerve branching from the posterior tibial nerve. The PAN always splits into two branches at its most distal portion, but occasionally this split continues more proximally. The PAN is dissected free from any surrounding connective tissue, care being taken to leave the blood supply to the nerve intact.

f) Laminectomy

A mid-line skin incision is made from the second lumbar vertebra to the base of the tail. The skin is retracted and the longissimus dorsi muscles are separated from the lumbar multifidus muscles and retracted. The multifidus muscles are cleared from the vertebral column and removed. The dorsal parts of the fourth and fifth lumbar vertebrae (L4 and L5) are removed using small bone nibblers. This is achieved by raising the vertebral column. This procedure opens the dorsal aspect of the inter-vertebral joints and ensures the spinal cord remains on the floor of the vertebral canal. One jaw of the nibblers is inserted between the vertebrae, and the dorsal aspect of the vertebra along with the dorsal longitudinal ligament is removed with the nibblers.

g) Spinalisation

This is achieved in the same manner as the laminectomy. An incision is made from T11 to T13 and the dorsal aspect of T12 is removed. The spinal cord is injected throughout its depth, with 0.1 ml lignocaine, before sectioning. This prevents excessive activity descending in the spinal cord when it is sectioned. A small section of spinal cord is removed to ensure complete transection.

h) Securing the animal in the experimental frame.

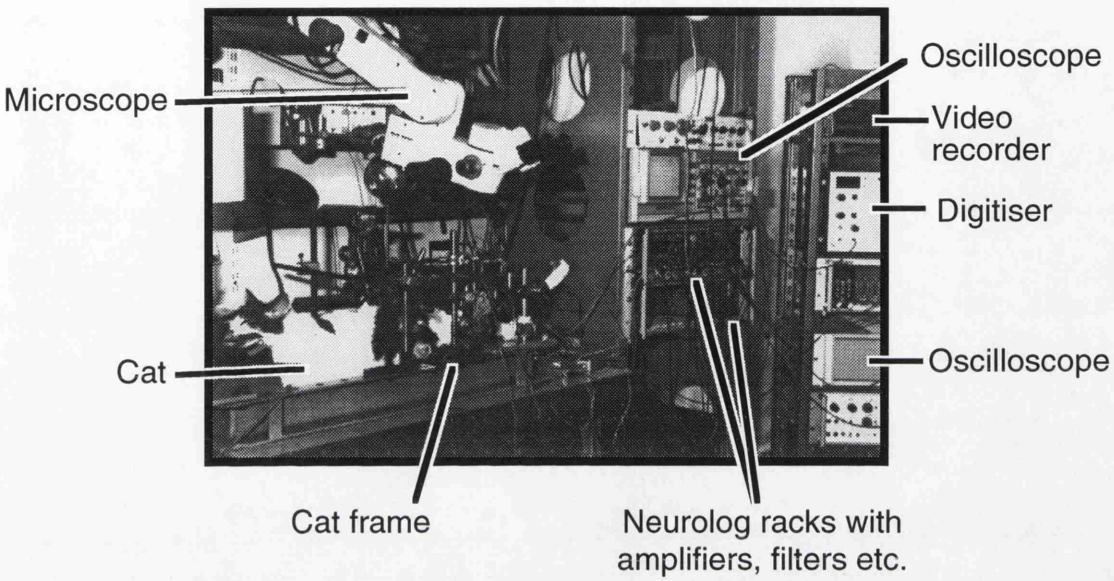
After all the surgery has been performed the animal is transferred to the experimental frame. Knitting needles are passed through the inter-spinous ligaments behind the shoulders and in front of the hips. The spinal supporting needles and head holder are held securely in the frame. The left leg is secured by the paraffin pool with the knee slightly bent and supported by the base of the frame.

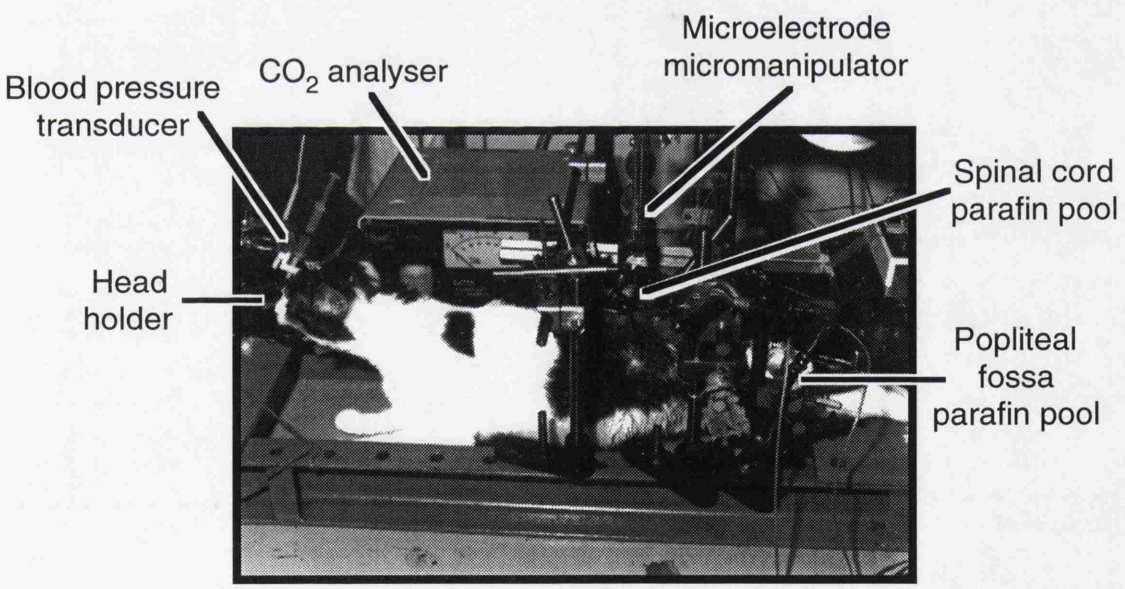
With experiments involving stimulation of the spinal cord, the spine is held more rigidly with clamps gripping the dorsal processes of the spinal vertebrae (Fig. 24). There is also a micromanipulator incorporated into the frame to allow precise positioning of the stimulating electrode.

i) Creation of paraffin pools

Paraffin pools are formed around the popliteal fossa (Fig. 25) and, when present, around the laminectomy. Both pools are formed by stitching the edges of the skin incision to brass rings. The rings are big enough to provide access to the PAN or spinal cord in the pool. The skin is pulled high enough to form a pool deep enough to cover all the nerves. The pools are filled with liquid paraffin (previously equilibrated with 0.9% saline) at thirty eight degrees centigrade, the temperature of which is

Figure 24: The experimental arrangement, showing the cat held in the frame during an experiment involving spinal stimulation. The cat is decerebrate and spinalised, a laminectomy has been performed and the PAN has been exposed. Paraffin pools have been created around the laminectomy and the popliteal fossa. A tungsten microelectrode is held in the micromanipulator above the spinal cord.


Microscope
Oscilloscope
Video recorder
Digitiser
Cat
Oscilloscope
Cat frame
Neurolog racks with amplifiers, filters etc.

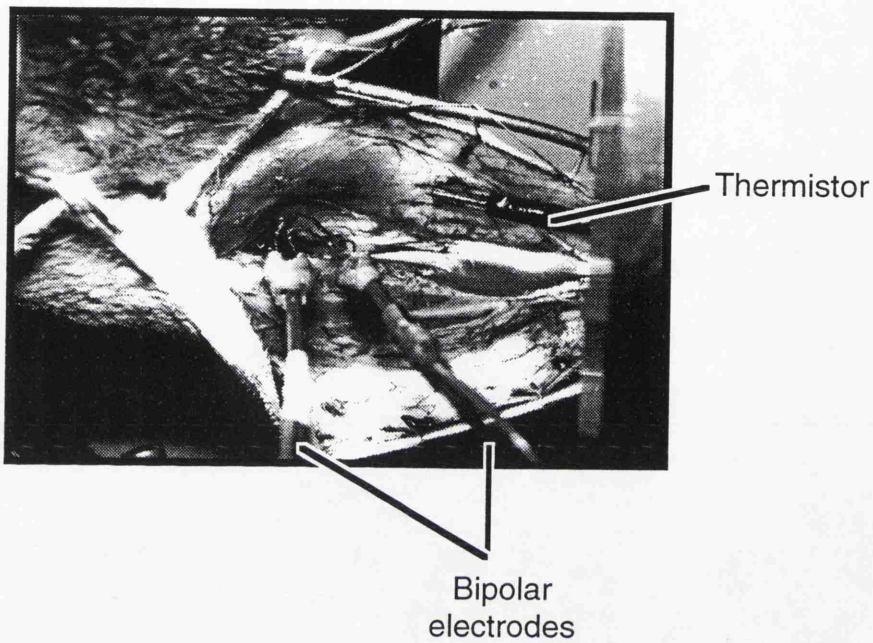

Blood pressure transducer
CO_2 analyser
Microelectrode micromanipulator
Spinal cord parafin pool
Head holder
Popliteal fossa parafin pool

maintained by the heating lamp which is thermostatically controlled by a thermistor in the pool.

j) Microdissection of nerve filaments

Lateral gastrocnemius (see later) or PAN filaments are dissected further to enable recording of single units. This is achieved by placing the free end of the nerve on black silk to provide a contrasting background. As much connective tissue as possible is removed. A high power dissecting microscope (Wild Heerbrugg M650) is used to view the teasing of all the connective tissue from the nerve with very fine forceps (No. 5 forceps ground to very fine points). The nerve is then split by using the fine forceps to pick up two halves of the nerve and tease them apart. This process is repeated with each resulting filament until a single unit is found. This is determined by placing each filament over recording electrodes and observing the spontaneous or evoked neuronal discharge. Uniformity of size and shape of the spike and inter-spike intervals of greater than 1ms indicate a single unit. Triggering the oscilloscope with the spike will produce a quiet period for at least 1ms following the trigger, if a single unit is present.

Figure 25: The popliteal paraffin pool with the PAN dissected into two halves. Each half of the PAN is placed on a bipolar electrode. An earth electrode is placed at the bifurcation of the two halves of the PAN during the experiment.


Thermistor
Bipolar
electrodes

2.1 Recording and processing muscle (electromyogram) and nerve (electroneurogram) potentials

Action potentials from the PAN or lateral gastrocnemius nerves are recorded with bipolar platinum electrodes. These potentials are amplified by a factor of one or two thousand with a Neurolog AC preamplifier (NL104). The preamplifier is set to make differential recordings between the two parts of the electrode by adjusting the input switch to the A-B position. The balance control is adjusted to minimise common mode signals such as mains interference or shock artifacts present in the recording. The low resistance of the electrodes limits any reduction in frequency response and obviates the need for a headstage. The output from the preamplifier is filtered to between three hundred and three thousand hertz by a Neurolog filter (NL125). The output from the filter is lead to a digitising oscilloscope (Tektronix 5A 18N dual trace amplifier with 5B 25N time base amplifier and 5223 double beam with memory) and also to a PCM-4/8 digitiser (PCM8) (Medical Systems Corp.). The information is digitised for storage on magnetic tape.

Muscle potentials are recorded with dual wire needle electrodes and processed in the same fashion as nerve potentials, other than the filter setting restricting the output frequency to between one hundred and one thousand hertz.

2.2 Electrical stimulation of the PAN and spinal cord

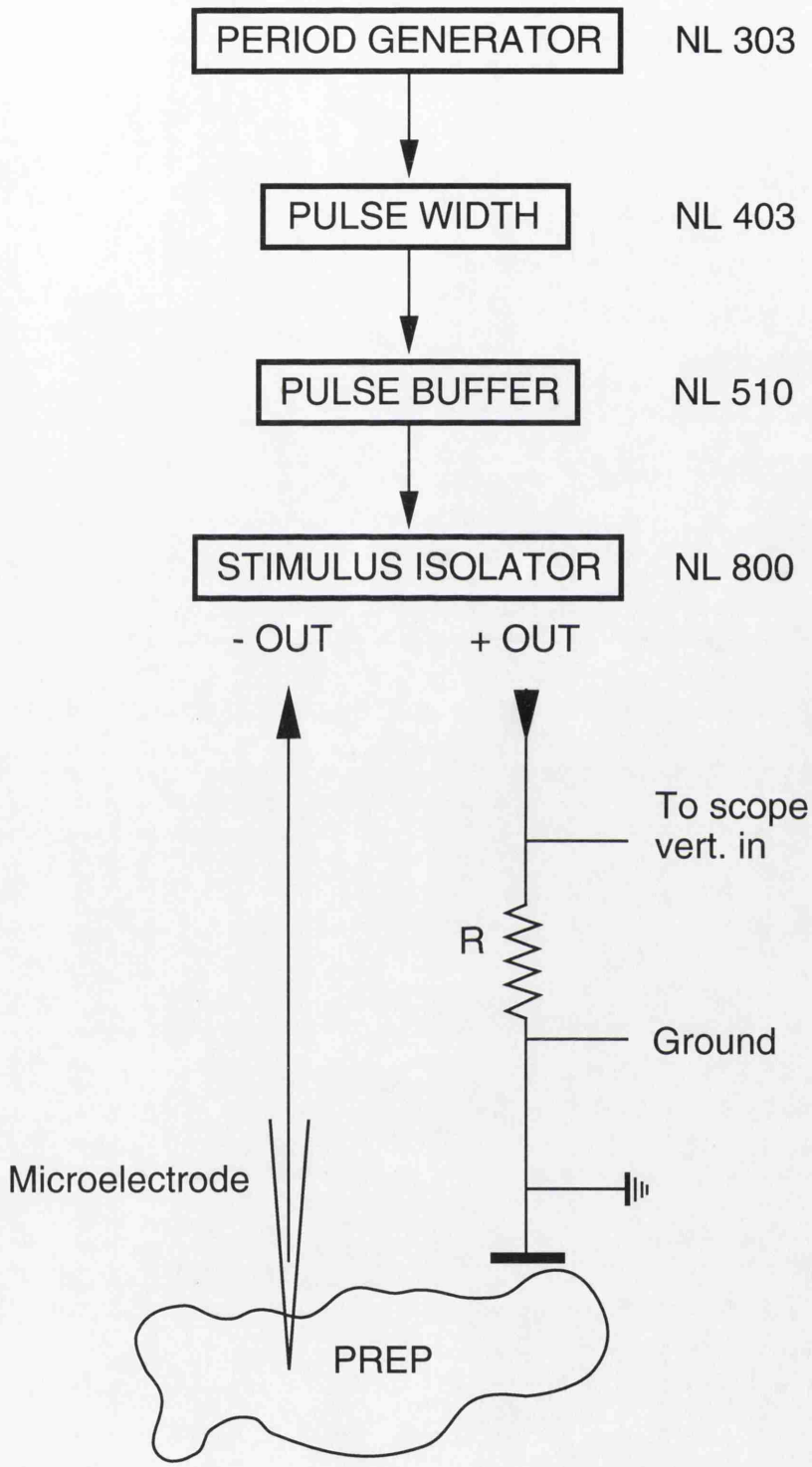
The constant current electrical stimulus to the PAN or spinal cord is delivered by a Neurolog stimulus isolator (NL800), a Neurolog pulse buffer (NL510) providing the appropriate input drive pulses. The NL800 power switch is turned on, the output range is selected and the desired output amplitude is produced at the two output terminals by adjusting the amplitude dial (10 turn) on the NL510. The pulse buffer is driven by TTL pulses from a Neurolog delay-width module (NL403), which can be set to deliver pulses of varying width. The NL403 is triggered by TTL pulses from the Neurolog period generator (NL303).

The maximum voltage which can appear across the output terminals of the stimulator is limited by the internal battery supply of approximately forty one volts. This voltage determines the maximum output current for a given load resistance, according to Ohm's law (V=I.R). As the load resistance of the PAN is unknown, the actual output current can be measured by placing a resistor of known resistance (13 Ohms) in series with the stimulating bipolar electrode. Measurement of the voltage across this resistance allows determination of the actual output current (Fig 26).

This circuit is also used to measure the current delivered to the spinal cord by the tungsten microelectrodes. The current passing through the electrode is measured as above. The stimulating electrode can also be connected to an NL100

headstage, which allows measurement of the voltage across the electrode. Knowledge of both the current through and voltage across the microelectrode allows the electrode impedence to be calculated. Microelectrodes are glass coated tungsten. The terminal characteristics of these metal microelectrodes can be represented by a capacitor and resistor in parallel; the distribution of charge into a double layer at the electrode-electrolyte interface providing the capacitive component. As there are both resistive and reactive components, the electrode impedence is frequency-dependent and electrodes have much lower effective series reistances for narrow pulses than than for DC inputs. The pulse width of 0.1 ms, used with spinal stimulation, is sufficiently short to allow currents of up to 400μA to be delivered by the stimulus isolator (a typical tungsten microelectrode with a DC resistance of 200 Mohms will have an impedance of only 100 kohms for a 0.1 ms pulse, which limits the stimulator output to 400μA if the battery is at 40V), which is sufficient for activation of the spinal terminals of afferents. The measurement of electrode impedance gives an indication of the quality of the electrode tip.

Figure 26: A schematic diagram of the circuit used to stimulate nerves or the spinal cord. Measurment of the voltage drop across the resistor (R) gives a measurement of the stimulating current. When stimulating peripheral nerves, the microelectrode is replaced with a bipolar electrode.


PERIOD GENERATOR
NL 303
PULSE WIDTH
NL 403
PULSE BUFFER
NL 510
STIMULUS ISOLATOR
NL 800
- OUT
+ OUT
To scope
vert. in
R
Ground
Microelectrode
PREP

2.3 Frequency response of rapidly and slowly adapting joint afferents

a) Objectives

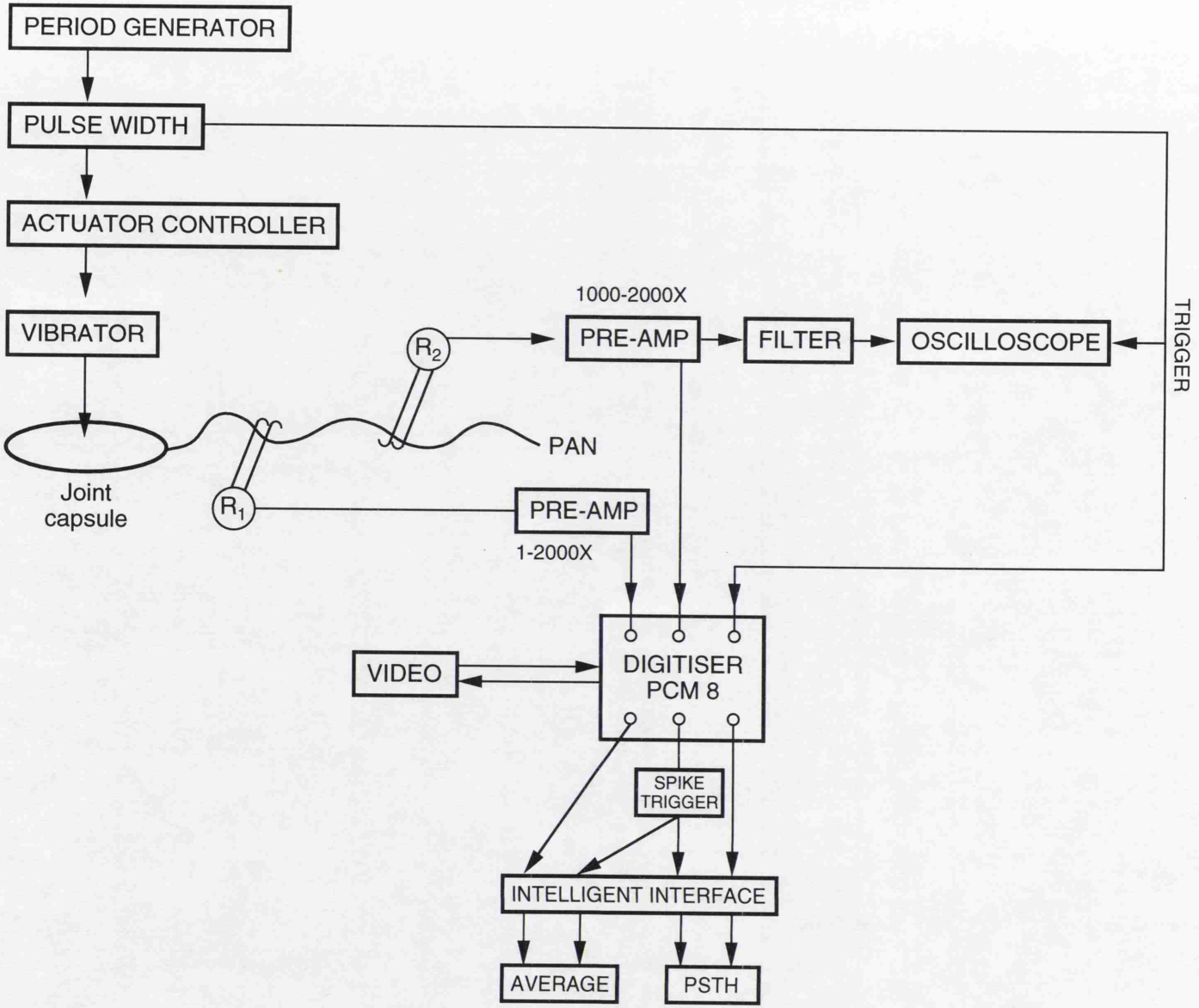
In recent studies involving restricted mechanical stimulation (R.M.S.) applied perpendicularly to the joint capsule of the posterior aspect of the cat knee, potent reflex effects on quadriceps motor units were observed (Baxendale *et al*, 1988; Ferrell *et al*, 1990). However, the nature of the receptors which were activated with this form of mechanical stimulation, and were therefore responsible for these effects, was not investigated. The present study aimed to determine whether slowly and rapidly adapting afferent fibres can be distinguished by R.M.S. at various frequencies of vibration. Electrical stimulation of articular nerves is of limited usefulness in studies of the differential effects of group II joint afferents as it excites both slowly and rapidly adapting fibres, since there is no clear separation of their axon diameters (Boyd & Davey, 1968) and therefore of their electrical thresholds.

b) Methods

Cats are deeply anaesthetised with pentobarbitone (45mg/kg i.p.) and anaesthesia maintained with additional doses as required. Some cats had been decerebrated under gaseous

anaesthesia for a previous experiment, and only required further anaesthesia to eliminate movement. The PAN is dissected free, sectioned proximally and the distal segment microdissected into filaments. Individual afferent units can then be recorded by placing individual filaments over bipolar platinum recording electrodes. The intact distal portion of PAN is placed over another bipolar recording electrode to allow the conduction velocity to be calculated by peri-stimulus averaging, using the spike recorded from the filament as the trigger. The removal of the medial gastrocnemius muscle during the PAN disection exposes the posteromedial aspect of the joint capsule. The popliteus muscle is tenotomised and extirpated to eliminate any activity of popliteal afferents in the PAN. The search stimulus consists of prodding of the capsule with a glass rod, tip diameter ~1mm. The units are identified as joint afferents by their response to punctate pressure on the capsule surface, and further classified as slowly adapting by a maintained discharge to sustained indentation elicited with the probe in a clamped position, or by sustained discharge on extension of the knee joint. The mechanical probe is then placed on the receptive spot and the probe advanced until just below the discharge threshold of the unit. The probe is then switched on at a frequency of 1Hz and the amplitude of displacement adjusted until the unit responds with one impulse to each indentation (ratio of 1 : 1). Frequencies are then increased. The probe is 1mm. in diameter and driven by an electromechanical vibrator (Ling dynamics

Figure 27: A schematic diagram of the circuit used to activate joint receptors and record from the PAN afferents.
R1 = Whole PAN recording
R2 = Single unit recording.


PERIOD GENERATOR
PULSE WIDTH
ACTUATOR CONTROLLER
VIBRATOR
Joint capsule
R_2
R_1
PAN
1000-2000X
PRE-AMP
FILTER
OSCILLOSCOPE
TRIGGER
PRE-AMP
1-2000X
VIDEO
DIGITISER PCM 8
SPIKE TRIGGER
INTELLIGENT INTERFACE
AVERAGE
PSTH

model no. V101) which is in turn driven by an amplifier (actuator controller). The amplifier is triggered by a rectangular TTL pulse of 2ms width, emanating from a variable delay and pulse width Neurolog module (NL403). The pulse width unit is triggered by TTL pulses from a period generator (NL303) (Fig. 27). This generator can deliver pulses at frequencies from 0.01 Hz to 1,000,000 Hz, selected by combining the settings on two rotating switches. A pulse width of 2ms is selected to drive the vibrator because this width produces the most suitable combination of movement amplitude with the smallest reduction in movement amplitude of the vibrator tip with increasing frequencies of vibration. The amplitude of displacement of the vibrator probe is adjusted by altering the potentiometer on the actuator controller. The displacement of the mechanical probe at various frequencies is calibrated with a compliant displacement transducer (Grass) and also by viewing the displacement of the probe tip relative to calibrated etchings on a glass slide.

c) Recording and analysis

Single unit action potentials are recorded, via one channel of the PCM8, onto magnetic tape. The multi-unit recording of the more distal electrode, which is recording from the whole PAN, is recorded onto magnetic tape via another channel of the PCM8. The output of the period generator (NL303), which triggers both

the vibrator and oscilloscope, is recorded via a third PCM8 channel (Fig. 27).

This data which is collected on magnetic tape is subsequently analysed when the analogue signal is reconstituted by passing the digitised recording back through the PCM8. Each spike is then converted into a single short duration (15μS) pulse of plus 2.3 volts (TTL pulse) by passing the analogue signal through a Neurolog spike trigger (NL201). A window is set on the spike trigger, between two voltage levels, and if the voltage of the tip of the spike falls within this window a TTL pulse is generated. The monitor output socket provides a display of the input signal with lower and upper levels of the window superimposed. The output is led to one channel of a 1401 intelligent interface (Cambridge Electronic Design Ltd.), while the TTL pulses from the period generator are led to a separate channel. The output from the interface can now be analysed by computer. The MRate programme (Cambridge Electronic Design Ltd., November 1986) is used to analyse the spike processor output of TTL pulses in relation to the output of TTL pulses from the period generator i.e. the afferent neurone discharge in relation to displacement of the mechanical probe. Using the post stimulus time histogram (PSTH) setting on the MRate programme, the histogram is triggered with each pulse from the period generator. Each subsequent afferent discharge is recorded at a time relevant to the trigger, until the next pulse from the period generator re-triggers the histogram and the process is repeated. One set of

values on the histogram is termed a "bin" and the period of time relating to this set of values is termed the "bin width". The bin width can be varied, and is set for each experiment to encompass all relevant discharges in the period of interest.

The Signal Averager programme (Cambridge Electronic Design Ltd., November 1986) is used to measure the afferent discharge at one location on the nerve, in relation to the same discharge at a different location. The single unit recording is converted into a series of TTL pulses by the spike trigger as described above, and each pulse triggers the sweep of the averager. The raw analogue signal from the more distal bipolar electrode is led into a separate channel of the 1401 interface and this signal is plotted for a set time around each trigger. Each time the sweep is re-triggered the overall average value of all the sweeps is plotted. The analogue signal from the distal bipolar electrodes is therefore averaged relative to the single unit discharge, and only events tightly time-linked to the single unit discharge will not be reduced to imperceptible voltage levels by subsequent sweeps. The action potential travelling orthidromically up the PAN from the joint will be recorded, along with other potentials, by the more distal electrodes at a fixed time interval prior to being recorded as a single unit by the more proximal electrode. This spike in the distal electrode recording will therefore not be reduced in size by subsequent sweeps and is therefore identified as the same spike as that triggering the sweep. This gives an accurate measure of the time taken for the action potential to

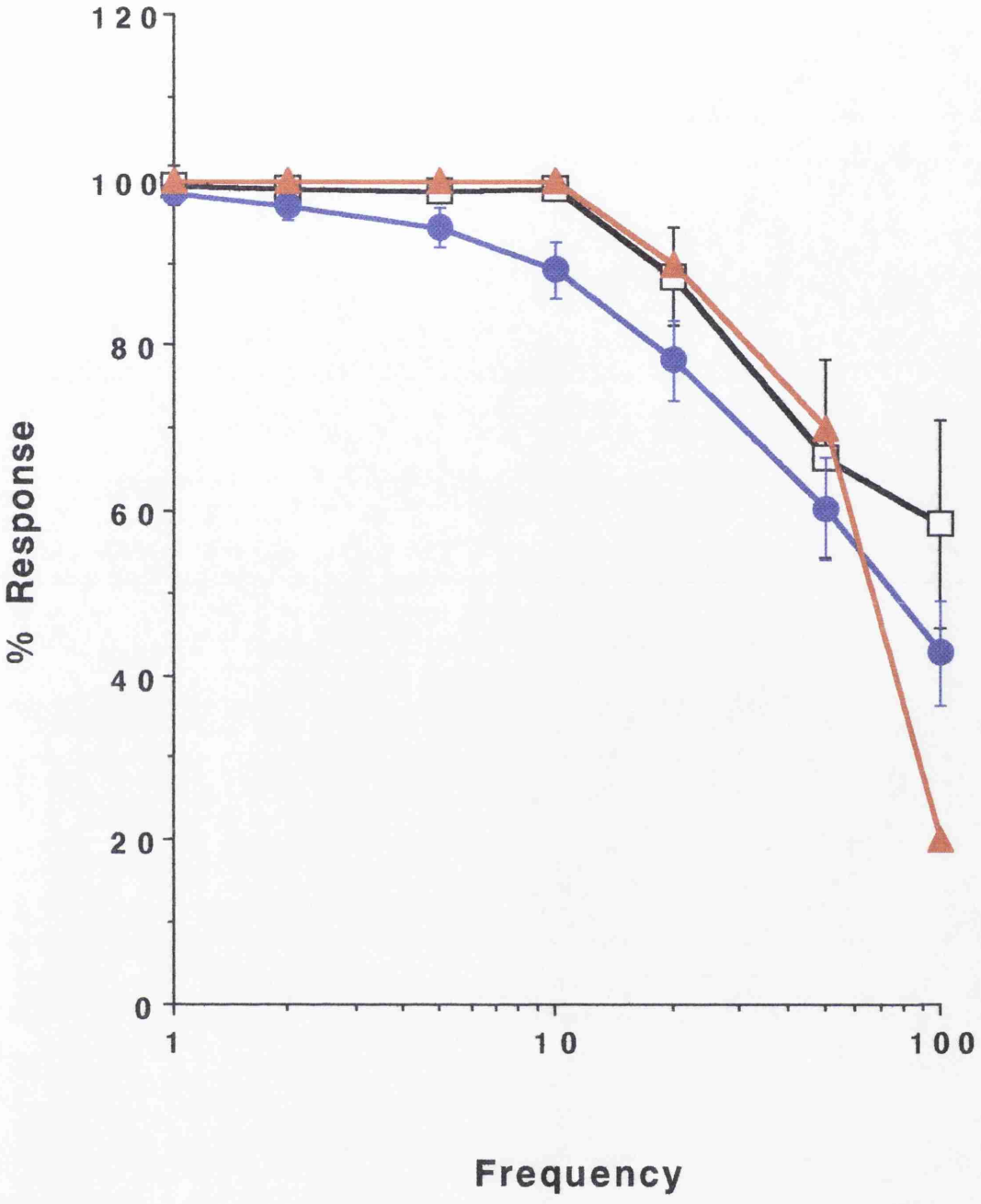
travel between the closest two points of the two electrodes. The conduction velocity of the afferent nerve is calculated by dividing this distance by the travelling time.

d) Results

Of 61 afferents recorded in 12 experiments, 46 were classified as slowly adapting and 15 were rapidly adapting. Nine (60%) of the rapidly adapting fibres could be activated by a 5g von Frey bristle, compared to only 21 (46%) of the slowly adapting fibres. The rapidly adapting receptors all had larger receptive fields than any of the slowly adapting receptors. The conduction velocities of the rapidly adapting afferents (n=9) ranged from 40 to 85m/s with a mean (± SEM) of 57.8 ± 7.6m/s whilst the slowly adapting afferents (n=15) ranged from 35 to 75m/s (49.3 ± 4.5m/s). This overlap of conduction velocities between the two groups of afferents is in good agreement with previous findings (Boyd & Davey, 1968).

The response of the different classes of afferent to various frequencies of capsule indentation was similar (Fig. 28). This was almost at a ratio of 1:1 up to 10Hz, and then decreased directly with an increase in the log of the frequency. Both rapidly adapting and slowly adapting units could respond at frequencies of 100Hz, although a greater percentage of rapidly adapting units respond at this frequency.

Figure 28: Responses (means ± SEM) of rapidly adapting PAN afferents (❐) and slowly adapting PAN afferents (●) to restricted mechanical stimulation (RMS) of the posterior region of the cat knee joint capsule as a function of the frequency of capsule indentation. Responses are described as percentages of the maximum (100%) where a 1:1 ratio occurs. Displacement of the transducer as a percentage of the maximum is also shown (▲).


120
100
80
60
40
20
0
% Response
1
10
100
Frequency

The maximal amplitudes that could be delivered by the probe with the rectangular waveform applied to the vibrator were 500μM at 1-10Hz, 450μm at 20Hz, 350μM at 50 Hz and 100μM at 100Hz. Displacement amplitudes measured with the transducer were the same as when viewed under the microscope. In one experiment different waveforms (sinusoid, square, rectangle and triangle) from a signal generator (Feedback, PGF605) were applied to the vibrator, and individual receptors were driven by the different waveforms. There was no difference in response to different waveforms.

2.4 Reflex effects of group IV joint afferents on γ-motoneuron excitability

a) Objectives

Electrophysiological studies have shown that group II (Aβ) joint afferents can exert powerful short latency reflex actions on α-motoneurones (Baxendale *et al,* 1988; Ferrell *et al,* 1990) and that these effects change when tested against a background of articular nociceptive activity (Baxendale, Ferrell & Wallace, 1989). Electrical stimulation of group II joint afferents has also been shown to elicit mixed excitatory and inhibitory actions on γ-motoneurones in the cat hind limb (Johansson *et al,* 1986). Acute knee joint inflammation enhances the spontaneous activity of flexor γ-motoneurones and their responsiveness to limb movement (He *et al,* 1988), but the reflex response to other inputs and the effect on extensor γ-motoneurones has not been investigated. Data are presented here concerning the effect of electrical stimulation of group IV joint afferent fibres on extensor γ-motoneurone excitation, previously induced by electrical stimulation of group II joint afferents.

b) Methods

Eight adult cats of either sex were anaesthetised with nitrous oxide and halothane (1-3%) in oxygen. The carotid arteries are

tied before decerebration is performed by intercollicular section of the brain stem and removal of the hemispheres. Following decerebration the anaesthetic is discontinued and the animals are paralysed with pancuronium bromide (Pavulon, Organon Technica, 0.2mg/30 mins.) and artificially ventilated. A spinal section is performed at the level of the twelfth thoracic spinal segment. Blood pressure, end-tidal CO_2, rectal temperature, and the temperature of liquid paraffin pools covering nervous tissue are monitored and kept within normal limits. The PAN is dissected free and placed over platinum stimulating electrodes distally, and recording electrodes proximally. One of the nerve fascicles supplying the lateral head of the gastrocnemius/soleus muscle is desheathed and filaments are dissected to enable the recording of single γ-motoneurone units using fine platinum electrodes. The exposed tissues are bathed in warm paraffin oil previously equilibrated with 0.9% saline. Axonal conduction velocities of the spontaneously active γ-motoneurones are determined by pretrigger averaging of the recordings made from bipolar electrodes on the lateral gastrocnemius nerve (30-40mm from the trigger site), triggered from impulses recorded at the peripheral site. The criteria used for identifying γ-motoneurones are based on axonal conduction velocities and discharge characteristics (Ellaway & Trott, 1978). Neurones with conduction velocities below 40m/s which are spontaneously active and have a spike to noise ratio of less than four to one are selected as γ-motoneurones. The firing rate of these neurones can

usually be altered by rubbing the skin over the fibular tarsal bone (Davey & Ellaway, 1989), although this was not taken as a prerequisite for γ-motoneurone identification. α-motoneurones, on the other hand, have conduction velocities above 40m/s, do not fire spontaneously, do not respond to rubbing of the skin, and generally have a signal to noise ratio of greater than ten to one in a well dissected nerve.

Nerve impulses in the lateral gastrocnemius nerve are simultaneously viewed on an oscilloscope and recorded on magnetic tape, in the same manner as that described above for PAN afferents. The discharge of each γ-motoneurone isolated is examined during electrical stimulation of the PAN. This electrical stimulus is a constant current pulse 0.2ms wide, the intensity of which is increased until a period of increased probability of γ-motoneurone firing occurs (Fig. 29A). The threshold of the most excitable PAN afferents is determined by increasing the stimulus current until the early part of an A-wave can be seen in the PAN recording. This current is noted as the PAN threshold current, and further increases in current are referred to in multiples of the PAN threshold. Bipolar electrodes situated 19mm to 35mm proximal to the stimulating electrodes are used to record from the PAN. Neurones in the lateral gastrocnemius nerve which do not show obvious periods of excitation following PAN stimulation are discarded.

Those γ-motoneurones exhibiting periods of increased excitability in response to the above test stimulus to the PAN are

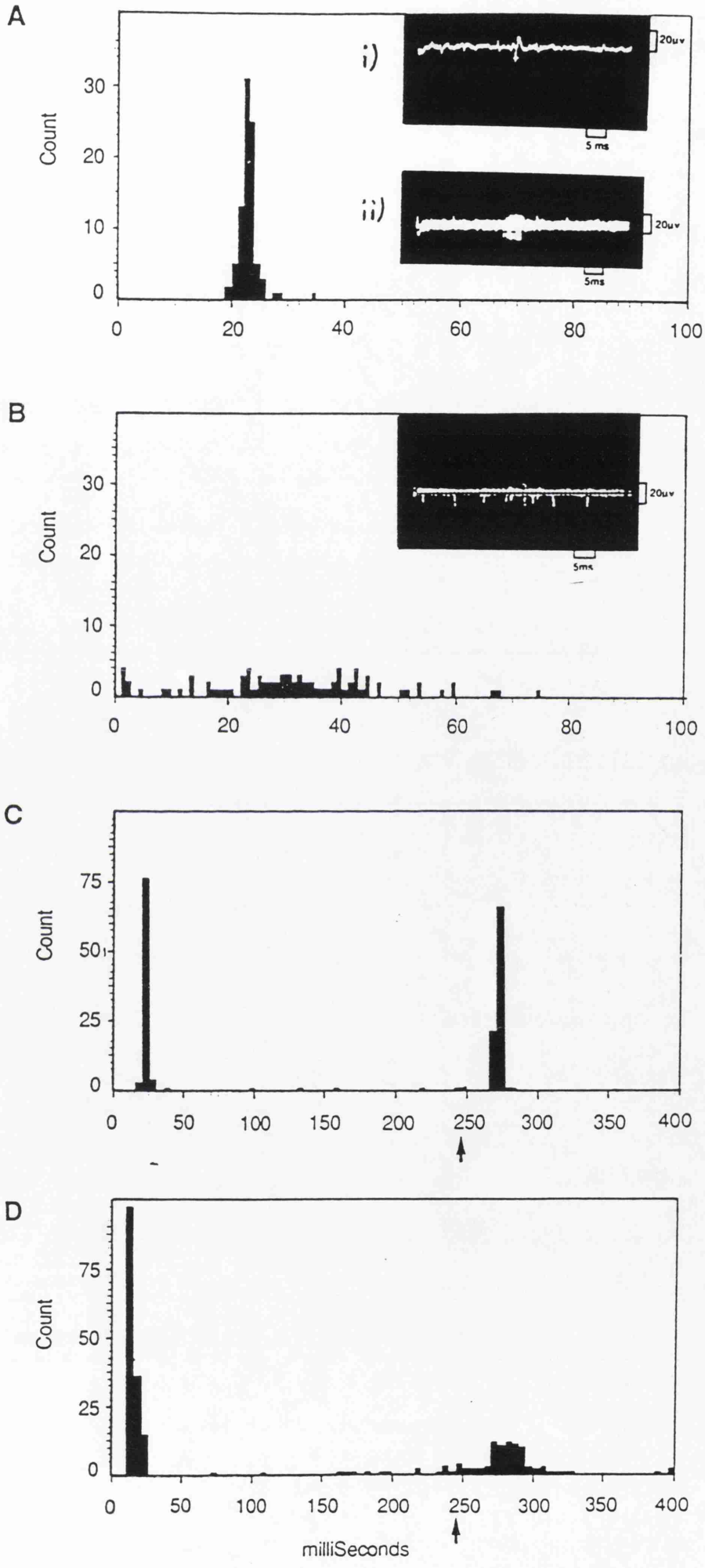
Figure 29: Post stimulus time histograms (PSTH) of the response of a γ-motoneurone to electrical stimulation of PAN.

A: Control experiment involving a low intensity (50μA) conditioning stimulus to PAN, followed by another 50μA (test) stimulus after a 240ms delay. The sweep was triggered by the second (test) stimulus. Inset (i) shows a single sweep recording from the neurone whilst inset ii shows twenty superimposed sweeps. Linkage between the stimulus and the neurone is clearly evident in the PSTH (100 sweeps). Mean firing rate = 2.5 impulses/s. Bin width = 1ms.

B: As in **A** but with the preceding conditioning 50μA stimulus pulse train replaced with a high intensity (supramaximal) conditioning stimulus. Mean firing rate = 1.74 impulses/s. Linkage has been much reduced (also seen in the inset i).

C: Same unit and parameters as in **A**, but the sweep now triggered by the 50μA conditioning stimulus (time 0), with the test stimulus denoted by the arrow. Bin width = 5ms.

D: As in **B**, but the sweep triggered by the high intensity conditioning stimulus.


A
Count
30
20
10
0
0
20
40
60
80
100
i)
20uv
5 ms
ii)
20uv
5ms
B
Count
30
20
10
0
0
20
40
60
80
100
20uv
5ms
C
Count
75
50
25
0
0
50
100
150
200
250
300
350
400
D
Count
75
50
25
0
0
50
100
150
200
250
300
350
400
milliSeconds

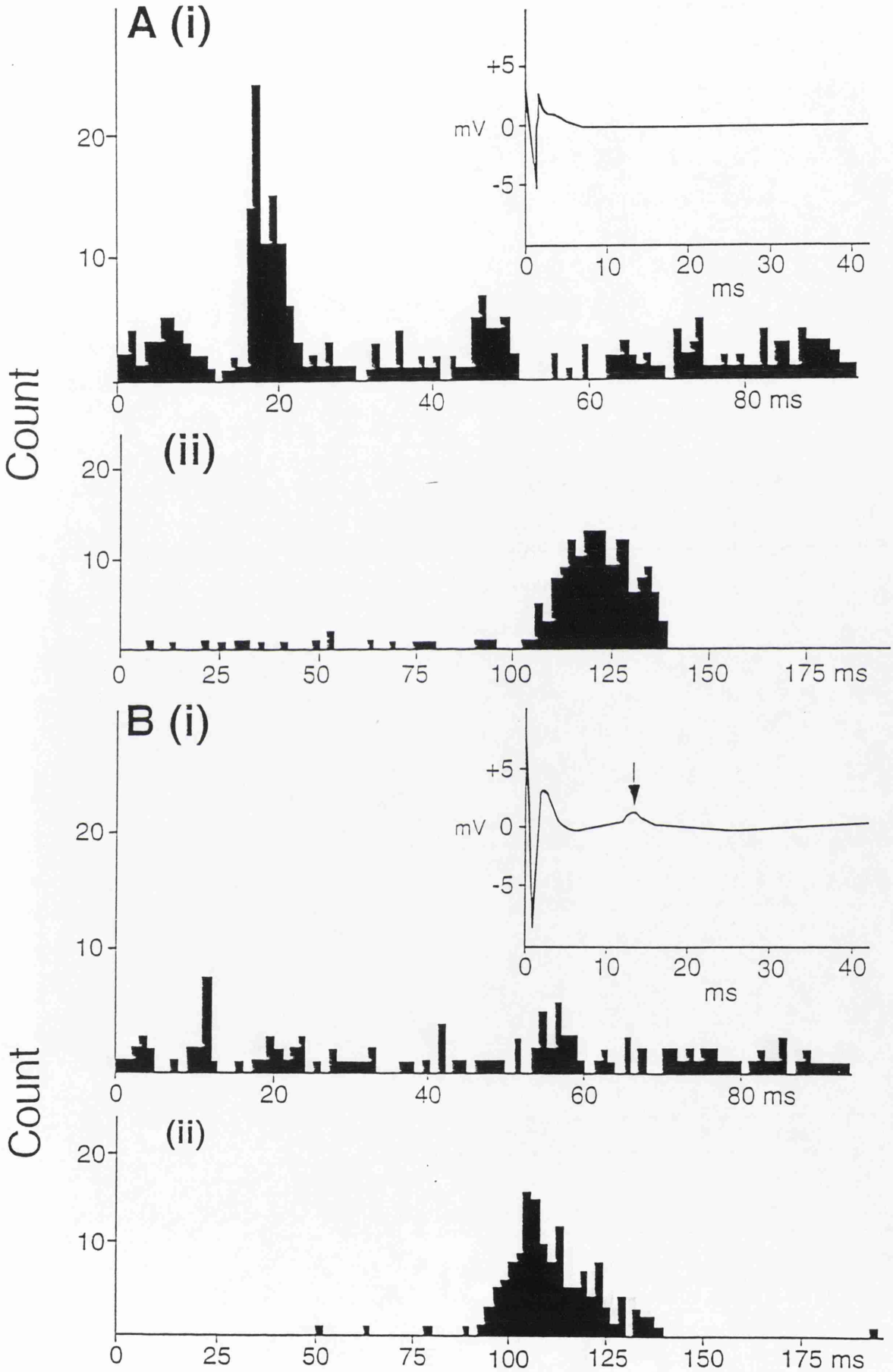
used to monitor the effect of a conditioning stimulus to PAN, which precedes the test stimulus. This conditioning stimulus is of maximum current intensity, 0.5ms wide, and precedes the test stimulus by periods varying from 75ms to 1s. This stimulus is sufficient to activate all group IV joint afferents, as indicated by the size of the C wave in the compound action potential being maximal (Fig. 30B). The frequency of both stimuli is between 0.5 and 2Hz, and the conditioning to test intervals are, of course, limited by this frequency of stimulation. The frequency of PAN stimulation is adjusted to maintain a stable firing rate in the γ-motoneurone and is limited to this range of frequencies as faster repetition rates significantly enhances γ-motoneurone discharge rate when high intensity stimulation (>group IV threshold) is used. Slow rates (0.5Hz) are used with neurones with the greatest tendency to increase firing rate in response to high intensity PAN stimulation, and also when intervals between conditioning and test stimuli became longer. A control experiment involves replacing the high intensity conditioning stimulus (Fig. 29D) with one of similar intensity to the test stimulus (Fig. 29C). This control procedure shows that any difference in response is due to recruitment of the highest threshold fibres in PAN by the high intensity conditioning stimulus.

The PAN stimulation is controlled by one period generator (NL303) which supplies two separate delay-width modules (NL403). The width of the test and conditioning pulses are

Figure 30:

A(i) Post stimulus time histogram(PSTH) of a γ-motoneurone, with inset of averaged PAN compound action potential (100 sweeps) during period of low intensity conditioning stimulation of PAN. PSTH sweep triggered by test stimulus. PAN average triggered by conditioning stimulus.
(ii) Interval histogram of the same γ-motoneurone during the period of low intensity stimulation of PAN shown in A(i).

B(i) Post stimulus time histogram of the same γ-motoneurone as in A, with inset of PAN compound action potential during a period of high intensity conditioning stimulation of PAN. C-wave denoted by arrow. Conduction velocity at this point is 1.5m/s.
(ii) Interval histogram of the same γ-motoneurone during the period of high intensity conditioning stimulation of PAN shown in B(i).


A (i)
+5
mV 0
-5
0 10 20 30 40
ms
20
10
0 20 40 60 80 ms
Count
(ii)
20
10
0 25 50 75 100 125 150 175 ms
B (i)
+5
mV 0
-5
0 10 20 30 40
ms
20
10
0 20 40 60 80 ms
Count
(ii)
20
10
0 25 50 75 100 125 150 175 ms

independently controlled by these modules, and the interval between test and conditioning pulses is set by introducing a delay to the output of the module supplying the test stimulator. Stimulation is provided by two constant current stimulators/pulse buffers (Neurolog NL 800/NL 510) driven by the delay-width modules. The frequency of stimulation is set by the period generator (NL303) (Fig. 31).

c) Recording and analysis

Recorded data is subsequently digitised and analysed by computer to prepare post stimulus time histograms (PSTHs). A spike discriminator (NL201) is used to convert the analogue recording of the γ-motoneurone into TTL pulses, each time the spikes pass through the window of the discriminator. These pulses are led to one channel of the PCM8 digitiser, while pulses from each of the delay-width modules supplying the pulse buffers are led to another two channels (Fig. 31). The total number of spikes can then be displayed in relation to the electrical stimuli to PAN by displaying γ-motoneurone discharge in a PSTH triggered by the electrical stimulus to the PAN (MRate; Cambridge Electronic Design Ltd., November 1986).

The spontaneous discharge rate of γ-motoneurones is measured by counting the total number of times the neurone fires over a one hundred second period, and expressing this as impulses per second. The MRate programme is also used in the

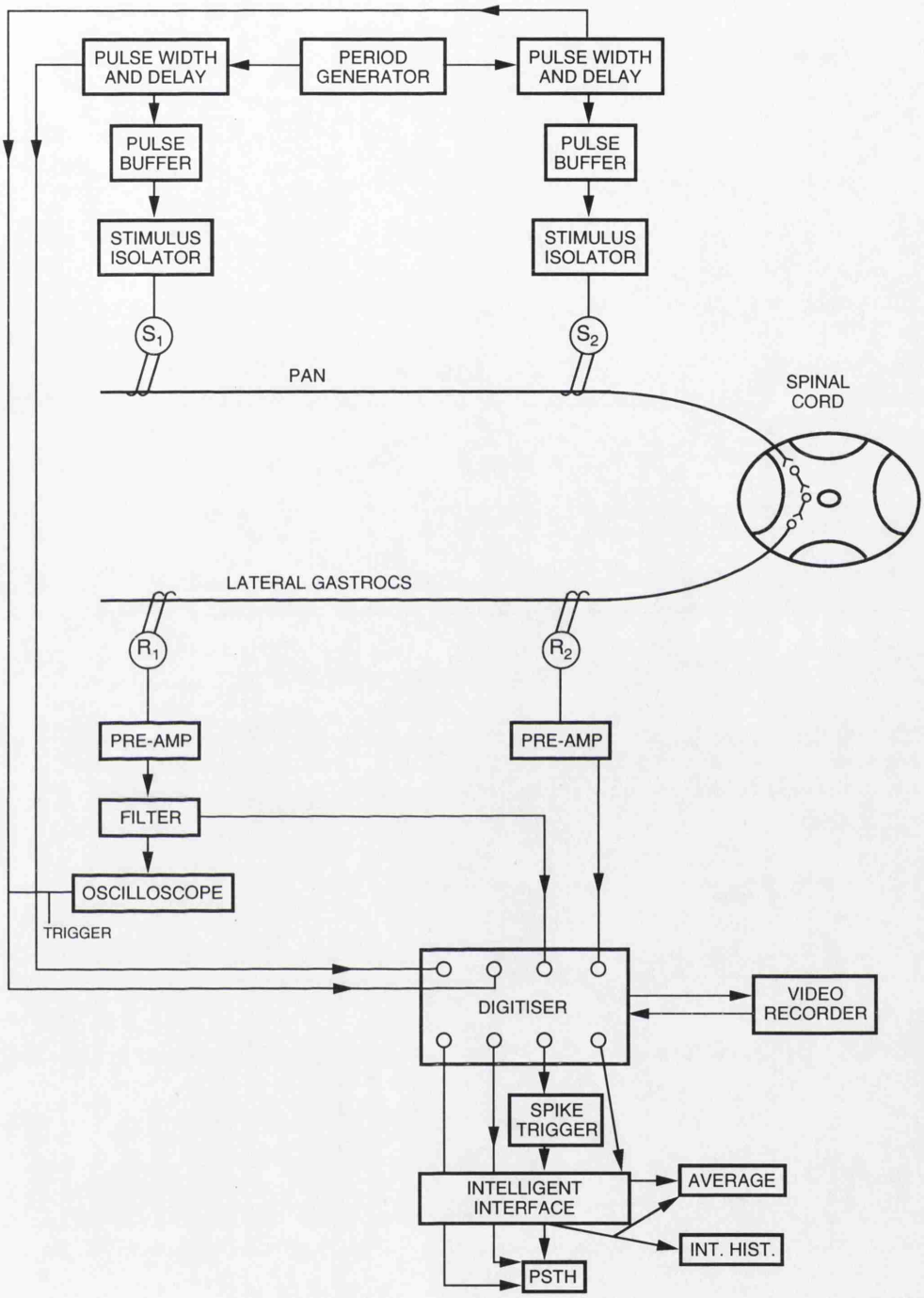
Figure 31: A schematic diagram of the circuit used to stimulate the PAN afferents electrically, while recording γ-motoneurone discharge in the lateral gastrocnemius nerve.

R1 = Single unit recording

R2 = Multi-unit recording

S1 = Test stimulus

S2 = Conditioning stimulus


PULSE WIDTH AND DELAY
PERIOD GENERATOR
PULSE WIDTH AND DELAY
PULSE BUFFER
PULSE BUFFER
STIMULUS ISOLATOR
STIMULUS ISOLATOR
S1
S2
PAN
SPINAL CORD
LATERAL GASTROCS
R1
R2
PRE-AMP
PRE-AMP
FILTER
OSCILLOSCOPE
TRIGGER
DIGITISER
VIDEO RECORDER
SPIKE TRIGGER
INTELLIGENT INTERFACE
AVERAGE
INT. HIST.
PSTH

interval setting to produce an interval histogram of γ-motoneurone discharge. The discriminated γ-motoneurone discharge is led to the 1401 interface and is used to trigger the interval histogram. Each time the γ-motoneurone fires the histogram is reset and the interval is recorded. This information provides details of the instantaneous firing rate of the neurone and also confirms the single unit status. With single units there are no intervals less than 1ms, due to the refractory period of the nerve.

d) Results

Stimulation of the PAN elicites an excitatory reflex response in twenty (54%) of the thirty seven spontaneously discharging neurones originally dissected for recording. If no response is observed the nerve filament is discarded before the conduction velocity is measured, so it is not certain that all unresponsive neurones are γ-motoneurones. However, this figure is similar to results from previous work (Johansson *et al*, 1986, Baxendale *et al*, 1993). The responsive neurones had conduction velocities ranging from $13m.s.^{-1}$ to $38m.s.^{-1}$ with a mean (±S.E.M.) of 24 ± $1.97m.s.^{-1}$. The stimuli required to elicit an excitatory reflex response in γ-motoneurones range from the threshold (T) of the PAN compound action potential to thirty times threshold (30T) with a mean of 4.6 ± 1.5T. The latency to the onset of the

excitatory response of the γ-motoneurones range from 8 to 35ms after PAN stimulation, with a mean of 20.5 ± 1.8ms.

The excitatory reflex response is abolished or reduced in fourteen neurones (70%), by the high intensity conditioning stimulus (Figs. 29B, 29D), whereas the low intensity conditioning stimulus has little effect on the reflex response (Figs. 29A, 29C). This reduction in the reflex response is not a consequence of alteration of the discharge frequency of the γ-motoneurone, as the intervals between successive discharges are similar during test and conditioning stimulation (Fig. 30). The C-wave component of the compound action potential (Fig. 30B, inset), recorded from PAN during conditioning stimulation, reached a maximal size indicating all electrically excitable neurones are excited. The number of impulses recorded following the test stimulus when this is preceded by a high intensity conditioning stimulus is expressed as a percentage of that recorded after a low intensity conditioning stimulus (control). This provides a measure of the reduction in the response following high intensity conditioning. The reflex excitability of the γ-motoneurones varies with the time interval between the conditioning and test stimuli (Fig. 32). The reduction in reflex excitability is abolished when the time interval between the two stimuli becomes too great. If an arbitrary level of 75% return to the original level of excitation is taken, then the range of inter-stimuli times required for this level to be achieved in different γ-motoneurones is from 100ms to >1s (mean ± S.E.M. = 280 ± 59.6ms) (Fig. 33). The peak

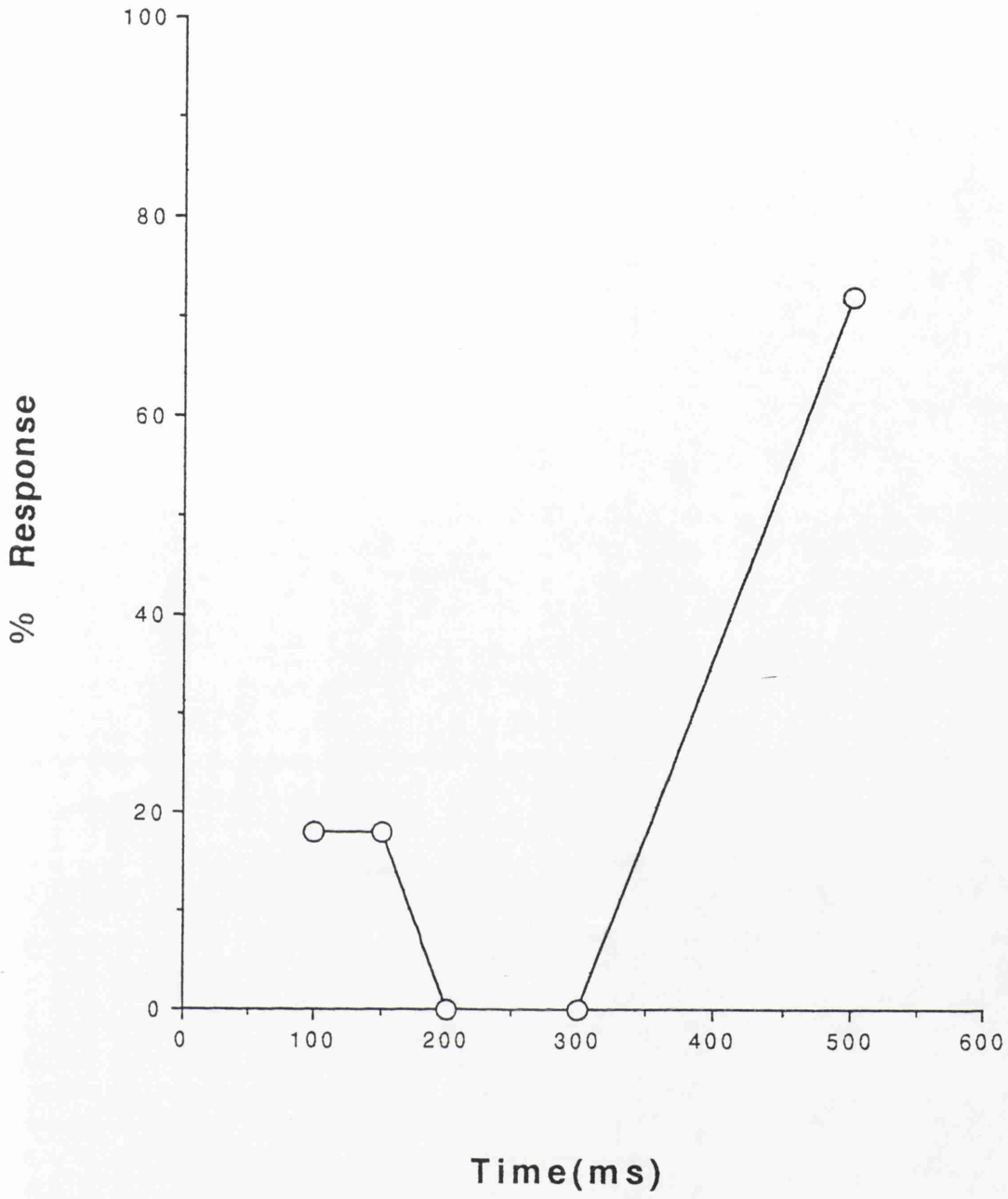


Figure 32: The abscissa represents the conditioning to test interval. The ordinate represents the percentage of the control response with each inter-stimuli time interval for an individual γ-motoneurone.

depression of excitation for each γ-motoneurone ranges from complete abolition of the response to 46% of the control.

With three of the remaining neurones, the preceding high intensity conditioning stimulus results in a larger excitation than that observed with the low intensity conditioning stimulus. However, in these neurones the high intensity stimulus produces a period of depressed activity before this period of excitation. The period of excitation is also time-linked to the high intensity pulse, in contrast to all the other neurones which have the periods of excitation linked to the test stimulus.

With three neurones (15%) the excitation is reduced or abolished by the control conditioning stimulus, which is the same amplitude as the test stimulus required to produce excitation.

With all neurones there is an excitation linked to the conditioning stimulus, both with high and low intensity stimulation. The low intensity conditioning stimulus therefore acts as a control to show that the firing of the γ-motoneurone itself does not result in a refractory period with resultant depression of excitation.

The mean firing rate of the γ-motoneurone is increased by high intensity stimulation at 0.5Hz to 2Hz, in nine cases and decreased in eleven. The overall mean change in firing rate (±S.E.M.), expressed as a percentage of the original firing rate, is 113.6% ± 12.2

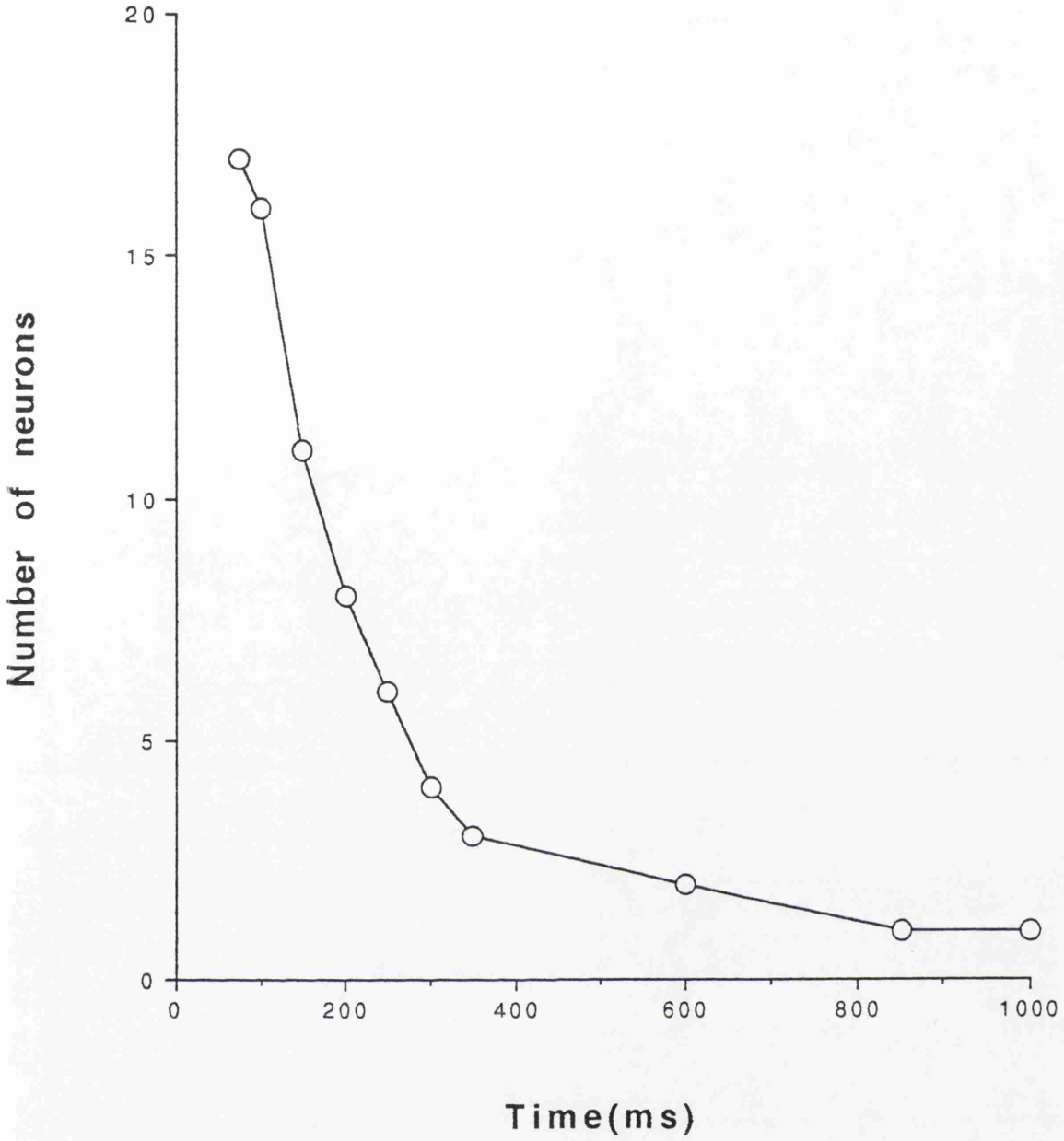


Figure 33: The abscissa represents the condition to test interval. The ordinate represents the number of neurones which show a reduction of at least 25% of the initial response, at each inter-stimulus time interval. From a total of seventeen neurones tested, all show a reduction in response with a time interval of 75ms, while only one neurone shows a reduction with an inter-stimulus time interval of 1s.

2.5 Reflex effects of group IV joint afferents on α-motoneurone excitability

a) Objectives

In a previous investigation it was demonstrated that discrete repetitive mechanical stimulation of the dorsal aspect of the cat knee joint capsule caused short latency excitation of quadriceps motor units (Baxendale *et al*, 1988; Ferrell *et al*, 1990). This effect arose from capsular mechanoreceptors with low thresholds to mechanical stimulation and was reversibly abolished by application of a local anaesthetic directly to the posterior articular nerve. The present study was performed to investigate the extent to which soleus motor units are affected by this form of mechanical stimulation and establish whether any reflex effects arising from these articular mechanoreceptors are significantly altered during activation of nociceptors from the same joint.

b) Methods

Experiments are performed on adult cats anaesthetised with a gaseous mixture of O_2/ N_2O/halothane. Blood pressure is monitored throughout via a cannula inserted into the left carotid artery. End-tidal CO_2 is also continuously monitored. A mid-collicular decerebration is performed following which the

anaesthetic is discontinued. Spinalisation is performed at T_{12}. The popliteus muscle is tenotomised and removed to eliminate popliteal afferents. Motor unit recordings are obtained via intramuscular needle electrodes whilst PAN recordings are obtained via bipolar platinum electrodes. Soleus is tenotomised and its tendon connected to a tension transducer. Sufficient tension is applied until tonic discharges are obtained in 2-3 motor units. Repetitive mechanical indentation of the posterior aspect of the joint capsule is achieved by means of the previously described vibrator, consisting of a probe (tip diameter 1mm) attached via a flexible cable to a vibrator coil whose frequency is set at 5-10Hz. The amplitude of indentation is increased until 3-5 PAN afferents are securely time-locked to the stimulus. Data is recorded on magnetic tape for subsequent analysis. Nociceptor activation is achieved by application of a droplet of 1% capsaicin directly to PAN. The capsaicin solution consists of: 0.02g capsaicin (Sigma) in 0.1ml ethanol, 0.1ml cremophore (Sigma) and 1.8ml of 0.9% saline.

In some experiments nociceptors are activated by electrical stimulation of PAN via a pair of silver wire electrodes. This conditioning stimulus is timed to occur in advance of the mechanical stimulus applied to the joint capsule, and this time interval is adjustable. The intensity of the stimulus is set at two levels: a low intensity conditioning stimulus, sufficient to elicit a group II wave in the PAN compound action potential, acted as the "control" whilst the test stimulus was of an intensity which

resulted in a group IV (C) wave in the PAN compound action potential.

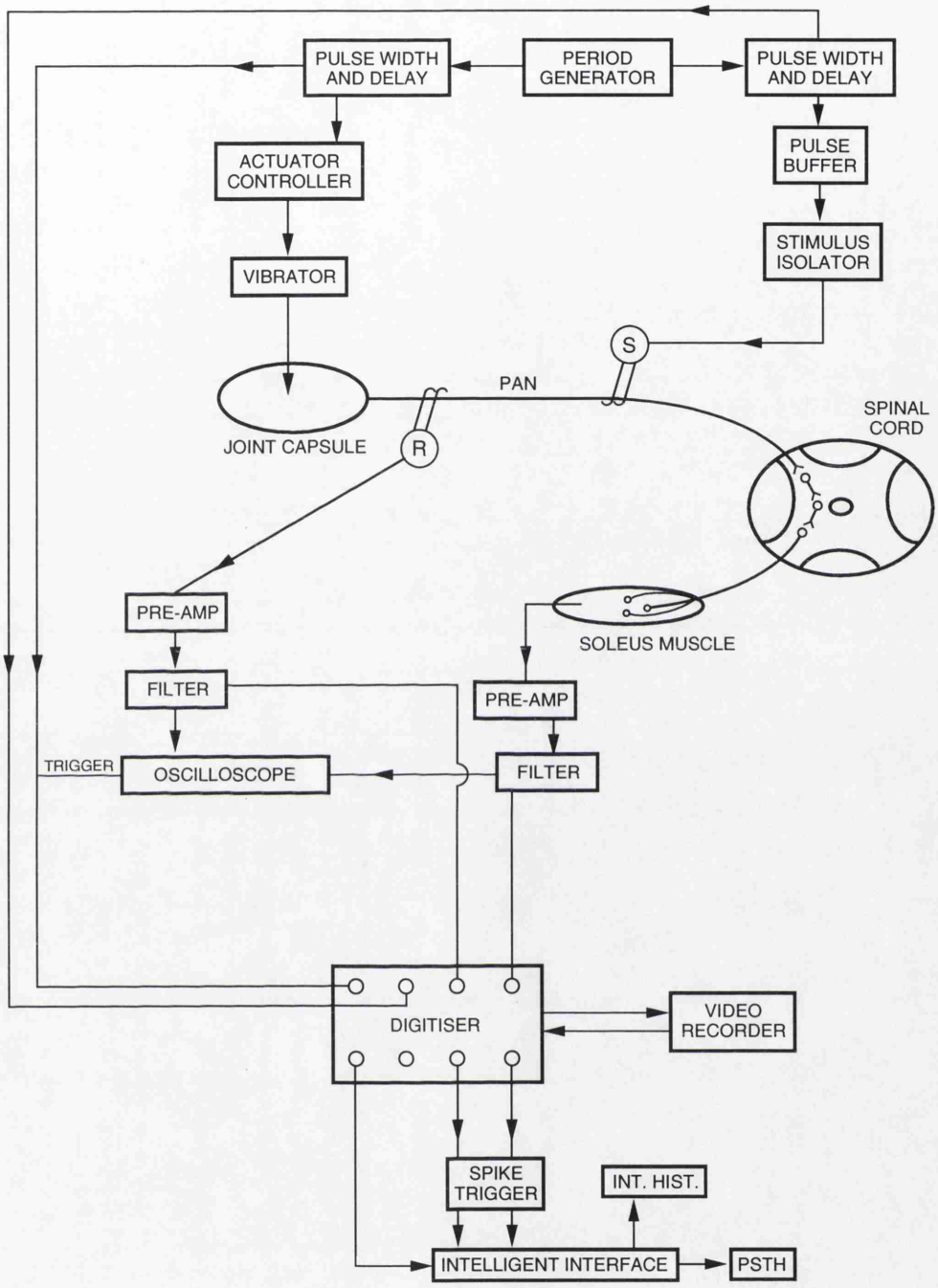
The period generator (NL303) triggers two separate delay-width modules (NL403), one supplying the pulse buffer/stimulus isolator (NL510/NL800) and the other supplying the vibrator actuator (Fig. 34). The intensity of electrical stimuli, the amplitude of the vibrator displacement and the delay between the two is achieved as previously described.

c) Recording and analysis

Electromyogram recordings are digitised, and recorded on magnetic tape along with trigger pulses from the delay-width modules. Subsequent analysis involves discrimination of the motor unit analogue signal with the spike processor (NL201). Post stimulus time histograms of the motor unit firing are created relative to the mechanical stimulation of the joint capsule i.e. the probe trigger is used to trigger the sweep of the histogram. Comparison of the linkage of motor unit firing during low threshold PAN conditioning stimulation, with linkage during high threshold PAN stimulation reveals the effect high threshold afferents have on low threshold afferent induced motor unit excitability.

The MRate programme is used in the interval histogram setting to monitor the instantaneous firing rate of the motor units. The analogue recording of motor unit firing is

Figure 34: A schematic diagram of the circuit used to stimulate the joint receptors mechanically while recording electromyograms from the gastrocnemius/soleus muscle.
R = Recording of the PAN afferent discharge in response to mechanical stimulation of the joint capsule.
S = Electrical conditioning stimulation of the PAN


PULSE WIDTH AND DELAY
PERIOD GENERATOR
PULSE WIDTH AND DELAY
ACTUATOR CONTROLLER
PULSE BUFFER
VIBRATOR
STIMULUS ISOLATOR
S
PAN
SPINAL CORD
JOINT CAPSULE
R
PRE-AMP
SOLEUS MUSCLE
FILTER
PRE-AMP
TRIGGER
OSCILLOSCOPE
FILTER
DIGITISER
VIDEO RECORDER
SPIKE TRIGGER
INT. HIST.
INTELLIGENT INTERFACE
PSTH

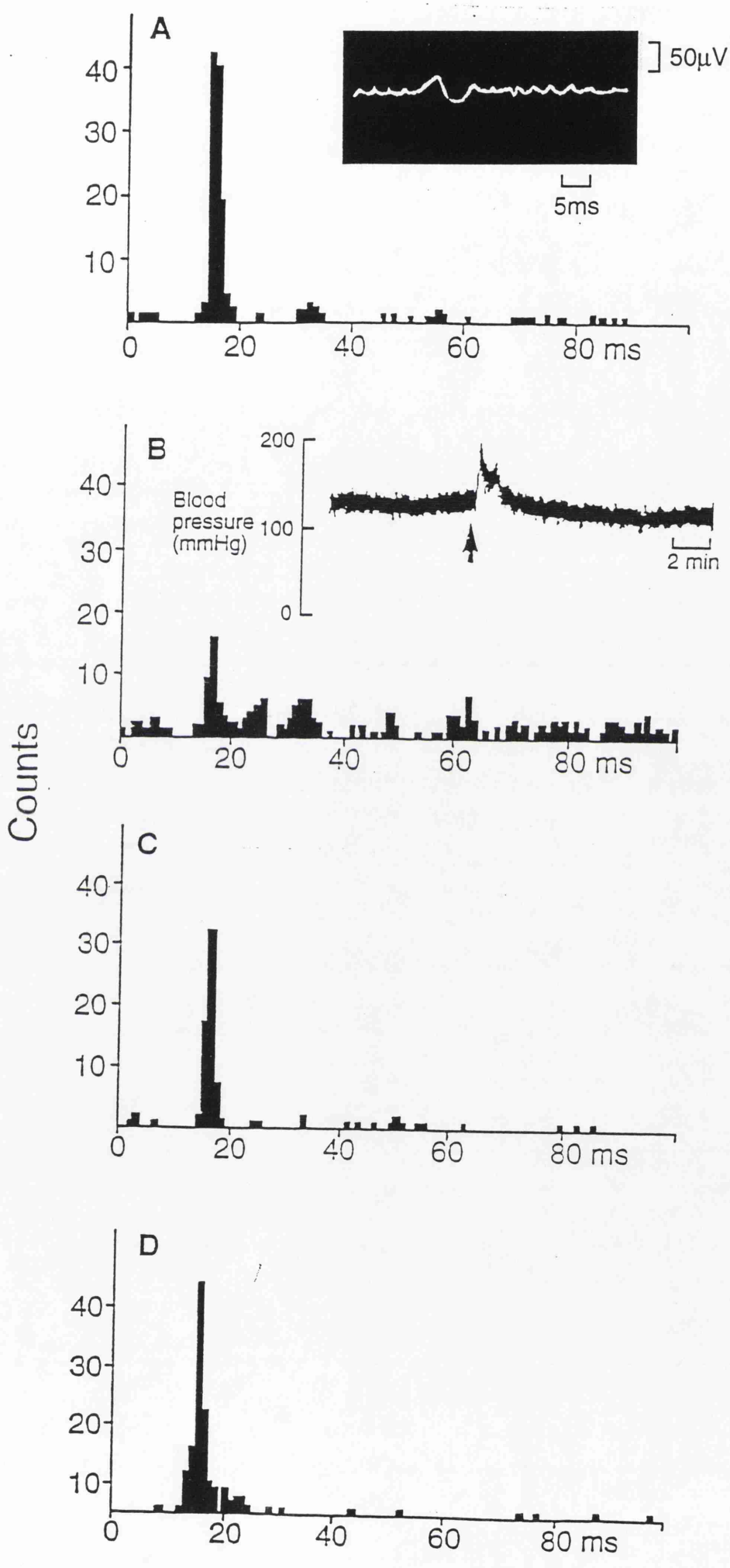
discriminated into TTL pulses by the spike discriminator. The output from the discriminator is processed by the MRate programme to chart each interval between sequential potentials of a motor unit (Fig. 34).

d) Results

Restricted mechanical indentation of the joint capsule, sufficient to excite between one to five group II low-threshold mechanoreceptors, results in an increased probability of discharge of soleus motor units (Fig. 35A) with a response latency of 15 ± 1.4ms (mean ± SEM; n=15). As in a previous study (Baxendale, Ferrell & Wood, 1988), it is found that this excitatory effect can be reversibly eliminated by a droplet of 2% lignocaine placed on PAN.

Application of a droplet of 1% capsaicin solution directly to PAN whilst recording the response to the probe stimulus clearly reduces this excitation (Fig. 35B). Capsaicin is known to activate nociceptive afferents potently, but not low threshold mechanoreceptors (Holzer, 1991). The PAN recording shows no alteration in group II discharge, but capsaicin application clearly activates articular nociceptors as this results in a rise in arterial blood pressure (inset in fig. 35B). Shortly after this rise is observed, the motor unit continues to discharge but its firing is no longer linked to the mechanical stimulus. This effect is specific to PAN as application of capsaicin to the medial

Figure 35: Post stimulus time histograms (PSTH's) showing reflex excitation of a soleus motor unit (inset) by repetitive indentation (5Hz) of the dorsal aspect of the cat knee joint capsule (A). The same motor unit shows reduced excitation following application of capsaicin to PAN (B), which also produces a pressor response (inset). This record was obtained thirty seconds after capsaicin application. Application of capsaicin to medial gastrocnemius muscle nerve (C) or the sural nerve (D) did not significantly alter reflex excitability. All PSTH's are produced from 100 sweeps.

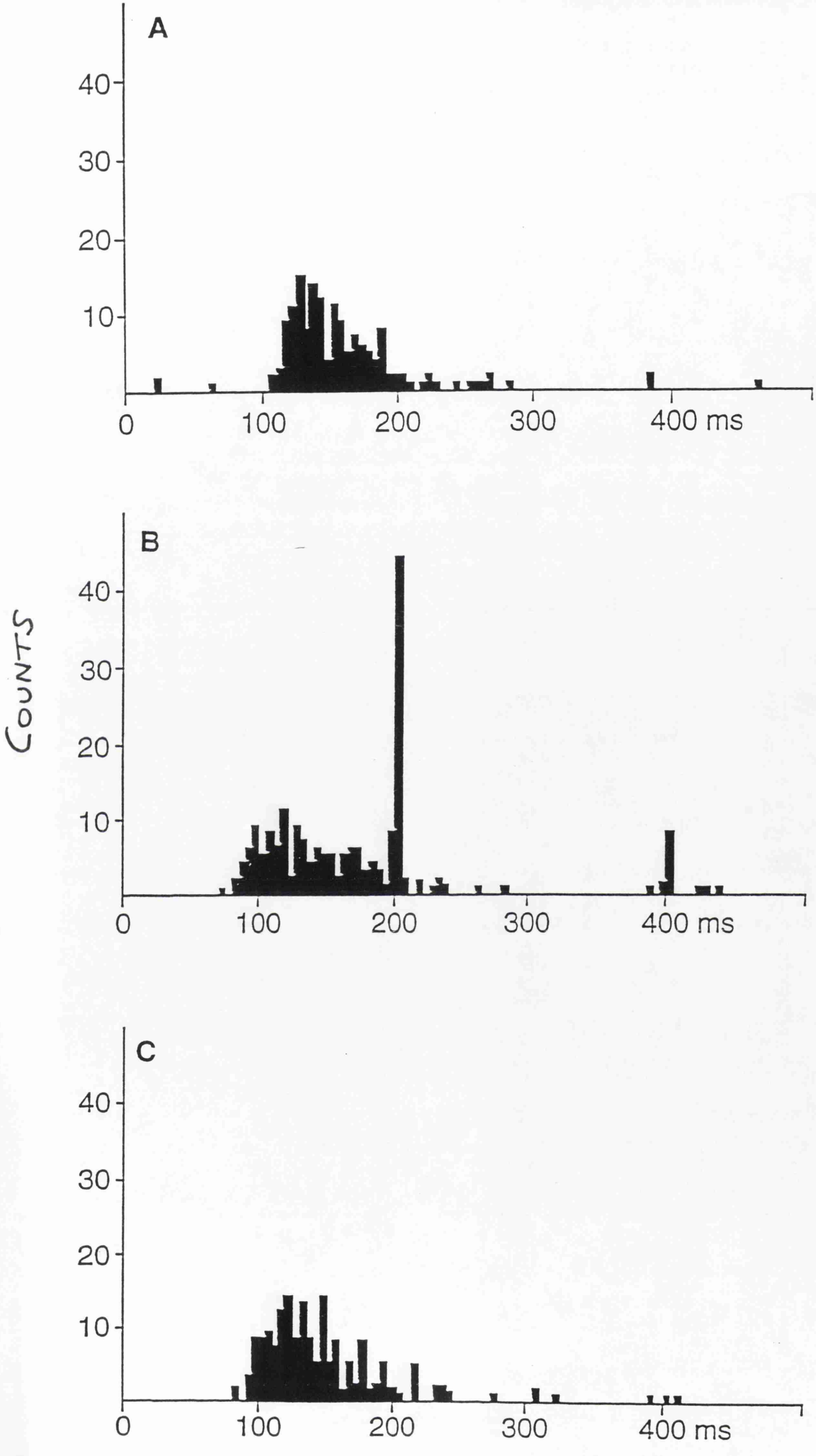

A
50μV
5ms
40
30
20
10
0
20
40
60
80 ms
B
200
Blood
pressure
(mmHg)
100
0
2 min
Counts
C
D

gastrocnemius muscle nerve (Fig. 35C) or the sural nerve (Fig 35D), although producing clear pressor responses, fails to inhibit the excitation of soleus motor units by PAN mechanoreceptors.

The reduction in the excitatory drive to soleus motor units with capsaicin application to PAN is not due to direct inhibition of soleus motor unit excitability by nociceptors as motor units continue to discharge spontaneously during capsaicin treatment, at rates close to their control values (Fig. 36). This is important, as the background EMG activity will strongly influence the response to other inputs (Bedingham & Tatton, 1984). These interval histograms show that the motor unit prior to intervention discharges tonically (Fig. 36A) and when the 5Hz probe stimulus is applied a sharp peak appears at 200msec superimposed on the background firing rate (Fig. 36B). This motor unit showed periods when it was linked one to one to the stimulus and other periods when it discharges spontaneously even though the mechanical stimulus is still being applied. Shortly after application of capsaicin this peak disappears, but the motor unit continues to discharge (Fig. 36C) at much the same rate as under control conditions (Fig 36A).

Activation of PAN afferents by capsaicin suffers the disadvantage that only one application is possible. Further application fails to elicit inhibition or raise blood pressure. This is likely to be due to nociceptor desensitisation which is a recognised effect of capsaicin (Holzer, 1991). In view of this, experiments were performed to apply a more controllable

Figure 36: Interval histograms showing the spontaneous discharge of a soleus motor unit without appplication of any stimuli (A). Repetitive capsule indentation at 5Hz resulted in the motor unit showing periods of synchronisation to the stimulus superimposed on the spontaneous discharge (B). The motor unit showed periods of one-to-one linking with the mechanical stimulus to the joint capsule, and other periods of spontaneous discharge. Capsule indentation following capsaicin application to PAN no longer synchronises the discharge to the stimulus (C) and the spontaneous discharge rate is similar to the control value (A).

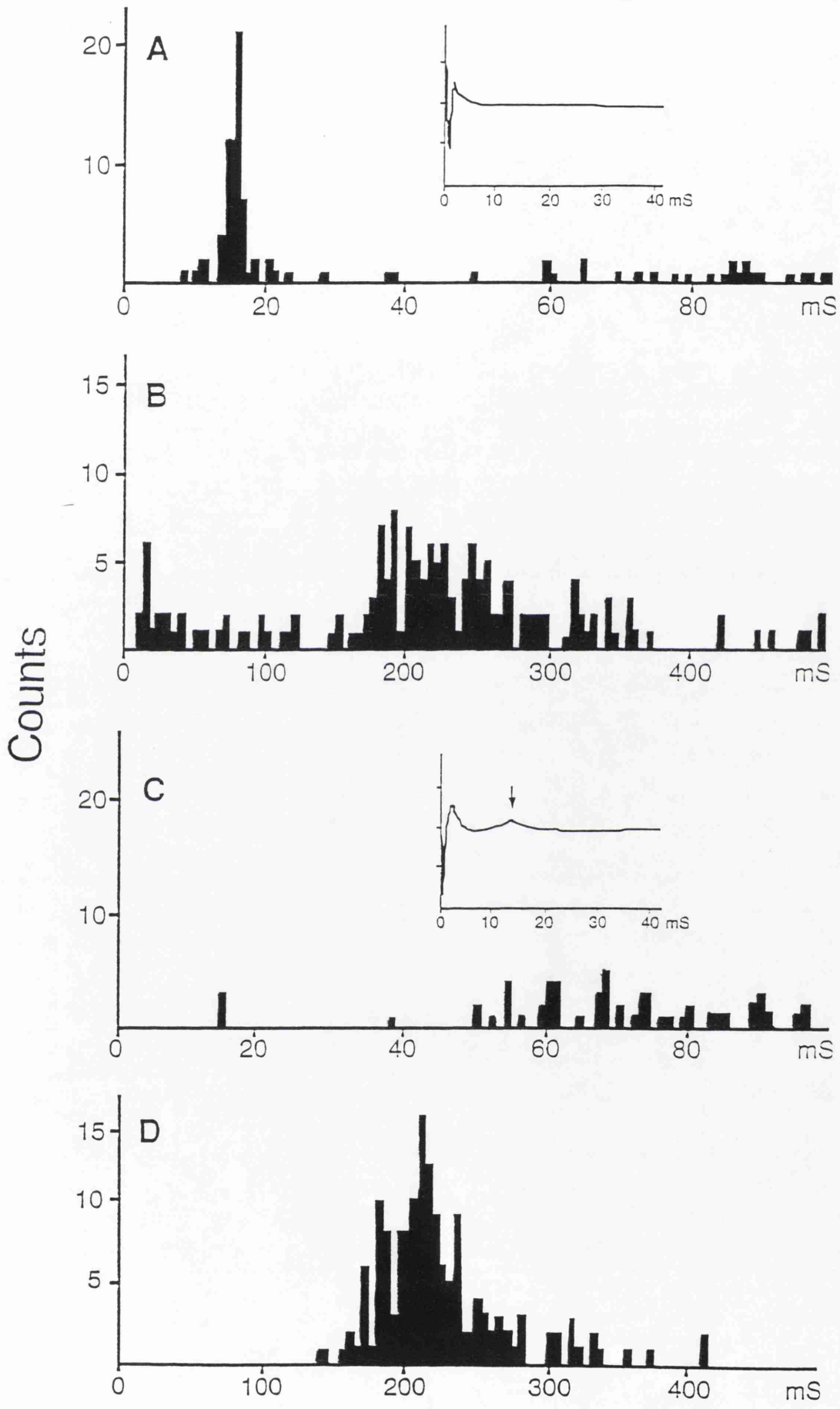

A
40
30
20
10
0
100
200
300
400 ms
B
COUNTS
40
30
20
10
0
100
200
300
400 ms
C
40
30
20
10
0
100
200
300
400 ms

nociceptive input by supramaximal electrical stimulation of PAN. This stimulus precedes the mechanical stimulus to the joint capsule and its intensity is adjusted so that it is either sufficient to maximally activate group II afferents (control conditioning stimulus) or it is increased to include group IV afferents (test conditioning stimulus). The control stimulus is mostly ineffective at reducing the reflex excitation of soleus motor units (Fig. 37A) whereas the group III/IV stimulus produces effects consistent with the experiments using capsaicin (Fig. 37B).

Aswell as a reduction in the group II excitation of the motor units there is also a longer latency period of postsynaptic inhibition of the motoneurones as evidenced by a period of reduced activity in the PSTH's.

By varying the interval between the conditioning electrical stimulus to PAN and the mechanical stimulus to the joint capsule, the time course of the response can be examined (Fig. 38). From this it is clear that the least inhibition is obtained when group II or group IV afferents are excited and greatest when group III afferents are recruited. Relative to the threshold of the conditioning stimulus, the inhibition obtained when the stimulus is less than 2.5 times threshold (75 ± 17.1%) is considerably less than when greater than 2.5 times threshold (14 ± 6.6%; means ± SEM; n=7).

Figure 37: (A) PSTH's showing the response of a soleus motor unit to capsule indentation conditioned by a preceding electrical stimulus to PAN just above group II threshold, with the PAN compound action potential shown in the inset. The conditioning stimulus was delivered 5ms before the test stimulus.
(B) Interval histogram of the spontaneous discharge of the same motor unit shown in A.
(C) Reflex excitation was abolished when the conditioning stimulus was above group IV threshold, as judged by the C wave in the PAN compound action potential (inset). The conditioning stimulus was delivered 20ms before the test stimulus.
(D) Interval histogram of the spontaneous discharge of the unit shown in C.


A
B
C
D
Counts
20
10
15
5
0
20
40
60
80
100
200
300
400
mS
0 10 20 30 40 mS

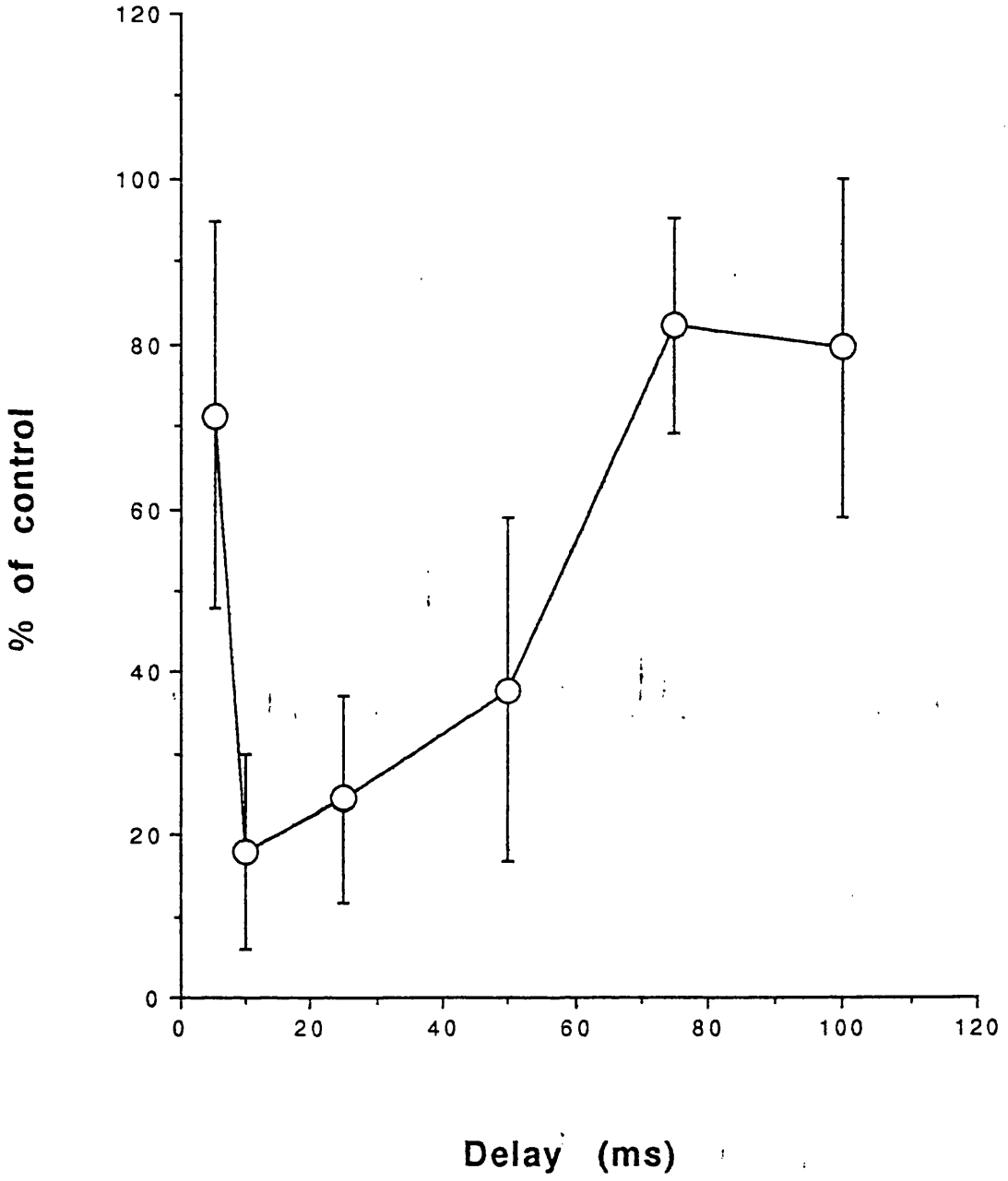


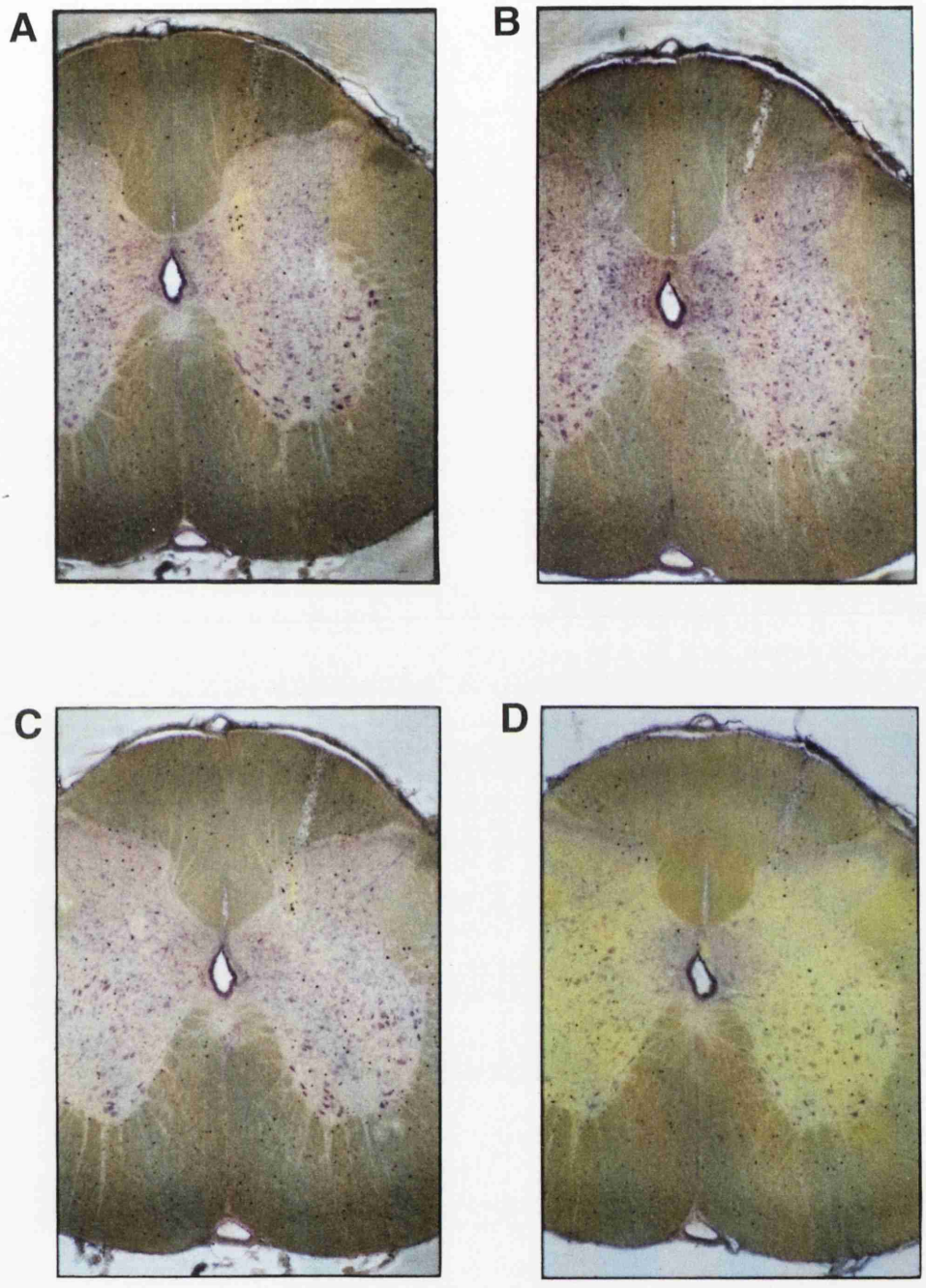
Figure 38: Time course of inhibition of reflex excitability by conditioning stimuli to PAN at increasing intervals (mean ± SEM; n=7). The abscissa represents the time interval between the conditioning stimulation and the tap to the capsule. The ordinate represents the percentage of the response without conditioning stimulation.

2.6 Spinal interactions of low and high threshold joint afferents

a) Objectives

The results of the above experiments indicate that high threshold joint afferents (group III or group IV) inhibit the excitability induced in both γ- and α-motoneurones by activity in group II joint afferents. This inhibition must occur at the intraspinal terminals of the group II primary afferents, or at interneurones interposed between the primary afferents and the motoneurones. Direct inhibition of the motoneurones is unlikely, as the firing rate is unaffected by high threshold PAN afferent activity. If there is any effect, it is an increase in firing rate rather than a decrease as would be expected with direct inhibition. There is also no obvious decrease in excitation of motoneurones by other pathways e.g. skin stimulation still excites γ-motoneurones and muscle stretch excites α-motoneurones. The present experiment is to determine if inhibition of group II joint afferent pathways by high threshold afferents occurs at the intraspinal terminal of the primary afferent i.e. presynaptic inhibition. This is usually caused by depolarisation occuring at axo-axonic synapses onto or near the terminal boutons of the primary afferent fibre (Eccles, Eccles & Magni, 1961; Schmidt, 1971). This primary afferent

Figure 39: Serial sections from the sixth lumbar segment of the spinal cord. The cord is embedded in celloidin and 100μM sections cut before staining with aqueous thionine. The electrode tract can clearly be seen extending into the left dorsal horn. Sections A, B, C and D are cut in sequence from rostral to caudal and are viewed from the rostral aspect.


A
B
C
D

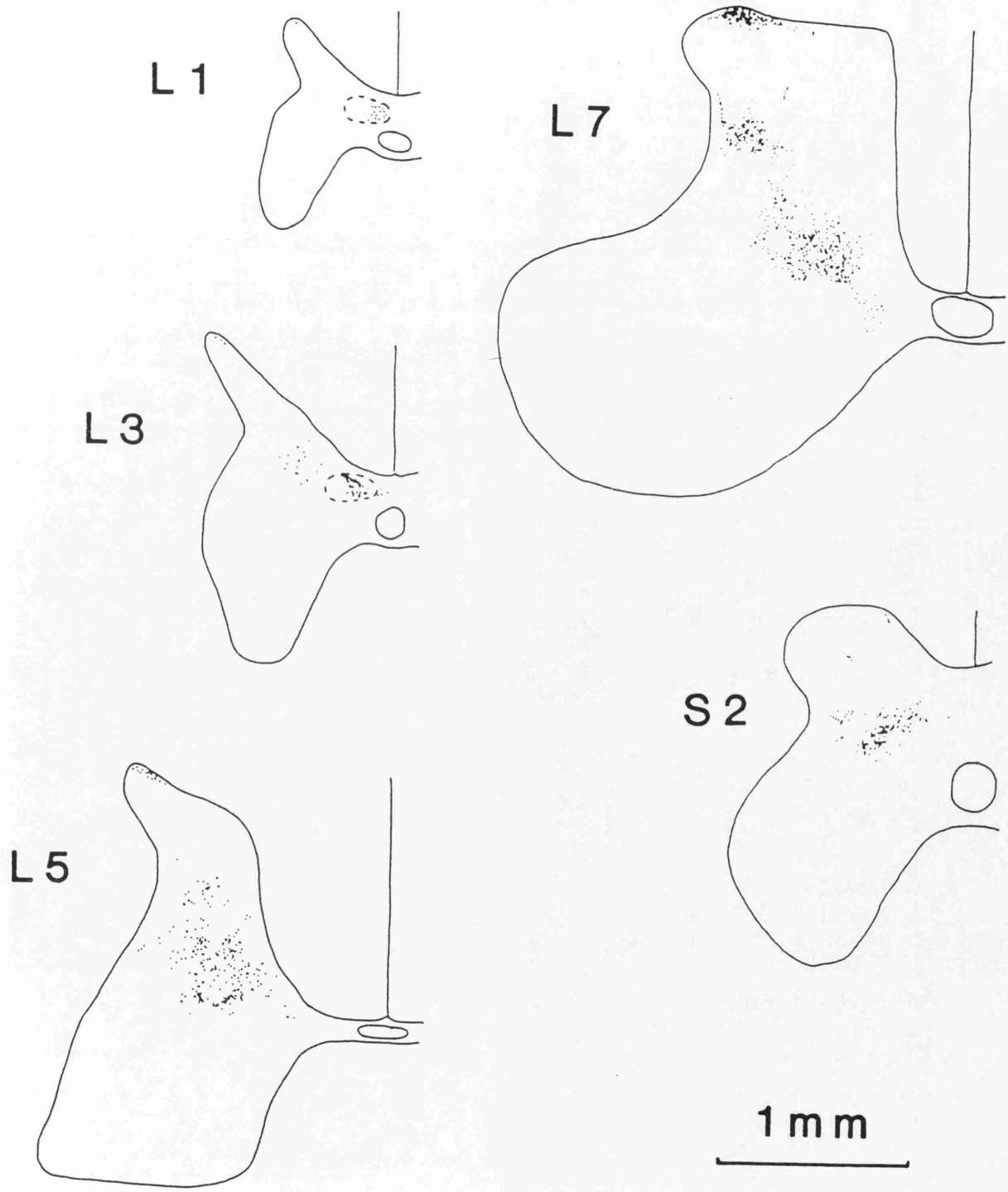
In fourteen cats the whole PAN is used for both stimulating and recording while in six cats the PAN is split, one half being used for recording and the other for stimulating. In six cats the ipsilateral sural and medial gastrocnemius nerves are dissected for stimulating, and in two cats the contralateral PAN is dissected and stimulated. The sural and gastrocnemius nerves are exposed during the previously described procedure for PAN dissection. The sural nerve can be seen lying beneath the popliteal fat pad, and the gastrocnemius nerve is exposed during removal of the medial gastrocnemius muscle.

Blood pressure, end-tidal CO_2, rectal temperature, and the temperature of liquid paraffin pools covering nervous tissue are monitored and kept within normal limits.

The spinal cord is stimulated by a tungsten microelectrode. The electrode is held in a micromanipulator which positions the electrode at various positions between the fifth lumbar and second sacral spinal segments. Intraspinal stimuli are applied just to the left of the dorsal median sulcus at depths of between one and three millimetres. The electrode tracts are seen in spinal cord sections fixed in celloidin and stained with aqueous thionine (Appendix 1). The example shown (Fig. 39) is taken from L6. The location of the electrode is consistent with the location of the spinal terminals of the PAN afferents (Fig. 40).

The tungsten electrodes are insulated along their length apart from the tip. The microelectrodes are used to apply stimulus pulses of negative current to excite the central terminals of

Figure 40: Camera lucida drawing of the spinal projections of the PAN afferents in the segments L1 to S2. In each segment data are superimposed from 4-5 adjacent sections of two experiments (Craig, Heppelmann & Schaible, 1987).


L 1
L 7
L 3
S 2
L 5
1 m m

afferent fibres. Stimulation is provided by a constant current stimulator (NL 800) driven by a pulse buffer (NL 510). The microelectrode is connected to the negative terminal of the stimulator and the positive terminal is connected to the animal. A delay-width (NL403) module is triggered by a TTL pulse from the pulse generator to provide a pulse, 600ms after triggering, and 0.2ms in duration, to the pulse buffer. The frequency of stimulation is set by the period generator (NL303) (Fig. 41).

Conditioning volleys in PAN, gastrocnemius and sural nerves are evoked by a train of five stimuli at 10Hz, with a pulse width of 0.5ms, the last of which precede the spinal test stimuli by 100ms. The period generator triggers a delay-width module set at a pulse width of 600ms and zero delay. The output is led to the "gate in" input of a pulse generator (NL300). No output pulses are produced when the "gate in" input is held at a low logic level; when the input goes high, pulses are produced, with the first pulse delayed by one period. Therefore, with the period of the NL300 pulse generator set at 100ms, a 600ms pulse from the NL 403 delay-width module generates five pulses at 10Hz. A toggle switch on the pulse generator selects an output pulse width of 0.5ms. A zero delay setting on the NL403 supplying the conditioning pulse stimulator, and a delay setting of 600ms on the NL403 supplying the spinal electrode stimulator, produces a delay of 100ms from the last conditioning pulse to the spinal stimulus. Triggering pulses from the NL403 modules are monitored on an oscilloscope to verify the correct delay and

number of conditioning pulses. Conditioning stimulation is provided by a separate pulse buffer and stimulus isolator connected to bipolar platinum stimulating electrodes (Fig. 41).

Spinal stimulation proceeds at one hertz and the current through the microelectrode is adjusted until an antidromic potential is recorded in the PAN approximately one hundred percent of the time. The conditioning stimulation is then turned on and the effect on the antidromic potentials is observed.

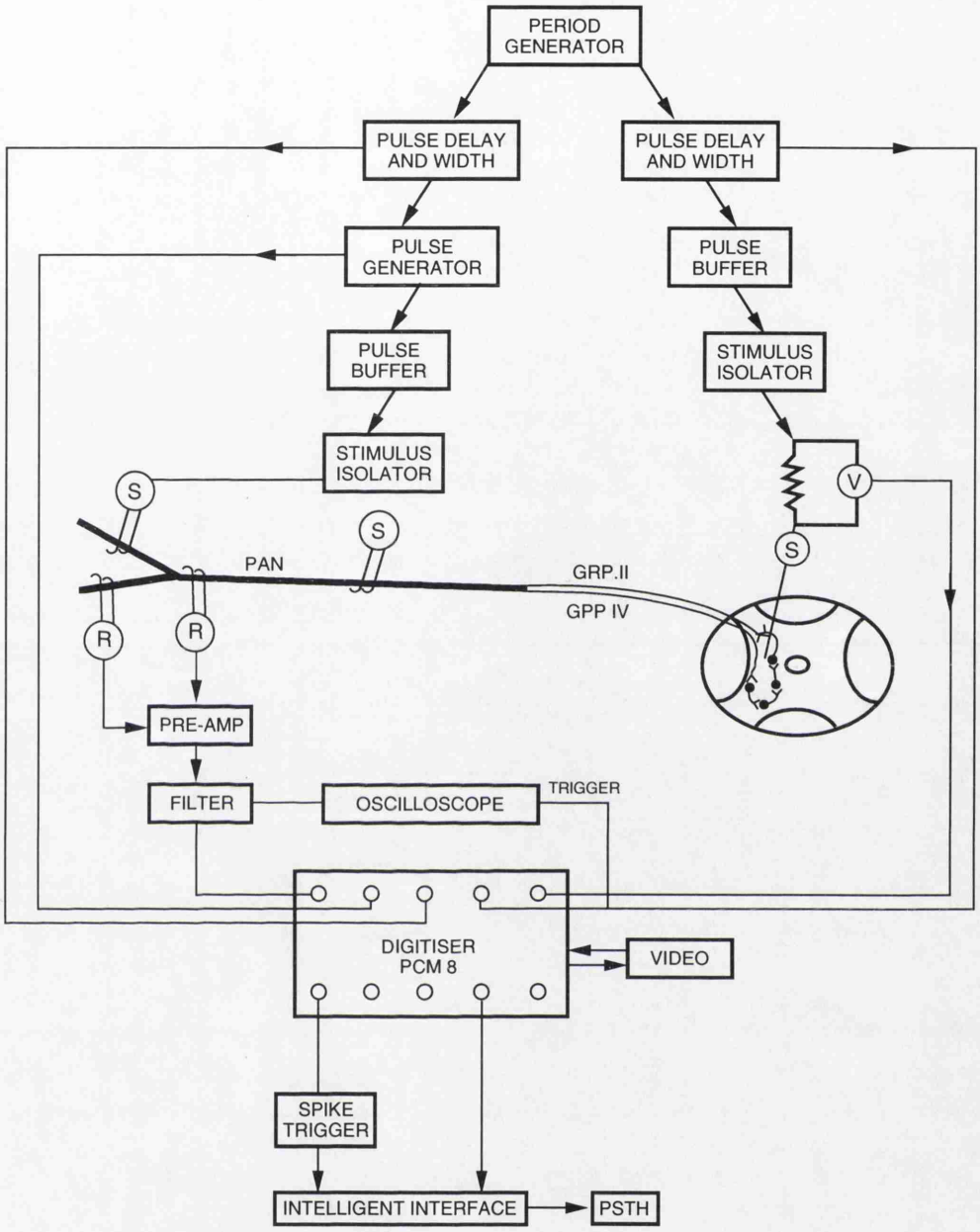
Where the PAN is split, recording of antidromic impulses and conditioning stimulation is in separate halves of the PAN. In some experiments there is recording and stimulation of the sciatic nerve and dorsal roots corresponding to the spinal segments being stimulated. In five experiments capsaicin is applied to PAN.

At the end of six experiments the dorsal roots from L5 to S3 are cut proximally and placed in a group, on top of bone wax which has been spread over the dorsal aspect of the cord. The bone wax serves as an insulator to ensure there is no stimulation of any PAN afferents which may have been left in contact with the spinal cord. The microelectrode is then manoeuvred onto the dorsal roots and these are stimulated, and antidromic potentials recorded, as described for the spinal cord. In three experiments the sciatic nerve is dissected by making a four centimetre incision from just below the greater trochanter of the femur. The sciatic nerve is exposed by blunt dissection between gluteus maximus and biceps femoris and freed from surrounding

Figure 41: A schematic diagram of the circuit used to stimulate the central terminals of the PAN afferents, while recording antidromic potentials in the PAN. Conditioning stimuli are applied either to the whole PAN proximal to the recording electrode, or the PAN is split, one half being used to record from and the other to stimulate.
R = Recording electrode
S = Stimulating electrode

The following sequence of stimulation is repeated at 1Hz:-
S-0.5s-C-0.1s-C-0.1s-C-0.1s-C-0.1s-C-0.1s-S

S = Spinal stimulus
C = Conditioning PAN stimulus


PERIOD GENERATOR
PULSE DELAY AND WIDTH
PULSE DELAY AND WIDTH
PULSE GENERATOR
PULSE BUFFER
PULSE BUFFER
STIMULUS ISOLATOR
STIMULUS ISOLATOR
S
S
S
V
PAN
GRP.II
GPP IV
R
R
PRE-AMP
FILTER
OSCILLOSCOPE
TRIGGER
DIGITISER PCM 8
VIDEO
SPIKE TRIGGER
INTELLIGENT INTERFACE
PSTH

connective tissue. It is suspended on bipolar stimulating electrodes and covered with liquid paraffin. The sciatic nerve is stimulated and the compound A-wave recorded in the PAN, with and without conditioning stimulation.

c) Recording and analysis

Single unit antidromic action potentials are recorded in the PAN as previously described (Fig. 41). Subsequent analysis with the MRate programme, to produce post stimulus time histograms, is used to compare the discharge of neurones in relation to spinal stimulation, with and without conditioning stimulation. With dorsal root and sciatic nerve recordings, compound potentials are produced, the size of which is assessed with the signal averager programme. Comparison in the size of compound potentials are made with and without conditioning stimulation.

d) Results

Intraspinal stimulation induces antidromic action potentials in the PAN. The latency from stimulation to recording of the potential is consistent with group II conduction velocities, as only latencies of less than 6ms from spinal stimulation to arrival of the potential in PAN are considered. The current in the spinal electrode is adjusted to be just sufficient to give approximately a

Figure 42: Post stimulus time histogram of antidromic firing recorded in the PAN, relative to spinal stimulation (100 sweeps). The analogue recording is shown in the inset (10 sweeps). An action potential is recorded 3ms after each spinal stimulus. This indicates a conduction velocity in the PAN afferents of 50m/s.

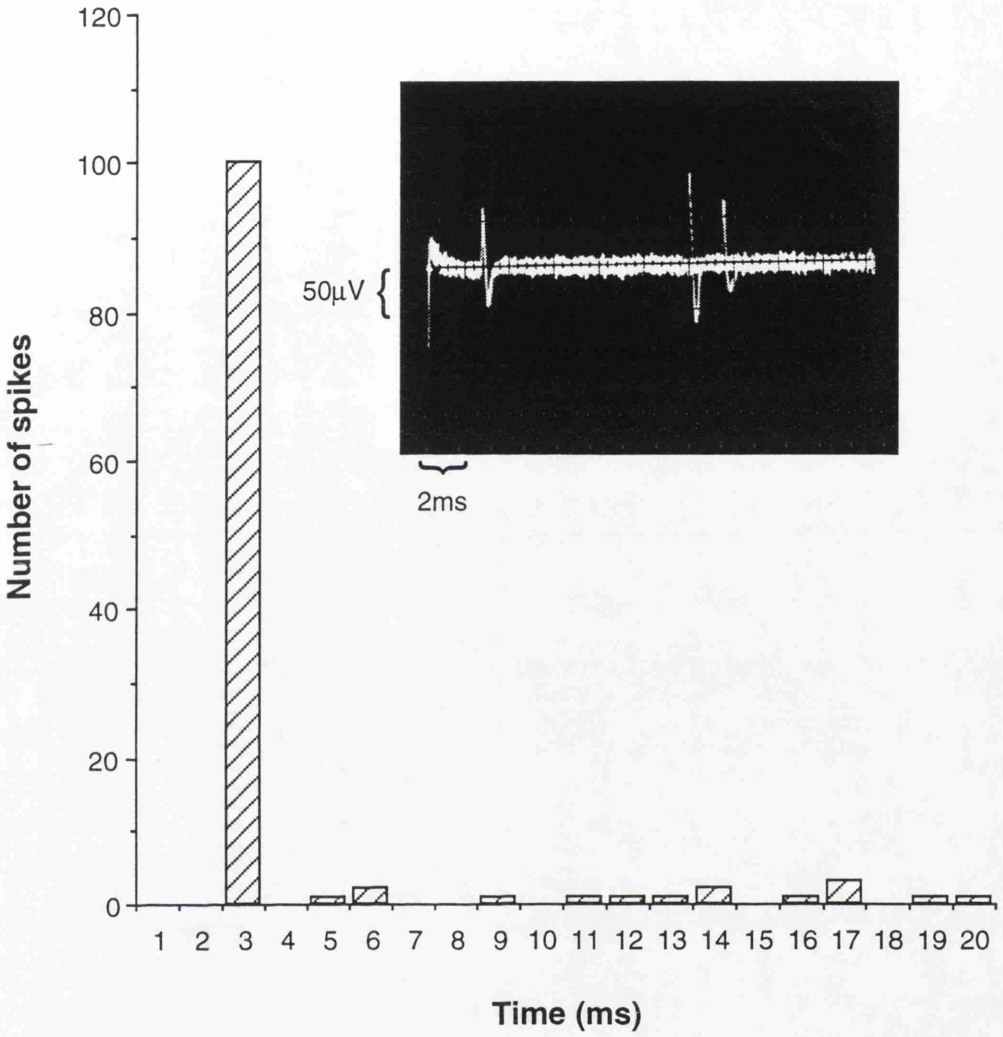
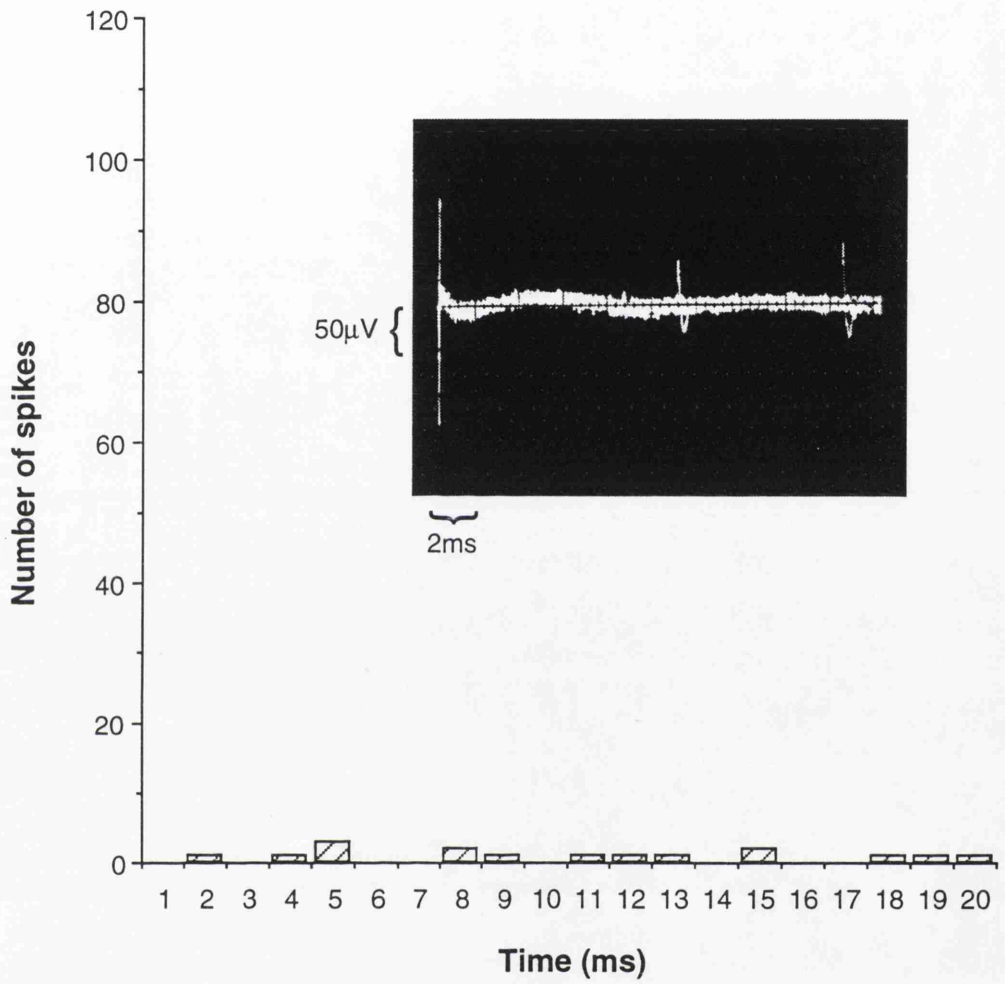

120
100
80
60
40
20
0
Number of spikes
50μV
2ms
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time (ms)

Figure 43: As in figure 42, but with five conditioning stimuli applied to the PAN from 500ms to 100ms before the spinal stimulation. The previous response to spinal stimulation has been abolished, although continuing background firing indicates that the nerve is not refractory


120
100
80
60
40
20
0
Number of spikes
50μV
2ms
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time (ms)

one hundred percent response in PAN (Fig. 42). In the majority of experiments, the PAN conditioning stimulus completely abolishes the antidromic potential in PAN, and increasing the spinal current is ineffective in making it return (Fig. 43). In the remainder the percentage response to spinal stimulation is reduced (Figs. 44 & 45). With any excitation that is completely abolished during whole PAN stimulation, increasing the current in the spinal electrode fails to re-establish the PAN excitation, sometimes even after the conditioning stimulus has been turned off. When the conditioning stimulus only reduces the percentage of firing, it is possible to return to 100% by increasing the spinal current by 347% ± 213 (mean ± S.E.M.). Conditioning stimulation parameters, sufficient to excite group II fibres only, have no effect.

The latency from conditioning stimulation to spinal stimulation is sufficient to prevent refractoriness of the group II afferents. This is also confirmed by a continuing spontaneous background discharge of the afferent in many experiments. However, to exclude the possibility of a direct effect on the group II afferents by the conditioning stimulus rendering them unresponsive, the PAN is split into two halves for six experiments. One half of the PAN is used for recording and the other for conditioning stimulation. The result is similar to whole PAN experiments, although slightly weaker. In the split PAN, if the firing returned, an increase in spinal current of 186% ± 133 was required.

Figure 44: Post stimulus time histogram (100 sweeps) with inset of recording (10 sweeps), showing a consistent response of an afferent fibre to spinal stimulation. The current through the spinal microelectrode is 5.2μA. The latency from spinal stimulation to the appearance of the action potentials in the PAN is 4ms, indicating a conduction velocity of 40m/s.

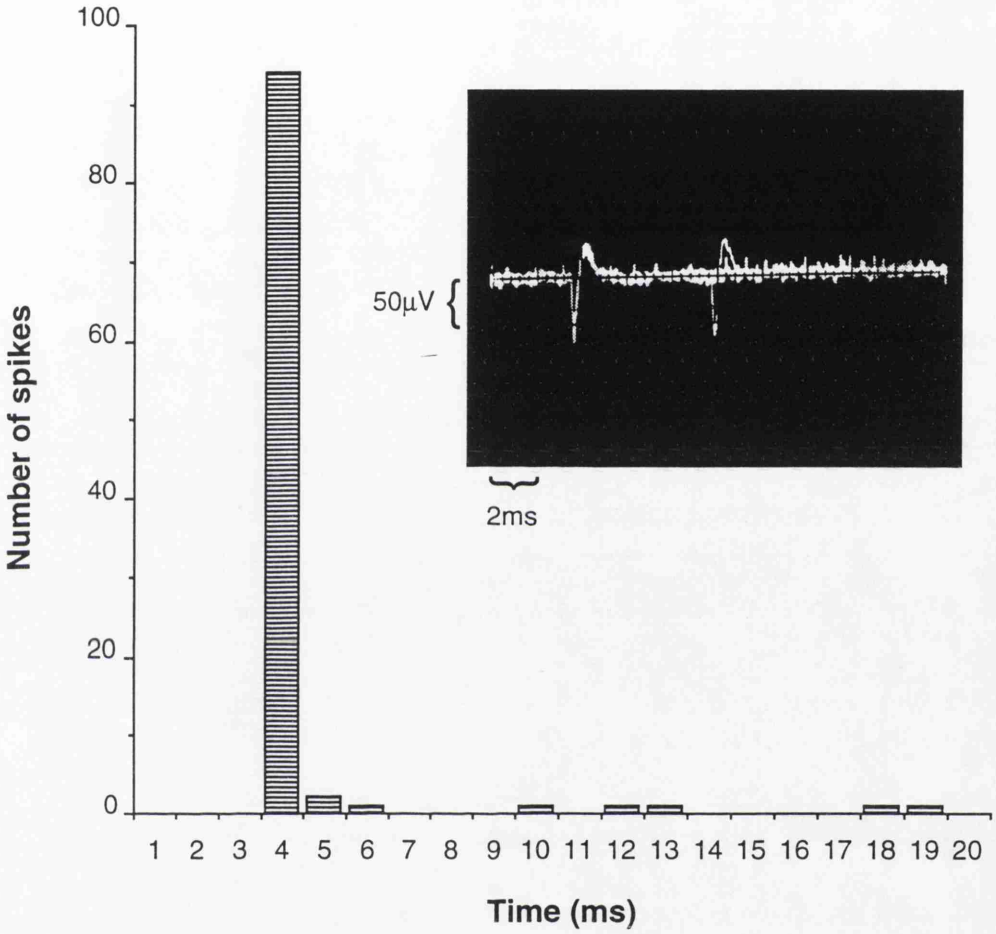
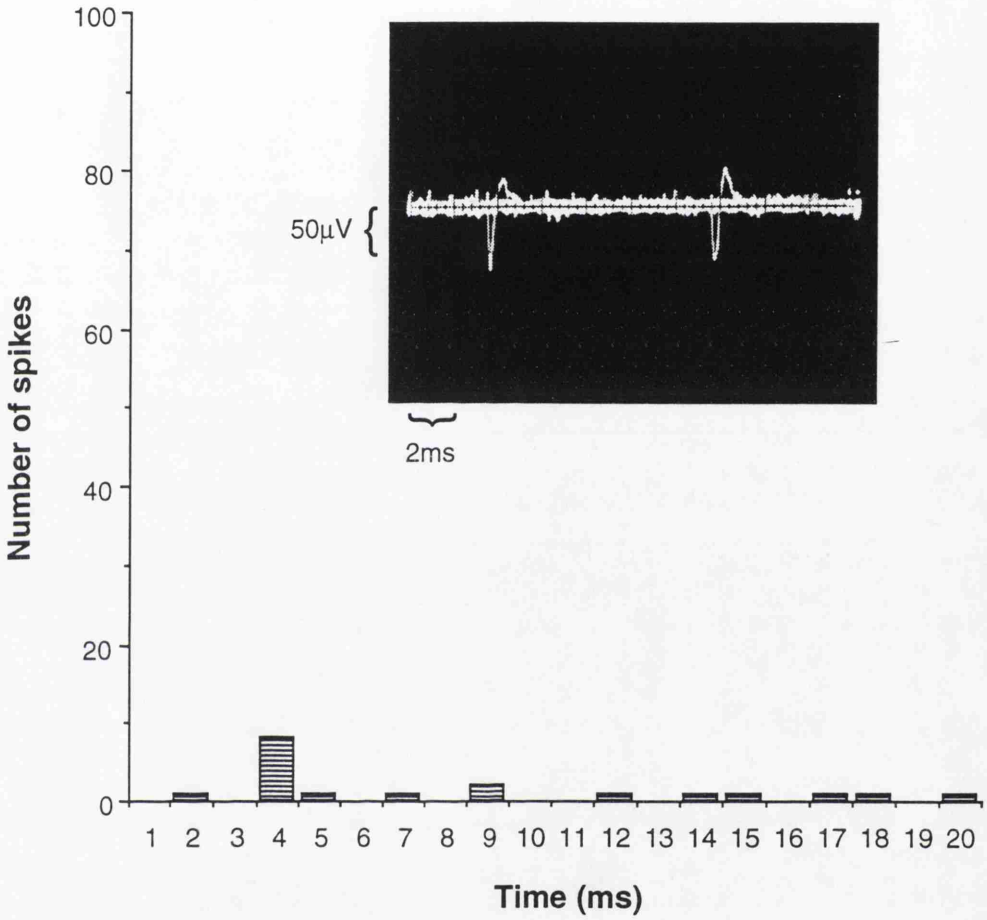

100
80
60
40
20
0
Number of spikes
50μV
2ms
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time (ms)

Figure 45: As in figure 44, but with conditioning stimulation applied to the PAN. The response to spinal stimulation is reduced from 94% to 8%. The response returns to control levels when the spinal current is increased to 29μA.


100
80
60
40
20
0
Number of spikes
50μV
2ms
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time (ms)

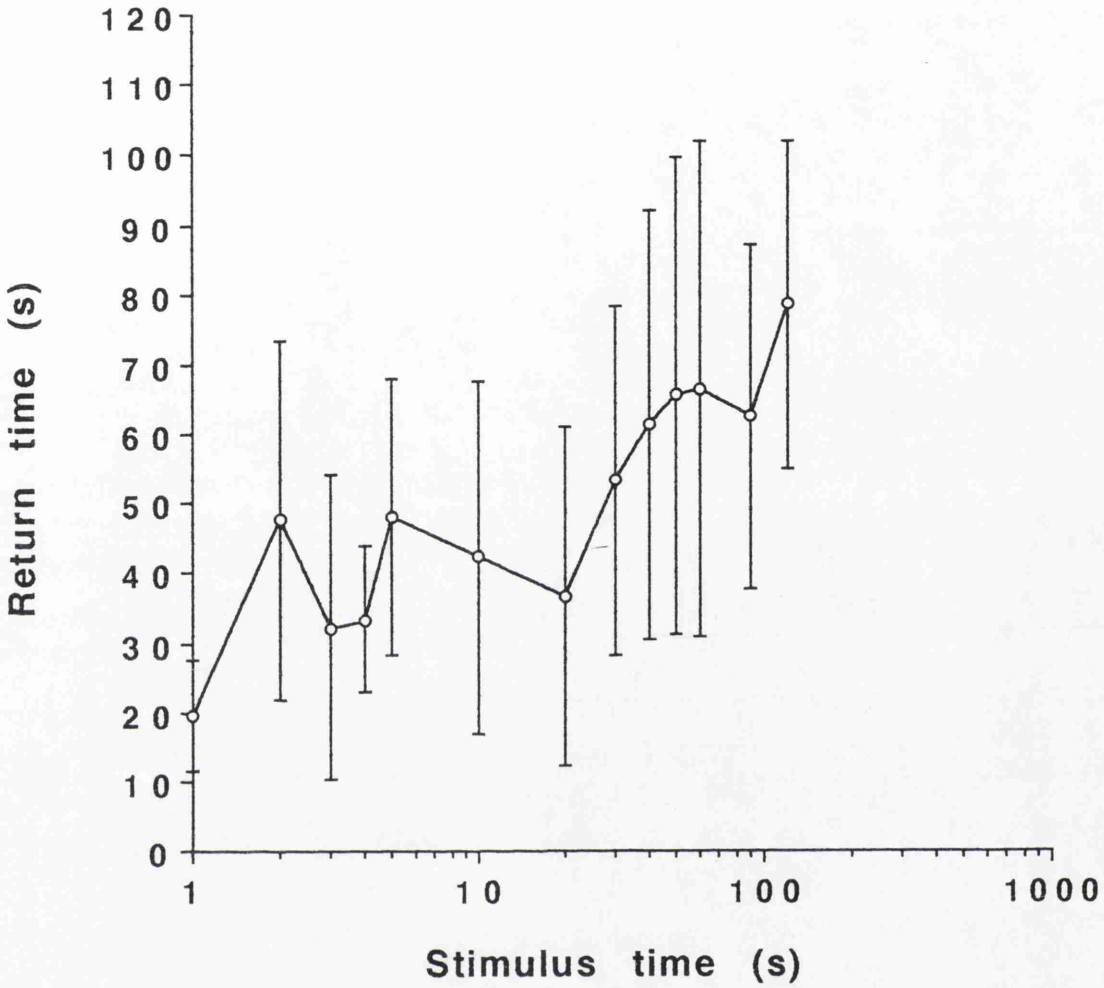
With PAN stimulation, the time taken for the antidromic potential to return after the conditioning stimulation has stopped is dependent on the duration of conditioning stimulation (Fig. 46). Some action potentials never return, especially after a long period of conditioning, and these are excluded from the graph.

With conditioning stimuli in the sural and gastrocnemius nerves it was always possible to return firing in PAN by increasing the spinal current (Sural=16.1% ± 7.5; Gastroc=23.3% ± 7.3).

Electrical stimulation of the whole PAN produces the greatest effect perhaps because the greatest number of group IV fibres are being activated simultaneously (Table 3). With the split PAN a smaller number of group IV fibres will be activated, especially if some have been damaged during the splitting of the nerve. With application of capsaicin, not all group IV afferents are activated and the firing will be asynchronous. Sural and gastrocnemius produce the weakest effect suggesting the input from these nerves is less than from PAN. Stimulation of contralateral PAN has no effect.

In two out of six experiments, stimulating the dorsal roots resulted in excitation of PAN afferents which was inhibited by conditioning stimulation (Fig. 47). The roots were cut centrally. Stimulation of the cut sciatic nerve produced an A-wave in PAN which was unaffected by conditioning stimulation (Fig. 48). This suggests there may be hyperpolarisation of group II afferents in the dorsal root ganglion.

Figure 46: The abscissa represents the duration of conditioning stimulation. The ordinate represents the time taken for the ratio of spinal stimulation to antidromic potentials in the PAN to return to control levels (mean ± SEM), after the conditioning stimulation has stopped. Some action potentials do not return, even after extended periods, and these are excluded from the graph. Increasing the spinal current has little effect on the time taken for the response to return.


120
110
100
90
80
70
60
50
40
30
20
10
0
Return time (s)
1
10
100
1000
Stimulus time (s)

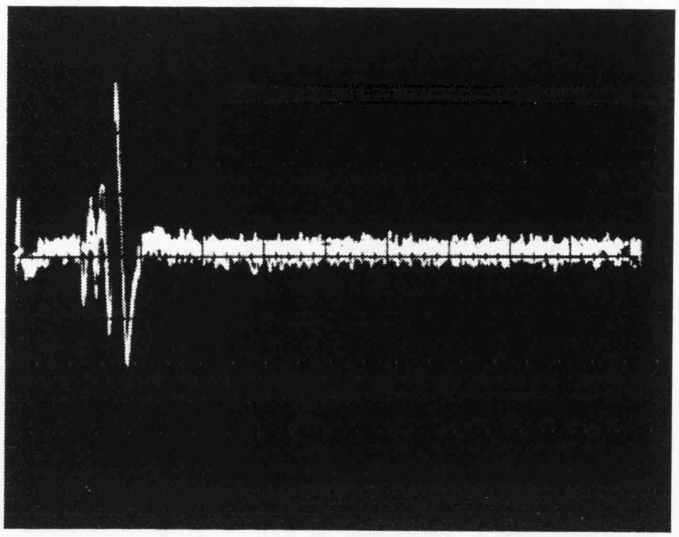
	Mean	S.E.M.	Total (n)	No effect	Completely abolished
Whole PAN	11.3	3.37	36	0	21
Split PAN	21.1	6.87	18	0	9
Capsaicin	32.2	18.33	5	1	2
Gastrocs.	42.9	13.92	11	4	2
Sural	69.2	10.07	8	5	1

Table 3: The control level of firing in PAN to spinal stimulation approximates one hundred percent. The table represents the percentage of the control response during conditioning stimulation.

Hyperpolarisation is produced in the spinal terminals of group II PAN afferents by group IV PAN, gastrocnemius and sural afferents. There may also be some interaction in the dorsal roots. These results show that both electrical and chemical stimulation of group IV PAN afferents result in hyperpolarisation of ipsilateral group II fibres. Stimulation of group IV sural and gastrocnemius fibres produces a weaker hyperpolarisation, while stimulation of the contralateral PAN has no effect. Hyperpolarisation is most probably the result of a depolarisation of neurones persistently depolarising the primary afferents, i.e. hyperpolarisation is the result of the reduction of an on-going PAD.

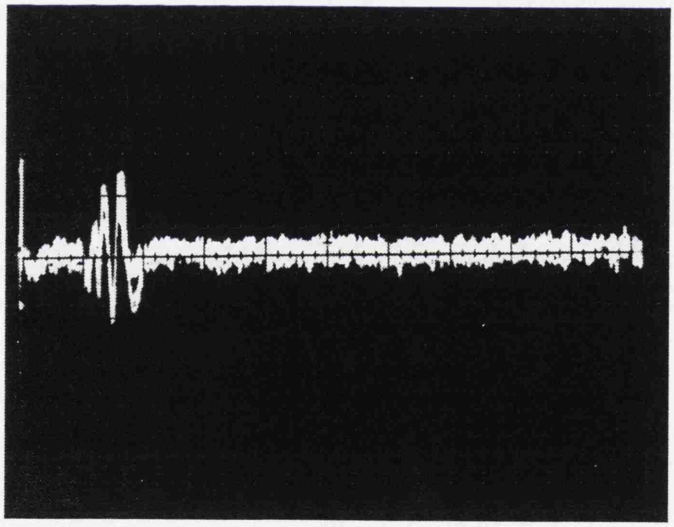
Figure 47: Recording from the PAN during dorsal root stimulation central to the dorsal root ganglion, without PAN conditioning stimulation (A) and with PAN conditioning stimulation (B) (10 sweeps). A decrease in the size of the wave can be seen with the conditioning stimulation. The dorsal roots are sectioned proximal to the stimulating electrode.

A



= 50μV = 2ms

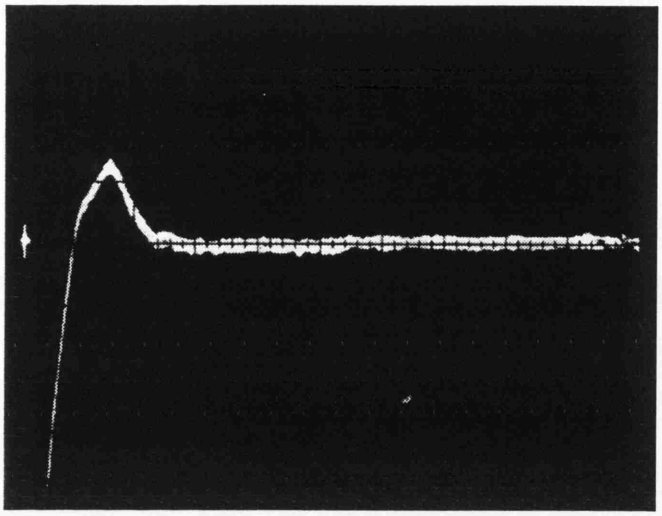
B



= 50μV = 2ms

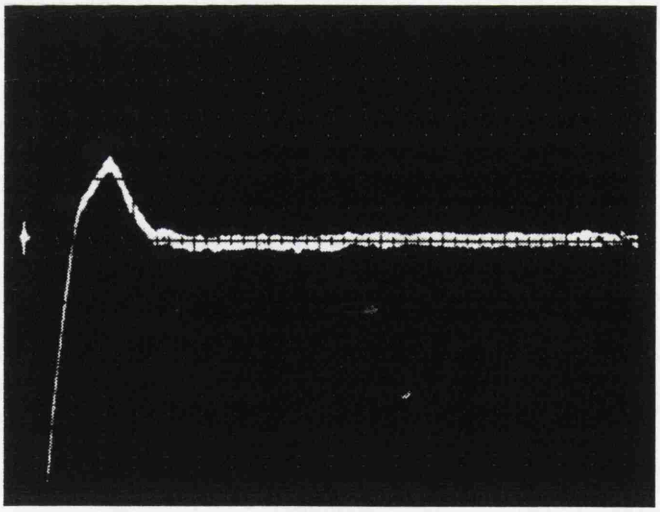
Figure 48: Recording from the PAN during sciatic nerve stimulation, without PAN conditioning stimulation (A) and with PAN conditioning stimulation (B) (10 sweeps). The sciatic nerve is sectioned proximal to the stimulating electrode. There is no change in the size of the A-wave.

A



} = 50μV = 1ms

B



} = 50μV = 1ms

b) **Neurogenic inflammation**

2.7 Protein extravasation induced by substance P in normal rat knees

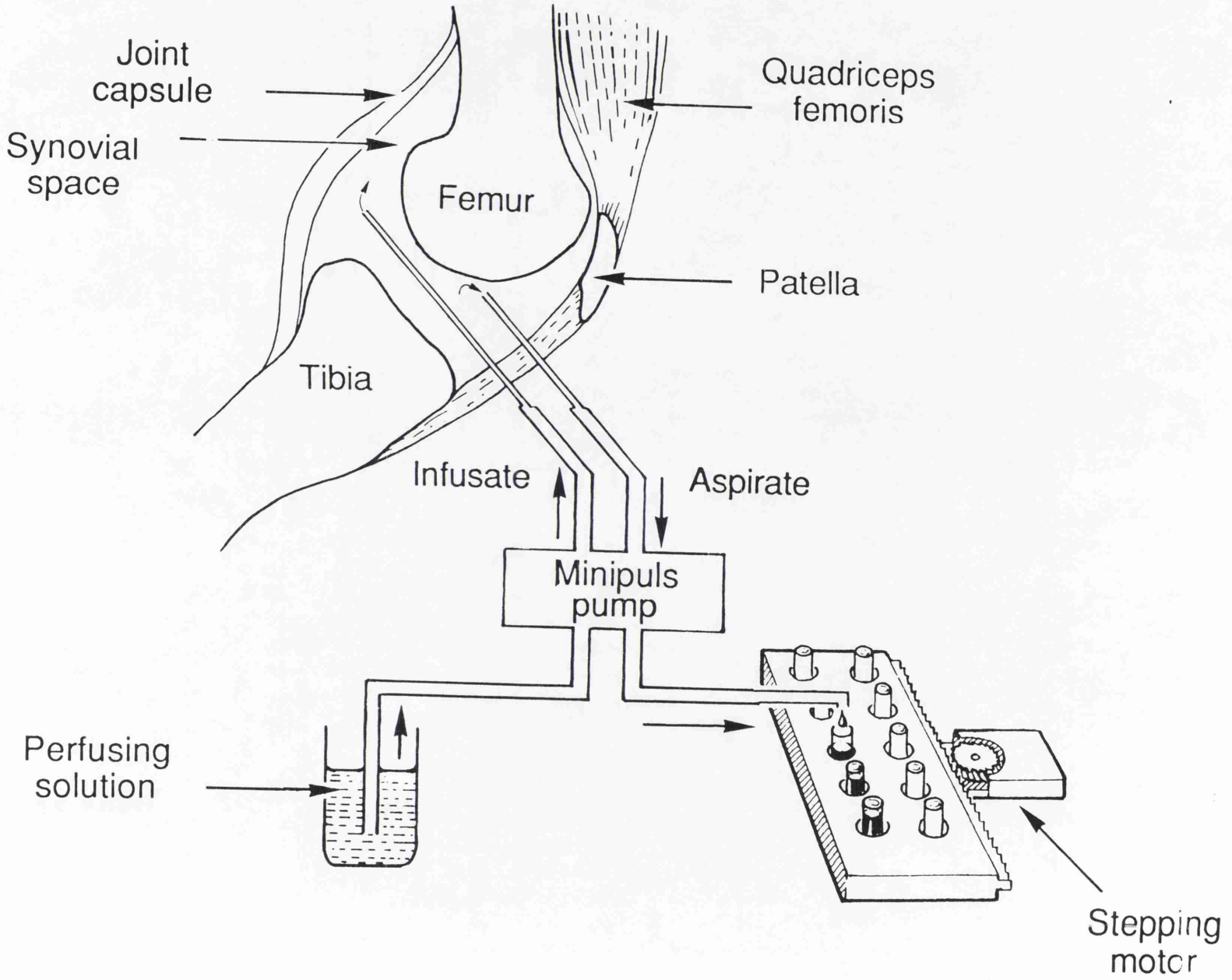
a) Objectives

In recent experiments performed in the rat knee joint it was observed that single intra-articular injections (volume = 0.2ml) of SP over a range of concentrations of 4 to 200 μM produced an inflammatory response as measured by plasma protein extravasation into the joint capsule (Lam & Ferrell, 1989a; 1989b; 1990). Protein extravasation into the synovial cavity, assessed indirectly by measuring the leakage of Evans blue dye from the vascular compartment, has also been shown to occur in the rat knee in response to intra-articular perfusion with SP (Kidd *et al*, 1990b), the technique being sensitive down to concentrations of SP in the perfusate of 10μM. The present study extends these investigations by using a more sensitive technique to measure directly the protein content of fluid collected from the knee joint during perfusion with SP, as well as following the time-course of the inflammatory response.

b) Methods

Experiments were performed in male Wistar rats (350-500g) deeply anaesthetised by intraperitoneal injection of urethane (1.13g/kg) and diazepam (2.5mg/kg). Rats are placed in dorsal recumbency, the skin over the knee is excised and two needle (29 gauge) are inserted into the synovial cavity. The inflow needle is positioned such that it passes between the femoral condyles to lie with its tip in the posterior region of the cavity. The outflow needle is inserted through the capsule medial to the patellar tendon, with its tip in the anterior region of the knee joint cavity. Normal saline (0.9%) is perfused into the synovial cavity (Gilson minipuls) at a rate of 0.1ml/4min and the effluent from the joint collected every four minutes (Fig. 49). Saline perfusion is continued for one hour, after which the perfusate is changed to one consisting of saline in which SP is dissolved in different concentrations. This second perfusion is continued for up to one hour. A control experiment involves changing to a saline solution without any dissolved SP. In most animals both knees are used. After all the samples have been collected, reagents are added to measure the protein content of each sample. The method is based on the observation that in alkaline solution, protein reacts with the quaternary ammonium salt benzyldimethyl{2-[2-(p-1,1,3,3-tetramethylbutylphenoxy) ethoxy]ethyl} ammonium chloride (benzethonium chloride) to produce turbidity (Iwata & Nishikaze, 1979). Samples (0.1ml)

Figure 49: Experimental arrangement for the perfusion of rat knees and subsequent collection of the effluent.


Joint capsule
Synovial space
Femur
Quadriceps femoris
Patella
Tibia
Infusate
Aspirate
Minipuls pump
Perfusing solution
Stepping motor

are mixed with final concentrations of 0.5mol/L NaOH in 33mol/L tetrasodium EDTA (0.4ml) and 2% benzethonium chloride (0.1ml). Standard protein samples are included with each experiment (20-500mg/L) so that each experiment has its calibration curve, to minimise any variability in the test. The amount of protein recovered is calculated by comparing the absorbency of the samples at 360nm (LKB Ultrospect II) with that of the calibration curve. Comparisons are made between pairs of solutions to check that any change in turbidity was not due simply to the presence of SP in the perfusate. The pairs of solutions were: saline vs saline plus SP; saline plus protein vs saline plus protein and SP; and synovial effluent plus saline vs synovial effluent plus saline and SP. Data is presented as the maximum change in protein after transfer to the test perfusate (mean ± S.E.M.).

In a different set of experiments Evans blue (100mg/kg) is injected into the external jugular vein of the rats. This is achieved by exposing the vein surgically before injecting Evans blue with a twenty five gauge needle. Saline and SP are then perfused through the knee joint as described above, and the absorbency of the perfusate from the joint measured in the spectrophotometer at 620nM.

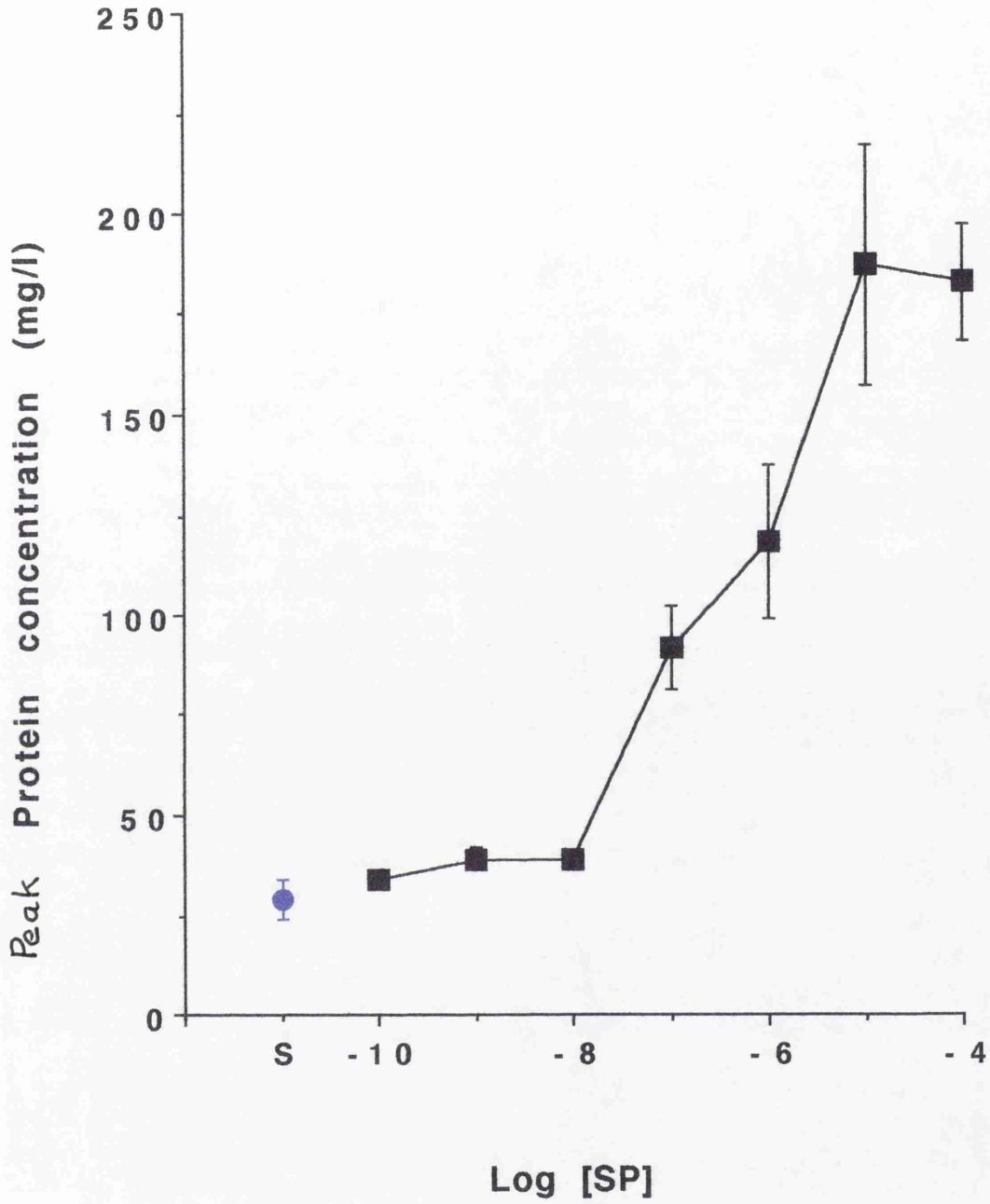
The Mann-Whitney U test (appendix 2) is employed for comparisons between protein values, as these values are often positively skewed within a group. Differences are considered significant if the P values are 5% or less.

c) Results

Perfusion of the knee joint cavity with SP produces a dose dependent response over the range of 10nM to 10μM (Fig. 50). SP perfusions at concentrations of 100pM, 1nM and 10nM all show a higher protein concentration in the effluent than that occurring with the control (saline) solution but this is not significant. A concentration of 100nM SP gives a significantly greater response than that due to 10nM ($p<0.005$) but increasing the concentration of SP to 1μM does not lead to a significant difference compared to 100nM. A further increase to 10μM produces a significant rise ($p<0.01$) but the response levels out with the next concentration (100μM). There is no difference in absorbency between the samples containing saline alone and saline plus SP, nor is there any difference between the samples containing saline plus protein and saline plus protein and SP. Saline added to control effluent samples gives marginally higher absorbency readings in some cases than SP added to control effluent samples. Thus, any rise in turbidity observed after switching the perfusate from saline alone to one containing SP must have resulted from a biological action of SP in causing plasma protein extravasation into the synovial cavity.

The response to SP perfusion of the joint shows tachyphylaxis in that a repeat perfusion of SP fails to elicit further plasma extravasation, even when the SP concentration in the perfusate is increased by a hundred fold. This necessitates data collection

Figure 50: Protein extravasation into the synovial cavity of the rat knee in response to intra-articular perfusion with different concentrations of substance P (SP). **S** represents value obtained with control (saline) perfusion. Values are means ± SEM; n = 5.


250
200
150
100
50
0
Peak Protein concentration (mg/l)
S
-10
-8
-6
-4
Log [SP]

using a single SP perfusion per knee. In a small number of rats, one knee is continuously perfused with saline whilst the contralateral knee is perfused with SP. In these animals a small rise in protein concentration occurs in the saline-perfused side, but only when the perfusate to the contralateral knee contains SP in a concentration of 100μM. With concentrations of 10μM or lower, no protein extravasation is observed in the contralateral knee joint.

A notable feature of the present experiments is the transient nature of plasma protein extravasation in response to SP perfusion. Although this perfusion is maintained in some cases for up to an hour, protein extravasation peaks at about 12-16 min from the onset of perfusion and then declines over a similar time course, even with the highest concentration of SP (Fig. 51). There is an obvious dose dependency in protein extravasation, both in terms of the peak response and the time course of the response over a SP concentration range of 100pM to 100μM (Fig. 51). To check that the transient response is not due to an artefact associated with the protein measurement technique, additional experiments were performed in five rats where the extravasation of plasma protein was assessed by the leakage of Evans blue dye (Kidd *et al*, 1990). Although, in our hands, responses to SP concentrations below 100μM can not be obtained, transient extravasation at this concentration is again observed, confirming our present results, but suggesting that the Evans blue technique is less sensitive.

2.8 Protein extravasation induced by substance P in acutely inflamed rat knees

a) Objectives

In the above experiments, perfusion of the rat knee joint with SP resulted in protein extravasation into the synovial cavity. The response was dose dependent from 100nM to 10μM, with each dose producing a steady rise in protein levels for 12-16 minutes, which then fell sharply over an equivalent period despite continued SP perfusion. Articular C-fibres have been shown to be more sensitive to physiological stimulation when surrounding tissues are inflamed (Gilbaud *et al*, 1985; Grigg, Schaible & Schmidt, 1986; McMahon & Koltzenburg, 1990), and this may result in SP being released in greater quantities in inflamed tissues. Thus, the actions of SP in these tissues is relevant in understanding its role in joint inflammation, and the present study investigates the effects of intra-articular perfusion of SP in acutely inflamed rat knees.

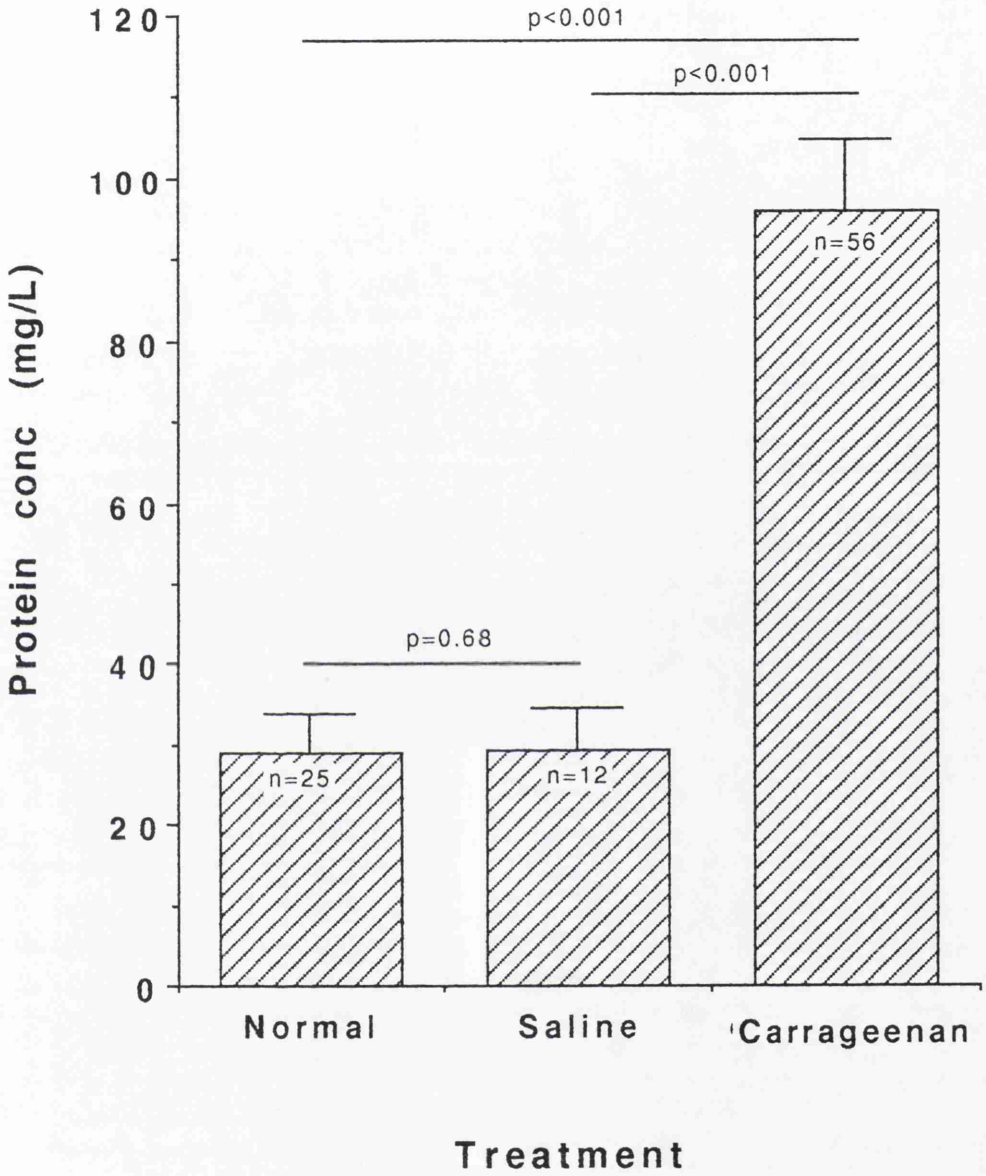
b) Methods

Experiments were performed in male Wistar rats (350-500g), deeply anaesthetised by intraperitoneal injection of diazepam (2.5mg/kg) and intramuscular Hypnorm (0.1mg/kg). The rats are then injected with 0.2ml of 2% carrageenan in the anterior and

posterior cavities of both knee joints and allowed to recover. Twenty-four hours later, anaesthesia is re-introduced by intraperitoneal injection of urethane (1.13g/kg) and diazepam (2.5mg/kg). The rats are placed in dorsal recumbency and the knees perfused with saline for an hour and thereafter by a perfusate containing SP in differing concentrations. Only one concentration of SP is tested in individual animals. Perfusate samples are collected over four minute intervals. The protein content of each sample is measured by protein micro-turbidimetry as previously described (Iwata & Nishikaze, 1979). Using 4ml of the NaOH/EDTA mixture and 1ml of benzethonium chloride is only sensitive to 50mg/L of protein but has a linear calibration curve up to 500mg/L of protein. This is in comparison to a sensitivity of 5mg/L and a linear calibration up to 200mg/L when volumes are used as in the previous experiments on normal knees. As higher levels of protein are present in the inflamed joints, the larger volumes of reagents are used. In a smaller separate group of rats, both knee joints are injected with 0.2ml of 0.9% saline instead of 2% carrageenan 24h prior to the start of experiments to serve as controls.

The Mann-Whitney U test is employed for comparisons between protein values. The figures represent means ± S.E.M. and differences are considered significant if the P values are 5% or less.

Figure 52: Basal plasma protein extravasation (mean ± SEM) during saline perfusion of the joint prior to substance P perfusion. Comparisons are between normal animals, animals whose joints were injected with sterile saline 24 hours previously and animals injected with 2% carrageenan 24 hours before.

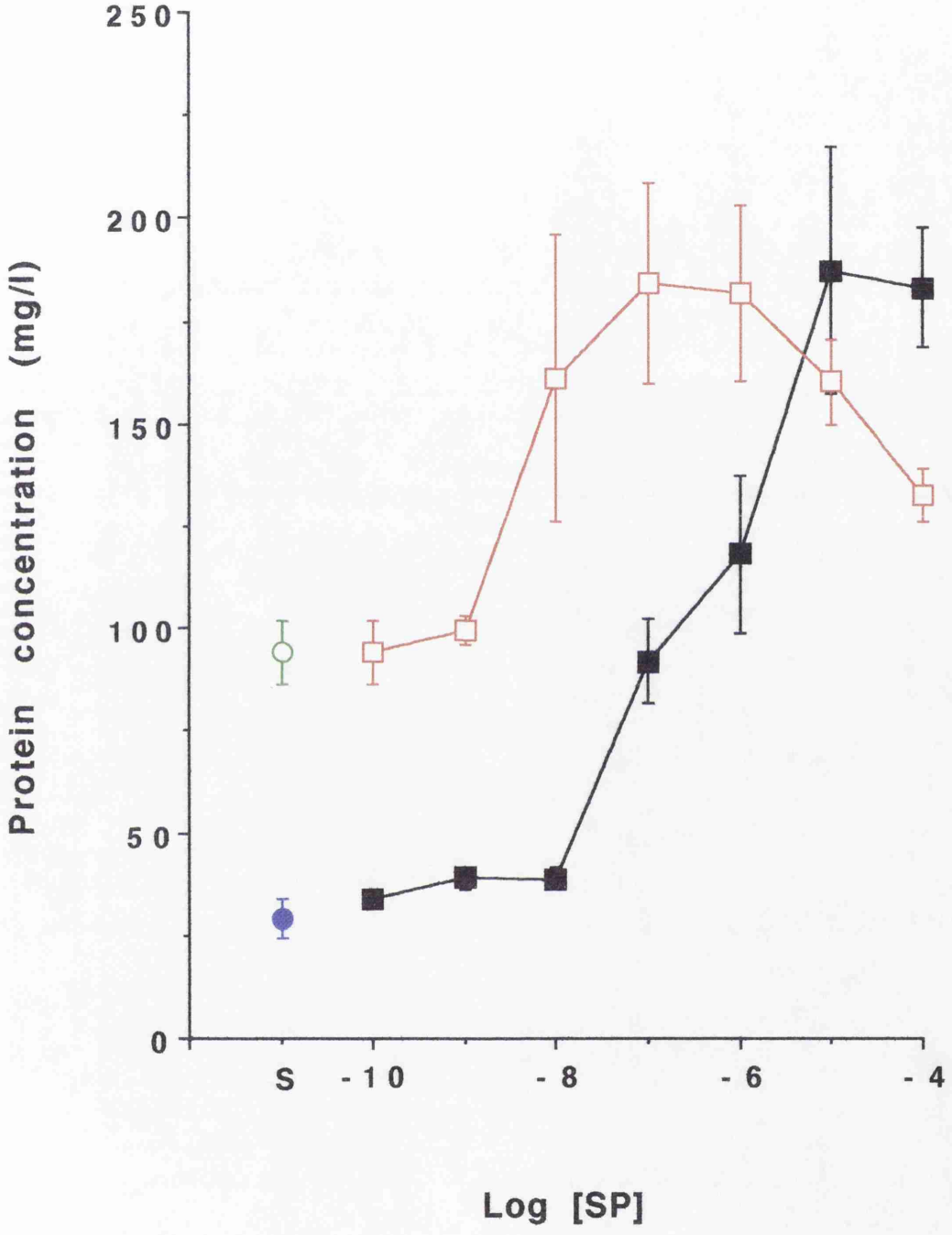

p<0.001
p<0.001
p=0.68
n=25
n=12
n=56
120
100
80
60
40
20
0
Protein conc (mg/L)
Normal
Saline
Carrageenan
Treatment

c) Results

Protein extravasation in the perfusate samples from rat knee joints were measured after saline perfusion for an hour. The mean basal protein extravasation values occurring in normal rat knee joints, in rat knee joints which had been previously injected with physiological saline, and in those injected with 2% carrageenan are shown (Fig. 52). As the normal and saline control rat knees produce similar basal levels of protein extravasation comparisons are made in subsequent studies between the normal and carrageenan-treated knees.

In normal rat knee joints, when the saline perfusate is switched to one containing SP in concentrations up to 10nM, no significant rise in protein extravasation is observed. However, when 100nM SP is perfused, protein extravasation is significantly ($P<0.01$) elevated in these animals. The effect is dose-dependent and the peak response achieved at a concentration of 10μM SP (Fig. 50). In rat knee joints which had been previously injected with carrageenan, the mean basal level of protein extravasation is significantly higher ($P<0.001$) than that obtained from normal rat knee joints (Fig. 52). The threshold SP concentration required to elicit a significant increase in plasma extravasation in these animals is 10nM instead of 100nM in the normal knees. Increasing SP concentrations to 100nM and 1μM produces slightly higher levels of plasma extravasation than that induced by 10nM, but the

Figure 53: Effect of intra-articular perfusion with differing concentrations of substance P on plasma protein extravasation in normal animals (■) and in those with carrageenan-induced acute joint inflammation (❐). **S** represents the protein level occurring during infusion with saline. At each concentration n = 5 for normal animals and n = 8 for carrageenan-treated animals.

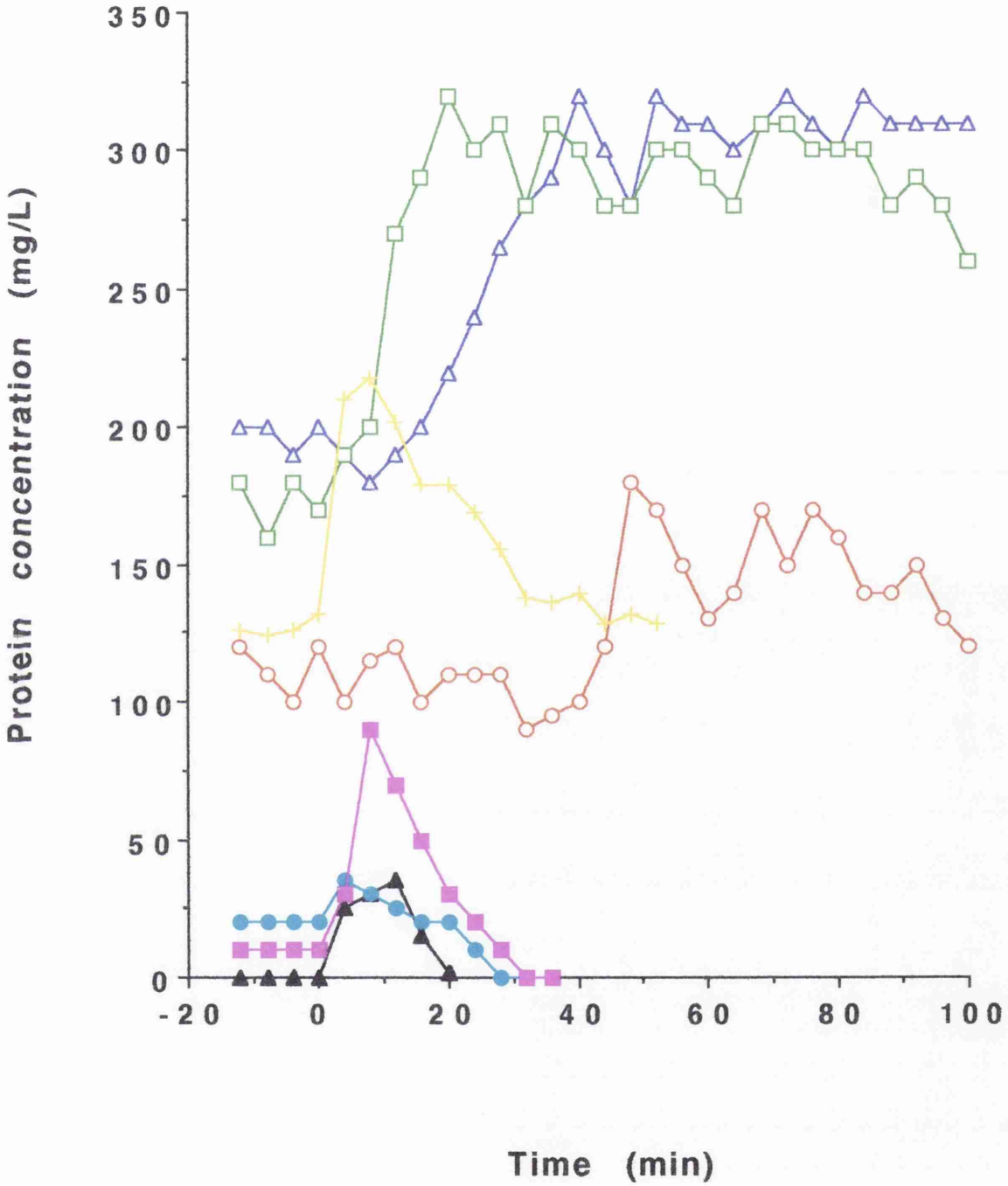

250
200
150
100
50
0
Protein concentration (mg/l)
S
-10
-8
-6
-4
Log [SP]

differences are not significant. Further increases of SP concentrations to 10μM and 100μM do not produce any further rise in plasma extravasation. In fact, plasma extravasation produced by 100μM SP is significantly lower ($P<0.05$) than that produced by 100nM SP, which is the peak response (Fig. 53).

In normal rat knees, the responses to SP is transient, with protein extravasation reaching a peak about 12-16 minutes and then falling sharply again, returning to control values over an equivalent period despite continued perfusion with SP. In carrageenan-treated rat knees, the basal level of protein extravasation is higher and SP-induced plasma extravasation is more persistent, with the increased level of protein extravasation being maintained, in some experiments, all the way through the perfusion period lasting nearly two hours (Fig. 54). The sustained response to SP in carrageenan-treated knees is related to the basal protein extravasation level as it is found that the time taken for SP-induced plasma extravasation to decline to 80% of its peak value is positively correlated ($r=0.661$) with the initial protein level, i.e. the higher the basal protein extravasation level, the longer the period of time required for the SP-induced protein extravasation to drop to 80% of the peak response.

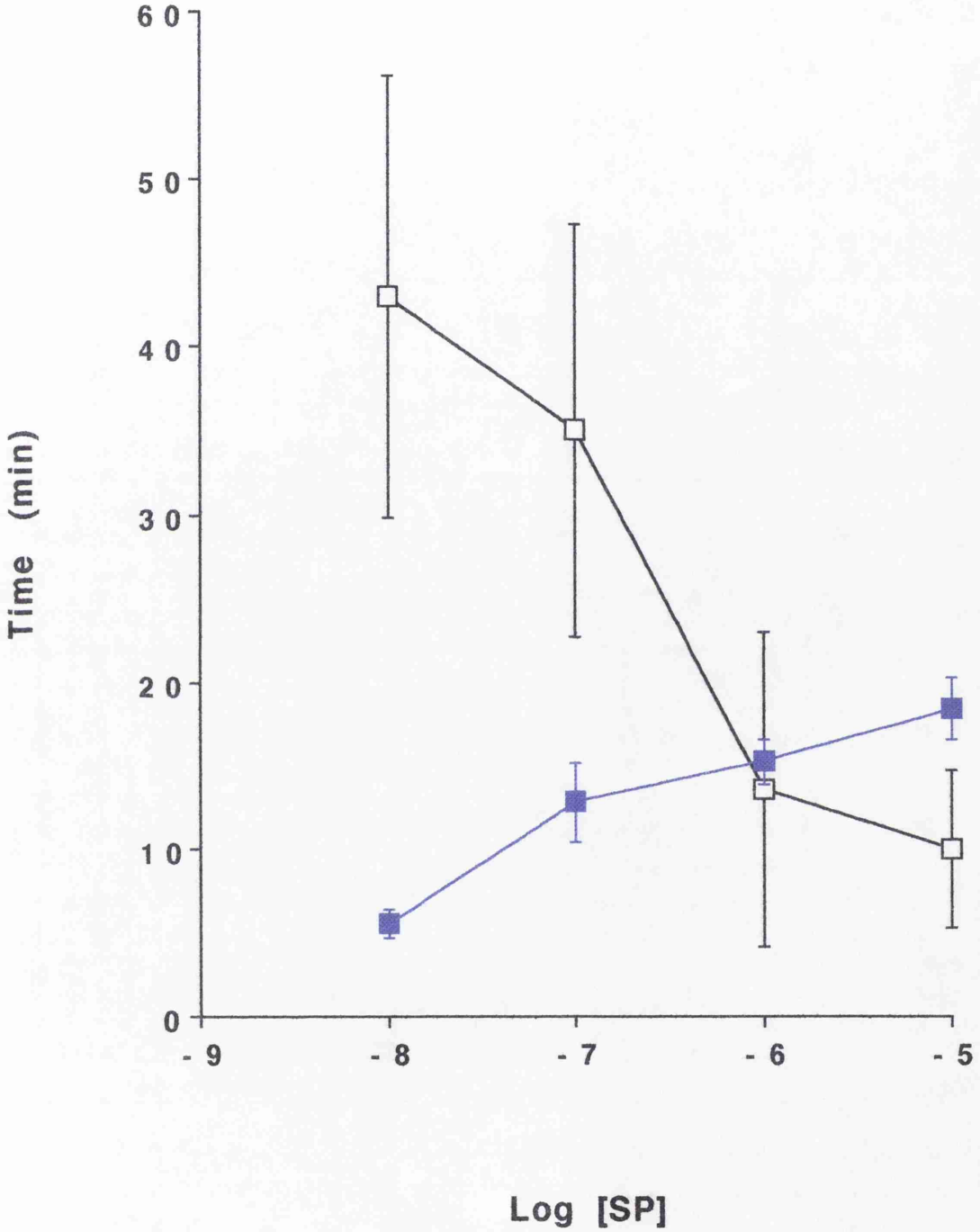
The time required to reach the maximum responses in both the normal and carrageenan-treated animals are similar (10-20min) when high concentrations of SP (1μM and 10μM) are perfused. However, with lower concentrations of SP (10nM and

Figure 54: Time course of substance P-induced plasma extravasation in normal animals (closed symbols) and in carrageenan-treated animals (open symbols). In each case three concentrations were used: 10nM (○), 100nM (Δ), and 1μM (❑). The response of one animal administered a perfusate containing inflammatory cells followed by the same perfusate with 1μM SP is also illustrated (⊣).


Protein concentration (mg/L)
350
300
250
200
150
100
50
0
-20
0
20
40
60
80
100
Time (min)

100nM), the carrageenan-treated animals require much longer periods (35-40min) to reach their maximum responses, whereas in the normal animals the time required is slightly reduced (5-12min) (Fig. 55).

Figure 55: Representation of the time taken for the substance P-induced plasma extravasation response to reach its maximum at different concentrations of SP (mean ± SEM) in normal animals (■) and in carrageenan-treated animals (□). At each concentration n = 5 for normal animals and n = 8 for carrageenan-treated animals.


60
50
40
30
20
10
0
Time (min)
- 9
- 8
- 7
- 6
- 5
Log [SP]

2.9 Inhibition of neuropeptide induced protein extravasation in the rat knee by specific neurokinin receptor antagonists.

a) Objectives

Substance P induces protein extravasation in both normal and acutely inflamed joints. However the response to other neurokinins and the receptors involved in the extravasation have not been identified with the above technique. As NKA is present with SP in peripheral nerve terminals it is relevant to examine the extravasation produced by NKA. Novel, highly specific neurokinin receptor antagonists have been produced, and are used here to identify the types of neurokinin receptors present in the rat knee. The antagonist FK888 is highly specific for NK-1 receptors and will help to identify these receptors when perfused with SP, the endogenous NK-1 receptor agonist. The antagonist FK224 binds to both NK-1 and NK-2 receptors and will help to identify these receptors when perfused with SP and NKA, the latter being the endogenous NK-2 receptor agonist. As the use of NKA and antagonists indicated the presence of NK-2 receptors, the effect of the specific NK-2 receptor agonist [Nle^{10}]-neurokinin A_{4-10} was examined. The effect of the specific NK_2 antagonist (SR48968) with SP and NKA was also examined.

b) Methods

Experiments are performed in male Wistar rats (350-500g; in-house colony), deeply anaesthetised by intraperitoneal injection of urethane (2g/kg). The rats are placed in dorsal recumbency, the skin over the knee excised, and two needles (29 gauge) inserted into the synovial cavity as previously described. Normal saline (0.9%) is perfused into the synovial cavity through one needle at a constant rate of 25μL/min by a peristaltic pump (Gilson minipuls) and the effluent from the joint collected, via the other needle, into sample tubes every 4 minutes. Perfusion is continued for an hour and is thereafter changed to a perfusate containing differing concentrations of neurokinin antagonist. Only one concentration of antagonist is used with each knee. Perfusion is continued for twenty minutes with the antagonist before changing to a perfusate with the same concentration of antagonist, plus a concentration of agonist (SP or NKA). Only one antagonist/agonist concentration is used in each rat knee. The protein content of each sample is measured by protein micro-turbidimetry as previously described. Volumes of 0.1ml sample, 4ml NaOH/EDTA and 1ml benzethonium chloride are used in these experiments.

Dose response curves for SP, NKA and [Nle^{10}]-neurokinin A_{4-10} are determined by perfusing with saline for an hour and thereafter changing to differing concentrations of the agonist.

Protein levels shown for the agonists are the protein levels immediately before drug application subtracted from the maximal protein level after drug application. The protein levels produced in the presence of antagonists are expressed as a percentage of the level achieved with the agonist alone.

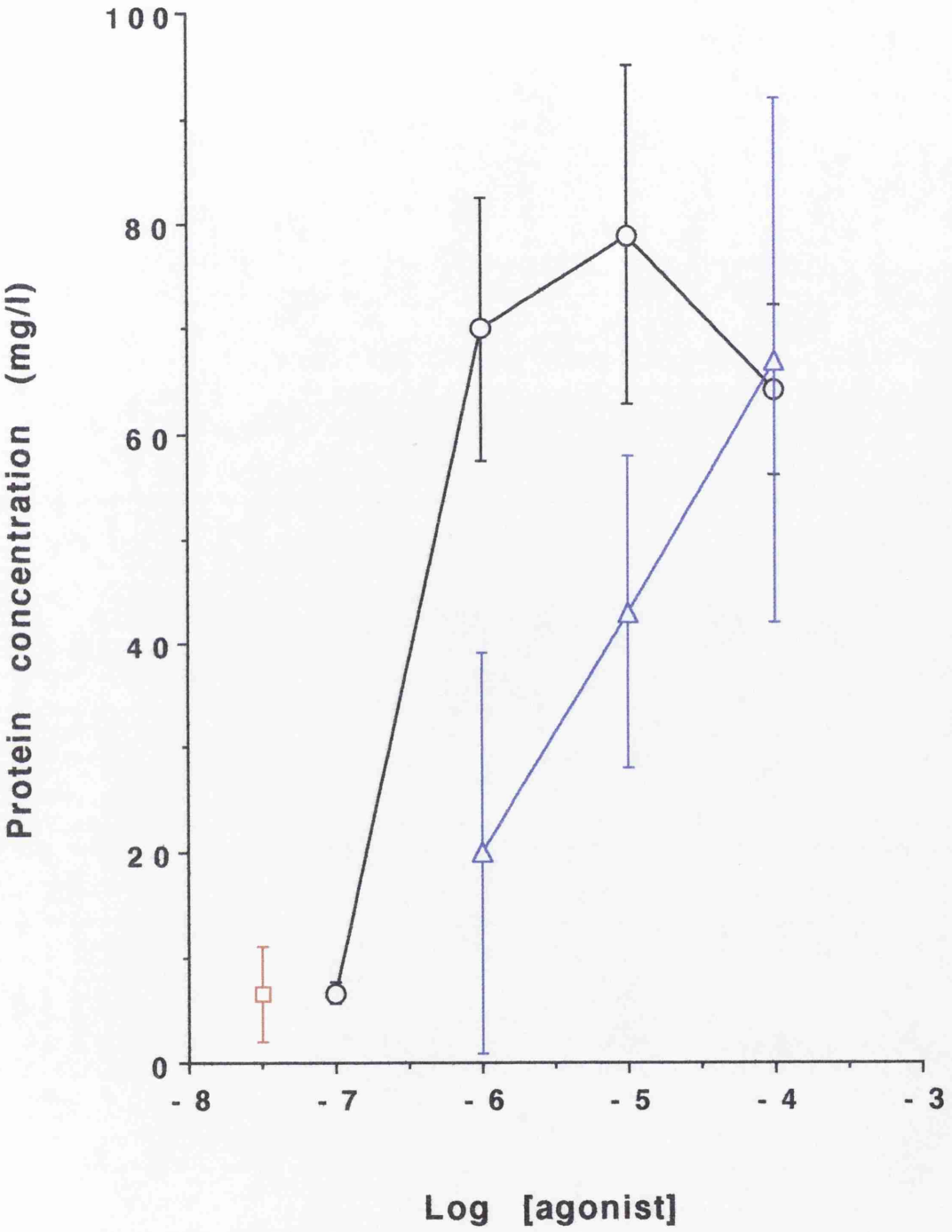
In some experiments, one of the carotid arteries is cannulated and the blood pressure is monitored continually while the knee is being perfused.

The Mann-Whitney U-test is employed for comparisons between protein levels and percentages. The figures represent means ± S.E.M. and differences are considered significant if the P values are 5% or less. The Kruskal-Wallis H test (appendix 3) is used for comparisons between the four sets of results for each concentration of agonist. The response to the agonist alone is compared with the agonist plus 1μM, 10μM or 100μM antagonist (Table 4).

c) Results

SP and NKA both produce a dose dependent increase in protein extravasation when perfused through the joint. SP and NKA are both dissolved in 0.9% saline, which itself, does not induce protein extravasation. NKA produces a significant rise in protein extravasation from 100nM to 1μM ($P < 0.005$), but increasing the concentration does not produce further significant changes (Fig. 56). The NK_2 agonist, [Nle^{10}]-neurokinin A_{4-10}, also

Figure 56: Protein extravasation into the synovial cavity of the rat knee in response to intra-articular perfusion with different concentrations of neurokinin A (NKA) (○) and [Nle^{10}]-neurokinin A_{4-10} (△). (□) represents value obtained with control (saline) perfusion. Values are means ± SEM; n = 5.


100
80
60
40
20
0
Protein concentration (mg/l)
- 8
- 7
- 6
- 5
- 4
- 3
Log [agonist]

induces extravasation with concentrations of 10μM producing significantly greater levels than control values ($P< 0.05$) (Fig. 56). Increasing the concentration does not produce further significant increases in extravasation. The time course of protein extravasation induced by NKA is similar to that induced by SP (Fig. 57).

Concentrations of 1μM ($P< 0.002$), 10μM ($P< 0.002$) and 100μM ($P< 0.001$) FK224 significantly reduce extravasation induced by 1μM SP, but only 100μM FK224 significantly reduces extravasation induced by 10μM SP ($P< 0.05$) (Fig. 58A). Increased levels of extravasation induced by 100μM SP, when combined with FK224, are not significantly different from extravasation induced by 100μM SP alone. FK224 has a pA_2 value of 9.8 when perfused with SP, according to the Schild plot (Fig. 58B). However, the gradient of the regression line is 0.255, which suggests this analysis is not applicable for assessing pA_2 values. Hence, calculating the pA_2 value for the 10μM concentration of SP gives a different value of 6.29.

Extravasation induced by NKA is also inhibited by FK224 (Fig. 59A). Extravasation induced by a concentration of 1μM NKA is inhibited significantly by 1μM, 10μM and 100μM concentrations of FK224 ($P< 0.005$). Reduction in extravasation induced by 10μM NKA is more significant with 100μM FK224 ($P< 0.01$) than with 1μM and 10μM FK224 ($P< 0.05$). There is no significant inhibition of 100μM NKA induced extravasation by FK224. FK224 has a pA_2 value of 9.7 when perfused with NKA (Fig. 59). However, the

Figure 57: Time course of protein extravasation into the synovial cavity of the knee in response to intra-articular perfusion of neurokinin A (NKA). At time 0 the control (saline) perfusate was changed to one containing NKA in concentrations of 10μM (Δ) and 1μM (○).

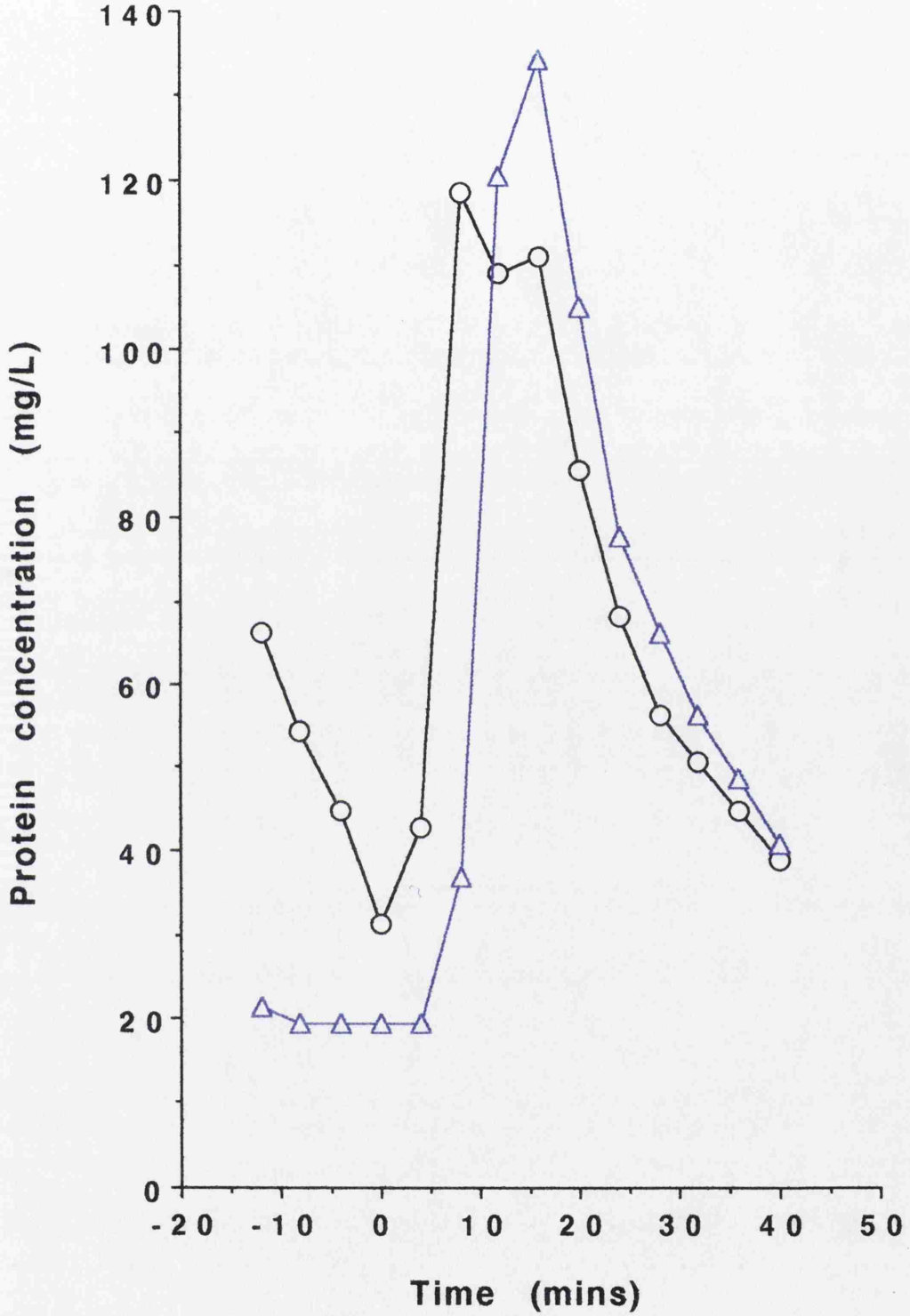
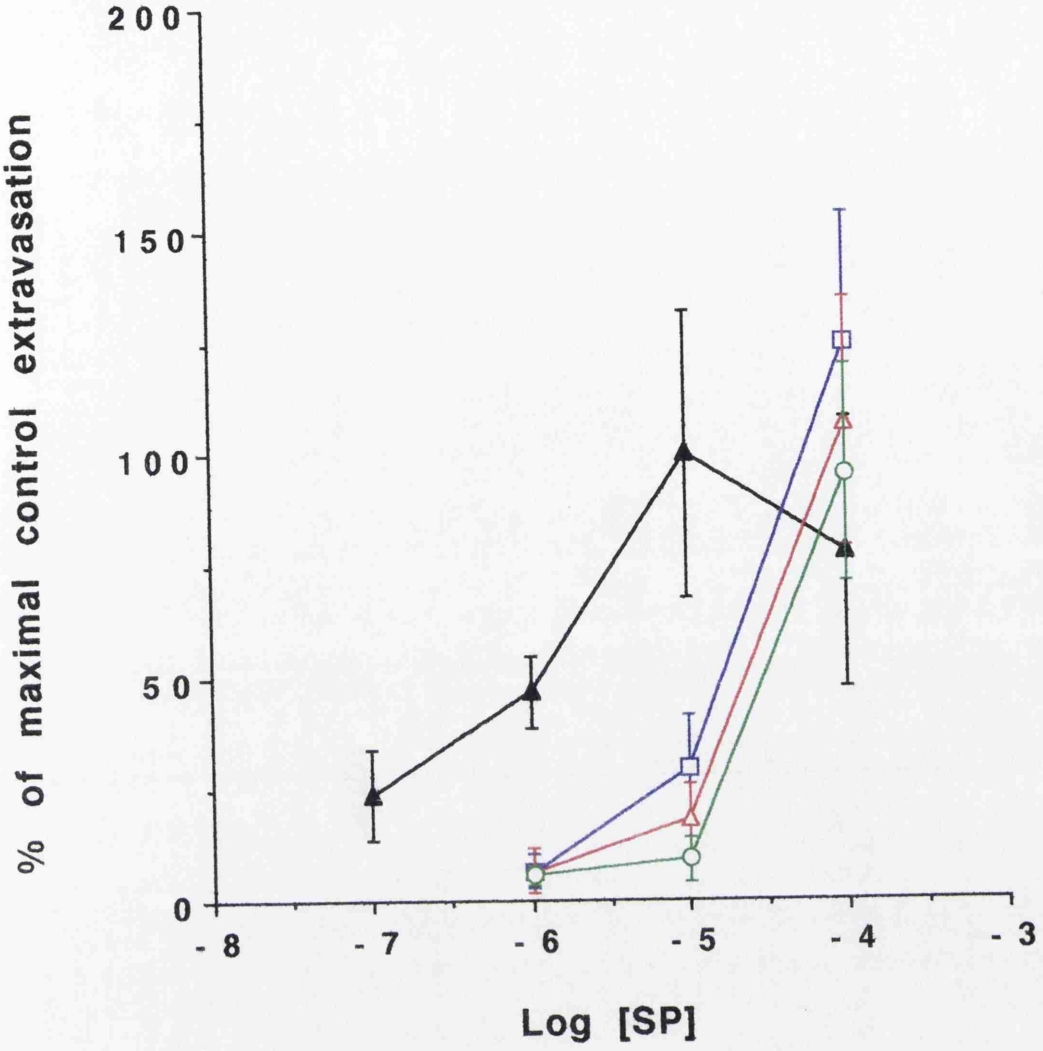

140
120
100
80
60
40
20
0
Protein concentration (mg/L)
-20
-10
0
10
20
30
40
50
Time (mins)

Figure 58: A. The percentages of protein extravasation induced by SP (▲) combined with 1μM (❑), 10μM(Δ) and 100μM (○) FK224. The values are calculated as a percentage of the maximal level of protein extravasation produced by SP alone. Values are means ± SEM; n = 7.

B. Schild plot using values from the above graph.


200
150
100
50
0
% of maximal control extravasation
- 8
- 7
- 6
- 5
- 4
- 3
Log [SP]

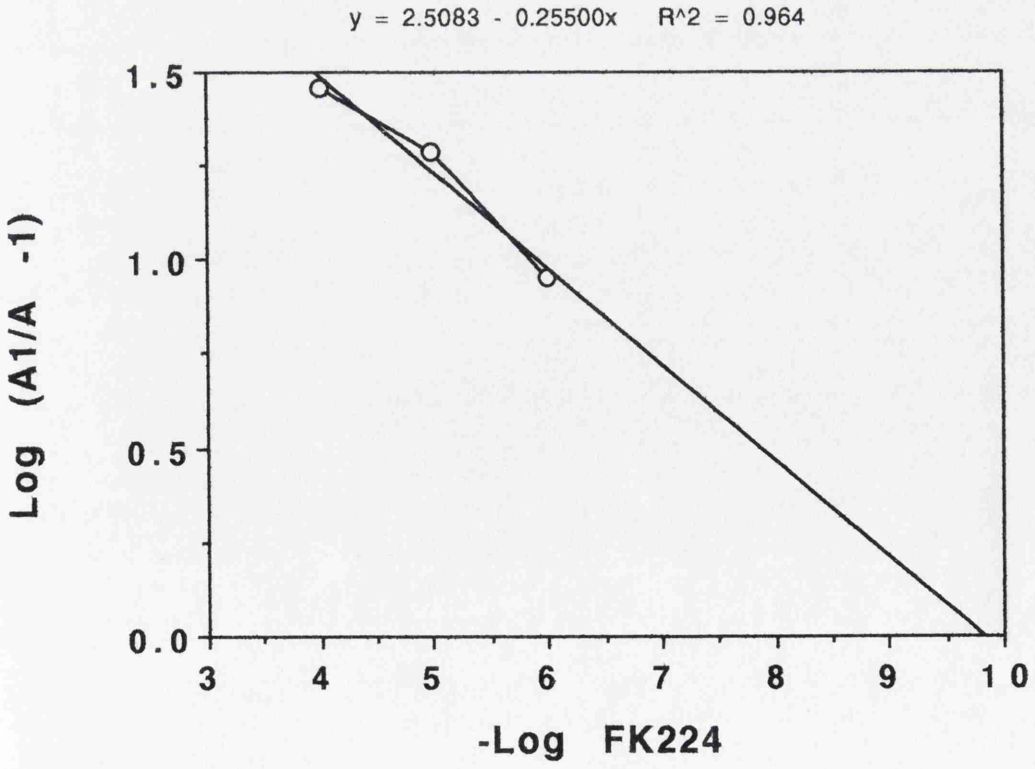
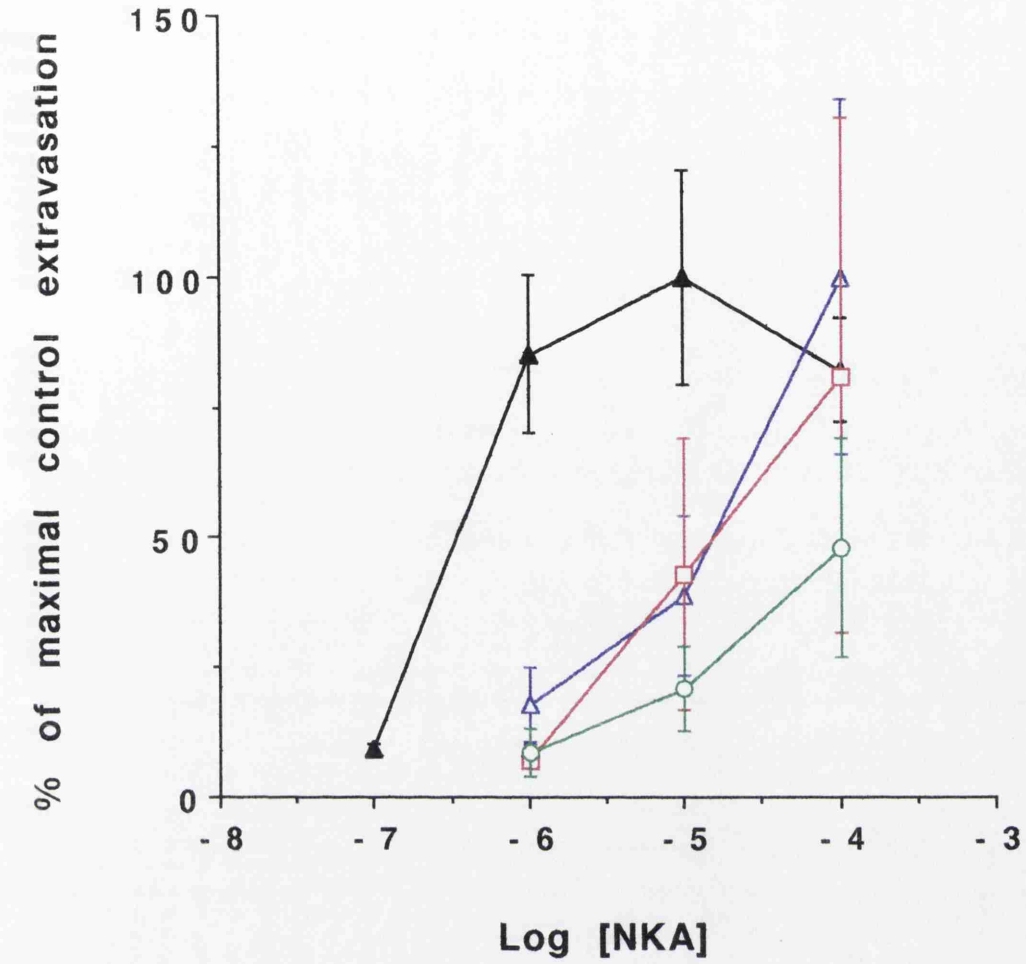
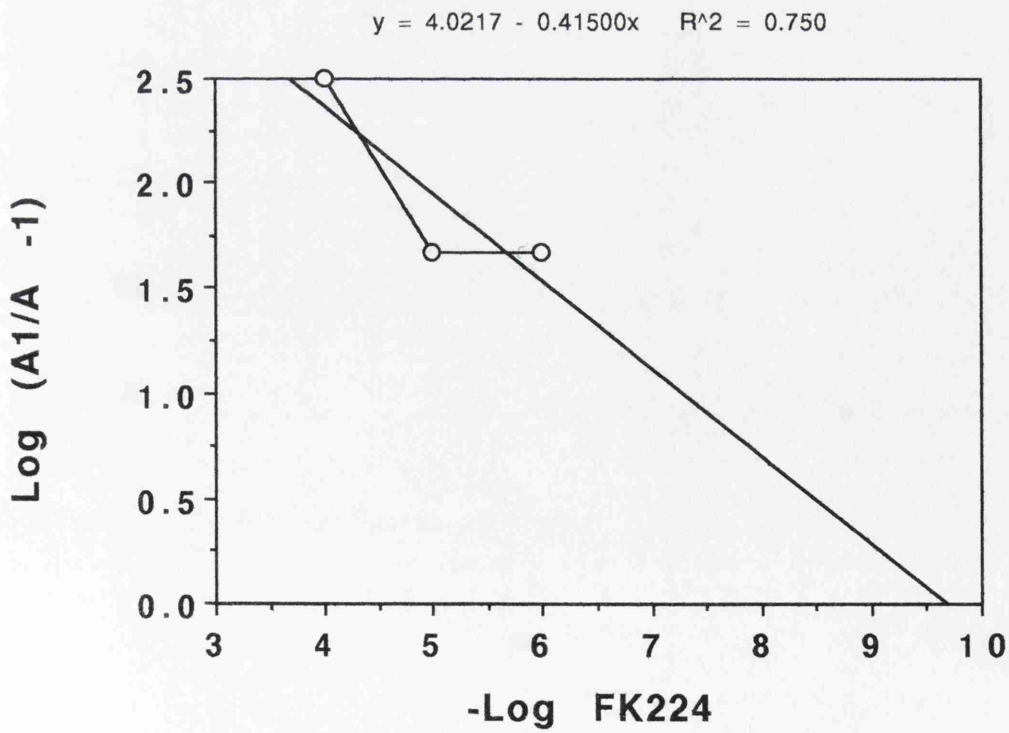

y = 2.5083 - 0.25500x R^2 = 0.964
1.5
1.0
0.5
0.0
Log (A1/A -1)
3
4
5
6
7
8
9
10
-Log FK224

Figure 59: A. The percentages of protein extravasation induced by NKA (▲) combined with 1μM (Δ), 10μM(❑) and 100μM (○) FK224. The values are calculated as a percentage of the maximal level of protein extravasation produced by NKA alone. Values are means ± SEM; n = 7.

B. Schild plot using values from the above graph.


150
100
50
0
% of maximal control extravasation
- 8
- 7
- 6
- 5
- 4
- 3
Log [NKA]


y = 4.0217 - 0.41500x R^2 = 0.750
2.5
2.0
1.5
1.0
0.5
0.0
Log (A1/A -1)
3
4
5
6
7
8
9
10
-Log FK224

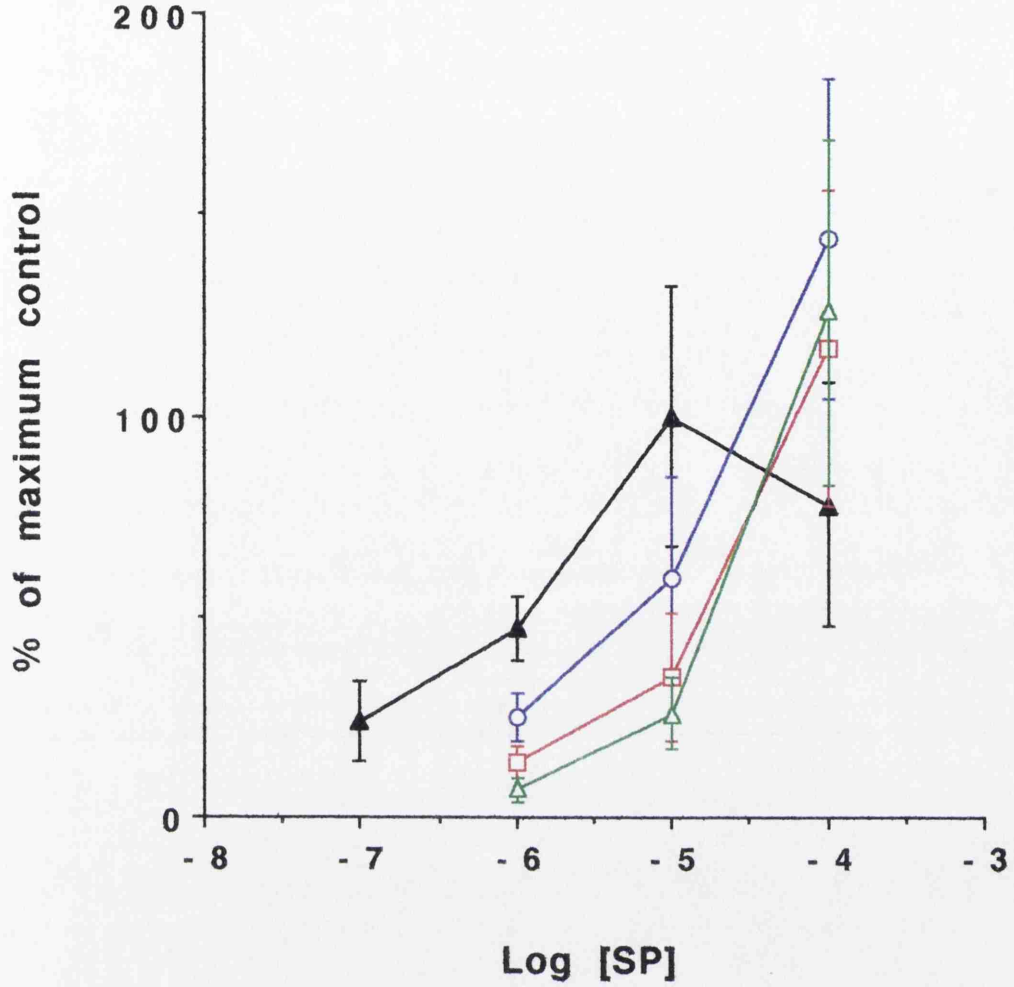
gradient of the regression line is 0.415, which suggests this analysis is not applicable for assessing pA_2 values. Hence, calculating the pA_2 value for the 10μM concentration of NKA gives a different value of 6.67.

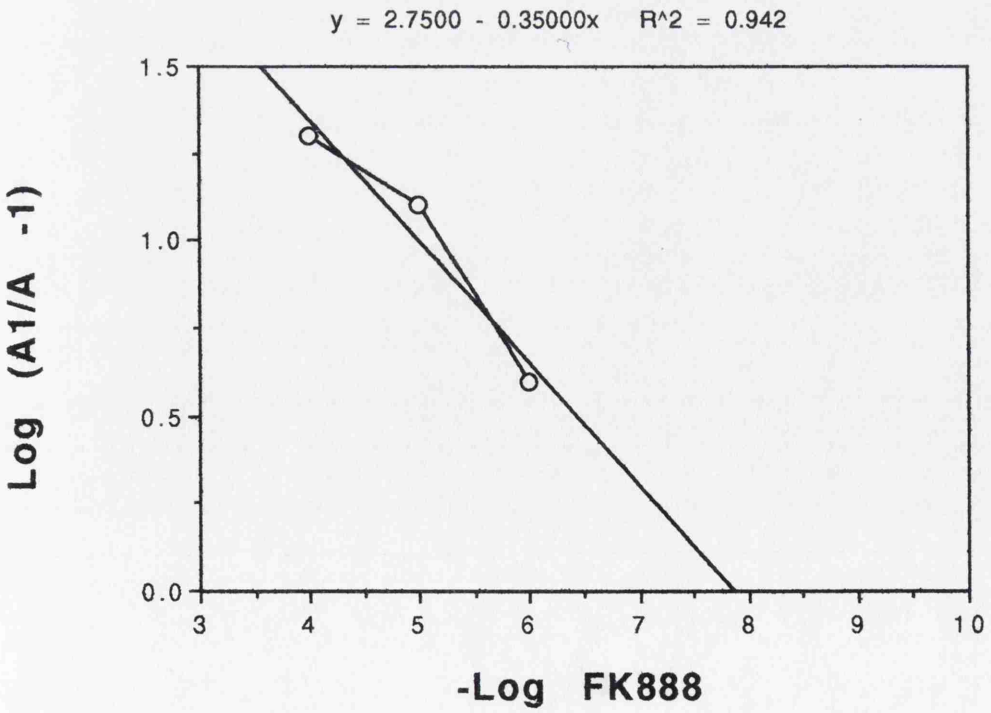
FK888 reduces protein extravasation, induced by SP, in a dose-dependent manner (Fig. 60A) but has no significant effect with NKA. Protein extravasation induced by 1μM SP is antagonised by 1μM ($P< 0.05$), 10μm ($P< 0.01$) and 100μM FK888 ($P< 0.001$). Extravasation induced by 10μM and 100μM SP is not altered significantly by FK888. FK224 reduces SP induced protein extravasation to a greater extent than FK888, although this is only significant at concentrations of 1μM of the antagonists and 1μM SP ($P<0.05$). FK888 has a pA_2 value of 7.8 when perfused with SP, according to the Schild plot (Fig. 58B). However, the gradient of the regression line is 0.35, which suggests this analysis is not applicable for assessing pA_2 values. Hence, calculating the pA_2 value for the 10μM concentration of SP gives a different value of 6.1.

Only the 1μM concentration of either SP or NKA has a significantly different population distributions when perfused with antagonist (Table 4). Individual concentrations of antagonist that produce a significanly different ($p>0.05$) response to other antagonist concentrations at the same agonist concentration will have a z-value greater than +/-1.64.

Figure 60: A. The percentages of protein extravasation induced by SP (▲) combined with 1μM (○), 10μM(❑) and 100μM (Δ) FK888. The values are calculated as a percentage of the maximal level of protein extravasation produced by SP alone. Values are means ± SEM; n = 7.

B. Schild plot using values from the above graph.


200
100
0
% of maximum control
- 8
- 7
- 6
- 5
- 4
- 3
Log [SP]


y = 2.7500 - 0.35000x R^2 = 0.942
1.5
1.0
0.5
0.0
Log (A1/A -1)
3
4
5
6
7
8
9
10
-Log FK888

Neither FK888, FK224 or saline infusion induce extravasation. The NK_2 antagonist SR48968 has no significant effect on either SP or NKA induced protein extravasation.

A transient fall in blood pressure is produced with perfusion of SP in a concentration of 100μM but not with lower concentrations of SP. This fall in blood pressure is abolished by previous perfusion with FK888 or FK224. All concentrations of the antagonists (1μM, 10μM & 100μM) prevent a fall in blood pressure. NKA does not produce a fall in blood pressure (Fig. 61) when perfused through the joint. This is in contrast to when it is administered intravenously (Murai *et al*, 1992a).

Figure 61: The blood pressure during perfusion with NKA, and SP with or without FK888 and FK224. Treatments are indicated on the trace.

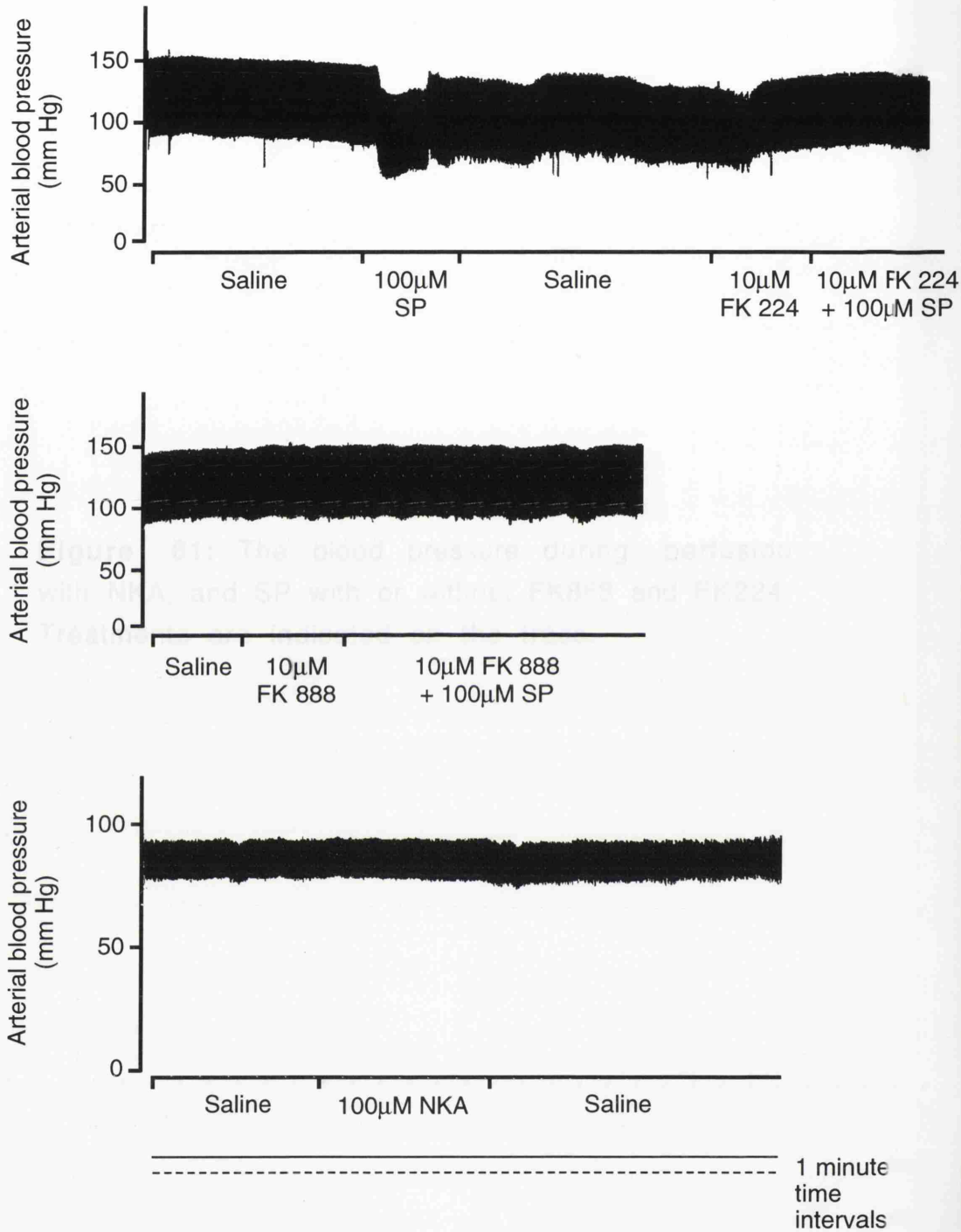

Arterial blood pressure (mm Hg)
150
100
50
0
Saline
100μM SP
Saline
10μM FK 224
10μM FK 224 + 100μM SP
Arterial blood pressure (mm Hg)
150
100
50
0
Saline
10μM FK 888
10μM FK 888 + 100μM SP
Arterial blood pressure (mm Hg)
100
50
0
Saline
100μM NKA
Saline
1 minute time intervals

Table 4: Kruskal-Wallis test adjusted for ties; three degrees of freedom

1μM SP + FK224		H = 13.81		p = 0.003	
10μM SP + FK224		H = 5.72		p = 0.127	
100μM SP + FK224		H = 1.45		p = 0.693	
FK224 conc. (μM)		**0**	**1**	**10**	**100**
z-values	1μM SP	3.71	-1.25	-1.35	-1.11
	10μM SP	2.02	0.29	-0.74	-1.57
	100μM SP	-0.88	1.01	0.19	-0.32

1μM SP + FK888		H = 14.93		p = 0.002	
10μM SP + FK888		H = 3.01		p = 0.390	
100μM SP + FK888		H = 0.98		p = 0.807	
FK888 conc. (μM)		**0**	**1**	**10**	**100**
z-values	1μM SP	3.37	0.45	-1.27	-2.55
	10μM SP	1.51	0.21	-0.69	-1.03
	100μM SP	-0.93	0.61	0.21	0.11

1μM NKA + FK224		H = 16.47		p = 0.001	
10μM NKA + FK224		H = 7.25		p = 0.065	
100μM NKA + FK224		H = 2.22		p = 0.528	
FK224 conc. (μM)		**0**	**1**	**10**	**100**
z-values	1μM NKA	3.77	0.11	-1.99	-1.88
	10μM NKA	2.61	-0.51	-0.87	-1.35
	100μM NKA	0.65	1.11	-0.67	-0.92

DISCUSSION

3.1 Frequency response of rapidly and slowly adapting joint afferents

Recent experiments, involving restricted mechanical stimulation (R.M.S.) of the posterior aspect of the joint capsule of the cat's knee, have demonstrated facilitatory reflex effects from group II joint afferents to quadriceps motor units (Baxendale *et al*, 1988). Also, both facilitatory and inhibitory responses are observed when R.M.S. is applied to the joint capsule while recording from γ-motoneurones (Baxendale *et al*, 1993). Although the measurement of conduction velocity classes these joint afferents as group II fibres, it is not clear if the R.M.S. preferentially selects rapidly adapting receptors. Electrical stimulation of group II PAN afferents produces both excitation and inhibition in γ-motoneurones, often in the same neurone (Baxendale *et al*, 1993). Both inhibitory and excitatory potentials are also observed in α-motoneurones with weak electrical stimulation of the PAN (Hongo *et al*, 1969; Lundberg *et al*, 1978). As electrical stimulation does not discriminate between rapidly and slowly adapting afferents, it is possible that rapidly adapting receptors evoke one type of response and slowly adapting receptors the other. This series of experiments therefore investigates the nature of the afferent fibres which are being excited by the R.M.S..

Both slowly and rapidly adapting afferents were sensitive to the R.M.S., and both showed a similar trend of decreasing sensitivity to increasing frequencies of stimulation. It had been

expected that R.M.S. might excite the rapidly adapting paciniform endings more powerfully than the slowly adapting Ruffini and Golgi Mazzoni endings. This would have meant that this form of mechanical stimulation of the joint capsule could be used to activate predominately one class of receptor. Free nerve endings, which give rise to thinly myelinated (Group III or Aδ) and unmyelinated (Group IV or C) fibres were excluded in this study on the basis of their conduction velocities (2.5-20 m/s and <2.5 m/s respectively).

Although a reduction in amplitude of the stimulus at higher frequencies may be responsible for a decrease in the responsiveness of the receptors, the slowly adapting receptors may also be showing a genuine depression of responsiveness. With maintained pressure, slowly adapting receptors have been shown to respond with increasing frequencies of neuronal discharge in response to increasing pressure (Grigg & Hoffmann, 1982; Grigg *et al*, 1982). Therefore, with an intermittent pressure, the receptor will only follow the mechanical stimulus frequency if this frequency is less than the maximum possible neuronal discharge frequency at that pressure.

The relation of the receptor to surrounding tissues is also important, and this could result in different responses to the same frequencies of stimulation in different areas. There is a finite time required for the necessary displacement of structures surrounding the receptor to apply sufficient force across the receptor to give a response. The viscoelastic properties of joint

capsules will therefore alter the response of receptors, as a different compliance of the joint capsule, as well as the distance from underlying hard tissue (e.g. bone) will mean that given displacements will produce differing reactive forces and vice-versa (Pubols, 1982a). Also repeated constant displacement, or force, can result in reduced discharges of both slowly and rapidly adapting units, which can vary within one tissue (Pubols, 1982b). The position of the joint itself will alter the stress in the capsule which will alter the sensitivity of some afferents to compression (Grigg *et al*, 1982). These factors may account for dissimilar results observed in a previous study of the response of receptors in the hip (Aloisi *et al*, 1988). In that study the response of hip joint afferents to a sinusoidal vibratory stimulus did not detail the response at different frequencies for all of the receptors, but suggested that rapidly adapting receptors were more sensitive to higher frequencies of vibration than to lower frequencies. In contrast, slowly adapting receptors were poorly sensitive to the mechanical sinusoidal stimuli, with some receptors failing to respond to frequencies greater than 1 Hz.

Failure of the rapidly adapting afferents to follow the stimulus at high frequencies is most probably due to the reduction in amplitude of the stimulus at higher frequencies. It is feasible that some slowly adapting receptors were mistaken for rapidly adapting receptors in this study, and this could account partly for the reduction in response of the rapidly adapting receptors at high frequencies. This is because the

tissues surrounding the receptor could provide the adaption by way of the compliance of the tissue reducing the force applied across the receptor. Although the receptor itself does not adapt, it may appear to do so under the maintained pressure used here to discriminate slowly and rapidly adapting receptors. Receptors with this type of response have been observed previously and were classed as "phasic" receptors (Burgess & Clark, 1969a). However, it would not be possible to mistake rapidly adapting receptors for slowly adapting receptors. If a single unit displays a maintained response to maintained pressure then it must be slowly adapting. As some slowly adapting receptors were one hundred percent active at frequencies of 100 Hz, it can be said with confidence that rapidly and slowly adapting receptors cannot be activated independently at frequencies of 100 Hz or below.

It is not possible to produce a true tuning curve for joint receptors without equipment which will maintain a given displacement at high frequencies of activation. However, the objectives of this study have been satisfied, in that it has been ascertained that both rapidly and slowly adapting receptors have the potential to be activated by the frequency of R.M.S. used in previous studies.

Stimulus parameters required for R.M.S. to activate predominately rapidly adapting afferents will require frequencies greater than 100Hz to eliminate slowly adapting afferents, and maintained displacements at these frequencies to

maintain the response of the rapidly adapting afferents. The nature of the tissue being stimulated and the nature of the tissue encompassing the receptor are probably more important factors in adaption of receptors at the frequencies used here (Caton & Petoe, 1966).

3.2 Reflex effects of group IV joint afferents on γ-motoneurone and α-motoneurone excitability

A) The technique

Electrical stimulation of peripheral nerves has been used widely to investigate reflex actions from primary afferents (Matthews, 1972). This is due to the easy control of an electrical stimulus and the possibility of estimating central latencies from the synchronous arrival of the afferent volley in the spinal cord. Differences in the axon diameters of afferents from different receptors and the consequent differences in excitability to electrical stimuli allows relatively selective stimulation of some groups of afferents. However, with group IV afferents, the slow conduction velocity creates a widely dispersed arrival of the C-wave in the spinal cord. This, together with the relatively small number of axons in the PAN, makes it difficult to detect the arrival of the afferent volley in the spinal cord. In this situation it is more effective to record the occurrence of a C-wave by recording from a site on the PAN proximal to the stimulating electrode. The latency to arrival in the spinal cord is then calculated from known conduction velocities and distances. Selective electrical stimulation of group IV afferents is difficult without also stimulating larger axons. It is therefore necessary to incorporate a control procedure which involves stimulation of group II afferents only. The effect of the group III/IV afferents

car then be determined. Activation of the receptor rather than their afferent fibres is more selective and more physiological. Hence restricted mechanical stimulation is used to excite proprioceptors and topical capsaicin to the PAN selectively excites nociceptive afferents.

The introduction of monosynaptic test reflexes to assess motoneuronal excitability (Renshaw, 1941; Lloyd, 1946) opened the way for studies on excitatory and inhibitory convergence onto motoneurones. By systematically varying the intervals between the conditioning and test stimuli, it was possible to study the time course of the synaptic actions brought about by the conditioning volleys. These studies were followed by intracellular recording of synaptic potentials in motoneurones (Eccles, 1953).

Similar techniques are used in the experiments presented here. A test stimulus to group II PAN afferents discloses a di/trisynaptic excitatory pathway from the afferents to both α-motoneurones and γ-motoneurones. This excitatory pathway can be conditioned by previous excitation of group IV PAN afferents. With γ-motoneurones the excitatory pathway from group II/III PAN afferents is inhibited by group IV afferent stimulation while group II α-motoneurones are inhibited by group III/IV joint afferents. The variability in group III/IV conduction times makes it difficult to make an exact estimation of the number of synapses involved in the group III/IV pathway. However, the

effective conditioning to test interval indicates that this is a polysynaptic pathway.

B) γ-motoneurones

Previous findings (Johansson *et al*, 1986; Baxendale *et al*, 1993) that electrical stimulation of group II axons in the PAN of the cat knee joint raises the probability of discharge of some γ-motoneurones are confirmed. It is also shown that this effect can be reduced or abolished by a conditioning electrical stimulus that excites all of the axons in the PAN. These effects are most probably mediated via afferent axons from the joint capsule, although some PAN afferents do originate from receptors in the popliteus muscle, in some animals. The majority of these muscle afferents are group I (Lindstrom & Takata, 1972; McIntyre *et al*, 1978a). Responsiveness of γ-motoneurones to electrical stimulation of group I muscle afferents is low, and of the very weak effects observed, inhibition is four times as frequent as excitation (Appelberg *et al*, 1983a). Therefore it is unlikely that muscle afferents in the PAN contribute to the response observed here.

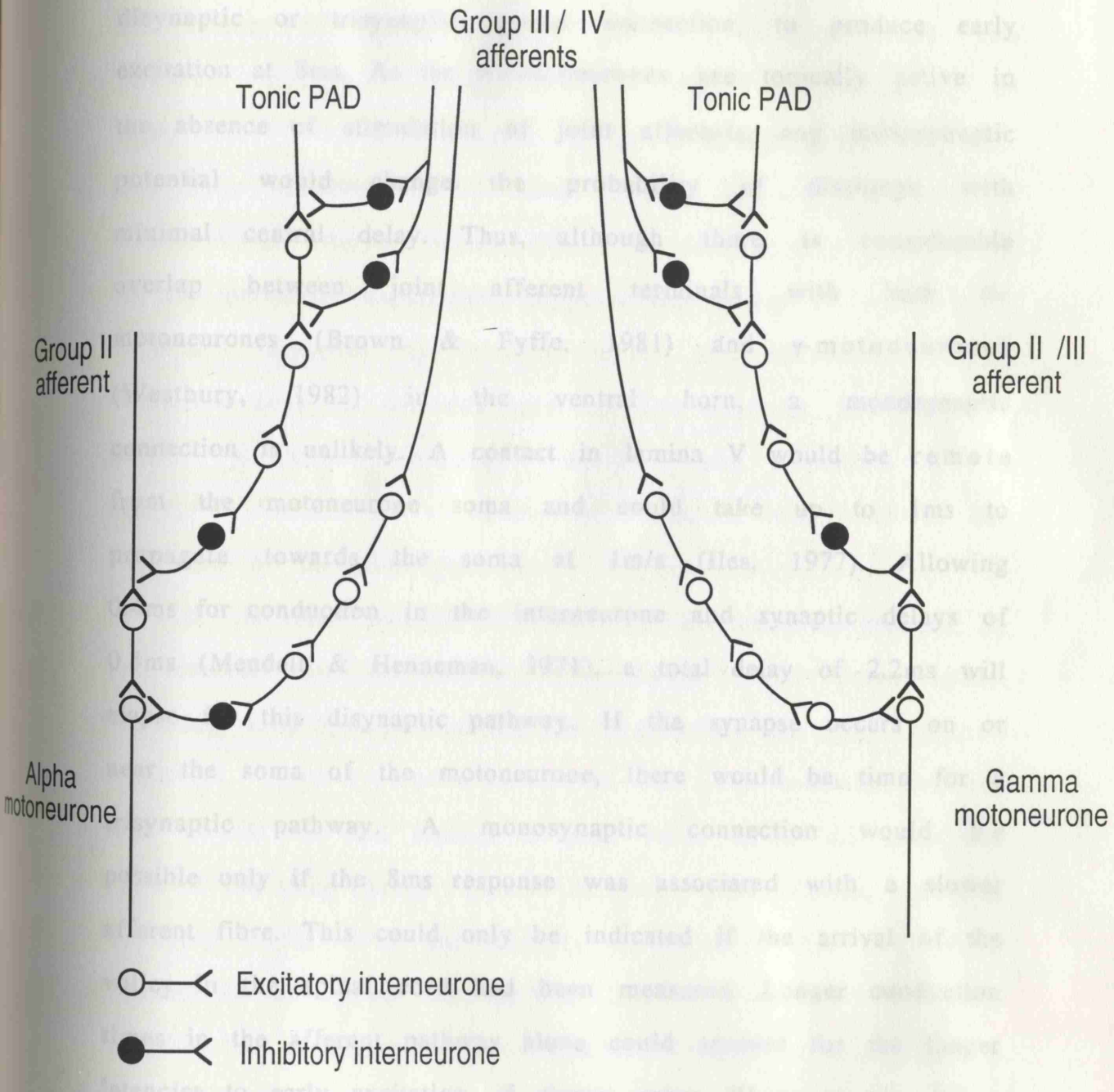
The thresholds for an excitatory response suggest that group II afferents in the PAN have an excitatory influence on LGS γ-motoneurones. These are similar to previous results (Baxendale *et al*, 1993) where thresholds as low as 1.5T excited γ-motoneurones. However, with four neurones excitation was only

observed at stimulation intensities greater than eight times the threshold of the PAN compound action potential, which suggests that recruitment of group III/IV axons was required. This could account for the less tightly coupled excitation in comparison to previous results (Baxendale *et al*, 1993). The fact that the overall discharge rate of most of the γ-motoneurones could be increased by high intensity stimulation of the PAN is also indicative of an excitatory link between group IV afferents and γ-motoneurones.

As the γ-motoneurones tested are spontaneously active, they are already in an excitable state and only need a relatively small excitatory post synaptic potential from the group II/III PAN afferent axons to result in an action potential. In contrast, an increase in discharge rate of the γ-motoneurone over a sustained period, is more likely to be the result of a greater overall input from the group IV PAN afferent axons, which results in a longer period of excitation. The longer time course and extended stimulation required for group IV excitation could be due to a longer pathway, involving more interneurones and requiring temporal summation on to the motoneurone. In contrast the short latency, narrow period of excitation induced by group II axons suggests few interneurones and limited convergence on the motoneurones. The fact that a conditioning stimulus could abolish the excitatory reflex effect, and yet also excite the γ-motoneurone (if the stimulation frequency is too high) suggests a strong inhibition of group II/III pathways by group IV axons, without direct inhibition of the motoneurone itself (Fig. 62).

Figure 62: Spinal neuronal network involving joint afferents and motoneurones, depicting pathways disclosed by the present study.

1) γ-motoneurones are excited by group II/III joint afferents through a di- or trisynaptic pathway.
2) α-motoneurones are excited by group II joint afferents through a di- or trisynaptic pathway.
3) γ-motoneurones are excited by group IV joint afferents through a polysynaptic pathway.
4) α-motoneurones are inhibited postsynaptically by group III/IV joint afferents through a polysynaptic pathway.
5) Group III/IV afferent stimulation inhibits excitation induced in motoneurones by group II afferent stimulation. This may be by inhibiting a tonic PAD pathway.


Group III / IV
afferents
Tonic PAD
Tonic PAD
Group II
afferent
Group II /III
afferent
Alpha
motoneurone
Gamma
motoneurone
Excitatory interneurone
Inhibitory interneurone

Afferent conduction time in the fastest group II axons would be approximately 2.1ms (150mm at 70m/s) and efferent conduction time for the particular γ axon is 3.7ms (140mm at 38m/s). This leaves a central delay of 2.2ms, and infers a disynaptic or trisynaptic central connection, to produce early excitation at 8ms. As the γ-motoneurones are tonically active in the absence of stimulation of joint afferents, any monosynaptic potential would change the probability of discharge with minimal central delay. Thus, although there is considerable overlap between joint afferent terminals with both α-motoneurones (Brown & Fyffe, 1981) and γ-motoneurones (Westbury, 1982) in the ventral horn, a monosynaptic connection is unlikely. A contact in lamina V would be remote from the motoneurone soma and could take up to 1ms to propagate towards the soma at 1m/s (Iles, 1977). Allowing 0.4ms for conduction in the interneurone and synaptic delays of 0.4ms (Mendell & Henneman, 1971), a total delay of 2.2ms will elapse for this disynaptic pathway. If the synapse occurs on or near the soma of the motoneurone, there would be time for a trisynaptic pathway. A monosynaptic connection would be possible only if the 8ms response was associated with a slower afferent fibre. This could only be indicated if the arrival of the volley in the spinal cord had been measured. Longer conduction times in the afferent pathway alone could account for the longer latencies to early excitation, if slower group III axons are being stimulated (e.g. 150mm at 5m/s = 30ms.). In addition, excitation

from group III axons could follow a different central pathway with longer central delays. The threshold level is consistent with group III stimulation in experiments with long latencies to early excitation, and there is significant correlation between threshold and latency to early excitation ($R=0.613$, $p<0.01$, $n=20$). The stimulus threshold used suggests that the majority of experiments involved stimulation of axons in the group II range. The large variation in the time interval between stimuli (i.e. condition-test interval) which results in depression of facilitation in any particular experiment, suggests that group IV axons form inhibitory synapses by various routes and therefore inhibit excitation over an extended interval. The variation in group IV afferent peripheral conduction times will also contribute to this effect, if afferents of a variety of diameters contribute to the inhibition of the same pathway.

With the three neurones in which the excitation is reduced by the control test stimulus, it is possible that refractoriness, including after-hyperpolarisation, is responsible for the reduced excitation. After-hyperpolarisation in the cat α-motoneurones lasts 40-200 ms and reaches a peak value 10-20 ms after the onset of the spike (Brock, Coombs & Eccles, 1952; Eccles, Eccles & Lundberg, 1958). With the majority of neurones this was not a problem as the control test stimulus had no effect.

With the remaining three neurones a period of excitation is linked to the conditioning stimulation. This period of excitation then masks the response to the test stimulus.

Whether reflex excitation of the γ-motoneurones from the group II afferents constitutes a positive or negative feedback depends on the location of the receptors in the joint capsule. It is possible that the inhibition demonstrated previously in γ-motoneurones is generated by afferents with receptors on the opposite aspect of the joint to the receptors evoking excitation. For an effect to be observed with electrical stimulation, γ-motoneurones would be required to be influenced predominately by receptors from either the extensor or flexor aspects of the joint, rather than a mixture of both. It has been shown that stimulation of a small number of joint afferents has a more pronounced effect on motoneurones than whole nerve stimulation (Baxendale *et al*, 1987), indicating a mixed excitation and inhibition from joint afferents to motoneurones.

As dynamic γ-motoneurones are spontaneously active in spinal preparations and static γ-motoneurones are not, it is assumed that the majority of γ-motoneurones in these experiments are dynamic.

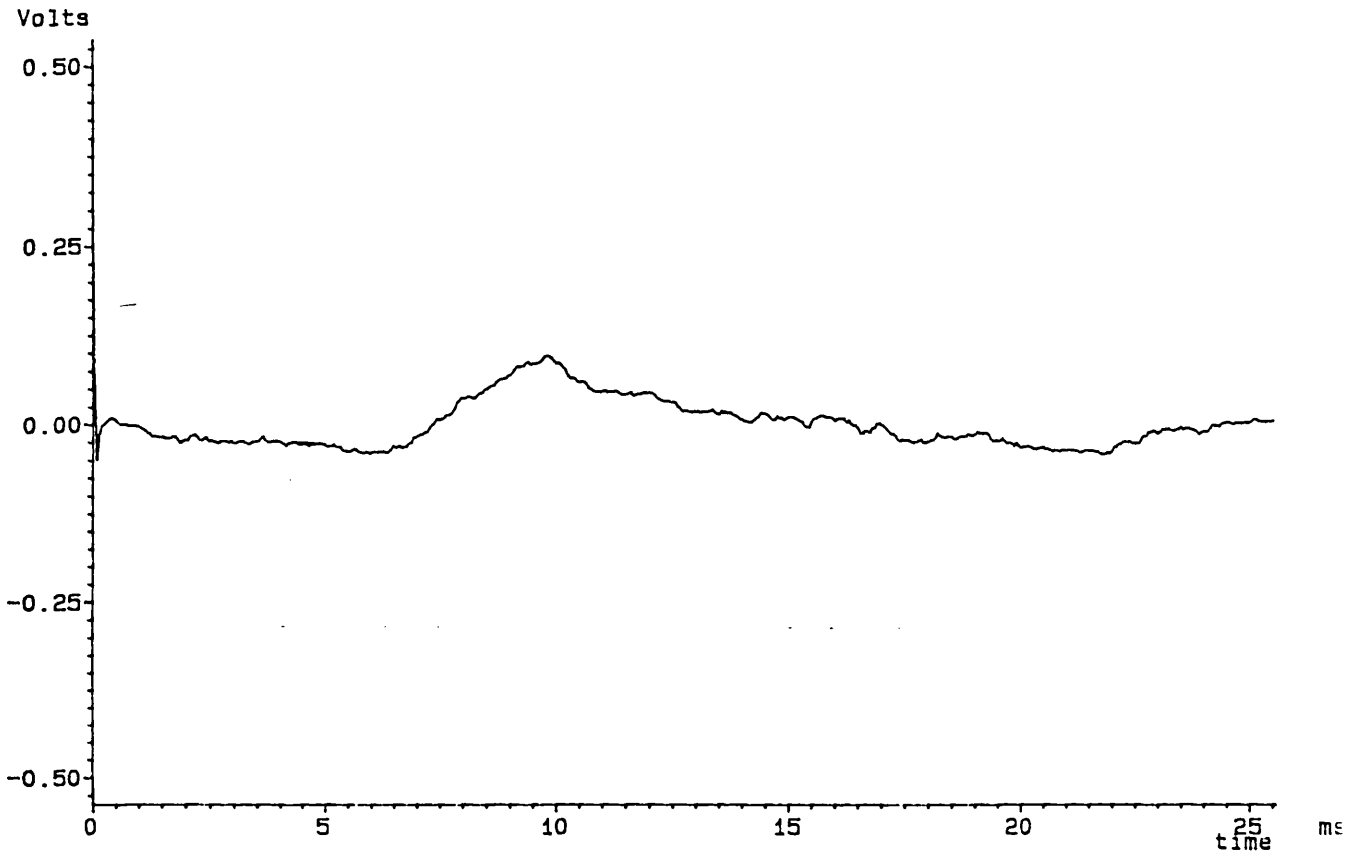
C) α-motoneurones

These results demonstrate that R.M.S., which activates rapidly and slowly adapting mechanoreceptors, but not nociceptors (Grigg *et al*, 1986), produces an excitatory response in gastrocnemius/soleus motor units. This extends previous observations where quadriceps motor units were reflexly excited

in the same way (Baxendale *et al*, 1988; Ferrell *et al*, 1990). These results are supported by intracellular recordings from lateral gastrocnemius/soleus α-motoneurones, which show excitatory post synaptic potentials in response to mechanical stimulation of the joint capsule or low threshold electrical stimulation of the PAN (Fig. 63)(Baxendale, Conway & Ferrell, unpublished data). The present results also clearly demonstrate that articular, but not cutaneous or muscle nociceptors exert a specific inhibitory effect which reduces the excitatory reflex actions of articular low-threshold mechanoreceptors. This is not direct inhibition of the motoneurone, as motor units continue to discharge during activation of the nociceptors.

The response of α-motoneurones to joint afferent stimulation is therefore very similar to the response of γ-motoneurones. The latency to excitation of the motor unit and the time course of the effective electrical conditioning stimulus are also similar. This suggests a similar pathway, if not the same interneurones, for the activation of both α- and γ-motoneurones. However, previous work in decerebrate cats (Davey & Ellaway, 1985; Connell, Davey & Ellaway, 1986) suggests that there is a significant separation of the interneuronal pathways to α- and γ-motoneuronal pools. Afferents acting on the same pathway would exert synaptic depolarisation of α-motoneurones both directly (α-route) and indirectly (γ-route via spindle afferents), and the discharge of α-motoneurones would accordingly depend on spatial facilitation of the two effects. With this organisation the γ-loop provides servo-

Figure 63: Intracellular recording of a lateral gastrocnemius motoneurone. An EPSP can be seen starting to rise at approximately 7ms after the electrical stimulation of the PAN at twice threshold for the most excitable fibres. This latency is consistent with a trisynaptic pathway.


Volts
0.50
0.25
0.00
-0.25
-0.50
0
5
10
15
20
25
time
ms

assistance to movements (Matthews, 1972). Coactivation of α- and γ-motoneurones under physiological conditions has been previously demonstrated. This includes recordings from respiratory muscle (Eklund, von Euler & Rutkowski, 1964) and jaw muscle (Lund *et al*, 1979; Taylor & Cody, 1974) spindle afferents, as well as during locomotion in decorticated and decerebrated cats (Perret & Berthoz, 1973). A very tight coupling in the co-activation of α- and γ-motoneurones has also been observed in humans (Valbo, 1970a; 1970b; 1971; 1974), although the extent to which skeletofusimotor (b) fibres contribute is not known.

Although it can be concluded that parallel contraction of extra- and intra-fusal fibres depends on α-γ-coactivation, the quantitative contribution made by muscle spindle afferents during voluntary muscle contraction is uncertain. The contribution of muscle spindle afferents to activation of the α-motoneurone is quite small (Valbo *et al*, 1979), although it has been shown that voluntary effort directed at α-motoneurone alone is not sufficient to activate muscles fully (Hagbarth *et al*, 1986). In the absence of activity in the gamma system, the range of voluntary movement at a joint is reduced, as well as the force. The dynamic γ-motoneurones control the sensitivity of the primary endings to stretch, while only static fibres are able to increase the firing of primary and secondary endings during muscle shortening (Lennerstrand & Thoden, 1968).

flow from spindles in spite of muscle shortening (Kuffler & Hunt, 1952) and a small contribution to α-motoneurone excitatory drive would be less important in this context. However, rather than being considered as a follow-up length servo, coactivation of α- and dynamic γ-motoneurones will prime the primary endings to stretches of small amplitude during movement, sensitising them to small perturbations and irregularities in the movement.

Results presented here are consistent with data demonstrating an increase in both flexor and extensor EMG activity in response to activity of low threshold joint afferents in the awake cat (Marshall & Tatton, 1990). These authors demonstrate that joint receptors modulate both short and long latency muscle responses to passively-imposed knee movements, as demonstrated by a reduction in the EMG response when local anaesthetic is introduced into the joint cavity. A reduction of activity in the sciatic and femoral nerves commensurate with a reduction in EMG activity may have arisen from reduced spindle afferent activity as a result of reduced excitation of γ-motoneurones by the joint afferents, again consistent with the present results.

D) Possible reflex pathways

In spinal cats a common reflex pathway exists from cutaneous and joint afferents and from group II and III muscle afferents (Eccles & Lundberg, 1959). These afferents, referred to as flexor reflex afferents (FRA's), excite motoneurones projecting to flexor muscles and evoke a mixture of excitation and inhibition in extensors (Bergmans & Grillner, 1969; Voorhoeve & van Kanten, 1969). The same afferents may also have other separate reflex connections that do not belong to the common reflex pathway (Holmqvist & Lundberg, 1961). Although high threshold joint afferents produce classical FRA reflex effects, it is uncertain if reflex effects produced by low threshold joint afferents are mediated by convergence on the system of interneurones utilised by the FRA's (Lundberg, 1979). Activation of joint proprioceptors (Ekholm, Eklund & Skoglund, 1960), or electrical stimulation of the PAN at less than twice threshold (Lundberg *et al*, 1978), can produce a characteristic flexor facilitation and extensor inhibition, and although the FRA effect produced by electrical stimulation of the PAN sometimes requires stimulation of high threshold fibres (Eccles & Lundberg, 1959), much summation is required for activation of the FRA pathway and the low threshold contribution cannot be excluded. In the acute spinal animal, volleys in ipsilateral FRA's evoke polysynaptic EPSP's in flexor motoneurones and IPSP's in

extensor motoneurones, as well as other actions not involving the common FRA pathway.

As an excitation of α-motoneurones has been demonstrated here, and inhibition demonstrated previously, low threshold joint afferents must have mixed inhibitory and excitatory influences on extensor α-motoneurones. It should also be remembered that although the lateral gastrocnemius/soleus muscle is a physiological extensor, it acts as a flexor at the knee joint, and it is therefore reasonable to expect mixed results with stimulation of different afferents. The latency from group II joint afferent stimulation to reflex excitation of the motoneurones suggests a di- or tri-synaptic pathway while the longer delay to postsynaptic inhibition of α-motoneurones and excitation of γ-motoneurones involves a longer pathway. Group III or IV joint afferents may utilise the FRA pathway in inhibiting the α-motoneurones or exciting the γ-motoneurones postsynaptically (in spinal preparations the FRAs activate dynamic γ-motoneurones more effectively than static (Bergmanns & Grillner, 1969)). A more direct pathway from group II afferents excites the motoneurones. This pathway may involve interneurones common to other pathways e.g. inhibition of interneurones mediating group I non-reciprocal inhibition of motoneurones (Harrison & Jankowska, 1985)

When high threshold joint afferents are excited by excessive stretch to the joint capsule it is probable that low threshold afferents will also be excited. As the majority of these high

threshold afferents produce excitation of flexor motoneurones it would seem prudent for the same afferents to inhibit excitation induced in extensor motoneurones by low threshold afferents. The inhibition of low threshold afferents by high threshold afferents can therefore be seen as an extension of the flexion reflex.

It should be remembered that these experiments are only involved with motoneurones which show a period of excitation in response to stimulation of low threshold joint afferents. There is also inhibition of both α- (Lundberg *et al*, 1978; Harison & Jankowska, 1985) and γ-motoneurone (Johhansson *et al*, 1986) in response to stimulation of low threshold joint afferents, and this effect was not examined in these experiments.

3.3 Presynaptic interactions of joint afferents

Electrical stimulation of the spinal cord elicits antidromic action potentials in the PAN, with a delay consistent with group II conduction velocities. Conduction in sympathetic efferents can be excluded as action potentials travelling in these neurones would require more than 6ms to reach the peripheral recording site. Similarly tendon organ afferents and muscle spindle primaries from popliteus can be excluded on the basis of conduction velocity. No action potentials were recorded with a latency from spinal stimulation of less than 2.1ms. Allowing 0.2ms for activation of the central terminal in the spinal cord and for a peripheral conduction velocity of more than 70m/s, group I potentials would all arrive at the recording site before 2.1ms. This only leaves the very small proportion of muscle spindle secondaries present in the PAN of some cat knee joints to be confused with the PAN group II joint afferents. The PAN was dissected to its termination in the joint capsule, which not only facilitates splitting of the nerve, but also virtually eliminates the possibility of muscle afferents, as the recording site is distal to the entry of muscle afferents.

Conditioning stimulation consisting of a single pulse to the PAN is ineffective with a condition to test interval of less than 60ms. This indicates that group IV afferents are involved in the hyperpolarisation of the group II central terminals. As it is difficult to estimate the central conduction time and the number

of synapses involved, it is possible that group III afferents are also involved. Decreasing the condition to test interval further to 35ms and applying a train of five stimuli at 200Hz, produces depolarisation of the group II central terminals on some occasions. This is not in agreement with previous findings that group II PAN afferents do not depolarise the central terminals of other group II PAN afferents (Jankowska *et al*, 1993). It is, however, an indication that the group II PAN afferents are not refractory after conditioning stimulation of the PAN.

The hyperpolarisation observed with high intensity conditioning stimulation of the whole PAN is very powerful in that it is always effective, the antidromic potential is completely abolished (i.e. zero percent response to spinal stimulation) in the majority of cases, increasing the spinal current usually has no effect in returning the antidromic potential, and the effect is maintained for some time even after the removal of the conditioning stimulation. If hyperpolarisation is in effect the removal of a tonic PAD, this suggests that the tonic PAD itself has a powerful depolarising action if its removal is to have such a pronounced effect. As group II afferents are under the continual influence of some, as yet undisclosed, presynaptic inhibition, it would be of interest in future experiments to selectively remove inputs to the joint afferents and to examine the reflex effects on motoneurones in these conditions. It would also be worthwhile to repeat the experiment in a non-spinalised preparation in case the tonic PAD is only evident in spinalised preparations.

Spinalisation was performed in these experiments to prevent changes in blood pressure with conditioning stimulation.

Splitting the PAN or applying capsaicin will result in a smaller number of group IV afferents being activated. There is also a less pronounced effect (with conditioning stimulation) on the group II spinal threshold in these situations, which suggests that each group II afferent must receive inputs from a lot of group III/IV afferents. As the number of group III/IV afferents which are excited decreases, the effect on the group II afferents also decreases. If each group II afferent received inputs from a limited number of group III/IV afferents, it would be expected that after the PAN is split, conditioning stimulation would have no effect in situations where the particular group III/IV afferents related to the group II afferent being stimulated are not being conditioned. In fact, although the response to conditioning stimulation is weaker in the split PAN, there is always some effect. This suggests a wide input from group III/IV afferents. It is possible that these afferents focus on a central system of interneurones responsible for producing a tonic PAD. Activation of virtually any group III/IV afferent would therefore have the potential to inhibit the tonic PAD to some extent. The system of interneurones involved in producing the PAD may involve a large degree of divergence to affect all the group II afferents. If there is a degree of convergence of group III/IV afferents onto interneurones early in this pathway,

this would account for the powerful effect of the conditioning stimulation.

In previous experiments we have shown that stimulation of group III/IV afferents inhibits excitation of motoneurones induced by group II afferent stimulation. This suggested the possibility of PAD and subsequent presynaptic inhibition. In these experiments we have disclosed a hyperpolarisation which would therefore suggest a presynaptic facilitation. However, if the conductance of the afferent terminal is reduced to such an extent that the action potential cannot be propagated, this will result in a presynaptic inhibition and hyperpolarisation. Presumably this would involve a decrease in the conductance of either sodium or calcium channels

Primary afferent hyperpolarisation (PAH) (Hongo *et al*, 1972; Lund, Lundberg & Vyklicky, 1965; Lundberg & Vyklicky, 1966; Mendell, 1972; Rudomin, Nunez, Madrid & Burke, 1974) or positive dorsal root potentials (Cangiano, Cook & Pompeiano, 1969; Hodge, 1972; Levy & Anderson, 1974; Mendell, 1970; Mendell & Wall, 1964) have been observed previously. Of particular interest is the description of PAH produced in large cutaneous afferents by small cutaneous afferents (Mendell & Wall, 1964). The P wave produced in cord dorsum potentials by PAD is not evident following electrical stimulation of the PAN (Schaible *et al*, 1986). This may be because the depolarisation of the largest afferent fibres, which is responsible for the P wave, is not sufficiently numerous to be detected. However it could also

be due to the concurrent processes of depolarisation and hyperpolarisation providing opposing neural generators which will have neutralised each other at the level of the cord dorsum.

The prolonged action of the hyperpolarisation suggests the release of a substance which remains active after the removal of the stimulus. The proportional relationship between the duration of the conditioning stimulus and the duration of the hyperpolarisation after the removal of the stimulus suggests the accumulation of such a substance during the conditioning stimulation. The most likely candidate to satisfy these conditions is the extracellular accumulation of potassium ions. Intraspinal extracellular potassium increases from a baseline value of 3mM up to 15mM during repetitive stimulation of mixed nerves or dorsal roots (Krnjevic & Morris, 1972; 1974; 1975; Somjen & Lothman, 1974; Lothman & Somjen, 1975). Although the specificity of PAD argues against potassium accumulation as a mechanism for presynaptic depolarisation, evidence suggests the existence of a specific, synaptic and non-specific, potassium component in PAD (Jiminez *et al*, 1983). As GABA is the principal transmitter involved in PAD, iontophoretic application would be expected to reduce the intraspinal threshold of the fibres normally exhibiting PAD. Iontophoretic application of GABA reduces the intraspinal threshold of group Ia fibre terminals, but increases the threshold of rubrospinal and vestibulo-spinal fibres, despite the ability of cutaneous afferents to depolarise rubrospinal terminals (Rudomin *et al*, 1980; 1981). It was

concluded that the depolarisation of the rubrospinal fibres produced by the cutaneous volleys was not GABAergic, but most likely due to potassium accumulation in the dorsal horn as a consequence of the repetitive stimulation of the cutaneous nerves (Rudomin *et al*, 1981). If the extracellular potassium concentration is raised in the spinal cord region associated with the interneurones producing tonic PAD in group II joint afferents, then there will be a general depolarisation and presynaptic inhibition of these interneurones. This would account for the divergent effect of group III/IV conditioning stimulation on group II intraspinal thresholds, as well as the time course of the response. With conditioning stimulation in the sural or gastrocnemius nerves there is a much weaker hyperpolarisation of the group II afferents. There is also often no effect at all. This suggests a more limited interaction between the group III/IV afferents from these nerves and neurones effecting PAD. It is possible that axo-axonic synapses are responsible for this more specific action of sural and gastrocnemius nerves compared to the ipsilateral PAN.

In a small number of control experiments excitation of the group II PAN afferents by stimulation of the dorsal roots (sectioned centrally) is partly inhibited by group III/IV PAN conditioning stimulation. It is possible that inhibitory somatic synapses exist in the dorsal root ganglion, although further work is required here to confirm these findings.

The functional significance of a presynaptic inhibition of group II afferents by group III/IV afferents probably originates as a protective reflex. This idea is reinforced by the lack of any response to stimulation of the contralateral PAN. Damage to the joint will activate joint nociceptors which will produce presynaptic inhibition of group II joint afferents. The excitatory input to extensor motoneurones from group II joint afferents will in turn be reduced. Presumably, as all group II afferents are affected by group III/IV stimulation, afferents from both the flexion and extension sides of the joint are equally inhibited. Also afferents with reflex connections to flexor muscles will be inhibited to the same degree as afferents influencing extensors. It would be of interest, therefore, to examine the effect of group III/IV afferent stimulation on excitation induced in flexors by group II afferent stimulation as well as the effect of group III/IV stimulation on inhibition induced by group II joint afferents.

3.4 Neurogenic inflammation

A) The technique

The measurement of protein extravasation in the knee joint of the rat is used as an indicator of acute inflammation. The acute inflammatory process is characterised by three main stages:-

1. Vasodilatation and increased blood flow to the damaged area.
2. Increased vascular permeability with leakage of plasma from the microcirculation into the surrounding tissues.
3. Migration of phagocytic leukocytes from the microcirculation into the surrounding tissues

Following release of vasoactive mediators there is an increase in blood flow to the damaged area due to dilatation of arterioles and the filling of newly opened capillaries and venules in the region. Increased permeability of capillaries and post-capillary venules then develops, allowing the leakage of plasma from the circulation. The increased permeability is the result of contraction of the endothelial cells lining the microcirculation causing the opening up of cell junctions and fenestrae (Fig. 64). At the same time the elevated hydrostatic pressure in the circulation due to increased blood flow, and the elevated osmotic pressure in the surrounding tissues due to accumulation of

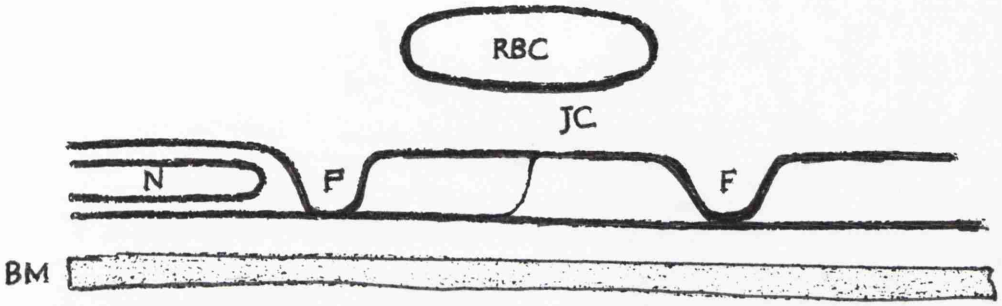
plasma and the cytoplasmic constituents of dead cells, result in the leakage of more fluid from the circulation. Because of plasma loss the blood becomes viscous and slows, resulting in disruption of the normal axial flow of leukocytes. The leukocytes marginate, coming into contact with the endothelial surfaces and then emigrate via open cell junctions to squeeze their way into the surrounding tissues.

The measurement of protein as an indicator for inflammation is therefore based on the principle that as inflammation develops the increased vascular permeability will allow leakage of plasma proteins from the circulation, which can be measured by the microturbidity technique. Of course, as the nature of the protein is not identified, it is not certain that all extravasated protein originates from the circulation. However previous studies using Evans Blue to label plasma proteins have indicated that at least a proportion of these proteins do originate from the circulation (Lam & Ferrell, 1991b). Also, in normal and inflamed joints, it has been shown that albumin constitutes the single largest protein fraction and the non-albumin proteins are also plasma derived (Levick, 1981; Knox *et al*, 1988). Subsequent experiments, using Evans blue (Cruwys *et al*, 1992), have succesfully confirmed the time course of SP-induced extravasation demonstrated here, and therefore confirm the origin of the protein as from the vascular compartment.

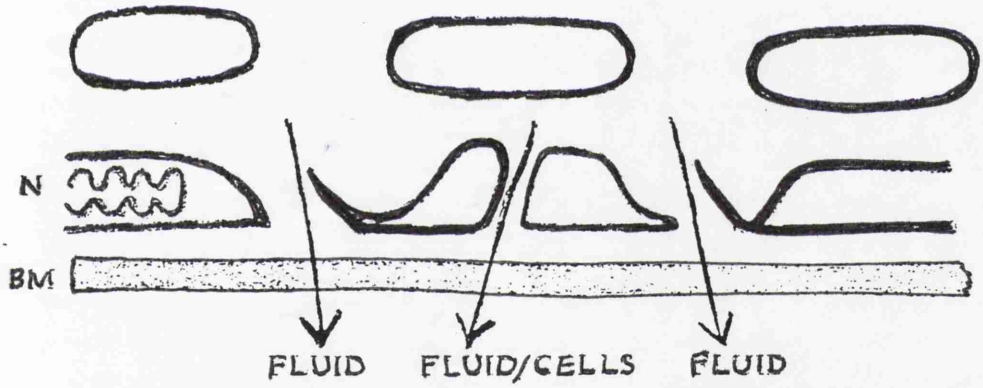
Original experiments performed here, involving labelling of plasma protein with Evans Blue or radioactive isotopes and

Figure 64: Fenestrated capillary permeability is regulated by endothelial contraction.

CAPILLARY PERMEABILITY



Wall of post-capillary venule. RBC, red blood cell. N, nucleus, F, fenestrae. JC junctional complex. BM, basement membrane



subsequent perfusion of the joint with SP, were unsuccessful. Presumably this is because only a proportion of the plasma protein is labelled and therefore only a proportion of the protein extravasated is measured. By measuring all of the protein in the extract from the joint perfusion, most of the extravasated protein will be measured, making this technique more sensitive.

Protein concentration is measured by reacting with benzethonium chloride to produce a turbid solution, the absorbance of which is then correlated to known protein concentrations. This method was chosen in preference to other similarly sensitive methods because it only requires a small volume of the test fluid, it does not react with peptides and it is cheap.

The main disadvantage of joint perfusion is the large variability in the response. This is probably due to the variation in the placement of the needles as well as the variation in response inherent in individual animals. The greatest response will be attained if the inflow needle delivers SP to as large an area of responsive vessels as possible, and if the outflow needle is in a position to remove all of the resulting extravasated protein. The positively skewed results suggest that this set of circumstances only exist in the minority of occasions, each concentration of drug tending to produce one or two large responses and a cluster of small responses. Presumably these large responses signify the true potential of the level of extravasation if the needles are near the optimal position.

To standardise the response in future experiments, it would be advisable to attain a true maximal response, for each experiment, with a standard substance e.g. 10% formalin. The response to different concentrations of SP could then be expressed as a percentage of the maximal response which each experiment is capable of producing.

B) SP promotes protein extravasation by its action at specific receptors

- When SP is perfused through the normal joint, the resulting level of protein extravasation is dependent on the concentration of SP in the perfusate. The minimum concentration of SP producing a significant effect is 100nM, which suggests that SP is exerting its effect by binding to a specific receptor. With drugs acting non-specifically, relatively high doses or concentrations are necessary to reach the physically or chemically effective partial pressure, osmotic pressure or ion concentration. These drugs exert their effect in a simple physical or chemical way which is non-specific, e.g. all liposoluble volatile substances may produce narcosis and all soluble macromolecular compounds raise the osmotic pressure.

On the other hand, various chemicals exert their effect by binding to specific receptors. This is demonstrated by the low doses or concentrations of the chemical required to produce an effect, and the high degree of specificity related to the molecular

structure of the chemical. This highly specific interaction of the drug molecule with a specifically arranged site on the target structure is similar to enzyme-substrate, or antigen-antibody interactions. The high pD_2 value of 7.0 (Fig. 50) suggests SP has a high affinity for receptors inducing protein extravasation in the rat knee joint. This is a lower affinity than that displayed by SP in other preparations e.g. guinea-pig isolated ileum (Fujii *et al*, 1992; Morimoto *et al*, 1992), but is a higher affinity than other known drug-receptor interactions e.g. noradrenaline induced rat vas deferens contraction (Ariens, 1960).

The importance of the structure of SP in producing a response is exemplified by the lack of response to substances structurally related to SP, e.g. the SP antagonist [D-Arg^1, D-Trp^{7,9}, Leu^{11}]-SP (Folkers, *et al*, 1984) has a similar structure to SP, but virtually no agonistic tendencies. This high degree of structural specificity again suggests specific binding of SP to a receptor. However, perfusion with high concentrations of unrelated chemicals such as 10% formalin or ammonium hydroxide also produces protein extravasation. The relatively high concentrations of these chemicals required to produce a response suggests they are by-passing the receptors which bind to SP, producing a non-specific response. Although the maximal response of these different systems may be the same if a final common path is utilised, the affinity of SP for specific receptors reduces the concentration of SP at which the response will occur.

In the perfusion experiments, only the concentration of the drug used and the degree of protein extravasation are known. All the other steps can be represented as a black box containing a number of events of which the drug-receptor interaction is only one. To give sufficiently precise information concerning this one step the experiment would have to be arranged in such a way as to minimise interference with the other steps. This would necessitate as simple an approach as possible e.g. isolated cells or tissue, blockade of all but the studied receptors, inhibition of drug destruction. The null method of irreversible receptor blockade (Furchgott, 1966) was not used to provide a more accurate measure of affinity as no irreversible NK receptor blockers are available. Also the concentration of SP in the joint cavity cannot be determined accurately, as the concentration of SP in the extract, and the volume of fluid in the joint cavity before perfusion commences, are not measured. The concentration of drug at the receptor may also differ significantly from the concentration in the joint cavity. As the time course of the response was an important part of the experiment, there was a limit to the time allowed for equilibrium and ubiquitous drug concentrations to be reached.

Although, in vivo, we cannot determine the precise affinity of SP for receptors, or the concentration of SP at receptors following release from nerve terminals, these factors can be circumvented by assessing the efficacy of nerve stimulation in producing protein extravasation. Several experiments were undertaken to

examine this point. Perfusion of rat knees with saline proceeded as previously described. The saphenous nerve was dissected and stimulated electrically, such that all axons in the nerve were activated. As the saphenous nerve gives rise to the MAN it was hoped that the release of SP from the MAN peripheral afferent terminals would give rise to an extravasation of protein. Unfortunately no protein extravasation was recorded. This negative result could be due to insufficient release of SP by the afferent nerve terminals or a mis-match in the location of release sites and receptors, resulting in too low a concentration of SP at the receptors. From the perfusion experiments it is known that enough receptors exist in the joint to exert a measurable response. Although the response to SP perfused through the joint is measurable by this technique, if insufficient receptors are activated by SP released from nerve stimulation, then a more sensitive technique is required. Although the release of a quantity of protein is indicative of an inflammatory response, it is possible that with release of SP from nerve terminals, a quantity of protein extravasated is indicative of a greater degree of inflammation than when SP is perfused through the joint. This is because the SP could be released at sites more relevant to the general pathogenesis of inflammation in the joint.

C) An increase in endothelial permeability during NK perfusion is responsible for protein extravasation.

Synovial fluid is an ultrafiltrate of plasma into which hyaluronate is secreted (Levick, 1984). A disequlibrium between plasma-protein concentration in synovial fluid and plasma suggests that the intervening synovial intima and capillary wall acts as an imperfect ultrafilter, allowing synovial fluid to attain an average of 42% of plasma levels of protein (Knox *et al*, 1988). The permeability of a membrane to a solute is described by the osmotic reflection coefficient of the membrane (σ). The latter depends on pathway width relative to solute radius and on any charge interactions (Pappenheimer, Renkin & Borrero, 1951). It is defined as the ratio of the osmotic pressure exerted by the solute across a test membrane to that exerted across a perfect semi-permeable membrane (Curry, 1984) and can be estimated by measuring the ratio of osmotic to hydraulic changes (an exact calculation has to take into account volume flow into the joint (Levick, 1981)) . σ for plasma albumin is approximately 0.8 in the rabbit knee (Knight, Levick & McDonald, 1988), which demonstrates that fenestrated capillaries are as impervious to protein as are continuous capillaries (Johnson & Hanson, 1966). The blood-joint barrier to proteins can be regarded as two layers in series; the synovial intima and the capillary endothelium (Simkin & Pizzorno, 1979). The relative contribution of these two layers to the resistance to diffusion can be assessed by Fick's law

of diffusion, which states that the ratio of resistances of the layers to a very small solute, whose diffusion is negligibly restricted within either layer, is $(A_1/l_1)/(A_2/l_2)$, where A is equivalent pore area and l is pathlength in membranes 1 and 2. A_1/A_2 may be estimated from the ratio of the area of intimal intracellular matrix (McDonald & Levick, 1988) to the area of endothelial diffusion, consisting of intercellular junctions (Landis & Pappenheimer, 1963) and fenestrae (Levick & Smaje, 1987). The distance (l) from the capillary plexus to the joint surface averages 10μm (Knight & Levick, 1984) and the thickness of the endothelial barrier is significantly less (0.1μm for intracellular junctions and 4nm for fenestral membrane- but fenestrae maybe 100nm due to glycocalyx and basement membrane (Levick, 1989)). Substituting these figures for area and length indicates that the capillary wall only accounts for 25-75% of the total resistance. However, when the radius of the solute is taken into consideration, solutes with a radius of between one and eight nanometres are restricted principally by the endothelium (Levick, 1981). Albumin is therefore restricted principally by the endothelium, while for larger proteins, such as globulins, the intimal layer dominates the net resistance to exchange.

Although the degree of permeability of the blood-joint barrier is sufficient to give protein concentrations approximating 42% of plasma levels, the control perfusion experiments performed here demonstrate that this extravasation does not happen sufficiently quickly to maintain a measurable level of protein in the

perfusate. Pre-infusion levels of protein in the rat knee joint will approximate 25 g/l, which, allowing for a ten-fold dilution by the perfusate, is approximately ten times greater than the largest concentration of protein extravasated during peptide perfusion. Perfusion of the joint with SP or NKA results in an increased level of protein in the perfusate, which must result from an increase in the rate of diffusion of protein across the barrier. As NK receptors are located principally on the endothelium, the increase in permeability is most likely to occur at this level. The principle oncotic agent, albumin, is the main protein molecule restricted by the endothelium and an increase in permeability will therefore involve a decrease in σ_{alb}. This increase in permeability of the endothelium can arise from 1) increase in the size of endothelial channels e.g. fenestrae 2) a reduction in endothelial fibre matrix density 3) a greatly increased rate of vesicular transport. The time course of the response favours the first option.

Fluid exchange between blood and the synovial cavity should be described by Starling's hypothesis of fluid exchange (Starling, 1896) and will depend on the hydraulic and osmotic pressures in the synovial capillaries and joint cavity. The effect of capillary blood pressure (Knight & Levick, 1985), plasma colloid osmotic pressure (COP) (Knight *et al*, 1988) and intra-articular hydrostatic pressure (Levick, 1979) on synovial fluid exchange are consistent with Starling's hypothesis of fluid exchange. However, as plasma proteins can slowly permeate the blood-

joint barrier, the osmotic pressure exerted by them depends not only on the concentration difference on either side of the semi-permeable membrane, but also on the osmotic reflection coefficient of the membrane (σ). As the size of endothelial gaps increase, then the greater will be the contribution of bulk flow of fluid to the net transport of proteins and solvents. The amount of solvent extravasated will have a limited effect on protein concentration measured, as it will be minimal compared to the 0.1 ml of saline perfused through the joint. It should also be noted that albumin exerts less of an oncotic pressure in the joint than in the blood stream, due to - a low protein local ultrafiltration stream from fenestrae diluting the protein (Levick, McDonald & Knight, 1991).

An increase in intraarticular pressure above yield pressure (9.5 cm H_2O) results in an increase in hydraulic permeability of synovium (Knight & Levick, 1985) which is due to a marked increase in interstitial area (McDonald & Levick, 1988) and a reduction in extravascular path length (Levick & McDonald, 1989a; 1989b). Joint pressures were not recorded during perfusion of the joints and it may be possible that there was an increase in pressure in some experiments. Although minimal extravasation was recorded during the control perfusion, if the intimal resistance is reduced then the endothelium becomes the rate limiting step for diffusion. It is possible, therefore, that large proteins which would not normally show an increase in the rate of diffusion in response to increased endothelial

permeability, do so when the intima is compromised. As these proteins (e.g. globulins) are too large to pass through fenestrae, this would necessitate an increase in the rate of vesicular transport.

D) SP evokes desensitisation

Progressive decline and block of the effect have been described in various cases when an agonist is acting in high concentrations over an extended period e.g. the muscle end plate of frog sartorius repolarises spontaneously despite the continued presence of acetylcholine. Explanations of this phenomenon of desensitisation are inactivation of the receptor itself or exhaustion of some limited substances specifically required for the reaction, following binding of the agonist and the receptor. It is also possible that the variable being measured becomes depleted or the rate of degradation of the agonist increases.

The reactive form of the receptor is able to form a drug-receptor complex which may then dissociate back to the receptor and unbound agonist. However, the receptor bound with the agonist may also undergo very slow change, becoming inactivated. The reactivation of the receptor also proceeds slowly. In this way, if all the receptors are in the reactive state at the start of the reaction, the effect progressively declines because the concentration of reactive receptors diminishes as long as the rate of inactivation exceeds that of reactivation (Fig.

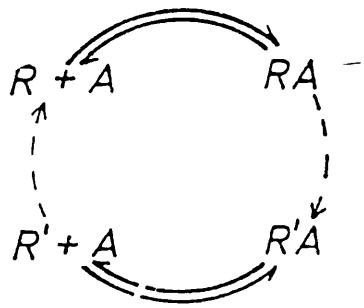
65). This receptor-inactivation theory (Gosselin, 1977) accommodates the major elements of both the occupation (Clark, 1933) and rate (Paton, 1961) theories of drug-receptor interactions. The receptor-inactivation theory also predicts that the latent period between addition of drug and peak response decreases with dose (Gosselin, 1977). This is consistent with the time to peak response in the normal animals (Fig. 55)

The main second messenger system coupled to activation of the three known neurokinin receptor types is stimulation of phospholipase C leading to phosphoinositide breakdown and elevation of intracellular calcium. The large number of potential targets for phosphorylation in the C-terminal, cytoplasmic region of the receptor may be related to desensitisation (Hersey & Krause, 1990). There may also be depletion of some elements of the second messenger system e.g. calcium. The events following second messenger activation remain unclear and it is uncertain if there could be any depletion of substances between the second messenger system and activation of the response.

The depletion of albumin or the blockage of dilated fenestrae by large molecules are not likely as protein extravasation continues in the inflamed joints.

Desensitisation is a feature common to many NK-1 receptor mediated events and is more intense than for NK-2 or NK-3 mediated responses (Moskowitz *et al*, 1987). In the present experiments both SP and NKA have the same time course of protein extravasation. This could be because NKA is exerting the

Figure 65: The free receptor (R) and agonist (A) interact to form an active receptor complex (RA). This may become inactivated (R'A) before dissociation takes place (R'+A). The relative rates of activation and deactivation dictate the rate of desensitisation.


R + A
RA
R' + A
R'A

majority of its effect at NK-1 receptors. With both SP and NKA the response decays over a period of twelve minutes after the maximum and does not reappear with continued perfusion of the agonist. The time course of the decline in response in the joint is similar to the time course of the effect on blood pressure. Following one hour of saline perfusion there is a response to re-introduced SP or NKA, but this response is less than the previous peak. This could be in part because of incomplete removal of SP during the saline perfusion, providing some continued desensitisation.

E) Inflammation increases the sensitivity to SP and reduces desensitisation

The response of a tissue to stimulation by an agonist is a product of the affinity and intrinsic efficacy of the agonist and the amplification of the effect by the tissue. The intrinsic efficacy and the tissue response are sometimes described together as the intrinsic activity, although this term also originally represented the efficacy alone (Ariens, 1954) (this would only be true if a linear relationship exists between receptor occupancy and tissue response). The affinity of an agonist is the tenacity with which it binds to its receptor or, in statistical terms, the probability of binding to a free receptor at any given instant. The intrinsic efficacy is the inherent property that imparts the biological signal to the receptor to result in a response and is a property of

the agonist, not the tissue. Tissues are amplifiers of the effects of agonists and can vary dramatically in their threshold and maximal limits of amplification. The observed response is what the tissue does with the signal from the receptor.

It follows, therefore, that the increased sensitivity of the inflamed tissues to SP is either the result of increased amplification of the signal by the inflamed tissues, or an up-regulation of the receptors. The dose-response curve for protein extravasation with SP perfusion in the inflamed joint displays a shift to the left. The pD_2 of SP in the inflamed joint is 8.5 compared to a pD_2 value of 7.0 in the normal joint (Fig. 53). However, because of tissue amplification systems for receptor stimuli, the concentration of agonist that produces half the maximal response is not necessarily the agonist concentration that occupies half the receptor population. A left-shift is therefore not necessarily the result of increased affinity.

An amplification by the tissues could result in a response being detectable at lower concentrations of SP, as well as increasing the response to more concentrated SP. This amplification could be due to an increase in the effective concentration of the agonist at the receptors or to an increase in the number of effective receptors. Blood vessels are more permeable in inflamed joints than in normal joints as demonstrated by the degree of protein extravasated in the inflamed joints (Fig. 52). As protein can diffuse out of the vessels more readily in the inflamed joint, peptides should be able to

diffuse into the vessel more readily and achieve greater concentrations inside the capillaries. This is because both peptides and albumin have their rate of diffusion limited by the capillary fenestrae, which will be dilated if albumin is diffusing out more rapidly. As the majority of the NK-1 receptors are located on the endothelial cells it is possible that the effective concentration of the agonist at these receptors is increased in the inflamed joint by SP diffusing through the fenestrae. This may increase the total number of effective receptors, thereby amplifying the response. It is also possible, that with some endothelial cells already contracted and fenestrae open as a result of the inflammation, a further contraction of endothelial cells in response to SP perfusion produces a disproportionate increase in the size of the fenestrae as a result of the tension in the endothelium i.e. the tissue response is non-linear. An increased amplification by the tissue would also result from an activation of the second messenger system. If SP and carageenan share similar second messenger systems it is possible that this system has been primed by the introduction of carageenan. Subsequent stimulation by SP would then produce a greater effect, i.e. each binding of SP to a receptor will produce a greater response in the inflamed joint. Dilatation of blood vesels in the inflamed joint may also be responsible for both the protracted time course and increased sensitivity. CGRP, a known vasodilator, has been shown to inrease the sensitivity and prolong the extravasation produced by SP perfusion (Green *et al*, 1992). Also,

as SP partly mediates its inflammatory response through mast cells, it is possible that an increase in activity of the mast cells in the inflamed joint will increase the sensitivity to SP perfusion. NK-1 agonists which are devoid of mast cell degranulating effects (Drapeau *et al*, 1987) could be used to distinguish the mast cell component of the response to SP in the inflamed joint.

It is also possible that the sensitisation to SP in the inflamed joint is due to up-regulation of the receptors. Drug binding involves diffusion to the receptor followed by formation of the drug-receptor complex. The association rate constant for diffusion to the receptor (k_{D1}) is influenced by various factors, including the viscosity of the medium and the radius of the target on the receptor. A decrease in the viscosity of the medium in proximity to the receptor as a result of extravasation, or an increase in the radius of the receptor target as a result of conformational changes would both increase the diffusion rate. A drug-receptor complex is formed when intermolecular forces between the agonist and receptor modify diffusion rate constants. Various attractive and repulsive intermolecular forces create a distance of minimum free energy at which the agonist will reside in the influence of the receptor. It will only escape the force field of the receptor when it gains enough kinetic energy by thermal agitation to do so. An up-regulation of the receptor will result from a speeding up of this process and will depend mainly on an alteration in the charge or structure of the

receptor leading to altered electrostatic or hydrophobic forces. A change in temperature will also affect the rate of dissociation.

It would appear at first sight that SP has a similar maximal response in both normal and inflamed joints (Fig. 53). However, it should be noted that these results represent the increase in protein extravasation with introduction of SP, rather than a measure of the total protein concentration. The increase in protein levels with SP perfusion are then added to the mean basal level of protein extravasation with saline perfusion in the inflamed joints. As resting protein levels are higher in the inflamed joint, the increase in protein extravasated is greater with low concentration of SP in inflamed joints in comparison to normal joints but is less with high SP concentrations. However the maximal level of total protein extravasation is the same in normal and inflamed joints and it is probable that this is the maximal level of protein extravasation that the joint is capable of producing. In other words the maximal response for both the normal and inflamed joints is not a consequence of the saturation of receptors with SP but is a saturation of the capability of the vessels in the joint to extravasate protein. Both normal and inflamed joints therefore show the same maximal response of total protein extravasated. This could be verified by high concentrations of substances not acting at receptors e.g. formalin

The time taken for the SP-induced protein extravasation to reach the peak response in normal animals is longer for higher

concentrations of SP (Fig. 55). This may involve the latency to maximal receptor response as mentioned above, as well as a longer period required for equilibrium to be reached with higher concentrations. In inflamed joints the situation is reversed, high concentrations of SP taking the shortest time to produce a peak response. More careful analysis shows this to be due to the maintained raised protein levels together with the fluctuations in the raised levels. With low SP concentrations the fluctuations in the protein levels are often as great as the initial response and so the peak level can be virtually any time at random, so long as the level remains moderately raised. With more concentrated SP there is a more definite drop in protein levels after the initial rise such that subsequent fluctuations do not encompass the true peak.

The desensitisation of the response, which takes effect over twenty minutes in the normal joint, takes place over an hour or more in the inflamed joint. If after uniting with SP the receptors themselves undergo a change to form a non-reactive form in the normal joint (Fig. 65), it is possible that in the inflamed tissue the step returning the receptor to the sensitised form is accelerated.

If depletion of second messengers or other substances leads to the desensitisation, then it is possible that in the inflamed joint the process for the replenishment of these substances has been accelerated. Presumably if carageenan utilises a similar second messenger system to SP, a process for the replenishment

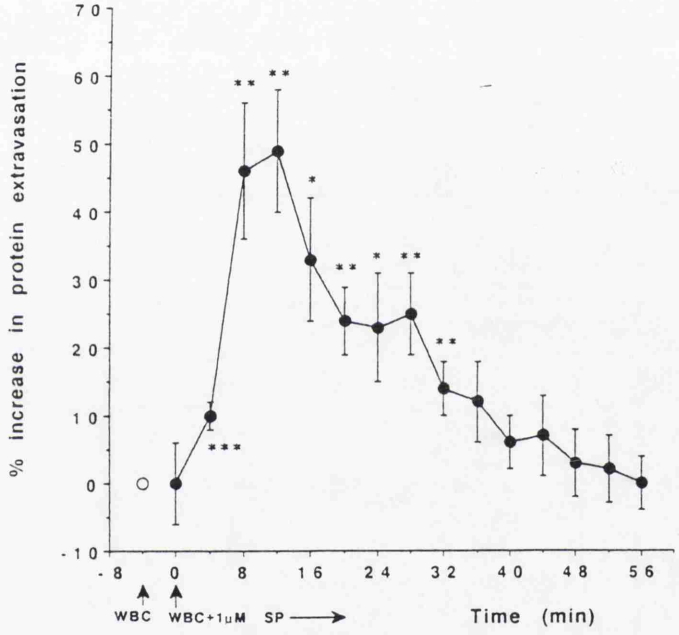
of the substances involved could have evolved over the twenty four hours during the establishment of the inflammation. The correlation between basal protein extravasation levels and the time taken for a reduction in SP-induced protein extravasation suggests that the rate of desensitisation is inversely proportional to the degree of inflammation.

This change in duration of the response could also be due to an increase in the number of available receptors as a result of increased permeability of the endothelium. This could mean that although a proportion of the receptors are desensitised, a significant proportion remain to enable the response to continue.

The sustained response to SP in the carageenan-treated animals could be due to the presence of greater numbers of inflammatory cells in the synovial fluid, as these are known to be involved in the inflammatory response to carageenan (Di Rosa, 1972). To test this hypothesis, a series of normal knees are perfused with a suspension of inflammatory cells to which SP (1μM) is added. The response for a single animal (Fig. 54) and for the mean of five animals (Fig. 66) indicate clearly that the response is transient in the presence of inflammatory cells.

In the inflamed joint, greater levels of protein extravasation are produced and remain elevated over a more extended period than in normal joints. In other words the total area under the protein-time curve is much greater i.e. the total protein extravasated is much greater in inflamed joints. If protein extravasation is an indication of acute inflammation, then SP has

Figure 66: Relative change in protein extravasation from basal level during perfusion with white blood cells (WBC) and during perfusion with a similar perfusate containing SP (1μM) in normal animals. Means ± SEM. n = 5.
* = p<0.05; ** = p<0.01; *** = p<0.001.


% increase in protein extravasation
70
60
50
40
30
20
10
0
-10
-8
0
8
16
24
32
40
48
56
WBC
WBC+1μM SP
Time (min)

a greater inflammatory effect in inflamed joints than in normal joints. Also increased levels of intraarticular albumin *per se* will increase synovial interstitial fluid viscosity and exert a pericapillary oncotic pressure (Levick *et al*, 1991). As σ_{alb} is reduced, an increased transsynovial flow of albumin and solvents will increase the pressure in the joint (Knight *et al*, 1988) with the consequent deleterious effects on joint function. Therefore, not only is protein extravasation an indication of inflammation, but is also pathogenic in its own right.

F) Both NK-1 and NK-2 receptors are involved in protein extravasation in the rat knee joint

Experiments with receptor selective agonists (Abelli *et al*, 1989; Jacques *et al*, 1989; Abelli, 1991; Lam & Ferrell, 1991b) and antagonists (Eglezos *et al*, 1991; Lembeck *et al*, 1992; Xu *et al*, 1992) have demonstrated that NK-1 receptors are responsible for protein extravasation. The results of the experiments presented here demonstrate that NK-2 receptors may also be involved in protein extravasation in the rat knee joint.

Both SP and NKA produce the same degree of protein extravasation over the same time-course. In the first series of experiments determining a dose-response curve for SP, the pD_2 value was 7.0, while in the second series of experiments the pD_2 value was 5.9; although the difference in the protein values from the two dose-response curves is not significant. The difference in

the values is due partly to the different volume of reagents used to measure protein concentration. This alters the sensitivity of the test, and as the basal levels of protein below the threshold level detectable by the test are taken at the threshold level, when subtracted from the peak response, this reduces the maximum increase in the level of protein extravasation. Also, in the second series of experiments urethane alone was used for anaesthesia, while in the first series of experiments urethane and valium were used. It has been shown that urethane decreases the level of neurokinin induced protein extravasation in the rat (Kerouac & Couture, 1987). As the inclusion of valium reduces the amount of urethane required it is possible that the level of urethane has an effect on the second series of SP dose-response experiments. The series of experiments producing the NKA dose-response curve are produced under the same conditions as the second SP dose-response curve. NKA has a pD_2 of 6.4 on this curve which is a higher affinity than SP under the same conditions. As NKA is the natural endogenous agonist for NK-2 receptors, these values suggest the existence of NK-2 receptors. All natural TKs are capable of acting as full agonists at all of the NK receptors but with different affinities. Although SP is the natural endogenous ligand at NK-1 receptors and NKA is the natural endogenous ligand at NK-2 receptors, NKA shows a higher affinity for NK-1 receptors than SP for NK-2 receptors(Abelli *et al*, 1989; Advenier *et al*, 1992). Thus, depending on the relative densities of the two classes of

receptor, NKA may have a greater affinity with a mixed bag of NK-1 and NK-2 receptors. The protein extravasation induced by perfusion with the NK-2 agonist [Nle^{10}]-neurokinin A_{4-10} supports a claim for the contribution of NK-2 receptors in protein extravasation. It is possible that the NK-2 agonist cross-reacts with NK-1 receptors, but the response to concentrations as low as 10μM makes it unlikely that this is the only receptor responsible.

The NK-1 and NK-2 receptor antagonist FK224, antagonises both SP and NKA induced protein extravasation. The ability of high concentrations of FK224 to completely inhibit protein extravasation at low agonist concentrations suggests that only NK-1 and NK-2 receptors are involved in this process. Unfortunately it was not possible to dissolve sufficiently high concentrations of FK224 to completely abolish the response to higher concentrations of the agonists. In fact, although the response was not significantly different, there was an increase in the mean response to 100μM SP when perfused with FK224. There was a larger increase for the less concentrated solution of FK224 than for the more concentrated solutions. Blood pressure recordings taken during SP perfusion show that there is a transient fall in blood pressure during perfusion with 100μM SP, which has a similar time-course to protein extravasation in the joint. It is therefore possible that the true effect of perfusion with 100μM SP is diminished by the coincident fall in blood pressure. Previous perfusion with all concentrations of FK224

used in these experiments abolishes the transient fall in blood pressure produced by 100μM SP. This could therefore explain the greater level of protein extravasated when FK224 perfusion precedes 100μM SP perfusion. This effect could be greatest with 1μM FK224 because, although the blood pressure is prevented from falling, there is only a minimal antagonistic effect in the joint at this concentration of antagonist. More concentrated levels of FK224 antagonise the response in the joint as well as preventing a fall in blood pressure and so the level of protein extravasation is less than with lower concentrations of FK224. However, it is uncertain to what extent a reduction in blood pressure has an effect on extravasation. Hypotension has been shown to have an inhibitory effect on tachykinin-induced extravasation in some preparations (Chahl, 1979) but not in others (Couture & Kerouac, 1987). To ascertain the true effect of lowering the blood pressure, future experiments should include an analysis of protein extravasation at different blood pressure levels. The large variability in extravasation experiments may also be due to a variability in the blood pressure in different experiments.

FK888 reduces extravasation induced by SP to the same extent as FK224. The only significant difference is a greater inhibition of extravasation by FK224 at 1μM concentrations of both the agonist and antagonist. This suggests that SP induces extravasation by binding predominately to NK-1 receptors, as FK888 is a highly specific NK-1 receptor antagonist. Protein

extravasation induced by NKA is unaffected by FK888. This suggests that NKA can produce extravasation by binding to receptors other than NK-1 receptors. It is most likely that NKA is binding to NK-2 receptors in this situation. However, perfusion with the NK-2 specific antagonist SR 48968 has no significant effect on protein extravasation induced by either SP or NKA. As the results with FK888 and SP show, SP is acting predominately at NK-1 receptors and so SR 48968 would not be expected to influence the results of SP induced extravasation. However results with NKA and SR 48968 suggest that NKA can induce protein extravasation by binding to either NK-1 or NK-2 receptors. A greater affinity for NK-2 receptors would be balanced by a greater density of NK-1 receptors to give similar degrees of protein extravasation with NKA. Hence NKA induced extravasation is only blocked by the NK-1 and NK-2 antagonist, FK224. Future experiments could include both FK888 and SR 48968 with NKA perfusion to ascertain if both NK-1 and NK-2 receptors are involved.

The presence of two receptors could account for the gradient of the line in the Schild plot analysis being less than one. The reduction in extravasation by the lowest concentration of the antagonists is not followed by similar further reductions as the concentration of the antagonist is increased by identical logarithmic steps. Initially the agonist will be displaced from the receptor to which it has the least affinity, as well as being displaced from its natural receptor. As the concentration of the

antagonist increases, the agonist only remains bound to its natural receptor and subsequent similar decreases in the level of extravasation require greater increases in the concentration of the antagonist. This is because the agonist has a greater affinity for its natural receptor and therefore takes more concentrated levels of antagonist to be displaced. Hence the dose response curves show decreasing right shifts, which gives a gradient of less than one in the Schild plot. With heterogenous receptor populations nonlinearity and a slope less than unity will be most evident at dose ratios less than ten. Different Schild regressions for the same antagonist with different agonists are also indicative of a heterogenous receptor population.

Schild regressions can also have slopes of less than unity if an agonist removal system is operative. However this is because the increased concentrations of agonist required for a response in the presence of antagonist will eventually saturate the uptake mechanism, resulting in an underestimation of the antagonism. Solubility and financial restrictions restricted concentrations used here to the same level both in the presence and absence of antagonist, so the same degree of saturation of uptake would be observed at all concentrations of antagonist. In this situation underestimation of antagonist potency at high concentrations could be made if the antagonist displays concomitant receptor and uptake blockade. This would also explain the increased response at high agonist concentrations, if uptake saturation by

the agonist and blockade by the antagonist combined to give maximal effects.

Failure of the system to reach equilibrium for any other reason will also yield a Schild regression of less than unity. As there is no period of maintained protein extravasation in normal animals it is likely that desensitisation occurs before equilibrium is reached, and so the maximal response does not represent equilibrium.

3.5 The contribution of joint afferent nerves to the pathogenesis of arthritis.

a) Central reflexes

If the reflex actions of low-threshold articular mechanoreceptors are protective in nature, then it is clear that enhanced articular nociceptive discharge, such as that occuring in inflammatory joint disease, will result in abolition of these protective reflex mechanisms with consequent aggravation of joint injury.

In the analysis of a typical ski injury, it is calculated that 34ms will pass between the loading of a cruciate ligament and ligament rupture, while 89ms will elapse between loading of the ligament and the initiation of a protective reflex (Pope *et al*, 1979). The latter latency agrees with the latency from stimulation of the ligament to the shortest latency of an EMG in the sartorius muscle (Stener & Petersen, 1962). These latencies would suggest that the reflex response is too slow to be protective. Results presented here indicate a latency from activation of group II afferents to excitation of either α- or γ-motoneurones to be of the order of 15ms. This would be quick enough to initiate a protective reflex provided sufficient muscle tension rises quickly enough to overcome the forces acting at the joint. The group II joint afferents respond to movement of the joint before this becomes noxious, and contribute to the

excitation of motoneurones accordingly. If the response of the muscles around the joint is not sufficient to stabilise the movement, more stress will be placed on the joint structures, activating nociceptors. However, by this time the cause is lost, the reflex being too slow to protect the joint structures. It is significant that the initial excitation of extensor motoneurones by the proprioceptors is inhibited when nociceptors are activated. Presumably by this stage it is not worth while for the muscles to resist the movement activating the nocieceptors, but better to be inhibited, thereby relaxing joint structures. This inhibition of extensor activity is consistent with other FRA's.

The level of activity in the α-motoneurone pool is dependent on descending commands, autogenic and heterogenic reflexes (Nichols, 1987). This activity mediates muscle stiffness, together with intrinsic muscle stiffness determined by the viscoelastic properties of the muscle (Hoffer & Andreassen, 1981). Of all the factors contributing to joint stability, including physical restraint by ligaments and friction, the load imposed on the joint is paramount (Markolf *et al*, 1981). A considerable proportion of this load comes from muscle contraction (Markolf, Graff-Radford & Amstutz, 1978). It is probable, therefore, that the reflexes from joint afferents are very important in the continuous regulation, or presetting, of the muscle tone of muscles surrounding the joint. It is also possible that joint deformities, which are so prevalent in articular disease, could result from alteration in reflex excitability of muscles acting over the

affected joint. Activity in nociceptors, such as found in joint inflammation, will lead to a disfacilitation of motoneurones by proprioceptors. This may lead to a general imbalance in muscle tone of muscles surrounding the joint, especially during movement of the joint. This imbalance may lead to an accumulation of minor injuries to the joint, accentuating the inflammation and producing further disfacilitation.

An imbalance is more likely to occur than atony of the muscles, as although disfacilitation of motoneurones is produced by activity in nociceptors, γ-motoneurones are also excited by nociceptors. Also, ān increase in activity of mechanoreceptors is responsible for muscle wasting and weakness associated with joint inflammation (Stokes & Young, 1984). Reflex inhibition in the absence of pain, and the ability of joint effusions to inhibit muscle contractions, implicate mechanoreceptors in the reflex inhibition of muscle contraction (Stokes & Young, 1984; Jones, Jones & Newham, 1987; Iles, Stokes & Young, 1990). An inhibition of these group II afferents would therefore be expected to increase the reflex excitation of the extensor muscles rather than inhibit them. However, as we have also shown excitation of extensor motoneurones by group II joint afferents, inhibition of these afferents is likely to have a complex result, with possible imbalance in the overall tension of muscles around the joint.

As joint receptors provide sensory feedback throughout the entire range of joint movement (Ferrell, 1980; 1985; Marshall &

Tatton, 1990), it is possible that these receptors may modify reflex-mediated stiffness at all joint angles. As the response characteristics of somatosensory cells in the brain are unsuited to monitoring joint position, and the majority of these cells are rapidly adapting (Yin & Williams, 1976), it is probable that the principle function of the joint receptors is not to provide an awareness of joint position, but in reflex regulation of joint stiffness at the spinal level, as well as through a long latency pathway to the cortex.

b) Peripheral inflammation

If pain is perceived in a joint, then nociceptive afferents are active. Neuropeptides are being released at the central synapses to act as neurotransmitters, and also in the periphery, from the afferent peripheral terminals. Under normal circumstances the peripheral release of the neuropeptides SP and NKA will result in a transient inflammation. However, if the joint is already inflamed, the release of SP will promote a more persistent inflammation. Therefore, if a joint suffers an injury which instigates an inflammatory response, the subsequent release of SP will potentiate the inflammation and perhaps delay or prevent the healing process. The quantitative contribution made by the neuropeptides to the overall pathogenesis remains uncertain. In certain individuals it is possible that the inflammation produced by the neuropeptide release is sufficient

to span the interval from the original acute inflammation to a persistent form of arthritis. What would have been an acute injury turns into a chronic problem in susceptible individuals.

As both NK-1 and NK-2 receptors are involved in the peripheral inflammation, it would be of value to assess the efficacy of NK-1 and NK-2 receptor antagonists in the treatment or prevention of arthritis. As both SP and NKA are involved at the central synapse and therefore in the segmental reflexes and the perception of pain in higher centres, it would also be of value to asses the efficacy of these antagonists in preventing muscle atrophy, deformation of joints and the perception of pain.

c) General Conclusion

Joint proprioceptive and nociceptive afferents play a role in maintaining the integrity of the joint by exerting effects on muscle tone (via proprioceptors) and blood vessel calibre and permeability (via nociceptors). An acute injury will evoke the appropriate response to reduce movement and initiate the healing process. However, if for whatever reason the inflammation becomes chronic, there is an adverse effect on proprioceptive feedback (Ferrell, Crighton & Sturrock, 1993) and an altered response to neuropeptides, thereby potentiating the disease process.

Appendix 1

Spinal cord histology.

At the end of the experiment, the spinal cord is dissected from the cat and fixed in formol saline. After several days the spinal cord is processed and embedded in celloidin using the following method.

Celloidin embedding.

B.D.H. NECOLOIDINE. = 8% CELLOIDIN. (DILUTE WITH ETHER.)

Method.

1.) 70% Alcohol. = 3 days. (Change daily.)

2.) 90% Alcohol. = 3 days. (Change daily.)

3.) Absolute alcohol. = 5 days. (Change daily.)

4.) Abs.alcohol/Ether. = 24 hours.

5.) 2% Celloidin. = 7 days.

6.) 4% Celloidin. = 7 days.

7.) 8% Celloidin. = 4 days.

8.) Embed in 8% Celloidin.

Wrap paper around the top of a wooden block to form a mould deeper than the length of spinal cord to be embedded. Place the specimen into the mould and fill with celloidin. Put the mould, together with a small beaker of ether, into a desiccator and leave for several hours. The ether atmosphere promotes the dispersion

of bubbles introduced during handling. Replace the ether with chloroform and reseal the desiccator. The chloroform atmosphere hardens the block without the formation of a hard outer skin and soft centre. Replace the chloroform when necessary as the hardening takes several days. The paper mould may be stripped away after several days to aid hardening. When the block is completely set it has the consistancy of hard rubber. Blocks are stored in 70% alcohol prior to sectioning.

Sectioning.

The embedded spinal cord is then sectioned using an M.S.E. base sledge microtome. Serial sections are cut at 100μm and stored in 70% alcohol prior to staining.

Staining.

Free floating sections are stained individually using the following technique.

Stain.

0·1% Aqueous Thionine.

Method.

1.) Running tap water. = 5 minutes.

2.) 0·1% Thionine. = 2-5 minutes. (Control microscopically.)

3.) Running tap water. = wash till clear.

4.) Dehydrate in 70 then 90% alcohol.

5.) Transfer to clearing xylene then pure xylene.

6.) Mount in D.P.X.

Result.

Nuclei and nerve cell bodies: blue to purple.

(Chang, M., 1936)

Microscopy.

Sections were examined using a Zeiss Axiophot microscope and photomicrographs taken on Kodak Ektachrome 60.

Appendix 2

The Mann-Whitney U Test.

1. Null Hypothesis (H_0): The population relative frequency distributions for A and B are identical (where B is control or lesser response and A is treatment or greater response).

2. Alternative hypothesis (Ha): The population relative frequency distribution for A is shifted to the right of the relative frequency distribution for population B (i.e. a one-tailed test).

3. Test statistic:

$$U_A = n_1 n_2 + \frac{n_1(n_1 + 1)}{2} - W_A$$

Where: W_A is the rank sum for sample A relative to sample B.
n_1 is the number of observations in sample A.
n_2 is the number of observations in sample B.
Ranks of ties are averaged.

4. Rejection region: For a given value of α (probability of a type I error), reject H_0 if $U_A \leq U_0$ where $P(U_A \leq U_0) = \alpha$. (i.e. U_0 is selected value of U corresponding to desired level of α for corresponding sample sizes)

5. Assumptions: Samples have been randomly and independently selected from their respective populations.

Appendix 3

Kruskal-Wallis H Test for Comparing k Population Distributions.

1. Null Hypothesis (H_0): The k population distributions are identical

2. Alternative hypothesis (Ha): At least two of the population distributions differ in location

3. Test statistic:

$$H = \frac{12 \sum n_i[R_i - R]^2}{n(n+1)}$$

Where: n_i is the number of observations in group i.

n is the total sample size.

Ri is the average of the ranks in group i.

R is the average of all the ranks.

4. Rejection region: Reject H_0 if $H > \chi^2_\alpha$ with (k-1) degrees of freedom (i.e. under the null hypothesis, the distribution of H can be approximated by a χ^2 distribution with k-1 degrees of freedom).

5. Assumptions: The k samples are randomly and independently drawn. There are five or more measurements in each sample.

The following z-values are also printed for each group

For group i,

$$z_i = \frac{R_i - (n+1)/2}{\sqrt{(n+1)n/n_i - 1)/12}}$$

Under the null hypothesis, z_i is approximately normal with mean 0 and variance 1. The value of z_i indicates how the mean rank, R_i, for group i differs from the mean rank, R, for all n observations.

Appendix 4

Drugs and reagents

1. Urethane (Ethyl Carbamate, Sigma) 25% in distilled water
2. Hypnorm (Fentanyl/Fluanisone, Janssen)
3. Vallium (Diazepam, Roche)
4. Pavulon (Pancuronium Bromide, Organon Teknika)
5. Fluothane (Halothane, ICI)
6. Capsaicin (Fluka) in saline
7. Benzethonium Chloride (Sigma) in saline
8. Substance P (ICI) in saline
9. Neurokinin A (Novabiochem) in saline
10. FK888 (Fujisawa) in 100% alcohol and then saline
11. FK224 (Fujisawa) in 100% alcohol and then saline
12. SR48968 (Sanofi Recherche) in 100% alcohol and then in saline

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