In the name of God

The beneficent The merciful

Neural and local regulation of blood flow and synovial fluid PO_2 in the rabbit knee joint

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by

Hamid Najafipour B.Sc., M.Sc.

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Institute of Physiology

Faculty of Medicine

University of Glasgow

In the name of Allah

The Beneficent the Merciful

And indeed We created man of an extract of clay.

Then We made him a small life-germ in a firm resting place.

Then We made the life-germ a clot, then We made the clot a lump of flesh, then We made in the lump of flesh bones, then We clothed the bones with flesh; then We did grow it into another creation; so blessed be Allah, the best of creators.

Then verily after that you shall die.

Then on the Day of Judgement you shall be raised.

And indeed We made above you seven paths (heavens); and neither, of the creation, We are heedless.

Holy Qur'an

Chapter 23

Verses 12-17

Dedicated

to

my family

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Summary

Synovial fluid, in addition to its lubricating action of moving structures, provides nutrients to avascular structures such as articular cartilage (McKibbin & Maroudas, 1979), and also to the knee ligaments (Renzoni *et al.*, 1984), within the joint . As synovial fluid formation is critically dependent on synovial blood flow (Levick, 1987), those factors which regulate flow in the synovial vascular bed are clearly important in this process. Although the presence of sympathetic nerve fibres innervating articular blood vessels has been demonstrated in dogs (Cobbold & Lewis, 1956b) and cats (Ferrell & Cant, 1987; Khoshbaten & Ferrell, 1990b), and rabbits (Khoshbaten & Ferrell, 1990a), the receptors mediating this regulatory function are not known in most cases. Moreover, it is not quite clear what happens to the role of sympathetic nervous system or to its receptors on joint blood vessels in inflammatory joint diseases. In recent years attention has been focused on the role of the endothelium in the regulation of blood flow to different vascular beds by releasing local vasodilator or vasoconstrictor factors (for review see Moncada *et al.*, 1991), but this role and its importance in regulation of blood flow to the normal and inflamed joints has not been investigated so far. Other local mediators, such as prostaglandins (PGs), have been shown to be present in synovial fluid from inflamed joints (Blackham *et al.*, 1974, O'Byrne *et al.*, 1990). Whether these are produced by joint blood vessels, or play any role in regulation of blood flow to normal and inflamed joints is not known yet, although they are found to be effective on some vascular smooth muscles (for review see Horton, 1979).

An important nutrient which must reach intra-articular structures via the synovial fluid is oxygen. The partial pressure of oxygen in

synovial fluid (P_sO_2) from diseased human joints has been measured, by aspiration of an effusion, and such aspirates have shown very low P_sO_2 values (Falchuk, Goetzl & Kulka, 1970; Lund-Olesen, 1970; Treuhaft & McCarty, 1971; Richman, Su & Ho, 1981), but the P_sO_2 of the normal synovial fluids has not been reported (Levick, 1987) probably due to the small volume of synovial fluid in the normal joint, which is difficult to aspirate. Although oxygen in synovial fluid is derived from the synovial blood flow, the relationship between these two has not been investigated.

The first objective of this research was to measure knee joint blood flow quantitatively and also assess the effect of sympathetic nerves in the regulation of joint blood flow. Secondly, to investigate and characterize the type of adrenoceptors mediating the sympathetic control of joint blood flow, and other mediators involved in nerve mediated changes in articular blood flow. Thirdly, to assess the possible role of the endothelium in regulation of joint blood flow and modulation of sympathetic nerve-mediated changes in joint blood flow. Finally to perform all the mentioned procedures in an experimentally induced acutely inflamed knee joints to determine the effect of inflammation on these regulatory mechanisms and factors.

The second objective of this study was to measure, in both normal and inflamed knee joints, the synovial PO_2 directly in its place; and also assess the correlation between the joint blood flow and oxygen tension in the synovial fluid and the extent to which this correlation may be affected by the process of inflammation.

Experiments were performed on rabbits. Acute inflammation was induced by intra-articular injection of carrageenan. Quantitative

measurement of joint blood flow was conducted by the radiolabelled microsphere technique. Relative changes in blood flow were assessed by laser Doppler flowmetry, and a polarographic oxygen elecrode (and oxygen meter) was used to measure synovial PO_2 and its changes during the course of experiments.

The results of this investigation indicate that: 1). The microsphere technique and laser Doppler flowmetry are suitable methods for quantitative and continuous measurement of joint blood flow respectively, and the process of inflammation increases joint blood flow significantly. Despite this increase in blood flow, synovial PO_2 which in normal joint is much lower than the arterial PO_2, even decreases more in the inflamed joints. 2). Sympathetic nerves innervate the blood vessels of the posterior capsule of the knee joint and play an important role in regulation of joint blood flow. α_2 adrenoceptors predominate in this vascular bed and mediate vasoconstrictor responses to nerve stimulation. No evidence of purinergic co-transmission was obtained. The process of inflammation reduced the effectiveness of sympathetic nervous system in regulation of joint blood flow. 3). Nerve-mediated vasodilator responses appeared to have two components, a β-adrenoceptor component which is mediated by postjunctional β_1 adrenoceptors which found to predominate in this vascular bed, and a substance P mediated component which is produced by the release of neuropeptide, substance P, presumably from the unmyelinated sensory nerve endings. 4). Vascular endothelium keeps the joint blood vessels of both normal and inflamed joints in a state of active dilatation by release of nitric oxide (NO) and therefore plays a major role in local regulation of joint blood flow. NO also counteracts the sympathetic vasoconstrictor responses, but it showed no modulatory effect on nerve-mediated vasodilator responses. 5). Prostaglandins are important local factors in

regulation of joint blood flow in both normal and inflamed joints. They seemed to have no modulatory effect on sympathetic regulation of blood flow to this vascular bed. PGE_2 receptors are present on knee joint blood vessels but they down regulate during the process of inflammation. 6). A polarographic oxygen electrode provided a new and suitable method for quantitative and continuous measurement of oxygen partial pressure in the synovial fluid of both normal and inflamed knee joints. A high correlation between changes in joint blood flow and synovial PO_2 was found in both joints.

CHAPTER ONE

General introduction and literature review

A: Introduction

I. Blood vessels and regulation of blood flow

Blood vessels are the tubing of a closed network in the body called the "vascular system" in which the blood flows from the heart towards the tissues and back from them to the heart. The rate of flow in each part of this network depends on the pressure produced by the heart and vessel resistance offered to the flow, with the latter being a more effective tool for the body to control the former in one side and the flow past the vessel in the other side. Therefore, changes in vessel resistance leads to changes in blood flow to the region supplying by that vessel i.e. its circulation. The peripheral circulation is essentially under dual control, 1) centrally by the nervous system and 2) locally in the tissue by environmental conditions in the immediate vicinity of the blood vessels, with circulating hormones also influencing the blood vessel calibre. The relative importance of these is different in various organs and tissues. For example, in some parts of the body like skin and splanchnic regions, nervous control of blood flow predominates, whereas in the others such as the heart and brain, neural regulation plays a minor role and local regulation is much more important. But in general the fine control of blood vessels is brought about largely by competition between centrally directed vasoconstrictor nerves and vasodilator effects of locally released factors and produced metabolites.

The vessels chiefly involved in regulating the rate of blood flow throughout the body are referred to as resistance vessels (i.e. small arteries and arterioles), since these offer the greatest resistance to the

flow of blood pumped by the heart and hence, are important in the maintenance of blood pressure and also regulation of flow in different tissues and organs (Berne & Levy, 1981).

Vascular smooth muscle, which is quite thickened in the wall of resistance vessels, is the tissue responsible for the control of total peripheral resistance, arterial and venous tone, and the distribution of the blood flow throughout the body. The smooth muscle of the resistance vessels is often spontaneously active, providing "basal tone". The level of tone can be affected by extrinsic nerves and local factors. Activation of the sympathetic nerves either directly or reflexly enhances vascular resistance. In contrast to the sympathetic nerves the parasympathetic nerves tend to decrease vascular resistance, but they innervate only a small fraction of the blood vessels in the body, mainly in certain viscera and pelvic organs. Some circulatory and local substances such as catecholamines, histamine, acetylcholine, serotonin, angiotensin, adenosine, prostaglandins and, local environmental changes (e.g. temperature changes and level of tissue O_2 and CO_2) alter the contractile state of vascular smooth muscle. The response of vascular smooth muscle may be different from one tissue to another or from the same tissue under different physiological conditions. For example, some agents elicit vasodilatation in some vascular beds and vasoconstriction in others. The phenomenon of autoregulation of flow (the increased vasodilatation that compensates for a primary decrease in pressure and flow through a circuit and the converse changes) is a product of myogenic tone of the resistance vessels (Berne & Levy, 1981).

A precapillary sphincter action regulates blood flow through the capillary network. This action is controlled primarily by the local factors, but there is also an extrinsic nerve supply.

Changes in the lumen of the capacitance vessels (venules and veins) have a profound effect on venous capacity and hence on venous return and cardiac output, with little effect on resistance to flow. The smooth muscle of the capacitance vessels is controlled by extrinsic vasoconstrictor nerves. Apart from the spontaneous activity of the longitudinal muscle coat found in large veins, venous smooth muscle is generally quiescent (Berne & Levy, 1981).

Thus, in general, those components of the vascular circuit which subserve no local function, namely the large arteries and capacitance vessels, are controlled exclusively by extrinsic nerves, and show little spontaneous activity. On the other hand the smaller arteries are under tonic neurogenic control of sympathetic noradrenergic vasoconstrictor fibres, which are governed by discharge from the medullary vasomotor centre. Decrease discharge of these fibres produce a fall in tone of the vessels or vasodilatation without the involvement of specific vasodilator nerve fibres. In the precapillary resistance vessels vasodilatation is also affected by release of various local factors and cell metabolites.

II. Synovial PO_2

Synovial fluid is known to have a variety of different functions, an important one of which is to provide nutrients to avascular structures such as articular cartilage within the joint (McKibbin & Maroudas, 1979). The joint cartilage is oxygenated by diffusion of oxygen from the capillaries of the synovial membrane through the synovial tissue and

joint fluid. However, synovial fluid does not contain hemoglobin and thus lacks buffering capacity for oxygen. Due to small amount of oxygen in physical solution, the synovial fluid PO_2 (P_sO_2) is extremely sensitive to changes of blood flow to the joint as synovial fluid formation is critically dependent on joint blood flow. As the synovial cavity is essentially an enlarged interstitial space (Bauer *et al.*, 1940, Edwards *et al.*, 1981), the synovial fluid is a plasma ultrafiltrate which its formation is under the same rules of interstitial fluid formation, i.e. simplified as Starling's hypothesis of trans-capillary ultrafiltration (Starling, 1896). Based on this hypothesis the factors favouring synovial fluid formation are: capillary blood pressure, extra-capillary colloid osmotic pressure, the total area of filtration and hydraulic conductance per unit of area, and the factors opposing its formation are: plasma colloid osmotic pressure, extra-capillary hydrostatic pressure, and the degree of semipermeability of capillary wall to plasma proteins. Oxygen is a highly diffusable molecule which can pass physiological membranes easily and also moves along with the bulk flow of fluids. Therefore the partial pressure of oxygen in synovial fluid depends on its partial pressure in plasma as well as local cellular oxygen consumption. These parameters may be affected by inflammation of the synovial membrane which is likely to affect the factors influencing synovial fluid formation or increase the diffusion distance and the rate of oxygen consumption of the joint tissues.

III. Sympathetic nervous system

The sympathetic nervous system consists of thoraco-lumbar outflow of preganglionic neurones passing via rami comunicates to make synaptic connections with postganglionic neurones in the

paravertebral or prevertebral ganglia. The sympathetic chain, which consists of a bilateral system of paravertebral ganglia joined by longitudinal connectives, extends rostrally to the upper cervical region and caudally to the lower sacral level, but receives no efferent contribution from the spinal nerves at these extremes. Those postganglionic axons which pass out of the sympathetic chain to supply peripheral vascular beds and other superficial structures such as sweat glands pass via grey rami comunicates to the spinal nerves and run with them. In addition, there are some other nerve fibres which arise from the motor cortex of the cerebrum and pass through the hypothalamus and the ventral medulla before they join the other sympathetic outflow in the spinal cord. This group is named the sympathetic cholinergic dilator system (Folkow *et al.*, 1961; Green and Kepchar, 1959; Uvans, 1960), and are activated in "Alarm-Defence" reaction. There are also some reports that cholinergic sympathetic fibres can produce local vasodilatation of vessels in the skin of the face and neck (Holton & Rand, 1962; Hertzman, 1959; Folkow, 1955). Classically preganglionic fibres of both types of sympathetic nerves release acetylcholine as neurotransmitter, but at their postganglionic nerve endings the neurotransmitter for sympathetic vasoconstrictor fibres is noradrenaline whereas for sympathetic vasodilator fibres it is acetylcholine (Berne & Levy, 1981).

IV. Aim of this study

Joints are essential for movement and the knee joint is the largest joint in the body and one of the most important which has received extensive attention due to the prevalence of inflammatory joint disease. Synovial fluid, in addition to its lubricating action of moving structures

provides nutritional support to avascular structures such as articular cartilage (McKibbin & Maroudas, 1979), and also to the knee ligaments (Renzoni *et al.*, 1984), within the joint . As synovial fluid formation depends critically on synovial blood flow (Levick, 1987), it follows that those factors which regulate flow in the synovial vascular bed are very important in this process. These factors have been considered in detail by Levick (1984). The composition and dynamics of synovial fluid have been reviewed in another article in more detail (Levick, 1987). The measurement of synovial blood flow is difficult because it is not a discreet organ, has multiple vessels supplying it and there is heterogeneity in the capillary distribution and therefore in blood flow at different depths. Therefore, even most suitable methods of blood flow measurement have some limitations in estimating joint blood flow precisely. More importantly, little is known about the factors involved in regulation of synovial blood flow. Although the presence of sympathetic nerve fibres innervating articular blood vessels has been demonstrated in dogs (Cobbold & Lewis, 1956b), cats (Ferrell & Cant, 1987; Khoshbaten & Ferrell, 1990b), and rabbits (Khoshbaten & Ferrell, 1990a), the receptors mediating this regulation are unknown in most cases. Moreover, it is not quite clear what happens to the role of the sympathetic nervous system or to its receptors on joint blood vessels in inflammatory joint diseases. On the other hand, in recent years attention has been focused on the local factors released especially by endothelium such as endothelium derived relaxing factor (EDRF) by different vascular beds (for review see Moncada *et al.*, 1991) which has been shown to have very important role in the regulation of tissue blood flow (and systemic blood pressure), but its presence and importance in regulating blood flow to normal and inflamed joints has

not been investigated so far. The other local mediators, prostaglandins (PGs), have been shown to be present in synovial fluids of inflamed joints (Blackham *et al.*, 1974; O'Byrne *et al.*, 1990), but whether these are produced by joint blood vessels, or play any role in regulation of blood flow to the normal and inflamed joints is not known yet, although they are produced by some other blood vessels, and found to be effective on the vascular smooth muscle of other vascular beds (for review see Horton, 1979).

An important nutrient which must reach intra-articular structures via the synovial fluid is oxygen . The partial pressure of oxygen in synovial fluid (P_sO_2) from diseased human joints has been measured, the technique used invariably involved aspiration of an effusion and such aspirates have shown very low P_sO_2 values (Falchuk, Goetzl & Kulka, 1970; Lund-Olesen, 1970; Treuhaft & McCarty, 1971; Richman, Su & Ho, 1981). As it has been very difficult to aspirate the synovial fluid of a normal joint (probably due to its small volume and negative intra-articular pressure), the P_sO_2 of the normal synovial fluid has not been reported. Although oxygen in synovial fluid originates from the synovial blood flow, the relationship between these two has not been investigated.

The first aim of this study was to measure knee joint blood flow quantitatively and assess the effect of sympathetic nerves in regulation of blood flow to the posterior capsule of the knee joint by a suitable qualitative but continuous method. Secondly, to investigate and characterize the type of adrenoceptors mediating the sympathetic control of joint blood flow and some other receptors which are involved in nerve mediated regulation of articular blood flow. Thirdly, to assess the

role of endothelium in regulation of joint blood flow by investigation of the possible local regulatory/modulatory roles of factors such as EDRF and PGs, and also their possible modulatory effect on sympathetic regulation of joint blood flow. Finally, performing all these procedures in an experimentally induced acutely inflamed joint in order to find out what changes the process of inflammation may produce on the mentioned regulatory mechanisms and factors.

The second purpose of this study was to measure, in both normal and inflamed knee joints, the synovial PO_2 directly in situ; and also assess the correlation between the joint blood flow and oxygen tension in the synovial fluid and the extent by which this correlation may be affected by the process of inflammation. The significance of low PO_2 values in synovial fluid aspirated from diseased joints is difficult to interpret as there are no values from normal joints to allow comparisons.

B: Literature review

I. Sympathetic nervous system and the concept of adrenergic receptors

In general, sympathetic nerves innervate most of vessels of the body, although in varying degrees. The degree of innervation ranges from sparse in the cerebral blood vessels to dense in skin. The density of innervation of the skeletal muscle and gastrointestinal tract is intermediate between the extremes represented by cerebral and skin blood vessels. The large arteries and veins are sparsely supplied whereas small arterioles are densely innervated (Berne & Levy, 1981).

Receptors are cellular constituents by which most drugs exert their specific effects on the tissue by forming a bond, generally reversible, with them. The role of the receptor is to recognise a chemical signal and to discriminate between such a signal and other molecules*. The receptor concept was first proposed by Langley in 1878. The presence of receptors in an anatomical site determines the selective nature of many drug effects. One of the main roles of receptors is where the sympathetic nerves make synapses with actual tissues (e.g. vascular smooth muscle), and neurotrasmitter(s) are released from the nerve endings and after passing the synaptic clefts, bind with the receptors either on the post- or pre-synaptic membrane.

The chemical neurotrasmission theory has been proposed from the beginning of the twentieth century and became the second theory of neurotransmission beside that of electrical transmission.

* Ross & Gilman, 1980.

In 1905 Langly showed that, there is similarity between the rise in blood pressure which was noted when adrenal extract was injected *in vivo* (Oliver & Schafer, 1895) and pressor response obtained after stimulation of sympathetic nerves. This rise in blood pressure remained after denervation, indicating a site of action on the effector system and not via the nerves. The chemical which was responsible for the pressor response of the adrenal extract was termed adrenaline because of its close association with the adrenal medulla.

In 1905, Elliott was the first to suggest that adrenaline was liberated from the sympathetic nerve endings when they were stimulated and the released adrenaline then acted on the responsive cells (Elliott, 1905). Barger and Dale tried different compounds related to adrenaline and pointed out that noradrenaline more closely mimicked the effect of sympathetic stimulation (Barger & Dale, 1910).

The first conclusive evidence that chemical trasmission occurs during nerve stimulation was obtained by Loewi in 1921. He set up two frog hearts and perfused them in such a way that the perfusate flowed from the first heart to the second one. When he stimulated the sympathetic nerves innervating the first heart, he observed that the heart beat and contractility of both hearts had been increased. So he concluded that the nerve endings of the first heart were liberating a substance which he named "Acceleranstoff" (Loewi, 1921), and that this substance was transported via the perfusate to cause the observed effects on the second heart. The close similarity between the action of acceleranstoff and that of adrenaline on the frog tissue led to investigation which eventually proved adrenaline to be the sympathetic transmitter in the frog.

However, in mammals the sympathetic neurotrasmitter was shown to be noradrenaline (NA) rather than adrenaline (see Burnstock, 1969). Von Euler in 1946 showed that the main sympathetic transmitter was NA since very little adrenaline was present in sympathetically innervated tissue whereas the concentration of NA was very high by comparison. He also showed a decrease in NA level following sympathectomy of the tissue, indicating NA association with nerve rather than muscle.

Ahlquist (1948) was the first to classify adrenoceptors into alpha and beta based on the rank orders of potency of adrenaline and NA, and isoproterenol to stimulate receptors and on the ability of Dibenyline (phenoxybenzamine hydrochloride) and Dichloroisoproterenol (DCI) to block their effects, and replaced the older classification based on excitatory and inhibitory effects (Ahlquist, 1948). Ahlquist found that adrenaline was the most potent stimulator for one receptor, called alpha (α) type, whereas isoproterenol was the least potent. Isoprotrenol was the most potent for the other receptor called the beta (β) type, and NA was the least potent. Phenoxybenzamine and phentolamine selectively blocked the action of the alpha-receptors, and DCI selectively blocked the beta-receptors.

The adrenergic receptors differ in their physical and chemical properties to such an extent that their affinities for various adrenergic agents also differ. In general the effect of alpha adrenergic receptors is excitatory and that of the beta adrenergic receptors is inhibitory. One important exception is beta receptors in the myocardium, which are excitatory in nature. Isoproterenol, by activating the myocardial beta-receptors, increases heart rate and force of contraction.

This theory was further strengthened by the use of DCI by others (Powell & Slater, 1958). This drug was chosen as a selective inhibitor of beta-receptors. Thus the terminology of alpha- and beta-receptors became accepted and lies at the basis of adrenergic neurotransmission research.

The discovery of pre-synaptic alpha-adrenoceptors followed the multiple actions of the alpha-adrenoceptor blocking agent phenoxybenzamine and led to the pharmacological subdivision of alpha-adrenoceptors (Brown & Gillespie, 1957). Presynaptic alpha-adrenoceptors have a different mode of action as they do not produce direct contraction or relaxation of smooth muscle but mediate inhibition of transmitter release from sympathetic nerve ending via a negative feedback mechanism (Langer, 1974; Westfall, 1977; Vizi, 1979). Langer proposed that pre- and post-synaptic alpha-receptors were not identical. He suggested that post-synaptic receptors to be named as $alpha_1$ and pre-synaptic receptors as $alpha_2$ Following the definition by Langer (1974), studies on relative potencies of different alpha-agonists and antagonists at the pre- and post-junctional sites were carried out.

Starke *et al.* (1975a) were the first to report the drug yohimbine displayed a preferential blockade of the presynaptic receptors as opposed to post-synaptic receptors in the rabbit pulmonary artery. Other studies using agonists showed that alpha-agonists varied widely in their pre- and post-synaptic potencies (Starke *et al.*, 1975b,c). In the rabbit pulmonary artery, metoxamine was the most potent agonist tested at the post-synaptic receptors, whereas oxymetazoline was the most potent pre-synaptically. They concluded that it may be due to a structural differences in the two subtypes. Just as yohimbine has been introduced

as a selective alpha$_2$-antagonist, prazosin was discovered to be a selective alpha$_1$-antagonist (Cambridge *et al.*, 1977). Therefore, by using different agonists and antagonists potencies, in time it has been possible to identify and classify the subtypes of alpha-adrenoceptors in cardiovascular system, and elsewhere.

Moulds and Jauernig in 1977 discovered that prazosin acts as a competitive antagonist at alpha-adrenoceptors but had selective effects on different vascular bed (e.g. peripheral beds such as the palmar digital artery were resistant to prazosin).

It was Docherty and McGrath (1980) who finally concluded that there are two types of post-synaptic alpha-adrenergic receptors on vascular beds named alpha$_1$ and alpha$_2$ subtypes (Docherty *et al.*, 1979; Docherty & McGrath, 1980). The classification of post-synaptic alpha$_2$-adrenoceptors was much aided by the discovery of a more specific and potent selective alpha$_2$-antagonist rauwolscine (Weitzell *et al.*, 1979).

Since then, vascular post-junctional alpha$_2$-adrenoceptors have been clearly demonstrated in many animal *in vivo* preparations (for review see McGrath 1981,1982,1983, Timmermans & Van Zwieten 1981,1982).

Minneman and co-workers have subdivided α_1- adrenoceptors into α_{1a} and α_{1b} subtypes based mainly on ligand binding studies (Han *et al.*, 1987). The antagonists benexathian and WB 4101 were found to have high affinity for the α_{1a}-ligand binding sites of rat vas deferens, but low affinity for the α_{1b} sites also found in rat vas deferens. The high affinity site for benexathian and WB 4101 correlated with the α-adrenoceptor mediating contractions to noradrenaline of the rat vas

deferens, whereas low affinity site correlated with the α-adrenoceptor mediating contractions to noradrenaline of the rat spleen (Han *et al.*, 1987). Other compounds with high affinity for α_{1a} site were 5-methyl-urapidil and (+)-niguldipine, whereas the α_{1b} was sensitive to inactivation by chloroethylclonidine. In this classification, the classical α_1-adrenoceptor antagonist prazosin was found to be non-selective between subtypes.

α_2-adrenoceptors have been subdivided into α_{2A}, α_{2B} and α_{2C} in ligand binding studies based on the relative potencies of a series of ligands, particularly prazosin and ARC 239 (Bylund 1988). Prazosin and ARC 239 showed high affinity for the α_{2B} ligand binding site of rat lung and kidney, and low affinity for the α_{2A} site of human platelet. In terms of relative affinities, prazosin was 5 times less potent than yohimbine at α_{2B} sites, 250 times less potent at α_{2A} sites and 40 times less potent at the proposed α_{2C} sites of the OK cell line (Bylund, 1988). The anatomical distribution and physiological function of the subtypes of α_2-adrenoceptors have yet to be elucidated.

In 1967 Lands and his co-workers found that using sympathetic amines (beta-agonists) on both isolated tissues and whole animals produced different responses. Thus he and his colleagues showed that there were two different beta-adrenoceptors, termed as $beta_1$ and $beta_2$ (Land *et al.*, 1967). Cardiac inotropic and chronotropic effects were mediated by β_1-adrenoceptors, and vasodilator and bronchodilator effects by β_2-adrenoceptors. Up until 1972 it was widely believed that there were two types of beta-adrenoceptors (Furchgott, 1972).

Later on, studies in cat, guinea-pig, rat and human heart muscle using selective agonists such as RO363 and procaterol and antagonists such as CGP 20712A and ICI 118551 clearly demonstrated the presence of functional β_2-adrenoceptors in cardiac muscle (see Molenaar & Summers, 1987; Jones *et al.*, 1989).

During the past few years an increasing amount of evidence has accumulated indicating that, in addition to the β_1- and β_2-adrenoceptors, a further subtype may exist. The new β-adrenoceptor concept was provided by development of lipolytically selective agonists (Arch *et al*, 1984), the most potent of them BRL 37344, which stimulates lipolysis of rat brown adipocytes with 400- and 20-fold selectivity compared with β_1- and β_2-responses respectively (see Zaagsma & Hollenga, 1991). This kind of adrenoceptor is named the atypical- or β_3-adrenoceptor and has been found in many other tissues, particularly in gastrointestinal system e. g. the smooth muscle from guinea pig ileum and stomach fundus, rat colon and rabbit jejunum, previously all classified as β_1-adrenoceptors (see Daly & Levy, 1979).

The presence of cardiac β_3-adrenoceptors has been suggested by the observation that some partial agonists related to pindolol stimulate the heart at concentrations appreciably greater than those required to block β_1- and β_2-adrenoceptors. These responses were resistant to blockade by propranolol and selective β_1- and β_2-adrenoceptor antagonists and were susceptible to blockade by bupranolol. The existence of all three subtypes of β-adrenoceptors has been confirmed independently by molecular biology techniques (Dixon *et al.*, 1986; Frielle *et al.*, 1987; Emorine *et al.*, 1989).

In summary, by classification the adrenergic receptors are two types, alpha and beta receptors which are subdivided to two subtypes α_1- and α_2-adrenoceptors and three subtypes β_1-, β_2- and β_3- (atypical) adrenoceptors. α_2-adrenoceptors are located either on pre- or post-synaptic membrane, whereas α_1- and β-adrenoceptors are known to be on post-synaptic membrane. α_1 is itself divided to α_{1a} and α_{1b}, and α_2 is divided to α_{2A}, α_{2B} and α_{2C} (fig 1).

II. Neurotransmitters and related substances

Neurotransmitters are substances synthesized and stored in nerves, and released during nerve activity from its nerve terminals. Then they diffuse across the junctional cleft to reach and bind to specific receptors on the post-synaptic membrane, which leads to change in activity of the post-synaptic cell.

Until 1970's it was believed that each neurone synthesizes and releases only one neurotransmitter, (Dale's principle). During early 1970's there was growing evidence of plurality of transmission mechanisms. In 1976, an article appeared in Neuroscience "Do some nerve cells release more than one transmitter?" (Burnstock, 1976). Nowadays, co-transmission is widely accepted and has been shown that almost all nerves release more than one neurotransmitter (Burnstock, 1986). Some examples are: co-existence and release of NA with ATP from sympathetic nerves supplying the vas deferens (Westfall *et al.*, 1978; Burnstock, 1983; Sneddon & Burnstock, 1984a), and from several blood vessels (Su, 1975,1983; Muramatsu *et al.*, 1981; Seneddon & Burnstock, 1984b; Hicks *et al.*, 1985); release of NA or Ach, or a mixture of them from a single sympathetic neuron *in vitro* (Bunge *et al.*,

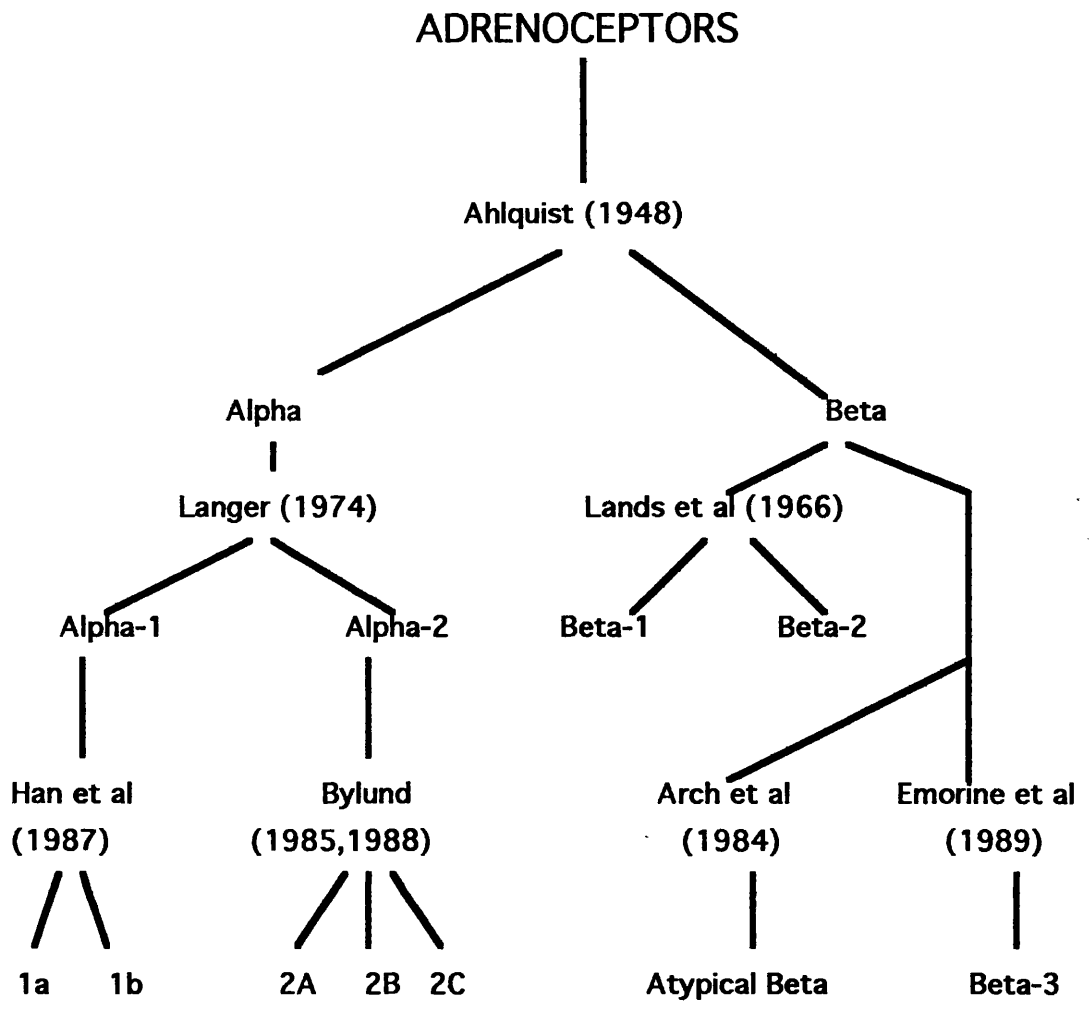


Fig 1.1. Subclassification of adrenoceptors (from J. Docherty 1991)

1978; Furshpan *et al.*, 1976). Sympathetic transmission involving NA, Ach, and a purinergic transmitter has also been demonstrated in cultured sympathetic nerves forming junctions with heart muscle cells (Potter *et al.*, 1983). A detailed account of the evidence for co-existence of Ach and NA with ATP can be found in Burnstock (1982). NA and ATP act as synergic neurotransmitters via post-junctional receptors, as well as exerting modulatory effects on each other via pre- and post-junctional mechanisms (Burnstock, 1985).

More recently, co-existence of neuropeptide Y (NPY) and several other peptides has been observed (McDonald, 1988). It displays a wide variety of functional activities depending on the site of release and its co-transmitter, particularly catecholamines. NPY has been found to be extensively distributed throughout the cardiovascular system in many species (Edvinsson *et al.*, 1983; Schon *et al.*, 1985; Allen *et al.*, 1986).

It has been known, for a relatively long time, that the predominant neurotransmitter released in sympathetically innervated mammalian tissues such as heart and arteries is NA, whereas the adrenal gland releases much more adrenaline than NA (Burnstock, 1969; Antone & Sayre, 1962).

The other catecholamine, dopamine, has been found in high levels in the pacemaker region (sinus venosus) of the frog heart, and it has been suggested that dopaminergic nerve fibres may supply this region of the heart (Angelakos *et al.*, 1965). Similarly, high levels of this sympathetic neurotransmitter has also been found in sino-atrial node of the mammalian heart (Angelakos, 1963; Angelakos *et al.*, 1965). Dopamine also produces vasodilatation of renal, mesenteric, coronary,

and intracerebral arteries, which is not antagonized by propranolol but is selectively attenuated by dopaminergic blockers, haloperidol and phenothiazines. Such specifications suggest the presence of specific dopamine receptors (Goldberg, 1974).

III. Regulation of joint blood flow

Little information is available in the literature about the physiological regulation of blood flow to the joints. This raises two questions: firstly, how much is the blood flow to the joint and secondly, how is this regulated?.

a. Measurement of joint blood flow

Synovium is the most important structure of the joint which has the richest capillary bed and is the main point of synovial fluid formation (Knight & Levick, 1983a,1984). However, as synovium is not a discreet organ, and has a multiplicity of vessels supplying it, it is difficult to measure its blood flow. So far, different indirect and direct techniques has been used to estimate the blood flow to the whole joint or its structures, although none of them is quite satisfactory.

Horvath and Hollander (1949) were the first to try to estimate joint blood flow in man indirectly by a calorimetric method, i. e. by measurement of intra-articular temperature. This technique has been criticized by Greenfield *et al.* (1951) as temperature measurements cannot be converted into blood flow values without knowledge of the temperature of the blood entering or leaving the tissue.

Bonney *et al.*(1952) applied a plethysmograph to the knee segment to measure the joint blood flow. This technique can not separate

intercompartmental blood flow, and may measure the flow of other structures around the joint as well.

Cobbold and Lewis (1956a,b,c) measured dog knee joint blood flow directly by using bubble and electromagnetic flowmeters, and showed the effect of heating, cooling, and drugs such as adrenaline and NA and Ach on joint blood flow. They concluded that heating and Ach increased blood flow, but cooling and the other two chemicals decreased joint blood flow. The results of these techniques are also complicated by the complexity of both arterial and venous anastmoses between muscles, periostium, epiphysis, and synovium (Leiw & Dick, 1981).

The clearance technique, which is a quantitative but indirect method of blood flow estimation and is based on the rate of disappearance of a rapidly-diffusing radiolabelled solute from the synovial cavity, has been used especially in man for measurement of joint blood flow by different investigators (Harris & millard, 1956; Harris *et al.*, 1958; Dick *et al.*, 1970; Wallis *et al.*, 1985). Although it is a safe and relatively reliable method of measuring blood flow in man, it still has some disadvantages such as the possibility of recirculation of radioactive substance (~10% does not leave the blood in first circulation- Dick *et al.*, 1970), and non-homogeneity of capillary distribution in joint structures which can not be differentiated by this technique.

Radiolabelled microsphere technique has been used more recently for measurement of animal joint blood flow (Christensen *et al.*, 1982; Bunger *et al.*, 1983,1984). With this method the number of microsphere counts in the tissue provides an estimation of the tissue blood flow. This

is a quantitative and accurate technique in blood flow measurement but it lacks continuous measurement, and is not applicable to humans.

The newest method used for measurement of joint blood flow, which is a non-invasive and continuous method of blood flow estimation, is laser Doppler flowmetry which has been used in both human (Gebroreck *et al.*, 1989), and animals (Ferrell & Khoshbaten, 1989; Khoshbaten & Ferrell 1990b; Ferrell & Najafipour, 1992). This technique is based on the principle that coherent light scattered by moving red blood cells experiences a frequency shift that is proportional to the number and velocity of red blood cells flowing through the tissue under measurement. The disadvantage of this method is the non-quantitative nature of this measurement.

b. Sympathetic regulation of joint blood flow

Cobbold and Lewis (1956b) were the first investigators who showed the sympathetic innervation of joint blood vessels. In a study on dog knee joint blood vessels, they observed that traction of the lumbar sympathetic chain reduced knee joint blood flow. In addition, they found that section of the articular nerve supply resulted in increased blood flow, suggesting that these vessels possess sympathetic vasoconstrictor "tone". In other experiments Cobbold & Lewis (1956c) found that close intra-arterial injection of noradrenaline produced vasoconstriction, indicating the presence of α-adrenoceptors.

Using the ^{133}Xe clearance technique and both α- and β-adrenergic agonist and antagonists, Dick *et al* (1971) obtained evidence for contribution of sympathetic nervous system in regulation of blood flow in the normal and diseased human articular vascular beds. In a

study on the knee joint of the cat, electrical stimulation of the posterior articular nerve caused an initial vasoconstriction of the blood vessels followed by a longer lasting vasodilatation (Ferrell & Cant, 1987). This finding was confirmed in another study by Khoshbaten & Ferrell (1990b). In a recent study using an isolated perfused rabbit knee joint preparation electrical stimulation of the joint capsule produced vasoconstriction which was substantially reduced by adding phenoxybenzamine to the perfusate, or pretreatment of the animal with reserpine which depletes the nerve endings of NA (Ferrell & Khoshbaten, 1990a). In a very recent study on posterior capsule of the rabbit knee joint *in vivo*, using laser Doppler flowmetry, the sympathetic innervation of joint blood vessels was investigated. The infusion of α-adrenoceptor antagonist phenoxybenzamine and α_2-adrenoceptor antagonist rauwolscine both blocked the vasoconstrictor responses to electrical stimulation of posterior articular nerve and converted it to vasodilatation (Najafipour & Ferrell, 1993).

c. Local regulation of joint blood flow

Apart from central regulation of joint blood flow which is mostly under the control of sympathetic nervous system, and is probably in the favour of the regulation of systemic blood pressure, local regulation of joint blood flow seems more important from a nutritional point of view. Many factors produced locally by the joint tissues or simply the end products of tissue metabolism and joint activities affect the rate of activity in vascular smooth muscle to change the vessel calibre and as a result the blood passing through it.

Bonney *et al* (1952), using a plethysmographic method in man, reported an increase in blood flow of the knee joint by increasing the temperature of the limb segment under study. Cobbold and Lewis (1956a), by using a bubble flowmeter in the dog, showed the effect of heating and cooling on the knee joint blood flow. They concluded that increasing temperature to 60°C increased the blood flow by 15-57%, and applying ice to the joint decreased the blood flow by about 60%. Lindstrom (1963), using vital microscopy, found that, at intra-articular temperatures in the range of 36 to 37°C, the capillary blood flow was discontinuous in the microcirculation of the rabbits' knee joint. When the articular temperature was increased to 40°C, generalized vasodilatation occured. Further increment in temperature resulted in cessation of blood flow in the capillary bed, with corpuscles flowing rapidly through the AV anastomoses. With local hypothermia, a reduction in arteriolar and venous calibre was noted. As intra-articular temperature fell to the range of 20 to 22°C, discontinuous corpuscular flow became prominant, until finally there was complete cessation of flow. Externally applied heat has also been shown to increase the clearance rate of radioactive xenon in animal and human studies (St Onge *et al.*, 1971), presumably reflecting vasodilatation of the synovial blood vessels.

Movements of the joint have been shown to have considerable effects on joint blood flow. Blood flow through the knee segment, as measured by venous occlusion plethysmography, was shown to be increased upon resisted rhythmic contraction of the calf and thigh muscles (Lindstrom, 1963). He inspected synovial vasodilatation in the opened joint after exercise. He also noted that after two weeks of

immobilization of rabbit knee joint, the synovial tissue was distinctly paler than it was on the control side. A longer period of immobilization resulted in generalized reduction in both calibre and number of the capillary plexuses in the inner most synovial tissue layer. More recently, by the microsphere technique, blood flow to the synovium has been reported to be increased by 3 times in the knee and 7 times in the wrist by exercise (Simkin *et al.*, 1986). In contrast, however, Xe clearance has been reported to be reduced by exercise (St Onge *et al*, 1971). As xenon clearance may largely reflect the flow through articular adipose tissue (Phelps *et al.*, 1972), it seems possible that differential changes occur in blood flow to areolar synovium and articular fat (Levick, 1987).

Intra-articular pressure is another important factor in local regulation of joint blood flow (Knight & Levick, 1982). This factor affects trans-synovial flow and hence the volume of fluid in the cavity; and supra-normal pressures affect the hydraulic conductance of synovium. In 1929 Muller discovered that synovial fluid pressure is several mmHg sub-atmospheric (negative) in human and canine joints at natural relaxed joint angles (Muller, 1929), a discovery which fits with the impression that the synovial cavity is a "collapsed" space. The ''negativity" of pressure in synovial cavity of the normal joints has been confirmed in recent studies on human knee (-1.6 to -5mmHg, Spencer *et al.*, 1984; Baxendale & Ferrell, 1985), cat knee (Wood & Ferrell, 1985), rabbit limb joints (Wigren *et al.*, 1975; McDonald *et al.*, 1986), and dog limb joints (Bunger *et al.*, 1983,1984; Nade & Newbould, 1983), except wrist (+1.3 mmHg, Simkin & Benedict, 1985). The negativity has been attributed to trans-synovial efflux of fluid upon flexion and the stimulation of lymph flow by movement (Levick, 1983).

Increased synovial fluid pressure and volume resulted in impairment of synovial blood flow above a critical effusion pressure (Jayson & Dixon, 1970; Lunch *et al.*, 1983). It has been demonstrated in dog (Phelps *et al.*, 1972) that even relatively small changes in intra-articular pressure (9 to 17mmHg) can cause an appreciable decrease in ^{133}Xe clearance. This has been confirmed by the studies of Knight and Levick (1982) on the relationship between volume and pressure in rabbit knee, which has revealed that the sub-atmospheric part of the volume-pressure curve is very steep, i.e. a small increase in volume causes a high increase in pressure (Fig 2).

The end products of metabolism, such as lactate, CO_2, H^+, accumulation of K^+, and also hypoxia, have been shown to be important local regulators of blood flow and give rise to vasodilatation. Probably one reason for exercise-induced vasodilatation is the higher rate of production of these substances in tissues around the synovial cavity. The importance of this type of blood flow regulation in the normal joint is not clear, but in situations which impair joint blood flow and thus the wash out of these substances, their role is probably more important.

The production of some local regulatory factors by joint tissues or joint blood vessels *per se*, may play an important role in regulation of joint blood flow. It has been shown that many blood vessels in different species have the capacity to synthesize prostaglandins from arachidonic acid (Tuvemo & Wide, 1973; Aiken 1974; Terragno *et al.*, 1975) and these agents change the vascular resistance (Blumberg *et al.*, 1977) and modulate the adrenergic mediated effects on blood vessels *in vitro* (Smith & McGrath, 1991). Dick *et al.* (1976), investigated the relative effects of exogenous prostaglandins (PGs) on the canine synovial

* (Berne & Levy, 1981).

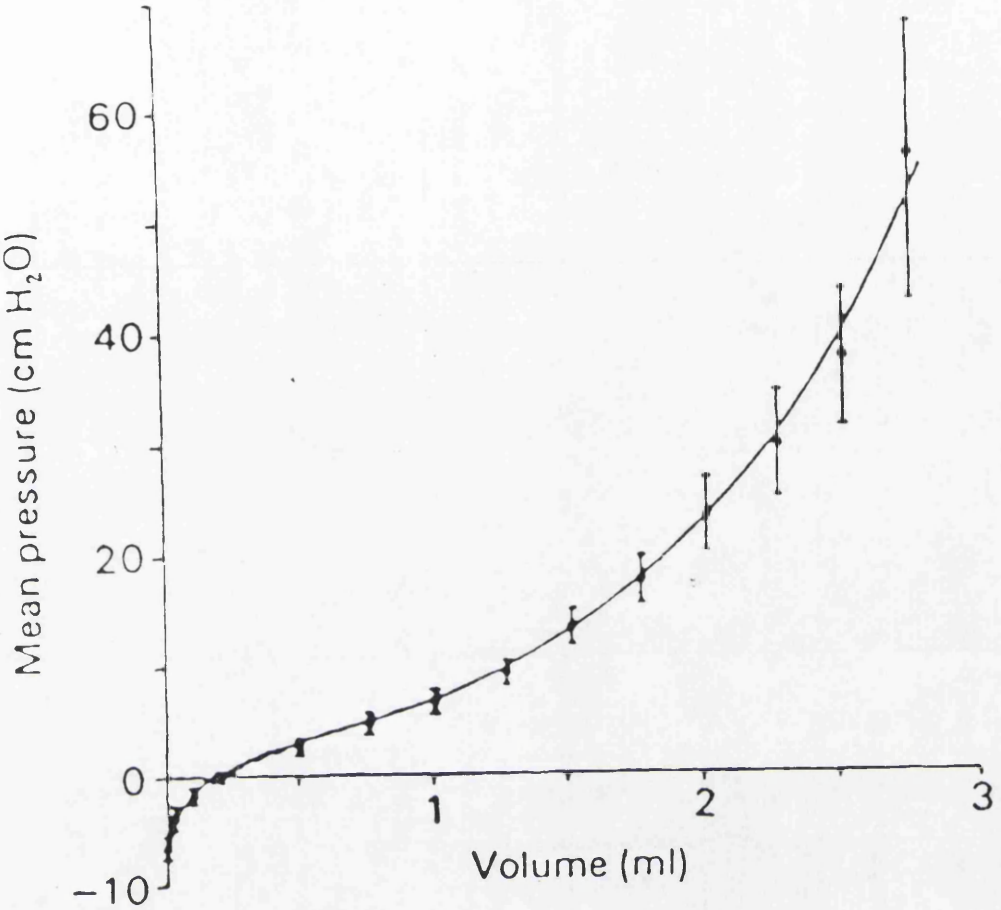


Fig. 1.2. Relation between mean pressure and injected volume of non-absorbable fluid in 10 rabbit knees. Note the steep physiological part of the curve at sub-atmospheric pressures. (From Knight and Levick, 1982)

microcirculation, and the PGE series were found to be the most potent vasodilators and $PGF_{1\alpha}$ had vasoconstricting effect. PGE_1, has also been shown to increase synovial vascular permeability in dog (Grennan *et al.*, 1977). Although it has been shown that the synovial fluid of the inflamed joints contain a high level of prostaglandins (Blackham *et al.*, 1974; O'Byrne *et al.*, 1990), the role of these agents in regulation of blood flow in normal and inflamed joints is not clear.

In 1980, Furchgott and Zawadzki demonstrated that the vascular relaxation induced by acetylcholine was dependent on the presence of endothelium and provided evidence that this effect was mediated by a labile humoral factor, later known as endothelium derived relaxing factor, EDRF (Furchgott & Zawadzki, 1980). Release of this factor was observed under basal conditions as well as after stimulation with ACh (Griffith *et al.*, 1984; Martin *et al.*, 1985), substance P and bradykinin (Furchgott, 1983). Nitric oxide (NO), formed by endothelial cells lining blood vessels from the amino acid L-arginine, accounts for the biological actions of EDRF (Palmer *et al.*, 1987, 1988). Production of NO by the vascular endothelium has been recently discovered to play a very important role in the physiological regulation of blood flow to different organs of the body (see Moncada *et al.*, 1991) and as a modulator of the action of the sympathetic nerves and many other vasodilator or vasoconstrictor substances (Toda & Okamura, 1990; Whittle *et al.*, 1989; Gardiner *et al.*, 1991). Whether NO is produced by joint blood vessels, or its probable modulatory effect on sympathetic regulation of articular blood flow in the normal and inflamed joints is not known.

IV. Oxygen in synovial fluids

The survival of avascular joint cartilage is dependent on the presence of oxygen in synovial fluid which originates, by diffusion of oxygen, from the capillaries of the synovium. The partial pressure of oxygen in synovial fluid (P_SO_2) from diseased human joints has been measured, by aspiration of an effusion, and has been found to have very low values (Falchuk, Goetzl & Kulka 1970; Lund-Olesen, 1970; Treuhaft & McCarty, 1971; Lund-Olesen, 1970; Richman, Su & Ho, 1981), however, the P_SO_2 of the normal synovial fluid has not been reported (Levick, 1987). Although oxygen in synovial fluid originates from synovial blood flow, the relationship between these two has not been investigated.

a. Measurement of synovial PO_2

The study of synovial fluid respiratory gases began when easily reproducible measurements of pH, PO_2, PCO_2, and HCO_3^- by blood gas analyzers became available. This machine can measure the PO_2 following injection of an aspirated body fluid. As the volume of synovial fluid in a normal joint is quite small, and the intra-articular pressure is negative (Muller, 1929), it has been not possible to aspirate enough fluid from a normal joint. Any attempt to increase the volume or pressure of the normal synovial cavity before aspiration would contaminate the synovial fluid with atmospheric oxygen and make true PO_2 measurement impossible. Therefore, the normal synovial PO_2 seems has not been reported (Levick, 1987). On the other hand, as the volume and pressure of the synovial fluid of the inflamed joint is increased due to extra effusion of plasma into the joint cavity, many

reports are available in the literature about the measurement of synovial PO_2 of diseased joints.

Lund-Olesen (1970) measured P_SO_2 of 103 samples of synovial fluid from diseased human knee joints. He found the lowest P_SO_2 values in the synovial fluid of rheumatoid arthritic group of patients, in some cases as low as zero, with the mean value of 26.53 mmHg. P_SO_2 was inversely correlated with PCO_2 and directly correlated with pH of the synovial fluids.

Goetzl *et al.*, (1971), investigated the O_2 consumption of normal and arthritic human knee joints, and found that oxygen consumption in resting rheumatoid knees is several fold greater than that of controls, and is accompanied by increase in blood flow to the inflamed joint. They proposed the existence of a "circulatory-metabolic imbalance" in diseased joints as the main reason for intra-articular hypoxia in these patients.

Treuhaft & McCarty (1971), examined the synovial fluid pH, lactate, oxygen and carbon dioxide partial pressure in various joint diseases. They found large variations of PO_2 between different diseases and among individuals of the same diagnosis.

In 1981, Richman, Su & Ho, aspirated the synovial fluid from the knee joints of 22 patients with different joint diseases, mostly with diagnoses of rheumatoid arthritis and osteoarthritis. They measured the volume, PO_2, and other metabolic correlates of the synovial fluids and noted an overall reciprocal relationship between PO_2 and volume of the aspirated fluids. The PO_2 ranged from 0-78 mmHg and aspirated volume from 5-100 ml.

CHAPTER TWO

Materials and methods

A: Materials

I. Instruments

1. Dissecting microscope M650 Wild Heerbrugg (Switzerland)
2. Dissecting table, Palmer (England)
3. Ventilator pump (Palmer (England)
4. Pressure transducer, Elcomatic EM751 (Scotland)
5. Clock, PYE (England)
6. Balance, Metler AE50 European Instrument, Oxford
7. Water bath, Grant Instrument LTD (England)
8. Slow infusion Pump, SRI (United Kingdom)
9. Infusion pump, Watson-Marlow (England)
10. Cordless Cautery, Warecrest. C28. (England)
11. Laser Doppler flowmeter - MBF3, Moor Instruments (England)
12. Thermalert model TH-6D, Harvard (U.S.A.)
13. Neurolog system; Pressure amplifier, AC-DC amplifier, Delay-Width, Spike trigger, Filters, Pulse buffer, Digital-Width, Pulse Generator, (England)
14. Multi-trace polygraph, Lectromed (England)
15. Rate meter, Type2750, Devices Sales LTD (England)
16. Oscilloscope 5103N (England)
17. AC stimulator, Harvard (U.S.A.)
18. DC stimulator Type2533, Devices Sales LTD (England)
19. Oxygen electrode (Diamond General, U.S.A.) and oxygen meter (Strathkelvin Instruments, Scotland)

20. Microdrive system, Narashigi (Japan)

21. Video tape recorder, Panasonic (Japan)

22. Shaker, Heidolph (W. Germany)

23. Nescofilm, Bando-chemical IND. LTD (Japan)

24. Polythene cannulae in sizes 2-5FG, Portex LTD (England)

25. Syringes sizes 1-20ml, Plastipack (Ireland)

26. Surgical instruments and operating table accessories (from different sources)

27. BGM blood gas analyzer, Allied Instrumentation Laboratory (U.K.)

II. Drugs

1. Pentobarbitone sodium, Sagatal; May & Baker LTD, Dagenham, (England)

2. Adrenaline hydrochloride (Evans)

3. L-Phenylephrine hydrochloride (Sigma)

4. Clonidine hydrochloride (Sigma)

5. Phenoxybenzamine hydrochloride (Smith Kline & French)

6. Prazosin hydrochloride (Sigma)

7. UK-14304 bitartarate (5-bromo-6[2-imidazoline-2-Ylamino]-quinoxaline) (Pfizer)

8. Rauwolscine hydrochloride (Roth)

9. α,β methylene adenosine 5'-triphosphate, lithium salt; (Sigma)

10. YM-12617 ((-)-(R)-5-[2-[[2-(o-ethoxyphenoxy)ethyl] amino] propyl]-2-methoxybenzene-suphonamide hydrochloride; Yamanouchi Pharmaceutical, (Japan).

11. (±) Isoprenaline hydrochloride (Isoproterenol, Sigma)

12. Salbutamol (Sigma)

13. Terbutaline sulphate (Bricanyl, Astra)

14. Dobutamine hydrochloride, Dobutrex, Eli Lilly (England)

15. DL-Propranolol hydrochloride (Sigma)

16. Atenolol (Sigma)

17. ICI118551 hydrochloride, Cambridge research biochemicals, (England)

18. Substance P antagonist, D-pro^4 D-Trp^{7,9,10} SP$_{4-11}$,Peninsula laboratories Inc., (U.S.A.)

19. Carrageenan (Sigma)

20. Nω-nitro-L-arginine methyl ester hydrochloride, L-NAME; (Sigma)

21. L-arginine (Sigma).

22. Indomethacin (Sigma)

23. Prostaglandin E_2 (PGE_2) (Sigma)

24. Diazepam BP with benzyl alcohol and sodium benzoate, Valium, Roche, (England)

25. Hypnorm (Janssen Pharmaceutical Ltd)

26. ^{57}Cobalt radiolabelled microsphere (Dupont)

27. ^{113}Tin radiolabelled microsphere (Dupont)

28. ^{153}Gadolinium radiolabelled microsphere (Dupont)

29. ^{46}Scandium radiolabelled microsphere (Dupont)

30. Normal saline

31. Heparinized saline (5 units/ml)

32. Paraffin liquid, BDH, (England)

B: Methods

I. Surgical Anatomy of the knee joint

It is conventionally supposed that each articulation is enclosed in a capsule formed by a sheet of strong connective tissue extending from one bone to other and completely enclosing a cavity containing the articular surfaces. Each of these surfaces is covered by a very thin layer of cartilage and the cavity contains a small amount of lubricating liquid, the synovial fluid, secreted from the lining of the capsule, the synovium (fig 2.1).

The articulation in the knee forms a hinge-joint, though movement is not rigidly confined to one plane. The structure is more complex than that of other joints (e.g. hip joint). The capsule is attached to the femur around the edges of the condyles and to the patellar surface and similarly to the tibia round the edges of its plateau. Outside the capsule, stout medial and lateral bands, the tibial and fibular collateral ligaments, hold the two bones together. The capsule is also attached to four small bones which have articular sufaces taking part in the formation of the joint, namely the patella and three other sesamoid bones embedded in the two heads of the gastrocnemius and in the popliteus muscles respectively. The patellar ligament forms part of the capsule and the joint is transversed by the tendons of origin of the popliteus and of the extensor digitorus longus.

Within the joint the femur and the tibia are held together by anterior and posterior cruciate ligaments, both attached to the femur

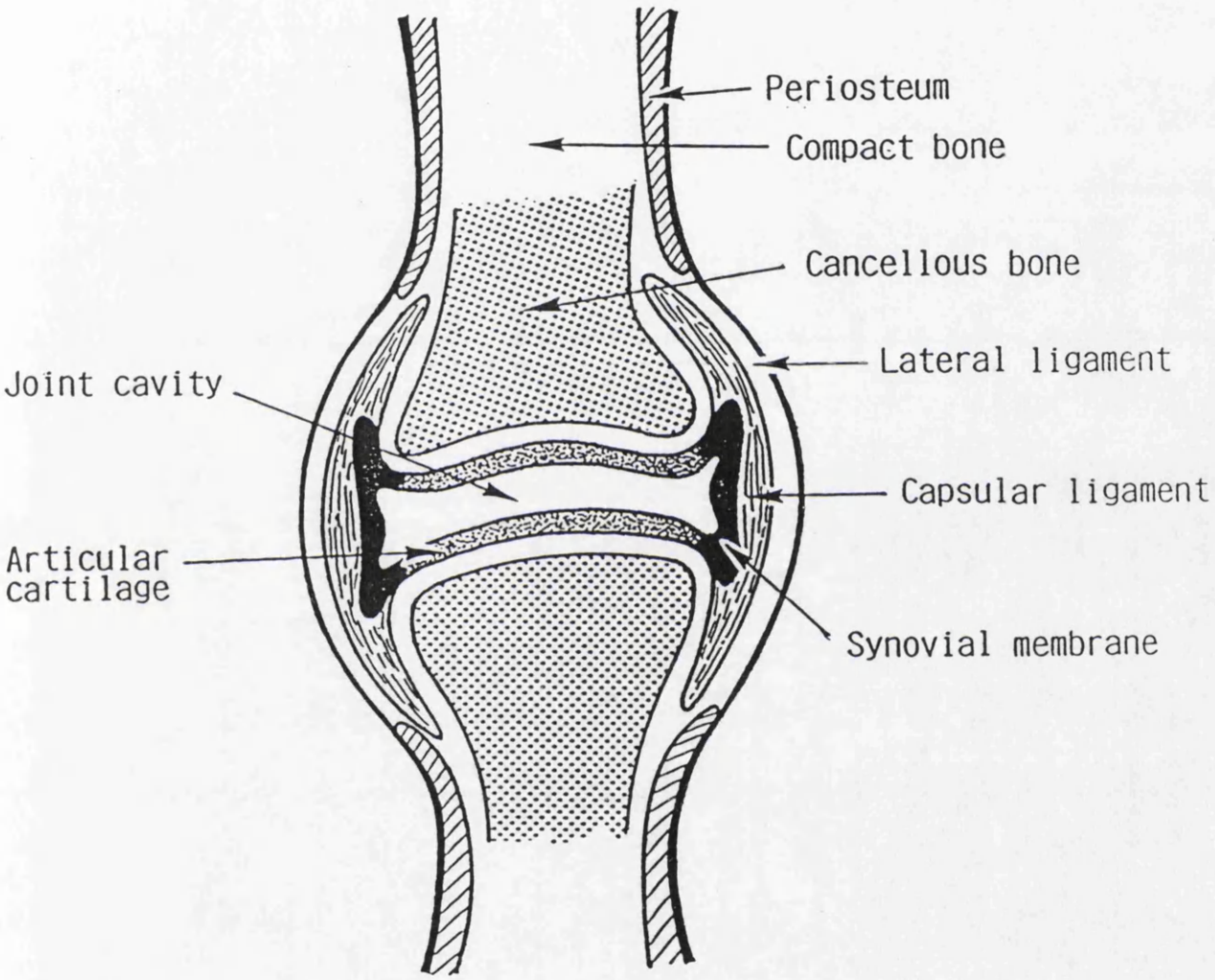


Fig. 2.1. The structural view of a diarthroidal joint, showing synovial cavity and membrane.

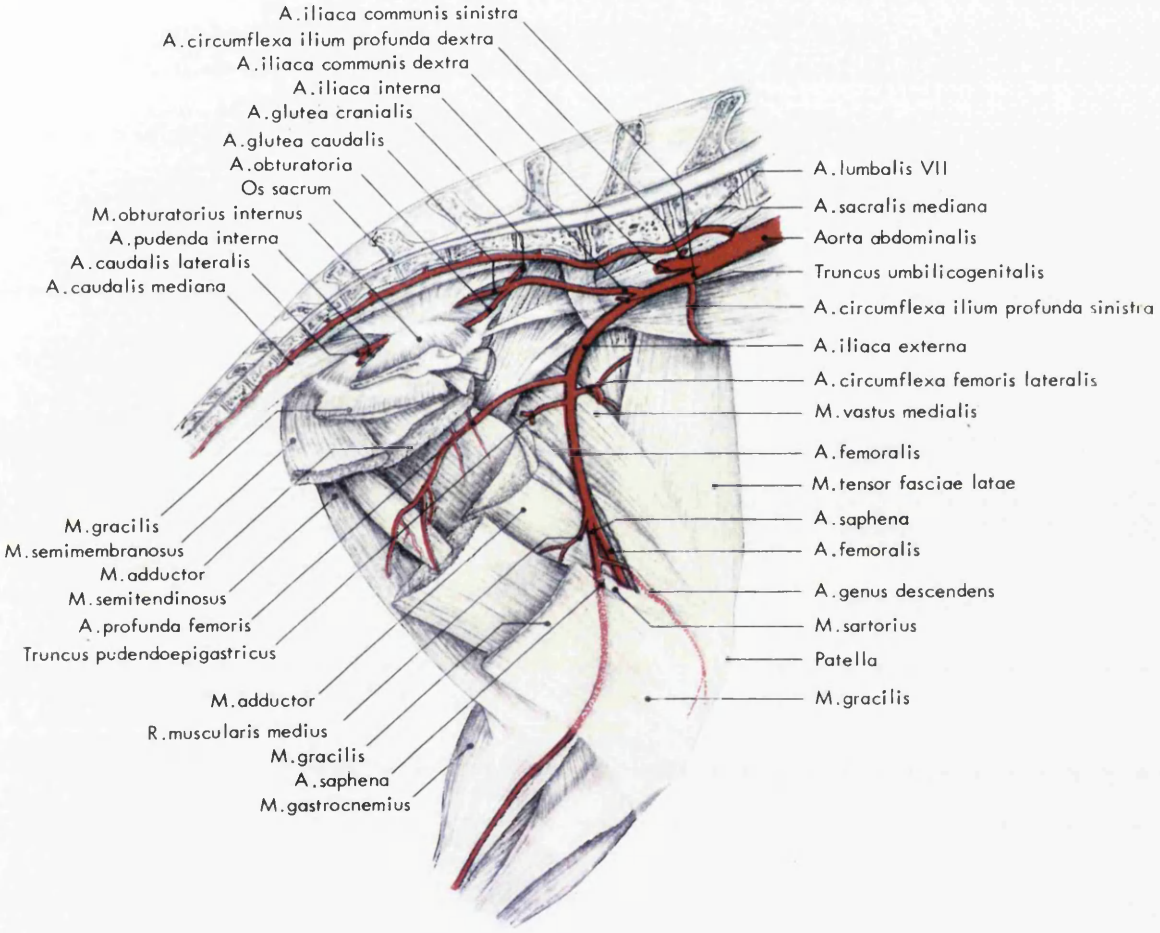
and in the intercondyloid fossa. Curved cushions of cartilage, the medial and lateral menisci, are inserted between the opposed condyles and are held in place by additional ligaments. The medial meniscus is attached to the tibia only but the lateral meniscus is connected to both tibia and femur, the ligament to the femur lying behind the posterior cruciate ligament.

The capsules at the knee joint should be exposed by removing the muscular attachments about it as thoroughly as is practicable. the arteries, veins and nerves of the knee joint region are as following:

a. Arteries

The femoral artery transverses the medial surface of the thigh, beginning at the dorsal side of the inguinal ligament, where it continues from the external iliac artery. (fig 2.2). Immediately distal to the inguinal ligament, it gives off posteriorly the deep artery of the thigh (artery profunda femoris). The latter passes to the dorsal side of the pectineus and adductor brevis muscles and distributed to the posterior proximal portion of the limb, chiefly to the adductors longus and magnus (fig 2.3). A second branch, the lateral circumference artery (a. circumflexa femoris lateralis), is given off from the anterolateral wall. It passes between the second head of the rectus femoris and vastus lateralis, on one hand, and the two portions of the vastus intermedius, on the other hand. It supplies various parts of the quadriceps femoris group. A third branch of femoral, the superficial epigastric artery (a. epigastrica superficialis), given off medially, and passing to the abdominal wall, has been divided. At the beginning of the distal third of the thigh, a small branch, the descending genus artery (a. genu

Fig. 2.2. The arteries of the pelvis and of the thigh. The medial aspect of rabbit hind limb (Barone et al 1973, atlas of rabbit anatomy).

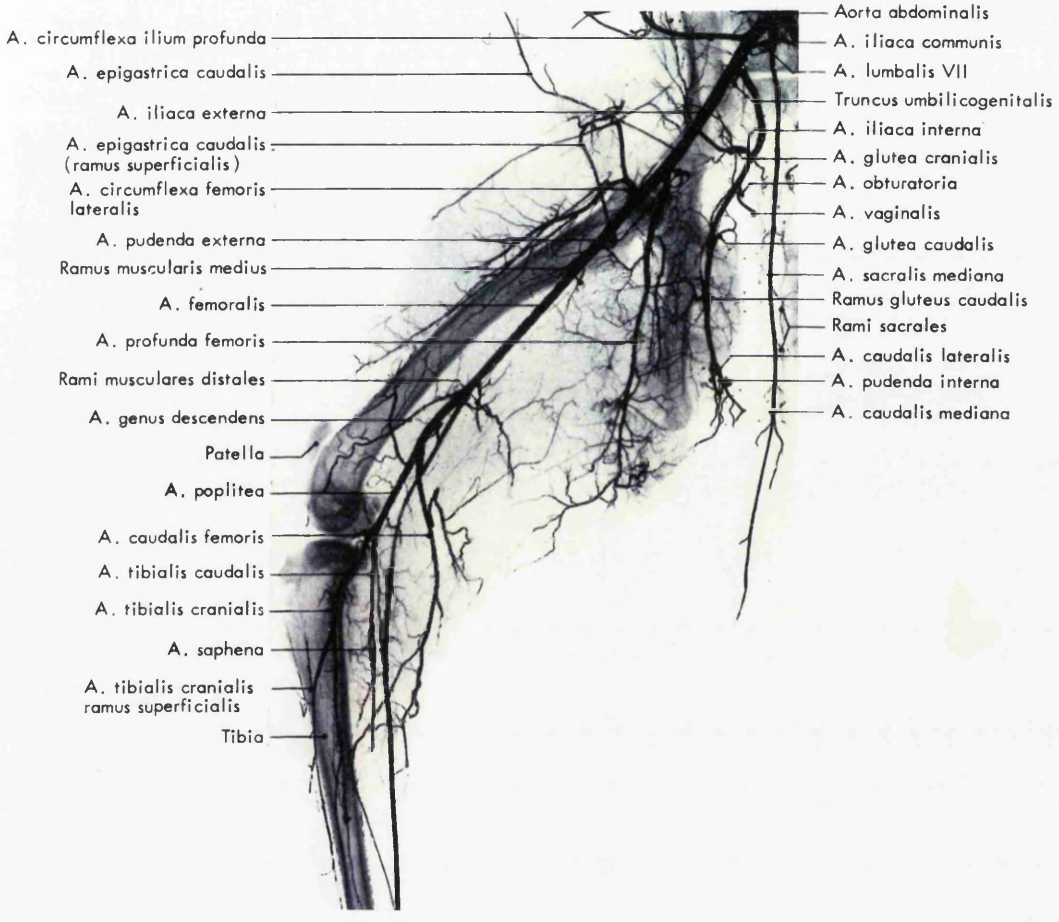


ARTERIAE PELVIS ET FEMORIS (FACIES MEDIALIS).

Artères du bassin et de la cuisse (face médiale).

Arteries of the pelvis and of the thigh (medial aspect).

Fig. 2.3. Radiograph of arteries of pelvis and hind limb of rabbit (Barone et al 1973, atlas of rabbit anatomy).


A. circumflexa ilium profunda
A. epigastrica caudalis
A. iliaca externa
A. epigastrica caudalis (ramus superficialis)
A. circumflexa femoris lateralis
A. pudenda externa
Ramus muscularis medius
A. femoralis
A. profunda femoris
Rami musculares distales
A. genus descendens
Patella
A. poplitea
A. caudalis femoris
A. tibialis caudalis
A. tibialis cranialis
A. saphena
A. tibialis cranialis ramus superficialis
Tibia
Aorta abdominalis
A. iliaca communis
A. lumbalis VII
Truncus umbilicogenitalis
A. iliaca interna
A. glutea cranialis
A. obturatoria
A. vaginalis
A. glutea caudalis
A. sacralis mediana
Ramus gluteus caudalis
Rami sacrales
A. caudalis lateralis
A. pudenda interna
A. caudalis mediana

descendens), passes over the medial condyl of the femur to the anterior part of the knee joint (fig 2.3). At about the point of this vessel a large branch, the great saphenous artery (a. saphena magna), arises from the posterior wall and passes across the medial surface of the distal end of the adductor magnus, and through the tendon of gracilis, to the medial surface of the leg. Another large branch from the femoral artery, the caudal femoral artery (a. caudalis femoris) originates at popliteal fossal region and runs superficially between the two bellies of gastrocnemius muscle and gives some branches to supply gastrocnemius muscle and popliteal fat pad before it moves towards the lower parts of the leg. The femoral artery passes between the adductors longus and magnus, continuing as the popliteal artery (a. poplitea).

The popliteal artery, the continuation of the femoral, passes between the medial head of the gastrocnemius on the one hand and the lateral head of the plantaris on the other, reaching the anterior surface of the popliteus muscle, and afterwards the anterior surfaces of the tibia and fibula by passing between their proximal ends. It distributes branches to the muscles and joint about the knee-joint, including a branch to the distal portion of the vastus lateralis, which is given off near the same point as the small saphenous artery. It then continues as the anterior tibial artery (a. tibialis cranialis). The other branch which runs posteriorly is called as posterior tibial artery (a. tibialis caudalis).

As has been described by Liew and Dick (1981), the sources of the arterial blood supply to the knee joint are multiple. The vessels from the popliteal artery and other vessels from the bones and muscles adjacent to joint anastomose freely to form a complex network around

knee joint between the capsule and the synovium at their attachment to the epiphysial line.

b. Veins

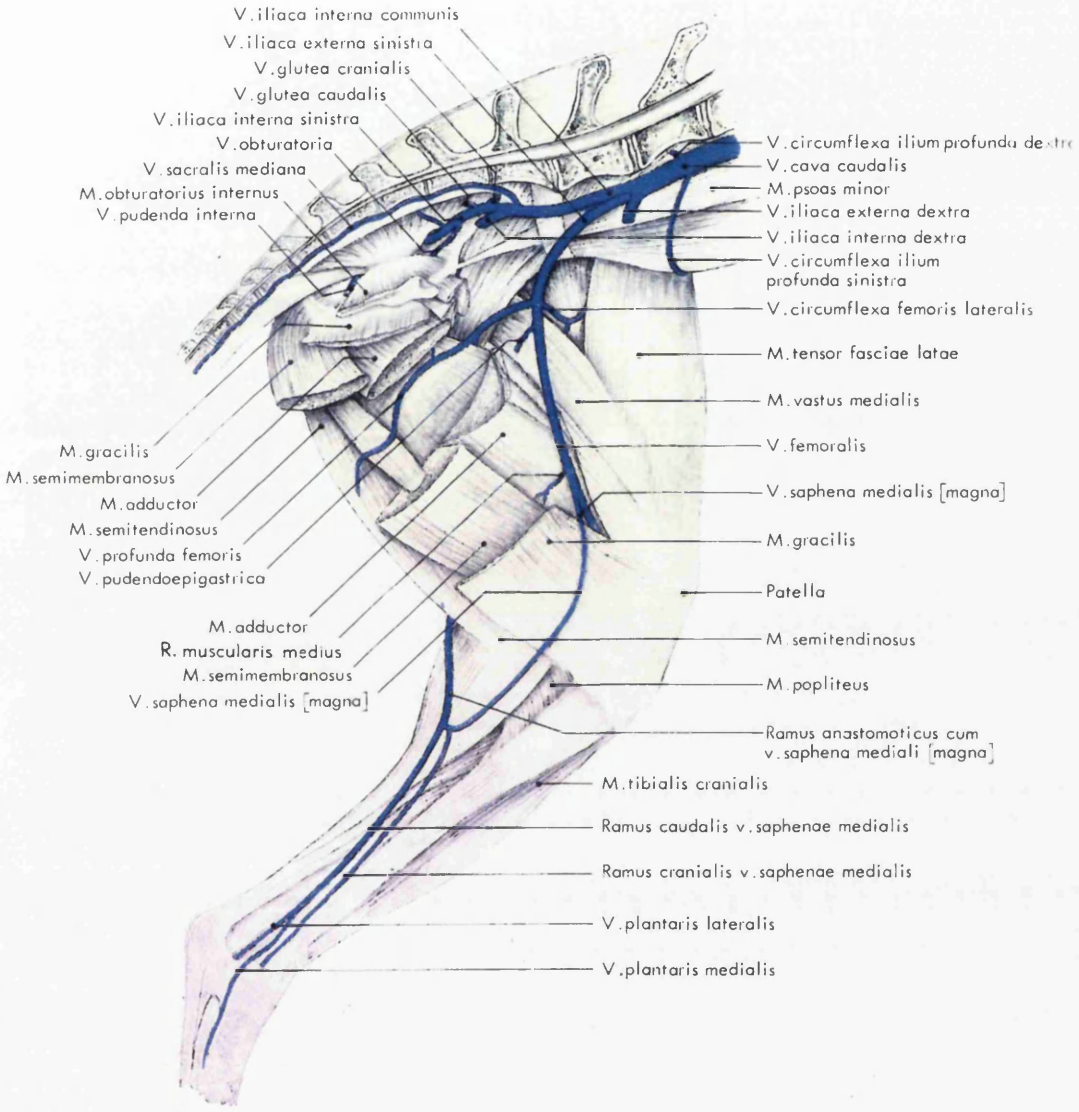
The femoral vein transverses the medial surface of the thigh in company with the femoral artery. It begins at the proximal end of the lower third of the thigh as continuation of the popliteal vein, which accompanies the corresponding artery. Its tributaries comprise the great saphenous, superficial epigastric, lateral circumflex, and the deep vein of the thigh. (fig 2.4).

The great saphenous vein, a large tributary of the femoral, accompanies the corresponding artery and great saphenous nerve. It is a continuation of the posterior tibial vein from the plantar surface of the foot. The popliteal vein, the root of the femoral, accompanies the corresponding artery in popliteal fossa. It receives the small saphenous vein from the posterior margin of the lateral head of the gastrocnemius, where this vein has been formed by tributaries accompanying the distal branches of the small saphenous artery.

c. Nerves

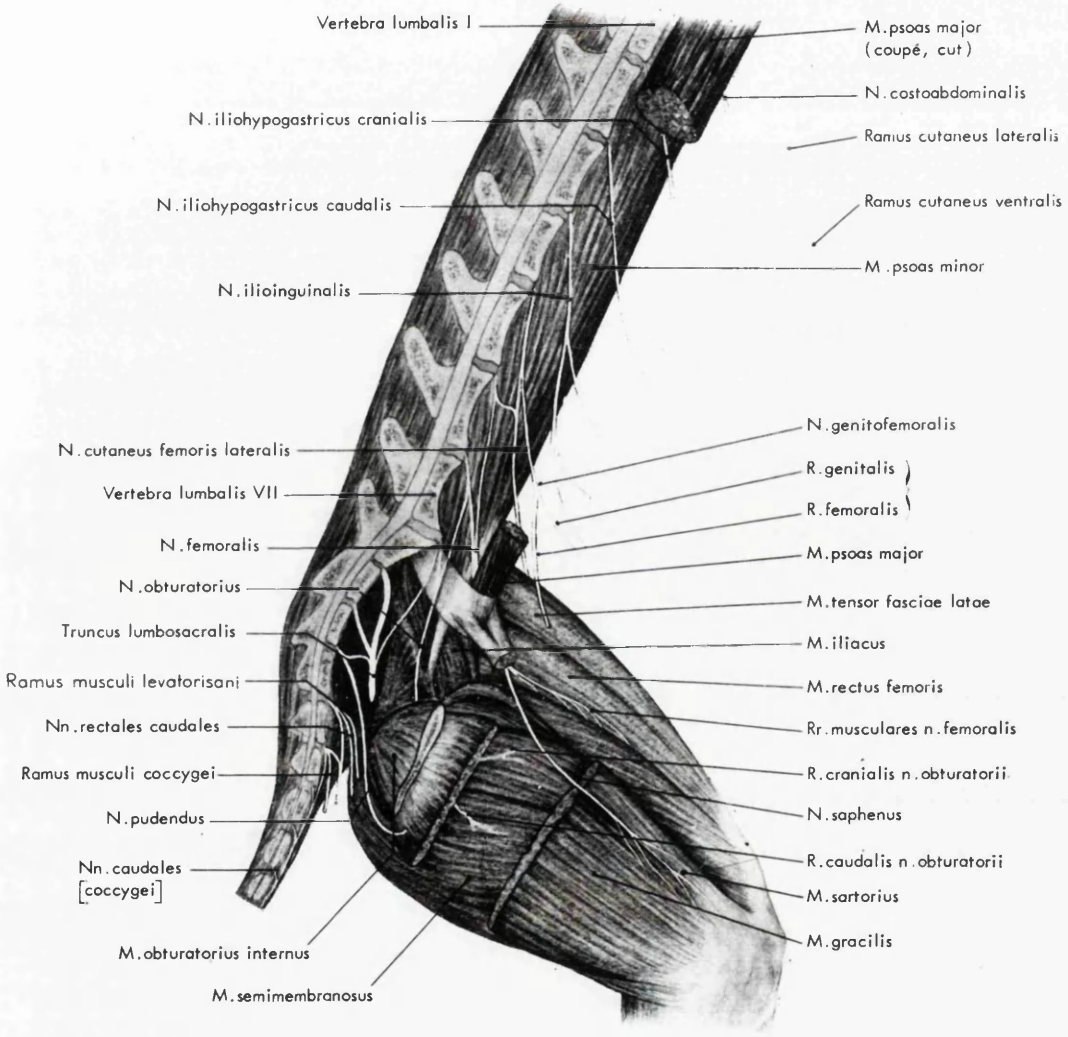
The femoral nerve (n. femoris) arises from the lumbo-sacral plexus, chiefly from the fifth and sixth lumbar nerves (fig 2.5). Its position between the psoas major and iliacus muscles has been shown in figure 2.5. Immediately beyond the inguinal ligament it divides into two portions, one of which is distributed to the muscles of the anterior side of the thigh, while the other, the great saphenous nerve (n. saphenous major), passes to the medial surface of the thigh and leg in company

Fig. 2.4. The veins of the pelvis and the thigh. The medial aspect of rabbit hind limb (Barone et al 1973, atlas of rabbit anatomy).



VENAE PELVIS ET MEMBRI PELVINI (FACIES MEDIALIS).
Veines du bassin et du membre pelvien (face médiale).
Veins of the pelvis and of the hindlimb (medial aspect).

Fig.2.5. The femoral nerve and its origin and anatomical position. The medial aspect of rabbit hind limb (Barone et al 1973, atlas of rabbit anatomy).



PLEXUS LUMBOSACRALIS.
Plexus lombo-sacré.
Lombosacral plexus.

first with femoral artery and afterwards with the great saphenous artery. The sciatic nerve (n. ischiadicus), formed chiefly from the seventh lumbar and first sacral nerves, appears laterally in the greater sciatic notch. It passes backward beneath the pisiformis muscle, and then turns and extends distal through the thigh, where it lies on the lateral surfaces of the adductors magnus and longus (fig 2.6). It distributes branches to the posterior musculature of the thigh. In the proximal portion of the thigh it divide into two chief branches, which are closely associated as far as the knee. The anterior branch is the peroneal nerve (n. peronaeus), the posterior branch, the tibial nerve (n. tibialis). The lesser saphenous nerve is a branch given off from the tibial above the knee joint. the posterior articular nerve (PAN) which supplies the dorsal aspect of the knee joint branches off from the tibial nerve just below the knee joint.

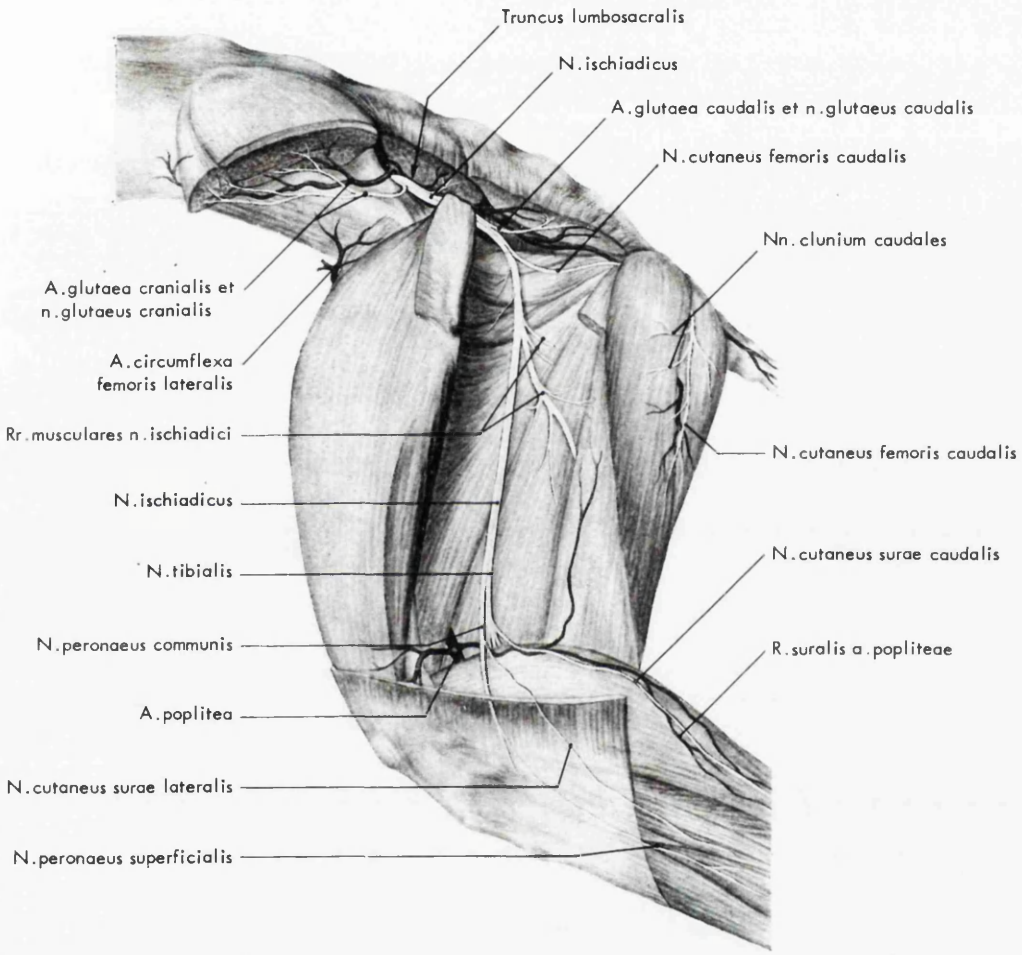
II. Animal preparation

175 adult albino New Zealand rabbits of either sex weighing between 1.9-4.5 Kg were used.

a. Anaesthesia

All animals were anaesthetized initially by intra-muscular injection of hypnorm (0.15 ml/Kg) and intra-peritoneal injection of diazepam (1.5 mg/Kg). Thereafter anaesthesia was maintained using 1% halothane in a mixture of O_2/N_2O (20/80), delivered through a face mask or later on through the tracheal cannula, during the surgical procedures. No surgical procedure was performed until reflex withdrawal of the limb on pinching the paw had been abolished. In most groups of the animals on completion of surgery, the gaseous anaesthetic

Fig.2.6. The sciatic nerve (N. ischiadicus) and its branches. The lateral aspect of rabbit hind limb (Barone et al 1973, atlas of rabbit anatomy).



NERVUS ISCHIADICUS.
Nerf sciatique.
Sciatic nerve.

was discontinued and a slow continuous infusion of pentobarbitone (0.25-0.5mg/min depending on the weight of the animal) administered throughout the remainder of the experiment via a cannula inserted into the left jugular vein. In some other groups the gaseous anaesthetic regime was continued until the end of the experiment.

b. Tracheotomy

The fur on the neck was shaved with fur clippers and a skin incision made from the hyoid bone to the suprasternal notch. The skin was retracted and the pretracheal muscles were separated by blunt dissection to expose the trachea. Any overlying connective tissue were cleared by blunt dissection, and a thread was looped around the trachea. The trachea was then lifted by this thread and an incision was made between two trachea rings using cautery to decrease the risk of bleeding. A glass trachea cannula of appropriate size was then inserted into the trachea and tied in position with the thread. Any bleeding, during the surgical procedure, was prevented by coagulating the bleeding point by electrical cautery. All animals were breathing spontaneously throughout the experiments through the tracheal cannula but in some instances it was necessary to use the respiratory pump temporarily.

c. Carotid artery and jugular vein cannulation

In all experiments, either left or both common carotid arteries were cannulated to permit monitoring of arterial blood pressure, or withdrawal of arterial blood samples and/or insertion of a cannula into the left ventricle. The carotid was first freed from surrounding connective tissues and adjacent vagus nerve, then its distal portion was ligated with a thread. Another thread was used to make a loose tie

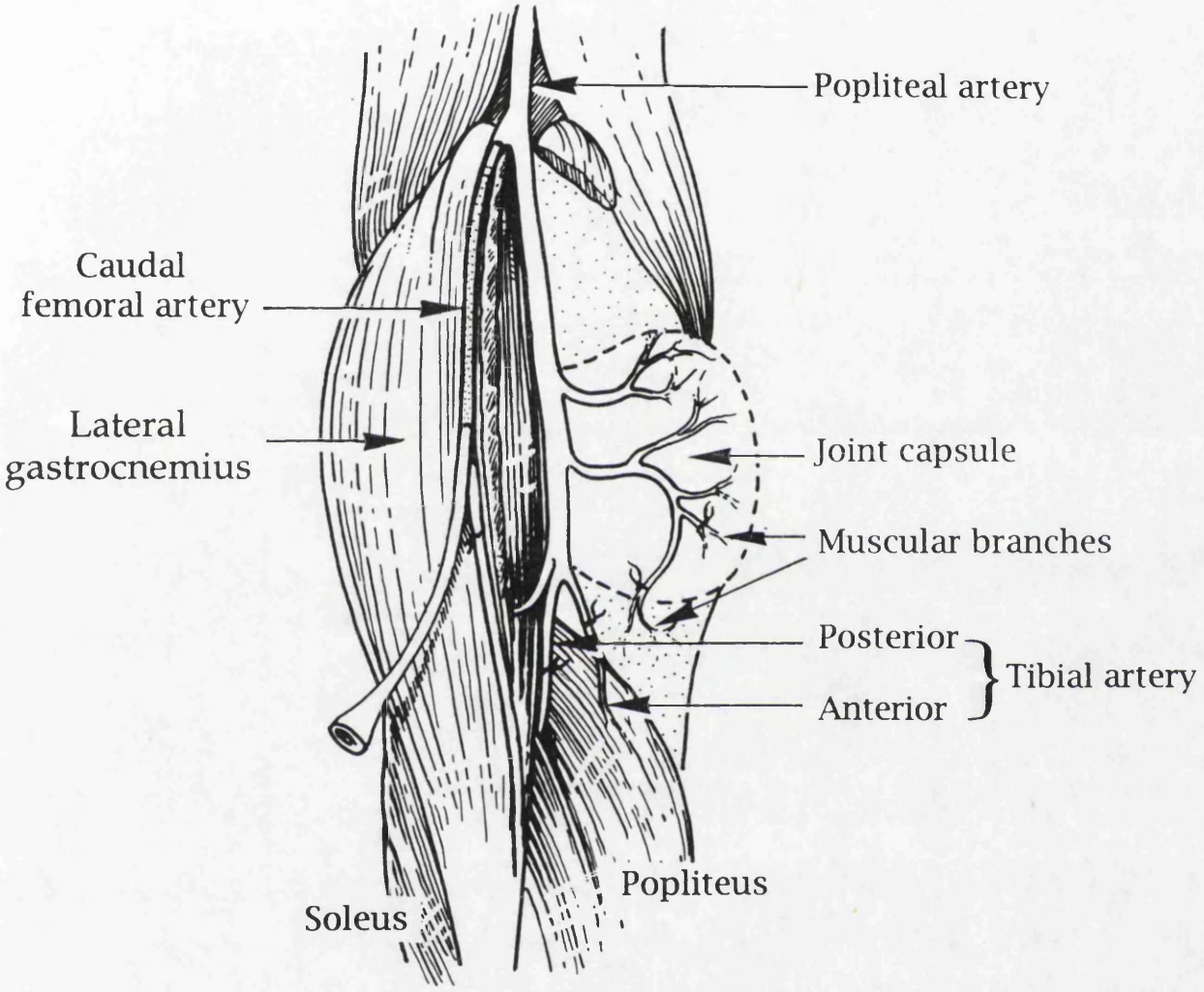
around the artery proximal to the ligature. Another about 15cm thread was used to make the second loose tie around the artery about 3cm proximal to the ligature, and proximal blood flow was stopped by pulling the free ends of this thread. Sometimes an arterial clamp was used instead of this thread. A small incision was made between the ligature and the first loose tie, and a heparinized cannula was inserted into the artery and secured in place by tightening the two loose ties. Finally, the free ends of the first ligature were also used to make another tie around the cannula, and then the cannula was flushed out with heparinized saline and connected to the pressure transducer.

In some animals one of branches of the left jagular vein, which lies very close under the skin in lateral aspect of the neck, was freed from the surrounding tissues and cannulated by the same technique as the carotid artery. This cannula was used for slow infusion of pentobarbitone in those experiments in which this anaesthetic was used for maintaining anaesthesia after surgical procedures.

d. Caudal femoral artery cannulation

In most of experiments in which intra-arterial administration of drugs close to the knee joint was necessary, a 25G heparinized polythene cannula was inserted into the caudal femoral artery (fig 2.3 & 2.7) and advanced until the tip was located close to the main branch (popliteal artery). The drug were then released into the blood stream through this cannula before the the origin of joint branches to ensure effective and even distribution of the drug in joint blood vessels.

Fig.2.7. Diagramatic representation of the arterial supply of the dorsal aspect of the rabbit knee joint . The popliteal fat pad and the medial belly of gastrocnemius muscle haye been removed. The picture also shows the cannulation of the caudal femoral artery.


Popliteal artery
Caudal femoral artery
Lateral gastrocnemius
Joint capsule
Muscular branches
Posterior
Anterior
Tibial artery
Popliteus
Soleus

e. Temperature regulation

The animal's temperature was maintained at around 37°C by means of a thermostatically controlled heated operating table during surgical and experimental procedures. Body temperature was controlled by a rectal thermometer connected to the thermostatic system.

III. Surgical procedures and stimulating and recording techniques

a. Surgical procedures in the popliteal fossa region

After shaving the fur of posterior aspect of the leg over gastrocnemius muscle, popliteal fossa and thigh muscles, an incision was made in the skin from the achilles tendon to about one third of the thigh region. The skin flaps were freed by blunt dissection and retracted by using clamps connected to the elastic strings and pulled and secured to the edges of the operating table. The underlying connective tissues and popliteal fat pad were removed by precise blunt dissection or cutting by cautery. The larger vascular branches in dissected tissues were firstly ligated by fine thread and then cut, while the small vessels were directly coagulated and cut by cautery. To get access to the posterior articular nerve (PAN) and posterior capsule of the knee joint, the medial belly of gastrocnemius muscle was removed by blunt dissection or cautery, starting from its lower tendon (achilles side) towards its upper tendon connecting to the femoral shaft.

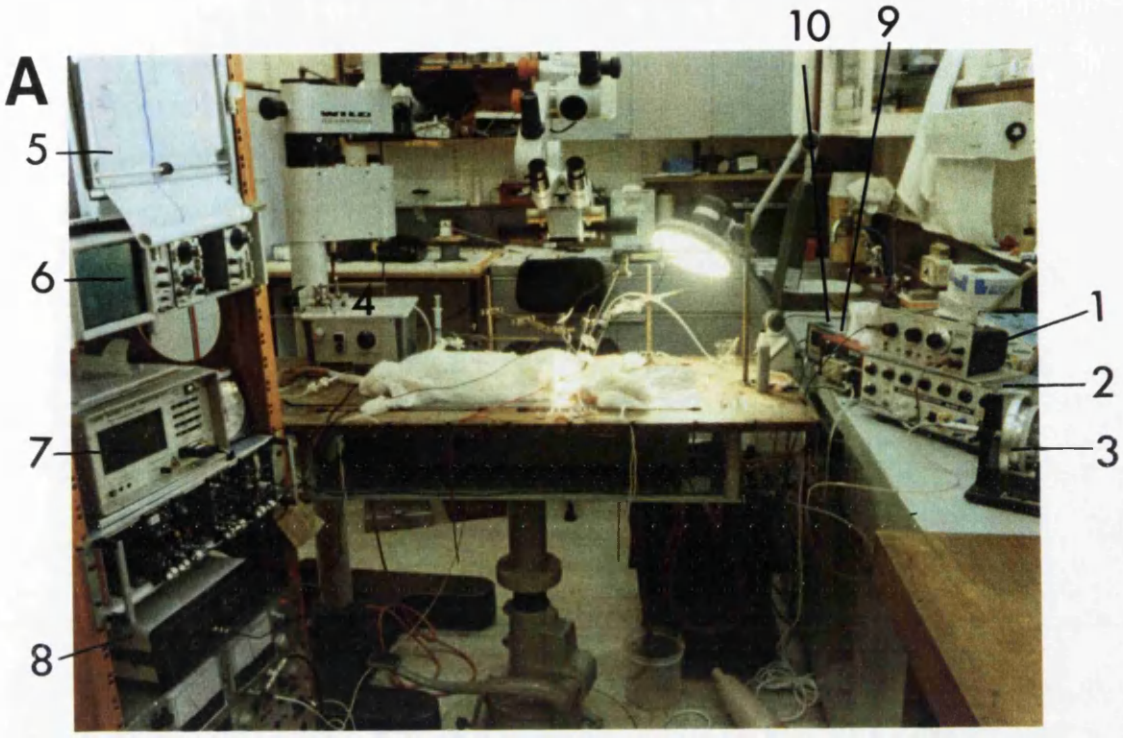
b. Posterior articular nerve dissection

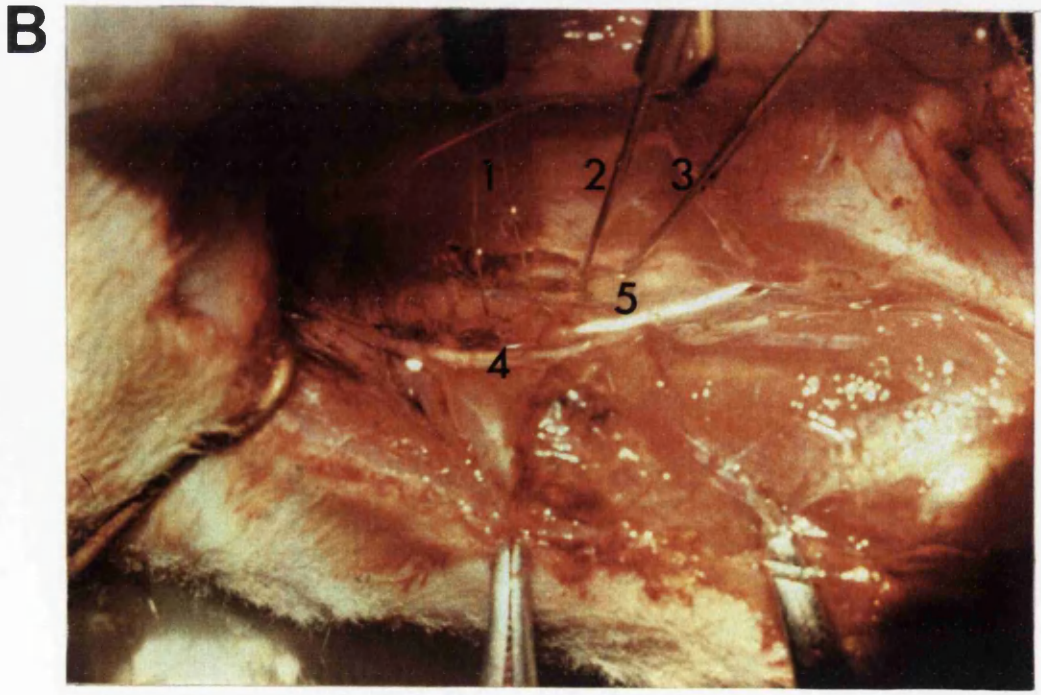
The posterior articular nerve (PAN), arises from the tibial nerve in the popliteal fossa (fig 2.8B) which is located between the two bellies of gastrocnemius muscle. The sympathetic fibres running in this nerve

Fig. 2.8.

A: Photograph shows the experimental set up and the position of the animal at the end of the surgical procedures. 1. Devices isolated stimulator; 2. Harvard isolated stimulator; 3. Microdrive system; 4. Anaesthetic infusion pump; 5. Pen recorder; 6. Oscilloscope; 7. Laser Doppler flowmeter; 8. Oxygen meter; 9. Slow infusion pump; 10.Thermalert TH-6D.

B: Photograph shows the posterior aspect of the rabbit knee joint with the popliteal paraffin pool. 1. Stimulating electrode under the PAN (PAN not clearly visible); 2. Laser Doppler probe; 3. Oxygen electrode; 4. Tibial nerve; 5. Posterior capsule.


A
10
9
5
6
4
1
2
3
7
8


B
1
2
3
5
4

innervate the blood vessels of the posterior region of cat (Khoshbaten & Ferrell 1990b) and rabbit (Ferrell & Najafipour 1992) knee joints. PAN was dissected free from connective tissues and accompanying blood vessels, under the operating microscope, for at least 1.5cm of its length, using a fine glass probe and very small dissecting scissors.

c. Popliteal fossa paraffin pool

After the dissection of PAN and cannulation of caudal femoral artery, the animal and the knee was held in a fixed position (fig 2.8A). Then in those experiments in which measurement of oxygen partial pressure in synovial fluid was performed, an oxygen electrode was inserted into the joint space (see below). To make a paraffin pool, threads were passed through the skin flaps and were retracted on either side and tied to the frame enabling warm paraffin oil to be poured into the formed space (fig 2.8B). The temperature of the pool was kept constant at 33°C using a heating lamp mounted above the pool which was automatically controlled by a thermocouple inside the pool and connected to a digital amplifier (thermalert TH-6D). This temperature was chosen as it was anticipated to be similar to that occuring in the synovial cavity (31-33°C) on the basis of previous experiments performed in this laboratory (Khoshbaten & Ferrell 1990a).

d. Locating nerve electrodes and recording from PAN

When PAN was dissected free, to prevent the somatic and systemic reflex effects of nerve stimulation, its proximal end was cut and then bipolar silver electrodes were placed on the distal end of the nerve and the stimulator (Harvard stimulator) was set to deliver trains of rectangular pulses with different duration, intensities and frequencies.

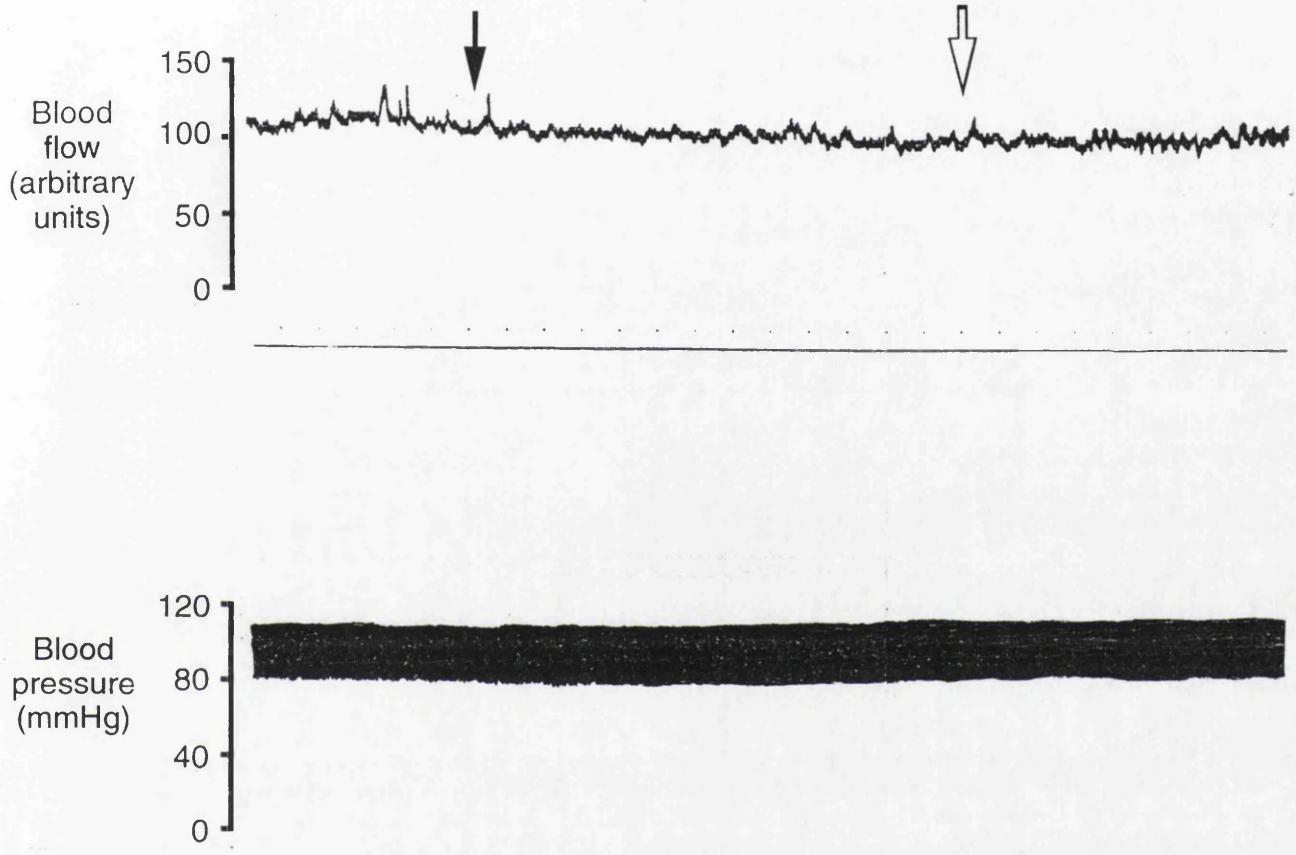
Transection of PAN had very small effects on basal joint blood flow (fig 2.9) (blood flow increased only by 2.4±2.1%, n=12), showing that sympathetic nerves had very little effect on the basal tone of rabbit knee joint blood vessels. In experiments in which electrophysiological recording from PAN was necessary, bipolar platinum recording electrodes were placed on the intact PAN and main nerve trunk (tibial nerve) was sectioned proximally about 3-5cm above the branching point of the PAN. Then, the distal end of the trunk was placed on bipolar silver stimulating electrodes and the stimulator (Devices stimulator) was set to deliver trains of rectangular pulses with different duration, intensities and frequencies. All the branches of the nerve trunk except PAN were sectioned to prevent contraction of the leg muscles. Finally, the compound action potentials were monitored on an oscilloscope (Tektronix 5103N) and recorded on video tape during the stimulation of the tibial nerve.

IV. P_SO_2 measurement

The most popular method of oxygen measurement in body fluids is using a gas analyzer. In this machine an aspirated fluid (normally blood) is injected to the machine in which three electrodes measure pH, PCO_2, and PO_2, and the results are displayed digitally. The oxygen electrode provides a current that is proportional to oxygen partial pressure (PO_2) in the injected fluid. The principles of oxygen measurement by this electrode are the same as those used by needle oxygen electrode described below. Although blood gas analyzers provide a quantitative measurement of PO_2 in the fluids, this is a non-continuous measurement and they are not able the measure the PO_2 of non-aspiratable fluids. Also there is always the risk of contamination of

Fig.2.9. Trace shows the blood flow signal from posterior knee joint capsule, recorded by a laser Doppler flowmeter, during the transaction of posterior articular nerve (PAN) (closed arrow), and close intra-arterial (in caudal femoral artery) injection of 0.25 ml physiological saline (open arrow) in a normal rabbit.

None of the above procedures had significant effects on joint blood flow. The time constant of the flowmeter was 3 seconds, and each point on the time scale represents one minute.


Blood flow (arbitrary units)
150
100
50
0
Blood pressure (mmHg)
120
80
40
0

the aspirating fluid by atmospheric oxygen. Therefore an alternative instrument was used to measure the synovial PO_2 which did not suffer from these disadvantages.

Oxygen tension in synovial fluid was monitored by means of a hypodermic needle O_2 electrode (Diamond General) which is a non-Clark style electrode with a built-in liquid junction electrode. This was connected to an O_2 metre (Strathkelvin Instruments) which provided a linear measure of PO_2 over a range of 0-160mmHg. The electrode was advanced into the synovial cavity through the postero-medial capsule (fig 2.8) by a microdrive system (Narashigi), to a such depth that the sensing elements of the electrode penetrating the capsule and located in the synovial fluid and not in contact with surrounding tissues.

a. Principles of Polarographic O_2 measurement

Polarographic oxygen sensors provide a current that is proportional to oxygen partial pressure (PO_2). This is accomplished by introducing negatively polarized (cathodic) noble metal sensors with reference to a silver chloride electrode into the tissue. The sensors interact with oxygen to produce a current from the oxygen reduction process:

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^-$$

The reaction goes to various degrees of completion, depending on the environment, the sensor metal, and the polarization potential. An increase in the polarization potential results in increase in current until electrons are provided at a sufficient rate to maintain the concentration

of oxygen on the surface of the oxygen sensor at zero. An increase in polarization potential defines the plateau region, where current is limited by diffusion of oxygen to the sensor, and is independent of the polarization potential (Fleckenstein & Weiss 1982) (fig 2.10A). The plateau is linearly related to the PO_2 and is defined by the solution of the oxygen diffusion equation (Stone *et al*, 1987).

$\nabla^2 PO_2 = 0$, subject to boundary conditions

$PO_2 = P_0$, far from the sensor

PO2= 0, at the sensor, with the current

$i = 4eSD\ dP/dn\ dA$

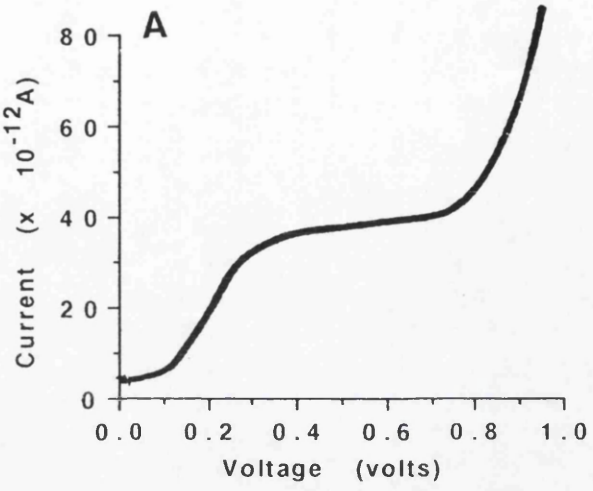
Where e is electric charge, S is the solubility of oxygen, and D is the diffusibility of oxygen (in cm^2/s). The silver chloride electrode, described as the reference electrode, is a silver wire with a porous coating of silver chloride. At this electrode oxidation takes place:

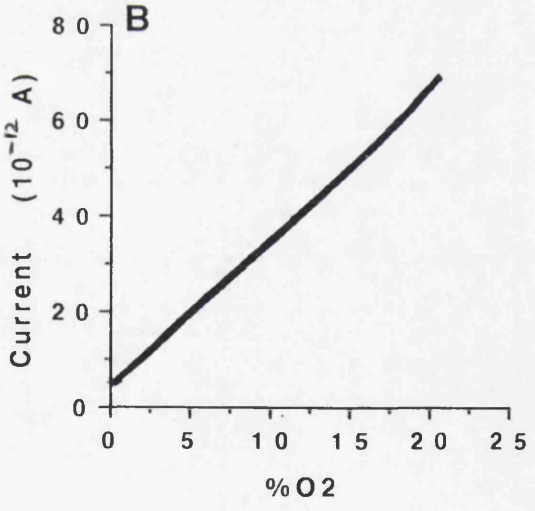
$$4Ag + 4Cl \rightarrow 4AgCl + 4e^-$$

With a constant concentration of chloride, and at a constant temperature, the polarographic current depends only on the concentration of oxygen (Fatt 1982). If the sensor is calibrated at two concentrations of oxygen, then the sensor current is a linear measure of the oxygen concentration. For use in tissue, isotonic saline provides a reliable fluid for calibration of the electrode (Cobbold 1974).

Fig. 2.10. **A**: Voltage-current relationship of the polarographic oxygen electrode in a media with constant PO_2. The current increases by increasing the voltage to ~0.4 volts. Between 0.4-0.8 volts the current is independent of voltage, but it increases again with voltages over 0.8 volts.

B: The relationship between the current produced by the polarographic oxygen electrode, at a constant voltage of 0.8 volts, and different partial pressures of oxygen in physiological saline at constant temperature. The current increases linearly by increasing in solution PO_2 (fig from the Diamond General manual leaflet).


A
80
60
40
20
0
Current (x 10-12A)
0.0
0.2
0.4
0.6
0.8
1.0
Voltage (volts)


B
80
60
40
20
0
Current (10-12 A)
0
5
10
15
20
25
%O2

Needle oxygen sensors are available with a single-tipped oxygen sensor on rigid metal needles which can be inserted into the tissue to measure oxygen partial pressure or concentration. When measuring the PO_2 in saline the current is proportional to the oxygen concentration , but the actual tissue oxygen tension is a function of the diffusivity of the tissue and the oxygen concentration.

b. voltage-current and PO_2-current relationship of oxygen sensors

The voltage-current relationship for a polarographic oxygen electrode is represented by the characteristic curve (fig 2.10A). In the region below approximately -0.5 volt, there is a reasonably linear voltage-current relationship. As the polarization voltage is increased beyond -0.5 volt, the current will tend to reach a plateau in which changes in voltage have little effect on current. In this plateau region the current is limited by the rate at which oxygen can diffuse to the cathode. As the voltage is increased above -0.8 volt, the current will again increase with voltage, due to the reduction of other elements in addition of oxygen. The electrode is normally operated with the polarization voltage set to the midpoint of the plateau region, in which case the current is diffusion limited. In a diffusion-limited condition, virtually all of the oxygen molecules reaching the cathode are immediately reduced, resulting in a zero oxygen concentration at the cathode surface and a current which is limited by the rate at which oxygen can diffuse to this zero concentration region. The diffusion rate is a function of the oxygen coefficient of the membrane and the media surrounding the cathode and the dissolved oxygen concentration which, in turn, is proportional to the oxygen partial pressure and temperature. The result

is that, for a constant temperature, current flow through the electrode will be directly proportional to the partial pressure (PO_2) of oxygen.

The plot of the relation between current and PO_2 (at a fixed polarization voltage) is called the standard curve (fig 2.10B), which for most electrodes is linear. The small current at zero PO_2 (called residual or dark current) results from factors such as electrical leakage through insulating materials in the system and reduction of oxygen which was absorbed into the electrode materials.

c. Calibration of the oxygen meter

The O_2 meter used (Strathkelvin Instruments) provided a linear measure of PO_2 over a range of 0-160mmHg. The equipment should be calibrated every day before the electrode inserted into the joint cavity. The top end of the range was calibrated by placing the tip of the O_2 electrode in physiological saline through which room air was bubbled for at least twenty minutes. The barometric and water vapour pressures were taken into account in adjusting the meter to give a reading corresponding to the calculated partial pressure of O_2 in atmospheric air. At the lower end of the range two calibration procedures were performed. One involved placing the electrode in a solution of 0.01M disodium tetraborate to which sodium sulphite was added. This gave a stable reading and the meter was then adjusted to read zero. Another method involved bubbling 100% nitrogen gas through saline. The vessel containing this solution was covered with cling film to minimise ingress of room air. This method also showed a reading very close to zero, and served as a control for the first calibration. All calibrations were performed at a temperature corresponding with that anticipated in the

synovial cavity (31-33°C) on the basis of previous experiments (Khoshbaten & Ferrell 1990a).

V. The measurement of joint blood flow

As mentioned in chapter one, the synovial circulation has been evaluated by measuring skin temperature, synovial fluid temperature, by plethysmography, electromagnetic and bubble flow meters, vital microscopy, as well as clearance of radioactive substances. All these methods have disadvantages - for example, sensitivity to environmental temperature, lipophilic property of xenon (Phelps *et al.,* 1972), introduction of radioactive substances, complicated measuring procedures, and the importance of taking the synovial fluid volume into account in clearance studies (Simkin & Nilson 1981). Furthermore, almost all of these methods are not suited for measuring rapid changes in the synovial microcirculation. Thus a method for measuring rapid microciculatory changes would be a useful tool in the investigation of synovial blood circulation physiology. The laser Doppler technique is a suitable method for evaluation of rapid changes in synovial blood flow. The only disadvantage of this method is that it provides non-quantitative measurement of blood flow, thus a reliable quantitative technique is also needed.

Two methods were employed to measure joint blood flow. A radiolabelled microsphere technique provided quantitative measurement of joint blood flow. The fundamentals and specifications of this technique, which is a well known reliable and widely used method in blood flow measurement, along with the results provided, will be discussed in next chapter.

Laser Doppler flowmetry was used to assess relative changes in joint blood flow. This method has been used as a reliable and accurate technique to measure blood flow of highly localized microcirculation of different tissues such as intestinal blood flow (Ahn *et al.*, 1985), cerebral blood flow (Busija *et al.*, 1981; Haberl *et al.*, 1989a,b), cutaneous blood flow (Holloway *et al.*, 1977), bone blood flow (Hellem *et al.*, 1983; Notzli *et al.*, 1989), retinal blood flow (Riva *et al.*, 1972), and articular blood flow in the rabbit (Khoshbaten & Ferrell, 1990a), cat (Khoshbaten & Ferrell, 1990b) and human (Geborek *et al.*, 1989).

a. The principles of laser Doppler flowmetry

The first attempt to measure blood flow by the laser Doppler technique was made in 1972 by Riva *et al.*, who studied red blood cell velocities in the retinal vessels of rabbits. From then this technique has been used increasingly to assess the blood flow to different tissues. The technique is based on the principle of the frequency shift of the laser light by moving particles. A light guide transmits a low-energy laser beam (diode laser, 3mW, lambda = 780nm) to the tissue surface under examination. The light enters the tissue where it is repeatedly reflected, refracted, and increasingly absorbed. This produces a region of virtually isotropic illumination. All erythrocytes passing through this region reflect some of the light and produce a shift in wavelength. Although the shift is quite small compared to the frequency of light, the method is practical because of the spectral purity of the laser light. A glass fibre running parallel to the light guide for the incident beam passes the reflected light to a photodetector where it is converted into an

electrical signal. Further processing of this signal finally produces the actual Doppler signal which represents the flow velocity of the erythrocytes (fig 2.11). The active specimen volume under the measuring head is a hemisphere with a diameter of approximately 1mm.

As this method senses only the blood flow in the smallest arterioles and capillaries it can provide information on changes in nutritive blood flow, not in absolute values but only as percentage changes relative to the initial signal. The important advantage is the straight-forward application of this technique without involving extensive experimental equipment. The measuring head does not need to be placed directly on the organ surface while the light beam penetrates into the tissue to a depth of approximately 1mm, thus permitting atraumatic measurement without any danger of oedema formation.

The electrical signal is the consequence of two variables, the velocity (speed) and the number (concentration) of moving red blood cells. The flux signal, which is a processed signal by the flowmeter of the speed and concentration signals (fig 2.12), is a representative of blood flow in the area under the tip of the laser probe. This signal is unitless and is expressed as arbitrary units.

b. Locating blood flow probe and recording

To monitor the relative changes in blood flow to the joint, A 0.9mm diameter fibre-optic probe connected to a laser Doppler flowmeter (Moor Instruments MBF3) was positioned just above the surface of the postero-medial aspect of the knee joint capsule (fig 2.8B). The probe was able to transfer an infra-red laser beam (780nm), produced by the flowmeter, to the tissue beneath the probe tip, and

Fig. 2.11. Laser-Doppler measurement of tissue perfusion. Laser light is typically delivered to tissue and returned to a detector by a fibre optic light guide. Light in tissue is diffusely scattered by stationary elements in the tissue. Such light reaches the detector without being Doppler-shifted. Photons that encounter moving red blood cells experience a Doppler shift.

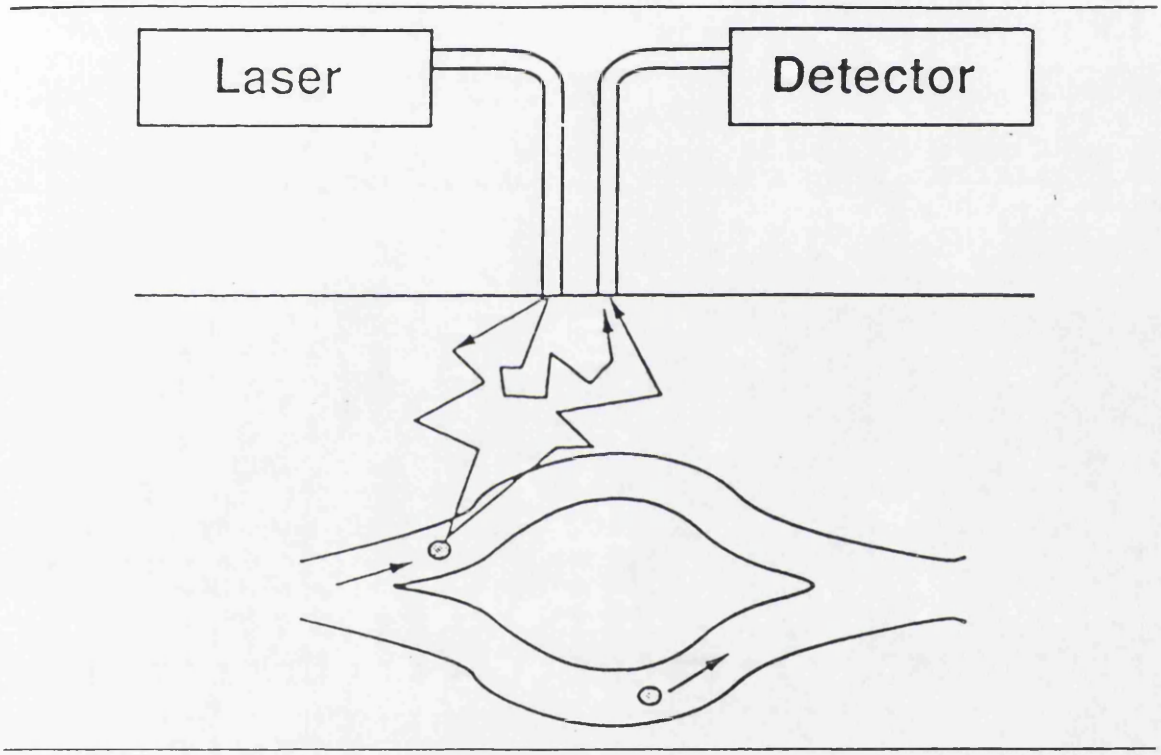
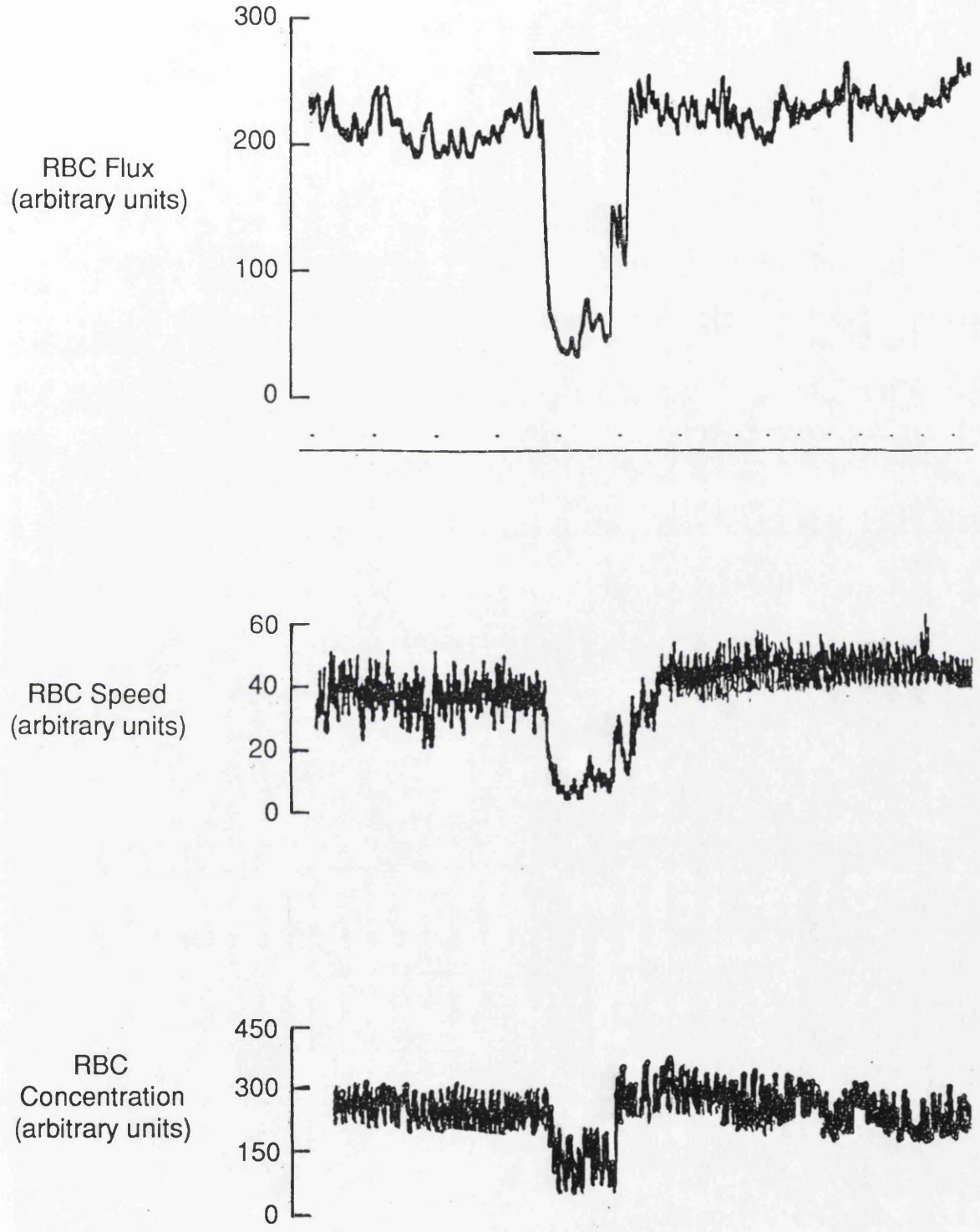

Laser
Detector

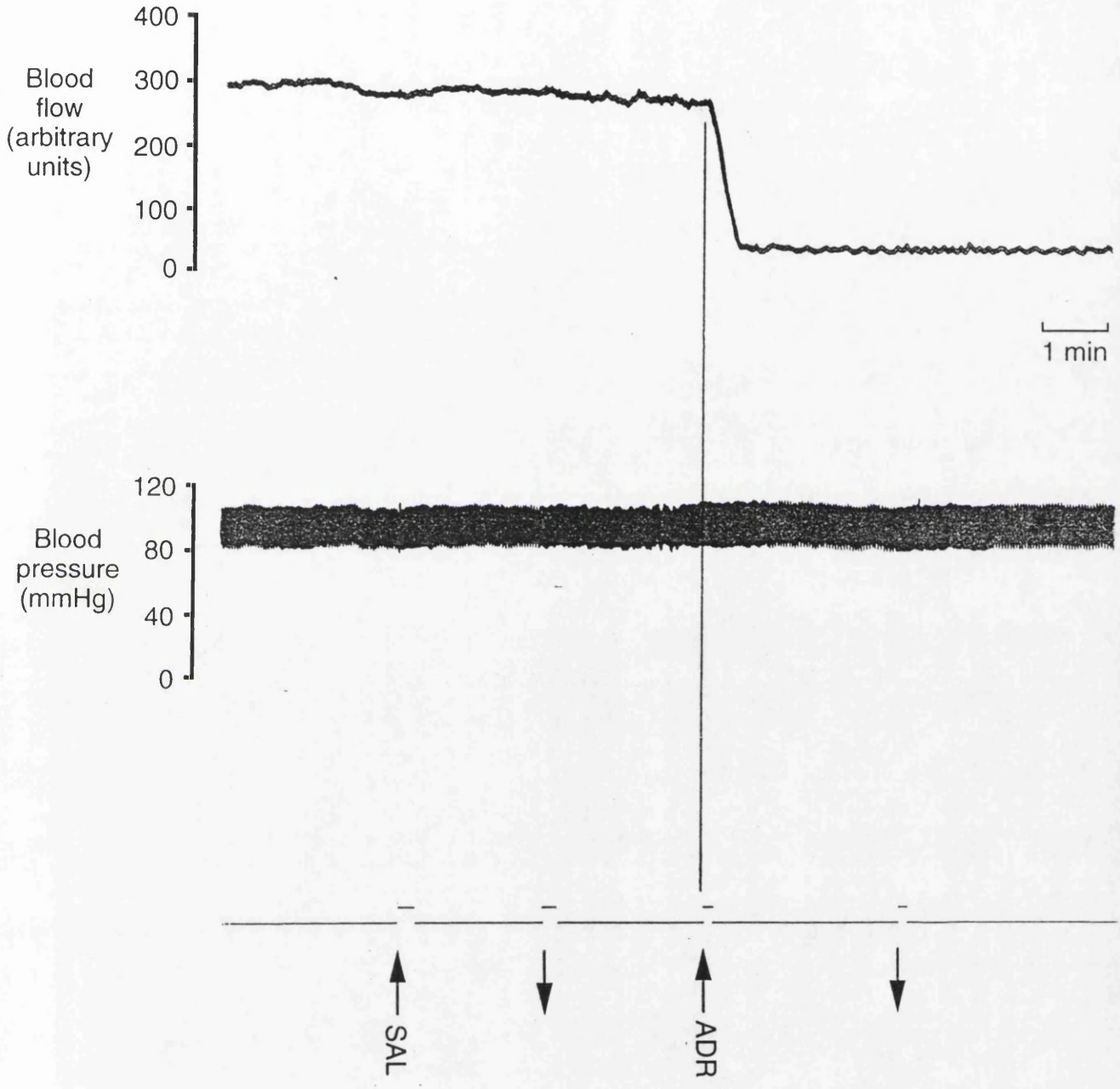
Fig. 2.12. Flux, speed and concentration signals of the laser Doppler flowmeter recorded from the posterior knee joint capsule of the rabbit. The flux signal is actually a processed signal of the other two. The popliteal artery was occluded, at black bar shown over the trace, by an arterial clamp. The time constant of the flowmeter was 3 seconds.


RBC Flux
(arbitrary units)
300
200
100
0
RBC Speed
(arbitrary units)
60
40
20
0
RBC
Concentration
(arbitrary units)
450
300
150
0

return the reflected light by the tissue to the flowmeter, providing a measurement of tissue blood flow. In preliminary experiments it was shown that alterations of blood flow in the synovium are detected with the probe in this position (fig 2.13). The delay time between close intra-arterial injection (see below, Perfusion technique) of 2.5nmol adrenaline and initiation of the vasoconstriction response examined in five knees was 8.4±0.68 seconds, whereas it was 5.4±1.24 seconds when 1nmol adrenaline was injected directly into the synovial cavity (P=0.03, paired t-test). This difference suggests that the initiation of response is most likely due to reduction of blood flow in the synovial membrane.

The equipment was set on a time constant of 3 seconds to minimise peak to peak fluctuations of the recorded trace. The signal from the flowmeter was recorded on one channel of a pen recorder and the speed and concentration signals were recorded, when it was necessary, on the other two channels. In most of the experiments, arterial blood pressure, heart rate, and synovial PO_2 were recorded on the other channels. The flowmeter was also able to record flux, speed, or concentration signals on a built-in chart recorder in the flowmeter. The flowmeter was checked for biological zero i.e. the reading when the animal was dead and the blood flow has stopped. In ten posterior capsules examined the flowmeter signal was found to be 9.4 ± 1.5 flux units two hours after the death of the animals at the end of the experiments. As this value was always below 10% of the flow readings during the course of the experiment and the results were not affected significantly, it was not taken into account for simplicity in calculation of percentage changes in blood flow.

Fig 2.13.Effect of intra-articular injection of 1nmol adrenaline (in 0.1ml saline) on posterior capsule blood flow in one normal joint. The very short delay between the injection and the initiation of response (~5 seconds in this animal) shows that the initiation of response is most likely due to the reduction of blood flow in synovium and not due to the diffusion of the drug to the most superficial layers of the capsule immediately under the probe. Upward arrows indicate injection (0.1ml) into and downward arrows indicate withdrawal (0.1ml) from the synovial space. SAL= saline, ADR= adrenaline.


400
300
200
100
0
Blood flow (arbitrary units)
1 min
120
80
40
0
Blood pressure (mmHg)
SAL
ADR

VI. Perfusion technique

Nearly all drugs were administered close intra-arterially through the cannula in the caudal femoral artery (fig 2.7). By this route of administration, the drugs were delivered into the blood stream about 2cm before the origin of joint branches to provide effective and even distribution of the drugs to joint blood vessels. This cannula was connected to a three-way stopcock and then to a slow perfusion pump (Watson-Marlow), to infuse saline or the antagonistic drugs continuously and by an adjusted constant rate during the course of experiment. When injecting agonists, the pump was stopped temporarily, the drug was injected through the stopcock and then the pump was switched on again to continue the infusion, and also wash out the rest of the agonist in the arterial cannula. All the agonists and antagonists were dissolved in saline to give the appropriate concentration. Agonists (by either concentration) were injected in volumes of 0.25 ml. Control injections of 0.25 ml of physiological saline had no effect on joint blood flow (fig 2.9)

VII. Induction of acute joint inflammation

In some experiments, acute inflammation was induced in one knee, by intra-articular injection of 1ml of 2% solution of carrageenan in sterilized distilled water into the joint cavity, 24 hours before the experiment. The other knee joint was injected by 1ml sterilized physiological saline to act as an internal control. Injection of carrageenan or saline was conducted by insertion of a 25G hypodermic needle through the middle of the patellar tendon into the joint cavity.

Carrageenan is a natural seaweed polysaccharide which is relatively nontoxic to the animal (Lowder & Gillard, 1976), and produces an inflammatory response which is localized and similar to that of the human rheumatoid arthritis (De Rosa, 1972; Gardner, 1960). It produces a rapid inflammatory response, and a single injection is sufficient for development of an acute inflammation within 24 hours (Gardner, 1960; Lowder & Gillard, 1976).

VIII.Termination of the experiment

At the end of experiment the anaesthetized rabbit was painlessly killed by intravenous injection of 1M KCl through the cannula in the jagular vein.

IX. Statistical analysis

The values expressed in all graphs and histograms are means $\pm$ standard error of mean (SEM). Two types of comparisons were performed. A student's paired t-test was used to compare between the results of a special treatment in a group of animals, or comparing the results of one knee with the other knee in a group. In situations in which having an internal control was not possible and two groups of animals as test and control were present, or the results of two different treatments in two groups has to be compared, a student's unpaired t-test was used. (An F test was used to test the assumption of homogeneity of variances). In situations in which the results of a group were not normally distributed, a non-parametric statistical test (e.g. Wilcoxon sign rank

test, or Mann-Whitney U test) was used. Differences between means were considered significant if the P values were 5% or less. *: 0.01< P< 0.05; **: 0.001< P <0.01; ***: P<0.001.

The comparison between correlations of P_SO_2 and blood flow in the normal and inflamed joints (in both situations, frequency/response and voltage/response profiles - page 81) was made using Fisher's Z' transformation (Phillips, 1978), which transforms *r* into Z' which is normally distributed. Then Z values were calculated from Z' values (found in appropriate statistical tables) based on the formula:

$$Z = \frac{Z'_1 - Z'_2}{\sqrt{\frac{1}{N_1 - 3} + \frac{1}{N_2 - 3}}}$$

Where Z'_1 and Z'_2 are Z' values corresponding to the normal and inflamed groups, N_1 and N_2 are number of paired data in normal and inflamed groups. Finally, P values were found in the Z table.

CHAPTER THREE

Joint blood flow measurement by radiolabelled microspheres

Summary

1. Experiments were performed to measure the blood flow of structures in and around normal and acutely inflamed knee joints and determine the effect of electrical stimulation of the posterior articular nerve (PAN) supplying the knee.

2. Radiolabelled microspheres (~ 16.5um) were used to measure blood flow to the joint structures quantitatively. This technique also showed absolute changes in blood flow due to surgical procedures and nerve stimulation.

3. Surgical exposure of the posterior aspect of the knee joint capsule resulted in an increase in flow restricted to this region in normal but not inflamed joints. Section of PAN had no significant effect on blood flow to both joints, suggesting that the basal tone in this vascular bed is not governed by sympathetic nerves travelling in PAN.

4. Electrical stimulation of PAN reduced the blood flow in the posterior region of both joints, but no alterations occurred in the anterior region or any other surrounding structures, suggesting that the neural supply is specific to the posterior joint capsule. PAN stimulation reduced the blood flow to the posterior capsule by 56% in the control knee and 45% in the inflamed knee.

5. The blood flow in the posterior and anterior capsule of the normal knee was 9.5±1.7 and 7.2±1.7 ml/min/100g tissue respectively. The process of inflammation at 24 hours increased the blood flow to the joint structures. The blood flow was increased from 17.1±3.4

ml/min/100g tissue in the posterior capsule of the control (saline injected) knees to 35.8±5.9 ml/min/100g tissue in the posterior capsule of the inflamed knees. In the anterior capsule it was increased from 13.8±3.3 to 24.7±5.6 ml/min/100g tissue, and in the popliteal muscle, patellar ligament, tibia, and femur, it was also proportionally increased.

6. The results indicate that: a) the radiolabelled microsphere technique is a valid and suitable technique for measurement of basal blood flow and absolute changes in joint blood flow. b) It confirmed once again the hyperaemic condition of the inflamed joints. c) PAN innervates the blood vessels of the posterior capsule but not blood vessels of any other surrounding tissue, and d) the process of inflammation decreased the sympathetic nerve-mediated vasoconstrictor responses of the blood vessels in the posterior capsule.

Introduction

Different methods of joint blood flow measurement and their advantage and disadvantages were reviewed in chapter two. Among those methods by which joint blood flow could be measured quantitatively, the radiolabelled microsphere technique was the most appropriate one. This technique was introduced by Rudolph and Heymann (1967), to measure regional blood flow. The microspheres were injected into the circulation and travel to the small peripheral vessels where they were trapped. The organ was then removed and the radioactivity measured. Repeated measurements could be made by using different nuclides and separating them by gamma spectrometry. This technique which is widely used by many investigators to evaluate other flow measurement techniques, is capable of blood flow measurement in a quantitative manner. Previous investigation in this laboratory has shown that electrical stimulation of the nerve supply to the anterior region of the rabbit knee joint results in constriction of blood vessels in both the joint tissues and in the adjacent long bones, as measured by radiolabelled microspheres (Ferrell, Khoshbaten & Angerson, 1990). Blood vessels in the cancellous bone of the distal femur (condyles) and proximal tibia (plateau) appeared to be innervated by vasoconstrictor fibres which reach their effectors via the articular nerves. The present study was performed using the posterior region of the rabbit knee joint as this is innervated by a discrete articular nerve (posterior articular nerve - PAN) which, when stimulated, produces constriction of knee joint blood vessels (Ferrell & Najafipour, 1992). The object of the present investigation was to measure the absolute blood flow to the

normal and inflamed knee joint structures, and to determine whether any differences would emerge in comparison to the anterior region. Another aim was to see whether other joint structures, in addition to posterior capsule, receive innervation from posterior articular nerve and to establish the basal blood flow value in an intact knee and determine whether surgical procedures affected this.

Methods

20 adult New Zealand white rabbits (2-3.5kg) were deeply anaesthetized, as judged by the absence of a flexor withdrawal response to a noxious stimulus applied to the forelimb, by an initial injection of hypnorm (0.15ml/kg I.P.; Janssen) and diazepam (1.5mg/kg I.P.; Valium, Roche) followed by a mixture of O_2/N_2O/halothane 1% delivered via a cannula inserted into the trachea. Both carotid arteries were cannulated. The right cannula was guided to the left ventricle and the left one was connected to a pressure transducer and to a slow withdrawal pump, which was a slow infusion pump rearranged to such a manner to withdraw blood samples. The presence of the cannula tip in the ventricle was confirmed by recording the left ventricular pressure by another pressure transducer. Arterial blood pressure was monitored throughout the experiment and blood flow measurements only taken in the presence of stable cardiovascular parameters. In each animal the right knee was left intact to provide a control knee joint whilst the other (experimental) knee underwent surgery. The surgical procedure in the popliteal fossa region, posterior articular nerve (PAN) dissection and stimulation, and placing laser Doppler probe over the posterior capsule for blood flow measurement, have been described in chapter two. Independent quantitative measurements of blood flow were obtained by intraventricular injection of radiolabelled microspheres (^{113}Sn, ^{57}Co, ^{153}Gd and ^{46}Sc; 16.5±0.1μm diameter; NEN-TRAC, New England Nuclear) with timed withdrawal of an arterial blood sample. The microspheres were suspended in 0.9% saline with 0.01% Tween 80 to prevent clumping. The vial containing the microspheres was shaken

vigorously immediately before withdrawing the microsphere solution into the syringe by a mechanical agitator. The syringe containing the microspheres was also vigorously agitated until immediately before injection.

For each measurement, 1ml of suspension, containing 1.5 x 10^6 microspheres, was injected over a period of 15sec into the left ventricle. A reference sample was withdrawn from the left carotid artery at a rate of 3ml/min for a period of 60sec, starting 5sec before the microspheres were injected. The blood sample was replaced by an equivalent volume of saline to maintain blood volume. This also helped to flush out the remainder of the microspheres in the cannula. As it was necessary to leave the femoral arteries intact on both sides, it was not possible to monitor blood pressure during microsphere injection. However, previous experiments (Ferrell *et al.*, 1990) involving three successive injections of a 1ml suspension of microspheres without withdrawal of an arterial blood sample but continuous measurement of arterial pressure have shown that systolic and diastolic blood pressures during and immediately after injection changed by less than 5mmHg. The blood pressure was not significantly different before and after microspheres injection. In a series of 10 rabbits the mean arterial pressure changed from 81±5.1mmHg before the first injection to 79.5±4.8mmHg after the last injection.

Two groups of animals were used: In the first group, which were the normal group, three injections of radiolabelled microspheres were administered: (1) When both knees were intact i.e. no surgical procedure was performed on the experimental knee. (2) At the end of surgical procedures on the experimental knee (before PAN

stimulation). (3) During nerve stimulation when the LDF signal reached its minimum value. The nuclides used in this group were ^{113}Sn, ^{57}Co and ^{46}Sc. In the second group (carrageenan-treated animals) four microsphere injections were given: The first two injections were under the same condition as in the first group (i. e. before and after the surgery to the experimental knee). (3) 10min after denervation of the experimental knee (PAN section); (4) During nerve stimulation when the LDF signal reached its minimum value. The nuclides used in this group were ^{113}Sn, ^{57}Co, ^{153}Gd and ^{46}Sc. The order of injection of microspheres was randomised. The method of carrageenan injection to the knee in order to produce acute inflammation, has been described in chapter two. The stimulus parameters were: amplitude 10V, width 1ms, train duration 1min and frequency 20 Hz.

10 minutes after completion of the last injection, the animals were administered an intraventricular overdose of pentobarbitone (200mg/kg) or molar KCl and tissue harvested from the following sites from both knees: popliteus muscle, distal femur (femoral condyles), proximal tibia (tibial plateau), posterior capsule (consisting of both synovium and overlying areolar and fibrous tissue), anterior capsule (comprising of synovium, infrapatellar fat pad and overlying fibrous tissue) and both kidneys. The latter were sampled as a check on the adequacy of mixing and distribution of microspheres, although Morris and Kelly (1980) have reported that intra-ventricular injection of microspheres results in even distribution according to blood flow measurement of different tissues and no significant differences were observed in various arteries. Tissue samples were weighed, placed in counting vials, and then counted (together with the reference blood

sample) in a 2-channel gamma counter (Packard 500C) with energy window settings appropriate for the nuclide used. Raw counts were corrected for crossover between channels, which was determined by counting pure samples of the two radionuclides.

Tissue blood flow was calculated using the equation:

$$TBF = C_x / C_r \text{ X RBF X } 100$$

Where TBF is tissue blood flow in ml/min/100g, C_x is counts per gram of tissue, C_r is total count in the reference blood sample. RBF is reference blood flow rate (rate of withdrawal of blood sample from reference artery in ml/min).

Data in the graphs and histograms are presented as mean±SEM. Analysis has been performed using non-parametric statistics; Mann-Whitney U test for the analysis of the data of the two independent groups (control vs inflamed joint), or control knee vs test knee in each group. Wilcoxon matched-pairs sign rank test was used for repeated measures of the flow in the experimental or control knee using different microspheres (i.e. between different stages of the experiment). Ten normal and ten carrageenan treated animals were used but the numbers of observations per joint (values in the figure legends) were less as some values were excluded due to asymmetric kidney counts suggesting incomplete mixing of microspheres in the blood.

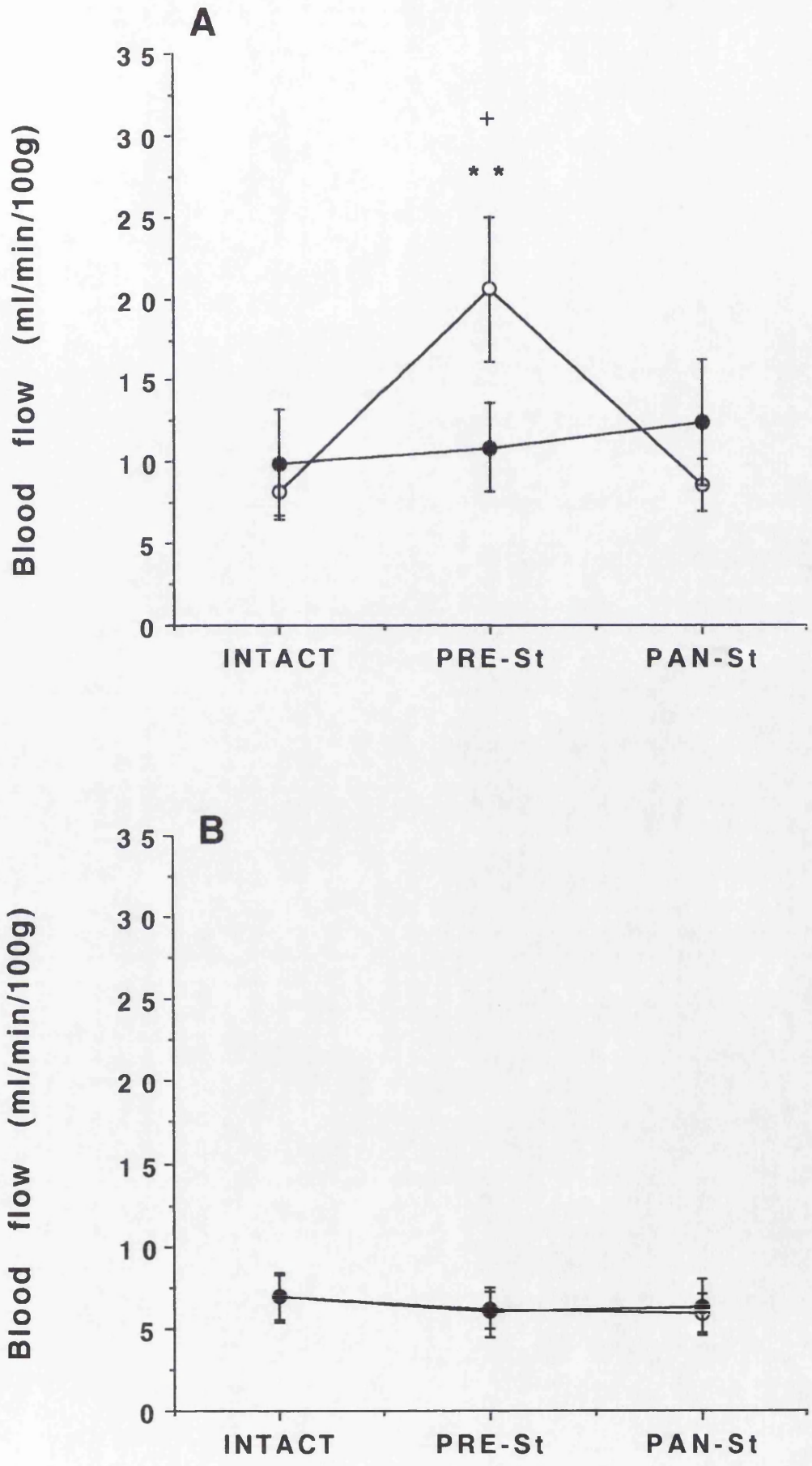
Results

I. Blood flow estimation in normal joints

a. Capsular blood flow

Blood flow estimation by microspheres showed that before surgery blood flow to the right and left posterior and anterior capsules was not significantly different, although it showed non-significant higher values in the posterior capsules compared to the anterior ones (fig 3.1). The surgical procedure to the popliteal fossa region of the left knee, which exposed the posterior capsule, as well as PAN dissection, increased the blood flow to this capsule significantly, but it had no effect on the blood flow to the contralateral capsule, or both anterior capsules (fig 3.1). This increase in blood flow is unlikely due to tissue denervation as PAN section showed no significant change in blood flow to the posterior capsule of the normal joint assessed by laser Doppler flowmetry (fig 2.9, chapter 2). Electrical stimulation of PAN, sufficient to maximally activate sympathetic efferent fibres in the nerve, decreased the blood flow to the left posterior region by about 60%, but no change was observed in the blood flow to the contralateral posterior capsule or both anterior capsules (fig 3.1). In fact the blood flow in these regions of the joints remained relatively constant throughout the experiment. This served as a useful control, indicating that no major haemodynamic changes occurred in the systemic circulation during the course of the experiment.

Fig 3.1. Changes in blood flow in the normal posterior (**A**) and anterior (**B**) joint capsule for right knees (closed symbols) and left knees (open symbols); both knees intact (INTACT), after surgery and PAN section of the left knee just before nerve stimulation (PRE-St), during left PAN stimulation (PAN-St). Means ± SEM; n = 8-10. ** = $P<0.01$ significant difference with INTACT (Wilcoxon test). + = $P<0.05$ significant difference between left and right knees (Mann-Whitney).


A
Blood flow (ml/min/100g)
35
30
25
20
15
10
5
0
+
* *
INTACT
PRE-St
PAN-St
B
Blood flow (ml/min/100g)
35
30
25
20
15
10
5
0
INTACT
PRE-St
PAN-St

b. Blood flow to peri-articular tissues

Examination of the blood flow to the other tissues harvested showed clear differences depending on tissue type (fig 3.2). Under resting conditions highest blood flow occured in bone with the popliteus muscle showing the lowest blood flow values. In none of the four tissues in fig 3.2 was there a significant difference between left and right sides during any of the phases of the experiment, nor was there any difference on a given side as a function of the phases. One conclusion is that none of these tissues is innervated by PAN.

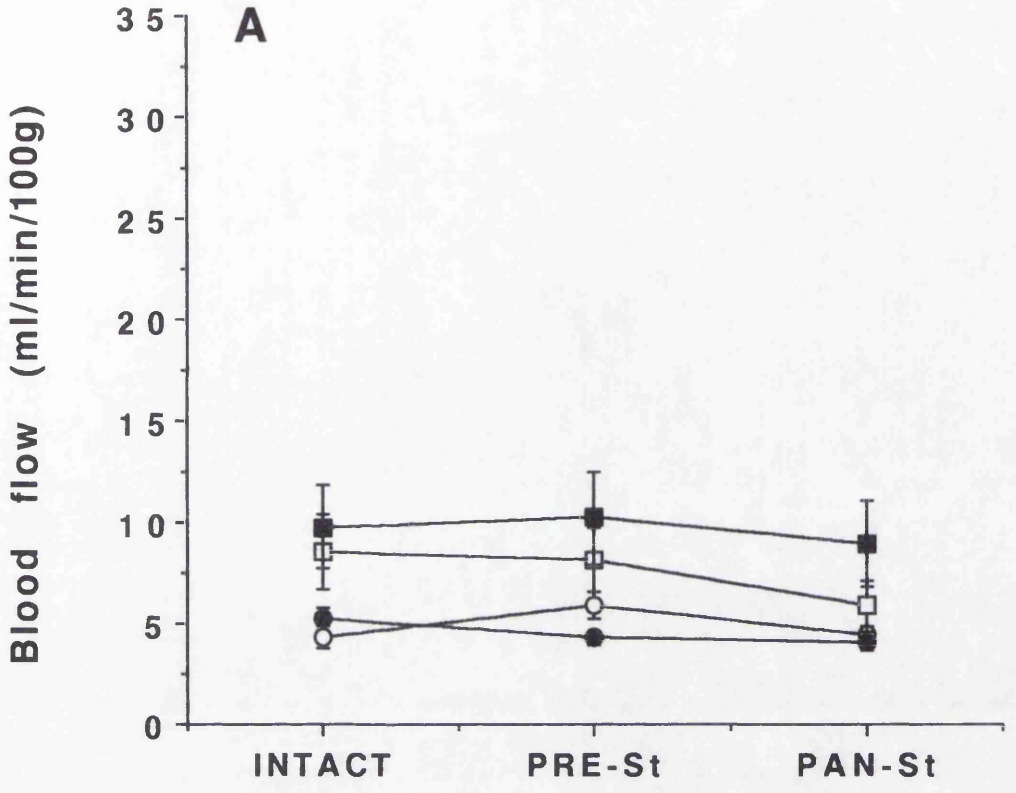
The blood flow to the kidneys was highest between the tissues measured in these experiments. No significant change in kidney blood flow, either left or right, was observed during the course of experiment (fig 3.3), nor the blood flow was significantly different between the two kidneys. This confirms the complete and even mixing of the microspheres with blood before they reach to the right and left joints.

II. Blood flow estimation in carrageenan-treated joints

a. Capsular blood flow

Before surgery, the blood flow to the anterior and posterior capsules of the inflamed (left) knee joint was almost double that of the corresponding capsules of the control (saline injected) knees (fig 3.4, phase 1). This confirms the hyperaemic condition of the inflamed knees. The inflamed joints also showed a different appearance (i.e. redness, swelling) compared to control joints. The joint circumference (around the patellar tendon and popliteal fossa) measured in five animals

Fig 3.2. Changes in blood flow in the **A**: popliteus muscles (circles) and patellar ligaments (squares) and **B**: femoral condyles (circles) and tibial plateau (squares) of the right knees (closed symbols) and left knees (open symbols); both knees intact (INTACT), after surgery and PAN section of the left knee just before nerve stimulation (PRE-St), during left PAN stimulation (PAN-St). Means ± SEM; n = 8-10.


A
Blood flow (ml/min/100g)
35
30
25
20
15
10
5
0
INTACT
PRE-St
PAN-St

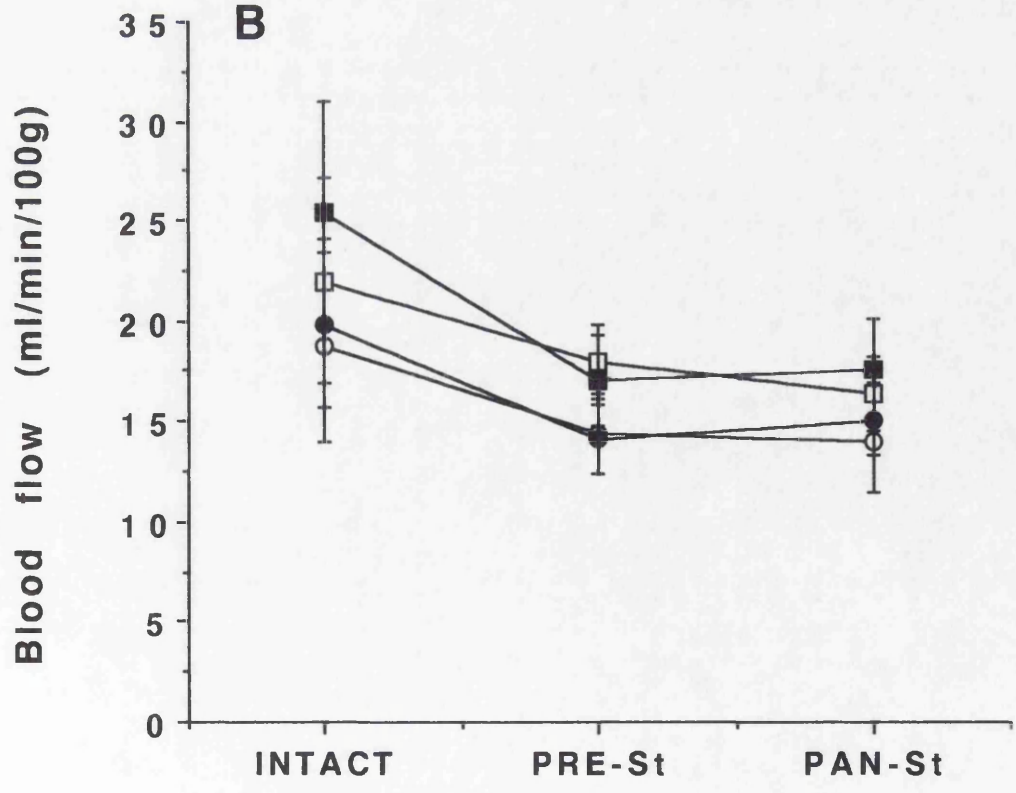

B
Blood flow (ml/min/100g)
35
30
25
20
15
10
5
0
INTACT
PRE-St
PAN-St

Fig 3.3. Changes in blood flow in the right (closed symbols) and left (open symbols) kidneys; before starting surgery of the left knee (INTACT), after surgery and PAN section of the left knee just before nerve stimulation (PRE-St), and during left PAN stimulation (PAN-St). Means ± SEM; n = 8-10.

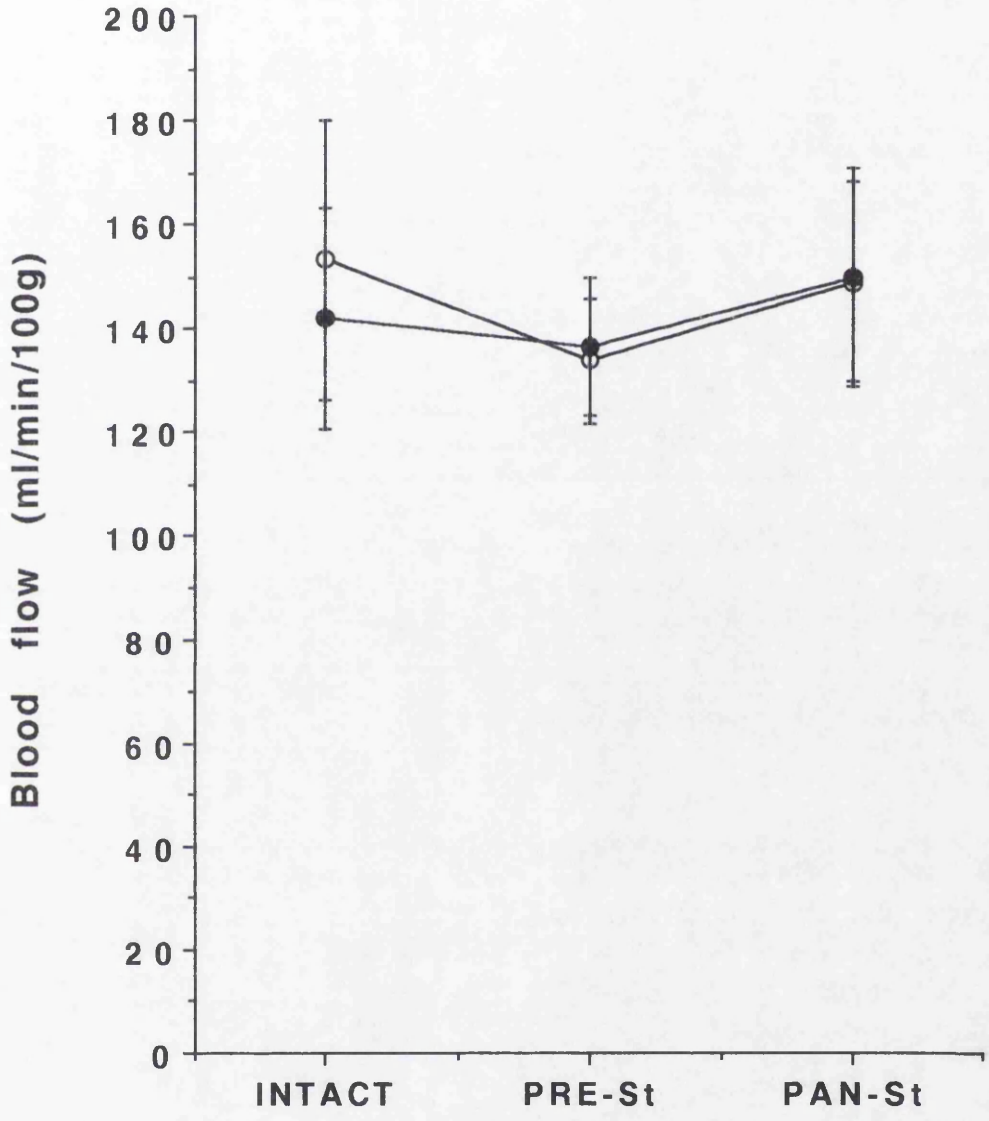
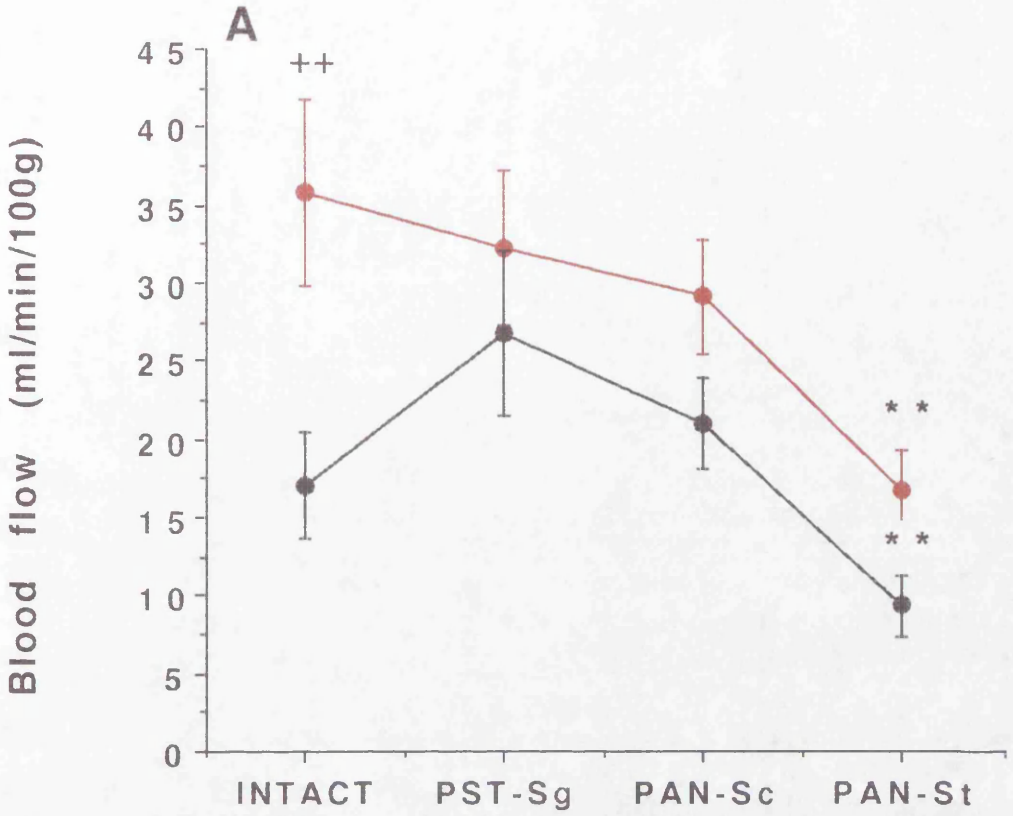
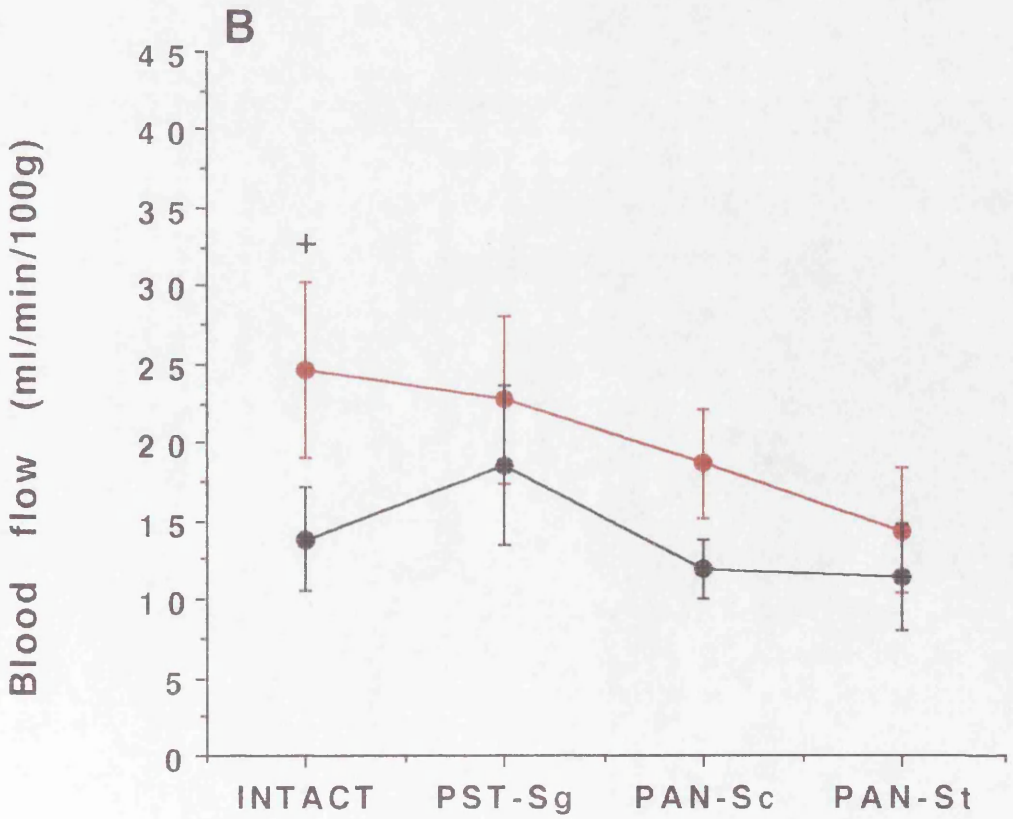

Blood flow (ml/min/100g)
200
180
160
140
120
100
80
60
40
20
0
INTACT
PRE-St
PAN-St

Fig 3.4. Changes in blood flow in the posterior (**A**) and anterior (**B**) joint capsule of the control knees (black symbols) and inflamed knees (red symbols); both knees intact (INTACT), after surgery to left knee (PST-Sg) and left PAN section (PAN-Sc), and during left PAN stimulation (PAN-St). Means ± SEM; n = 8-10. ** = P<0.01 significant difference with PAN-Sc (Wilcoxon test). ++ = P<0.01 significant difference between inflamed and control knees (Mann-Whitney).


A
Blood flow (ml/min/100g)
45
40
35
30
25
20
15
10
5
0
++
* *
* *
INTACT
PST-Sg
PAN-Sc
PAN-St


B
Blood flow (ml/min/100g)
45
40
35
30
25
20
15
10
5
0
+
INTACT
PST-Sg
PAN-Sc
PAN-St

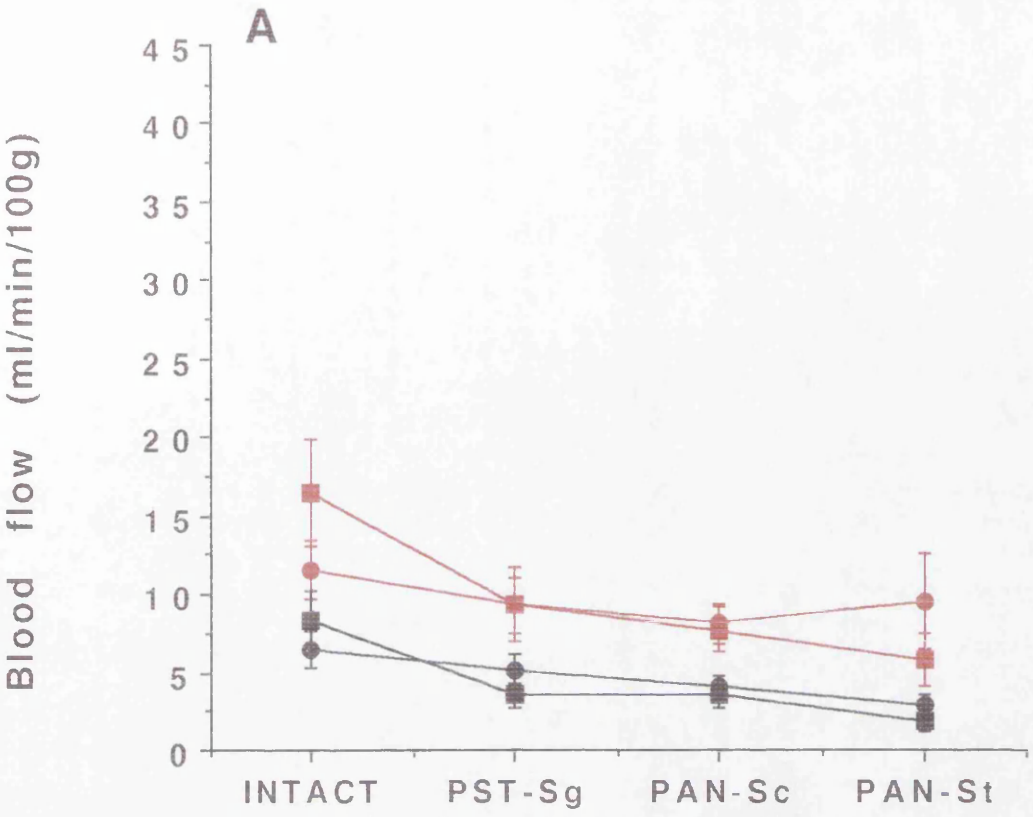
significantly increased during 24 hours after carrageenan injection (121.2 ± 4.1 mm before injection vs 136 ± 2.8 mm 24 hours after injection; $p<0.001$, $n=5$).

On the other hand, even in the control knee, capsular blood flow was double that occurring in the corresponding capsule of the normal group (fig 3.7A&B). This may reflect injury hyperaemia produced by the injection needle, as sterilized saline had been used. The surgical procedure on both knees, only produced a significant further increase in the blood flow to the posterior capsule of the control knee joint (fig 3.4, phase 2). As in the normal joint (fig 2.9, chapter two), PAN section caused no significant change in blood flow to the posterior or anterior capsules of either control or inflamed joints (fig 3.4, PAN-Sc). In both joints electrical stimulation of PAN, by the same parameters as in normal group, reduced the blood flow to the posterior region, but with different capabilities. Percentage reduction in blood flow was about 56% in the control and 45% in the inflamed joint, but no significant change was observed in the blood flow to the anterior regions (fig 3.4, PAN-St).

b. Blood flow to peri-articular tissues

Examination of the blood flow to other tissues harvested showed again differences depending on tissue type (fig 3.5). As observed before in normal joints, under resting conditions highest blood flow occurred in bone with the popliteus muscle showing the lowest blood flow values. In none of the four tissues in fig 3.5 was there a significant difference between different phases of the experiment or between the two joints (Mann-Whitney and Wilcoxon test). Non-significant difference between

Fig 3.5.Changes in blood flow in the **A**: popliteus muscle (circles) and patellar ligament (squares) and **B**: femoral condyles (circles) and tibial plateau (squares) of the right knees (black symbols) and left knees (red symbols); both knees intact (INTACT), after surgery to left knee (PST-Sg) and left PAN section (PAN-Sc), and during left PAN stimulation (PAN-St). Means ± SEM; n = 8-10.


A
Blood flow (ml/min/100g)
45
40
35
30
25
20
15
10
5
0
INTACT
PST-Sg
PAN-Sc
PAN-St

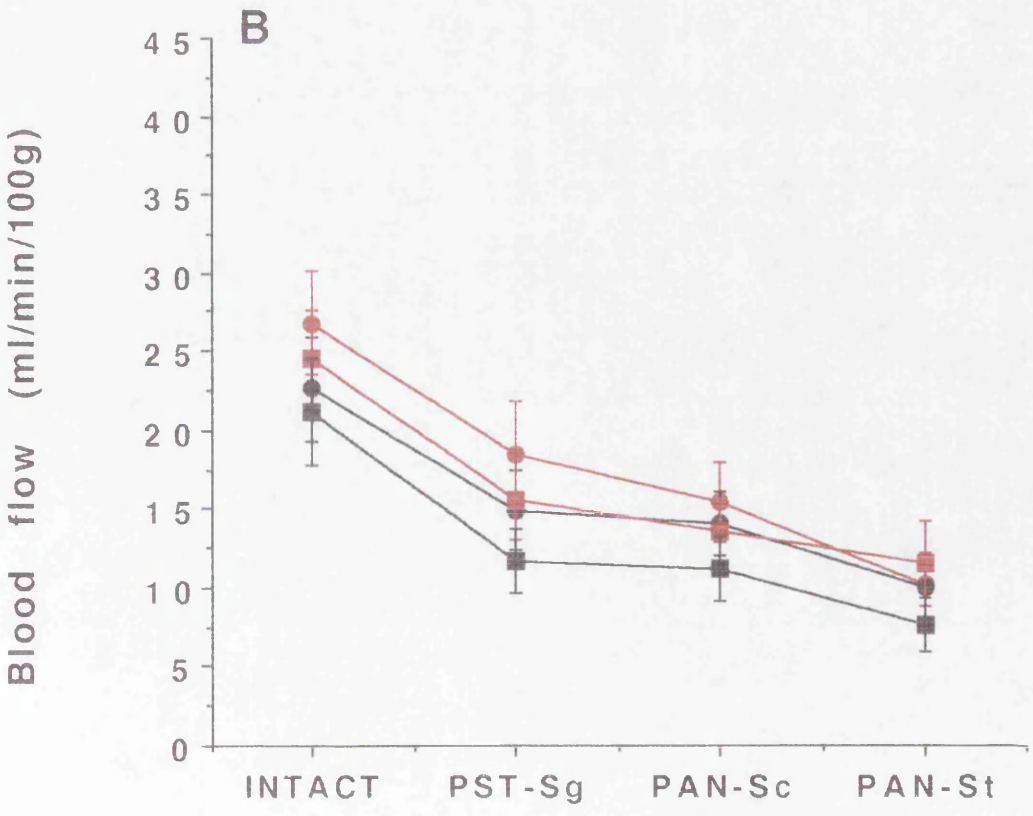
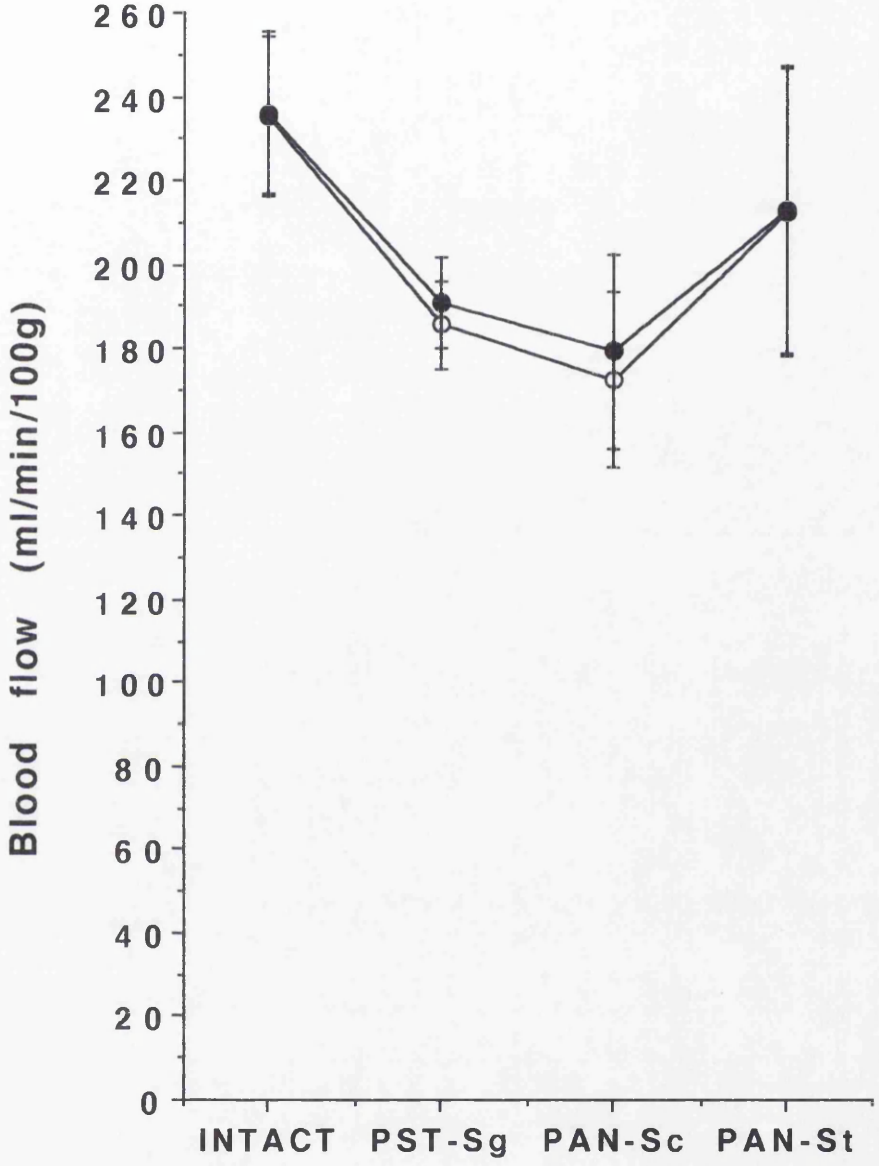

B
Blood flow (ml/min/100g)
45
40
35
30
25
20
15
10
5
0
INTACT
PST-Sg
PAN-Sc
PAN-St

Fig 3.6. Changes in blood flow in the right (closed symbols) and left (open symbols) kidneys; before starting surgery of the left knee (INTACT), after surgery to left knee (PST-Sg) and left PAN section (PAN-Sc), and during left PAN stimulation (PAN-St). Means ± SEM; n = 8-10.


Blood flow (ml/min/100g)
260
240
220
200
180
160
140
120
100
80
60
40
20
0
INTACT
PST-Sg
PAN-Sc
PAN-St

blood flow values before and after PAN stimulation (PAN-Sc vs PAN-St) suggests that none of these tissues is innervated by PAN.

Examination of the blood flow to the kidneys indicates that, as in the normal group, the blood flow in the right and left kidneys was not different (fig 3.6-INTACT). No significant difference was found between the two kidneys in any stage of the course of the experiments, which is important for validation of blood flow measurements.

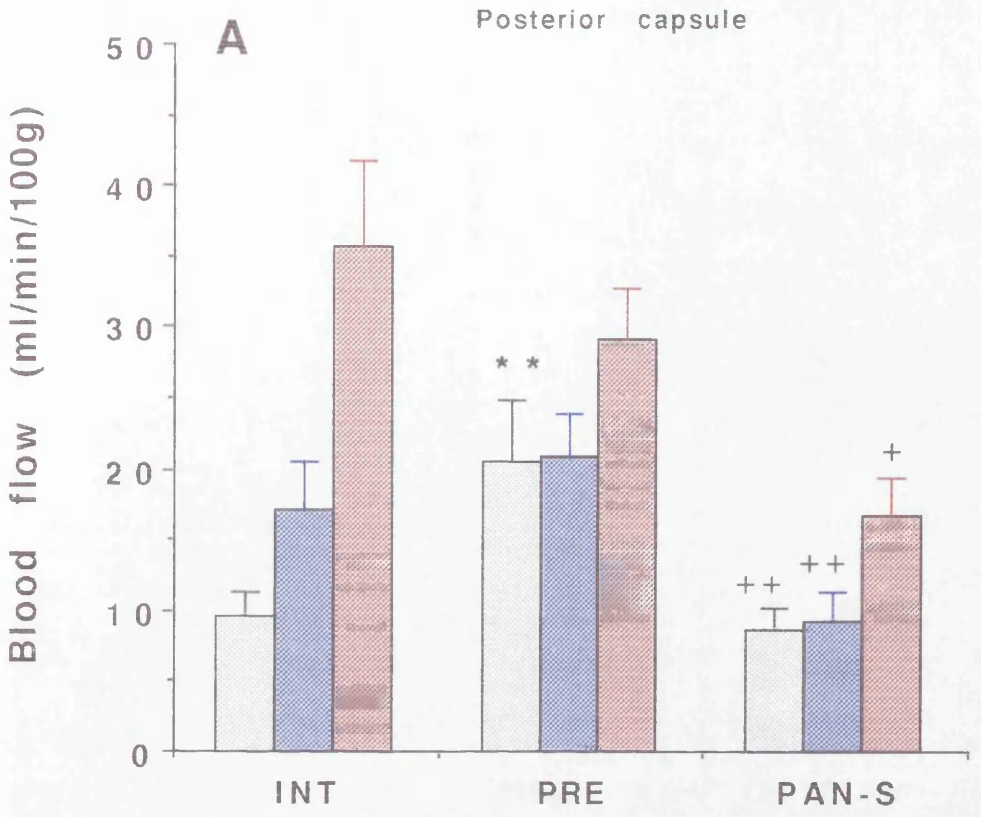
III. Comparison between normal and inflamed joints

The changes in tissues blood flow during experimental procedures in normal and inflamed joints are summarized and compared in figures 3.7-3.10. The blood flow to the posterior capsule of the normal knee increased significantly during the surgical procedures which leads to exposure of the capsule (fig 3.7A). Such an increase did not happen in the anterior capsule of the same knee (fig 3.7B) or any other tissue of the normal, control, and inflamed knees, even in the posterior capsule of the control and inflamed knees which also were exposed.

The blood flow to the anterior and posterior capsules of the saline injected (control) group showed a significant increase compared to the normal group ($P<0.05$, $n=8$) (fig 3.7), however none of the other tissues of the control knees showed differences in blood flow compared to the corresponding tissue in the normal knees (fig 3.8 & 3.9).

The process of inflammation produced a significant increase in blood flow to the posterior and anterior capsules, popliteal muscle and the patellar ligament of the inflamed knees, compared to the control knees. The tibia and femur showed no such increase in blood flow.

Fig 3.7. Blood flow measurement in **A**: posterior and **B**: anterior capsule of the normal (grey), control (blue) and inflamed(red) knee joints during different phases of the experiment; before starting surgery on the knee (INT), at the end of surgery and PAN section just before nerve stimulation (PRE) and during PAN stimulation (PAN-S). Means ± SEM; n = 8-10. ** = $P<0.01$ significant difference with intact (pre-surgical) conditions; + = $P<0.05$, ++ = $P<0.01$, significant difference with pre-stimulation conditions (Wilcoxon test). No significant alterations occurred in anterior capsule blood flow.


A
Posterior capsule
Blood flow (ml/min/100g)
50
40
30
20
10
0
* *
++
++
+
INT
PRE
PAN-S

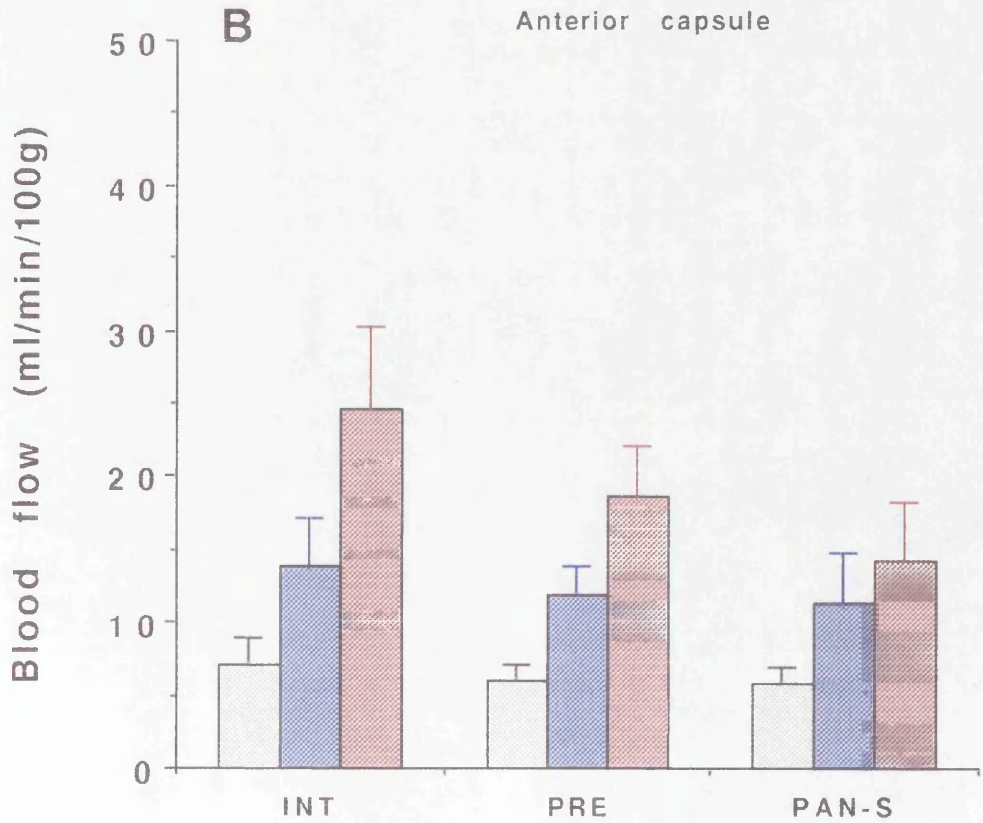
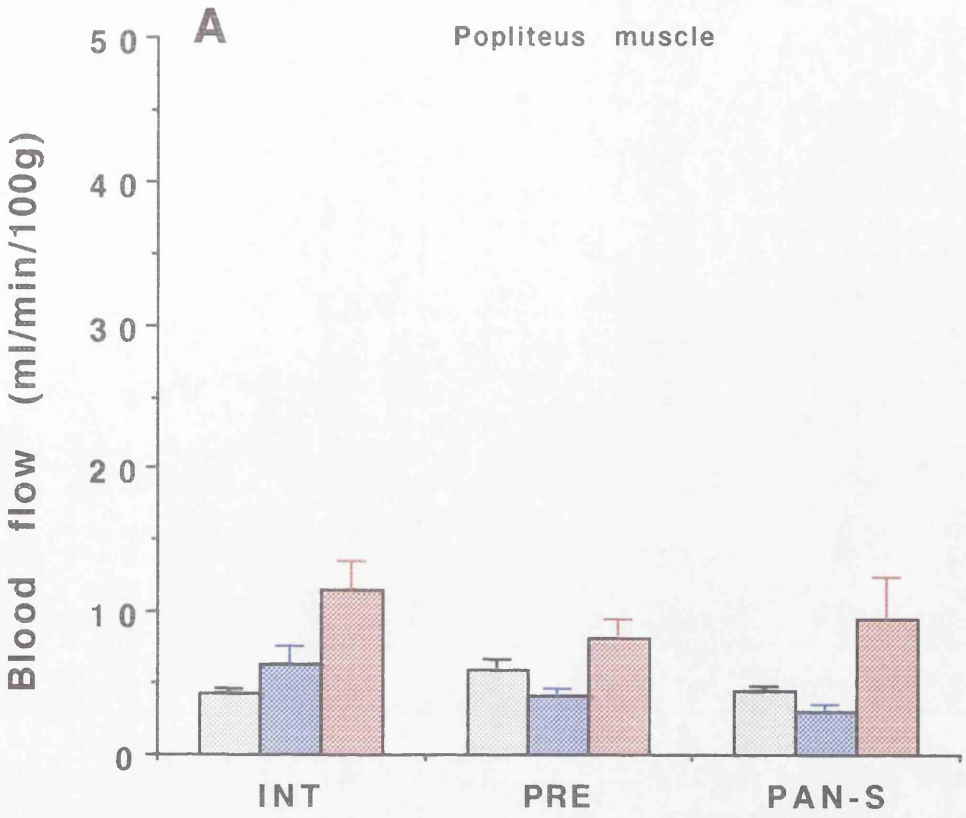
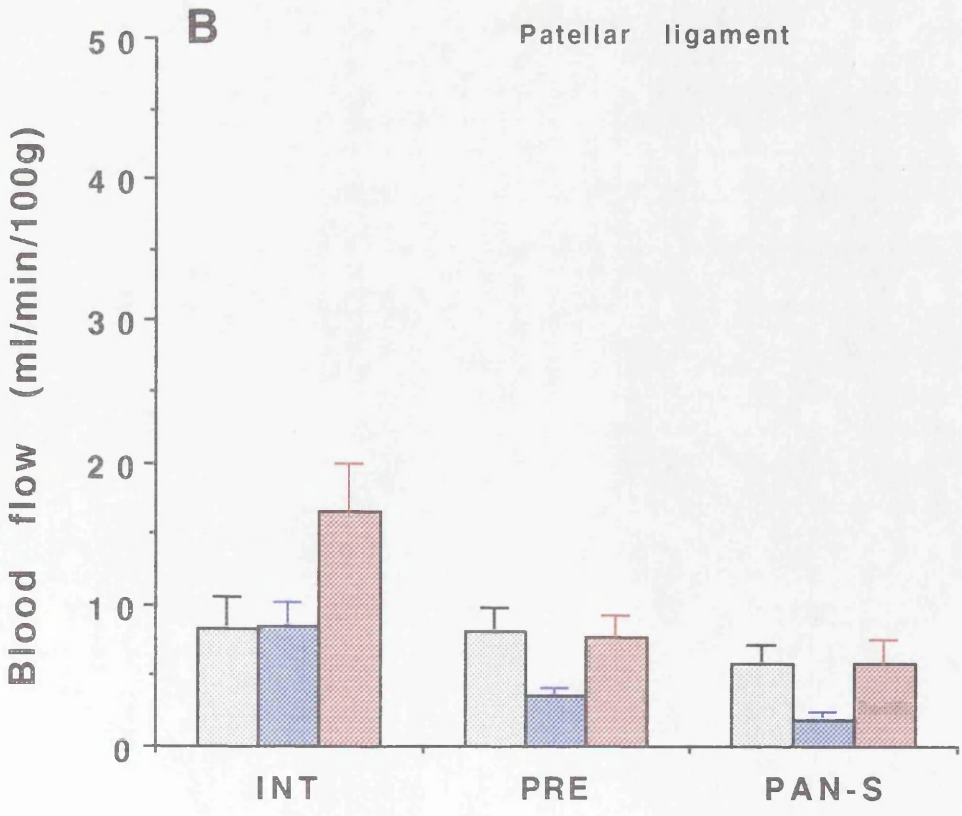

B
Anterior capsule
Blood flow (ml/min/100g)
50
40
30
20
10
0
INT
PRE
PAN-S

Fig 3.8. Blood flow measurement in **A**: popliteus muscle and **B**: patellar ligament of normal (grey), control (blue) and inflamed (red) knee joints during different phases of the experiment; before starting surgery on the knee (INT), at the end of surgery and PAN section just before nerve stimulation (PRE) and during PAN stimulation (PAN-S). Means ± SEM; n = 8-10. No significant alterations occurred between different stages in each group.

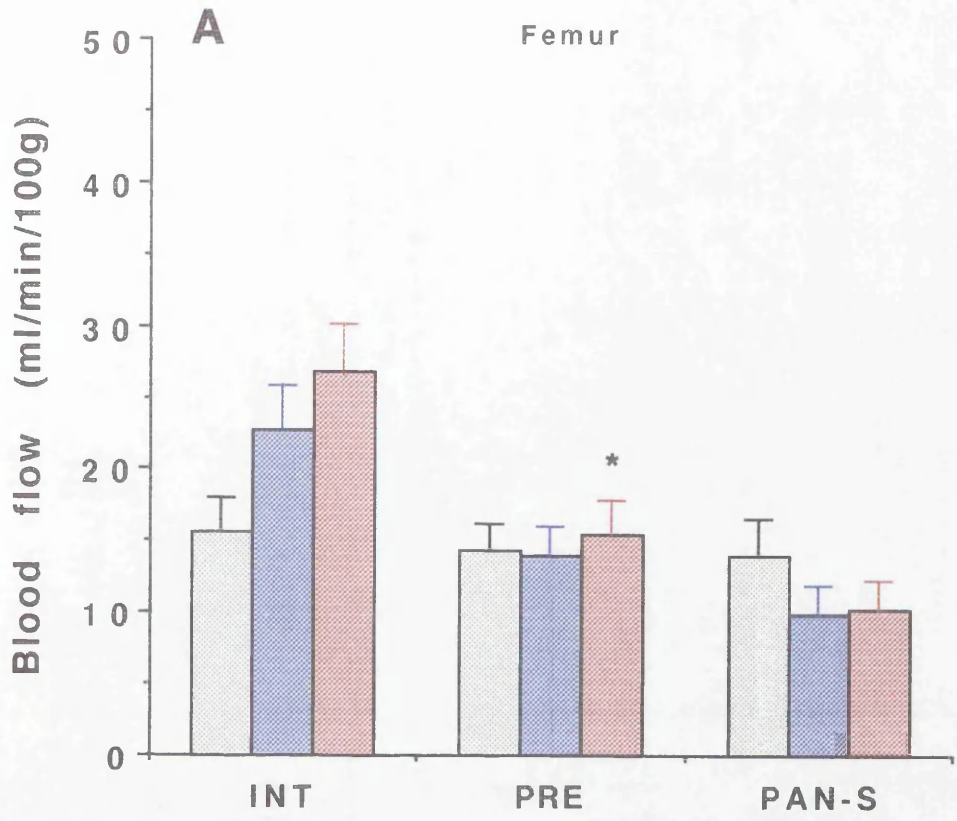

A
Popliteus muscle
Blood flow (ml/min/100g)
50
40
30
20
10
0
INT
PRE
PAN-S


B
Patellar ligament
Blood flow (ml/min/100g)
50
40
30
20
10
0
INT
PRE
PAN-S

Electrical stimulation of PAN produced a significant reduction in the blood flow to the posterior capsules of all knees but none of the other tissues, suggesting that this nerve only supplies the posterior capsule of the knee joint (PAN-S, figs 3.7-3.9).

Examination of the blood flow to the kidneys has shown a significant difference ($P<0.05$, $n=8$-10) between the normal and inflamed groups before starting the surgical procedure (fig 3.10, INT). However, in none of the experimental stages the blood flow showed significant changes in either kidneys. (see also fig 3.3).

Fig 3.9. Blood flow measurement in **A**: femoral condyle and **B**: tibial plateau of the normal (grey), control (blue) and inflamed (red) knee joints during different phases of the experiment; before starting surgery on the knee (INT), at the end of surgery and PAN section just before nerve stimulation (PRE) and during PAN stimulation (PAN-S). Means ± SEM; n = 8-10. * means $P<0.05$, significantly different from intact (INT); Wilcoxon test.


A
Femur
Blood flow (ml/min/100g)
50
40
30
20
10
0
*
INT
PRE
PAN-S

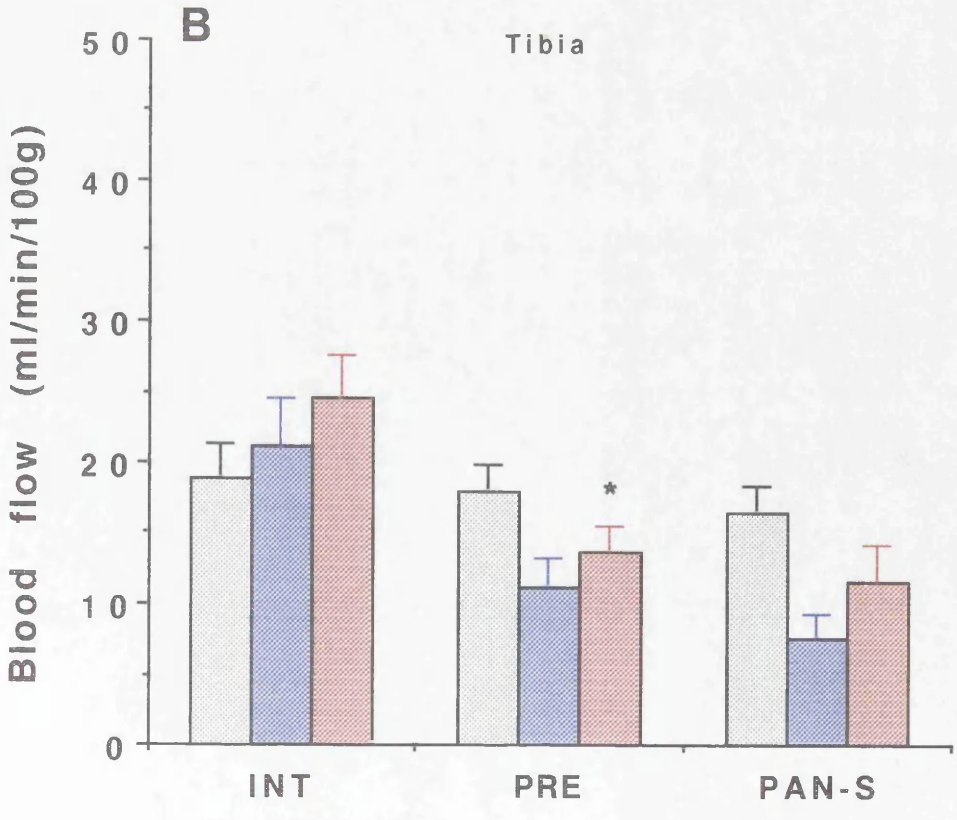
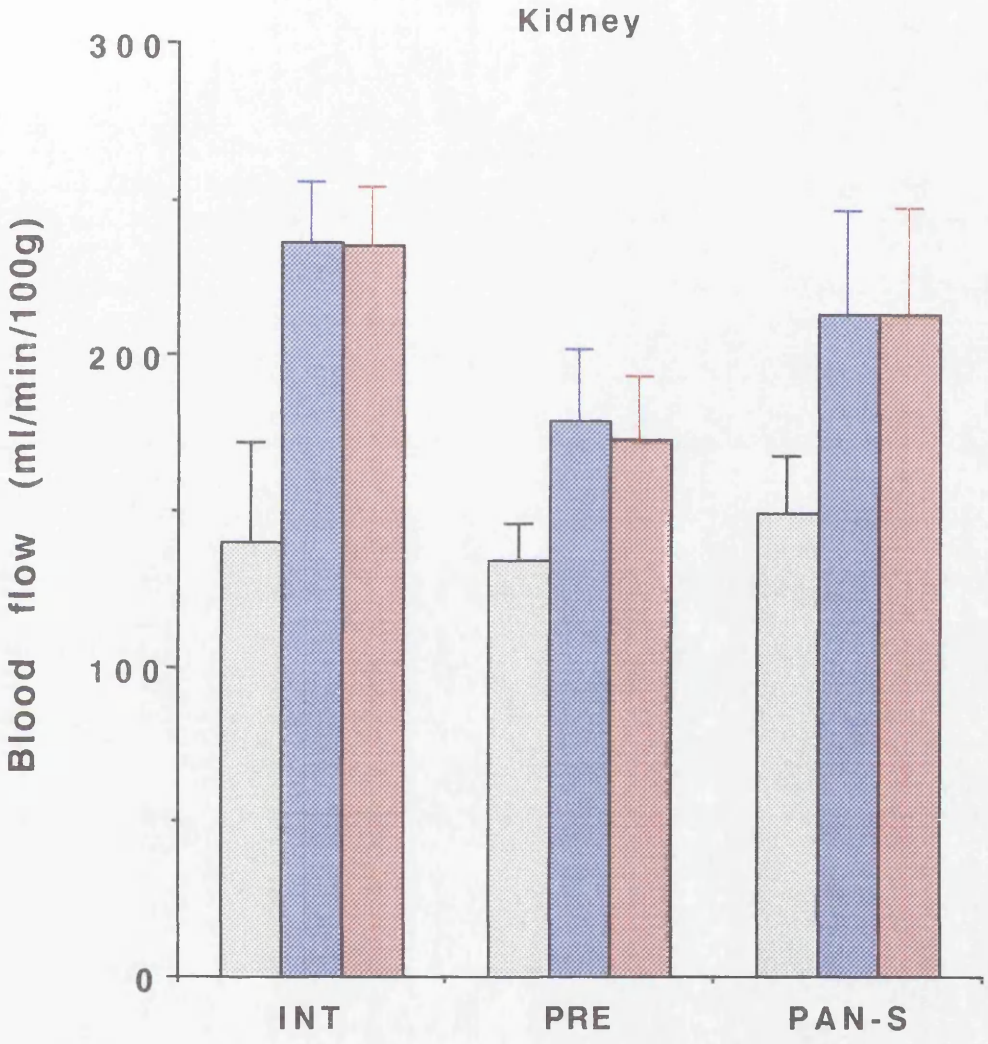

B
Tibia
Blood flow (ml/min/100g)
50
40
30
20
10
0
*
INT
PRE
PAN-S

Fig 3.10. Blood flow measurement in the left kidney of the normal (grey); and right (blue) and left (red) kidney of the carrageenan treated animal during different phases of the experiment; before starting surgery on the knee (INT), at the end of surgery and PAN section just before nerve stimulation (PRE) and during PAN stimulation (PAN-S). Means ± SEM; n = 8-10. Although the initial (INT) kidney blood flow of the carrageenan treated group (blue & red columns) was higher than the normal group (see text), no significant alterations occurred within each group between different stages of the experiment .


Kidney
300
200
100
0
Blood flow (ml/min/100g)
INT
PRE
PAN-S

Discussion

a. Method

The radiolabelled microsphere technique is a widely used method for blood flow measurement. It is a reliable method for quantitative blood flow measurement, and the total and regional blood flow can be determined. It is also possible to measure the blood flow to many organs and tissues simultaneously, and this can be done in both anaesthetized and conscious animals. Its main disadvantages are discontinuous blood flow monitoring and lack of flow measurement within small volume of tissue (e.g. a few cubic millimetres). Other disadvantages are the use of radio-isotopes and the need to sacrifice the animal or removing the tissue under the study which limits its clinical usefulness (Tothill, 1984). The repetitive measurement of flow in the organ or tissue is also limited. Some sources of error in measuring regional blood flow by this technique have been reviewed by Buckberg and his colleagues (1971). They concluded that using small number of microspheres, inadequate mixing of them before injection and disposition of the tip of the catheter in the heart could all be the sources of error in blood flow measurement. However, in the present study attempts were made to avoid or minimize these errors. The total number of spheres in each injection was $1.5X10^6$. According to a recent study, it was concluded that 0.5×10^6 spheres per Kg weight would be enough for an accurate estimation of blood flow in tissue such as bone (Li *et al.*, 1989), so it seems that the number of spheres used in these experiments was adequate. Checking that the tip of the cannula was in the left ventricle was carried out by monitoring ventricular pressure during the experiment except during the injection phase, and also at the end of

experiment by opening the heart. Finally, the adequate mixing of microspheres was done by shaking the solution containing microspheres thoroughly with a whirlimixer before injection, and it was also checked by examining both kidney counts.

b. Results

The present experiments demonstrate that exposure of the posterior aspect of the knee joint capsule and the associated surgery resulted in significantly increased blood flow. There could be two reasons for such increase in blood flow. The first possibility is loss of vasoconstrictor tone due to posterior articular nerve (PAN) section. However, laser Doppler flowmetry in the posterior capsule of the normal joint (fig 2.9) and microsphere technique in the control and inflamed joints (fig 3.4, PAN-Sc) showed no significant change in blood flow after nerve section, suggesting that the basal tone in this vascular bed in these anaesthetized animals is not greatly influenced by sympathetic nerves travelling in PAN. The second possibility is injury hyperaemia which is more likely as the increase in blood flow was specific to the posterior capsule of the operated knees (figs 3.1A & 3.4A) which was exposed and not to any of the other joint tissues examined which were not really exposed. The blood flow of the posterior capsule of the inflamed joint did not increase due to surgery probably because it had already been increased due to inflammation. Although subsequent PAN stimulation significantly reduced blood flow to the posterior capsule of the normal, control, and inflamed knees (figs 3.1A & 3.4A), it did not fall to the same extent, as the percentage of reduction compared to their pre-stimulation value was ~ 60%, ~56%, and ~45% in the normal, saline injected, and inflamed posterior capsules

respectively. The difference between reductions in inflamed and control joints just failed to reach significance. The actual nerve stimulation-mediated reduction in the inflamed joints may had been smaller than 45%, as drift in blood flow occured during the course of experiment in these joints but not in the normal and control joints (figures 3.1 & 3.4). If the drift is taken into account then the difference is likely to become significant. The difference between inflamed and control or normal joint could be the result of reduced effectiveness of sympathetic vasoconstrictor nerves due to inflammation as it was observed in other experiments using laser Doppler flowmetry (fig 6.4, chapter six). Recent experiments on rat knee joint using laser Doppler perfusion imaging (Lam & Ferrell, 1992) have also shown that nerve-mediated vasoconstriction is reduced in inflamed joints.

The magnitude of the constrictor response was significantly ($P<0.05$; Mann-Whitney) greater in the posterior region (56±4.3%; n=8) than in the anterior region (42.1±5.4%; n=9; from Ferrell *et al.*, 1990), perhaps due to a greater density of innervation posteriorly. However, although it was previously shown that stimulation of the rabbit saphenous nerve reduced bone blood flow (Ferrell *et al.*, 1990), there was little evidence of this with stimulation of the posterior articular nerve. None of the surrounding tissues appeared to have been affected by stimulation of this nerve, suggesting that PAN innervation is specific to the posterior capsule. This specificity is experimentally useful as it indicates that PAN stimulation will only affect joint capsule blood flow and not produce haemodynamic changes in surrounding tissues which could complicate the interpretation of the capsule data.

Blood flow to the control joint increased significantly 24 hours after saline injection compared to the normal joint (fig 3.7, INT). One reason could be the injury hyperaemia produced by insertion of needle into the joint space. However, the blood flow to both anterior and posterior capsules were increased whilst the needle had been inserted through the anterior capsule only. The other possibility could be contamination of the injected saline, however sterilized saline and syringes were used and the place of injection was also sterilized by a piece of cotton wool soaked in alcohol before injection. The more likely possibility is the transmission of the inflammation from the inflamed knee to the contralateral knee, as it has been shown that inflammation of one knee can produce a symmetrical inflammation of the other knee (Kidd *et al.*, 1989)

Although ligaments are generally regarded as having a low capillary density, the patellar ligament had surprisingly high blood flow compared to other tissues. This was perhaps due to removal of the ligament with underlying synovium attached. As the synovium is known to be densely vascularised (Knight & Levick, 1983), this could have artificially increased the true value for the ligament itself. This highlights a previously recognised problem (Levick, 1987): synovial blood flow is underestimated by techniques in which flow is measured per unit mass of tissue, as a result of the marked variation in capillary density of tissues investing the joint (Knight & Levick, 1983). Nevertheless the technique does permit comparisons of the effects of various interventions and also permits assessment of the effects of joint inflammation.

CHAPTER FOUR

Changes in synovial PO_2 and blood flow in normal and acutely inflamed rabbit knee joints following stimulation of the posterior articular nerve

Summary

1. Experiments were performed to measure the partial pressure of oxygen in the synovial fluid (P_SO_2) of normal and acutely inflamed rabbit knee joints and assess the extent to which this varied with changes in knee joint blood flow.

2. With the hypodermic needle oxygen electrode sited within the synovial cavity, mean (± S.E.M.) P_SO_2 values were 48.2 ± 3.1 mmHg (n=18) and 37.4 ± 3.6 mmHg (n=10) in normal and inflamed joints respectively, which differed significantly ($p< 0.05$).

3. P_SO_2 was found to decrease with increasing depth of penetration of the oxygen electrode in both normal and inflamed joint cavity. Lowest values were observed close to the articular cartilage.

4. Relative changes in blood flow were assessed using laser Doppler flowmetry. Electrical stimulation of the posterior articular nerve (PAN) of the knee resulted in vasoconstriction of knee joint blood vessels which was accompanied by decrease in P_SO_2. The frequency/response and voltage/response profiles to electrical stimulation of PAN, although differing in magnitude, showed a high degree of correlation between blood flow and P_SO_2.

5. The frequency-response profile to electrical stimulation of PAN shifted to the right in the inflamed joint, suggesting a reduction in the efficacy of the sympathetic nervous system in regulation of blood flow to the inflamed joints.

6. As judged by the conduction velocity, the vasoconstrictor response to nerve stimulation was mediated by unmyelinated nerve fibres, presumed to be sympathetic postganglionic fibres.

7. Although the microsphere technique revealed that the inflamed joint had the higher blood flow, P_sO_2 was lower compared to the normal joint.

8.The results of this study show significantly altered P_sO_2 and nerve mediated constrictor responses in the acutely inflamed joint. In view of the low P_sO_2 values occurring deep within the joint, avascular structures such as cartilage could be subject to injury if sustained reduction in synovial blood flow occurred.

Introduction

Synovial fluid which is the supplier of nutrients to avascular structures within the joint (McKibbin & Maroudas, 1979), is formed by synovial blood flow (for review see Levick, 1987).Therefore those factors which regulate flow in the synovial vascular bed are clearly important in this process. An important nutrient provided by synovial fluid is oxygen but the relationship between the partial pressure of oxygen in synovial fluid (P_sO_2) and synovial blood flow has not been investigated. There is good evidence in various species to show that nerves innervating joints contain sympathetic postganglionic fibres which constrict articular blood vessels during electrical stimulation (Cobbold & Lewis, 1956; Khoshbaten & Ferrell, 1990b). The ability to manipulate synovial blood flow by experimentally altering the calibre of articular blood vessels provides the opportunity to examine the extent to which P_sO_2 is affected by changes in blood flow.

An additional object of this study was to examine P_sO_2 in normal and acutely inflamed joints. The PO_2 of synovial fluid from diseased human joints has been measured, the technique used invariably involved aspiration of an effusion. Actual PO_2 values varied widely, but in many patients with rheumatoid arthritis it was observed that intra-articular PO_2 values were significantly lower than venous PO_2 values (Falchuk, Goetzl & Kulka, 1970; Lund-Olesen, 1970; Treuhaft & McCarty, 1971), with anoxia occuring in some cases (Lund-Olesen, 1970; Richman, Su & Ho, 1981) even though the affected joints were inflamed and therefore hyperaemic. To explain this paradox it has been suggested

that intra-articular hypoxia occurs due to a circulatory-metabolic imbalance (Falchuk, Goetzl & Kulka, 1970; Goetzl, Falchuk, Zeiger, Sullivan, Hebert, Adams & Decker, 1971). However, the significance of low PO_2 values in synovial fluid aspirated from diseased joints is difficult to interpret as there are no values from normal joints to allow comparisons. In addition, most of the measurements of PsO_2 from humans have been obtained from chronically diseased joints, so it is not known what has happened during the course of disease. In recent experiments from this laboratory (Khoshbaten & Ferrell, 1990a) it has been found that vasoconstrictor responses to electrical stimulation of sympathetic nerves to the joint were increased within eight hours of joint inflammation produced by injection of kaolin into the joint cavity. However, subsequent events and the changes in P_sO_2 were not investigated in that study.

The present study was performed to examine the actual values of P_sO_2 and how these are affected by changes in synovial blood flow in normal and acutely inflamed rabbit knee joints. This would provide baseline values for the examination of these parameters in experimentally induced chronic arthritis in future experiments.

Methods

a. Animal preparation and surgical procedures

Experiments were performed on 28 adult New Zealand rabbits (2.2-3.5 kg) which were anaesthetised initially by injection of diazepam (1.5mg/kg I.P.) and Hypnorm (Janssen; 0.15ml/kg I.M.) and thereafter with a 1% halothane/O_2/N_2O mixture during the surgical procedures. On completion of surgery, the gaseous anaesthetic was discontinued and a slow continuous infusion of pentobarbitone (0.25-0.5mg/min depending on the weight of the animal) administered throughout the remainder of the experiment via a cannula inserted into the left jugular vein. The trachea was cannulated, with the animals breathing spontaneously throughout the experiments. Deep anaesthesia was maintained throughout as judged by the absence of a flexor withdrawal response to noxious stimuli applied to the forelimb. Arterial blood pressure was monitored throughout the experiment by a cannula inserted into the left carotid artery.

The posterior articular nerve (PAN), which arises from the tibial nerve in the popliteal fossa, was dissected free, after removal of popliteal fat pad and the medial gastrocnemius muscle, and in most experiments transected proximally. Bipolar bright silver stimulating electrodes were then placed on PAN and pulse trains delivered. The stimulus parameters were: width 1ms, voltage 1-15V, frequency 0.5 to 70 pulses/s and pulse train duration of 90s. In some experiments platinum recording electrodes were placed on PAN whilst the stimulating electrodes were placed more proximally on the tibial nerve

which was transected rostrally along with all its distal branches except PAN. This arrangement allowed recording of the compound action potential from PAN (Digitimer Neurolog system) which was recorded on magnetic tape along with stimulus pulses and the signal from the flowmeter.

b. Blood flow measurement

A laser Doppler flowmeter (Moor Instruments MBF3) connected to a 0.9mm diameter fibre-optic probe provided a measure of relative changes in blood flow in the volume of tissue beneath the tip of the probe. Previous experiments have validated the use of this technique in assessing alterations in knee joint blood flow in both cats and rabbits (Ferrell, Khoshbaten & Angerson 1990). The probe was positioned just above the surface of the postero-medial aspect of the knee joint capsule. Experiments have shown that alterations of blood flow in the synovium are detected with the probe in this position (fig 2.13, chapter two). Alterations in blood flow are expressed as percentage change from control (0%) values occurring immediately before the test procedure.

c. Measurement of P_sO_2

Oxygen tension in synovial fluid was monitored by means of a hypodermic needle O_2 electrode (Diamond General) which was connected to an O_2 meter (Strathkelvin Instruments). This provided a linear measure of PO_2 over a range of 0 to 160mmHg. The method of calibration of the oxygen meter was detailed in chapter two. The electrode was advanced into the synovial cavity by means of a microdrive system (Narashigi). Alterations in oxygen tension are

expressed as a percentage change from control (0%) values occuring immediately before the test procedure.

d. Induction of inflammation

Acute knee joint inflammation was produced by intra-articular injection of 1 ml of 2% solution (W/V) of carrageenan (Sigma) in saline into the joint space through the anterior region (mid-patellar tendon) via a 24G needle, 24 hours before the experimental procedure.

e. Statistical analysis

The values expressed in the histogram and graphs are means $\pm$ SEM and are compared to control values using the student's paired or unpaired t-test as appropriate.

Results

a. Relationship between articular blood flow and P_SO_2

Insertion of the needle oxygen electrode through the posterior capsule to just within the synovial cavity showed some variation in the P_SO_2 values obtained in different animals, ranging from 25 to 72mmHg in the normal and from 20.5 to 52 mmHg in the test group. Overall, the mean value was 48.2 $\pm$ 3.1 mmHg in the normal and 37.4 $\pm$ 3.6 mmHg (SEM) in the inflamed joints and these were significantly different ($p=0.02$, unpaired t-test). The initial step in this investigation was to check that P_SO_2 was related to blood flow to the knee joint by examination of the effect of occlusion of the arterial supply to the joint, proximal to the articular branches, on blood flow and P_SO_2. As illustrated in figure 4.1, arterial occlusion for 90 seconds in a normal knee joint resulted in a predictable fall in both blood flow and P_SO_2, although with differing time courses. The failure to produce complete cessation of blood flow during the period of occlusion was probably due to incomplete occlusion of the artery, or to collateral arterial supply as the knee is known to have an extensive anastomotic arterial network (Liew & Dick 1981). It was noticeable that with the occlusion period chosen, there was little evidence of reactive hyperaemia in any of the preparations examined, and, when tested in a few cases, even longer periods of occlusion lasting five minutes still failed to give rise to hyperaemia.

PAN stimulation produced significant vasoconstriction which was clearly frequency dependent. Alterations in P_SO_2 clearly mirrored the

reductions in blood flow although the magnitude of these changes were smaller than the changes in blood flow (fig 4.2 & 4.3 & 4.4). Compared to the normal joint, the time course of changes in P_SO_2 in the inflamed joint was significantly longer. With maximal frequency of stimulation (10 Hz in normal and 30 Hz in inflamed) the time from the beginning of O_2 reduction to its full recovery, measured in six animals of each group was 8.3±0.92 minutes in normal, and 10.9±1.06 minutes in inflamed joints ($p<0.05$, unpaired t-test).

In the inflamed joint for both blood flow and P_SO_2, maximum reduction was observed at frequency of 30 pulses/s or over with little effect occurring below 1 pulse/s (fig 4.3). However in the normal joint maximum reduction of both blood flow and P_SO_2 happened at frequency of 10 pulses/s (fig 4.3). A sharp increase in response was seen over a short range of frequencies with a large increment occurring between 1 to 10 pulses/s. The percentage reduction in P_SO_2 followed the trend of blood flow changes although there was more variability in the former (fig 4.3B). P_SO_2 was highly dependent on blood flow with a correlation coefficient (Pearson's r) for the frequency/response relationship of 0.943 ($p<0.001$; t-test) in the normal and 0.81 ($p<0.001$; t-test) in the inflamed joint.

The voltage/response relationship (fig 4.4) was also highly correlated ($r = 0.977$; $P<0.001$ in the normal and $r = 0.77$; $p<0.001$, in the inflamed joint.). Comparison of the two correlation coefficients between normal and inflamed joints showed that these correlations differed significantly from each other ($P<0.001$ in each case).

Fig 4.1. Arterial occlusion for 90s (at black bar) resulted in sustained reduction in blood flow and fall in PO_2 of synovial fluid. O_2 electrode sited just within the the synovial cavity. PO_2 reduction has a longer time course than the blood flow changes. No evidence of reactive hyperaemia was seen on cessation of occlusion.

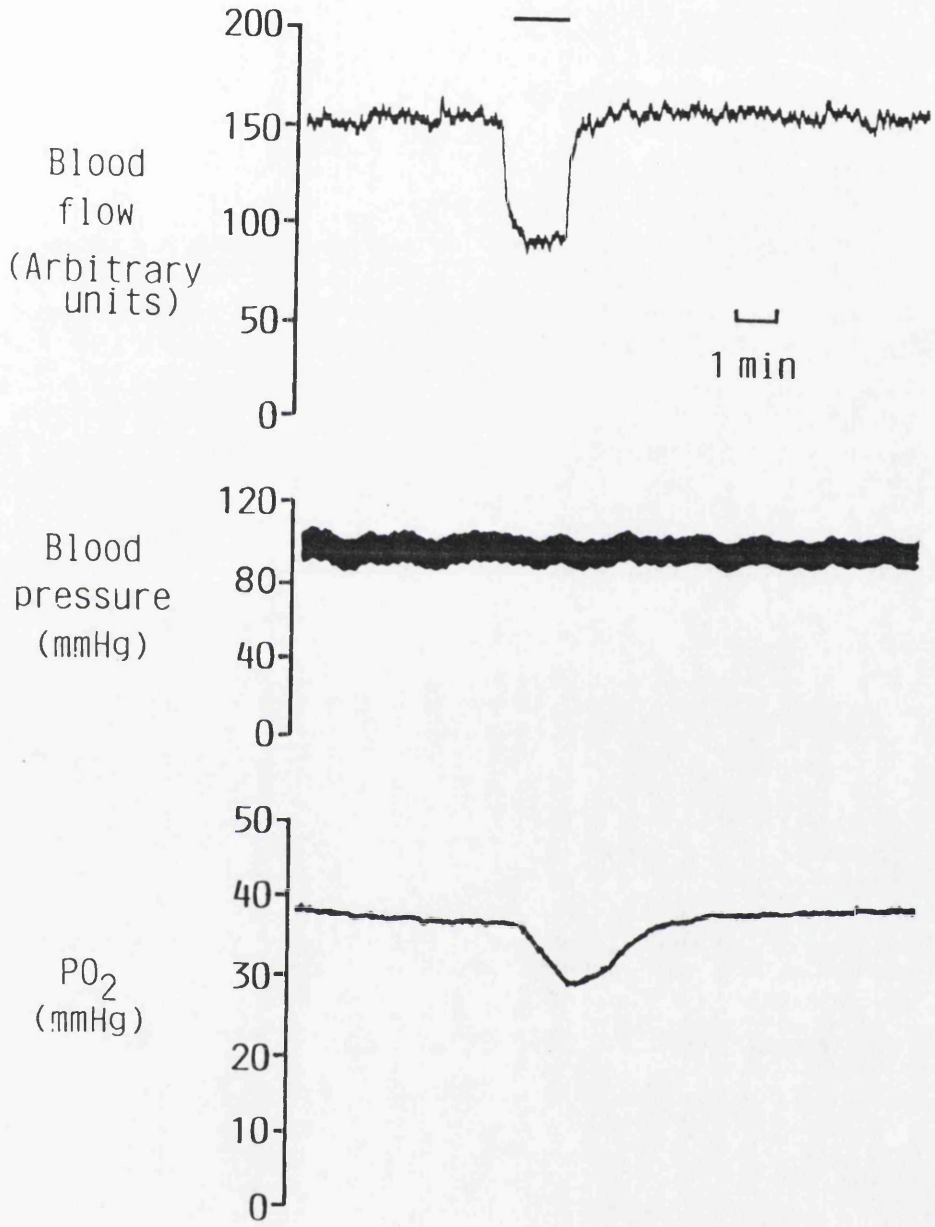

200
150
100
50
0
Blood
flow
(Arbitrary
units)
1 min
120
80
40
0
Blood
pressure
(mmHg)
50
40
30
20
10
0
PO_2
(mmHg)

Fig 4.2. Electrical stimulation of PAN (at black bars) in normal (A) and inflamed (B) rabbit knee joints produced a reduction in the flowmeter signal (upper trace) indicating vasoconstriction of joint blood vessels which was mirrored by reductions in the synovial fluid PO_2. Both responses were frequency dependent. The time course of changes in blood flow are similar* in both groups but recovery time for PO_2 changes is longer in inflamed joints. Stimulus parameters, 90s trains of pulses, 10V amplitude, 1ms width and frequencies of 5-70 pulses/s (shown over bars).

* e.g. analysis of the area of the time course changes in blood flow trace by the frequency of 20 Hz in which equal responses were produced in both groups (see next figure) measured in ten animals of each group showed no statistically significant difference between normal and inflamed joints (81.4±10.6 mm^2 in normal joint *vs* 68.8±10.4 mm^2 in inflamed; P= 0.203, unpaired t-test).

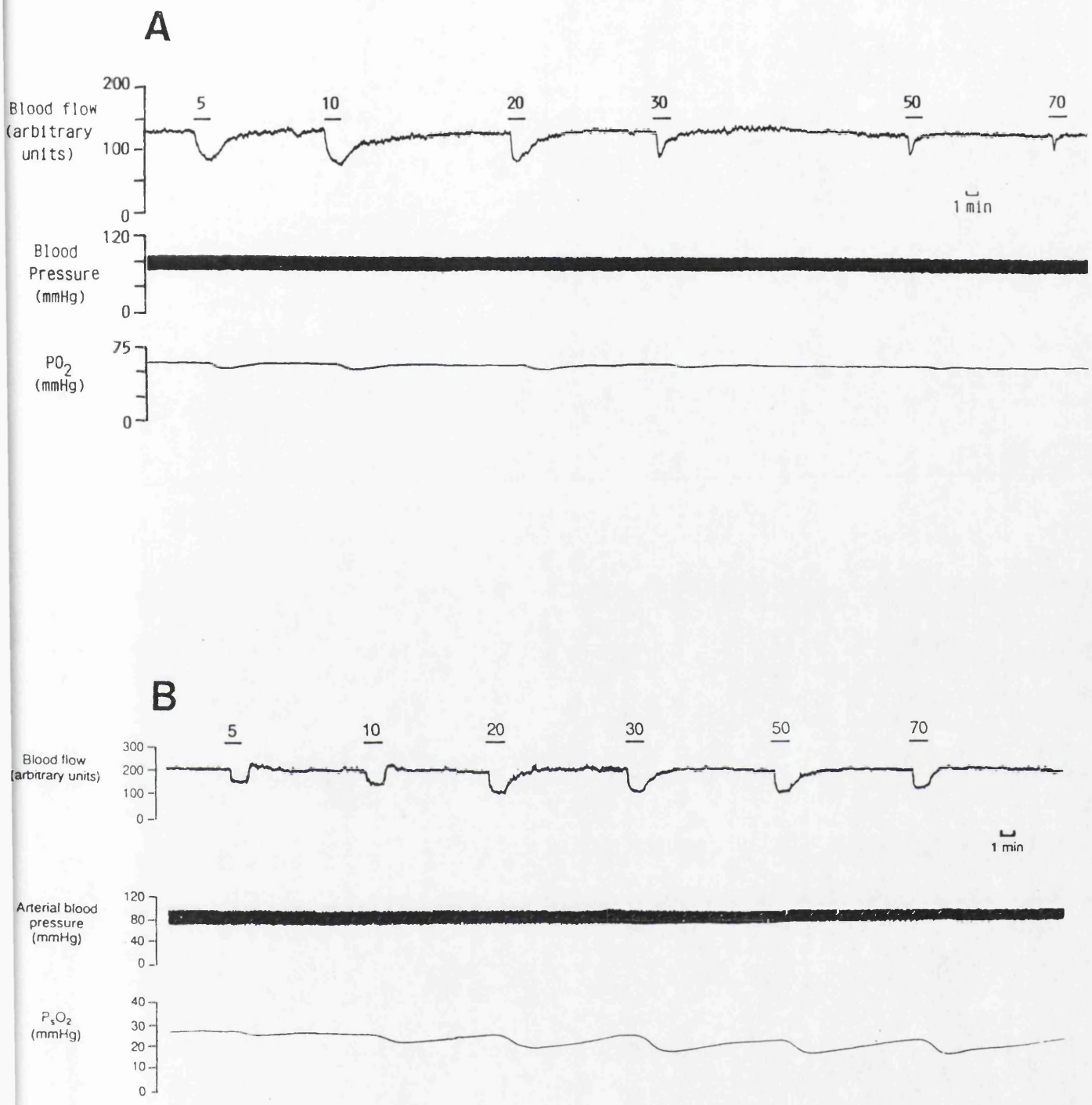
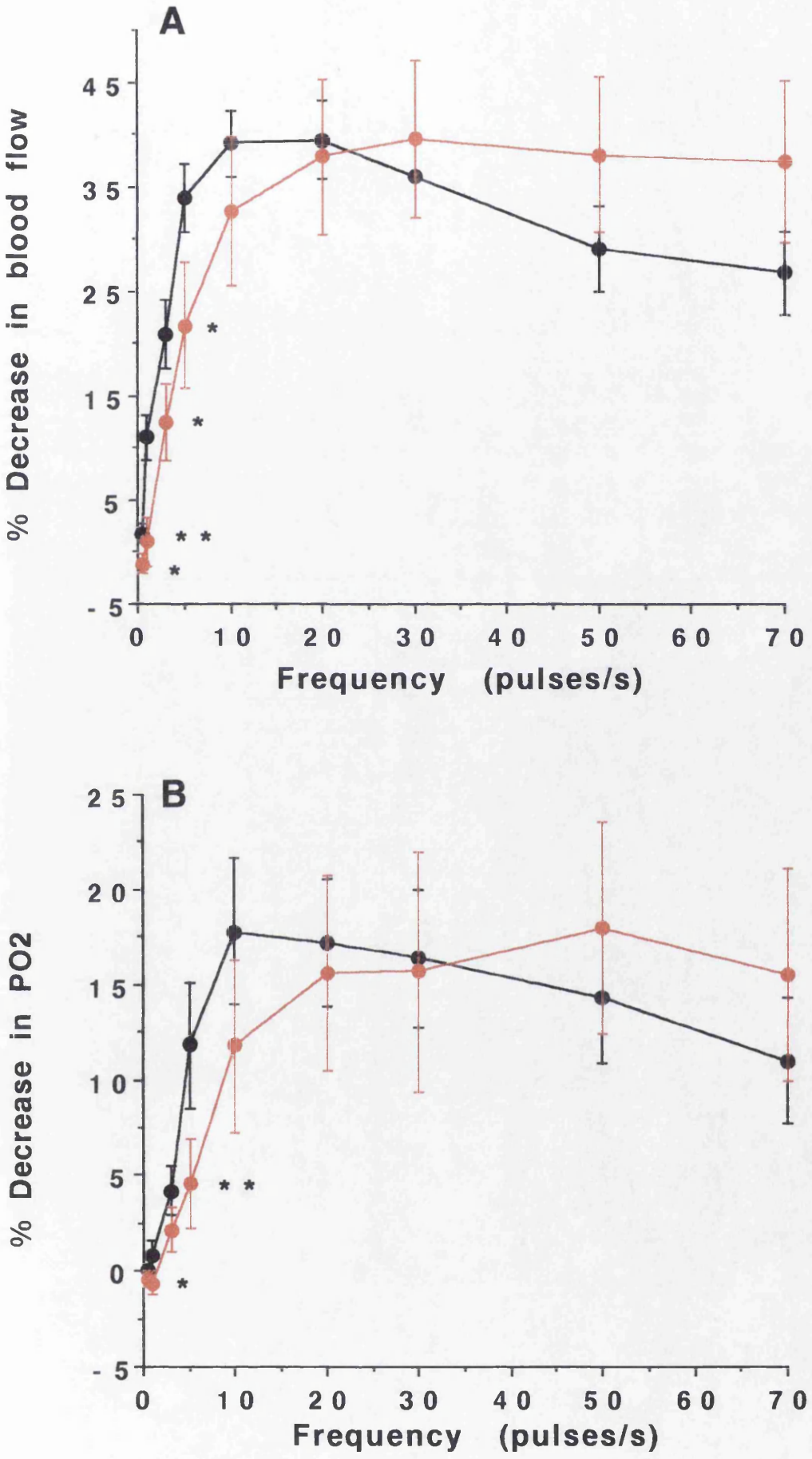

A
Blood flow (arbitrary units)
200
100
0
5
10
20
30
50
70
1 min
Blood Pressure (mmHg)
120
0
PO2 (mmHg)
75
0
B
Blood flow (arbitrary units)
300
200
100
0
5
10
20
30
50
70
1 min
Arterial blood pressure (mmHg)
120
80
40
0
PsO2 (mmHg)
40
30
20
10
0

Fig 4.3. **A**: Blood flow reductions (% decrease from control = 0%) as a function of frequency of stimulation of PAN (10V; 1ms; 90s train) in normal (black symbols) and inflamed (red symbols) joints. Significant differences occured at 1, 3 & 5 pulses/s.

B: Frequency dependent changes in synovial PO_2 - same stimulus parameters and symbols as in A. (significant differences occured at 1 & 5 pulses/s).

* = $p<0.05$; ** = $p<0.01$ - means differ significantly from the response in normal joint. n=13-17 in normal and n=10 in inflamed.


A
% Decrease in blood flow
45
35
25
15
5
- 5
0
10
20
30
40
50
60
70
Frequency (pulses/s)
B
% Decrease in PO2
25
20
15
10
5
0
- 5
0
10
20
30
40
50
60
70
Frequency (pulses/s)

At a fixed frequency of 10 pulses/s and pulse width of 1ms, in both groups the maximum constrictor response was obtained at an amplitude of 10 volts (fig 4.4). This suggests that at this voltage all unmyelinated fibres were recruited. This was confirmed in a smaller series of experiments on normal animals where electrophysiological recordings were obtained from PAN during electrical stimulation of the cut distal end of the tibial nerve after all branches except PAN were sectioned (fig 4.5). Electrical stimulation resulted in clear reduction of blood flow in the joint capsule but only when the stimulus strength was sufficient to recruit group IV (C) fibres, as shown by the C wave in the compound action potential. Stimulus strengths sufficient to recruit group II and group III afferents had no discernible effect on blood flow (fig 4.5).

b. Changes in P_SO_2 with depth of penetration into the synovial cavity

It was noticed early in the experiments that the position of the needle O_2 electrode was an important consideration, as values appeared to be lower with greater penetration. The PsO_2 values given above all refer to those obtained with the electrode just within the synovial space *i.e.* having just penetrated the capsule. Some additional observations were obtained where the electrode was advanced in small steps into the synovial cavity and the P_SO_2 measured with each displacement, as illustrated in fig 4.6.

It was consistently observed that the lowest P_SO_2 values occurred with the tip of the electrode having contacted the articular cartilage of the femoral condyle, with gradual reductions in between. These changes

Fig 4.4. **A**: Blood flow reductions as a function of amplitude of stimulation of PAN (1ms width; 10 pulses/s; 90s train) in normal (black symbols) and inflamed (red symbols) joints.

B: Voltage dependent changes in synovial PO_2 , stimulus parameters and symbols as in A.

n = 10-13 in normal, n=10 in inflamed.

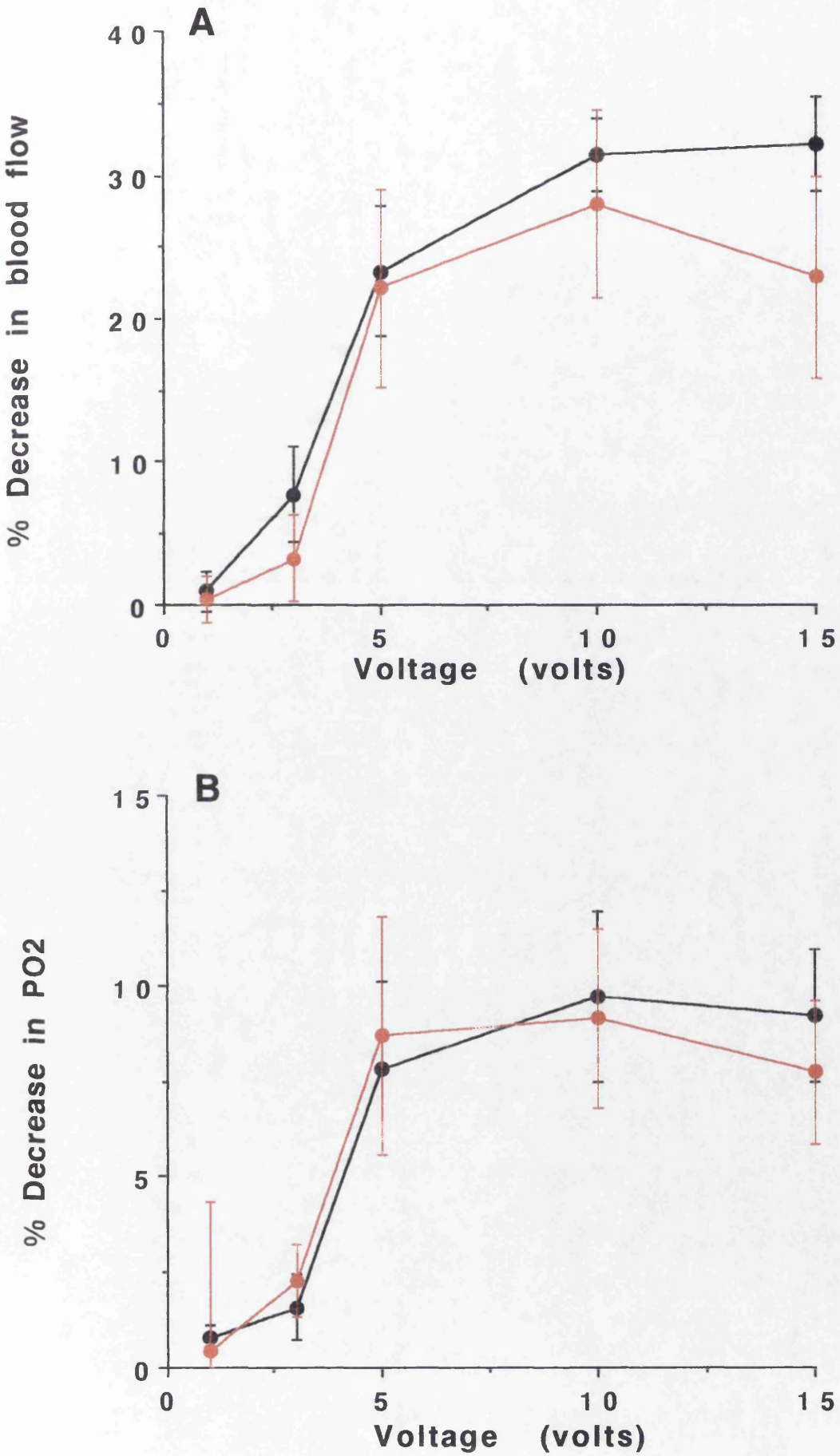

A
40
30
20
10
0
% Decrease in blood flow
0
5
10
15
Voltage (volts)
B
15
10
5
0
% Decrease in PO2
0
5
10
15
Voltage (volts)

Fig 4.5. Effect of tibial nerve stimulation on the blood flow in the normal joint at different pulse amplitudes (shown in volts under bars) but fixed frequency (10 pulses/s), width (1ms) and train duration (90s). Upper traces show compound action potentials recorded from PAN during stimulation. S indicates the time of occurrence of the stimulus. Vasoconstriction (in lower trace) coincides with appearance of the C wave, and the calculated conduction velocity at the peak of this wave (arrowed) was 0.7m/s.

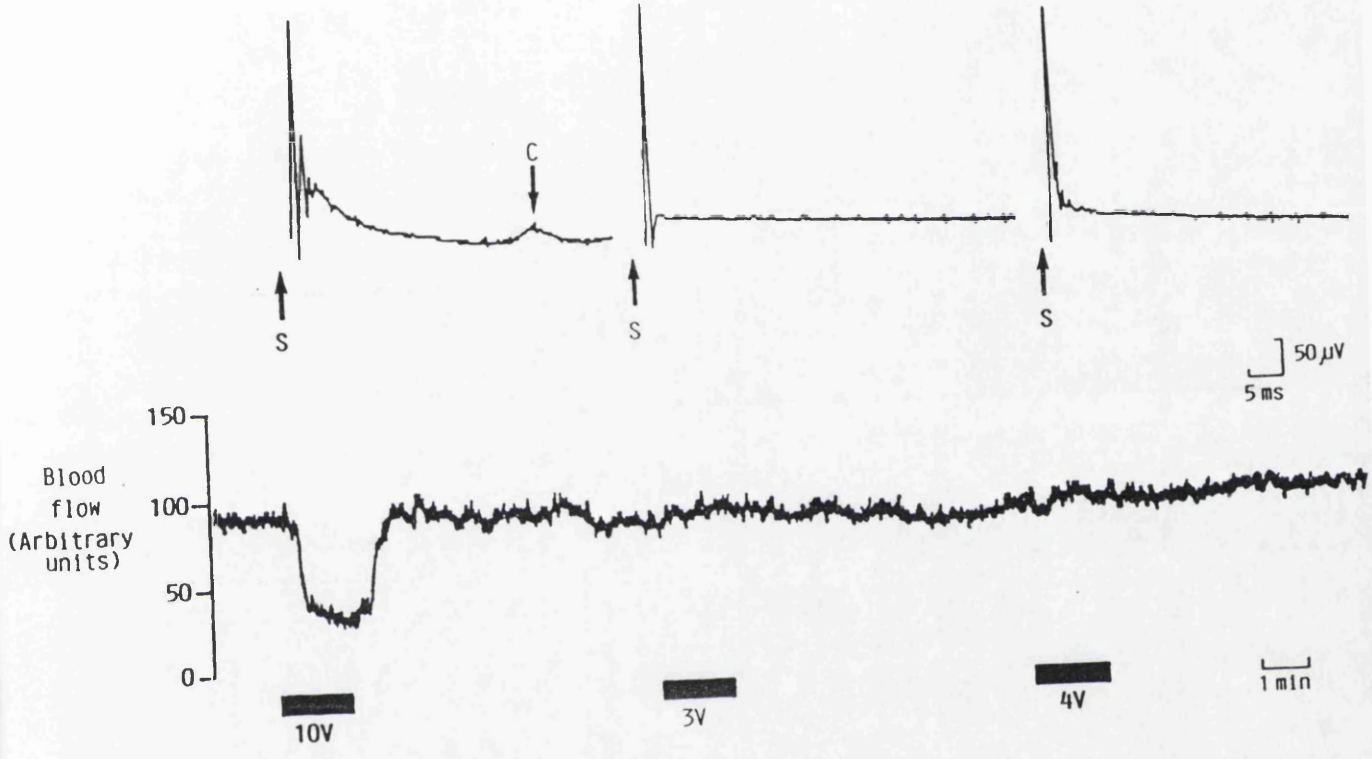
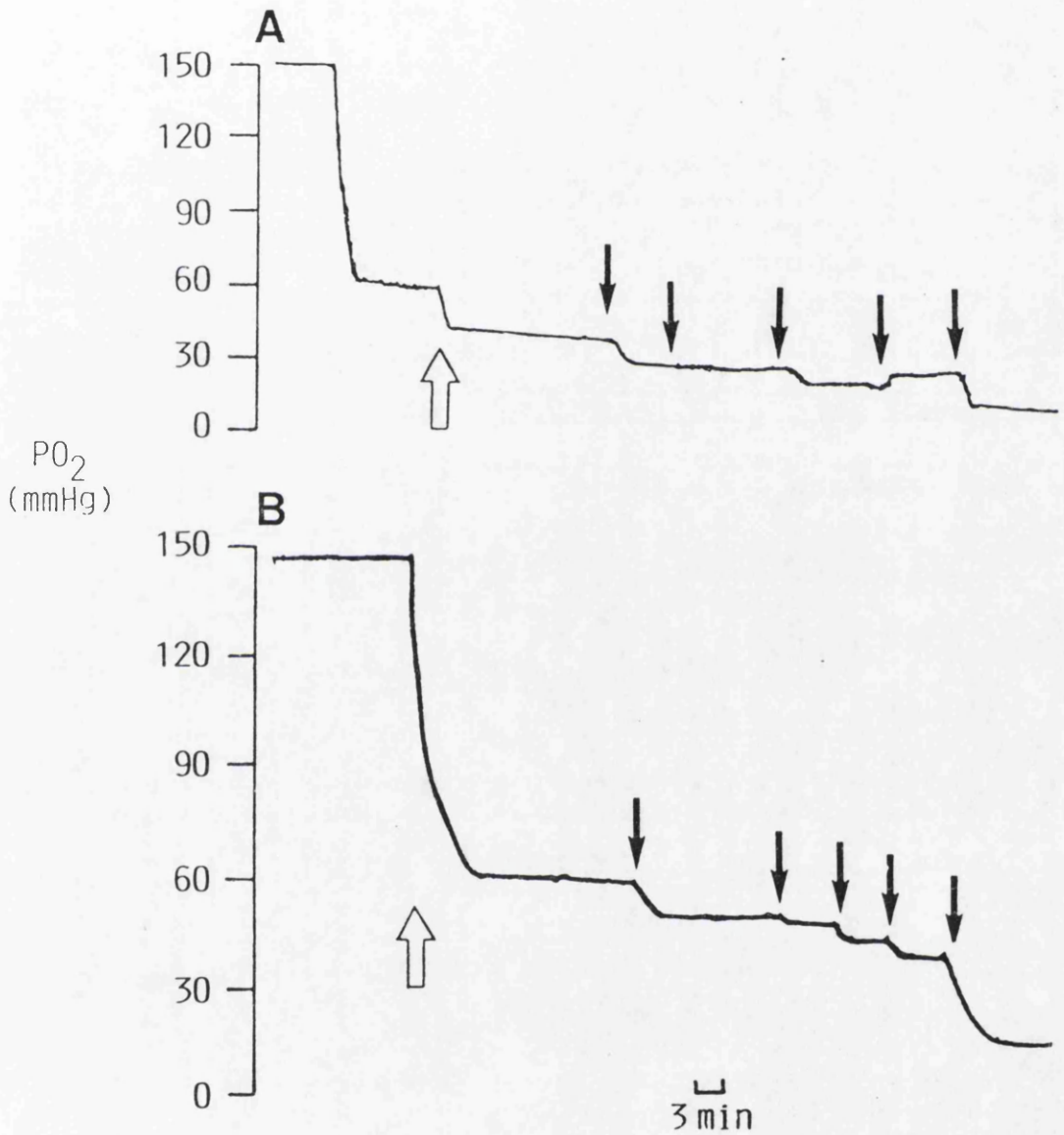

C
S
S
S
50 μV
5 ms
Blood flow (Arbitrary units)
150
100
50
0
10V
3V
4V
1 min

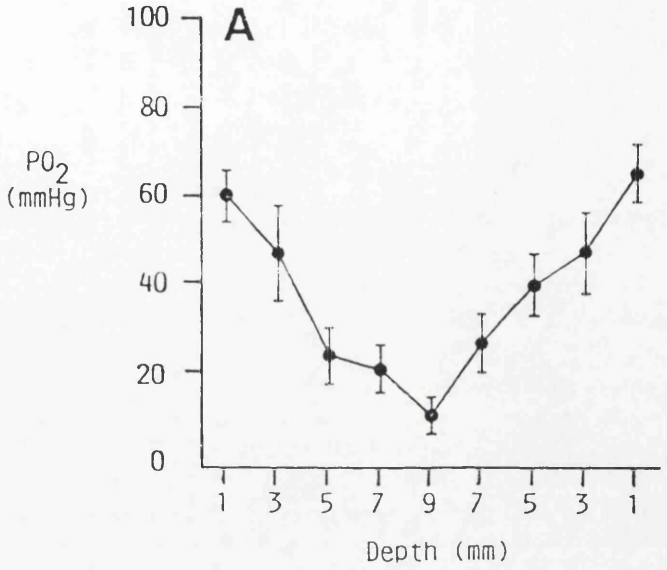
Fig 4.6. Synovial PO_2 recorded at different depths in the synovial cavity of normal knee in two rabbits. **A** - penetration through posterior capsule. **B** - penetration through anterior capsule. Downward pointing arrows indicate 2mm displacements of O_2 electrode further in the capsule or synovial cavity. The large open arrow indicates insertion of the sensing elements of the O_2 electrode into the joint capsule. The penetration through the posterior capsule was through a pool of saline in the popliteal fossa whereas in the anterior capsule the penetration was made from air directly into the capsule. After each displacement, several minutes were allowed to elapse to allow the signal to stabilise before the next displacement occurred.

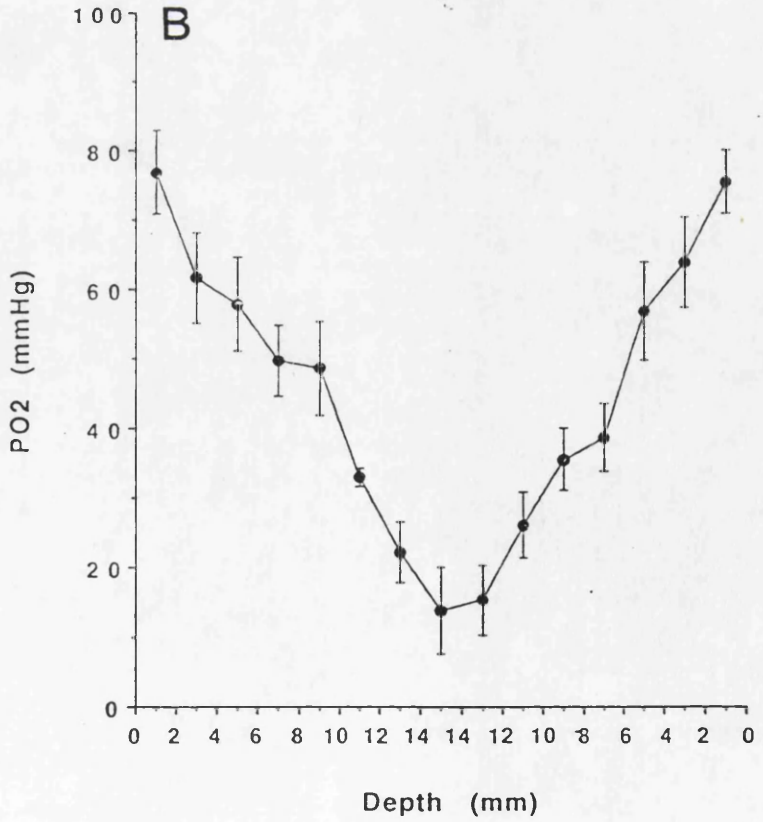

A
150
120
90
60
30
0
PO2
(mmHg)
B
150
120
90
60
30
0
3 min

in P_sO_2 were observed irrespective of whether the approach was made through the posterior capsule (upper trace) or the antero-lateral capsule (lower trace) (fig 4.6). The reverse changes in P_sO_2 were observed on withdrawal of the O_2 electrode. A consistent observation in the present experiments was that the PO_2 value in the saline pool formed in the popliteal fossa declined as the O_2 electrode tip neared the surface of the fibrous joint capsule, as illustrated in the upper trace. Control experiments with the electrode immersed to different depths in a beaker of saline showed no gradient, suggesting that the observed reductions *in vivo* resulted from oxygen uptake by the tissues exceeding the rate of oxygen diffusion through the solution from the air.

The same pattern of changes in P_sO_2 was observed in the penetration and withdrawal of the O_2 electrode in the inflamed joints although with longer depth of penetration and different P_sO_2 values (fig 4.7). Comparison of these P_sO_2 values with those obtained in normal animals shows that although the extent of penetration in inflamed joint is about six mm more, the deepest three points close to the cartilages have non-significantly different PO_2 values between normal and inflamed joint. However, the first point with the O_2 electrode just in the capsule had a significantly higher PO_2 value in the inflamed capsule (76.8 ± 5.8; $n=5$ vs 61 ± 6; $n=6$, $p<0.05$, unpaired t-test).

Fig 4.7. Synovial PO_2 measurement (means ± S.E.M.) at different depths of five normal (**A**) and six inflamed (**B**) knee joints with penetration through the anterior capsule during insertion followed by withdrawal of the needle O2 electrode. Each point indicates 2mm displacement of the O_2 electrode further in or out the capsule or synovial cavity. The extent of penetration was 6mm more in the inflamed joints due to their higher synovial fluid volume and tissue oedema.


A
100
80
60
40
20
0
PO2
(mmHg)
1 3 5 7 9 7 5 3 1
Depth (mm)


B
100
80
60
40
20
0
PO2 (mmHg)
0 2 4 6 8 10 12 14 14 12 10 8 6 4 2 0
Depth (mm)

Discussion

In the present study, P_sO_2 showed considerable variation in different animals of both groups, but with a significantly lower mean P_sO_2 value in the inflamed joint. One possible explanation for this variation might be differences in the depth of electrode penetration. However, attempts were made to ensure that the electrode was inserted through the same region of the posterior capsule and to the same depth to avoid any variations that might have occurred due to differences in the place or depth of penetration. In addition, the electrode configuration permitted estimation of the position of the sensing elements to within 2mm. Another possibility was hypoxaemia due to variations in the depth of general anaesthesia. However, in some of the animals the arterial blood gases were checked using a blood gas analyser and these were found to be normal. No systematic variation of the partial pressure of oxygen in arterial blood (P_aO_2) and P_sO_2 was found *i.e.* in some cases it was observed that an animal with a high P_sO_2 value had a P_aO_2 value towards the lower limit of normal whilst another animal with a low P_sO_2 value had a P_aO_2 value at the higher end of the range. The higher synovial volume or raised intra-articular pressure may be a reason for the lower P_sO_2 in inflamed joint. An inverse relationship has been found between synovial fluid PO_2 and its volume in the diseased human knee joint (Richman, Su, & Ho, 1981).

An interesting finding to arise from this study was that P_sO_2 decreased with increasing depth of penetration into the synovial cavity (fig 4.6 & 4.7). This may be related to the presence of fat pads within the joint as these have been shown to be less vascular than the synovium

(Simkin, Huang & Bendict 1986), and may present a limitation to oxygen diffusion or result in a tortuous path length for oxygen diffusion. An additional factor which could have contributed to lower P_sO_2 values deeper in the joint may have been the immobility of the knee in the present experiments. In the conscious freely-moving animal there may be more effective circulation of synovial fluid around the joint. Comparing the normal and inflamed joints, except for the deepest three points which had comparable values, the most superficial point showed significantly higher PO_2 values in the inflamed joint which may reflect its higher blood flow measured by microsphere technique (see chapter 3). The more rapid drop in P_sO_2 values toward the deeper parts of the inflamed joint could be due to the increase in diffusion distance, or enhancement of the rate of oxygen consumption by the diseased joint tissues (Svalastoga & Gronlund, 1989). Although it would be expected that P_sO_2 would be similar in the anterior and posterior regions of the joint, this may not be the case as there is evidence indicating that different areas of the rabbit knee joint are not hydraulically continuous (Knight & Levick, 1982). It could be that increase in inflamed knee joint blood flow is a compensatory mechanism to prevent further reduction in synovial PO_2, or to wash out extra CO_2 or hydrogen ions produced by the inflamed intra-articular tissues. Experiments have shown that when synovial PO_2 values dropped below 45 mmHg, intra-articular acidosis resulted (Richman, Su, & Ho, 1981). Another possibility is the release of more substance P or CGRP, which have been shown to be potent vasodilators of joint blood vessels (Lam & Ferrell, 1992), by unmyelinated sensory C-fibers of the inflamed joints. It has been demonstrated that in acute experimental knee joint inflammation, the proportion of articular C-

fibers displaying resting discharges is substantially greater than in normal joints (82% vs 36%), and that discharge frequencies of these fibres are also greater in the inflamed joints (Coggeshall, Hong, Langford, Schaible & Schmidt, 1983). Moreover it has been observed that passive movement in the normal working range of the joint activate a large population of C-fibres in the inflamed than in the normal joint (73% vs 13%) (Coggeshall *et al.*, 1983).

As expected, P_sO_2 was strongly correlated with variations in articular blood flow, but only when this involved vasoconstriction. No change in P_sO_2 was observed during any of the dilator periods which sometimes followed the constrictor response to PAN stimulation. This might have been due to the much smaller dilator response compared to the constrictor response or the differing time courses of change in P_sO_2 and blood flow (fig 4.2B). These differences were probably a reflection of the time required for oxygen utilisation by intra-articular tissues and perhaps some limitation to oxygen diffusion in the joint. The lack of reactive hyperaemia to arterial occlusion may reflect the rich anastomotic network around the joint or indicate a lesser degree of metabolic activity in synovial tissues of the immobilized joint.

Similarly to the normal joint, in the inflamed joint P_sO_2 was strongly correlated to articular blood flow (fig 4.2 & 4.3). However, this correlation was significantly less than that occurring in normal group, suggesting that other factors may influence blood flow, P_sO_2 or both. Although the time courses of changes in blood flow are comparable between the two groups, the recovery time for P_sO_2 is significantly longer in the inflamed joint (see results & fig 4.2).This difference may reflect the longer distance for diffusion of oxygen, or

increased rate of oxygen consumption by tissues of the inflamed joint (Svalastoga & Gronlund, 1989).

In a previous investigation using an isolated rabbit knee joint preparation it was shown that electrical stimulation of the joint capsule over a frequency range of 1 to 20 pulses/s produced a vasoconstrictor response which increased monotonically (Ferrell & Khoshbaten, 1990). The present experiments have extended the frequency range of stimuli and shown that *in vivo*, the maximum constrictor response occurred between 10-20 pulses/s with a decline in the response thereafter (fig 4.2A & 4.3A), presumably due to the inability of unmyelinated sympathetic postganglionic fibres to follow such high frequencies of stimulation. In contrast to the *in vitro* experiments of Ferrell & Khoshbaten (1990), more than 80% of the constrictor response occurred over the frequency range of 1-5 pulses/s in the present study (fig 4.3). This discrepancy may be due to the different forms of stimulation used in the two studies or may reflect differences between the *in vivo* and *in vitro* preparations. In addition, the methods used for assessing changes in blood vessel calibre differed, with an indirect technique (perfusion pressure change) being used in the latter. Ferrell & Khoshbaten (1990) measured changes in perfusion pressure as an indirect indicator of resistance whereas laser Doppler flowmetry was used in this *in vivo* study which is a more direct indicator of changes in vascular resistance. It is interesting that a large constrictor response occurred over the 1-5 pulses/s range as such a "high gain" system is well adapted to sympathetic postganglionic fibres which typically discharge at frequencies of 0.5-3 impulses/s (Janig, 1988). Although the maximum constrictor responses of blood vessels to PAN stimulation are almost

equal in both groups, this happened at a much higher frequency (30Hz vs 10Hz) in the inflamed joint, along with significantly lower responses below 10Hz (fig 4.3A). The reason for this shift of frequency-response curve to the right in the inflamed joint is not known at present, but the presence of more vasodilator substances, or nerve mediated release of less noradrenaline or more vasodilator mediators may play important roles. Change in property of postjunctional α-adrenoceptors is unlikely as the responses to different adrenoceptor agonists were found to be not different between normal and acutely inflamed joint blood vessels (fig 6.5, chapter six). The decrease in sensitivity of inflamed joint blood vessels to nerve stimulation observed in this study is in contrast to the findings of Khoshbaten & Ferrell, 1990 in which the responses were increased during eight hours of kaolin induced joint inflammation. This discrepancy may be due to different substances used for inflammation and/or longer time course of inflammation (24 hours) in this study. The present results are more in agreement with the findings of Dick *et al.* (1971) in which they observed evidence of loss of vasoconstrictor tone in chronically inflamed human joints.

Although the anatomical composition of the rabbit PAN has not been described, in two of animals this nerve was examined by transmission electron microscopy and found to contain both myelinated and unmyelinated nerve fibres. Electrophysiological recordings from PAN indicate that the constrictor response was correlated with the C wave in the compound action potential (fig 4.5) and the calculated conduction velocity of 0.7 m/s indicates that these were unmyelinated fibres. As other experiments have shown that the constrictor response can be blocked by pre-treatment with the α−adrenoceptor antagonist

phenoxybenzamine (see chapter 5), it is therefore likely that this response was mediated by sympathetic postganglionic fibres which are known to be unmyelinated (Janig, 1988).

In conclusion, the results of this study show that although in the acutely inflamed knee joint there is a significantly higher blood flow, P_sO_2 is lower than in the normal joint in the posterior region, with again lowest values occuring in the deepest parts of the joint cavity. The higher blood flow could compensate for the higher oxygen demands of the inflamed joint tissues, but the increase in synovial fluid volume and pressure which retards oxygen delivery and diffusion, along with the very small oxygen buffering capacity (due to lack of haemoglobin) of synovial fluid, leaves the deep structures of the joint at a high risk of damage if inflammation is sustained. In a rabbit *in vitro* articular cartilage model it was found that low PO_2 was detrimental to cartilage glycosaminoglycan metabolism resulting in inhibition both of cellular proliferation and synthesis of extracellular ground substance (Brighton, Lane & Koh, 1974; Lane, Brighton & Menkowitz, 1977).

The decrease in sensitivity of joint blood vessels to the action of sympathetic vasoconstrictor nerves, especially in physiological range (1-5 Hz), observed in this study may partly contribute to the hyperaemia of the inflamed joints.

CHAPTER FIVE

Sympathetic innervation and adrenoceptor profile of blood vessels in the posterior region of the rabbit knee joint

Section one

Sympathetic innervation and α-adrenoceptor profile

Summary

1. Experiments were performed to determine the nature of adrenoceptors mediating neurally-induced vasoconstriction of blood vessels in the posterior region of the rabbit knee joint capsule.

2. Electrical stimulation of the posterior articular nerve resulted in frequency dependent vasoconstriction which was maximal at 10Hz. This response was mediated predominantly by α_2-adrenoceptors as it was only slightly reduced by prazosin administration and was not only abolished but converted into a dilator response by the α_2-adrenoceptor antagonist rauwolscine. Further experiments using another specific α_1-adrenoceptor antagonist YM-12617 showed that the frequency/response curve in the presence of this antagonist did not differ significantly from control.

3. Neurally-induced vasoconstriction did not appear to have a purinergic component as it was unaffected by the P_{2X}-purinoceptor desensitiser α,β methylene ATP.

4. The rank-order of potency of α-adrenoceptor agonists given as a bolus by close intra-arterial injection was: adrenaline = UK-14304 > clonidine > phenylephrine, suggesting that the vasoconstrictor effects were mediated predominantly by postjunctional α_2-adrenoceptors.

5. The α_2 adrenoceptor antagonist rauwolscine converted the constrictor responses to close intra-arterial injection of adrenaline into a dilator response. The vasoconstrictor responses to UK-14304, clonidine and

phenylephrine were substantially inhibited by rauwolscine. The α_1 adrenoceptor antagonist prazosin failed to inhibit the vasoconstrictor responses to adrenaline, clonidine and UK-14304 and resulted in enhancement of their constrictor effects.

6. The enhancement of the responses to the α_1 and α_2 agonists by prazosin appeared to be specifically related to this agent as administration of YM-12617 did not show such enhancement. The dose/response curves to both clonidine and UK-14304 in the presence of YM-12617 did not differ significantly from control responses. Responses to phenylephrine were significantly reduced by YM-12617, indicating the presence of post-junctional α_1-adrenoceptors.

7. These results show almost complete reversal of the adrenoceptor profile compared to results obtained in an earlier *in vitro* study where responses were mediated predominantly by α_1-adrenoceptors with a small population of post-junctional α_2-adrenoceptors (Ferrell & Khoshbaten, 1989). This suggests that the differing environments *in vitro* may not completely reflect the conditions prevailing *in vivo*.

Introduction

The presence of sympathetic nerve fibres innervating articular blood vessels was first demonstrated by Cobbold & Lewis (1956b) who used an electromagnetic flowmeter to measure blood flow of the canine knee joint. They observed that traction of the lumbar sympathetic chain reduced knee joint blood flow and section of the articular nerve supply resulted in increased blood flow. In another study close intra-arterial injection of noradrenaline produced vasoconstriction, indicating the presence of α-adrenoceptors (Cobbold & Lewis, 1956c). In more recent experiments using an isolated perfused rabbit knee joint preparation, it was observed that the α_1-adrenoceptor agonist phenylephrine elicited potent vasoconstriction but the α_2-agonist clonidine was ineffective and another α_2-agonist UK-14304 exerted only small constrictor effects at high doses (Ferrell & Khoshbaten, 1989). Electrical stimulation of the joint capsule also produced vasoconstriction which was substantially reduced by adding the α_1-adrenoceptor antagonist prazosin to the perfusate, but inclusion of the α_2-adrenoceptor antagonist rauwolscine did not produce additional inhibition of the constrictor response (Ferrell & Khoshbaten, 1990a). These results indicate that in this preparation, sympathetic effects are mediated mainly via α_1-adrenoceptors which predominate in resistance vessels of the rabbit knee joint. Advantages of the *in vitro* preparation described above are the reduced number of variables which require to be controlled and that this model provides an indication of the overall vascular resistance of the joint capsule. The disadvantage of the *in vitro* technique is that the environment is

artificial and lacks many of the vasoactive agents which normally circulate in the blood stream and which could modify the response of adrenoceptors. Recent observations in both *in vitro* and *in vivo* models suggest that in the latter, effects mediated by postjunctional α_2-adrenoceptors are more readily detectable whereas *in vitro* these are difficult to detect and vasoconstrictor effects are mediated principally by postjunctional prazosin sensitive α_1-adrenoceptors (for review see McGrath *et al.*, 1989). Thus, the present study was performed in anaesthetized rabbits to examine nerve-mediated vascular responses and assess the nature of adrenoceptors which mediate vasoconstriction in the posterior region of the knee joint capsule *in vivo*.

Method

a. Animal preparation and physiological recordings

Experiments were performed on 30 adult New Zealand rabbits (2.2-3.5kg) which were anaesthetised and maintained as described in chapter four. The trachea was cannulated, with the animals breathing spontaneously throughout the experiments. Arterial blood pressure was recorded throughout the experiment by a cannula inserted into the left carotid artery. A laser Doppler flowmeter (Moor Instruments MBF3) provided a measure of relative changes in blood flow in the volume of tissue beneath the tip of the probe. The probe was positioned just above the surface of the postero-medial aspect of the knee joint capsule. Experiments have shown that alterations of blood flow in the synovium are detected with the probe in this position (fig 2.13, chapter two). Alterations in blood flow are expressed as percentage change from control (0%) values occurring immediately before each test procedure.

The posterior articular nerve (PAN), was dissected free and transected proximally. Bipolar bright silver stimulating electrodes were then placed on PAN and pulse trains delivered. The stimulus parameters were: width 1ms, voltage 10V, frequency 1-30 Hz and duration 90s. In previous experiments we have demonstrated that these stimulus parameters give rise to frequency dependent vasoconstrictor responses (Ferrell & Najafipour, 1992). As long as the laser Doppler probe remained in the same position, the constrictor responses remained stable and reproducible over several hours. At the end of experiment the animal was killed by intravenous injection of 1M KCl.

b. Drug administration

Drugs were administered by close intra-arterial injection via a polythene cannula (Portex 2FG) inserted into the caudal femoral artery (see chapter two) and advanced until the tip was located close to the main branch (popliteal artery) just before the origin of the joint branches. All non-articular branches of the latter were ligated. The α-adrenoceptor antagonists were administered as a continuous infusion (0.25ml/min; 10^{-4}M) via this cannula, with the duration of infusion being maintained for a minimum of 30min before α-agonists were administered or nerve stimulation applied. The antagonists were also administered intravenously via the cannula in the external jugular vein as a bolus (1-1.5 mg/kg body weight) prior to antagonist infusion. In some animals only one antagonist was administered whilst in others prazosin was administered first followed by rauwolscine. Other drugs were administered as a bolus injection into the caudal femoral intra-arterial cannula in a volume of 0.25ml. The adrenoceptor agonists were administered in random order. All drugs were dissolved in physiological saline. Control injections of 0.25ml saline were found to have no effect on blood flow (fig 2.9, chapter two). The drugs used and their sources were described in chapter two.

The values expressed in the graphs and histograms are means $\pm$ SEM and comparisons were performed using the student's paired or unpaired t-test as appropriate.

Results

a. Effect of nerve stimulation

Electrical stimulation of PAN consistently produced vasoconstriction which was unaffected by pre-treatment with α,β methylene ATP (fig 5.1A) which is a P_{2x}-purinoceptor desensitiser (Kasakov & Burnstock, 1983). The results are summarised in the histogram shown in fig 5.2 demonstrating that the mean values before and after injection of α,β methylene ATP were not significantly different (unpaired t-test).

Vasoconstriction elicited by PAN stimulation (fig 5.1B i) was, however, abolished by administration of phenoxybenzamine and converted into a dilator response (fig 5.1B ii, fig 5.2). Adrenaline (2.5nmole), given by close intra-arterial injection, produced vasoconstriction (fig 5.1B i) which was similarly altered by phenoxybenzamine (fig 5.1B ii, fig 5.2). In both cases the change in response was significant ($p<0.001$; unpaired t-test).

Vasoconstriction elicited by PAN stimulation was frequency dependent over the range 1 to 10Hz (fig 5.3). Administration of the α_1-adrenoceptor antagonist prazosin produced a significant reduction in this constrictor response at all frequencies except 30Hz. In contrast, the α_2-adrenoceptor antagonist rauwolscine completely reversed the response and produced significant vasodilatation during nerve stimulation.

As prazosin appeared to give smaller reductions than rauwolscine, suggesting that the constrictor response to PAN stimulation might be partially mediated by α_1-adrenoceptors, to further clarify the role of

Fig 5.1 **A**: Constrictor response to electrical stimulation of PAN (10V, 10Hz, 1msec width; 90s train; denoted by black bar over trace) is unaffected by pretreatment with the P_{2x}-purinoceptor desensitiser α,β methylene ATP (each dose = 2.5nmole in a volume of 0.25ml injected at the arrows).

B: i PAN stimulation (as in A) and close intra-arterial injection of adrenaline (2.5nmole at the arrow) produce constrictor responses.

B: ii 45min after onset of phenoxybenzamine administration, these responses are abolished and replaced by dilatations.

C: Responses to PAN stimulation (black bar; parameters as in A), and close intra-arterial injection of phenylephrine (PE), adrenaline (ADR), clonidine (CL) and UK-14304 (UK) prior to prazosin treatment. Dose for each agent = 2.5nmole.

D: As in C but 45 min after onset of prazosin administration. The responses are little affected.

E: Responses to PAN stimulation and the same agents as in C prior to rauwolscine.

F: As in E but 45 min after onset of rauwolscine administration. Responses to agents and nerve stimulation are substantially modified. See methods for details of administration of agonists and antagonists.

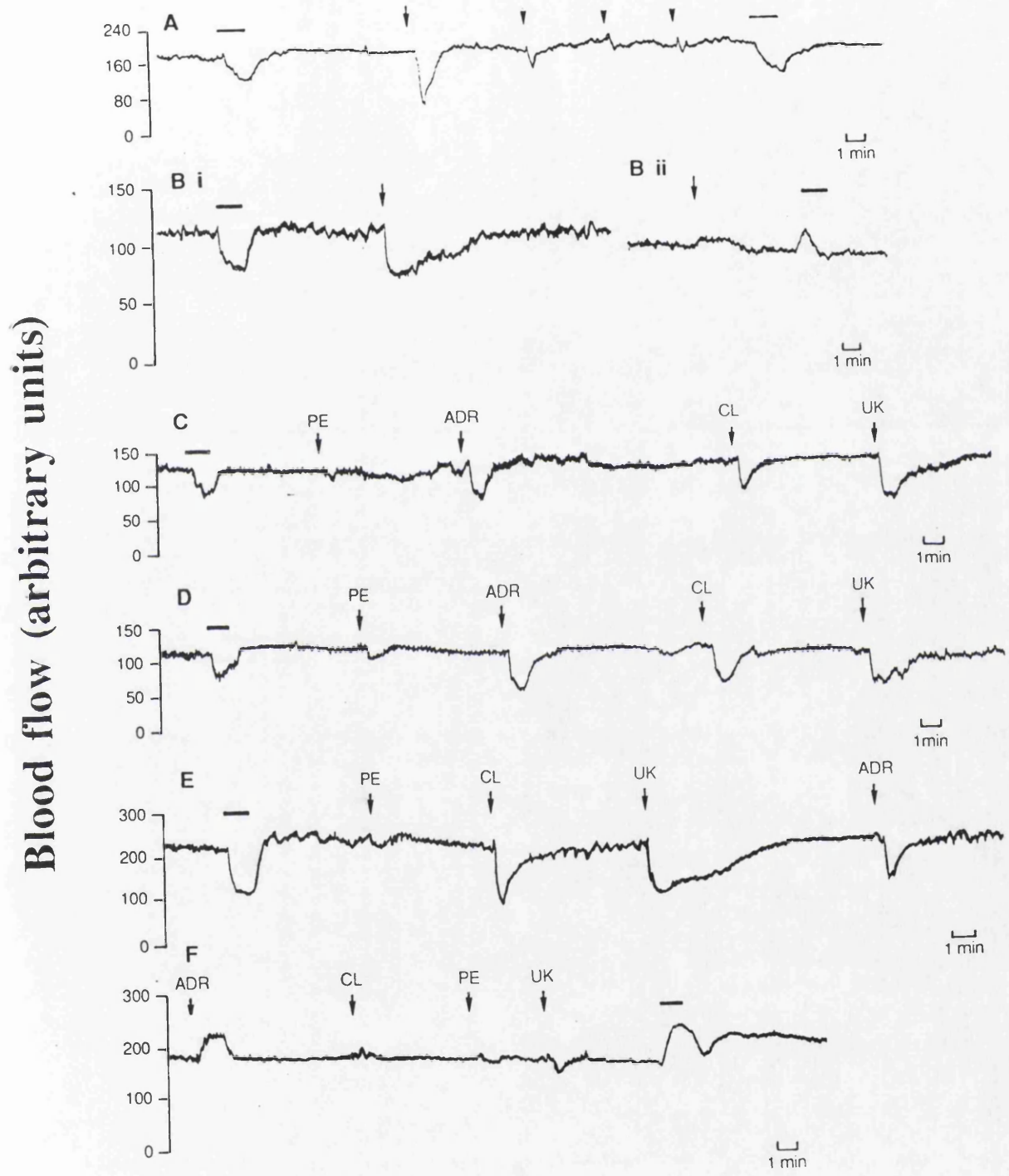
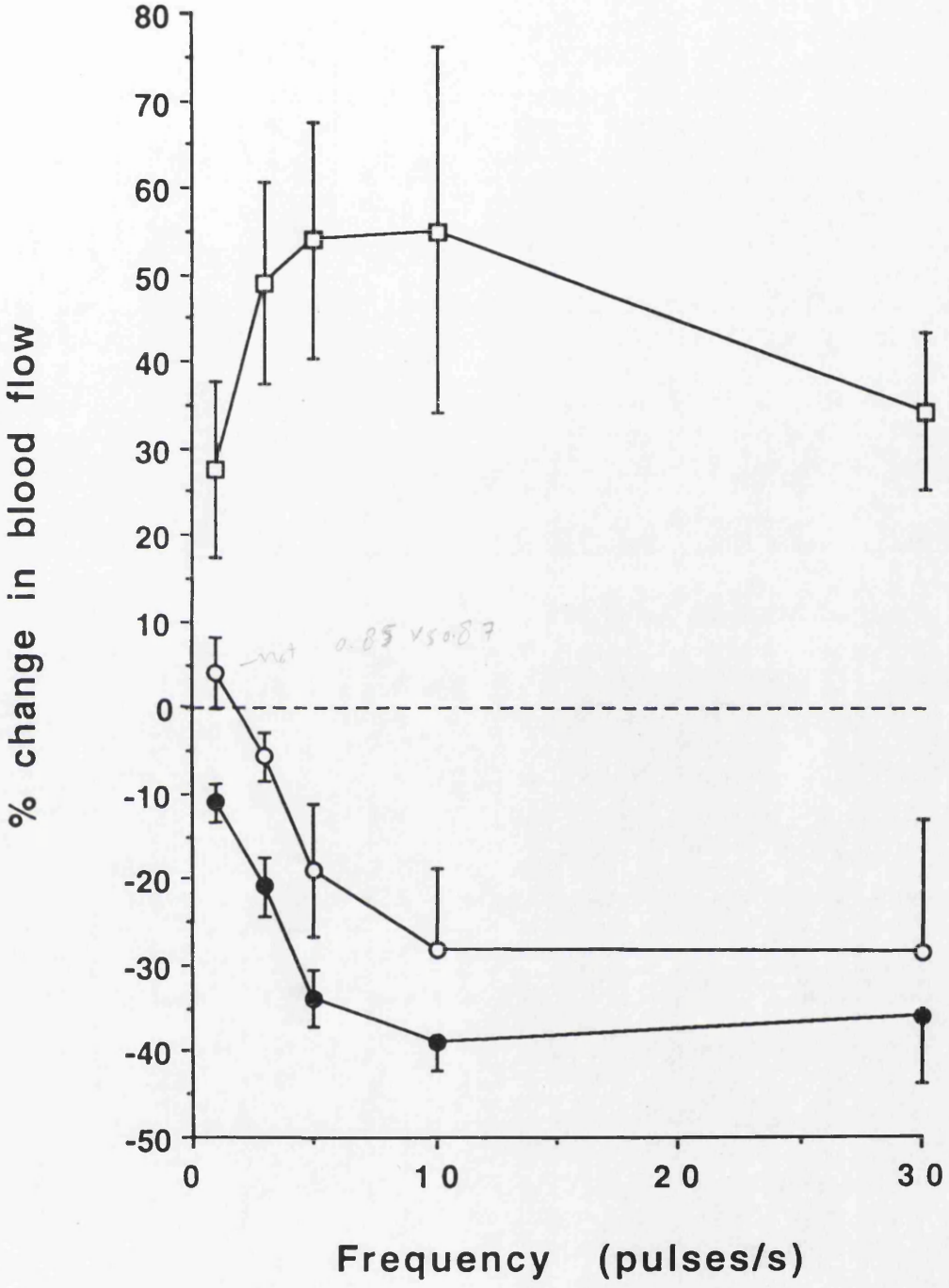

Blood flow (arbitrary units)
A
240
160
80
0
1 min
B i
B ii
150
100
50
0
1 min
C
PE
ADR
CL
UK
150
100
50
0
1min
D
PE
ADR
CL
UK
150
100
50
0
1min
E
PE
CL
UK
ADR
300
200
100
0
1 min
F
ADR
CL
PE
UK
300
200
100
0
1 min

Fig 5.2 . Vascular responses to PAN stimulation prior to treatment with α,β methylene ATP (NS CTL) and after four injections of this agent (NS αβmATP). PAN stimulation prior to treatment with phenoxybenzamine (NS CTL) produces vasoconstriction but during administration of phenoxybenzamine (NS PBX) this converts to vasodilatation. Close intra-arterial injection of 2.5nmole adrenaline also produces constriction prior to phenoxybenzamine (ADR CTL) and this also becomes a dilator response during treatment with this agent (ADR PBX). *** : means differ significantly from control ($p<0.001$).

% Change in blood flow
20
10
0
-10
-20
-30
-40
-50
-60
n=13
n=11
n=13
n=8
n=15
n=10
* * *
* * *
NS CTL
NS αβmATP
NS CTL
NS PBX
ADR CTL
ADR PBX

Fig 5.3. Frequency/response curves to electrical stimulation of PAN (10V, 1ms width, 1min train) under control conditions (closed circles; n=15-17), during prazosin administration (open circles; n=4), and during perfusion with rauwolscine (squares; n= 5). Means ± SEM. Prazosin produced a small inhibition of the constriction response which was significant at the first three doses (P= 0.003, 0.02 & 0.03, respectively) whereas rauwolscine completely blocked the response and transformed it to a vasodilatation at all doses.


80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
0
10
20
30
% change in blood flow
Frequency (pulses/s)

α_1-adrenoceptors, another selective α_1-adrenoceptor antagonist, YM-12617, (Honda *et al.*, 1985) was used in a smaller series of experiments. This showed that the frequency/response curve in the presence of YM-12617 only differed significantly from the control curve at the lowest frequency (fig 5.4), again highlighting the importance of α_2-adrenoceptors in nerve-mediated vasoconstriction.

b. Effect of α-adrenoceptor agonists & antagonists

In order to assess the nature of the adrenoceptors mediating these constrictor responses, selective α-adrenoceptor antagonists and agonists were employed. The α_1-agonist phenylephrine failed to show any effects at doses below 2.5nmole (fig 5.5D) whereas the α_2-agonists UK-14304 and clonidine both gave rise to constrictor responses at lower doses (fig 5.5B & C). The dose/response curves of these agents suggest that adrenaline and UK-14304 are equipotent (fig 5.5A & B), with clonidine giving rise to smaller responses which in general showed the same trend as those of UK-14304 (fig 5.5C).

The effect of the α_1-adrenoceptor antagonist prazosin was investigated as illustrated in fig 5.1C & 5.1D, which demonstrates that the constrictor responses to close intra-arterial injection of phenylephrine, adrenaline, UK-14304 and clonidine (all 2.5nmole) were not attenuated by prazosin administration. These results are detailed in figure 5.5 for different doses of each of the agonists which show that in the presence of prazosin the constrictor responses to clonidine, phenylephrine and UK-14304 across a range of doses were actually significantly greater than control values. This effect appeared to be specific to prazosin as repeating these experiments using YM-12617 at

Fig 5.4. Frequency/response curves to electrical stimulation of PAN (10V, 1ms width, 1min train) under control conditions (circles; n=4-6) and during administration of YM-12617 (triangles; n=4-6). Means ± SEM. The response was virtually unaffected by YM-12617.

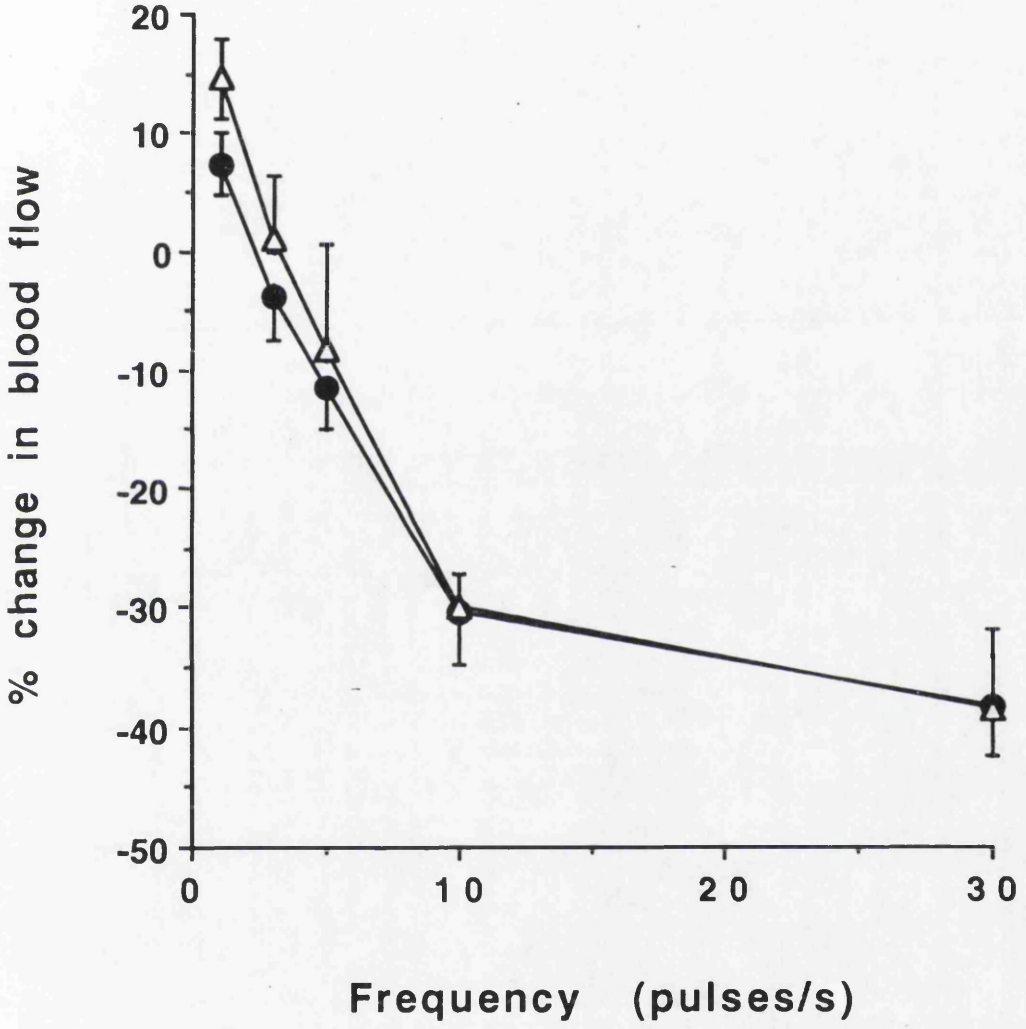
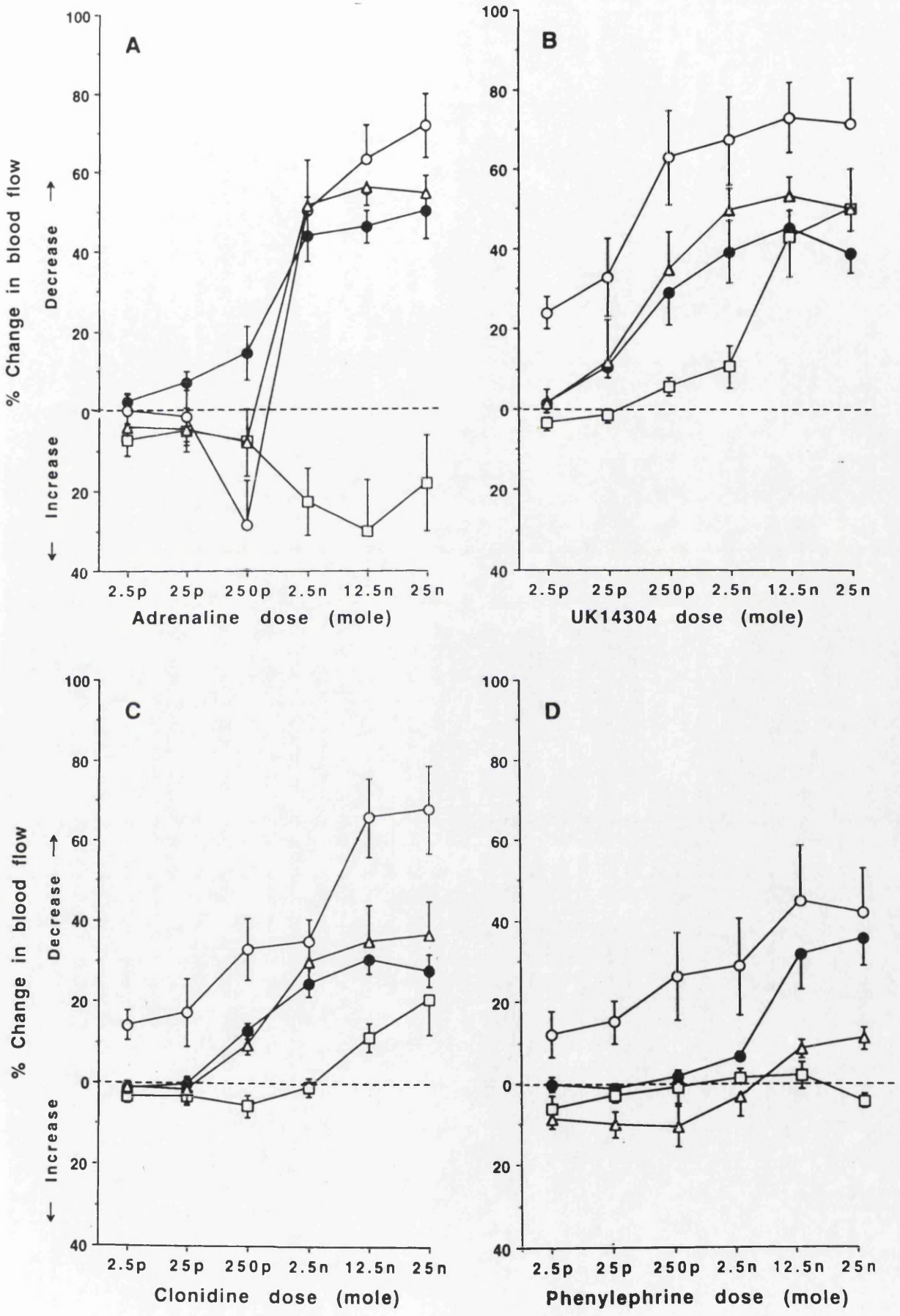

20
10
0
-10
-20
-30
-40
-50
0
10
20
30
% change in blood flow
Frequency (pulses/s)

Fig 5.5. Dose/response curves for close intra-arterial injection (0.25ml volume) of **A**: adrenaline, **B**: UK-14304, **C**: clonidine and **D**: phenylephrine under control conditions (closed circles; n=5-10), during administration of either prazosin (open circles; n=4), YM-12617 (triangles; n=4-6) or rauwolscine (squares; n=5). Means ± SEM. p = pico. n = nano.

YM12617 reduced the responses significantly at all doses of phenylephrine used (P= 0.03-0.002) and the first three doses of adrenaline (P=0.01, 0.01 & 0.002, respectively). Rauwolscine significantly reduced the responses to all agonists and in most doses (in the case of adrenaline at all doses with P= 0.01-0.003, in the case of UK14304 and clonidine at doses 25p to 2.5n with P= 0.01-0.003 and 0.002-0.0001, respectively; and in the case of phenylephrine only at last two doses with P=0.005 & 0.0005- unpaired t-test).


A
B
C
D
% Change in blood flow
Decrease →
← Increase
100
80
60
40
20
0
20
40
2.5p 25p 250p 2.5n 12.5n 25n
Adrenaline dose (mole)
UK14304 dose (mole)
Clonidine dose (mole)
Phenylephrine dose (mole)

similar intravenous doses (1-1.5 mg/kg) and perfusate concentrations (10^{-4}M) neither potentiated nor inhibited the actions of clonidine and UK-14304 (fig 5.5B & C). The constrictor response to phenylephrine was, however, significantly reduced by YM-12617 (fig 5.5D) suggesting the presence of α_1-adrenoceptors. The responses to adrenaline at doses below 2.5nmole were reduced by both prazosin and YM-12617, again indicating the involvement of α_1-adrenoceptors at these lower doses.

The α_2-adrenoceptor antagonist rauwolscine gave rise to pronounced inhibition of the responses to all the agonists. As illustrated in Fig 5.1E & F, the responses to PAN stimulation and close intra-arterial injection (2.5nmole in all cases) of phenylephrine, clonidine, adrenaline and UK-14304 were substantially inhibited by rauwolscine. Fig 5.5 shows that rauwolscine shifted the dose/response curves to the right and that adrenaline now gave rise to dilator responses. In all cases rauwolscine produced significant alteration in the responses.

Administration of the α-adrenoceptor antagonists produced some reduction in arterial blood pressure and consequently of synovial blood flow. Prior to administration of antagonists mean arterial blood pressure (±SEM) was 71±7 mmHg* (n=8). The bolus injection of antagonist produced an initial reduction of blood pressure and joint blood flow, but these partially recovered and levelled out by 40min at which time the test procedures were once again performed. Mean arterial blood pressure at this time for phenoxybenzamine, prazosin and rauwolscine was 54.3±4.4 (n=8), 60.8±3.7 (n=7) and 62±3 (n=7) mmHg respectively and these values did not differ significantly from each other although they did differ significantly ($P<0.0004$) from their controls

* Mean arterial pressure before prazosine administration was 71.4±4.3 mmHg and before rauwolscine it was 71±4 mmHg.

(unpaired t-test). Administration of YM-12617 decreased blood pressure from 82.8±5.6 to 77.7±5.9 ($P<0.05$; n=6; paired t-test)

Basal joint blood flow reduced by phenoxybenzamine from 127±13 to 88±8.2 arbitrary units, P=0.002; by prazosine from 249±9 to 207±13 arbitrary units, P=0.005; by rauwolscine from 221±13 to 176±24 arbitrary units, P=0.09; and by YM12617 from 230±7.6 to 177±16.9 arbitrary units, P=0.007; paired t-test.

Discussion

The results of this study have shown that electrical stimulation of the nerve supply to the posterior region of rabbit knee joint resulted in vasoconstriction which was mediated predominantly via α_2-adrenoceptors. Although purinergic co-transmission has been described in other vascular beds such as the urinary bladder (Kasakov & Burnstock, 1983), vas deferens (Sneddon & Burnstock, 1984) and colon (Hedlund *et al.*, 1983), no evidence was obtained in the present study to indicate that ATP has a role to play in mediating the actions of sympathetic vasoconstrictor fibres innervating the joint. The pronounced constrictor effect of the first injection of α,β methylene ATP (fig 5.1A) suggests the presence of P_{2x}-purinoceptors in these knee joint blood vessels although this could also have resulted from a direct depolarising effect of α,β methylene ATP on smooth muscle cells (Komori *et al.*, 1988). However, previous experiments in an isolated knee joint preparation showed both P_1 and P_{2x}-purinoceptors to be present, although relatively high doses of purines were required (Ferrell & Khoshbaten, 1990b). If these purinoceptors are indeed present, their functional role remains unclear.

Although phenoxybenzamine is not specific to α-adrenoceptors, the finding that rauwolscine gave rise to pronounced inhibition suggests that phenoxybenzamine was acting at these receptors. It was clear that the constrictor responses to nerve stimulation and the α_2-adrenoceptor agonists were mediated predominantly via α_2-adrenoceptors as these were substantially altered by rauwolscine but unaffected by YM-12617.

The effects of prazosin are difficult to interpret as it potentiated the responses to UK-14304, clonidine and phenylephrine but not the catecholamine, adrenaline. The mechanism underlying the potentiation is unclear at present, but was specifically related to prazosin as the structurally different α_1-adrenoceptor antagonist YM-12617 did not produce this effect. The potentiating effect of prazosin on the above α_1 and α_2- agonists meant that its expected inhibitory effect on phenylephrine could not be detected. However, the nerve-mediated vasoconstriction (fig 5.3) was significantly reduced by prazosin which could be explained by its lack of potentiation of the effect of neurally-released catecholamine, noradrenaline, leaving the inhibitory effects of prazosin on α_1-adrenoceptors unopposed. The vasoconstriction induced by phenylephrine at higher doses was reduced both with YM-12617 and rauwolscine (fig 5.5D), suggesting that phenylephrine may act at both α_1- and α_2-adrenoceptors at such high doses. The conversion of the constrictor responses to nerve stimulation and adrenaline into vasodilator ones suggests that the latter are normally masked by the more pronounced constrictor responses. In the case of adrenaline, the dilator response during blockade with phenoxybenzamine (and rauwolscine) could be explained by adrenaline acting on β-adrenoceptors. This would be consistent with the finding of Cobbold & Lewis (1956c) that close intra-arterial injection of noradrenaline produced greater vasoconstriction of dog knee joint blood vessels than adrenaline, suggesting the presence of β-adrenoceptors, although this was not discussed in their paper. Although vasoconstriction induced by adrenaline was not antagonised by prazosin or YM-12617 at doses $\geq$2.5nmole, both of these agents produced inhibition at lower doses, with rauwolscine being effective at all doses (fig 5.5A). This could be

interpreted as adrenaline acting at both α_1- and α_2-adrenoceptors, with the α_1-subtype having a lower activation threshold.

The dilator response which occurred with PAN stimulation during α-adrenoceptor blockade was probably mediated by substance P released from nerve endings of C afferent fibres present in PAN as in previous experiments on cat knee joint blood vessels it was shown that dilator responses following the initial constrictor responses to PAN stimulation were reduced by a substance P antagonist (Khoshbaten & Ferrell, 1990). The involvement of β-adrenoceptors is another possibility (see next section).

Although the administration of all the α-adrenoceptor antagonists resulted in similar decrease in arterial blood pressure and thus altered vascular resistance in the knee joint, the strikingly different responses to PAN stimulation and α-adrenoceptor agonists occurring during rauwolscine treatment contrasts with the lack of inhibitory effect of prazosin. This suggests that the differences between these agents reflects their differing effects on α-adrenoceptors in these vessels and is not secondary to altered vascular resistance.

In conclusion, the most striking finding of this study was that α_2-adrenoceptors play a major role in mediating the vasoconstrictor responses to α-agonists and nerve stimulation, whereas in previous *in vitro* studies these responses were predominantly mediated by α_1-adrenoceptors (Ferrell & Khoshbaten, 1989; Ferrell & Khoshbaten, 1990a). The mechanisms underlying this change are unclear, but may reflect the differences in the experimental conditions in terms of the artificiality of the *in vitro* preparation. It has been demonstrated in the

isolated rabbit distal saphenous artery that contractile responses to sympathetic nerve stimulation are substantially reduced by prazosin but a small residual component remains which is potentiated in the presence of angiotensin II (Dunn *et al.*, 1991). Thus, it is possible that circulating vasoactive substances which are present *in vivo* modify the response to nerve stimulation. However, in the present experiments the virtual absence of inhibition by YM-12617 coupled with the powerful effect of rauwolscine suggests that in this tissue the nerve-mediated constrictor responses are mediated predominantly by α_2-adrenoceptors. Evidence for α_2-adrenoceptor involvement in blood vessel contractions evoked by nerve stimulation has proved difficult to obtain in animal tissues, and when demonstrated, the effects were predominantly mediated by α_1-adrenoceptors (for review see Docherty, 1989). Only in the human saphenous vein, rabbit ear and saphenous vein have contractions evoked by nerve stimulation been shown to be mediated predominantly by α_2-adrenoceptors, although even in this preparation some inhibition by prazosin was observed at concentrations above 10^{-7}M (Docherty & Hyland, 1985). It could be argued that the difference in the results obtained in the *in vitro* and *in vivo* preparations may be related to different methodologies. The isolated knee joint preparation reflects changes of the resistance vessels in the whole capsule whereas laser Doppler flowmetry samples a small volume of tissue under the probe and therefore is more regionally specific. In addition, laser Doppler flowmeters are designed to sample flow in the microcirculation where it has been shown that α_2-adrenoceptors predominate whereas α_1-adrenoceptors are more common on the larger resistance arteries (Faber *et al.*, 1991).

Assuming 20% of body weight is extracellular fluid (including blood volume), for a rabbit with an average weight of 3Kg, the final concentration of the specific α-adrenoceptor antagonists administered by bolus injection and also close intra-arterial infusion for one hour would be in the range of 1.5×10^{-5} M. If the degradation of drug by the body is not taken into account it could be argued that this is a high concentration which could partly cross react with non-specific α-adrenoceptors. In the case of prazosin the potentiation of α-adrenoceptor-mediated vasoconstrictor responses is difficult to interpret but significant inhibition of the nerve-mediated responses (fig 5.3) could be partly due to inhibitory effects on α_2-adrenoceptors. In the case of rauwolscine the blockade of nerve- and α-agonist-mediated vasoconstrictor responses could partly be due to blockade of α_1-adrenoceptors. In both situations the final conclusion is that α_2-adrenoceptors predominate and are much more important in this vascular bed.

Section two

Sympathetic innervation

and

β-adrenoceptor profile

Summary

1. Experiments were performed to investigate the presence and nature of β-adrenoceptors in blood vessels supplying the posterior capsule of the rabbit knee joint.

2. Electrical stimulation of the posterior articular nerve (PAN) and close intra-arterial injection of adrenaline produced vasoconstriction which reversed to vasodilatation with administration of the α-adrenoceptor antagonist phenoxybenzamine. In almost all animals close intra-arterial injection of the β-adrenoceptor agonist isoprenaline resulted in vasodilatation.

3. Injection of the more selective β agonists dobutamine, salbutamol and terbutaline also produced vasodilatation with a rank potency order of isoprenaline > dobutamine > salbutamol ≥ terbutaline.

4. The β-adrenoceptor antagonist propranolol abolished the dilator responses to adrenaline and isoprenaline, and significantly reduced the dilator responses to PAN stimulation in phenoxybenzamine treated animals. Nerve-mediated vasodilatation was also reduced by the substance P antagonist D-pro^{4} D-Trp^{7,9,10} SP$_{4-11}$ suggesting that substance P contributes to this dilatation.

5. Dobutamine, a selective β_1-agonist produced vasodilatation which was abolished by administration of the selective β_1-antagonist atenolol.

Isoprenaline-induced vasodilatation was substantially reduced by atenolol.

6. The dilator response to isoprenaline appeared to be unaffected by the selective β_2-antagonist ICI118551, but the weak dilator responses to the selective β_2-agonists salbutamol and terbutaline were significantly reduced by this antagonist.

7. The results of this study suggest that β-adrenoceptors appear to be involved in the sympathetic regulation of rabbit knee joint blood flow, and that this is predominantly mediated via β_1 adrenoceptors.

Introduction

In their studies on dog articular blood vessels, Cobbold & Lewis, (1956b,c) studied the vasoconstrictor effects of sympathetic nerve stimulation and noradrenaline on blood flow to the knee joint. These authors also described vasoconstriction in response to adrenaline, but did not comment on the finding that noradrenaline produced greater vasoconstriction than adrenaline given in similar doses, suggesting a possible role for β-adrenoceptors. Using the ^{133}Xe clearance technique, Dick *et al.* (1971) obtained evidence for a contribution from both α- and β-adrenergic mechanisms in the regulation of blood flow in normal and diseased human articular vascular beds. In a previous study from this laboratory (Khoshbaten & Ferrell, 1990b), it was shown that electrical stimulation of posterior articular nerve (PAN) to the cat knee joint produced a rapid vasoconstriction followed by a more prolonged vasodilatation. Intra-articular administration of the substance P antagonist D-pro^4 D-Trp^{7,9,10} SP_{4-11} resulted in significant reduction of the vasodilator response but not complete blockade (Khoshbaten & Ferrell, 1990b). More recently it has been shown that the α-adrenoceptor antagonist phenoxybenzamine converted adrenaline and neurally-induced vasoconstriction to vasodilatation in rabbit knee joint blood vessels (section one, this chapter) suggesting a possible role for β-adrenergic receptors in the regulation of articular blood flow. The aim of this study was to determine directly whether β-adrenoceptors are present on rabbit posterior knee joint blood vessels, the subtype of these receptors, and whether they can mediate neurally-induced vasodilatation.

Joint vascular resistance was calculated by dividing mean arterial pressure by blood flow.

Methods

a. Animal preparation and physiological recordings

Experiments were performed on 33 adult New Zealand rabbits (2.2-3.5kg). Animals were anaesthetized and prepared for physiological recordings as mentioned in section one (α-adrenoceptor profile). Heart rate was monitored by a cardiotachometer triggered by arterial blood pressure. Blood flow was monitored by the laser Doppler flowmeter (Moor Instruments MBF3). Mean arterial blood pressure was calculated by adding 1/3 of pulse pressure to the diastolic pressure. Blood pressure values in histograms represent changes in blood pressure when changes in joint blood flow reached their peak following injection of adrenoceptor agonist or nerve stimulation.

The posterior articular nerve (PAN) dissection and stimulation was performed as mentioned in section one. At the end of experiment the animal was killed by intravenous injection of 1M KCl.

b. Drug administration

Except the substance P antagonist, all drugs were administered by the same route as mentioned in section one. The rate of infusion of β-adrenoceptor antagonists was based on their molecular weights. Propanolol was infused at 25μg/kg/min, atenolol at 22μg/kg/min and ICI118551 at 26μg/kg/min. Each of these were administered for 20min prior to, and throughout the test procedures. Only one β antagonist was infused in each animal. Phenoxybenzamine and rauwolscine were administered as a continuous infusion (3μg/kg/min and 2.8μg/kg/min

respectively) and a slow intravenous bolus injection of 1mg/kg body weight at the beginning of infusion period, with the duration of infusion being maintained for 45min before test procedures were performed, to ensure complete α-adrenoceptor blockade and allow the blood pressure to stabilise. Only one β antagonist was infused after phenoxybenzamine or rauwolscine in each animal. All drugs were dissolved in physiological saline. Adrenaline and all β-agonists were injected in random order and in volumes of 0.25 ml. Control injections of 0.25ml saline has been found to have no effect on blood flow (fig 2.9, chapter two). The substance P antagonist, D-pro^{4} D-Trp^{7,9,10} SP_{4-11}, was injected at a dose of 125μg in a volume of 0.2ml into the joint space through the posterior capsule 15min prior to the test procedure. Such administration was necessary due to limited supplies of this costly agent. The drugs used and their suppliers have been mentioned in chapter two.

The values expressed in the graph and histograms are means ± SEM and comparisons were performed using the student's paired or unpaired t-test as appropriate. All n values refer to the number of animals examined.

Results

a. Effect of isoprenaline injection

Close intra-arterial injection of the β_1, β_2 agonist isoprenaline elicited vasodilatation of posterior knee joint blood vessels. Out of a total of 25 rabbits examined, only three showed no response to injection of 2.5 nmole isoprenaline and these animals were excluded from the study. The isoprenaline dose-response curve (fig 5.6) shows that this dose is the most appropriate as larger doses could have significant depressor effects on systemic blood pressure which would tend to counteract the dilator effect of this agent on articular blood vessels. Thus, dilator responses to doses of isoprenaline larger than 2.5nmole illustrated in fig 5.6 may have been attenuated by the fall in systemic blood pressure which occurred with administration of these higher doses.

The choice of the appropriate dose of the more selective β-agonists, dobutamine, terbutaline, and salbutamol was also constrained, although these had smaller effects on blood pressure. However, larger doses of these agonists could not be used due to possibility of the agonist cross-reacting with other β-adrenoceptor subtypes.

b. Effect of PAN stimulation and different agonists and antagonists

PAN stimulation and adrenaline produced vasoconstriction, whilst the β-adrenoceptor agonist isoprenaline produced vasodilatation in the blood vessels of the posterior knee joint capsule (fig 5.7A). This

Fig 5.6. Dose/response curve for close intra-arterial injection (0.25ml volume) of isoprenaline (n=5). The points represent means ± SEM. The dilator responses to doses larger than 2.5 nmole may have been reduced by the depressor effect of the drug on systemic blood pressure at these doses. * = means differ significantly ($p<0.05$) from the smallest dose. p=pico, n=nano.

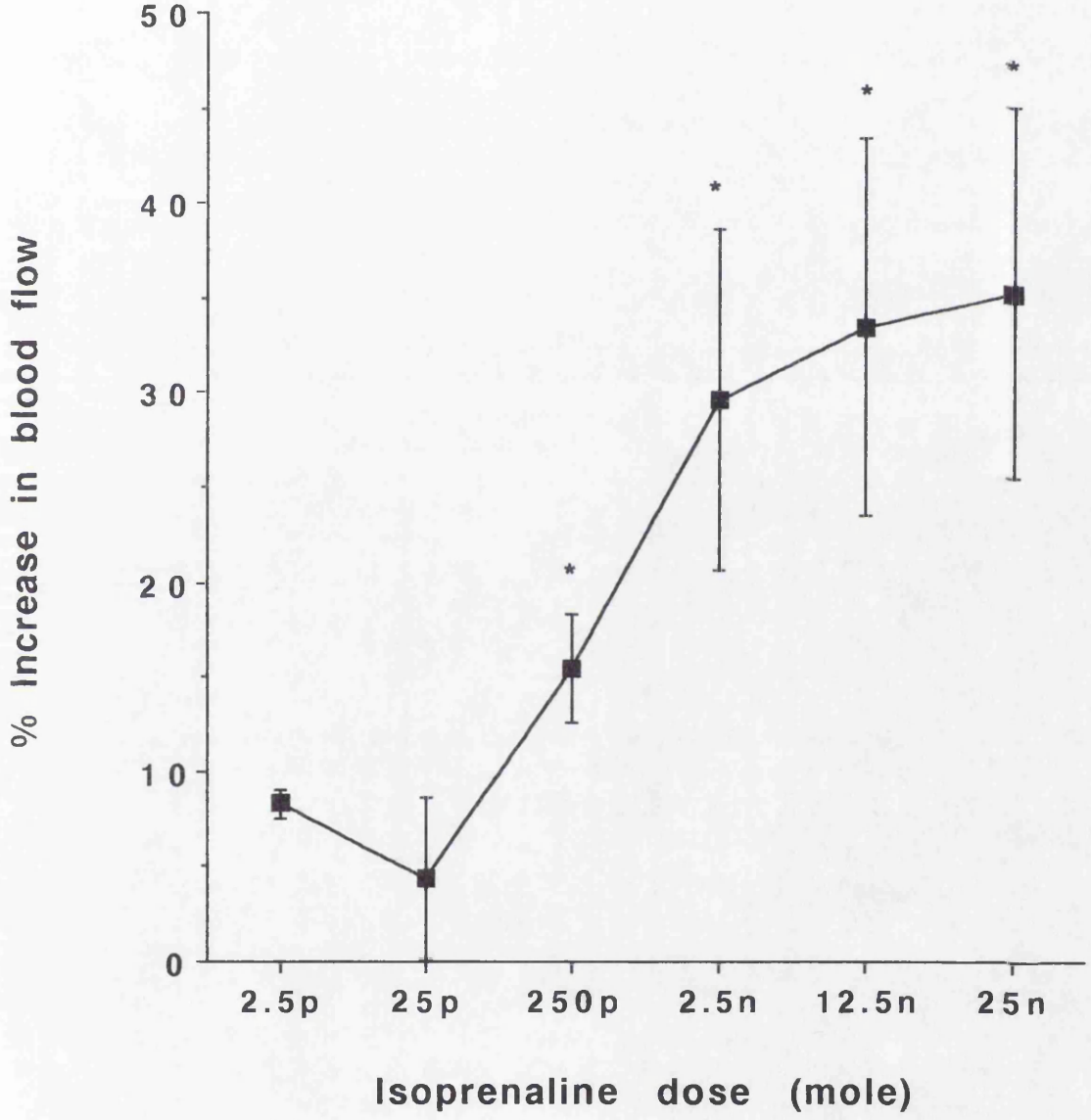
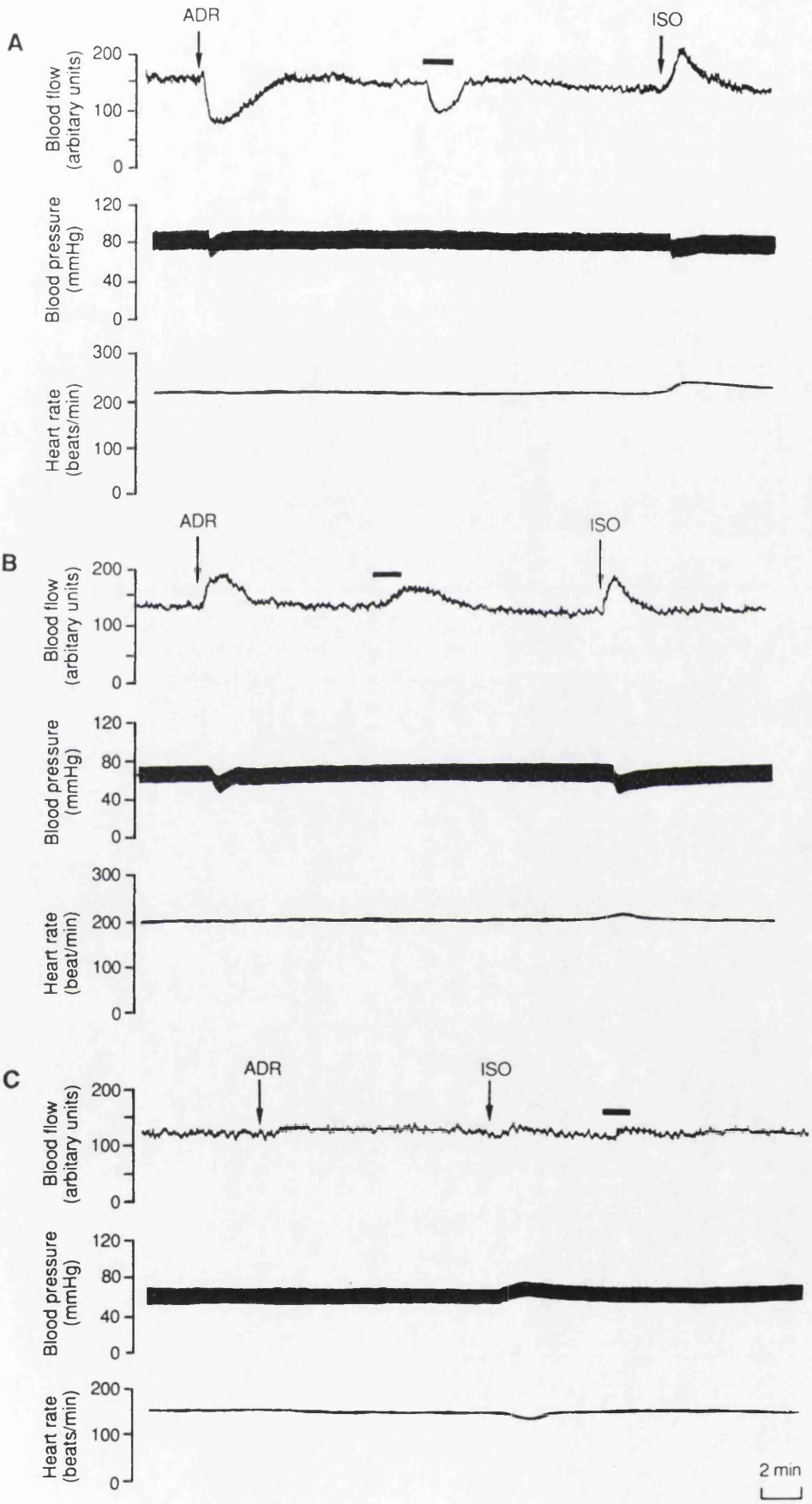

50
40
30
20
10
0
% Increase in blood flow
*
*
*
*
2.5p
25p
250p
2.5n
12.5n
25n
Isoprenaline dose (mole)

Fig 5.7. **A:** Posterior knee joint blood vessel, and cardiovascular responses to electrical stimulation of PAN (10V, 10Hz, 1msec width; 90s train; denoted by black bar over trace), and to close intra-arterial injection of 2.5 nmole adrenaline and isoprenaline. As PAN was centrally cut, nerve stimulation had no effect on systemic arterial blood pressure or heart rate whilst adrenaline and isoprenaline produced changes.

B: Responses to the same variables as in A and in the same animal but 45 min after administration of phenoxybenzamine (see methods). The constrictor responses have now been converted to vasodilatations.

C: Responses to the same variables as in B and in the same animal but 20min after close intra-arterial infusion of propranolol (see methods). The dilator responses were substantially inhibited by propranolol. Heart rate has also decreased because of β−adrenoceptor blockade.


A
ADR
ISO
Blood flow (arbitary units)
200
100
0
Blood pressure (mmHg)
120
80
40
0
Heart rate (beats/min)
300
200
100
0
B
ADR
ISO
Blood flow (arbitary units)
200
100
0
Blood pressure (mmHg)
120
80
40
0
Heart rate (beat/min)
300
200
100
0
C
ADR
ISO
Blood flow (arbitary units)
200
100
0
Blood pressure (mmHg)
120
80
40
0
Heart rate (beats/min)
200
100
0
2 min

vasodilatation indicates that β-adrenoceptors are present in this vascular bed, the results being summarized in fig 5.8A. The vasoconstrictor responses were abolished and converted to vasodilatation by treatment with the non-selective α-adrenoceptor antagonist phenoxybenzamine (fig 5.7B & 5.8A). Both test results are significantly different from their controls.

All dilator responses after phenoxybenzamine administration were virtually abolished by addition of the non-selective β-adrenoceptor antagonist propranolol (fig 5.7C) and in the case of adrenaline, the response once more reverted to a small vasoconstriction (fig 5.8A). All these responses were significantly different from their control values in phenoxybenzamine-treated animals.

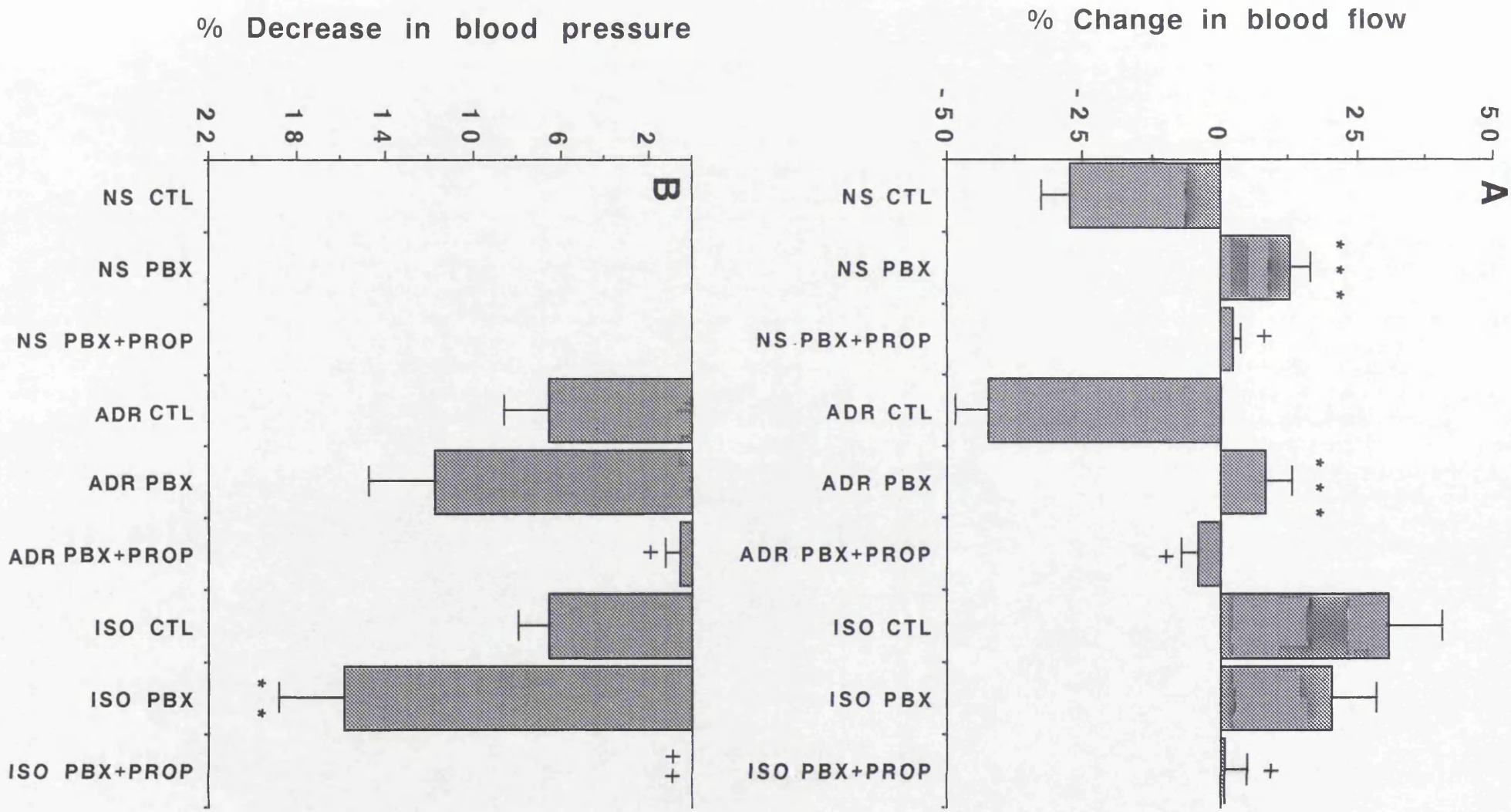
As PAN was cut centrally, stimulation of this nerve had no effect on systemic blood pressure or heart rate, whilst close intra-arterial injection of adrenaline and isoprenaline (each at a dose of 2.5nmole) had some transient effects (fig 5.7 & 5.8B) which were altered appropriately in some cases after using antagonists (fig 5.7C & 5.8B). PAN section had only a transient effect on blood pressure in some animals, and showed no significant effect on basal joint blood flow (see also chapter two).

Basal mean arterial pressure and basal blood flow to the joint were decreased by administration of phenoxybenzamine, and these differed significantly from their control values. The former decreased (±SEM) from 72.7 ± 5.2 to 55.5 ± 3.1 mmHg, ($p<0.004$, $n=10$) and the latter from 137.3 ± 14.2 to 112.6 ±15 flux units, ($p<0.01$, $n=10$). Subsequent administration of propranolol changed the mean blood

Fig 5.8 A: The constrictor response to PAN stimulation prior to treatment with phenoxybenzamine (NS CTL) is converted to vasodilatation by this agent (NS PBX). This dilator response is substantially reduced by propranolol (NS PBX+PROP). Similarly, close intra-arterial injection of 2.5nmole adrenaline (ADR CTL) produces constriction which is converted to dilatation in phenoxybenzamine treated animals (ADR PBX). Subsequent propranolol administration abolished this dilatation (ADR PBX+PROP). The dilator response to isoprenaline (ISO CTL) was not significantly affected by phenoxybenzamine (ISO PBX) but almost completely abolished by propranolol (ISO PBX+PROP).

B: Corresponding percentage changes in mean arterial pressure from pre-test basal values which transiently occurred under the same conditions as in A. No change in blood pressure was observed during PAN stimulation.

$*$ = means differ significantly from control (CTL) situation; + = means differ significantly from PBX. ** = $P<0.01$, *** = $P<0.001$, + = $P<0.05$.


% Decrease in blood pressure
22
18
14
10
6
2
B
NS CTL
NS PBX
NS PBX+PROP
ADR CTL
ADR PBX
ADR PBX+PROP
ISO CTL
ISO PBX
ISO PBX+PROP
% Change in blood flow
-50
-25
0
25
50
A
NS CTL
NS PBX
NS PBX+PROP
ADR CTL
ADR PBX
ADR PBX+PROP
ISO CTL
ISO PBX
ISO PBX+PROP

pressure to 56.6 ± 4 mmHg (n=7) and basal blood flow to 121 ± 16.5 flux units (n=7), which are not significantly different from phenoxybenzamine treatment. The calculated basal vascular resistance (see methods) changed from 0.616±0.10 units in control conditions to 0.518±0.76 units after phenoxybenzamine (P=0.08; n=10), and to 0.502±0.06 units after propranolol (P=0.25 compared to phenoxybenzamine treatment; n=7).

In order to assess the nature of the adrenoceptors mediating these dilator responses, more selective β-adrenoceptor agonists and antagonists were employed. The selective β_1-agonist dobutamine (2.5 nmole) exerted dilator effects on articular blood vessels whilst it had no effect on systemic blood pressure or heart rate at this dose (fig 5.9A). The two β_2-agonists salbutamol and terbutaline (2.5 nmole) produced weak dilator responses which were usually smaller than the responses to dobutamine and isoprenaline (fig 5.9A & 5.10A). Dobutamine had very small effects on systemic blood pressure in this dose, whilst isoprenaline and salbutamol were more effective (fig 5.9A,B & 5.10B).

The selective β_1-antagonist atenolol abolished the dilator responses to dobutamine ($p<0.05$, n=6), substantially attenuated the dilator responses to isoprenaline ($p<0.001$, n=10), but had little effect on responses to salbutamol and terbutaline (p=0.43 and p=0.26 respectively; n=10 in both cases; fig 5.10A).

Infusion of atenolol had small but significant effects on systemic blood pressure (from 67.5±3.5 to 61.8±3.2 mmHg, p=0.03, n=10) and reduced the heart rate (from 200±8.5 to 156.2±5.8 beats/min; $p<0.001$, n=10), and basal blood flow (from 150.5±13.8 to 132.4±11.4 flux

Fig 5.9. **A**: Trace shows the posterior knee joint blood vessel, and cardiovascular responses to close intra-arterial injection of 2.5 nmole of the β_1 agonist dobutamine (DOB), the β_2 agonists salbutamol (SAL) and terbutaline (TER) and the β_1,β_2 agonist isoprenaline (ISO) in a rabbit. All the agonists produced vasodilatation, but the potency rank order is isoprenaline > dobutamine > salbutamol $\geq$ terbutaline. Dobutamine had no effect on blood pressure at this dose but the other three were effective. Changes in HR are from the direct effect of the drugs on the heart or secondary to the changes in blood pressure.

B: The responses of the same animal as in A but 20min after onset of close intra-arterial infusion of the β_1 antagonist atenolol (see methods). All vasodilator responses are substantially decreased by this agent, especially in the case of dobutamine and isoprenaline. HR is decreased significantly (see results).

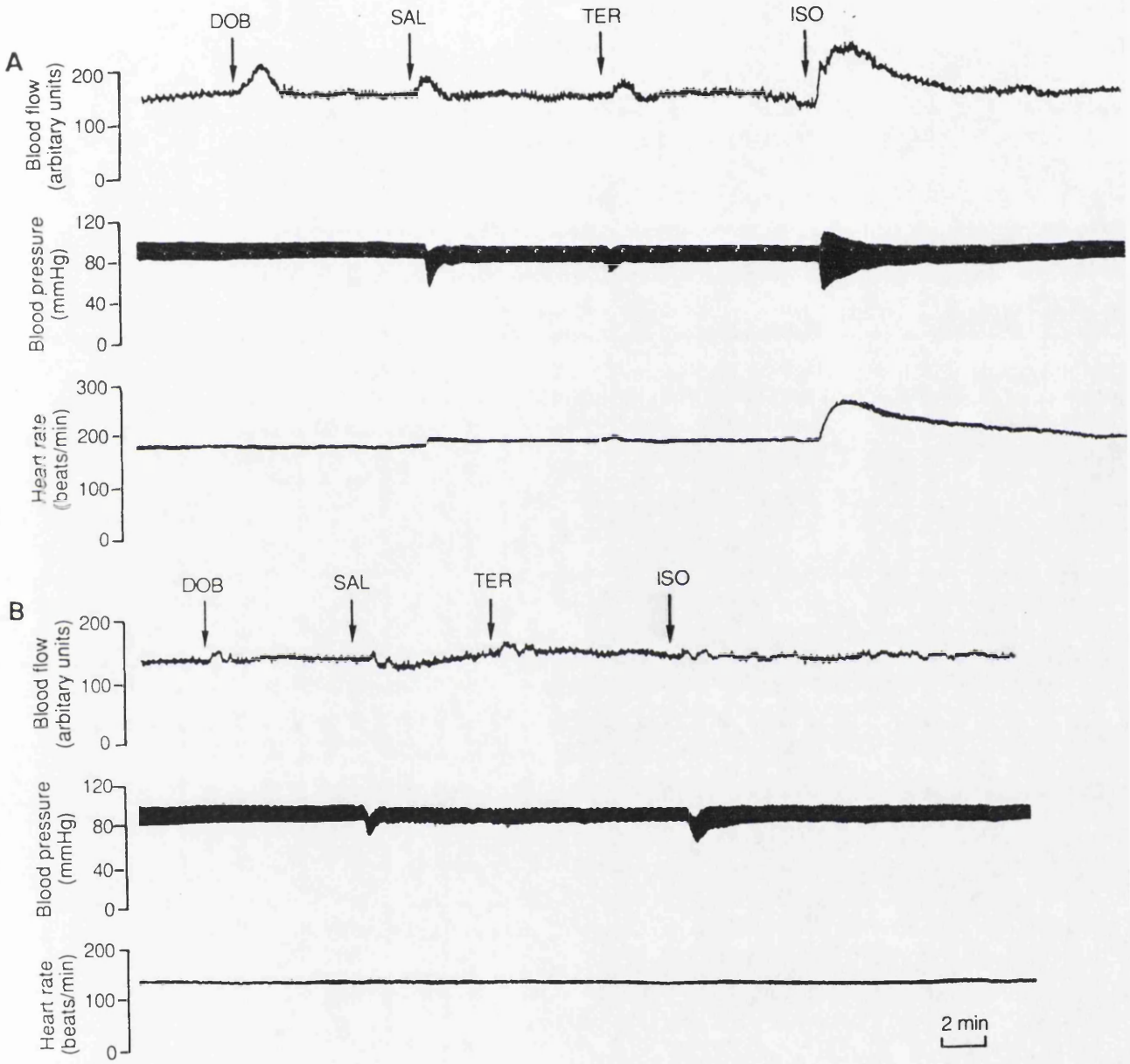
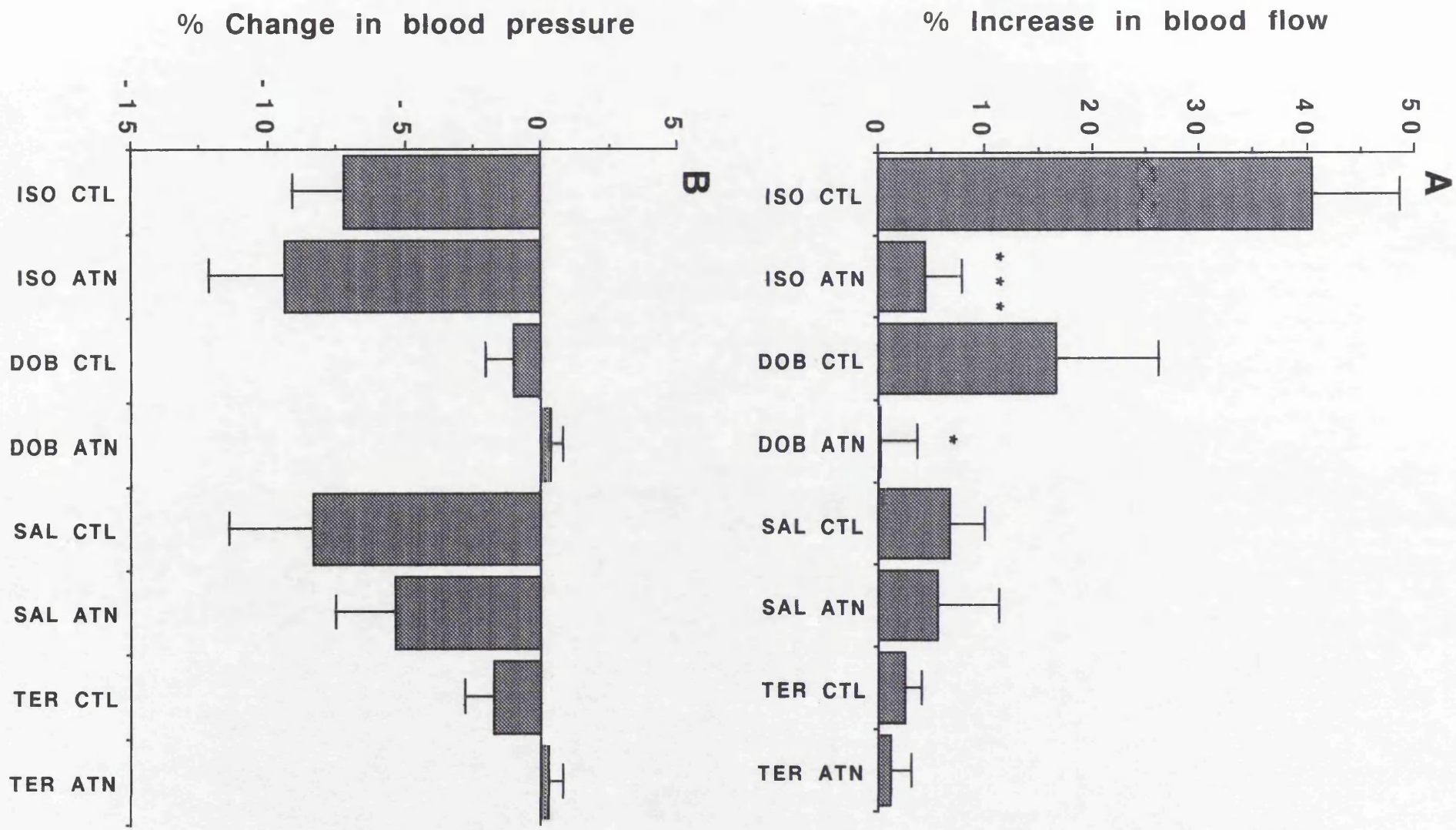

A
DOB
SAL
TER
ISO
Blood flow (arbitary units)
200
100
0
Blood pressure (mmHg)
120
80
40
0
Heart rate (beats/min)
300
200
100
0
B
DOB
SAL
TER
ISO
Blood flow (arbitary units)
200
100
0
Blood pressure (mmHg)
120
80
40
0
Heart rate (beats/min)
200
100
0
2 min

Fig 5.10. **A:** The dilator responses to close intra-arterial injection of 2.5 nmole isoprenaline (ISO CTL) and dobutamine (DOB CTL) are substantially decreased by atenolol infusion (ISO ATN and DOB ATN respectively). The weak dilator responses to salbutamol (SAL CTL) and terbutaline (TER CTL) were slightly decreased by atenolol (SAL ATN and TER ATN respectively) but the differences are not statistically significant. * = $p < 0.05$ and *** = $p<0.001$ For n values see text.

B: Percentage changes in mean arterial pressure, transiently lowered due to close intra-arterial injection of the β-adrenoceptor agonists mentioned in part **A**, from pre-test basal values. Changes in blood pressure with agonist administration after atenolol infusion are not significantly different from those values obtained prior to infusion of this β_1 antagonist.


% Change in blood pressure
-15
-10
-5
0
5
B
ISO CTL
ISO ATN
DOB CTL
DOB ATN
SAL CTL
SAL ATN
TER CTL
TER ATN
% Increase in blood flow
0
10
20
30
40
50
A
ISO CTL
ISO ATN
DOB CTL
DOB ATN
SAL CTL
SAL ATN
TER CTL
TER ATN
* * *
*

units, P<0.05, n=10). None of the blood pressure changes due to injection of the agonists were significantly different before and after atenolol infusion (fig 5.10B). Basal vascular resistance changed from 0.504±0.06 units in control conditions to 0.509±0.06 units after atenolol (P=0.45, n=10).

The β_2-antagonist ICI118551 did not significantly affect the dilator responses to isoprenaline and dobutamine in joint blood vessels (p=0.24, n=6 and p=0.17, n=6 respectively; fig 5.11 & 5.12A). However, this agent significantly reduced the dilator effects of salbutamol and terbutaline (p<0.01, n=6, and p<0.05, n=6 respectively) as summarised in fig 5.12A.

Infusion of ICI118551 inhibited the transient systemic effects of the β_2 agonists (fig 5.11B & 5.12B) and also had significant effects on basal systemic blood pressure and heart rate (from 65.7±5 to 73.5±6.2 mmHg, p<0.01, n=6, and 208±13.5 to 197±14 beats/min, p<0.05, n=6; respectively). Basal vascular resistance was not changed significantly with ICI118551 (from 0.558±0.06 units in control conditions to 0.565±0.06 units; P=0.89, n=6).

c. Nerve-mediated vasodilatation

Previous experiments showed that the more specific α_2-adrenoceptor antagonist rauwolscine is as potent as phenoxybenzamine in blocking vasoconstrictor responses to PAN stimulation but has much smaller systemic hypotensive effects (see section one). In a group of seven animals, rauwolscine was used to block the constrictor responses to PAN stimulation. The resultant dilator responses were significantly (p<0.05) reduced by subsequent atenolol infusion (fig 5.13). Intra-

Fig 5.11. **A:** Trace shows the effect of close intra-arterial injection of 2.5 nmole of dobutamine, isoprenaline, salbutamol, and terbutaline on posterior knee joint blood flow. Isoprenaline and dobutamine are more effective than the other two agonists.

B: The responses to the the same drugs in the same animal but 20min after onset of close intra-arterial infusion of the selective β_2 adrenoceptor antagonist ICI118551. Blood flow responses to dobutamine and isoprenaline were unaffected by the antagonist infusion whilst systemic blood pressure effects of agonists were abolished by ICI118551.

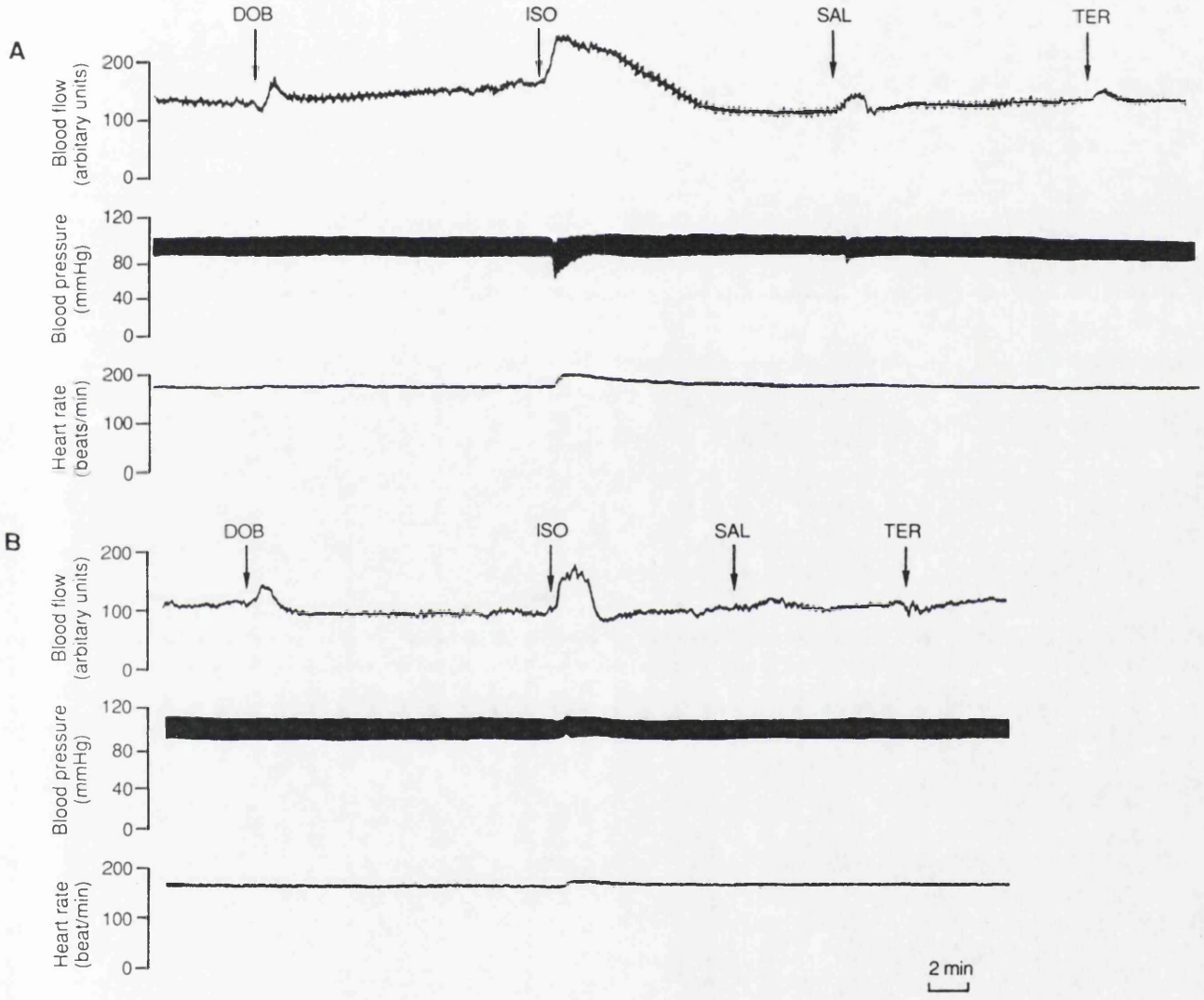

A
DOB
ISO
SAL
TER
Blood flow (arbitary units)
200
100
0
Blood pressure (mmHg)
120
80
40
0
Heart rate (beats/min)
200
100
0
B
DOB
ISO
SAL
TER
Blood flow (arbitary units)
200
100
0
Blood pressure (mmHg)
120
80
40
0
Heart rate (beat/min)
200
100
0
2 min

Fig 5.12. **A:** The dilator responses to isoprenaline (ISO CTL) and dobutamine (DOB CTL) in normal animals were not affected by infusion of ICI118551 (ISO ICI and DOB ICI respectively). However the dilator responses to salbutamol (SAL CTL) and terbutaline (TER CTL) were significantly reduced by this agent (SAL ICI, and TER ICI respectively). *, ** = means differ significantly ($p< 0.05$, $p< 0.01$ respectively) from CTL. For n values see text.

B: Percentage changes in mean arterial pressure from pre-test basal values, transiently lowered following close intra-arterial injection of the β-adrenoceptor agonists mentioned above. ICI118551 blocked all the changes in blood pressure produced by injection of β – agonists. *, ** = means differ significantly ($p< 0.05$, $p< 0.01$ respectively) from CTL.

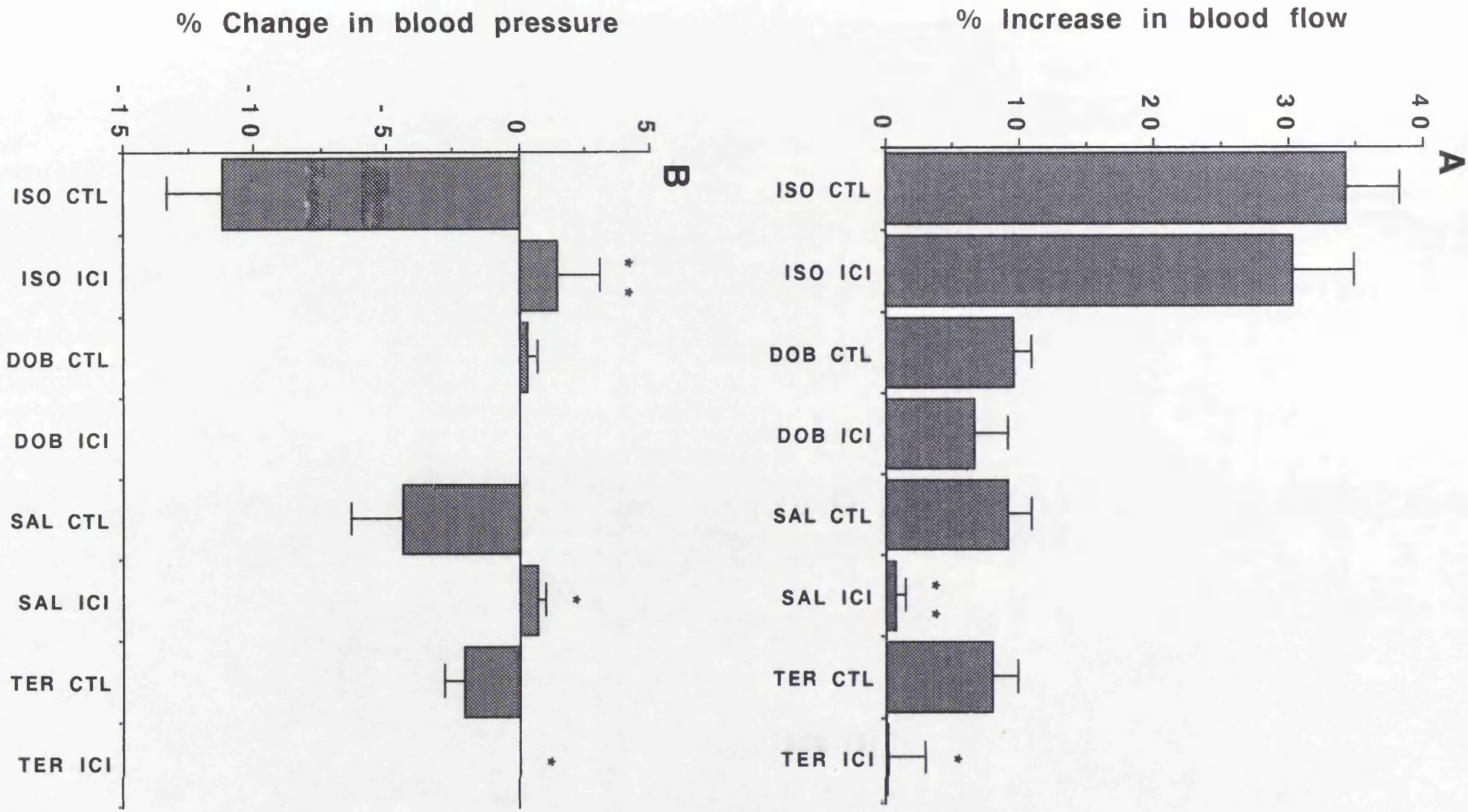
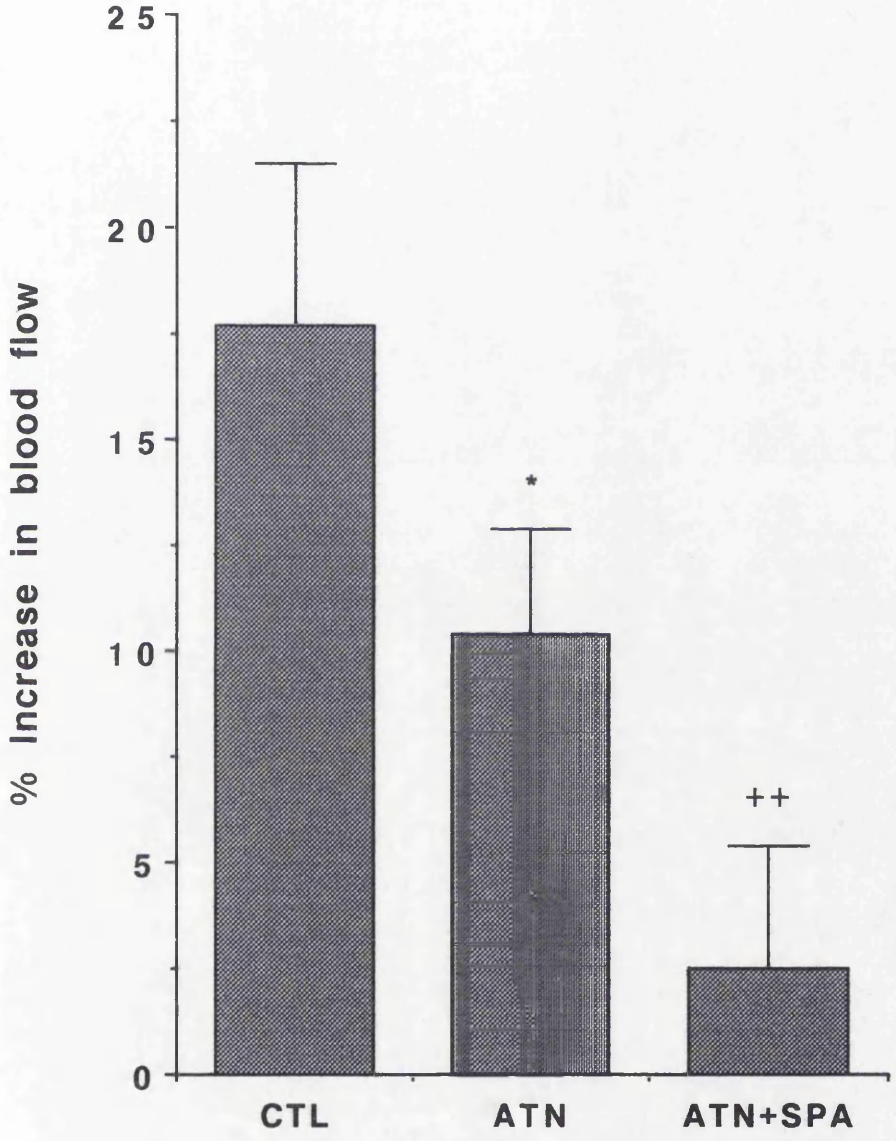

% Change in blood pressure
-15
-10
-5
0
5
B
ISO CTL
ISO ICI
DOB CTL
DOB ICI
SAL CTL
SAL ICI
TER CTL
TER ICI
% Increase in blood flow
0
10
20
30
40
A
ISO CTL
ISO ICI
DOB CTL
DOB ICI
SAL CTL
SAL ICI
TER CTL
TER ICI

Fig 5.13. Nerve-mediated dilator responses in rauwolscine-treated animals (CTL) are significantly decreased by atenolol infusion (ATN). The residual dilator response is further substantially reduced by intra-articular injection of 125μg of the substance P antagonist D-pro^{4} D-Trp^{7,9,10} SP_{4-11} (ATN+SPA). * = significantly different ($p<0.05$) from CTL; ++ = significantly different ($p<0.01$) from ATN. n=7.


% Increase in blood flow
25
20
15
10
5
0
*
++
CTL
ATN
ATN+SPA

articular injection of 125μg of the substance P antagonist D-pro^4 D-Trp^{7,9,10} SP_{4-11} produced further significant ($p<0.01$) reduction of the residual dilator response. Administration of rauwolscine decreased the basal mean blood pressure from 73 $\pm$ 4.7 to 65 $\pm$ 4.4 mmHg, and basal blood flow from 221 $\pm$ 13 to 176$\pm$24 flux units, ($p<0.04$ and $P=0.09$ respectively, n=7). Basal vascular resistance changed from 0.540$\pm$0.07 units in control conditions to 0.495$\pm$0.09 units after rauwolscine ($P=0.21$, n=7). The substance P antagonist had no effect on systemic blood pressure by this route of administration.

Discussion

The results of this study are consistent with previous findings that the vasoconstrictor responses to PAN stimulation and close intra-arterial injection of adrenaline can be reversed to dilator responses by administration of the irreversible α-adrenoceptor antagonist phenoxybenzamine (section one). The present study has extended this to demonstrate the presence of β-adrenoceptors in articular blood vessels. The β-adrenoceptor agonist isoprenaline potently dilated blood vessels in the posterior capsule of the knee joint (fig 5.6), despite its transient lowering effect on systemic blood pressure (fig 5.7) which tends to counteract the former effect. These results along with the fact that the dilator responses to adrenaline, isoprenaline and nerve stimulation were virtually abolished by propranolol suggests that β-adrenoceptors are present in this vascular bed. A previous study using an isolated rabbit knee joint preparation (Ferrell & Khoshbaten, 1989) failed to demonstrate the presence of β-adrenoceptors, but such *in vitro* results have to be interpreted with caution. As it was shown in section one, α_2-adrenoceptors predominated in posterior capsule blood vessels whereas the previous *in vitro* investigation has shown a predominance of α_1-adrenoceptors (Ferrell & Khoshbaten,1989). Such differences could arise due to the different methodologies used in these studies. Ferrell & Khoshbaten (1989) measured changes in perfusion pressure as an indirect indicator of resistance whereas laser Doppler flowmetry was used in this *in vivo* study in which changes in blood flow are indicators of changes in vascular resistance. On the other hand, the former technique is likely to reflect changes in the calibre of the larger

resistance vessels whereas the latter reflects changes in the smallest arterioles and in the capillaries. In addition, there may be differences in the responsiveness of blood vessels resulting from changes in vessel tone or the absence of circulating vasoactive substances *in vitro*. It is possible that the laser Doppler technique is more sensitive for detection of dilator responses or that β- adrenoceptors are present on smallest resistance vessels.

To investigate the nature of these β-adrenoceptors, more specific agonists and antagonists were employed. Although the dilator responses to dobutamine, a selective β_1-agonist (Weiner, 1980a), were smaller than the responses to isoprenaline (fig 5.9A), the potent inhibitory effect of the selective β_1-antagonist atenolol (Robertson, Kaplan, Caldwel & Speight, 1983) on the dilator responses to both of these agents (fig 5.9B) suggests that these effects are mediated via β_1 adrenoceptors. Atenolol cross-reacting with β_2 adrenoceptors is unlikely because the transient changes in blood pressure due to isoprenaline or salbutamol injection are not significantly different before and after atenolol (fig 5.10B), suggesting that β_2 adrenoceptors in other vascular beds are unaffected by atenolol. The difference in potency on blood flow of isoprenaline and dobutamine may reflect differences in their affinities for β_1 adrenoceptors or the additive β_2 effect of isoprenaline. The specificity for β_1 adrenoceptors of dobutamine and atenolol is evident from the negligible hypotensive effect of dobutamine, and inability of atenolol to block the depressor effects of isoprenaline and salbutamol (fig 5.9B & 5.10B), which are exerted via systemic β_2 adrenoceptors in other vascular beds, particularly in skeletal muscle. On the other hand, isoprenaline and salbutamol seemed to be equipotent based on their

hypotensive effects (fig 5.10B), but the former showed a more powerful vasodilating effect on joint blood vessels (fig 5.10A). This may reflect an additive effect on β_1 adrenoceptors.

Dobutamine is used in the treatment of cardiac failure mostly for its positive inotropic effect on the human heart. In the present study and at the dose used, it showed no chronotropic effect on the rabbit heart (fig 5.9A & 5.11A), although isoprenaline showed clear chronotropic effects. This could be due to dobutamine having less chronotropic effects (Tuttle and Mills, 1975), or may reflect species differences in β-adrenoceptor subtypes in the heart.

The β_2-agonists salbutamol and terbutaline appeared to be much less potent in joint blood vessels than isoprenaline and dobutamine (fig 5.9A & 5.10A). Although their dilator effects were somewhat decreased by atenolol, they did not differ significantly from control values (fig 5.10A). The β_2-antagonist ICI118551 failed to inhibit the dilator responses to isoprenaline and dobutamine (fig 5.11B & 5.12A), although the smaller dilator responses to salbutamol and terbutaline were significantly reduced by ICI118551. The latter finding may indicate that a minor population of β_2-adrenoceptors also exist in articular blood vessels. The specificity of ICI118551 is clear from its blocking action of the hypotensive effects of isoprenaline, salbutamol and terbutaline (fig 5.12B) whilst it exerts no direct effect on the positive chronotropic effect of isoprenaline on the heart (fig 5.11B). The longer lasting effect of isoprenaline on blood flow before ICI118551 infusion (fig 5.11A) could be related to its additive β_2-mediated effect on joint blood vessels, although the reduced isoprenaline time course of action following ICI118551 was not consistently observed.

Although infusion of most of the α and β adrenoceptor antagonists resulted in decreased basal blood flow to the joint, this is likely to have been a consequence of the decrease in perfusion pressure and not due to changes in joint vascular resistance as calculated resistance values before and during adrenoceptor blockade were not significantly different (see results).

The nerve-mediated vasodilatation which was observed after blockade of α-adrenoceptors appears to have two components. One of these was mediated via β_1-adrenoceptors as it was reduced by propanolol (fig 5.7C & 5.8A) and atenolol (fig 5.13). Although propranolol has been reported to have membrane stabilising effects at higher doses, its inhibitory effect on nerve-mediated vasodilator responses is unlikely to be due to this as these responses were also significantly reduced by the equivalent dose of atenolol which does not have membrane stabilising effects compared to propranolol (Weiner, 1980b). The other component, which remained after α and β adrenoceptor blockade, may have been produced by substance P released from the endings of sensory C-fibers travelling in PAN and therefore activated during PAN stimulation as the substance P antagonist D-pro^4 D-Trp^{7,9,10} SP_{4-11} significantly reduced the residual dilator responses (fig 5.13).

In summary, the results of this study suggest that the dilator responses to stimulation of sympathetic nerves present in PAN, which are unmasked after using the α-adrenoceptor antagonist phenoxybenzamine or the α_2-adrenoceptor antagonist rauwolscine, are predominantly mediated via postjunctional β_1 adrenoceptors. Although β_1 adrenoceptors are well known to be present in heart muscle and

vascular smooth muscle of the coronary vessels, there are few reports of their presence in the vascular smooth muscle of more peripheral vascular beds. The rat pulmonary artery (O'Donnell & Wanstall, 1981) and jugular vein (Cohen & Wiley, 1978) have been shown to contain both types of β-adrenoceptors. As β_1-adrenoceptors can be activated by noradrenaline, it has been suggested that these (tissues) may be "functionally innervated" by sympathetic fibres (O'Donnell & Wanstall, 1984). If this is the case, it is possible that these receptors may have a role in modulating the constrictor response to noradrenaline (mediated via α-adrenoceptors) to prevent cessation of blood flow to the joint during activation of the sympathetic nervous system. Whether this is of relevance in inflammatory joint disease has not yet been investigated.

CHAPTER SIX

Local regulation of blood flow in the rabbit knee joint

Section one

Role of nitric oxide in the regulation of joint blood flow and synovial PO_2

Summary

1. Experiments were performed to investigate in normal and acutely inflamed rabbit knee joints the role of nitric oxide in the regulation of joint blood flow as well as its modulation of sympathetic vasoconstriction.

2. Close intra-arterial infusion of Nω-nitro-L-argenine methyl ester (L-NAME) a nitric oxide (NO) production inhibitor, reduced basal joint blood flow, measured by laser Doppler flowmetry, by 36.4±5.1% in normal and 21.4±7.8% in carrageenan inflamed knee joints. Mean systemic arterial blood pressure was increased by 20±3.1% and 17.9±2% in normal and test animal groups respectively. Mean joint vascular resistance was increased by 101% in normal and 69% in carrageenan treated animals.

3. Vasoconstrictor responses to electrical stimulation of the posterior articular nerve (PAN) were significantly smaller in the inflamed joint compared to normal. Infusion of L-NAME for 45min resulted an increase in vasoconstrictor response by 78.1% in normal and 78.9% in inflamed joints.

4. Subsequent close intra-arterial infusion of L-arginine failed to return the enhanced vasoconstrictor responses induced by L-NAME infusion to their control levels in both normal and test animal groups, but partially restored blood flow changes (in normal joints from 72% to 84% of the basal value, in inflamed joints from 80% of the basal value to 93.5%).

5. Synovial fluid PO_2, measured by insertion of an oxygen electrode into the synovial cavity, increased in the normal joint during L-NAME infusion from 62.4±5.7 mmHg to 66.7±6.4 mmHg and finally to 73.8±8.2 mmHg following L-arginine infusion. In the inflamed joint it was decreased by L-NAME from 55.1±4.8 mmHg to 48.5±11.5 mmHg and increased by L-arginine to 52.7±10.1 mmHg.

6. In both normal and inflamed joints the vasoconstrictor responses to close intra-arterial injection of 2.5 nmole of the α_1-agonist phenylephrine and 250 pmole of the α_2-agonists clonidine and UK 14304 were increased significantly by L-NAME infusion but not completely restored to control values by L-arginine infusion.

7. The results of this study show that NO maintains the vessels of both normal and inflamed joints dilated and plays a major role in regulation of their basal tone and their basal blood flow. Also, NO plays an important role in modulation of sympathetic nerve mediated effects on joint blood flow. The rate of NO production does not appear to be increased by the process of acute inflammation.

Introduction

Previous results have shown a high degree of correlation between blood flow and synovial fluid oxygen tension in normal and acutely inflamed rabbit knee joints (see chapter four). It is therefore important to examine those factors which influence joint blood flow, and thus have an important effect on oxygen delivery to the avascular structures deep inside the joint. Production of nitric oxide (NO) by the vascular endothelium has been recently discovered to play a very important role, as a local factor, in physiological regulation of blood flow to different organs in the body (for review see Moncada *et al.*, 1991), but the role of NO in joint blood flow regulation has not yet been investigated. It has also been shown that sympathetic innervation to the joint plays an important role in modulation of blood flow and this effect is reduced in carrageenan induced acute inflammation with a significant reduction in synovial fluid PO_2 by the inflammatory process (see chapter four). Microsphere experiments showed that basal blood flow to the acutely inflamed rabbit knee joint is significantly higher than normal joint (see chapter three), but the possible role of NO on this alteration in blood flow is not clear. Whether NO modulates sympathetic constrictor influences in articular blood vessels has also not been examined.

The aim of this study was to determine, in normal and acutely inflamed rabbit knee joints, whether NO plays a role in regulation of basal blood flow and if this changes with joint inflammation. A second objective was to determine if NO has a role to play in modulating sympathetic vasoconstriction of articular blood vessels. Finally, to

examine the extent to which synovial PO_2 is changed by inhibition of NO production in normal and acutely inflamed rabbit knee joint blood vessels.

Methods

a. Animal preparation and physiological recordings

Experiments were performed on 34 adult New Zealand rabbits (2.2-3.5kg) which were anaesthetised initially by injection of diazepam (1.5mg/kg I.P.) and Hypnorm (Janssen; 0.15ml/kg I.M.) and thereafter maintained with a 1% halothane/O_2/N_2O mixture during the surgical procedures and throughout the remainder of the experiment. The trachea was cannulated, with the animals breathing spontaneously throughout the experiments. Arterial blood pressure was monitored through a cannula inserted into the left carotid artery. A laser Doppler flowmeter (Moor Instruments MBF3) was used to monitor the changes in posterior knee joint capsule. Alterations in blood flow and P_sO_2 are expressed as percentage change from control (0%) values occurring immediately before each test procedure. Joint vascular resistance was calculated by dividing mean arterial pressure by joint blood flow. The method of measurement of PO_2 in the synovial fluid has been described in chapter four.

The posterior articular nerve (PAN) dissection and stimulation was performed as mentioned in the previous chapter. At the end of experiment the animal was killed by intravenous injection of 1M KCl.

b. Drug administration

Drugs were administered by the same route as described in chapter five. L-NAME and L-arginine were administered by close intra-arterial infusion at a rate of 3mg/kg/hour and 6mg/kg/hour respectively,

for at least 15min prior to, and throughout test procedures. α-adrenoceptor agonists phenylephrine, UK 14304, and clonidine were injected in volumes of 0.25 ml. All drugs were dissolved in physiological saline.

c. Induction of acute joint inflammation

The method of production of acute joint inflammation has been described in chapter four. 1 ml sterile saline was injected into one knee joint of a separate series of animals (n=10) to serve as a control. This was necessary as it has been shown that inflammation of one knee can produce a symmetrical synovitis of the contralateral knee (Kidd *et al.*, 1989), invalidating the use of an internal control.

d. Statistical analysis

The values expressed in the graphs and histogram are means ± SEM and comparisons were performed using the student's paired or unpaired t-test as appropriate. All n values refer to the number of animals examined. P values of 5% or less were considered significant.

Results

a. Effect of L-NAME and L-arginine infusion on arterial blood pressure, joint blood flow, and joint vascular resistance

In both groups of animals infusion of L-NAME for one hour raised the systemic blood pressure by about 20% (fig 6.1 & 6.2). In contrast, and despite the high degree of correlation between blood flow and blood pressure, the basal blood flow to the joint was decreased between 22% in the inflamed (test) joints and 36% in the control joints. Basal vascular resistance was increased in the control group by ~100% and in the test group by ~70%. None of the above values in test group was significantly different with its corresponding value in the control group.

Subsequent infusion of L-arginine for one hour had no effect on blood pressure in both groups (in the control group from 81.8±4.3 mmHg to 79.3±5.7 mmHg, n=10; and in test group from 80.6±5.7 mmHg to 79±6.5 mmHg, n= 10). However blood flow reductions partially recovered during L-arginine infusion (in control group from 172±20 arbitrary units to 201±27 arbitrary units; p=0.1, n=10; and in test group from 105±11.9 arbitrary units to 123.2±13.4 arbitrary units; $p<0.01$ n=10). Infusion of L-arginine decreased the basal vascular resistance to 78.8±27% above the original (pre-L-NAME) value in control and 38.4±14% in test group ($P=0.16$ and $P<0.001$ respectively, compared to the L-NAME treatment).

Fig 6.1. **A:** Changes in joint basal blood flow and synovial PO_2 in a saline injected (control) rabbit knee during one hour of close intra-arterial infusion of L-NAME (LN) at a rate of 3mg/kg/hour. Systemic blood pressure was increased by L-NAME but blood flow was decreased. P_sO_2 started to recover after an initial decrease.
Each dot on the time scale represents one minute.

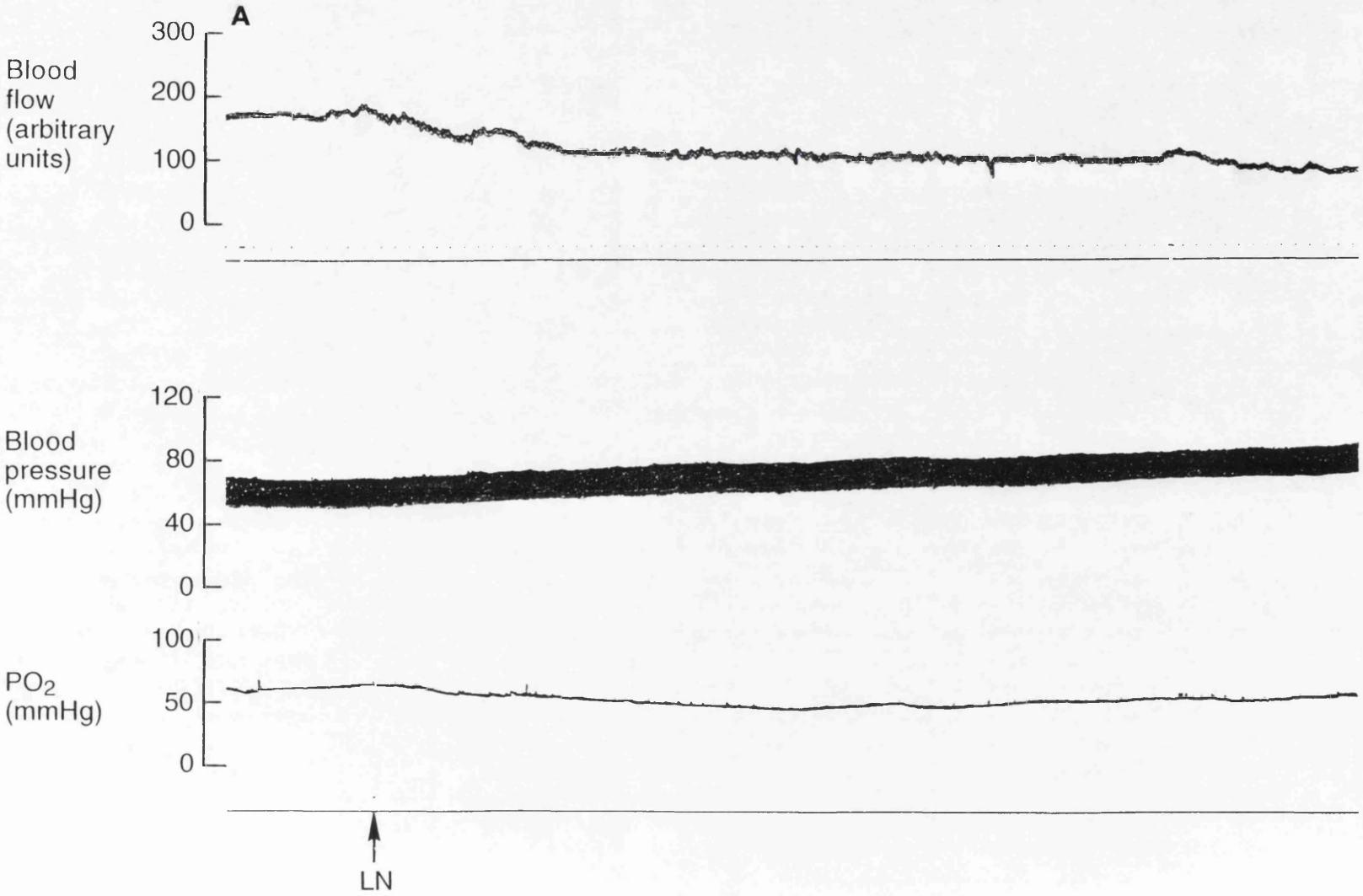

A
300
200
100
0
Blood flow (arbitrary units)
120
80
40
0
Blood pressure (mmHg)
100
50
0
PO_2 (mmHg)
LN

Fig 6.1. **B:** Changes in joint basal blood flow and synovial PO_2 in a saline injected (control) rabbit knee during one hour infusion of L-arginine (LA) at a rate of 6mg/kg/hour following L-NAME infusion. Systemic blood pressure does not recover, blood flow partially recovered and P_sO_2 continued to rise following an initial decrease. Each dot on the time scale represents one minute.

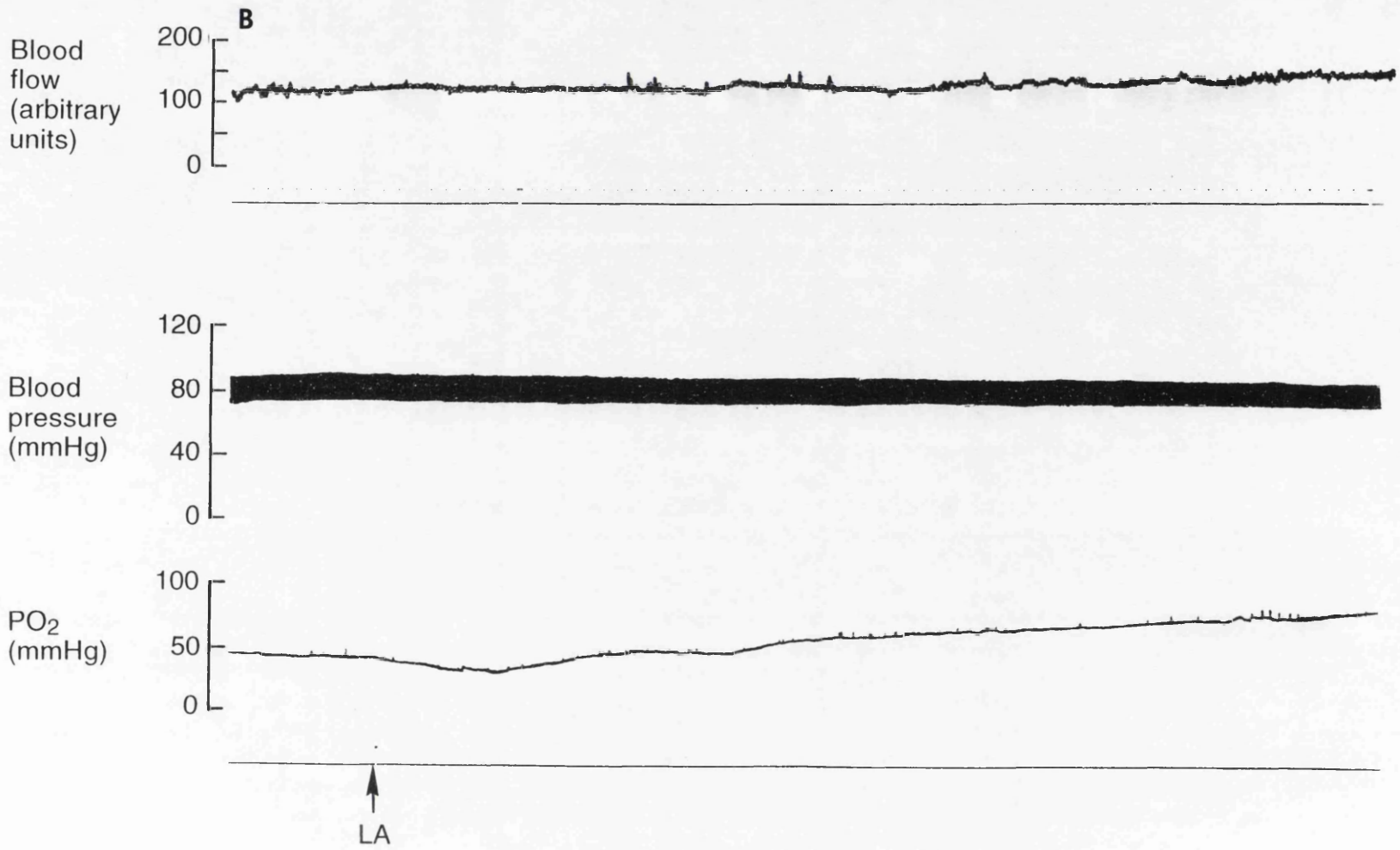
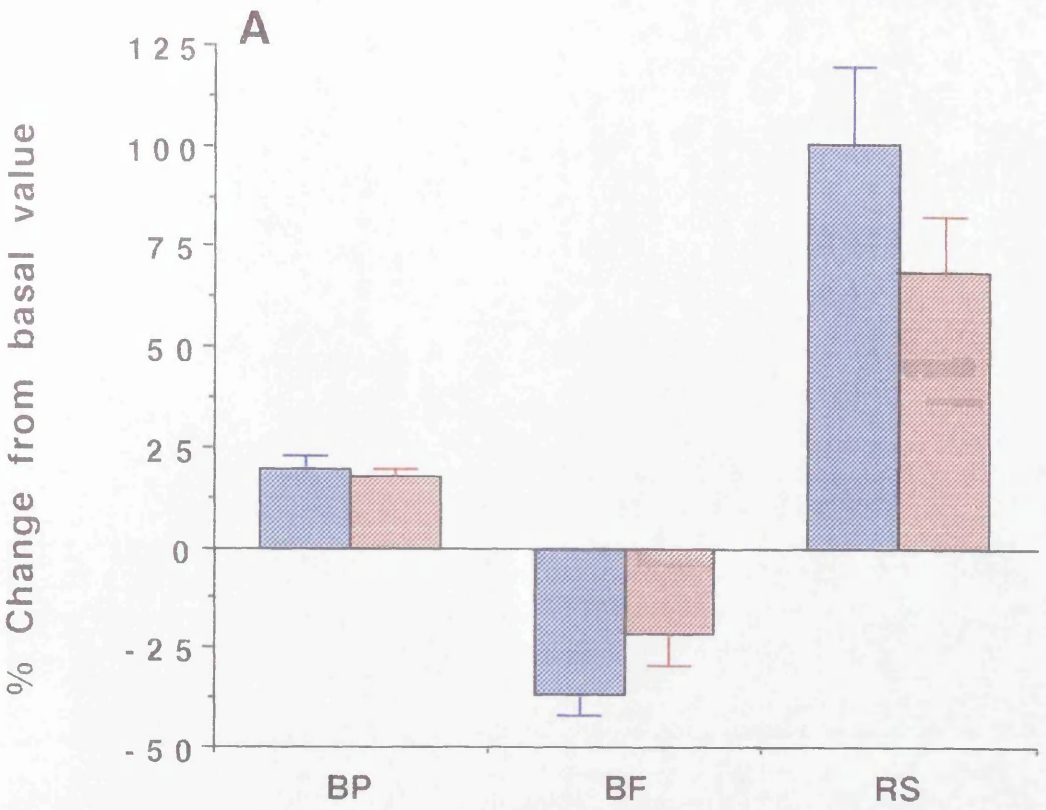
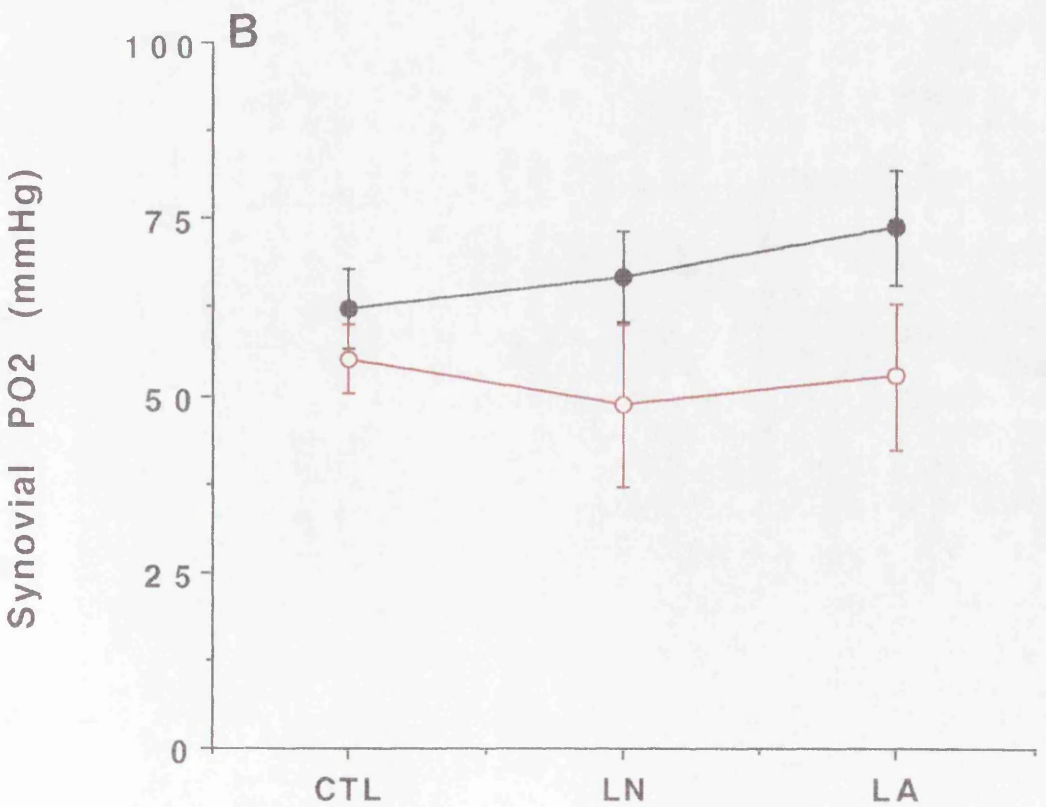

B
Blood flow (arbitrary units)
200
100
0
120
Blood pressure (mmHg)
80
40
0
100
PO_2 (mmHg)
50
0
LA

Fig 6.2. **A:** Percentage change in mean arterial blood pressure (BP) ,posterior capsular blood flow (BF) and joint vascular resistance (RS) from basal (pre-infusion = 0%) values during close intra-arterial infusion of L-NAME in control group (blue) and animals with inflamed joints (red). Although in both groups blood pressure rose, blood flow decreased due to inhibition of nitric oxide production with a resultant increase in joint vascular resistance.
(n=9 animals in control group, and n=8-10 in the test group).

B: Changes in synovial PO_2 following close intra-arterial infusion of L-NAME (LN) or subsequent L-arginine infusion (LA), each for one hour, in saline injected (black symbols) and inflamed (red symbols) knees. CTL refers to pre-infusion value. n=8 in saline injected and n=6 in inflamed.


A
% Change from basal value
125
100
75
50
25
0
-25
-50
BP
BF
RS


B
Synovial PO2 (mmHg)
100
75
50
25
0
CTL
LN
LA

b. Effect of L-NAME and L-arginine infusion on P_sO_2

Although blood flow was decreased by L-NAME infusion in both groups of animals, P_sO_2 showed a slight increase in normal joints along with a moderate decrease in inflamed ones (fig 6.2B). None of these changes were significantly different from their control values. P_sO_2 was increased in both groups by subsequent L-arginine infusion although these changes were again not statistically different.

c. Effect of L-NAME infusion on nerve-mediated responses

Electrical stimulation of PAN in the control (saline injected) joint produced a sharp reduction in posterior capsule blood flow which was followed by a longer lasting vasodilatation after cessation of stimulation (fig 6.3A). The vasoconstrictor response was increased sharply with L-NAME infusion while the vasodilator response was unaffected (fig 6.3B) suggesting that the latter is unlikely to be dependent on nitric oxide (NO) production. The magnitude of vasodilator response was $19.1\pm1.6\%$ before and $26.6\pm5\%$ 45min after initiation of L-NAME infusion ($P=0.07$, $n=8$). Two out of ten animals did not show any vasodilatation. No significant changes were also found in test group. There was a rapid rise in vasoconstrictor response during the first fifteen minutes of L-NAME infusion with a slower rise after that, leading to the maximum effect observed 45 minutes after the onset of infusion (fig 6.3B & 6.4).

Although control (pre-infusion) constrictor responses in inflamed joints were significantly smaller than normal joints (fig 6.4), infusion of

Fig 6.3 **A:** Posterior knee joint blood flow and systemic arterial blood pressure responses to electrical stimulation of PAN (10V, 10Hz, 1msec width; 60s train; denoted by black bar over trace), and to close intra-arterial injection of 2.5 nmole of the α_1 agonist phenylephrine (PE) and 250 pmole of the α_2 agonists clonidine (CL) or UK-14304 (UK) in a saline injected knee. As PAN was cut centrally, nerve stimulation had no effect on systemic blood pressure.

B: Responses to the same variables as in A and in the same animal but during close intra-arterial infusion of a nitric oxide inhibitor, L-NAME, at a rate of 3mg/kg/hour for one hour. Systemic blood pressure and the constrictor responses both increased, but the basal blood flow decreased.

C: Responses to the same variables in B and in the same animal but during subsequent infusion of L-arginine at a rate of 6mg/kg/hour for one hour. The responses were not changed substantially by L-argenine infusion.

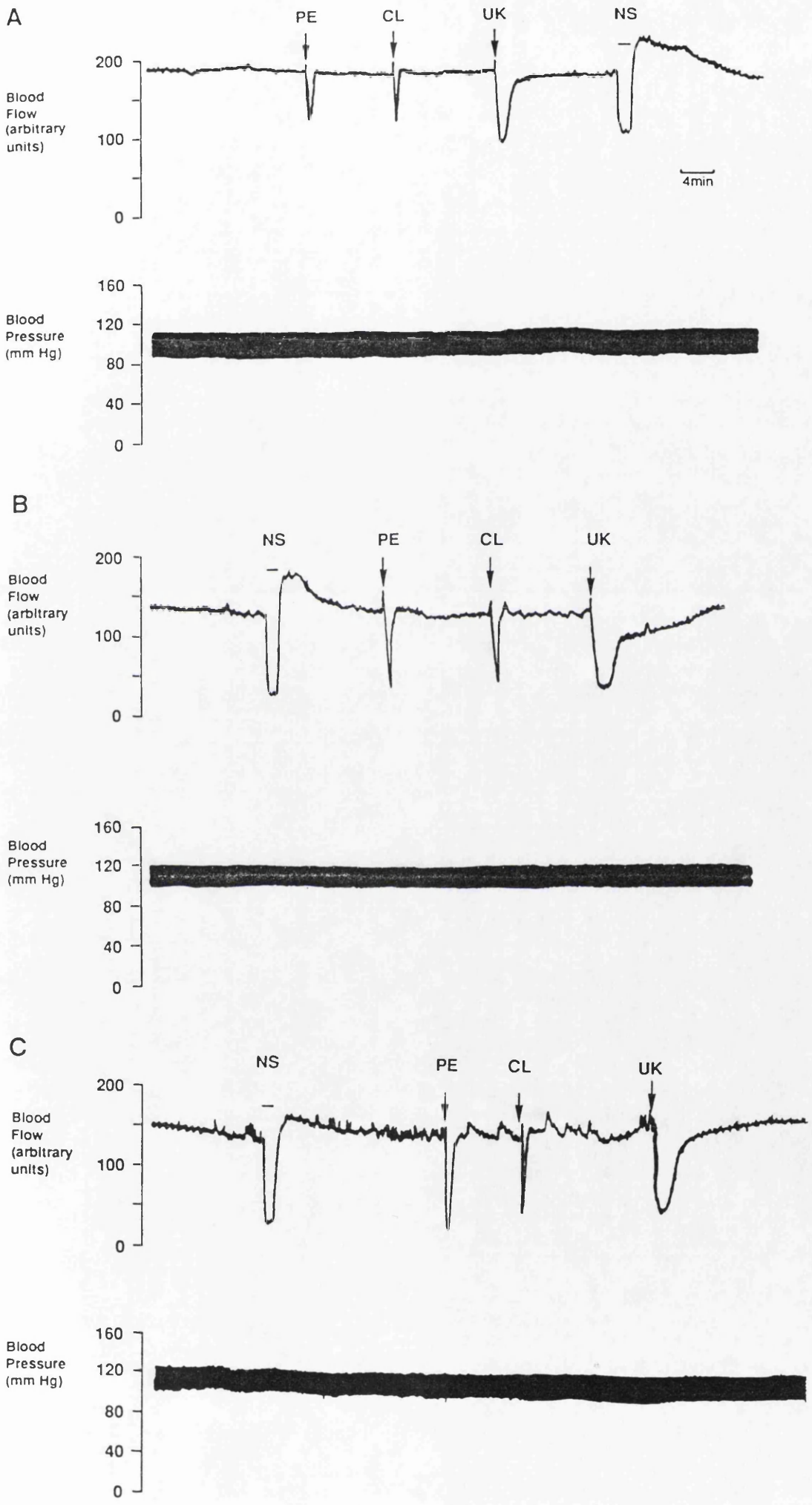
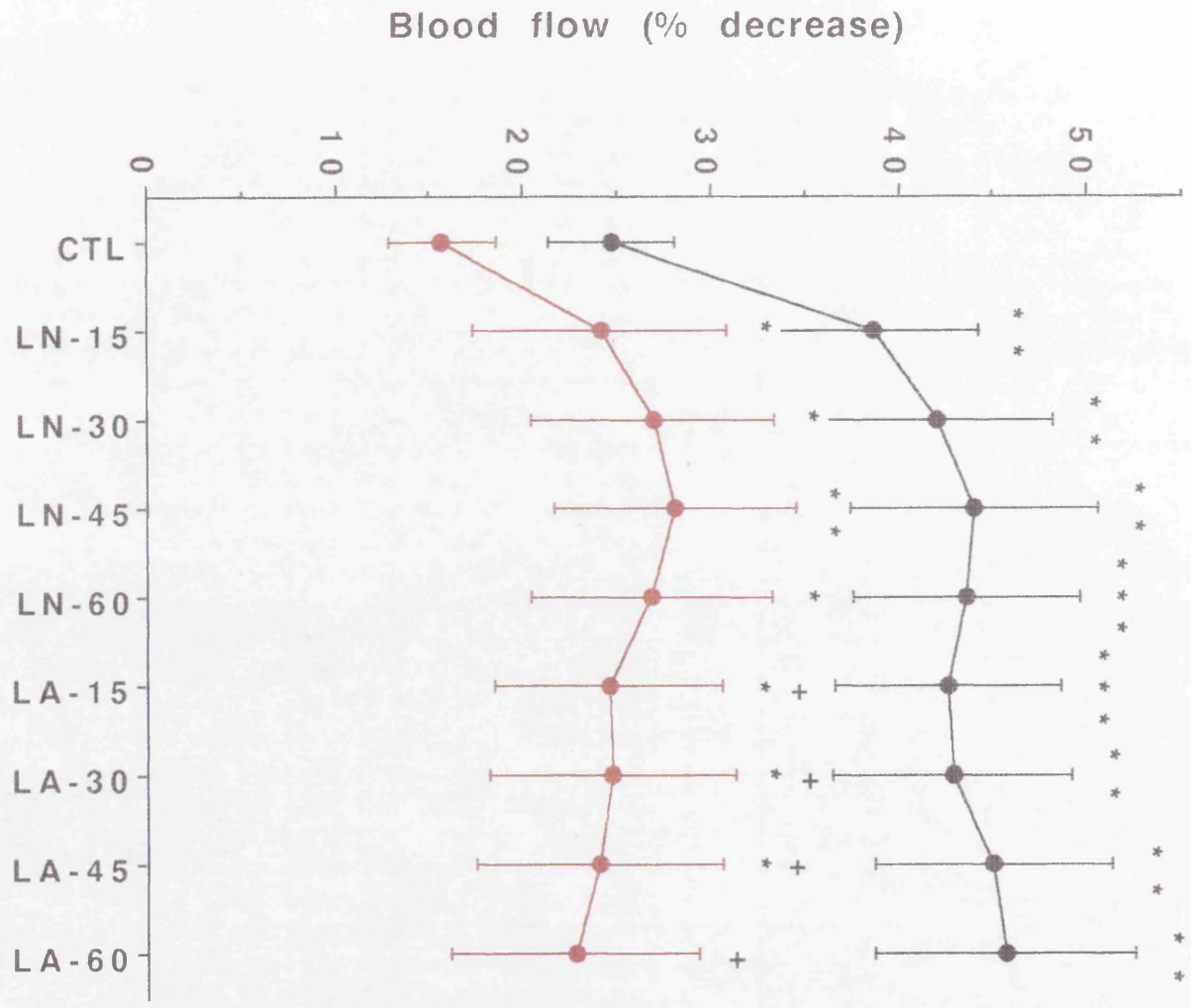

A
PE
CL
UK
NS
200
100
0
Blood
Flow
(arbitrary
units)
4min
160
120
80
40
0
Blood
Pressure
(mm Hg)
B
NS
PE
CL
UK
200
100
0
Blood
Flow
(arbitrary
units)
160
120
80
40
0
Blood
Pressure
(mm Hg)
C
NS
PE
CL
UK
200
100
0
Blood
Flow
(arbitrary
units)
160
120
80
40
0
Blood
Pressure
(mm Hg)

Fig 6.4. Effect of close intra-arterial infusion of L-NAME (3mg/kg/hour) and subsequent infusion of L-argenine (6mg/kg/hour) each for one hour on nerve-mediated vasoconstrictor responses in saline injected (black symbols) and inflamed (red symbols) knee joints. Responses increased to a maximum during 45 minutes of L-NAME infusion but were not restored in the control joint after 60 minutes of L-argenine infusion. CTL = pre-infusion response.

* means significantly different from CTL response; + means significantly different from peak response. + & * , **, ***, = $p<0.05$, $p<0.01$, and $P<0.001$ respectively; n=10 for both groups and all points.


Blood flow (% decrease)
0
10
20
30
40
50
CTL
LN-15
LN-30
LN-45
LN-60
LA-15
LA-30
LA-45
LA-60

L-NAME increased the response in both groups. Comparison of the normal and inflamed knees shows that the increases in the constrictor response to nerve stimulation followed similar time courses. The percentage increase in response from control during 45 minutes was 78.1% in normal and 78.9% in the inflamed joint (fig 6.4).

d. Effect of L-arginine infusion on nerve-mediated responses

Subsequent infusion of L-arginine, the natural precursor of NO production, at double the rate of L-NAME infusion and maintained for one hour, failed to restore the nerve-mediated constrictor response to its basal value in both normal and inflamed joints (fig 6.3C & 6.4), although L-arginine seems to be more effective in the inflamed one. Infusion of L-arginine had also no significant effect on the vasodilator response. The magnitude of vasodilator response was 20.8+3.9% (above the control value) in this situation which was not significantly different from its value after L-NAME infusion (P=0.15, n=8).

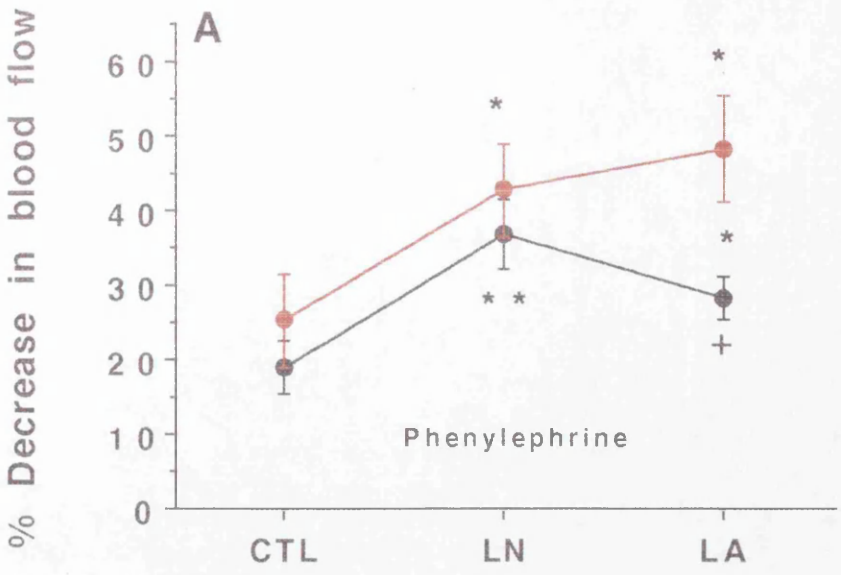
e. Effect of L-NAME and L-arginine infusion on responses to α - agonists

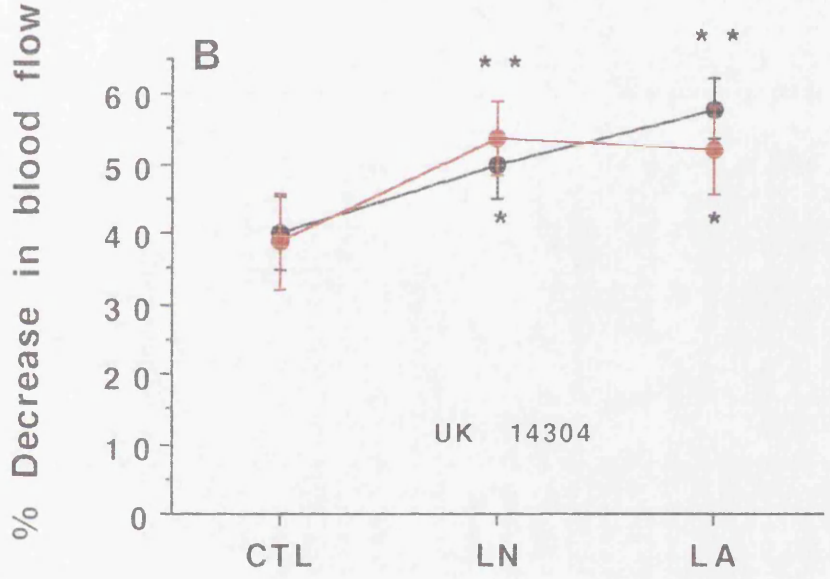
To investigate whether the rise in constrictor response due to L-NAME infusion was specific to nerve mediated responses or whether NO may also play a role in modulation of joint blood vessel responses to α- agonists, effective doses of different α-agonists were injected close intra-arterially. Injection of 2.5 nmole of the α_1-agonist phenylephrine, and 250 pmole of the α_2-agonists, clonidine and UK-14304, produced vasoconstriction comparable to that elicited by nerve stimulation (fig

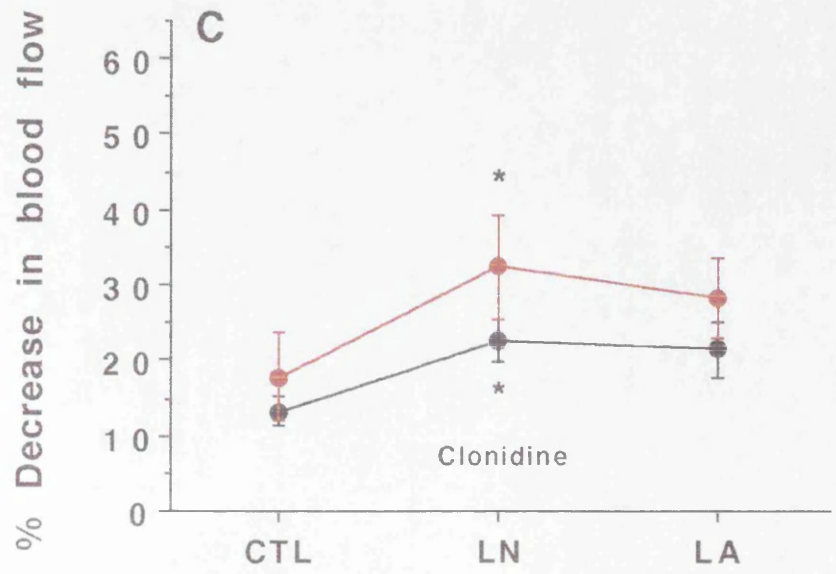
6.3A) although with different time courses. These constrictor responses were increased significantly after one hour of L-NAME infusion in both control and inflamed knees (fig 6.5), and again infusion of L-arginine failed to bring the responses back to the original level (except for phenylephrine in control joints). In some cases such as the effect of UK-14304 in control and phenylephrine in inflamed joints, the responses continued to rise during L-arginine infusion (fig 6.5). The reason for selection of a higher dose of phenylephrine was that previous results have shown that α_2-adrenoceptors predominate in blood vessels of the posterior region of the rabbit knee joint (see chapter five), so a higher dose of phenylephrine is needed to elicit comparable vasoconstrictor responses.

Fig 6.5. Responses of posterior capsule blood vessels to close intra-arterial injection of 2.5 nmole phenylephrine (**A**), 250 pmole UK-14304 (**B**) or clonidine (**C**) (all in 0.25 ml volumes) before infusion (CTL), or 60 minutes after infusion of L-NAME (LN), or 60 minutes after subsequent infusion of L-argenine (LA) in saline injected (black symbols) and inflamed (red symbols) knee joints. n=10 for both groups and all points.

* means significantly different from CTL response; + means significantly different from peak response. * , + = $p<0.05$, **, = $p<0.01$


A
% Decrease in blood flow
60
50
40
30
20
10
0
Phenylephrine
CTL
LN
LA


B
% Decrease in blood flow
60
50
40
30
20
10
0
UK 14304
CTL
LN
LA


C
% Decrease in blood flow
60
50
40
30
20
10
0
Clonidine
CTL
LN
LA

Discussion

The results of this study have shown that nitric oxide (NO) plays an important role in the regulation of basal blood flow to the normal and acutely inflamed rabbit knee joint. The fact that inhibition of NO production by L-NAME reduced the basal blood flow between 20 to 35% (fig 6.2) despite the rise of blood pressure by about 20%, which would be expected to increase blood flow by the same extent, suggests that NO is produced continuously and extensively in this vascular bed, and therefore keeps the joint blood vessels in a state of active vasodilatation under basal (resting) conditions. A previous study has shown a very high positive correlation between joint blood flow and blood pressure (Khoshbaten, 1989). The marked increase in joint vascular resistance in both groups (fig 6.2) is another indicator of continuous basal release of NO in this vascular bed. The finding that reduction in blood flow to the inflamed joint is not significantly different from the reduction of flow to the normal joint (fig 6.2), and also the non-significant difference between change in vascular resistances, suggests that the process of inflammation at 24 hours probably does not alter NO production rate and this agent is not responsible for the higher basal blood flow found in acutely inflamed knee joints (see chapter three).

Despite the high correlation between P_sO_2 and joint blood flow, the changes in P_sO_2 did not follow completely the changes in blood flow with L-NAME infusion (fig 6.1). In fact P_sO_2 started to decrease along with decrease in blood flow at the beginning of infusion period but in

most cases it started to rise after a few minutes (see fig 6.1), leading to non-significant difference in P_sO_2 values after one hour of L-NAME infusion, although the changes in blood flow were significant. The reason for this finding is not clear at the moment, but as animals were on gaseous anaesthetic containing O_2 in these experiments the possible higher level of arterial PO_2 could retard the decrement in P_sO_2. The non-significant difference between P_sO_2 values in normal and inflamed joints before L-NAME infusion in this study (fig 6.2B) may have the same cause. Another possibility is the reduction in oxygen consumption of the articular tissues by L-NAME. The complete recovery of P_sO_2 (fig 6.2B) by L-arginine infusion despite the partial recovery of blood flow supports this hypothesis but it needs more investigation.

The sympathetic vasoconstrictor responses in blood vessels of both normal and acutely inflamed joints were markedly increased by L-NAME infusion (fig 6.4) with 70% of the rise in response occurring during the first 15 minutes of the infusion period. This finding, which suggests an important role for NO in modulation of sympathetic nerve mediated responses, is consistent with the finding of Gardiner, Kemp & Bennett (1991b), which showed that NO was involved in transducing the vasodilator effect of circulating adrenaline, and that L-NAME amplifies the hypertensive effect of endogenous adrenaline. Again, non-significant differences between the rate and extent of rise in neurally mediated responses in normal and inflamed joints, compared with their own control responses (fig 6.4), suggests that the process of inflammation within 24 hours did not alter the role of NO in modulation of these nerve-mediated responses. The finding that the basal vasoconstrictor response (CTL) and all other values in the time response

curve (fig 6.4) were significantly smaller in inflamed joint is consistent with the previous results (chapter four) that the process of inflammation reduces the efficacy of the sympathetic nervous system in regulating blood flow to the inflamed rabbit knee joint. This is perhaps a compensatory mechanism to reduce the effectiveness of sympathetic nerves which have been shown to play a facilitating role in initiation and progression of the inflammatory process in arthritis (Levine *et al.*, 1988) and is probably contributes to the hyperaemic condition of inflamed joints. The smaller control vasoconstrictor responses in the control group, compared to the responses in the normal animals (at 10 pulses/s) observed in the previous experiments (chapters four and five), is probably due to the halothane anaesthesia used in these experiments as it has been shown that halothane causes some prejunctional inhibition of neurotransmitter release from sympathetic nerve endings (Muldoon *et al.*, 1975).

The other finding of this study was that the potentiating effect of L-NAME is not limited to the neurally mediated vasoconstrictor response as the responses to specific α_1- and α_2- agonists were also potentiated by inhibition of NO production (fig 6.5). This finding is in contrast with the findings of Toda, Yoshida, & Okamura (1991) who showed in endothelium denuded dog temporal artery strips the contraction responses to noradrenaline were not affected by inhibition of NO synthesis. The reasons for this discrepancy could be the absence of endothelium, *in vitro* versus *in vivo* conditions, difference in the vascular beds examined and the animal species, or the different NO synthesis inhibitors employed in the two studies. The first reason seems to be more likely as the main source of NO production is the endothelial

layer cells of the blood vessels. Infusion of L- arginine, the natural precursor of NO production, failed to abolish the hypertensive effect of L-NAME. This is in contrast with the reports on the reversal effect of L-arginine on pressor effects of NO synthesis inhibitors in rat and rabbit (Whittle *et al.*, 1989; Rees *et al.*, 1989) .The reason for this difference is probably due to the usage of another L-arginine analog (L-NMMA) which seems to be less potent than L-NAME, and higher dose of L-arginine used (3 times of L-NMMA dose) in their study.

Although Toda, Minami & Okamura (1990), and Toda & Okamura (1990a,b) have reported that L-arginine was able to reverse the suppressive effect of NO synthesis inhibitors on nerve stimulation induced relaxation in the dog cerebral artery, the nerve mediated vasodilatation following vasoconstriction in this study (fig 6.3) was not found to be NO mediated as it was resistant to L-NAME infusion. The previous experiments (see chapter five) have shown that this vasodilatation is partly substance P mediated and partly β-adrenoceptor mediated although in rat the β−mediated effects of adrenaline and salbutamol have been shown to be partly dependent upon endothelium-derived nitric oxide (Gardiner, Kemp & Bennett, 1991a,b).

Regarding the effect of L-arginine on the potentiating effects of L-NAME, only the enhanced nerve-mediated vasoconstrictor responses in the inflamed joint and phenylephrine-mediated responses in the normal joint were significantly reduced by L-arginine infusion (fig 6.4 & 6.5). The reason could be that the rate of degradation of L-NAME in the body is so low that one hour is not sufficient to reduce the concentration of this agent to a level where its effect can be overcome by L-arginine or a higher dose of L-arginine is needed.

In conclusion, this study showed, in both normal and inflamed joint blood vessels, NO plays an important role in regulation of knee joint blood flow and consequently synovial PO_2. As well as from the endothelial layer of blood vessels, NO may be released from the sympathetic post ganglionic nerve terminals or another tissue, during nerve stimulation, to counterbalance the vasoconstrictor effects of neurally released noradrenaline and neuropeptide Y. More recently it has been shown that in endothelial denuded aortic rings, NO still is produced by transmural electrical stimulation of the rings (Toda, Yoshida & Okamura, 1991). Moreover, spontaneously released NO may also counterbalance the constrictor effect of circulating adrenaline and probably the other naturally occurring vasoconstrictors to ensure adequate blood flow and oxygen necessary for survival of those joint tissues which already are at risk of oxygen deficiency due to low synovial PO_2 values present in both normal and inflamed rabbit knee joints (see chapter four).

Section two

Role of prostaglandins in regulation of joint blood flow

Summary

1. Experiments were performed to investigate in normal and acutely inflamed rabbit knee joints the role of prostaglandins in the regulation of joint blood flow as well as their modulation of sympathetic vasoconstriction.

2. Close intra-arterial injection of PGE_2 produced a dose dependent vasodilatation in control joints but the responses in the inflamed joints were much smaller. Close intra-arterial infusion of indomethacin significantly increased the responses of the control joints to PGE_2 but had no effect on the responsiveness of the inflamed joints.

3. Nerve-mediated vasoconstrictor responses did not change with close intra-arterial infusion of indomethacin either in control or in inflamed joints.

4. Infusion of indomethacin decreased basal joint blood flow, measured by laser Doppler flowmetry, by about 35% in normal and 17% in inflamed joints. Systemic blood pressure was elevated significantly only in the control group.

5. The results of this study show that although prostaglandins have a role to play in regulation of basal blood flow in both normal and acutely inflamed rabbit knee joints, do not appear to play a significant role in modulation of sympathetic vasoconstrictor responses. PGE_2 receptors are functional in normal knee joint blood vessels but they may be disabled by the process of inflammation.

Introduction

It has been shown that blood vessels in different species have the capacity to synthesize prostaglandins (PGs) (Tuvemo & Wide, 1973; Aiken, 1974; Terragno, Crowshow & McGiff, 1975). Dick, Grennan & Zeitlin (1976), using ^{133}Xe clearance technique, have shown that exogenous PGs E_1, E_2 and $F_{2\alpha}$ dilate the blood vessels in the dog knee joint. Synovial fluid from inflamed joints has been found to contain high levels of PGs (Blackham *et al.*, 1974; O'Byrne *et al.*, 1990). There is evidence suggesting that PGs modulate adrenergic mediated effects in some isolated blood vessels (Smith & McGrath, 1991) and change the rabbit mesentric vascular resistance in vitro (Blumberg *et al.*, 1977), and dog coronary vascular resistance in vivo (Dusting *et al.*, 1978). Whether PGs are produced by articular blood vessels in the rabbit and their effects on these are unknown. It is possible that hyperaemia of the acutely inflamed joint (see chapter three) is mediated in part by PGs.

The aim of this study was to determine whether PGs play a role in regulating basal joint blood flow in the normal rabbit knee joint and if this is changed by the process of acute inflammation. The second objective was to investigate if PGs have a role to play in modulating sympathetic vasoconstriction of articular blood vessels in both normal and acutely inflamed rabbit knee joints.

Methods

a. Animal preparation and physiological recordings

Experiments were performed on 17 adult New Zealand rabbits (2.2-3.5kg) which were anaesthetised as mentioned in section one. On completion of surgery, the gaseous anaesthetic was discontinued and a slow continuous infusion of pentobarbitone (0.25-0.5mg/min depending on the weight of the animal) was administered throughout the remainder of the experiment via a cannula inserted into the left jugular vein. The trachea was cannulated, with the animals breathing spontaneously throughout the experiments. Arterial blood pressure was monitored through a cannula inserted into the left carotid artery. A laser Doppler flowmeter (Moor Instruments MBF3) was used to monitor the changes in the posterior knee joint capsule. Alterations in blood flow are expressed as percentage change from control (0%) values occurring immediately before each test procedure. Joint vascular resistance was calculated by dividing mean arterial pressure by joint blood flow.

The posterior articular nerve (PAN) dissection and stimulation was performed as mentioned in previous chapter. The stimulus parameters were: width 1ms, voltage 10V, frequency 20 pulses/s and duration 60s. In previous experiments we have demonstrated that, with the other parameters constant, PAN stimulation at this frequency (20 pulses/s) gives rise to equally maximal vasoconstrictor responses in both normal and acutely inflamed joints (see chapter four).

b. Drug administration

Drugs were administered by the same route as mentioned in chapter five. Indomethacin was infused close intra-arterially at a rate of 0.2 mg/kg/hour for 30min prior to, and throughout test procedures. PGE_2 was dissolved initially in ethanol (1mg/ml) and then diluted in physiological saline and kept on ice before being warmed and injected in volumes of 0.25 ml. PGE_2, carrageenan and indomethacin were obtained from Sigma.

c. Induction of acute joint inflammation

The method of production of acute joint inflammation has been described in chapter four. 1 ml sterile saline was injected into one knee joint of a separate series of animals (n=7) to serve as control. This was necessary as it has been shown that inflammation of one knee can produce a symmetrical synovitis of the contralateral knee (Kidd *et al.*, 1989), invalidating the use of an internal control.

d. Statistical analysis

The values expressed in the graphs and histograms are means $\pm$ SEM and comparisons were performed using the student's paired or unpaired t-test as appropriate. All n values refer to the number of animals examined. P values of 5% or less were considered significant.

Results

a. Effects of PGE_2 and indomethacin

As PGE_2 has been shown to be one of the major prostaglandins present in synovial fluid of inflamed joints (Blackham *et al.*, 1974), it was most likely that blood vessels in the normal joint contain receptors to this agent. Close intra-arterial injection of PGE_2 elicited a dose dependent vasodilatation in the normal joint (fig 6.6A) but the responses in inflamed joints were very small even with highest dose (2.5nmol) used in these experiments (fig 6.6C). This dose was sufficient to produce transient systemic effects on blood pressure in some animals (fig 6.6B).

Close intra-arterial infusion of the prostaglandin production inhibitor indomethacin for a minimum of 30min significantly enhanced the effect of exogenous PGE_2 on normal joint blood vessels (fig 6.6B & 6.7A). The responses in the inflamed joint did not differ before and after indomethacin (fig 6.6D & 6.7B).

b.Effect of indomethacin infusion on joint blood flow, arterial blood pressure and joint vascular resistance

In both groups of animals, infusion of indomethacin for one hour reduced joint blood flow, but the magnitude of reduction was significantly higher in the normal joint (fig 6.6A&C and fig 6.8). Systemic blood pressure rose about 5% in the control group and, although small, this rise was statistically significant compared to the very small rise occuring in the test group (fig 6.8). Basal vascular

Fig 6.6. **A**: Posterior knee joint blood flow and systemic arterial blood pressure responses to electrical stimulation of PAN (10V, 20Hz, 1msec width; 60s train; denoted by black bar over trace), and to close intra-arterial injection of different doses of prostaglandin E_2 (PGE_2) in a saline injected knee. As PAN was centrally cut, nerve stimulation had no effect on systemic blood pressure. Infusion of the PG production inhibitor indomethacin (0.2mg/kg/hour) was started at the arrow point (IND). Basal blood flow was decreased due to indomethacin infusion.

B: Responses to the same variables as in A and in the same animal but during close intra-arterial infusion of indomethacin. The nerve-mediated constrictor and dilator responses did not change, but the response to exogenous PGE_2 substantially increased.

F=femtomol, p=picomol, n=nanomol.

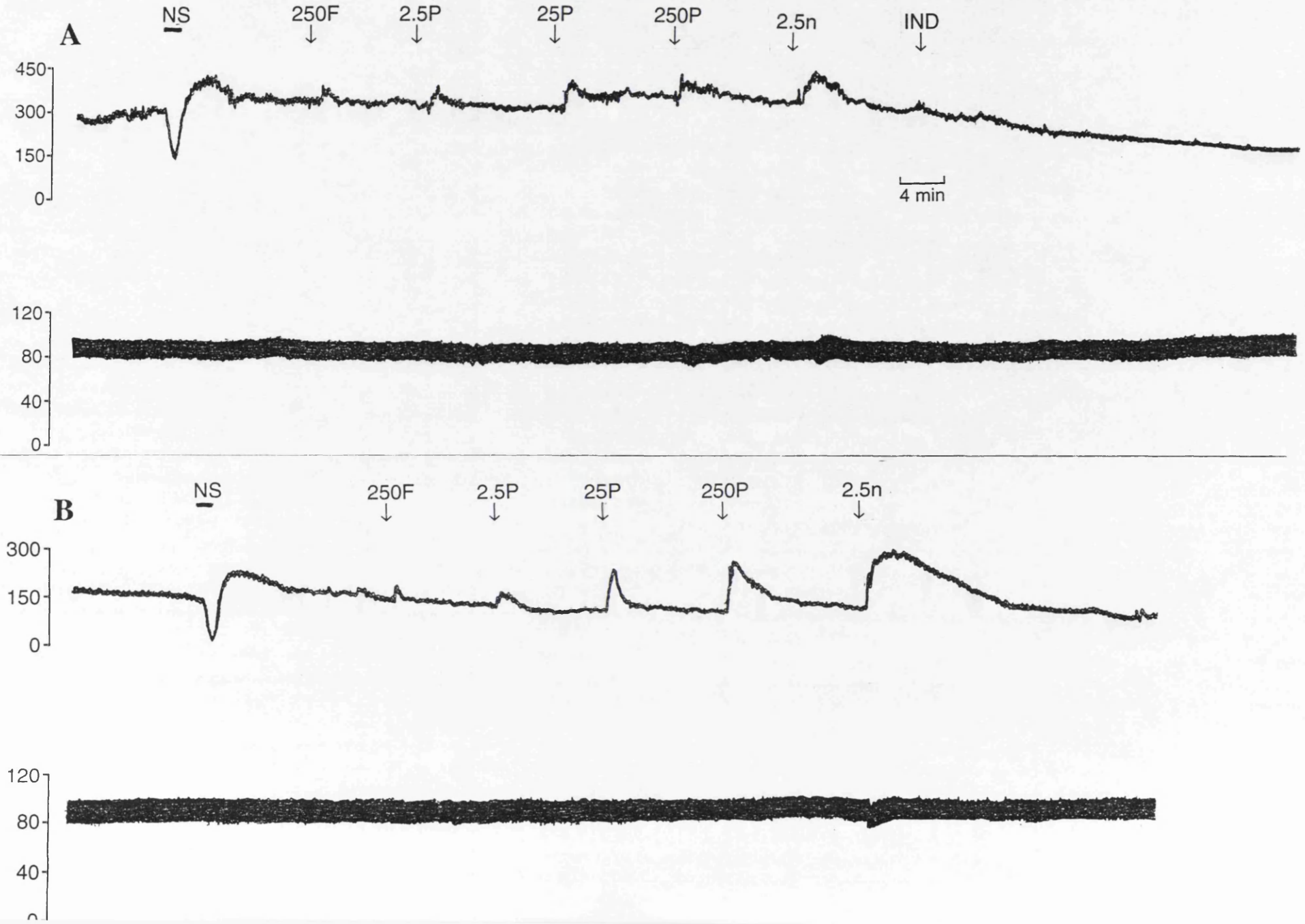

A
NS
250F
2.5P
25P
250P
2.5n
IND
450
300
150
0
4 min
120
80
40
0
B
NS
250F
2.5P
25P
250P
2.5n
300
150
0
120
80
40

Fig 6.6. **C&D:** Responses to the same variables as in fig 6.6.A but in a carrageenan induced acutely inflamed joint before (**C**) and during (**D**) infusion of indomethacin. The responses to exogenous PGE_2 were abolished due to inflammation and not potentiated by subsequent indomethacin infusion. The nerve-mediated constrictor response did not change, and the nerve-mediated dilator response was not present in this animal.

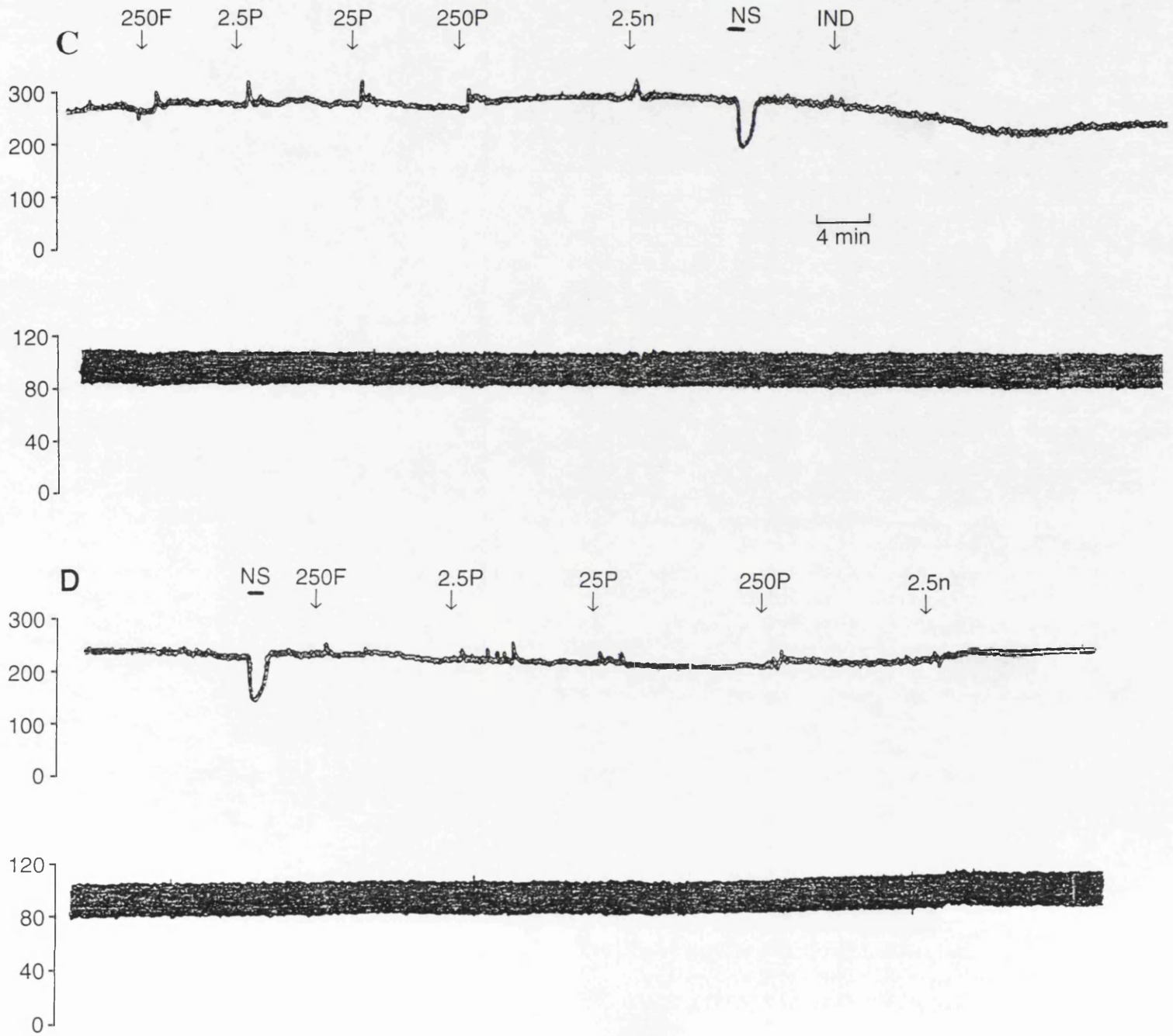
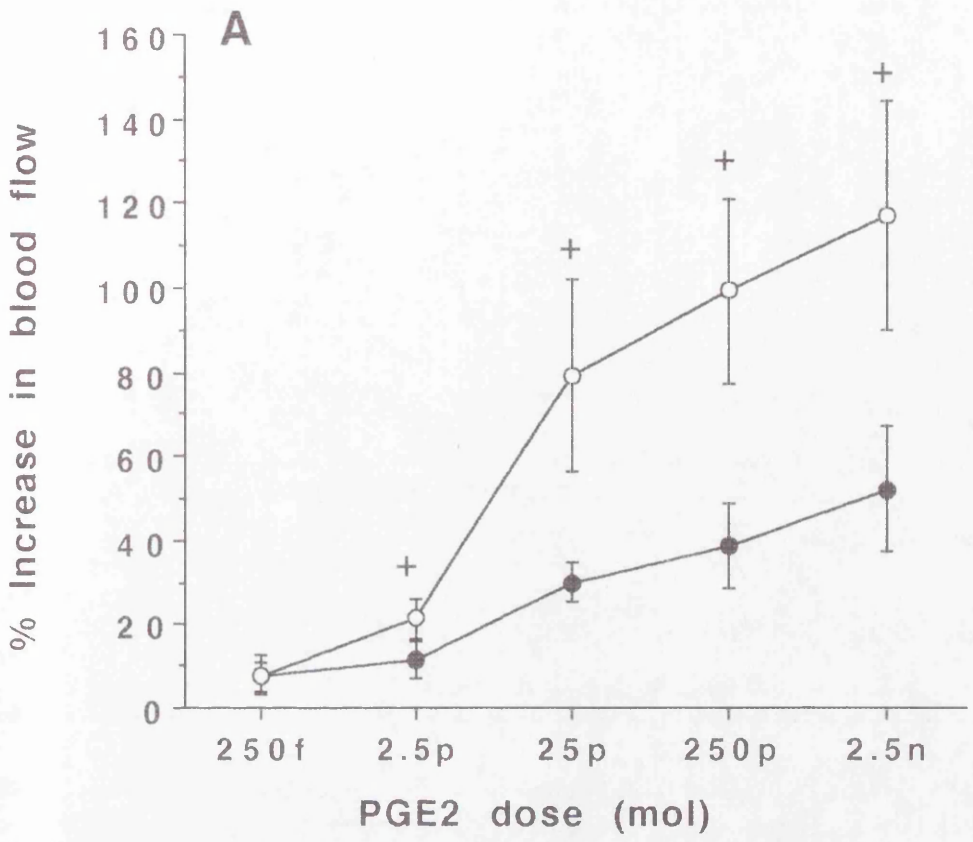

C
250F
2.5P
25P
250P
2.5n
NS
IND
300
200
100
0
4 min
120
80
40
0
D
NS
250F
2.5P
25P
250P
2.5n
300
200
100
0
120
80
40
0

Fig 6.7. PGE_2 dose-response curve before (closed symbols) and after (open symbols) close intra-arterial infusion of indomethacin (0.2mg/kg/hour for 30min) in the control (**A**) and carrageenan-induced acutely inflamed (**B**) knee joints. Responses in the control joint were potentiated by indomethacin infusion. + means significantly different from pre-infusion response; + = $p<0.05$, n=7 for control group and n=10 for inflamed group


A
160
140
120
100
80
60
40
20
0
% Increase in blood flow
+
+
+
+
250f
2.5p
25p
250p
2.5n
PGE2 dose (mol)

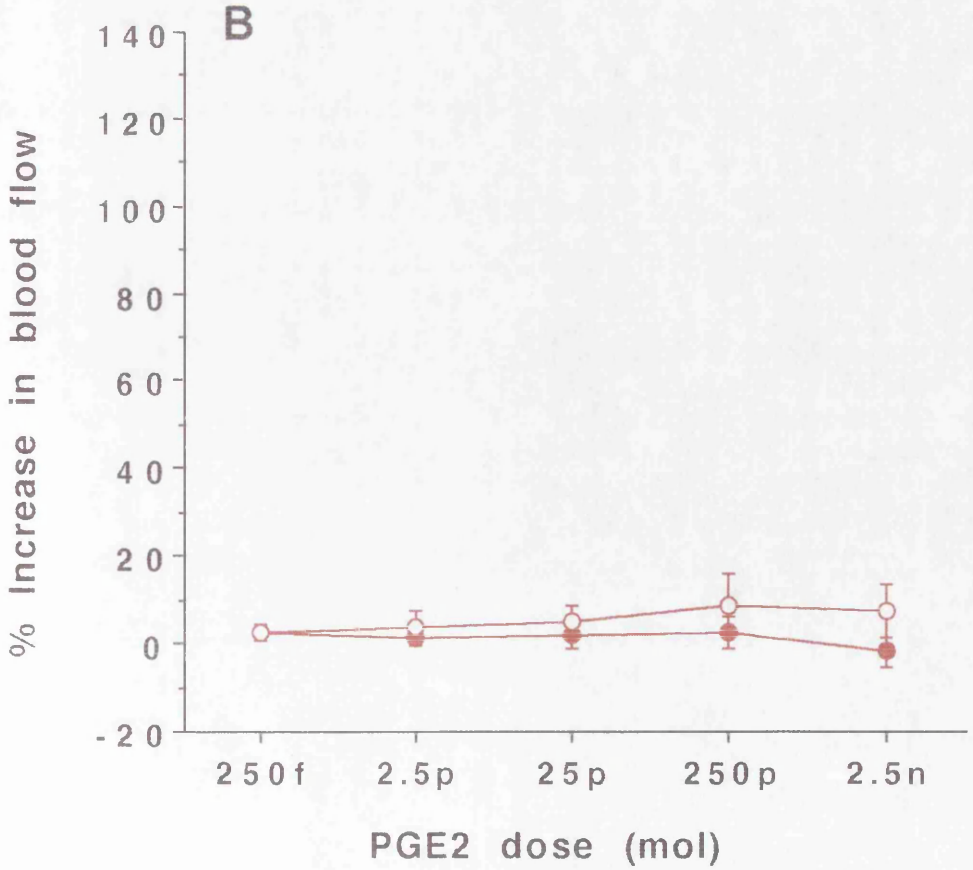
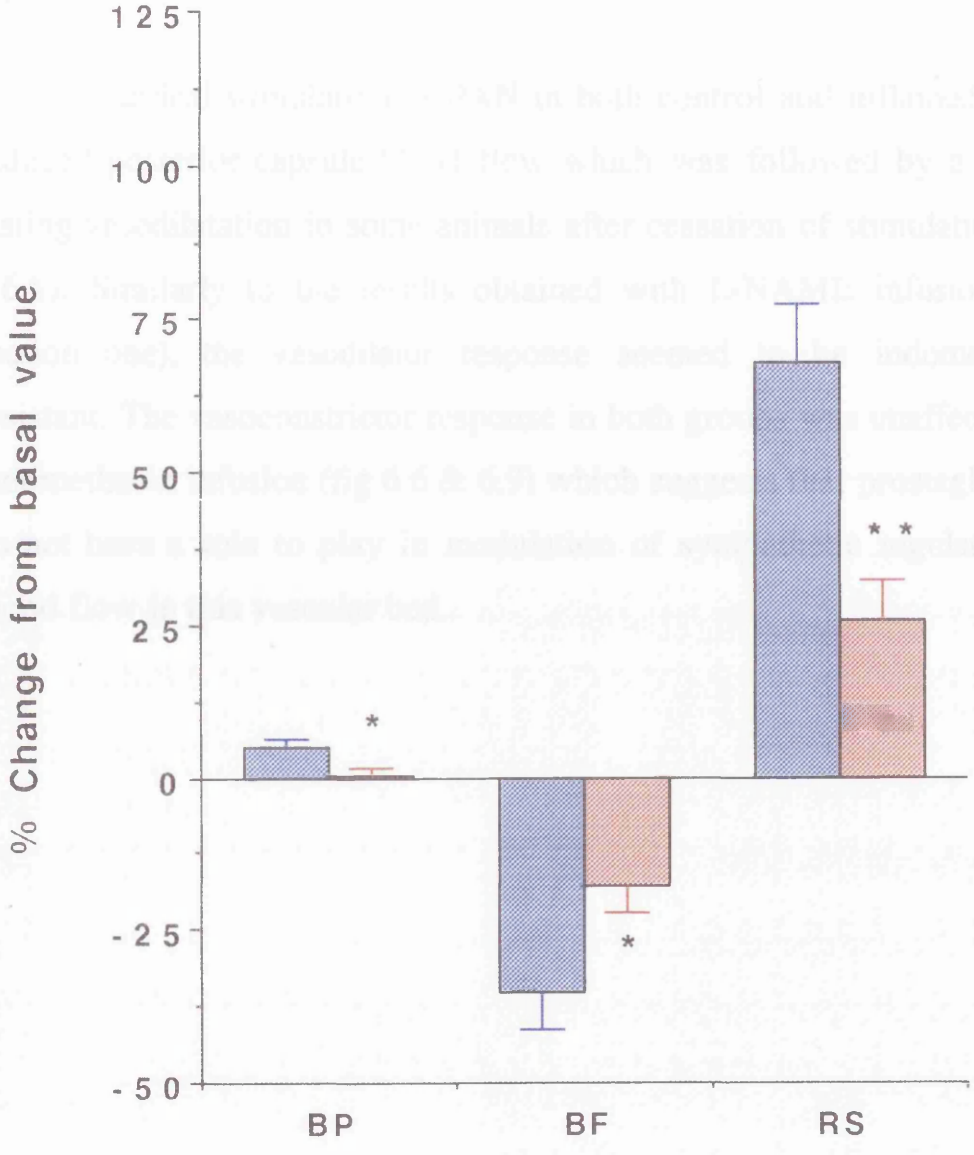

B
140
120
100
80
60
40
20
0
-20
% Increase in blood flow
250f
2.5p
25p
250p
2.5n
PGE2 dose (mol)

Fig 6.8. Percentage change in posterior capsular blood flow (BF), mean arterial blood pressure (BP) , and joint vascular resistance (RS) from basal (pre-infusion = 0%) values during close intra-arterial infusion of indomethacin for 60 minutes, in control animals (blue) and animals with inflamed joints (red).

* means significantly different from control group. * = $p<0.05$, **= $p<0.01$. N=7 in control, n=10 in inflamed.

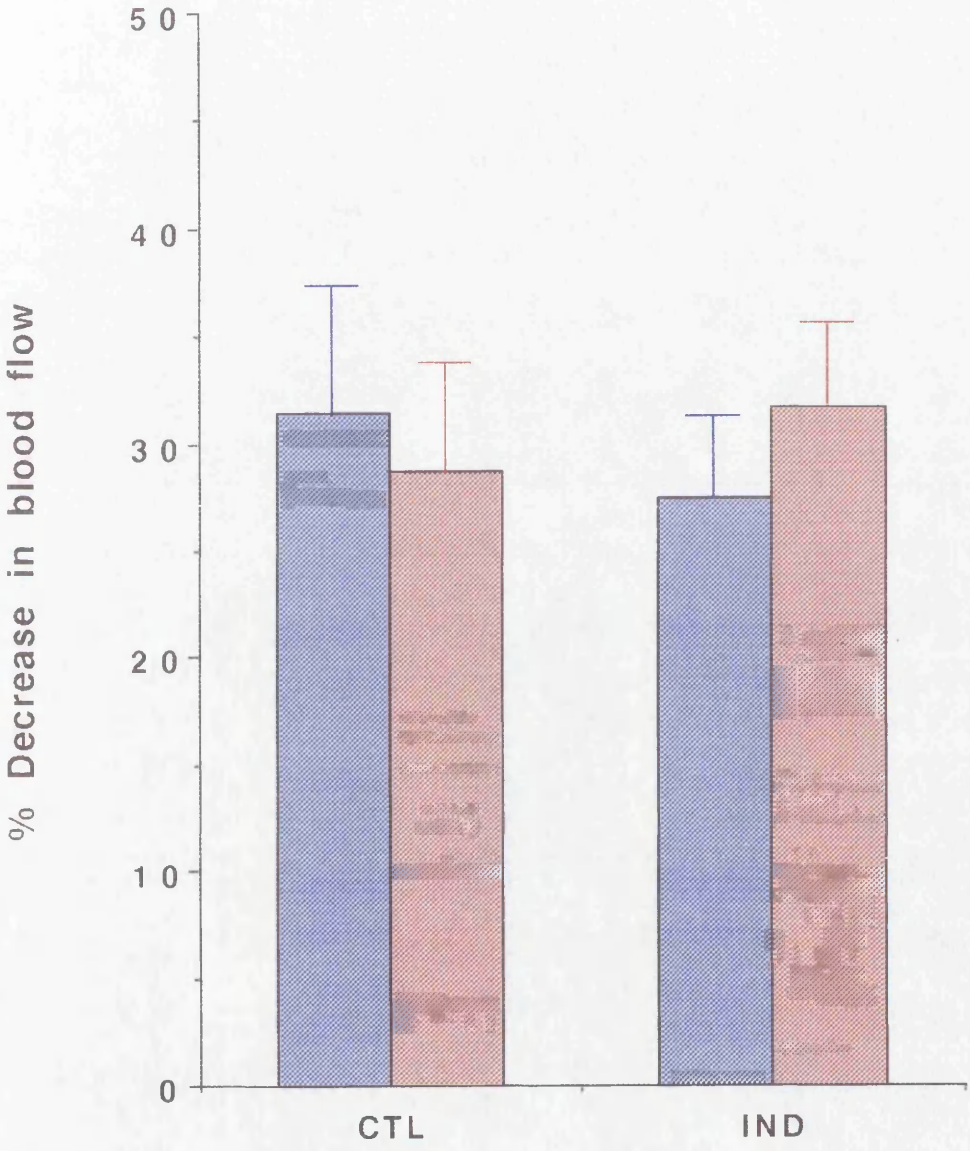

125
100
75
50
25
0
-25
-50
% Change from basal value
*
*
* *
BP
BF
RS

resistance was increased in both control and test groups with a significantly higher increase in normal joints.

c. Effect of indomethacin infusion on nerve-mediated constrictor responses

Electrical stimulation of PAN in both control and inflamed joints reduced posterior capsule blood flow which was followed by a longer lasting vasodilatation in some animals after cessation of stimulation (fig 6.6A). Similarly to the results obtained with L-NAME infusion (see section one), the vasodilator response seemed to be indomethacin resistant. The vasoconstrictor response in both groups was unaffected by indomethacin infusion (fig 6.6 & 6.9) which suggests that prostaglandins do not have a role to play in modulation of sympathetic regulation of blood flow in this vascular bed.

Fig 6.9. Responses of knee joint blood vessels to electrical stimulation of posterior articular nerve (PAN), (10 V, 20 Hz, 60s trains) before (CTL) and after close intra-arterial infusion of indomethacin (IND) for a duration of 45min, in control (blue) and acutely inflamed (red) knees. The constrictor response in both groups was not changed by indometh


% Decrease in blood flow
50
40
30
20
10
0
CTL
IND

Discussion

The results of this study have shown that prostaglandins (PGs) play an important role in regulation of joint blood flow to normal and acutely inflamed rabbit knee joints. The fact that inhibition of PG production by indomethacin reduced the basal blood flow between 18 to 35% (fig 6.8) suggests that PGs are produced continuously in this vascular bed, and therefore help to maintain joint blood vessels dilated under basal (resting) conditions. Although inflamed joint blood vessels seemed to lose their PGE_2 receptors or their sensitivity to PGE_2 (fig 6.6C & 6.7B) which could account for the lower reduction in their blood flow following indomethacin infusion, it is likely that some other types of PGs are still produced and function in inflamed joints. It is likely that supersensitization (Ross & Gilman, 1985) is responsible for the enhancement of vasodilator responses to exogenous PGE_2 observed in normal joints after inhibition of PG synthesis by indomethacin (fig 6.6B & 6.7A). The significant difference in joint vascular resistance between control and inflamed joints (Fig 6.8) also suggests that PGs are more important in flow regulation to the normal joint, although it has been found that the level of PGs in synovial fluid of an inflamed joint is much higher than the normal joint (Blackham *et al.*, 1974). These PGs are probably not produced by the blood vessels but more likely by inflammatory cells and nonvascular joint tissues, although the method of induction of inflammation in this study had been different (intra-articular injection of ovalbumin after sensitization with mycobacterium tuberculosis). The high level of PGs might be the reason for down regulation of PGE_2 receptors in blood vessels of the inflamed joints (fig

6.7B), as it has been shown that PGE_2 is one of the major PGs found in the synovial fluid of inflamed rabbit knees (Blackham *et al.*, 1974; O'Byrne *et al.*, 1990). The sympathetic vasoconstrictor responses in blood vessels of both normal and acutely inflamed joints were not changed by indomethacin infusion (fig 6.6 & 6.9). This suggests that PGs do not have role in modulating sympathetic regulation of blood flow to the normal and inflamed joints. Probably the loss of sympathetic tone found in blood vessels of inflamed human knees (Dick *et al.*, 1971) is not related to the higher level of PGs which probably exist in the synovial fluid of these joints. In contrast to the findings of this study, neural stimulation of the isolated rabbit portal vein (Greenberg., 1978) and vascularly perfused rabbit kidney (Davis & Horton, 1972), pancreas (Hamamdzic & Malik, 1977) and cat spleen (Hedqvist *et al.*, 1971) has caused release of a PG-like material. The reasons for this discrepancy are probably isolated versus *in vivo* conditions or different tissues used in their studies from this study. On the other hand, in these experiments PGs were traced in venous effusates after nerve stimulation and the origin of PGs was not necessarily from the blood vessels of these organs. Similarly to earlier experiments involving NO (section one of this chapter), the nerve-mediated dilator response was also not PG dependent (fig 6.6 B). As mentioned in section one this response is mostly substance P- and β-adrenergic-mediated. Other neuropeptides such as calcitonin gene related peptide (CGRP) could also contribute.

It could be argued that the increase in vascular response to exogenous PGE_2 in normal joints after indomethacin infusion (fig 6.7A) was due to the pre-constricted condition of the joint blood vessels. However, this could be responsible for only a small part of the increase in response as indomethacin infusion reduced joint blood flow by 35% but the dilator responses were increased by more than 150% at most doses.

In conclusion, this study showed that, in both normal and inflamed rabbit knee joint blood vessels, prostaglandins play an important role in regulation of basal blood flow, although they probably have no role in the modulation of sympathetic nerve-mediated vasoconstriction. PGE_2 receptors are present in normal joint blood vessels but, by an unknown mechanism, their number or sensitivity to PGE_2 is extensively decreased within 24 hours of induction of acute inflammation. This may have evolved as a compensatory homeostatic mechanism against inflammation, as it has been shown that PGE2 has proinflammatory effects and its level is very high in synovial fluid of inflamed joints (O'Byrne *et al.*, 1990).

GENERAL CONCLUSIONS

The findings of this study have revealed interesting, new and in some cases unexpected results about the regulation of joint blood flow, synovial fluid PO_2 (P_SO_2) and the correlation between these.

The first advantage was assessment of a new technique for measurement of P_SO_2 by an oxygen electrode and establishment of a baseline as there were no reported values from normal joints to allow comparisons. Another interesting finding to arise from this study, apart from the high correlation found between blood flow and P_SO_2, was that P_SO_2 decreased with increasing depth of penetration into the synovial cavity (fig 4.6 & 4.7) with resultant minimum PO_2 values close to the avascular cartilage in both normal and inflamed joints.

Despite the high correlation found between P_SO_2 and joint blood flow, during the inhibition of NO production with L-NAME the changes in P_SO_2 did not follow completely the changes in blood flow (fig 6.1). In fact P_SO_2 started to decrease along with decrease in blood flow at the beginning but in most cases it started to rise after a few minutes, leading to non-significant difference in P_SO_2 values after one hour, although the reductions in blood flow remained significant. The reason for this finding is not quite clear at the moment and needs more investigation.

The results of electrical stimulation of sympathetic nerves to the posterior capsule showed a shift of the frequency-response curve to the right, suggesting a reduction in the efficacy of the sympathetic nervous system in joint blood flow regulation. This was found to be not due to change in property of postjunctional α-adrenoceptors as the responses to

different adrenoceptor agonists were not different between normal and acutely inflamed joint blood vessels (fig 6.5, chapter six). This was in contrast to the findings of Khoshbaten & Ferrell (1990) in which the responses were increased during eight hours of kaolin induced joint inflammation, but were more in agreement with the findings of Dick *et al.* (1971) in which they observed evidence of loss of vasoconstrictor tone in chronically inflamed human joints. Further studies are required to determine whether sympathetic neurotransmission is affected by arthritis.

More interesting was the finding that α_2-adrenoceptors play the major role in mediating the vasoconstrictor responses to nerve stimulation and α-agonists, whereas in previous *in vitro* studies these responses were predominantly mediated by α_1-adrenoceptors (Ferrell & Khoshbaten, 1989; Ferrell & Khoshbaten, 1990a). The main reason probably is the property of laser Doppler flowmeters which are designed to sample flow in the microcirculation where it has been shown that α_2-adrenoceptors predominate whereas α_1-adrenoceptors are more common on the larger resistance arteries (Faber *et al.*, 1991). However, this could also be related to the different conditions *in vivo* versus *in vitro*.

The present study has demonstrated the presence of β-adrenoceptors in articular blood vessels, although again the previous study using an isolated rabbit knee joint preparation (Ferrell & Khoshbaten, 1989) failed to demonstrate the presence of these receptors. It is possible that the laser Doppler technique is more sensitive for detection of dilator responses or that β- adrenoceptors are present on smallest resistance vessels. Of greater interest was that these

receptors were found to be predominantly of the β_1 subtype. Apart from the heart muscle and coronary blood vessels, there are only few reports on the presence of β_1-adrenoceptors in the vascular smooth muscle of peripheral vascular beds. The rat pulmonary artery (O'Donnell & Wanstall, 1981) and jugular vein (Cohen & Wiley, 1978) have been shown to contain both types of β-adrenoceptors. The functional significance of β-adrenoceptor innervation in joint blood vessels remain to be elucidated.

Although microsphere experiments showed that basal blood flow to the acutely inflamed rabbit knee joint is significantly higher than normal joint (see chapter three), and also blood flow measurements by laser Doppler flowmeter showed that NO plays a major role in regulation of basal tone and basal blood flow of the normal and inflamed joints, NO was not found responsible for higher blood flow to the inflamed joints as the rate of NO production did not appear to be increased by the process of acute inflammation. Sympathetic vasoconstrictor responses of both normal and acutely inflamed joints were markedly increased by L-NAME which suggests an important role for NO in modulation of sympathetic nerve mediated responses and again, non-significant differences between the rate and extent of rise in neurally mediated responses in normal and inflamed joints (fig 6.4), suggests that the process of inflammation within 24 hours did not alter the role of NO in modulation of these nerve-mediated responses. However, full dose/response relationships for both L-NAME and L-arginine are now required for comprehensive analysis.

The results of this study have also shown that prostaglandins (PGs) play an important role in regulation of joint blood flow to normal

and acutely inflamed rabbit knee joints and that PGs are produced continuously in this vascular bed. However, the sympathetic vasoconstrictor responses in blood vessels of both normal and acutely inflamed joints were not changed by indomethacin infusion (fig 6.6 & 6.9) suggesting that in contrast to NO, PGs do not have role in modulating sympathetic regulation of blood flow to the normal and inflamed knee joints. PGE_2 receptors are present in normal joint blood vessels but, by an unknown mechanism, their number or sensitivity to PGE_2 is extensively decreased within 24 hours of induction of acute inflammation. Further studies are required to investigate this remarkable phenomenon. Of particular interest is whether long term administration of non-steroidal anti-inflammatory drugs (NSAID's) such as indomethacin alters this response.

For all the above it is clear that a great deal more research is required to elucidate the physiology and pathophysiology of synovial blood flow.

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