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Tachykinin regulation of basal synovial blood flow

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1 Experiments were performed to investigate the role of endogenously released tachykinins in the regulation of blood flow to the rat knee joint. Synovial perfusion was assessed by laser Doppler perfusion imaging, which permitted spatial measurement of relative changes in perfusion from control (pre drug administration), expressed as the percentage change. Most experiments were performed on the exposed medial aspect of the knee joint capsule.

2 Neither the selective tachykinin NK_1 receptor antagonist, FK888, nor the selective tachykinin NK_2 receptor antagonist, SR48968, significantly influenced synovial blood flow at doses of 10^{-12}, 10^{-10} and 10^{-8} mol. However, topical co-administration of these agents produced significant dose-dependent reductions in basal synovial perfusion of 6.3 ± 4.6, 12.0 ± 3.4 and $19.9 \pm 2.6\%$, respectively; $n = 29$. The non-selective tachykinin NK_1/NK_2 receptor antagonist, FK224, also produced significant (at 10^{-10} and 10^{-8} mol), but less potent, reductions in perfusion of 5.3 ± 4.0, 8.4 ± 2.2 and $5.9 \pm 2.8\%$, respectively; $n = 25$.

3 Topical administration of the α_1-, α_2-adrenoceptor antagonist phenoxybenzamine elicited a $31.3 \pm 6.2\%$ increase in blood flow which was substantially reduced to $10.4 \pm 3.8\%$ by co-administration of the FK888 and SR48968 (both at 10^{-8} mol; $n = 8–13$), suggesting that normally there is sympathetic vasoconstrictor 'tone' which is opposed by the vasodilator action of endogenous tachykinins.

4 One week after surgical interruption of the nerve supply to the knee joint, co-administration of FK888 and SR48968 (both at 10^{-8} mol) now produced slight vasodilatation ($6.7 \pm 4.6\%$; $n = 9$) which did not differ significantly from vehicle treatment. Depletion of tachykinins from sensory nerve fibres by systemic capsaicin administration also resulted in abolition of the vasoconstrictor effect of FK888 and SR48968 (both at 10^{-8} mol), with these agents only producing a slight vasodilatation ($2.5 \pm 5.3\%$; $n = 6$).

5 By use of a near infra-red laser source it was possible to image knee joint perfusion transcutaneously, the overlying skin being left intact. In this more physiological situation, close intra-arterial injection of the combination of FK888 and SR48968 (both at 10^{-8} mol) again elicited vasoconstriction ($48.8 \pm 16.2\%$ reduction in blood flow; $n = 4$).

6 These results indicate that endogenous tachykinins may be continuously released from sensory fibres innervating the joint. Basal release of tachykinins could therefore be an important physiological influence opposing sympathetic vasoconstrictor tone.

Keywords: Joint; substance P; tachykinins; blood flow; vasodilatation

Introduction

Substance P (SP) is an undecapeptide which is known to be present in unmyelinated sensory fibres innervating many structures including the rat knee joint (Konttinen *et al*., 1990). Though known to be the preferential endogenous ligand for tachykinin NK_1 receptors, SP can also act at the other tachykinin receptors. Neurokinin A (NKA) is the preferential endogenous ligand for tachykinin NK_2 receptors, but not exclusively so. Neurokinin B (NKB) is the preferential endogenous ligand for tachykinin NK_3 receptors, but again can also act at other tachykinin receptors. SP is known to be very widespread in the peripheral nervous system and NKA is also known to be present in the periphery but NKB is found predominantly in the central nervous system (Maggi, 1995).

Although SP is thought to play a role as a neurotransmitter in nociceptive pathways (Schaible *et al*., 1990; Neugebauer *et al*., 1994) it is known that four times as much of this tachykinin is transported peripherally than centrally (Keen *et al*., 1982), suggesting an important peripheral role. There is evidence to suggest that SP may have a pathophysiological role and could contribute to joint inflammation, as exogenously administered SP potently vasodilates synovial blood vessels (Lam & Ferrell 1991) and enhances their permeability (Scott *et al*., 1991). However, whether a physiological role for tachykinins in the regulation of synovial perfusion exists is presently unknown and was investigated in this study. There is considerable interest in the 'efferent' function of nociceptive afferent fibres with some authors re-evaluating the axon reflex theory (Szolcsányi, 1996), suggesting that peripheral release of sensory neuropeptides may not be confined to nociceptive stimuli and that the sensory and putative efferent functions of these fibres could be dissociated (Maggi, 1995). The aim of the experiments was to establish whether antagonizing the actions of tachykinins would affect basal synovial perfusion. The recent development of potent and selective antagonists acting at tachykinin NK_1 (Fujii *et al*., 1992) and NK_2 receptors (Advenier *et al*., 1992) offers the opportunity to investigate the role of endogenous tachykinins in regulating synovial perfusion and the tachykinin receptors which mediate their effects.

Methods

Adult male Wistar rats (250–400 g) were deeply anaesthetized by intraperitoneal injection of urethane (1.13 g kg^{-1}), placed in dorsal recumbency with the knee in the rest (mid) position and the medial aspect of the knee joint capsule was exposed. Arterial blood pressure was monitored continuously via a cannula inserted into a carotid artery to ensure that the animals remained normotensive throughout the procedures. Relative changes in joint perfusion were detected by laser Doppler perfusion imaging (LDI) which was originally developed to examine skin perfusion (Wårdell *et al*., 1993) but can also be

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used to investigate rat knee joint perfusion (Lam & Ferrell, 1993; Karimian *et al*., 1995). The LDI technique has been described in detail previously (Karimian *et al*., 1995). Briefly, this involved scanning a low power (1 mW) laser beam (633 nm) over the exposed medial aspect of the rat knee joint, collecting the backscattered Doppler-shifted photons in a photodetector in the scanner head, and processing these to generate a two dimensional image of knee joint perfusion. This required a scan time of approximately 1 min and as demonstrated previously this is sufficient time to capture vasodilator and vasoconstrictor responses (Karimian *et al*., 1995). The signal is processed to yield a flux signal in volts which was not normally distributed in the images and therefore the median was used as the measure of central tendency with the semi-interquartile range as the measure of variability. Measurements were taken from rectangular areas which included the knee joint but excluded surrounding regions and were slightly varied in size to accommodate differences in size of joints between animals. The joint capsule was exposed by removing an ellipse of skin overlying the knee and warmed (37°C) 0.9% saline was regularly applied to the exposed tissues to prevent dessication. Scans were obtained immediately before, then immediately after topical administration of vehicle or antagonists. The test image was then compared to the control image obtained immediately before application of the drug and expressed as a percentage change in perfusion (flux).

The following agents were used to antagonize the actions of tachykinins: FK888, a specific tachykinin NK_1 receptor antagonist; SR48968, a specific tachykinin NK_2 receptor antagonist and FK224, a non-selective tachykinin NK_1/NK_2 receptor antagonist. In some animals the α_1-, α_2-adrenoceptor antagonist phenoxybenzamine was administered topically, the purpose being to block postjunctional sympathetic adrenoceptors. After every application of each of the agents, the joint was washed by repeated topical application of warmed saline. This resulted in tissue perfusion returning close to the original control level of 3.2 [0.75] V (median [semi-interquartile range]) as shown by the flux values measured ten minutes after each intervention (in order: saline; vehicle; FK888 + SR48968; phenoxybenzamine; phenoxybenzamine + FK888 + SR48968; saline) which yielded values of 3 [1]; 3.4 [0.55]; 3.2 [0.55]; 2.9 [0.9]; 3.2 [0.75]; 3.3 [0.65] V, respectively. Friedman one way analysis of variance revealed no differences ($P=0.8$, $n=13$).

In four animals the effect of the tachykinin receptor antagonists was investigated in joints before exposure. This was achieved by transcutaneous imaging of knee joint perfusion with the same LDI equipment and methodology as described above but with a near infra-red (830 nm) laser source for these experiments. With this longer wavelength it is possible to scan over the depilated knee and detect alterations in perfusion of the underlying knee joint in response to vasoactive agents (unpublished observations). For this part of the present study, the distal saphenous artery which supplies the knee joint region was cannulated and the antagonists or vehicle were administered as a bolus by intra-arterial injection. To ensure that the procedure of exposing and cannulating the saphenous artery did not itself initiate tachykinin release from surrounding sensory nerve terminals by an 'axon reflex' mechanism, transcutaneous imaging experiments were performed in a further two animals. In these rats, the skin overlying the saphenous artery was anaesthetized by topical application of Emla 5% anaesthetic cream, and after 20 min, a bolus of xylocaine was injected into the skin surrounding the area over the artery to be cannulated and then over the artery itself before cannulation.

In nine rats the medial aspect of the rat knee joint was surgically denervated by resection of 5 mm of the saphenous nerve proximal to the knee joint under deep general anaesthesia with Hypnorm (0.1 ml 300 g^{-1}, i.m.) and under aseptic conditions. The animals then recovered from anaesthesia and seven days later, which is a sufficient period of time for nerve degeneration to occur distally, taken to the terminal experiment. Figure 1 illustrates the changes which occur in the distal segment of the nerve supplying the medial aspect of the rat knee joint one week after proximal resection. These nerve samples (10 mm length) were obtained close to the knee joint and were fixed with 2% gluteraldehyde in 0.1 M sodium cacodylate followed by 1% osmium tetroxide in the same buffer (Sabatini *et al*., 1963). The fixed specimens were dehydrated with graded alcohol, infiltrated with and then embedded in Araldite resin (Glauert & Glauert, 1958). Ultrathin sections taken at the midpoint of the excised nerve were mounted on 1 mm aperture grids and double stained with uranyl acetate (Stempak & Ward, 1964) and lead citrate (Reynolds, 1963) and thereafter examined by a Zeiss EM 109 electron microscope.

In another group of rats, capsaicin (50 mg kg^{-1}) was administered subcutaneously whilst the animals were under deep halothane (2%) anaesthesia. Four days later these animals were taken to the terminal experiment. Previous work has shown that this regime of systemic capsaicin administration results in substantial depletion in the content of tachykinin-like immunoreactivity (96% reduction) and substance P-like immunoreactivity (94% reduction) from the urinary bladder (Maggi *et al*., 1988).

Statistical analyses were performed by use of MINITAB software. By use of the Shapiro-Wilk test, the percentage data were found to be normally distributed and are expressed as means $\pm$ s.e.mean with two sample comparisons being by unpaired Student's *t* test. All quoted *P* values are two-tailed. Comparisons of three or more samples was initially by one way ANOVA with *post hoc* comparisons by the Tukey-Kramer test. Flux values were non-normally distributed and are therefore expressed as medians and the semi-interquartile range with statistical analysis being performed by non-parametric tests.

Materials

Urethane was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.), Hypnorm (fentanyl citrate 0.315 mg ml^{-1} and fluanisone 10 mg ml^{-1}) from Janssen (Buckinghamshire, U.K.) and both Emla cream (lignocaine and prilocaine eutectic mixture) and xylocaine (2%) from Astra Pharmaceuticals Ltd (Kings Langley, U.K.). The substance P antagonists, FK888 (N^2-[(4**R**)-4-hydroxy-1-(1-methyl-1H-indol-3-yl)carbonyl-L-prolyl]-*N*-methyl-N-phenylmethyl-3-(2-naphthyl)-L-alaninamide) and FK224 ({N-[N^2-[N-[N-[N-[2,3-didehydro-N-methyl-N-[N-[3-(2-penthylphenyl)-proprionyl]-L-threonyl]tyrosyl]-L-leucynyl]-D-phenylalanyl]-L-*allo*-threonyl]-L-asparaginyl]-L-serine-*v*-lactone}), were provided by Fujisawa Pharmaceuticals Co. (Osaka, Japan), and SR48968 ((S)-N-methyl-N[4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide) by Sanofi (Cedex, France). These antagonists were initially dissolved in 100% alcohol and subsequently diluted in 0.9% saline (alcohol content at highest concentration = 0.5%). The vehicle used consisted of ethanol (0.5%) in saline. Phenoxybenzamine (Dibenyline) was obtained from Smith Kline & French (Essex, U.K.), and diluted in 0.9% saline. Capsaicin was obtained from Fluka (Switzerland) and diluted in a vehicle consisting of 0.1 ml ethanol, 0.1 ml Tween and 1.8 ml 0.9% saline.

Results

Individual administration of either FK888 or SR48968, even at the highest dose (10^{-8} mol), did not significantly affect synovial perfusion compared to application of vehicle ($P=0.33$) and $P=0.9$, respectively, $n=29$; Figure 3a). Comparison of vehicle and 0.9% saline treatments did not reveal a significant difference ($P=0.72$, $n=29$). Although neither FK888 nor SR48968 individually affected synovial perfusion, combining them produced an obvious reduction in perfusion (Figure 2) which was clearly dose-dependent (Figure 3a). With both of these antagonists co-administered at 10^{-8} mol, a 19.9 ± 2.6% reduction in perfusion occurred which was highly significant

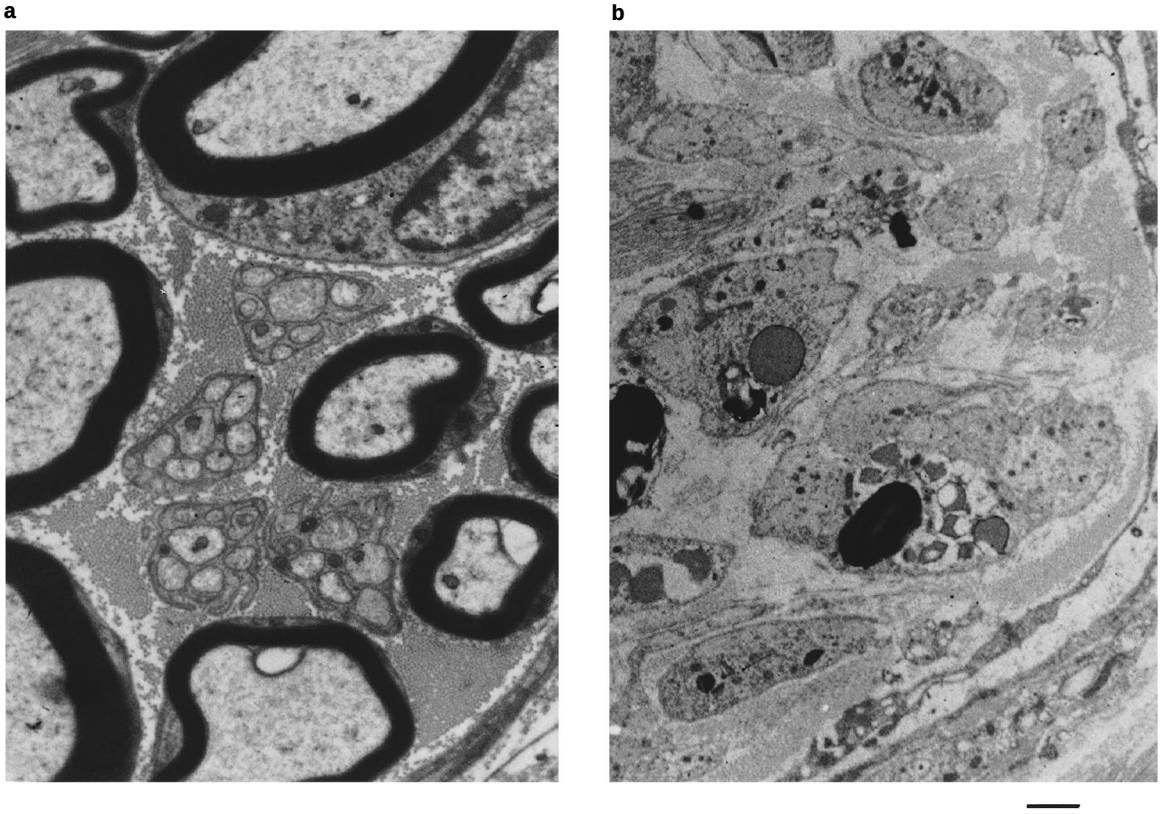

Figure 1 Transmission electron micrographs of a cross section through the articular nerve supplying the medial aspect of the rat knee joint under normal conditions (a), and one week after surgical resection of the saphenous nerve (b). The scale bar applies to both micrographs and represents 1 μm. Note presence of large myelinated nerve fibres and clusters of unmyelinated fibres in the normal nerve. The distal segment of the nerve following section of the saphenous nerve proximally shows gross degeneration with invasion of macrophages, lack of myelin sheaths and no visible evidence of unmyelinated fibres.

($P=0.001$; $n=29$) compared to vehicle. The reduction in perfusion at 10^{-10} mol was also significant ($P=0.005$; $n=29$) but not at 10^{-12} mol.

FK224 produced more modest vasoconstriction, but at both 10^{-10} and 10^{-8} mol this differed significantly from vehicle administration ($P=0.011$ and $P=0.025$, respectively; $n=25$).

These results suggest that normally there may be basal release of tachykinins in the joint which produces vasodilatation by acting on both tachykinin NK_1 and NK_2 receptors. Administration of the antagonists inhibits the vasodilator effect of endogenous tachykinins, resulting in vasoconstriction. The effect of the combined antagonists could not be ascribed to lowering of arterial blood pressure as this was found to be unaffected by these antagonists, either individually or in combination.

The vasoconstrictor effect of the antagonists could be due to unopposed sympathetic vasoconstrictor influences on articular blood vessels. To test this hypothesis, experiments were performed to examine the effect of the combined antagonists during α-adrenoceptor blockade. Application of the α_1-, α_2-adrenoceptor antagonist phenoxybenzamine (10^{-6} mol) elicited a $31.3\pm6.2\%$ vasodilatation (Figure 3b), indicating the presence of sympathetic vasoconstrictor 'tone'. This vasodilatation was significantly ($P=0.01$, $n=13$) reduced by 60% when co-administered with the combination of FK888 and SR48968 (both at 10^{-8} mol). This suggests that normally there is sympathetic vasoconstrictor 'tone' which may be counterbalanced by the vasodilator action of endogenous tachykinins. The effect of the different treatments (vehicle, administration of the combined antagonists, phenoxybenzamine alone and phenoxybenzamine plus the antagonists) was highly significant ($P<0.0001$, one way ANOVA). However, blood pressure, measured 30 s after drug administration (in order: saline, vehicle, FK888 + SR48968, phenoxybenzamine, phenoxybenzamine + FK888 + SR48968, saline; all antagonist doses as above), was unaffected, yielding mean values ($\pm$s.e.mean) of 90 ± 7, 87 ± 8, 87 ± 7, 86 ± 7, 84 ± 6 and 85 ± 7 mmHg with one way ANOVA revealing no differences ($P=0.99$; $n=13$).

To confirm that these actions were mediated by tachykinins released from sensory nerve fibres and not merely a non-specific vasoconstrictor effect of the antagonists, in nine rats deeply anaesthetized with Hypnorm the medial aspect of one knee was surgically denervated and one week later the tachykinin receptor antagonists administered. Although overall basal flux readings (median [semi-interquartile range]) in the denervated knees (2.02 [0.94] V) were lower than in the normal knee (3 [0.53] V), these did not differ significantly ($P=0.18$; Mann-Whitney test; $n=8-13$). In denervated joints the combination of FK888 and SR48968 (both 10^{-8} mol) did not significantly change blood flow compared to vehicle treatment (6.7 ± 4.6 vs $0.46\pm1.2\%$ change in flux, respectively). The findings of the denervation study were confirmed by further experiments in which rats were pretreated with systemic capsaicin to deplete sensory nerve endings of tachykinins. In these experiments, antagonist administration (both at 10^{-8} mol) again failed to elicit vasoconstriction but instead produced a slight vasodilatation ($2.5\pm5.3\%$; $n=6$). This response did not differ significantly from that occurring following surgical denervation.

It could be argued that removal of the skin and exposure of the joint capsule introduced an unphysiological artefact due to increased activity in capsular nerve endings, resulting in an artificially-induced release of tachykinins. To test whether tonic tachykinin release occurs under more physiologcal conditions, the FK888 and SR48968 combination (both at 10^{-8} mol) was administered by close intra-arterial injection to knee joints before removal of the skin overlying the joint. Compared to vehicle, the antagonists still elicted a vasocon-

triction (Figure 2IIa and b) under these more physiological conditions, with a mean reduction of $48.8 \pm 16.2\%$ which differed significantly from vehicle administration ($P = 0.03$; $n = 4$). Subsequent exposure of the joint capsule and administration of the antagonists also elicited vasoconstriction, with the vascular architecture closely corresponding with the images obtained before removal of the skin (Figure 2IIc and d). Essentially similar results were obtained in two rats where care was taken

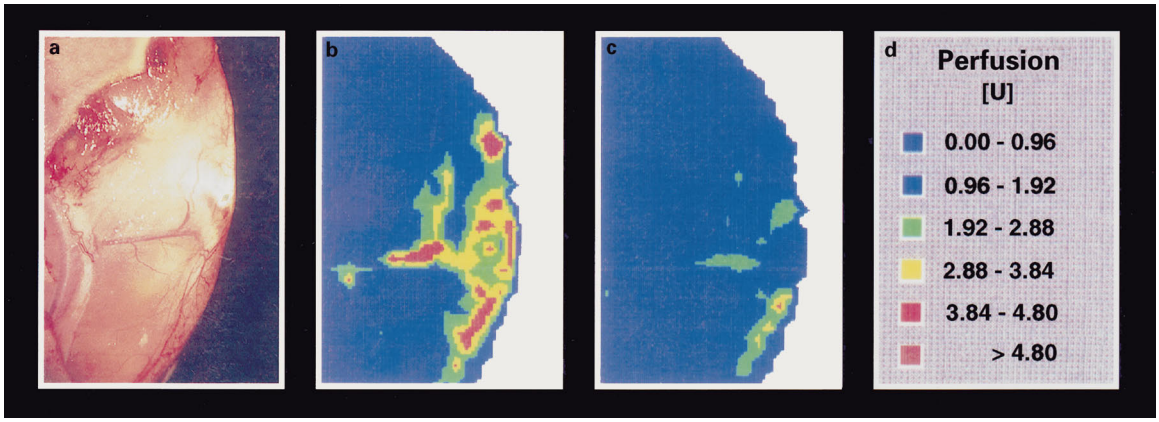

Figure 2I Photograph of the exposed knee joint (a) and laser Doppler perfusion image of the corresponding region before (b) and immediately after (c) topical application of the combination of the tachykinin NK_1 receptor antagonist FK888 and the tachykinin NK_2 receptor antagonist SR48968 (both 10^{-8} mol). The images are colour-coded (in volts) with highest perfusion in dark red and lowest flow in dark blue (d). A substantial reduction in perfusion is evident following administration of the antagonists.

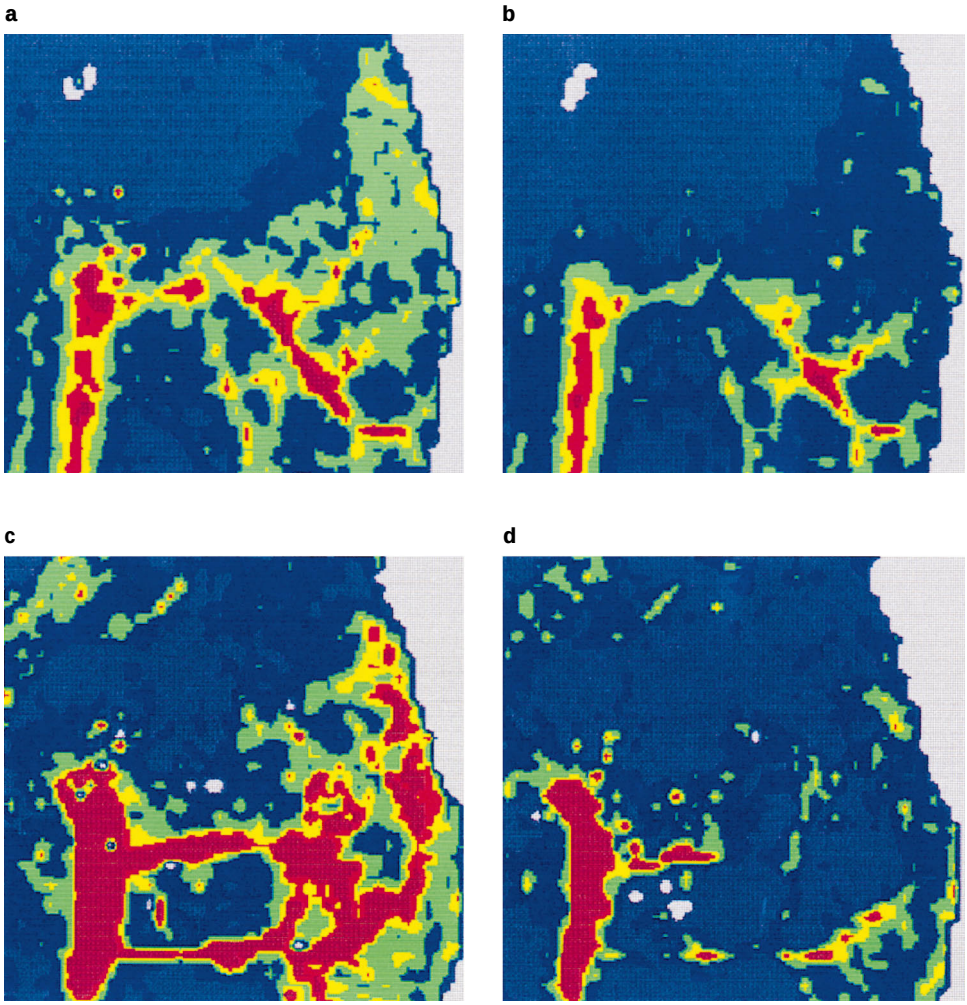

Figure 2II Laser Doppler perfusion images of the knee joint region with the overlying skin intact before (a) and after (b) close intra-arterial injection of the combination of the tachykinin NK_1 receptor antagonist FK888 and the tachykinin NK_2 receptor antagonist SR48968 (both 10^{-8} mol). The antagonists produce clear vasoconstriction. Excision of the overlying skin revealed the vascular architecture of the exposed joint capsule (c) which bears a close resemblance to the image obtained prior to skin removal (a). Repeat close intra-arterial injection of the combination of FK888 and SR48968 (both 10^{-8} mol) again resulted in vasoconstriction (d). Perfusion scale as above.

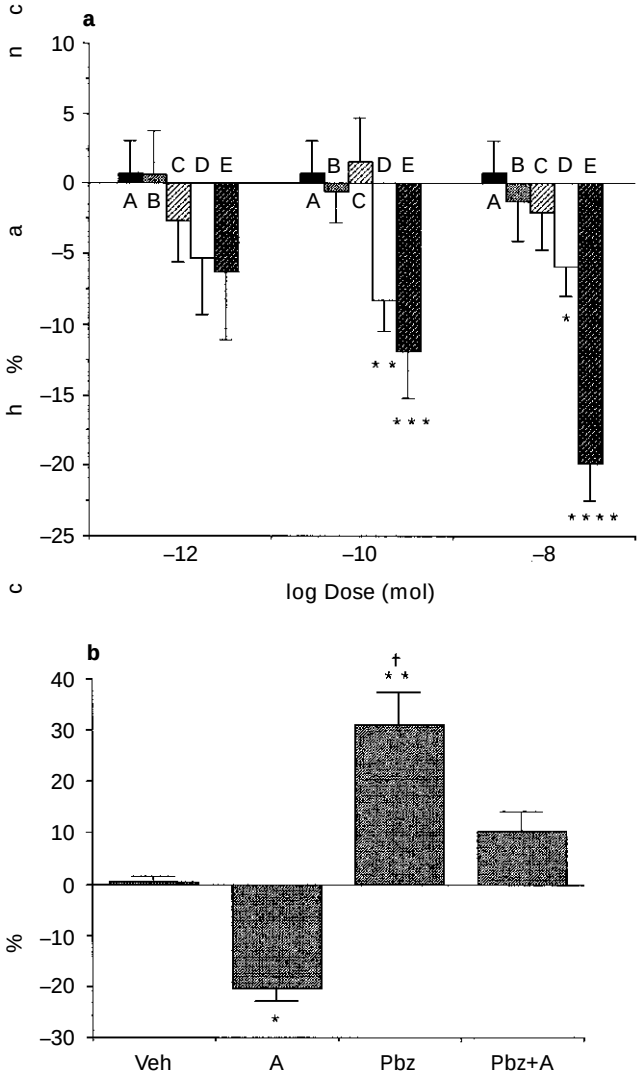

Figure 3 (a) Responses of knee joint vessels to application of the different antagonists at three different doses. Negative values indicate vasoconstriction. Column A, vehicle; B, SR48968; C, FK888; D, FK224; E, FK888 + SR48968. Comparisons are relative to the vehicle: ****$P \leqslant 0.001$; ***$P < 0.01$; **$P < 0.02$; *$P < 0.05$; $n = 25-29$. (b) Vasoconstriction of blood vessels in the normal knee joint in response to topical application of the combination of tachykinin NK_1 receptor antagonist, FK888, and the tachykinin NK_2 receptor, antagonist, SR48968, both at 10^{-8} (A). Application of phenoxybenzamine (Pbz) on its own produced significant vasodilatation which was substantially reduced when co-administered with the combination of antagonists (A + Pbz). $n = 8-13$. †$P < 0.01$, Pbz significantly different from Pbz + A. *$P < 0.02$, **$P < 0.01$, significantly different from vehicle (Veh).

to avoid eliciting the axon reflex by anaesthetizing all tissues before surgery. Under these conditions the antagonists (both 10^{-8} mol) again produced a reduction in blood flow ($41.5 \pm 15.5\%$).

Discussion

The results of this study suggest that tachykinins released from sensory nerve endings exert a tonic vasodilator influence on synovial blood vessels. This appears to be mediated by both tachykinin NK_1 and NK_2 receptors as administration of the individual selective tachykinin receptor antagonists failed to reduce this influence whereas either the combination of these antagonists, or the non-selective tachykinin NK_1 and NK_2 receptor antagonist, FK224, gave significant inhibition. It is particularly noteworthy that the combination of FK888 and SR48968 at 10^{-10} mol produced significant reduction in synovial perfusion, whereas individual administration of each of these agents even at a hundred fold higher dose failed to influence perfusion significantly. The combination of antagonists was capable of eliciting a vasoconstrictor response of synovial blood vessels in the more physiological condition of the skin covered knee, thereby confirming the validity of the results obtained in the exposed knee and suggesting that tachykinins do play a physiological role in the regulation of synovial perfusion. The possible release of endogenous tachykinins via 'axon reflex' mechanisms elicited by surgery or vessel cannulation was excluded by additional transcutaneous imaging experiments. In these experiments, the tachykinin receptor antagonists still caused a reduction in basal blood flow even when all tissues were anaesthetized before surgical intervention to preclude activation of the axon reflex. Although nociceptive afferents contain tachykinins, their tonic release from these axons does not appear to involve overt activation of these fibres. The knee was held in a non-noxious position and the animals were normotensive, thus no noxious stimuli were present to activate these fibres and this was confirmed by the experiment involving transcutaneous imaging of knee perfusion. In the absence of inflammation, most nociceptive afferents show little spontaneous activity, even with the joint capsule exposed (Schaible & Schmidt, 1983). These findings indicate that tachykinin release from peripheral sensory nerve endings may occur without invoking an axon reflex mechanism. Evidence to support this comes from the finding that capsaicin-induced plasma extravasation is not inhibited by pretreatment with tetrodotoxin (Szolcsányi, 1984).

The antagonist-induced vasoconstriction appears to result in part from unopposed sympathetic 'tone' as phenoxybenzamine administration resulted in vasodilatation, presumably due to blockade of sympathetic neurotransmission and thus loss of tonic vasoconstrictor influence. The substantial reduction in this vasodilatation when the antagonists were co-administered suggests that a significant component of this response may be due to endogenous tachykinins. This observation also suggests that in the absence of sympathetic tone there must be other sources of constrictor tone which the tachykinins oppose. These other sources of constrictor tone have not been investigated in this study but possibilities include the potent vasoconstrictor agent endothelin as well as inherent myogenic 'tone'. Although considered in isolation, it is possible that there exists interaction between sympathetic effects and tachykinin effects. However, examination of such interaction was beyond the scope of the present study.

The absence of a vasoconstrictor response to FK888 and SR48968 following surgical denervation suggests that this effect is mediated via tachykinins present in sensory nerve endings. Although the lack of function of the distal segment of the surgically interrupted nerve was not tested in this study, electron microscopy revealed gross degeneration of this segment of the nerve with no evidence of unmyelinated fibres. Under these conditions it is difficult to see how significant quanitites of tachykinins would still be present in the joint capsule. Supporting this is the observation that 48 to 72 h after pelvic nerve section, SP/NKA-LI levels in the rat isolated bladder become undetectable and this is paralleled by abolition of the functional response to capsaicin (Maggi *et al*., 1988; Santicioli *et al*., 1986). Our denervation study is further supported by the similar result obtained following systemic capsaicin administration, since this is known to deplete tachykinins selectively from peripheral sensory nerve fibres (for review see Holzer, 1991). Although we were unable to examine the extent to which systemic capsaicin treatment depleted tachykinins from the joint capsule, experience in other tissues by other researchers indicates that substantial reduction in immunoreactivity to tachykinins, including substance P, can be obtained with a capsaicin regime of 50 mg kg^{-1} (Gamse, 1982; Maggi *et al*., 1988). In the absence of endogenous tachykinins by either surgical denervation or capsaicin treatment, the tachykinin

receptor antagonists were no longer able to elicit vasoconstriction. These findings indirectly indicate that the antagonists do not of themselves exert a non-specific vasoconstrictor effect and strongly point to a neural source of tachykinins being responsible for the tonic vasodilator influence.

These findings provide strong evidence that tachykinins, and perhaps other sensory neuropeptides, under physiological conditions are released from nerve endings in the joint capsule. The present study was centred on the tachykinins, but there are data suggesting that calcitonin gene-related peptide (CGRP) could also be tonically released in the rat knee joint under similar experimental conditions to those described in this study (McMurdo *et al*., 1996). These observations suggest that neuropeptide release can occur in the absence of an axon reflex. Continuous release of tachykinins may to some extent oppose sympathetic vasoconstrictor 'tone' and this implies that under physiological conditions these peptides have an important regulator function. It is possible that inflammatory joint disease alters this function, thereby significantly interfering with synovial perfusion and perhaps contributing to the intra-articular hypoxia often prevailing in such disease states.

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