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Specific neurokinin receptors mediate plasma extravasation in the rat knee joint

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1 Plasma extravasation in the rat knee joint was induced by intra-articular injection of neurokinins and specific neurokinin receptor agonists.

2 Pronounced plasma extravasation was produced by substance P (SP, 4–185 μM) and to a lesser extent by neurokinin-B (NKB, 83–413 μM), whereas neurokinin-A (NKA, 88–440 μM) and calcitonin gene-related peptide (CGRP, 26–130 μM) had no significant effect.

3 The specific neurokinin$_1$ receptor agonist [Sar^9, Met(O$_2$)^{11}]-substance P (NK$_1$ agonist) in doses of 0.4–70 μM appeared to be more potent than SP in eliciting plasma extravasation. The neurokinin$_2$ receptor agonist [Nle^{10}]-neurokinin A$_{4-10}$ (NK$_2$ agonist) was not effective at 70 μM but produced a small and significant effect at 330 μM, whereas the neurokinin$_3$ receptor agonist [MePhe^7]-neurokinin B (NK$_3$ agonist) was without effect at 40 μM or 400 μM.

4 Injections of SP or NKA into the synovial cavity of the rat knee were equally effective in producing marked plasma extravasation in remote sites such as the forelimb and hindlimb paws.

5 Co-administration experiments showed that the effects of SP were synergistic with NKA or the NK$_1$ receptor agonist, but not with CGRP or the NK$_2$ receptor agonist.

6 The rank order of potency was NK$_1$ agonist $\geq$ SP > NKB > NK$_2$ agonist suggesting that NK$_1$ receptors mediate plasma extravasation in the rat knee joint.

Keywords: Neuropeptides; neurokinins; substance P; tachykinins; joint inflammation; calcitonin gene-related peptide (CGRP); plasma extravasation; neurokinin A; neurokinin B; neurogenic inflammation

Introduction

Substance P (SP), a neuropeptide contained in sensory (C) fibres (Hokfelt *et al.*, 1975), has long been implicated as the mediator of neurogenic inflammation (Jancso *et al.*, 1967; Lembeck & Holzer, 1979; Gamse *et al.*, 1980; Lembeck *et al.*, 1982). A family of peptides structurally related to SP, known as the tachykinins, have now been characterized. More appropriate nomenclature for these peptides is the neurokinin family, since these are synthesized and stored in nervous structures and act as neurotransmitters. The neurokinins include the physalaemin-like compound, neurokinin A (NKA, also called neuromedin L or substance K) and neurokinin B (NKB, also called neuromedin K) (Kimura *et al.*, 1983; Kangawa *et al.*, 1983; Minamino *et al.*, 1984). The occurrence of several neurokinins in mammalian tissues suggests the existence of different types of neurokinin receptors. Based on the rank order of potencies of the neurokinins in both pharmacological (Regoli *et al.*, 1987a,b) and biochemical (Buck *et al.*, 1984; Beaujouan *et al.*, 1984) studies, three distinct neurokinin receptor populations have been postulated: (1) the NK$_1$ (or SP-P) receptor at which SP is the agonist; (2) the NK$_2$ (or SP-E) receptor at which NKA is the agonist; and (3) the NK$_3$ (or SP-N) receptor at which NKB is the agonist.

It is now recognised that sensory afferents commonly contain more than one type of neuropeptide, with SP and calcitonin gene-related peptide (CGRP) being often co-localised in many types of nociceptive afferent fibres (Fischer *et al.*, 1985). It is also possible that other neurokinins such as NKA and NKB may be co-localised with SP in nerve fibres. SP has been shown to induce plasma extravasation when injected into the synovial cavity of the knee (Lam & Ferrell 1989a,b). This suggests that NK$_1$ receptors are present in articular tissues as SP is the preferential endogenous ligand for this receptor. However, endogenous neurokinins tend to show cross-reactivity with other neurokinin receptors (Drapeau *et al.*, 1987). The recent description of selective agonists for NK$_1$, NK$_2$ and NK$_3$ receptors (Drapeau *et al.*, 1987) offers the opportunity to assess the types of neurokinin receptors present in the knee joint. Thus, the present study is an attempt to identify the neurokinin receptor types mediating plasma extravasation by comparing the rank order of potency of the various neuropeptides (including CGRP) in the rat knee joint. Interactions between the different agents with SP were also assessed.

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Methods

Experiments were performed on male Wistar rats (~300 g) deeply anaesthetized by intraperitoneal injection of urethane (1.13 g kg^{-1}) and diazepam (2.5 mg kg^{-1}). Evans blue (75 mg kg^{-1}) was injected into the external jugular vein. The experimental procedure consisted of injection of 0.2 ml of the naturally-occurring neuropeptides or the specific neurokinin receptor agonists into the synovial cavity of one knee, the other being injected with 0.9% saline to provide an internal control. These were left in the joint for 4 h after which the animals were injected with Euthatal and exsanguinated. A 4 h period was chosen as the time course of neurokinin-induced plasma extravasation was not known and it also allowed comparisons with results of previous investigations (Lam & Ferrell, 1989a,b). The anterior and posterior portions of the knee joint capsule on both sides were dissected free from each rat. The amount of tissue obtained from each animal was small, necessitating pooling of samples from five rats. These samples were weighed and Evans blue extracted by a modified dye extraction technique (Harada *et al.*, 1971), details of which have been given previously (Lam & Ferrell, 1989a). The amount of dye recovered was calculated by comparing the absorbance of the fluid obtained at 620 nm (LKB Ultrospec II) with that of a standard curve prepared with known concentrations of Evans blue solution. As Evans blue binds to plasma proteins normally restricted to the vascular compartment, its presence in the capsule provides an index of altered vascular permeability.

In a group of rats, the effects of co-administration of SP with other naturally occurring neuropeptides and with the specific NK_1 receptor agonists were investigated. The procedures were the same as above with the combined volume of drugs co-administered remaining at 0.2 ml. In another group of rats, the effects of intra-articular injection of NKA and NKB on plasma extravasation in the rat hind paw were also assessed. Measurements of Evans blue content in rat paws were performed individually as the amount of tissues obtained from each paw are much greater than those obtained from the joint capsules. For each dose of the neuropeptides, 5 rat paws were used. Experiments involving measurements of Evans blue content from joint capsules were obtained from 3–6 groups of five rats. Data are presented as the mean difference (± s.e.mean) in Evans blue content between the control and the test knee in each group. The figures represent means ± s.e.mean and differences were considered significant if the P values were 5% or less (unpaired t test). Human α-CGRP was kindly donated by Celltech Ltd (Berkshire). All other drugs were purchased from Cambridge Biochemicals Ltd. In all cases physiological saline solution was used as the solvent.

Results

Plasma extravasation in the knee joint induced by naturally-occurring neuropeptides

Substance P (SP) injected into the synovial cavity produced plasma extravasation in a dose-dependent manner (Figure 1). The threshold for this response was 1 μg in 0.2 ml volume, representing a dose of 3.7 μM, with 50 μg (180 μM) giving a maximal response. CGRP and NKA in concentrations up to 132 μM and 440 μM respectively, were ineffective in eliciting plasma extravasation. These results are illustrated in Figure 1, which shows that plasma extravasation induced by CGRP and NKA did not differ significantly from the control situation when saline was injected into both knees. Significant plasma extravasation was produced with 80 μM NKB, but increasing the dose of NKB to 410 μM failed to produce further significant increase in the plasma extravasation (Figure 1). The maximum plasma extravasation produced by NKB was less than half of that produced by SP.

Plasma extravasation in the knee joint induced by neurokinin receptor agonists

The effects of intra-articular injection of different neurokinin receptor agonists were compared with the effects of SP. [Sar^9, $Met(O_2)^{11}$]-substance P was the chosen specific NK_1 receptor agonist in the present studies. [Nle^{10}]-neurokinin A_{4-10} and [$MePhe^7$]-neurokinin B were the chosen specific NK_2 and NK_3 receptor agonists, respectively. The NK_1 receptor agonist produced dose-dependent inflammatory responses which closely resembled those produced by SP (Figure 2). Although the NK_1 agonist appeared to be more potent than SP, their dose-response curves did not differ significantly. The NK_2 receptor agonist produced a small degree of plasma extravasation at 70 μM which was not significantly different from that produced by injection of saline alone. At a higher concentration of 330 μM, the NK_2 receptor agonist produced a significant plasma extravasation which was about 40% of that produced by SP (Figure 2). Two concentrations of the NK_3 receptor agonist were tested, at both 40 μM and 400 μM, but no significant plasma extravasation was observed (Figure 2).

Plasma extravasation in the rat paw induced by intra-articular administration of neurokinins

During the course of the experiments it was observed that although intra-articular injection of NKA produced no significant plasma extravasation in the knee joint, Evans blue extravasation was prominent elsewhere, especially in the skin of the forelimb and hindlimb paws. It was decided therefore to determine the extent of Evans blue extravasation in the hind-

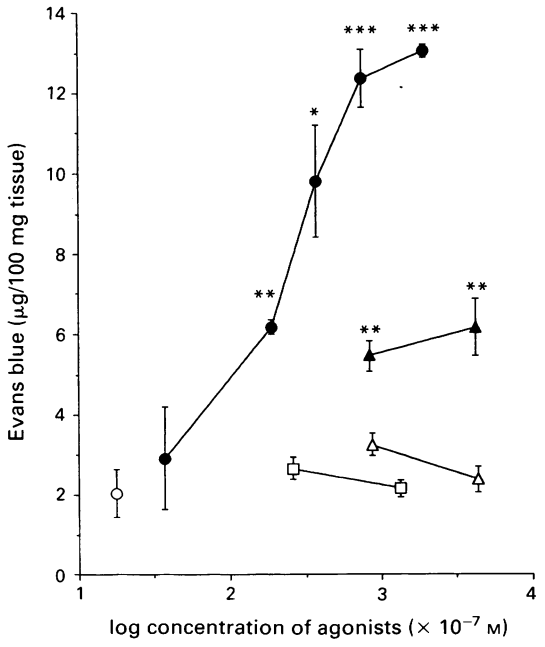

Figure 1 Effects of increasing doses of substance P (●), neurokinin A (△), neurokinin B (▲), and calcitonin gene-related peptide (□) on plasma extravasation into the knee joint capsule. Evans blue content represents the difference between the test (neuropeptide-injected) and the control (saline-injected) knee for each group of five animals. Mean of $n = 3$–4; vertical bars show s.e.mean. Significant difference from saline injection in both knees (○): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

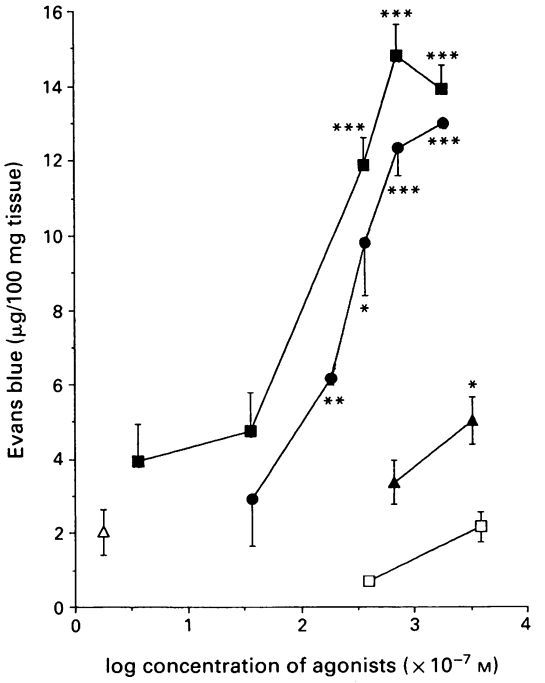

Figure 2 Effects of increasing doses of substance P (●), and neurokinin$_1$ (NK_1) (■), NK_2 (▲), and NK_3 (□) receptor agonists on plasma extravasation into the knee joint capsule. Evans blue content represents the difference between the test (neurokinin-injected) and the control (saline-injected) knee for each group of five animals. Mean of $n = 3$–4; vertical bars show s.e.mean. Significant difference from saline injection in both knees (△): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

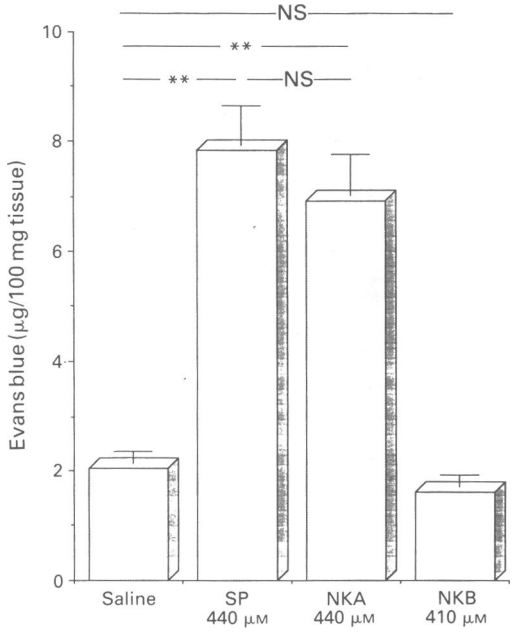

Figure 3 Effects of intra-articular injections of substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) on plasma extravasation into the contralateral hindlimb paw. Evans blue content represents plasma extravasation from five individual rat paws (Mean with s.e.mean shown by vertical bars). NS = no significant difference from saline control. Significant difference from saline control: * $P < 0.05$, ** $P < 0.01$.

limb paw along with that occurring in the knee joint capsules following intra-articular administration of the neurokinins. As illustrated in Figure 3, NKA at 440 μM, a concentration that had no effect in the knee joint, produced marked plasma extravasation in the hindlimb paw contralateral to the neuropeptide-injected knee. In contrast, NKB, which produced significant plasma extravasation in the knee joint capsules at 410 μM, had no effect on the paw at this dose (Figure 3). SP at the same concentration as NKA (440 μM) produced similar plasma extravasation in the paw (Figure 3).

Plasma extravasation in the knee joint induced by co-administration of substance P with naturally-occurring neuropeptides and neurokinin receptor agonist

The effect of a submaximal dose of SP (18 μM) on plasma extravasation was investigated with co-administration of CGRP, NKA, NKB, and with the specific NK_1 receptor agonist. CGRP (130 μM) had no significant effect on plasma extravasation on its own. When co-adminstered with SP, no alteration on the SP-induced plasma extravasation was observed (Figure 4a). As no effect was observed with this, the highest dose, lower doses were not used. Co-administration of SP with 400 μM NKA, which by itself was ineffective in producing a response, resulted in a small but significant increase on the SP-induced plasma extravasation (Figure 4b). Co-administration of SP with 400 μM NKB on the other hand did not affect the SP-induced plasma extravasation although NKB at this concentration was effective in producing plasma extravasation on its own (Figure 4c). The specific NK_1 receptor agonist which itself is a potent inflammatory agent, when co-administered with SP resulted in summation of the individual responses (Figure 4d).

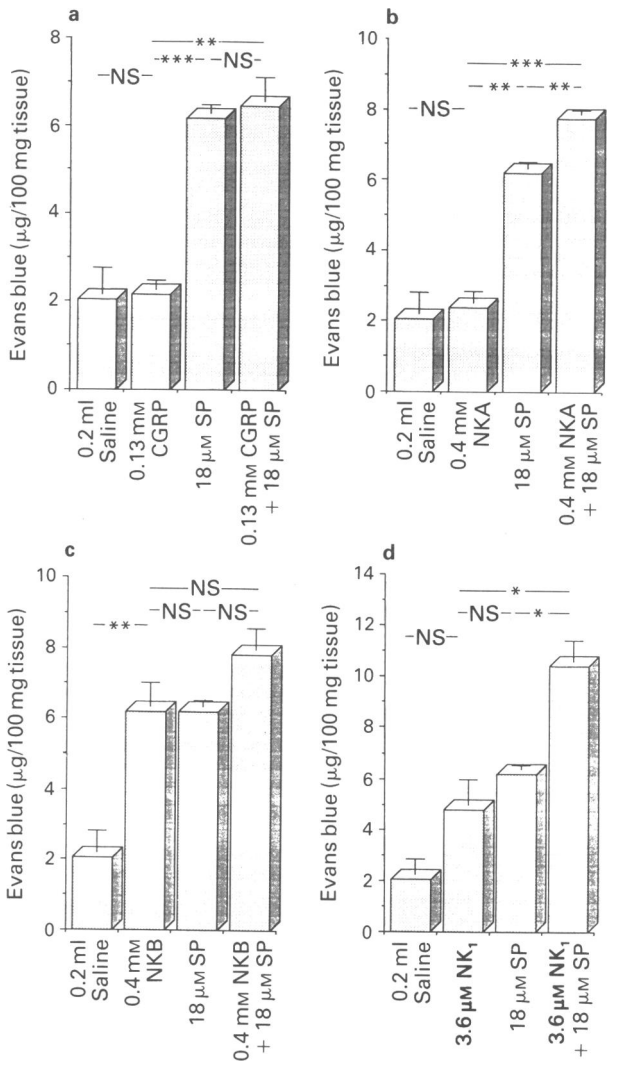

Figure 4 Effects of co-administration of substance P (SP) with (a) calcitonin gene-related peptide (CGRP), (b) neurokinin A (NKA), (c) neurokinin B (NKB), and (d) specific neurokinin$_1$ receptor agonist (NK_1) on plasma extravasation into the knee joint capsule. Evans blue represents the difference between the test (neuropeptide-injected) and the control (saline-injected) knee for each group of five animals. Mean with s.e.mean shown by vertical bars. $n = 3$. NS = no significant difference. Significant difference: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Discussion

Plasma extravasation in rat knee, induced by intra-articular injection of pro-inflammatory agents has been shown to have a significant neurogenic component (Lam & Ferrell, 1989a). The neuropeptide SP present in articular C-afferent fibres has been confirmed as an important mediator of the inflammatory process (Lam & Ferrell, 1989a,b). Marked plasma extravasation can be elicited by direct administration of SP into the synovial cavity of rat knee (Lam & Ferrell, 1989a,b) which can be inhibited by pretreatment with substance P antagonist D-Pro^4, D-Trp^{7,9,10} SP(4–11) (Lam & Ferrell, 1989a). The plasma extravasation induced by SP is thought to be mediated by interactions from the carboxyl terminus of the neuropeptide with specific vascular receptors (Foreman *et al.*, 1983). An N-terminal analogue of SP as verified in human skin (Foreman *et al.*, 1983) failed to induce plasma extravasation. The common C-terminal of the tachykinins suggest that they should all induce plasma extravasation as was found to be the case in studies on rat skin (Brain & Williams, 1989; Andrews *et al.*, 1989). The present studies on the rat knee have shown that this is not always the case, as among the neuropeptides tested, only SP and to a lesser extent NKB, were effective in producing plasma extravasation in the rat knee but not NKA or CGRP.

Antagonists of histamine and 5-hydroxytryptamine have been shown to inhibit partially SP-induced plasma extrava-

sation in rat knee (Lam & Ferrell, 1990) and skin (Brain & Williams, 1989). Inflammatory responses induced by NKA and NKB on rat skin were not affected by these antagonists (Brain & Williams, 1989). This suggests that SP-induced plasma extravasation but not that of the other neurokinins is partially dependent on stimulation of mast cell amine release, which is mediated via the N-terminal amino acids (Foreman *et al.*, 1983). In the rat knee, the importance of this N-terminus contribution is possibly greater and hence the difference in potency of SP from the other neurokinins (which share the same C-terminus and not the N-terminus) in eliciting plasma extravasation is even greater.

As SP is the preferential agonist on NK_1 receptors (Lee *et al.*, 1986), this suggests that inflammatory processes in the rat knee are probably mediated by NK_1 receptors. The lack of effect of NKA which is the preferential agonist on NK_2 receptors (Lee *et al.*, 1986) at a concentration as high as 440 μM suggest that the NK_2 receptor is not important in this response. NKB which is the preferential agonist on NK_3 receptors (Lee *et al.*, 1986) elicited plasma extravasation which was less pronounced than that produced by SP. This suggests that perhaps NK_3 receptors are also involved in this response, but as high concentrations of NKB (83–413 μM) were required, the effects could well be due to cross-reactivity of NKB on NK_1 receptors. Furthermore, the concentrations of NKB in the spinal cord and dorsal root ganglia are 35–40 times less than SP (Ogawa *et al.*, 1985), and the presence of NKB has not yet been demonstrated in the peripheral nerve fibres. These observations taken together do not favour the involvement of NK_3 receptors in the present inflammatory model.

Andrew and co-workers (1989) in their studies on plasma extravasation using a vacuum-induced blister model on rat footpad skin, reached a similar conclusion to ours and considered the NK_1 receptor to be the mediator in the inflammatory response. Plasma extravasation in the rat paw was also investigated in the present study which showed that at a high concentration (440 μM), NKA although not effective on the rat knee, was able to elicit a marked plasma extravasation in the rat paw. However, NKB (410 μM) which was effective at the knee joint was without effect on the rat paw. This could suggest that plasma extravasation at the two sites may be mediated by different types of neurokinin receptors. However, closer examination of the results suggests that both effects are more likely to be mediated by NK_1 receptors, as (i) the effect of NKA on the rat paw is apparent only at high concentrations which would possibly cross-react with NK_1 receptors, and (ii) SP which is potent at the knee joint also produced the most marked plasma extravasation in the rat paw. The reason for NKB being effective on the rat knee but not in the rat paw may be due to less of the neuropeptide being absorbed into the circulation or due to NKB being more susceptible to inactivation by peptidase present in the circulation, hence resulting in an insufficient amount reaching distant sites to produce a response.

In the present study, the receptor type mediating plasma extravasation in the rat knee joint was investigated further with specific neurokinin receptor agonists. The results showed that the specific NK_1 receptor agonist [Sar^9, $Met(O_2)^{11}$]-substance P was as, if not more, potent than SP as an inflammatory agent in the joint, hence confirming that the plasma extravasation is mediated principally by NK_1 receptors. The involvement of NK_2 receptors is unlikely as the endogenous NK_2 receptor agonist, NKA was without effect, and also the specific NK_2 receptor agonist [Nle^{10}]-neurokinin A_{4-10} showed only small responses even at a high concentration (330 μM). Thus, the plasma extravasation induced by the NK_2 agonist is more likely to be due to cross-reactivity of the NK_2 agonist on NK_1 receptors. The present studies on the specific NK_3 receptor agonist [$MePhe^7$]-neurokinin B have shown that it is ineffective as an inflammatory agent in the rat knee. Taken together these findings and earlier discussions on the endogenous NK_3 receptor agonist, NKB, lead to the conclusion that the NK_3 receptor plays no part in the present inflammatory model.

CGRP and SP when co-administered have been shown to be synergistic in their effects on plasma extravasation in rat skin (Brain & Williams, 1985; 1989). CGRP is a potent vasodilator and it is therefore not surprising to find that as a consequence of this action, CGRP can potentiate SP-induced plasma extravasation. However, the present study has shown no potentiation on the SP-induced plasma extravasation when CGRP was co-administered with SP. Thus, there are differences between the two different inflammatory models despite evidence to suggest that the NK_1 receptor is the common mediator in both cases. The differences could well be due to the facts that much higher concentrations of SP (μM instead of pM) and much longer duration of action (4 h instead of 30 min) of the drug at the site of injection were required to establish measurable inflammatory responses in the present model, which may have resulted in short-lived interactions being missed. It is known that both in rat (Brain & Williams, 1989) and in human skin (Brain & Williams, 1988), the vasodilator effects of CGRP can be inhibited when SP is present. This is thought to result from SP-stimulated mast cells releasing proteases which degrade CGRP and thus terminate its vasodilator activity (Brain & Williams, 1989). The fact that CGRP potentiated SP-induced plasma extravasation in the skin model (Brain & Williams, 1989) irrespective of whether the CGRP lost its activity prematurely due to proteolysis, suggests that CGRP is active as a vasodilator over the short period that SP is increasing microvascular permeability. In the present inflammatory model, as a consequence of the larger dose and longer duration of SP in the joint, the effect of proteases released by SP stimulation on mast cells may be much greater than in the skin model. Thus, degradation of CGRP would be greater in the present model resulting in no potentiation of the SP-induced plasma extravasation by CGRP.

It is interesting to note that CGRP was without effect on plasma extravasation in the rat knee joint despite its potent vasodilator effect which has been demonstrated by laser Doppler flowmetry in the rat knee (Grice *et al.*, 1990). This finding suggests that plasma extravasation is more dependent on changes in vascular permeability than changes in vascular tone in this model.

Co-administration of NKA with SP showed a slight additive effect on the SP-induced plasma extravasation, whereas no summation was observed when NKB was co-administered with SP. The magnitude of summation of NKA on the SP response was of the order of 1.56 μg Evans blue/100 mg tissue which was in fact less than the variability from injecting saline alone (2.04 μg Evans blue/100 mg tissue). Although this summation was shown to be significant statistically, the importance of this is doubtful as the present studies have shown that NKA is not an effective agent in inducing plasma extravasation in the rat knee. Moreover, NKB is a better agent than NKA in the rat knee and yet it did not alter the SP-induced response. Laser Doppler flowmetry studies (Grice, 1990) have also shown that the NK_2 receptor agonist which acts on the same receptors as NKA, is not as potent a vasodilator when compared to SP or the NK_3 receptor agonist which acts on the same receptors as NKB.

It was expected that when the NK_1 receptor agonist was co-administered with SP, the plasma extravasation induced by SP would be enhanced as the two agents were each effective in their own right. The present results confirm this view, thus providing further evidence that the effects of the NK_1 receptor agonist and SP resulted from their actions on NK_1 receptors in the rat knee.

In conclusion, the rank order of potency determined in the present inflammatory model was NK_1 receptor agonist $\geqslant$ SP > NKB > NK_2 receptor agonist, which is in keeping with that proposed for the NK_1 receptor (Regoli *et al.*, 1987a). Taken together the present studies indicate that the inflammatory effects of neurokinins in the rat knee joint are probably mediated by NK_1 receptors.

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