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Demonstration of a 'septide-sensitive' inflammatory response in rat skin

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1 Measurement of plasma protein extravasation induced by the natural tachykinins following intradermal administration in rat skin indicated equipotency between substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). The selective NK_1 receptor agonist, [Sar^9]SP sulphone was 10-100 times more potent than SP. The synthetic hexapeptide, septide, [pGlu^6, Pro^9]SP-(6-11), which has been proposed to act on a distinct NK_1 receptor subtype/binding site was equipotent with [Sar^9]SP sulphone.

2 The selective NK_2 receptor agonist [βA1a^8]NKA(4-10) (0.1-1 nmol) and the selective NK_3 receptor agonist, senktide (0.1-1 nmol) were both ineffective in producing oedema. The selective NK_2 receptor antagonist, SR 48, 968 (0.3 μmol kg^{-1}) had no significant inhibitory effects upon oedema induced by approximately equiactive doses of SP (0.2 nmol), septide (0.002 nmol), [Sar^9]SP sulphone (0.002 nmol), or NKB (0.3 nmol). These results together suggest that neither NK_2 nor NK_3 receptors are involved in oedema formation in rat skin.

3 The non-peptide tachykinin NK_1 receptor antagonist, RP 67,580 (1-3 μmol kg^{-1}), inhibited plasma protein extravasation induced by septide (0.002 nmol) to a greater extent than that to SP (0.2 nmol). RP 67,580 (1 μmol kg^{-1}) produced a significant inhibition of approximately 66% of the response to septide (0.002 nmol) only. Increasing the dose of RP 67,580 3 fold resulted in inhibition of the response to SP (0.2 nmol) and [Sar^9]SP sulphone (0.002 nmol) by approximately 66% and 64% respectively with the response to septide being inhibited by approximately 70%.

4 Co-administration of the nitric oxide (NO) synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) (0.1 μmol) with the relevant tachykinin, resulted in a significant attenuation of the oedema response to septide (0.1 nmol) producing only an approximate 56% inhibition of the response. The response to 0.2 nmol SP was unaffected whereas the response to a higher dose of 1 nmol was lowered by L-NAME but this did not reach significance.

5 Degranulation of mast cells, achieved by pretreatment with compound 48/80 (5 mg kg^{-1}) for 3 consecutive days, significantly inhibited the oedema responses to only high dose SP (1 nmol) and [Sar^9]SP sulphone (0.002 nmol). SP (0.2 nmol), septide (0.002 nmol), NKA (0.2 nmol) and NKB (0.3 nmol) were unaffected by this treatment.

6 RP 67,580 (0.3-3 μmol kg^{-1}) inhibited oedema induced by both 0.002 nmol and 0.1 nmol of septide. When using equiactive doses of SP only the response to the lower dose of 0.2 nmol SP was significantly inhibited, while RP 67,580 (3 μmol kg^{-1}) did not affect the response to 1 nmol SP.

7 These results suggest distinct mechanisms of action for SP and septide in producing plasma protein extravasation in rat skin. The response induced by septide is blocked by RP 67,580 and is both NO-dependent and mast-cell independent. In contrast the response to SP is only partially blocked by RP 67,580 and is NO-independent. These data support the existence of a distinct 'septide-sensitive' receptor/binding site and suggest that this site is involved in tachykinin-induced oedema formation in rat skin.

Keywords: Tachykinin; septide; skin inflammation; NK_1 receptor

Introduction

All of the features of an inflammatory response may be induced by various neuropeptides found in sensory C-fibres. Probably recognised as the most important inflammatory peptide found within these neurones is substance P (SP), which may cause increases in vascular permeability, vasodilatation and accumulation of inflammatory cells when injected into specific tissue sites (for review see, Payan 1989). These characteristics may all be termed pro-inflammatory and have been demonstrated in several different species including man (Foreman *et al.*, 1983; Brain & Williams, 1989; Perretti *et al.*, 1993). The immediate inflammatory actions of SP in the skin have been shown to be due both to activation of the tachykinin NK_1 receptor and also to a non-receptor-dependent induction of mast cell degranulation (Foreman *et al.*, 1991). Indeed SP-induced mast cell degranulation is known to be strictly dependent upon its N-terminal basic sequence, although the C-terminal end is also required in this property of SP (Devillier *et al.*, 1985). Other natural tachykinins, neurokinin A (NKA) and neurokinin B (NKB) or synthetic analogues which do not possess the N-terminal basic residues of SP, do not degranulate mast cells (Devillier *et al.*, 1989). It has also been demonstrated, with the aid of specific antagonists, that the activation of NK_1 receptors plays a central role in the development of neurogenic inflammation and this has consequently in turn been attributed to block of SP action (Lembeck *et al.*, 1992; Moussaoui *et al.*, 1993).

It is now well accepted that 3 distinct tachykinin receptors exist, termed NK_1, NK_2 and NK_3. These receptors have been defined according to the rank order of potency of the natural tachykinins. As expected, all of the natural tachykinins interact with all of these receptors; however, they may be differentiated according to preferential potency. The receptor through which SP is believed to exert its actions is the tachykinin NK_1 receptor, where SP is the most potent followed by NKA and then

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the least active being NKB. In turn NKA is the most potent of the natural tachykinins upon NK_2 receptors and NKB the most potent at NK_3 receptors (Maggi *et al.*, 1993).

The pharmacology of the NK_1 receptor subtype has become more complicated with the advent of several tools including selective agonists, primarily septide, and the antagonists RP 67,580 and CP96345, both non-peptide NK_1 receptor blockers. Differences in the potencies of the non-peptide antagonists to SP and septide in several studies *in vitro* (Petitet *et al.*, 1992), hand in hand with binding studies which show septide to possess a significantly lower binding affinity for the NK_1 receptor (Fardin and Garret, 1991; Fardin *et al.*, 1993) have been explained by the suggestion that distinct receptors may exist for these 2 substances i.e. that there is a 'septide-sensitive' receptor. It has also been suggested that these differences may be accounted for by different binding sites on the same receptor to one of which septide may show preferential binding (e.g. Pradier *et al.*, 1993).

Unlike *in vitro* studies in various species, septide has been demonstrated to be approximately 10 times more potent in producing plasma protein extravasation and bronchoconstriction than SP *in vivo* (Devor *et al.*, 1989; Floch *et al.*, 1993). These differences in potency could involve a differential sensitivity of SP and septide to degrading enzymes, yet the possibility that a septide-sensitive receptor/binding site is involved cannot be excluded. In the rat urinary bladder, *in vivo*, the inflammatory activity of septide is inhibited to a greater extent than that of SP by RP 67,580 (Montier *et al.*, 1994). In light of the growing attention to the role of tachykinins in inflammatory-disease states we have now investigated whether there is any role of the septide-sensitive receptor/binding site in skin inflammation. To do this we have used the rat skin oedema model, a model frequently used to investigate both pro- and anti-inflammatory activity of compounds not only with direct relevance to the skin but also as a general assay for assessment of inflammatory actions. We aimed to determine if differences in the potency of the non-peptide antagonist, RP 67,580 toward septide and SP may be visualized *in vivo*. We have also investigated whether there are differences in the mechanism of action of SP and septide.

Methods

Plasma protein extravasation

Male Wistar rats (250–290 g) were anaesthetized with sodium pentobarbitone (45 mg kg^{-1}) and the dorsal skin shaved. Following this Evans blue (10 mg kg^{-1}) was injected i.v. in the rat tail vein and 5 min later various tachykinins were administered intradermally each in a volume of 0.1 ml. A maximum of 6 different solutions were administered, each in duplicate. Five different tachykinins were injected into any one rat, the 6th solution being saline control. After a period of 30 min a blood sample was taken by intracardiac puncture and the animal killed. Each skin site was then punched out with a 17 mm diameter hole punch. The Evans Blue content of both plasma and skin samples were measured. To do this each skin sample was kept in formamide (4 ml) for 24 h at 60°C. After this time the amount of Evans blue in each site was determined by measurement of absorbence with a microplate reader (Biokinetics, E23 12E, Biotek Instruments) at a wavelength of 620 nm. The amount of Evans Blue contained in 1 µl of plasma was also determined.

Dose-response curves to SP, [Sar^9]SP sulphone, septide, NKA and NKB were constructed. The effect of 0.1 and 1 nmol doses of both [βAla^8]NKA(4-10) and senktide were also determined. In any one animal, dose-response curves were constructed for only one tachykinin with each animal representing one value of n for each dose tested. From these dose-response curves a submaximal dose of each agent was selected and used in all further experiments.

To determine the effect of the NK_1 antagonist, RP 67,580, upon responses to each of the above-mentioned tachykinins 0.3–3 µmol kg^{-1} of this drug was administered i.v. 5 min prior to application of intradermal agents. The inactive enantiomer RP 68,651 (3 µmol kg^{-1}, i.v.) was also tested to verify the selectivity of activity of RP 67,580. Involvement of NK_2 receptor activation in the oedema responses to the agonists was investigated by use of the selective antagonist, SR 48,968 (0.3 µmol kg^{-1}) given 5 min prior to application of i.d. stimuli. To ascertain the possible involvement of nitric oxide (NO) in the responses to the tachykinins the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) (0.1 µmol) was coinjected into the skin with the tachykinin. The effect of mast cell depletion upon the responses to chosen tachykinins was determined by comparing the oedema responses produced in animals treated with compound 48/80 (5 mg kg^{-1} on 3 consecutive days prior to use) to those in vehicle-treated controls.

Data and statistical analysis

Oedema is expressed in µl of plasma and statistical differences were calculated from the absolute values. Responses to tachykinins in drug-treated animals are expressed as % of the responses to these same agents in control vehicle-treated animals carried out at the same time. If drug treatment had no effect upon the responses to saline then values of tachykinin-induced extravasation used for statistical analysis were net values minus the response to saline; otherwise the values for saline are shown in the text. Statistical differences were calculated by the Newman-Keuls test where multiple comparisons were concerned and the Student's unpaired t test in all other instances.

Materials

[Sar^9]SP sulphone, senktide and NKB were all purchased from Peninsula (St Helens, England). Evans blue, sodium pentobarbitone, L-NAME and compound 48/80 were purchased from Sigma Chemical Co. (St. Louis, U.S.A.) and formamide from Merck (Dortmund, Germany). SP, NKA and [βAla^8]NKA(4-10) were all synthesized in the Chemistry Department of Menarini. RP 67,580 ((3aR, 7aR)-7, 7-diphenyl-2-[1-immuno-2-(2-methoxyphenyl)ethyl] po-hydroisoindol-4-one) was a generous gift from Dr C. Garret, Rhone Poulenc, Vitry, France and SR 48,968 ((S)-N-methyl-N-[4-(4-acetylamino-4-phenyl piperidino-z-(3,4-dichloro-phenyl)-butyl] benzamide), a kind gift from Dr X. Emonds-Alt, Sanofi Recherche, Montpellier, France. All solutions were made up freshly in sterile saline on the day of use.

Results

In agreement with other studies we have demonstrated that septide is approximately 10 times more potent than SP in inducing oedema formation in the skin (Devor *et al.*, 1989), as also was the analogue [Sar^9]SP sulphone (Figure 1). Both NKA and NKB were approximately equipotent with SP as can be seen in Figure 1. From these curves equiactive doses of each tachykinin were chosen corresponding to an approximate EC_{50} of 0.2 nmol for SP and used in the ensuing studies. Selective agonists for both NK_2 and NK_3 receptors were tested by use of [βAla^8]NKA(4-10) and senktide, respectively, both at doses of 0.1 and 1 nmol. Senktide (0.1 and 1 nmol) had no significant oedema-inducing actions above that achieved with saline ($n=6$ in each case). [βAla^8]NKA(4-10) had no effect at 0.1 nmol; at 1 nmol it induced an oedema of 23.5 ± 6.6 µl above saline ($n=6$) but this effect did not reach statistical significance.

RP 67,580 (1–3 µmol kg^{-1}) caused a dose-related inhibition of the responses to all of the natural tachykinins (Figure 2). RP 67,580 (1 µmol kg^{-1}) significantly inhibited by approximately 66% only the response to septide (0.002 nmol) ($n=6$) in comparison to the control vehicle-treated animals ($n=7$). At 3 µmol kg^{-1} the responses to both SP (0.2 nmol) ($n=5$) and

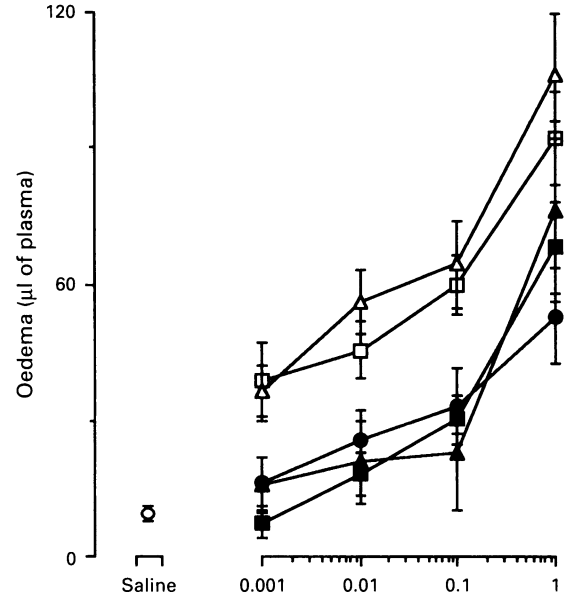

Figure 1 Dose-related induction of oedema formation in rat skin by a range of different tachykinins. Oedema responses to substance P (SP) are shown by (■) ($n=6$ for each point); [Sar^9]SP sulphone (△) ($n=5$); septide (□) ($n=6$), neurokinin A (●) ($n=6$) and neurokinin B (▲) ($n=5$). The effect of the vehicle alone, saline, is shown (○) ($n=28$). Each value is expressed as mean±s.e.mean.

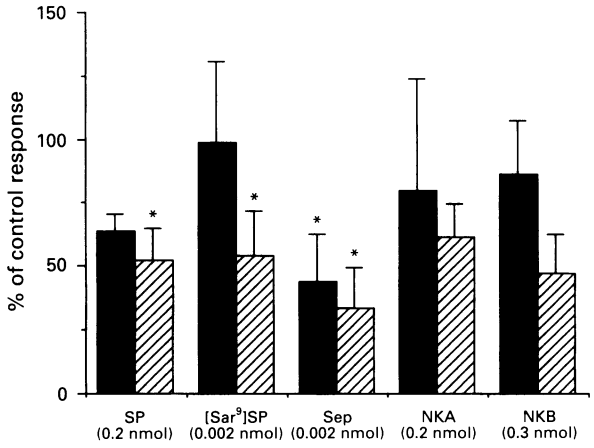

Figure 2 Effect of tachykinin NK_1 receptor blockade with the non-peptide selective antagonist, RP 67,580 (1–3 µmol kg^{-1}) upon oedema formation induced by a range of tachykinin agonists. The effect of RP 67,580 (1 µmol kg^{-1}) is shown by the solid column and the hatched columns represent the responses in animals treated with 3 µmol kg^{-1}. The response to each agent is shown as a percentage of the response achieved in control vehicle-treated animals for substance P (SP), [Sar^9]SP sulphone ([Sar^9]SP), septide (Sep), neurokinin A (NKA) and neurokinin B (NKB).

[Sar^9]SP sulphone (0.002 nmol) ($n=5$) as well as septide (0.002 nmol) ($n=5$) were significantly inhibited in comparison to control responses (Figure 2). The response to saline in animals treated with RP 67,580 (1 µmol kg^{-1}) was 5.0±3.1 µl ($n=7$) with 16.3±3.4 µl ($n=6$) in corresponding vehicle controls whereas in RP 67,580 (3 µmol kg^{-1}) treated animals the response was 11.5±4.5 µl ($n=5$) *vs* 16.9±4.0 µl ($n=5$) in vehicle controls. The inactive enantiomer, RP 67,581 (3 µmol kg^{-1}) had no significant effects upon the oedema induced by any of the tachykinins used in this study (results not shown).

The selective NK_2 receptor blocker, SR 48,986 (0.3 µmol kg^{-1}) had no significant effects upon the oedema responses to any of the tachykinins tested ($n=4$). The responses to SP (0.2 nmol), [Sar^9]SP sulphone (0.002 nmol), septide (0.002 nmol), NKA (0.2 nmol) and NKB (0.3 nmol) in drug and vehicle-treated animals were 58.9±10.0 and 45.1±9.8, 54.7±10.7 and 40.6±9.4, 52.9±5.7 and 51.9±14.4, 65.1±20.4 and 39.3±9.0, 59.8±21.4 and 49.6±9.2 µl of oedema, respectively. The response to saline was unaffected by this treatment.

Figure 3 shows the effect of L-NAME (0.1 µmol per site) upon the oedema responses to equipotent doses of SP and septide. Inhibition of NO synthase resulted in significant attenuation of the response to septide (0.1 nmol, $n=6$) producing only an approximate 56% inhibition of the response. However L-NAME did not significantly lower the responses to either doses of SP of 0.2 nmol ($n=11$) or 1 nmol ($n=6$). L-NAME had no effect on the response to saline; 19.5±3.4 and 20.6±3.5 µl in the presence and absence of L-NAME, respectively ($n=17$ in each case).

Since differences appeared to be present between the mechanism of the response to SP at different doses of this tachykinin, we compared the effect of RP 67,580 (0.3–3.0 µmol kg^{-1}) upon SP 1.0 and 0.2 nmol ($n=6$ in each case). Figure 4 shows that while the responses to septide (at both 0.002 and 0.1 nmol, $n=6$ in each case) were inhibited significantly by RP 67,580 at all of the doses tested only the response to SP (0.2 nmol) was significantly attenuated ($P<0.05$) by this drug treatment with the response to SP (1 nmol) being unaffected.

Compound 48/80 pretreatment had no significant inhibitory effects upon the responses to SP (0.2 nmol), septide (0.002 nmol) or NKB (0.3 nmol) ($n=6$ for each value) as shown in Figure 5. However, the responses to both SP (1 nmol) and [Sar^9]SP sulphone (0.002 nmol) were significantly attenuated ($P<0.05$, $n=6$ in each case). Figure 5 also shows that the response to saline was slightly but not significantly reduced.

Discussion

Recent studies have demonstrated the possible existence of a tachykinin septide-sensitive receptor or binding site with which the NK_1 receptor antagonists may interact. Several studies

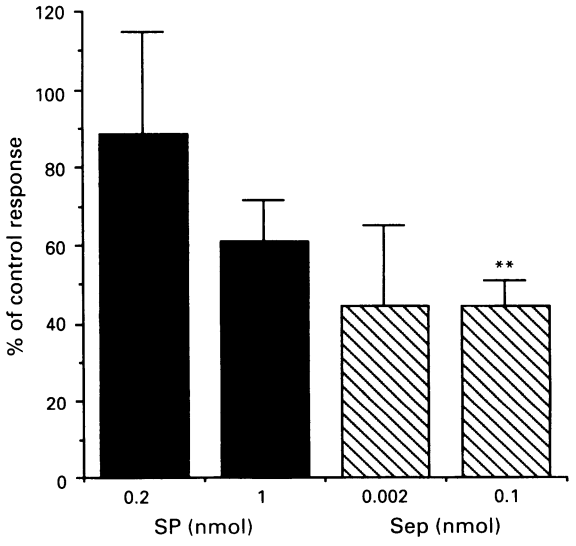

Figure 3 The effect of inhibition of NO synthase upon oedema induced by substance P (SP) and septide (Sep). NO-synthase inhibition was achieved by co-administration of the inhibitor L-NAME (0.1 µmol per site) with each tachykinin. Two doses of SP were tested 0.2 ($n=11$) and 1.0 nmol per site ($n=6$); equipotent doses of septide of 0.002 and 0.1 nmol respectively were also tested. The responses to each agent in combination with L-NAME are expressed as a percentage of the response in the absence of the enzyme inhibitor. Values shown are means±s.e.mean.

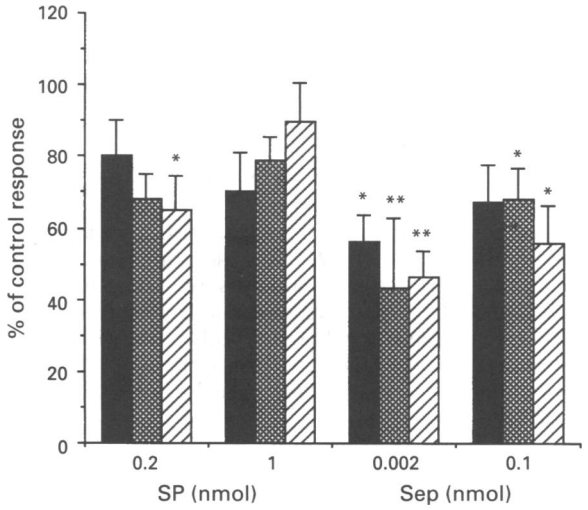

Figure 4 Comparison of the effect of RP 67,580 (0.3–3 μmol kg^{-1}) upon the oedema responses to different doses of the tachykinin NK_1 receptor agonists substance P (SP) and septide (Sep). The effect of 0.3 μmol kg^{-1} is shown by solid columns; 1 μmol kg^{-1} by cross-hatched columns and 3 μmol kg^{-1} by the hatched columns. Values shown are means ± s.e.mean of $n=5$, 6-8 and 6 respectively. Statistical significance was established with Student's t test for unpaired data and is shown by $^*P<0.05$ and $^{**}P<0.01$.

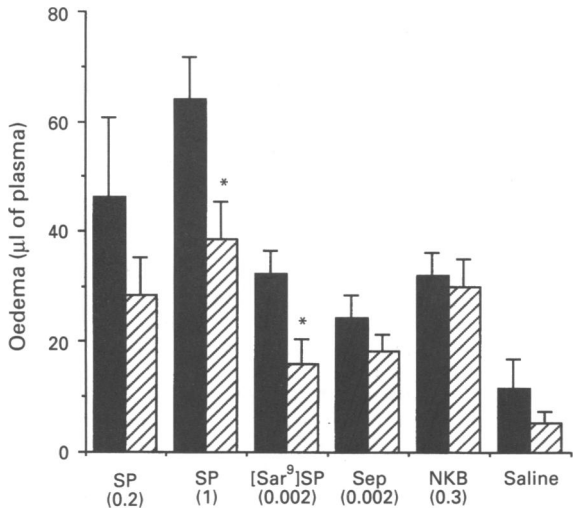

Figure 5 Effect of compound 48/80 pretreatment (5 mg kg^{-1}, once daily for 3 consecutive days) upon oedema induced by substance P (SP), [Sar^9]SP sulphone ([Sar^9]SP), septide (Sep), neurokinin A (NKA) and neurokinin B (NKB) and saline. All values shown are means ± s.e.mean $n=6$. Statistical significance was evaluated with Student's t test for unpaired data and shown as $^*P<0.05$.

have shown an apparent greater potency of the non-peptide antagonists in inhibiting responses to septide than to those produced by SP. We wished to assess whether tachykinins produce similar or different profiles of activity in skin oedema and whether the mechanisms of SP- and septide-induced oedema are similar.

SP, NKA and NKB all showed the same degree of potency in their oedema-inducing capacity in this study. Other studies have shown either an order of potency of SP > NKA = NKB (Andrews *et al.*, 1989) or, as was shown both by Gamse & Saria (1985) and by Couture & Kerouac (1987) NKB > SP > NKA. These differences can be attributed to experimental differences such as the supplier of animals. From our dose-response curves, approximately equipotent doses of each tachykinin were chosen for all the subsequent studies. In agreement with a previous study (Devor *et al.*, 1989) septide was found to be 10–100 times more potent than SP, with [Sar^9]SP sulphone showing approximately equal potency to septide. The oedema-inducing capacity of higher doses of these agonists could not be ascertained since pronounced systemic effects were observed with 10 nmol, such as intense blueing in both paws and ears and, in some cases, death. The lack of effect of senktide, indicates, in agreement with Devor *et al.* (1989), that activation of NK_3 receptors is not associated with plasma extravasation in the skin. Similarly, the NK_2 receptor agonist, [βAla^8]NKA(4-10), was ineffective indicating little role for the NK_2 receptor in tachykinin-induced oedema formation in rat skin. This was further supported by the fact that the non-peptide NK_2 receptor antagonist SR 48,968 had no significant effect upon the oedema response produced by NKA, the natural agonist at NK_2 receptors as it did not affect the responses to any of the tachykinins tested. In all, our data support the conclusion that plasma extravasation, in rat skin, is produced only via activation of NK_1 receptors.

Figure 2 very clearly shows the apparently preferential effect of the selective non-peptide NK_1 receptor antagonist, RP 67,580, upon the oedema responses produced by septide in comparison to either SP or the selective NK_1 agonist [Sar^9]SP sulphone. These findings suggest that RP 67,580 may be blocking a subtype of the NK_1 receptor or possibly a specific binding site at which septide acts preferentially. The possibility that this difference may arise from differential susceptibility to degradation of SP and septide seems unlikely since, the stable SP-analogue, [Sar^9]SP sulphone, was affected by RP 67,580 in a similar manner to SP, although displaying a potency very similar to that of septide. Therefore the activity of RP 67,580, in this system, is not simply being limited by the half life of the agonists.

It has been proposed that the vasodilator property of SP is dependent upon an intact endothelium and more specifically upon NO production both *in vitro* (Rees *et al.*, 1989) and *in vivo* (Whittle *et al.*, 1990). More recently it has also been shown that the oedema-inducing activity of SP in the rat skin was attenuated by NO synthase inhibition (Hughes *et al.*, 1990), implicating NO in SP-induced plasma protein extravasation. In contrast, a separate study failed to show any effect of N^G-nitro-L-arginine, another NO synthase inhibitor, upon plasma extravasation induced by either SP or [Sar^9]SP sulphone in the rat trachea, ureter or urinary bladder (Santicioli *et al.*, 1993). In the present study L-NAME (0.1 μmol) had no significant effect upon the response to SP (0.2 nmol) and although the higher dose of SP was dampened by NO synthase inhibition, this suppression fell just short of significance. The dose of the NO synthase inhibitor used in this study has previously been shown to be the maximum effective dose, inhibiting the NO-dependent inflammation induced by various other stimuli in rat skin (Ialenti *et al.*, 1992; Hughes *et al.*, 1992). The inhibitory effect of this dose of L-NAME was also reversed by the addition of L-arginine, indicating that the effect seen was specifically due to the inhibition of NO synthase. This dose had no effect on saline-induced oedema responses in our studies in agreement with Ialenti *et al.* (1992); higher doses were not used as it has been demonstrated that higher concentrations of L-NAME have an oedema-inducing capacity in the skin *per se* (Paul *et al.*, 1994). The responses to septide were attenuated by L-NAME with an approximate 43% inhibition of the response to 0.002 nmol and 57% inhibition of the response to 0.1 nmol. These results suggest that NO appears to be involved in the response to septide to a greater extent than SP, most importantly at equiactive doses of these agonists known to be acting at receptors sensitive to RP 67,580, highlighting and possibly supporting the suggested differences in receptor activation and hence different ensuing transduction mechanisms.

We also found that whilst the lower dose of SP was unaffected by pretreatment with compound 48/80 there was a significant inhibition of the extravasation response to 1 nmol SP, implying that SP activates mast cells at high doses whereas lower doses may activate only NK_1 receptors. Inhibition of the response to 1 nmol SP by compound 48/80 may explain the

inhibitory effects of L-NAME at this dose since it has been suggested that a mast-cell-derived NO may exist (Salvemini *et al.*, 1990). The lack of effect of compound 48/80 upon either septide or NKB indicates no mast cell component in these responses in agreement with the concept that mast cell degranulation induced by SP involves the basic N-terminal domain of the SP molecule which is not shared by septide. The dosing regime with compound 48/80 in rats has previously been shown to be an effective treatment for the chronic degranulation of mast cells (Saria *et al.*, 1984). Since neither the response to SP (0.2 nmol) nor septide (0.002) was affected by treatment with compound 48/80 we can fairly confidently presume that both tachykinins are acting through their respective receptor/binding site. This being the case, the differences in the effect of RP 67,580 and L-NAME upon these agonists support the postulate that SP and septide act at distinct receptor subtypes/binding site. It is important to note that SP at different doses employs different mechanisms to produce an effect; thus care should be taken when interpreting results with only one dose of this tachykinin and that comparison of SP to other tachykinins should only be carried out at doses when the tachykinin is known to be acting through the NK_1 receptor.

In conclusion the present findings indicate that a 'septide-sensitive' mechanism operates in determining plasma protein extravasation in rat skin with characteristics similar to those observed in other systems. Whether these differences reflect the existence of NK_1 receptor subtypes or a 'septide-sensitive' binding site on the NK_1 receptor cannot be decided at present. Clearly the responses to SP and septide differ with respect to mechanisms involved and especially intriguing is the greater contribution of non-mast cell-derived NO in the response to septide *vs* SP.

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