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# Sensory neuropeptide effects in human skin

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**1** Neuropeptides released from sensory nerves may account for cutaneous flare and wheal following local trauma. In 28 normal subjects we have studied the effects of four sensory neuropeptides given by intradermal injection on the forearm or back.

**2** All peptides caused a flare distant from the site of injection, presumably due to an axon reflex. Substance P (SP) was the most potent (geometric mean dose causing 50% of maximum flare, 4.2 pmol). Neurokinin A (NKA) was the next most potent with neurokinin B (NKB) and calcitonin gene-related peptide (CGRP) the least. The distant flare response to SP, NKA and NKB was maximal at 5 min and disappeared within 2 h.

**3** CGRP caused a local erythema over the site of injection at doses above 0.5 pmol which at higher doses lasted for up to 12 h.

**4** SP, NKA and NKB caused wheals at doses above 5 pmol with SP and NKB being the most potent. CGRP (up to 250 pmol) did not consistently cause wheal formation. There was no significant effect of coinjection of CGRP upon the response to SP although there was a tendency for an enhancement of the wheal response.

**5** The $H_1$-histamine antagonist terfenadine (60 mg orally) significantly inhibited the wheal and distant flare response to histamine (5 nmol) and NKA, but not that caused by NKB. The distant flare of CGRP was also reduced but the local erythema was unaltered.

**6** Aspirin (600 mg orally) significantly inhibited the distant flare response to SP, NKA and CGRP, but not that caused by NKB or histamine; the local erythema induced by CGRP was unaffected by aspirin. Aspirin also inhibited the wheal formed by NKA but not the wheal induced by the other substances.

**7** These results suggest that tachykinins cause a distant flare response partially via the release of histamine and cyclo-oxygenase products, but cause a wheal by a direct effect on the skin microvasculature. The order of potency SP > NKB > NKA suggests that an $SP_p$ or $NK_1$ receptor is involved in the wheal response. CGRP by contrast has a direct vasodilator effect which is very prolonged.

## Introduction

The cutaneous response to trauma and exposure to noxious stimuli involves local wheal formation due to alteration of vascular permeability and vasodilatation at the site of damage and a flare spreading from the site of injury (Lewis, 1927). The widespread flare and part of the wheal response are dependent on intact sensory nerves (Jancso *et al.*, 1968) and probably release of sensory neurotransmitters by an axon reflex (Chahl, 1979; Lembeck & Holzer, 1979; Gazelius & Olgart, 1980; Brodin *et al.*, 1981; Anand *et al.*, 1983; Lundberg *et al.*, 1983; Kenins *et al.*, 1984; Barlas *et al.*, 1985; Pernow, 1985). However, the identity of the transmitters which when released from the sensory nerves lead to these responses is not yet certain (Pernow, 1985). Both substance P (SP) (Eklund *et al.*, 1977; Samnegard *et al.*, 1978; Fuller *et al.*, 1987) and calcitonin gene-related peptide (CGRP) (Brain *et al.*, 1985), which are potent vasodilator substances, have been demonstrated in cutaneous sensory nerves (Dalsgaard *et al.*, 1983; Rosenfeld *et al.*, 1983), and, indeed, probably co-exist in the same nerves (Lundberg *et al.*, 1985) and may be released together (Brain & Williams, 1985). Tachykinins, with a similar structure to SP, neurokin-

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ins A (NKA) (Pernow, 1985) and B (NKB) (Laufer *et al.*, 1985) have been discovered in the mammalian nervous system and may also be present in cutaneous sensory nerves, so that their release may also contribute to the wheal and distant flare response (Pernow, 1985). SP has been shown to produce a wheal and distant flare response via the release of histamine from skin mast cells (Hägermark *et al.*, Fewtrell *et al.*, 1982; Foreman *et al.*, 1983; Saria *et al.*, 1983; Barnes *et al.*, 1986; Devillier *et al.*, 1986) and cause vasodilatation through release of cyclo-oxygenase products (Saria *et al.*, 1983). It is not yet certain whether all the skin sensory neuropeptides act similarly via the secondary release of vasoactive substances, such as histamine or cyclo-oxygenase products.

We have conducted a study comparing the potency of SP, CGRP, NKA and NKB in producing cutaneous effects. We monitored three cutaneous responses: first erythema at the site of injection (local erythema), second, flare spreading distal from the site of injection (distant flare) and finally wheal. We have also investigated possible interaction between the sensory neuropeptides and the mechanism by which these responses may occur by pretreating subjects with the non-sedative $H_1$-histamine receptor antagonist terfenadine or the cyclo-oxygenase inhibitor aspirin.

Parts of this work have been presented to the Physiological Society in December 1985 and the British Pharmacological Society in December 1986.

## Methods

### *Subjects*

Twenty eight subjects (7 female) aged 22 to 40 years, participated in the study; 11 (3 female) of the subjects were atopic. All subjects gave informed consent and the study was approved by Hammersmith Hospital Ethics Committee. Except for 3 female subjects who were taking oral contraceptives, all of the subjects were drug-free for at least 48 h before study and in particular were not taking non-steroidal anti-inflammatory agents or antihistamines.

### *Measurements*

Distant flare and local erythema area was measured by placing a cellophane sheet over the injection site and tracing around the edge of the flare with a felt tip pen. The wheal area was measured by drawing around the perimeter of the wheal with a ballpoint pen. Clear adhesive tape was then placed over the wheal and removed to keep a permanent record of wheal size. Both areas were calculated by using a computer assisted digitizing pad (Hewlett Packard, California, USA).

### *Protocol*

All doses of peptide and controls were given in a volume of 50 µl intradermally into the back or forearm with a 27 gauge short bevelled needle.

### *Dose-response studies*

Local erythema and distant flare was measured at 5 and 15 min and wheal at 10 min following intradermal injections of SP and CGRP at doses between 0.05 pmol and 0.5 nmol, NKA at doses from 2.5 pmol to 1 nmol and NKB from 5 pmol to 0.5 nmol. Full dose-response curves to all the peptides were carried out in the same 6 subjects. Ten subjects had simultaneous injections (in different sites on the back) of the same doses of SP and CGRP from 5 pmol to 0.5 nmol and 6 subjects had simultaneous injections of either NKA or NKB with equal doses of SP (5 pmol–0.5 nmol). Responses were monitored for up to 24 h.

### *Interaction studies*

In eight subjects, flare was measured at 5, 15 and 30 min and wheal at 10 min following the combined injection of either control, 5 or 50 pmol SP with either control, 5 or 50 pmol CGRP. Each subject received each combination of doses on the same study day.

### *Inhibitor studies*

Three groups of six subjects were studied on two or three occasions 50 min after orally ingesting either placebo, or aspirin (600 mg); and 3 h after taking terfenadine (60 mg) or placebo in a double-blind randomized manner. On each occasion subjects received an injection in the volar aspect of both forearms of CGRP (0.25 nmol), NKA (1 nmol), NKB (1 nmol) and histamine (5 nmol). SP (0.25 nmol) was studied after 600 mg aspirin and placebo alone, since its effects with terfenadine had been reported in a previous study (Barnes *et al.*, 1986).

### *Analysis of data*

Wheal and flare areas were compared by analysis of variance and by Student's $t$ test, with a Bonferroni correction to allow for multiple comparison (Wallenstein *et al.*, 1980). The data from the interaction study between SP and CGRP were further analysed by multifactor analysis of variance using the GLIM programme.

### *Materials*

SP (Sigma, Poole, UK) and NKA (Cambridge Research Chemicals, Cambridge UK) were dissolved

in 0.1 M acetic acid to give stock solutions of $10^{-3}$ M. CGRP (Bachem, UK) was dissolved in 20% heat-treated human serum albumin (HSA) and lyophilized in 5 μg aliquots. NKB (Cambridge Research Chemicals, Cambridge, UK) was dissolved in dimethylsulphoxide (DMSO) (BDH Chemicals, Poole, UK) to give a stock solution of $10^{-3}$ M. Histamine acid phosphate (Antigen, Eire) 1 mg $ml^{-1}$ was used as stock solution. Control solutions were 0.2% acetic acid for SP and NKA, 1% HSA for CGRP and 0.2% DMSO for NKB. All were diluted in normal saline before injection on the study day. Terfenadine 60 mg (Merrell Dow, London, UK), aspirin 600 mg and lactose placebo were made up in identical capsules in Hammersmith Hospital Pharmacy.

## Results

### *Dose-response studies*

Comparative dose responses for the distant flare at 5 min and the wheal at 10 min induced by the 4 neuropeptides are shown in Figures 1 and 2 and summarized in Table 1. SP at doses above 0.5 pmol caused a distant flare which was greater than that

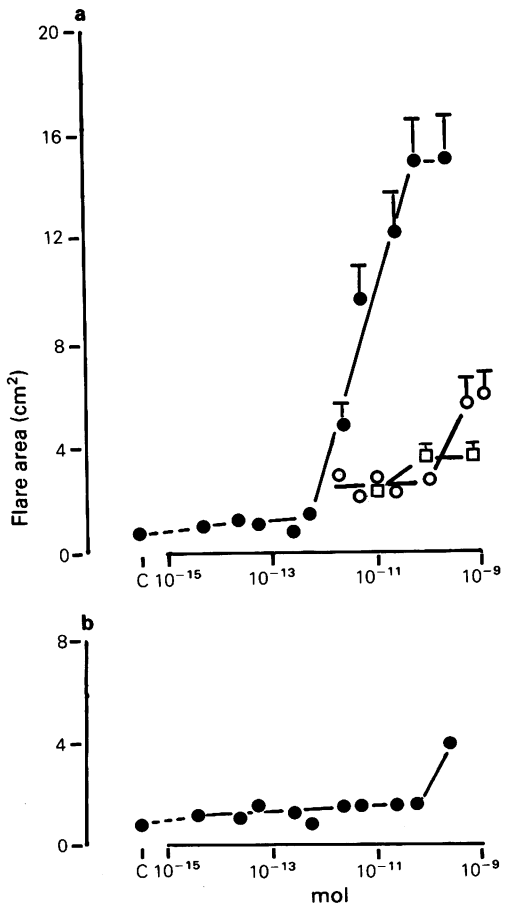


**Figure 1** Distant flare response to sensory neuropeptides in human skin: (a) shows the flare response to intradermal injections of substance P (●), neurokinin A (○) and neurokinin B (□); (b) shows the flare following intradermal injections of calcitonin gene-related peptide. All measurements were made at 5 min; the results are the mean from 10 subjects with s.e. shown by vertical lines.

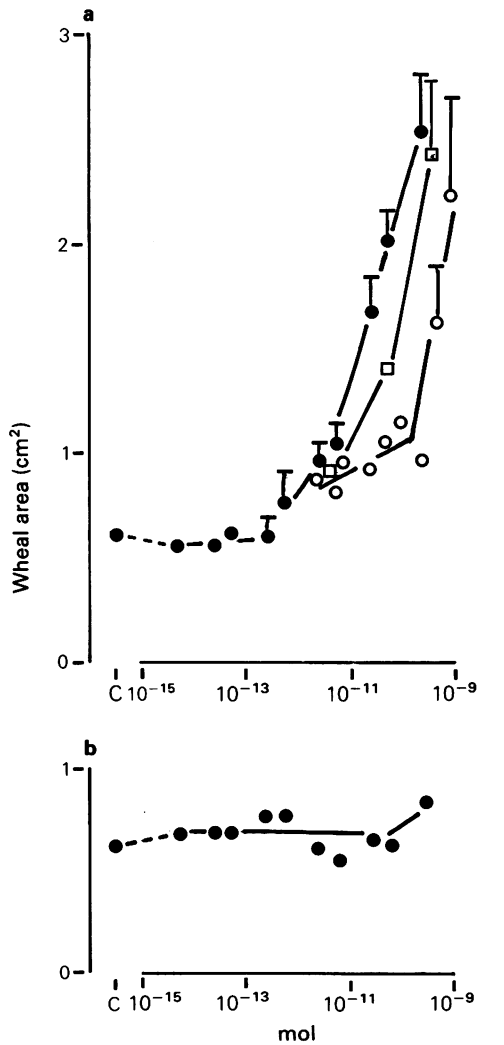


**Figure 2** Wheal response to sensory neuropeptides in human skin. (a) shows the wheal caused by intradermal injections of substance P (●), neurokinin A (○) and neurokinin B (□); (b) shows the wheal following intradermal injections of calcitonin gene-related peptide. All measurements were made at 10 min; the results are the mean from 10 subjects with s.e. shown by vertical lines.

**Table 1** A summary of the rank order of potency of sensory neuropeptides in human skin, and the effects of terfenadine (T) and aspirin (A)

| | | *Flare and erythema* | | | *Wheal* | |
|---|---|---|---|---|---|---|
| | | *A* | *T* | | *A* | *T* |
| Substance P | +++ | + | NT | ++ | 0 | NT |
| Neurokinin A | + | + | ++ | + | + | + |
| Neurokinin B | ± | 0 | 0 | ++ | 0 | 0 |
| Calcitonin gene-related peptide (distant flare) | + | ++ | ++ | 0 | 0 | 0 |
| Calcitonin gene-related peptide (local erythema) | ++ | 0 | 0 | 0 | 0 | 0 |

0 = no response; NT = not tested.

caused by the control injection. This distant flare was apparent 1 min after injection and reached maximum at 5 min. The response was maximal at 0.5 nmol with a geometric mean interpolated dose causing a 50% of the maximal response ($EC_{50}$) of 4.2 pmol (range 1.8–6.3 pmol, $n = 10$). Over a similar dose range SP produced a wheal greater than that caused by the control injection but this did not consistently reach a maximum at doses below 0.5 nmol. NKA was less potent than SP in causing a distant flare, this being observed at doses of 0.5 nmol and tending towards a plateau at doses above 1.0 nmol. NKA produced a consistent wheal in doses above 0.25 nmol but did not reach a maximum by 1 nmol. NKB was the least potent peptide at causing distant flare but was not significantly less potent than SP in causing a wheal. The distant flare response reached a plateau at 5 pmol, the maximum response being approximately only 20% of that achieved by SP. Doses above 5 pmol caused a wheal larger than that caused by the control and was of a similar potency to SP. CGRP had a similar potency to NKB and caused a distant flare but did not consistently produce a wheal area larger than the control value at any of the doses tested.

When the peptides were compared in the same individual, SP was significantly more potent in causing a distant flare. SP was also more potent than NKA and CGRP but not NKB at wheal formation.

The distant flare and wheal caused by injection of SP, NKA and NKB receded from their maximum at 5 and 10 min respectively and at the higher doses were measurable for up to 2 h for SP and NKA and 30 min for NKB.

CGRP at doses above 0.5 pmol produced a local erythema over the site of injection that could be observed at between 15 and 20 min after injection and at higher doses spread in a pseudopodal fashion from the site of injection, lasting for up to 12 h and had resolved by 24 h (Figure 3). The character of the local erythema caused by CGRP was different from the tachykinin-induced distant flare, being more intense and sapiginous.

*Interaction studies*

Figure 4a shows the distant flare produced by co-injection of SP and CGRP at 5 min. There was no significant difference in the distant flare elicited by SP in the presence of CGRP when compared to SP alone.

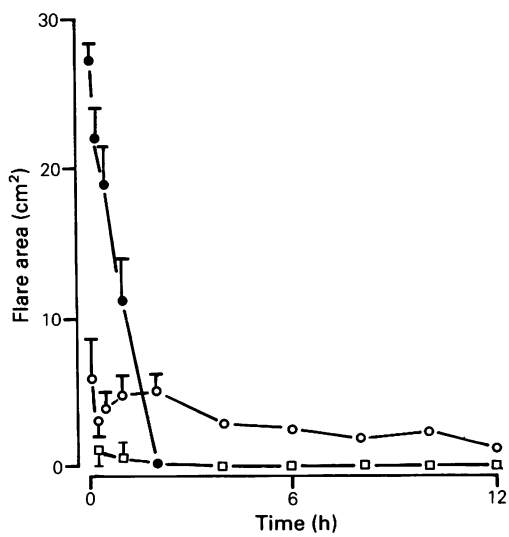


**Figure 3** Time-course of distant flare and local erythema response in 3 subjects following intradermal injection of substance P 0.25 nmol (●), calcitonin gene-related peptide 0.25 nmol (○) and control (□). The results are mean with s.e. shown by vertical lines.

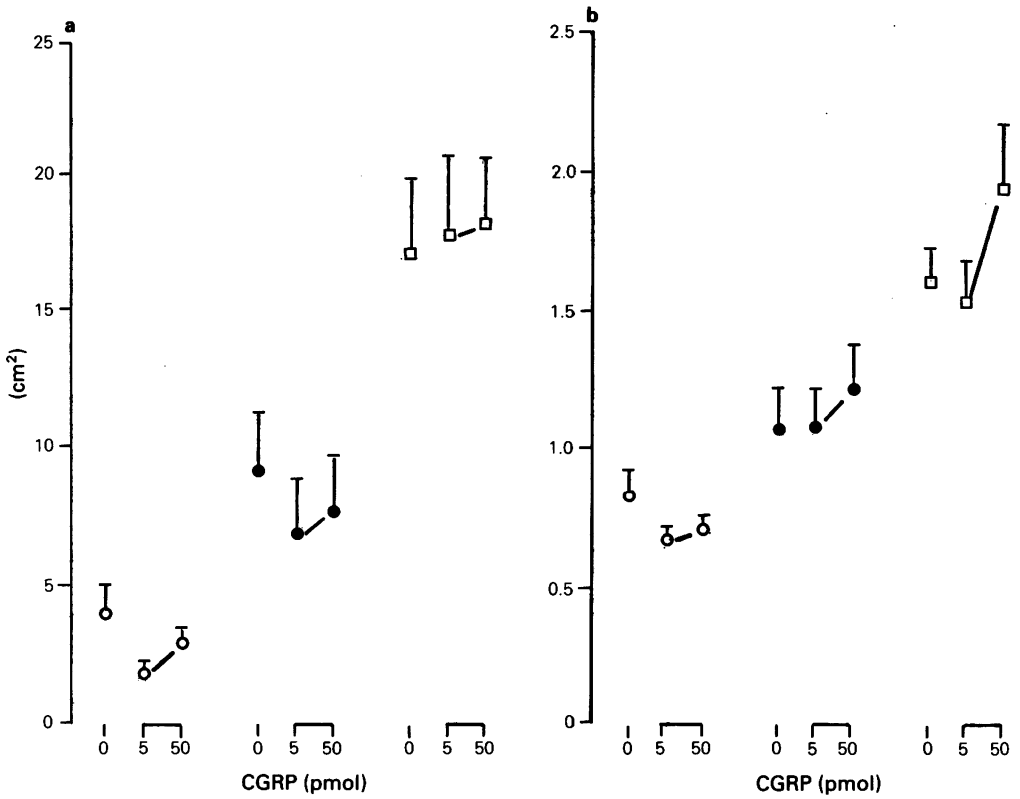


**Figure 4** Interaction between substance P and calcitonine gene-related peptide (CGRP). (a) Distant flare response 5 min after intradermal injections of mixtures of control (●), 5 pmol SP (○) and 50 pmol (□) injected simultaneously with calcitonin gene-related peptide. (b) Wheal area 10 min after intradermal injections of mixtures of control (●), substance P 5 pmol (○) and 50 pmol (□) simultaneously with calcitonin gene-related peptide. Means of 8 subjects with s.e. shown by vertical lines.

The local erythema produced by CGRP in the absence of substance P at 5 and 50 pmol CGRP was not observed when the peptide was co-injected with SP. Figure 4b shows that the 10 min wheal area measured following injection of 50 pmol CGRP combined with 50 pmol SP was 1.85 $cm^2$ compared with 1.2 $cm^2$ with 50 pmol SP alone. However, there was no significant interaction between the two peptides at any dose compared with SP alone when analysed by analysis of variance.

*Inhibitor studies*

The distant flare measured at 5 min (Figure 5a) following the injection of 5 nmol histamine was significantly ($P < 0.05$) inhibited by terfenadine (60 mg orally) but not aspirin (600 mg orally), distant flare area (mean ± s.d.), being 7.5 ± 2.1, 1.4 ± 1.0 and 8.4 ± 2.2 $cm^2$ after placebo, terfenadine and aspirin respectively. The distant flare following injection of 1 nmol NKA was significantly ($P < 0.05$) inhibited by both terfenadine and aspirin, mean distant flare area being 5.5 ± 3.0, 1.6 ± 0.6 and 3.4 ± 2.4 $cm^2$ on the three study days. The distant flare following the injection of 0.25 nmol CGRP was also significantly ($P < 0.05$) inhibited by both terfenadine and aspirin, mean distant flare area being 3.6 ± 4.0, 1.0 ± 0.6 and 0.8 ± 0.2 $cm^2$ on the three days respectively. The small distant flare following 1 nmol NKB was unaltered by either drug. Aspirin reduced the flare caused by 0.25 nmol SP from 11.9 ± 4.8 to 9.2 ± 4.1 $cm^2$ ($P < 0.05$). Local erythema 30 min following the injection of CGRP was not inhibited by either drug, the mean erythema area being 2.3 ± 1.3, 1.7 ± 1.4, 1.5 ± 1.3 $cm^2$ respectively on the three study days.

The wheal, 10 min (Figure 5b) after injection of

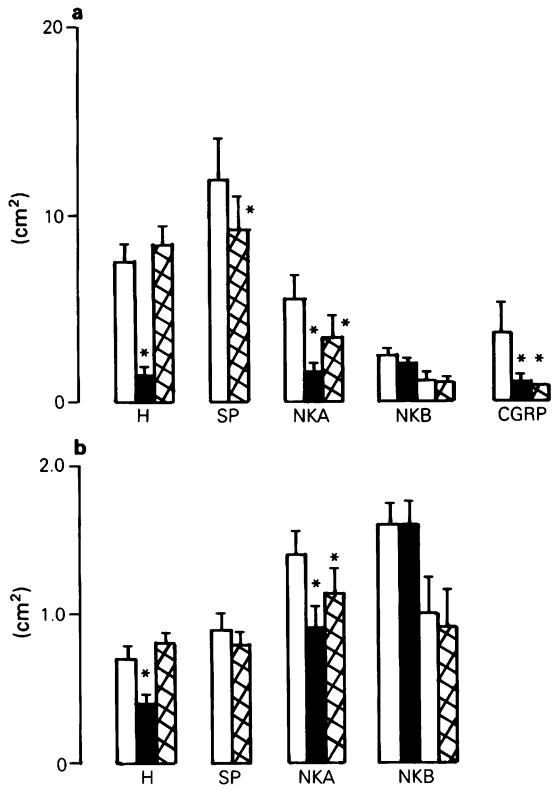


**Figure 5** (a) Distant flare area measured 5 min after intradermal injection of histamine 1 nmol (H), substance P 0.25 nmol (SP), neurokinin A 1 nmol (NKA), neurokinin B 1 nmol (NKB) and calcitonin gene-related peptide 0.25 nmol (CGRP) after treatment with placebo (open columns), terfenadine (60 mg) (closed columns) and aspirin (600 mg) (cross-hatched columns). (b) Wheal area measured 10 min after intradermal injection of H 5 nmol, SP 0.25 nmol, NKA 1 nmol and NKB 1 nmol. After treatment with placebo (open columns), terfenadine 60 mg (closed columns) and aspirin 600 mg (cross-hatched columns); 2 placebo columns for NKB are due to the studies being performed in different groups of subjects. Results are the mean of 6 subjects in each case; s.e.mean shown by vertical lines. *Signifies $P < 0.05$.

5 nmol histamine was significantly ($P < 0.05$) inhibited by terfenadine but not by aspirin, mean wheal area being $0.68 \pm 0.2$, $0.42 \pm 0.1$, $0.8 \pm 0.2$ cm$^2$ respectively for the three study days. The wheal following the injection of 1 nmol NKA was significantly ($P < 0.05$) inhibited by both terfenadine and aspirin, mean wheal area being $1.34 \pm 0.39$, $0.95 \pm 0.4$, $1.14 \pm 0.4$ cm$^2$ respectively on the three study days. Neither drug altered the wheal caused by 1 nmol NKB. Aspirin did not alter the wheal induced by 0.25 nmol SP. There was no effect by either drug on wheal area following injection of CGRP; however, this area was no different from that obtained from control injection in the same subjects.

## Discussion

This study shows that all 4 sensory neuropeptides were capable of eliciting a flare spreading distant from the site of injection, probably by stimulating an axon reflex. SP was most potent, being 100 times more potent than NKA, NKB and CGRP. However, the maximum response to NKB was less than that caused by the other neuropeptides. The neurogenic flare following injection of neuropeptide appears to be unrelated to the receptor specificity of the peptide. Foreman *et al.* (1983) have shown that this flare is due to the presence of one or more basic residues in the N-terminal end of the molecule. Flare was produced by SP, $SP_{1-9}$, substituted SP and eledoisin-related peptide but not $SP_{4-11}$, eledoisin or physalaemin. These results do not relate to the known response of tachykinin receptor which are activated by the C-terminal end of the peptide suggesting that flare may be the result of the basic nature of SP, rather than receptor-mediated mast cell degranulation. The inhibition of the flare of NKA and SP by terfenadine is consistent with the hypothesis that this flare is in part the result of histamine release from skin mast cells. The lower potency of NKA at causing histamine release is probably due to its lack of an N-terminal argenine making it a less basic molecule. The inhibition of flare by aspirin implies that a cyclo-oxygenase product is also involved. Several cyclo-oxygenase products may be potent vasodilators (Williams & Morley, 1973). SP is capable of releasing prostacyclin from endothelial cells *in vitro* (unpublished observations). Cyclo-oxygenase inhibitors will reduce the hypotensive effects of SP (Pals & Micalizzi, 1980) suggesting that this effect is dependent upon prostaglandin release. CGRP also caused distant flare response at high doses (250 pmol) which was inhibited by terfenadine and aspirin. The distant flare due to NKB is however independent of either histamine or cyclo-oxygenase products and may be due to direct sensory nerve stimulation by the peptide.

All three tachykinins tested were able to elicit wheal formation following intradermal injection. There was a different relative potency between the tachykinins in terms of wheal production when compared to distant flare production, since NKB and NKA were much more potent in wheal production, although SP remained the most potent. At least three sub-types of tachykinin receptor have been recognized, one which is preferentially stimulated by SP (SP-P or $NK_1$

receptor), a second by the peptide eledoisin and neurokinin A (SP-E on $NK_2$-receptors) and a third by neurokinin B (SP-N or NK3 receptors) (Laufer *et al.*, 1985). Our results would suggest that $NK_1$ receptors are responsible for the microvascular changes which lead to wheal formation, and are consistent with previous observations (Foreman *et al.*, 1983; Devillier *et al.*, 1986). The inhibition of NKA-induced wheal by terfenadine suggests that histamine release is involved, as in SP-induced flare (Barnes *et al.*, 1986). The inhibition of the NKA-wheal by aspirin indicates that cyclo-oxygenase products also contributed, although prostaglandins do not generally cause wheal formation in man without the presence of another inflammatory mediator (Williams & Morley, 1973). Neither histamine nor cyclo-oxygenase products are required for wheal formation by NKB and SP-induced wheal was likewise not inhibited by aspirin. CGRP, however, did not produce a wheal at the doses tested, which is consistent with previous reports (Brian *et al.*, 1986) although wheal has been reported by Piotrowski & Foreman (1986). As tachykinins and CGRP are co-localized in some sensory nerves (Lundberg *et al.*, 1983), their simultaneous release from the nerve may cause an additive response. Co-injection of CGRP and SP in the rat (Brain & Williams, 1985) results in a potentiation of skin oedema formation. However, we were unable to show a significant interaction between the two peptides in man in either wheal or distant flare response, although there was a tendency for a larger wheal on co-injection of two peptides.

As reported previously (Brain *et al.*, 1985), injections of low doses of CGRP can produce a local erythema at the site of injection. This erythema is slow in onset taking at least 15 min to occur but at higher doses spreads from the site of action in pseudopodal fashion, presumably due to lymphatic transport and persists for at least 12 h. The erythema was not seen with the other neuropeptides but is similar to that seen with high doses of prostaglandin $E_2$ (Crunkhorn & Willis, 1971). Unlike the distant flare, the local erythema induced by CGRP was not altered by pretreatment with either terfenadine or aspirin, suggesting that production of histamine or prostaglandins are not involved in the production of this flare. The prolonged erythema was, however, abolished by co-injection with SP at doses that caused wheal and flare and perhaps increases clearance of CGRP from the site of injection. The mechanism for this slow onset of a persistent erythema by a peptide that is rapidly cleared from the plasma is uncertain but probably due to a long lasting vascular dilatation.

There is now consistent evidence that release of a neurotransmitter from sensory nerves can be responsible for wheal and distant flare formation following trauma (Jancso *et al.*, 1968; Lembeck & Holzer, 1979; Gazelius & Olgart, 1980; Anand *et al.*, 1983; Lundberg *et al.*, 1983; Kenins *et al.*, 1984). This study suggests wheal formation following trauma may be due to release of a tachykinin, either SP, NKA or NKB rather than release of CGRP which does not lead to wheal formation. Distant flare formation following release of tachykinins would be dependent upon the secondary release of histamine and the formation of cyclo-oxygenase products, probably from dermal mast cells. However, other cell subtypes, such as endothelial cells (Crossman *et al.*, 1987), may be involved in the release of the cyclo-oxygenase products. Should the release of a neurotransmitter following trauma be involved in the genesis of antidromic sensory nerve stimulation and widespread vasodilatation leading to flare, then the present study would suggest that SP, which is the most potent neuropeptide at causing this effect, would be the most likely natural candidate. The effect of SP would be by a non-receptor mechanism leading to the release of histamine and cyclo-oxygenase products, either local to the site of trauma or distant in the area of flare. Previous work with the effect of terfenadine on capsaicin-induced flare would however, suggest that histamine is not released in the area of flare. These results are consistent with the role of neuropeptides in the genesis of cutaneous inflammation. However, further work with inhibitors is required to elucidate fully their role.

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