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Substance P-induced inflammatory responses in guinea-pig skin: The effect of specific NK_1 receptor antagonists and the role of endogenous mediators

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1 The sensory neuropeptide substance P (SP), when released from sensory nerves, has been implicated in the development of neurogenic inflammation. In the present study, using an *in vivo* model system, we have characterized and investigated the mechanisms underlying SP-induced leukocyte accumulation and oedema formation in the guinea-pig.

2 Intradermally injected SP (i.d., 10^{-13}–10^{-9} mol per site), induced a dose- and time-dependent accumulation of ^{111}In-neutrophils, ^{111}In-eosinophils and oedema formation as measured by the local accumulation of i.v. injected ^{125}I-albumin. The leukocyte accumulation evoked by SP was significant at 10^{-10} and 10^{-9} mol per site, whereas oedema formation was significant at the lowest dose tested (10^{-13} mol per site).

3 The NK_1 receptor antagonists, CP-96,345 (1 mg kg^{-1}, i.v.) and RP-67,580 (10 μg per site, i.d.), significantly attenuated the oedema formation induced by the lower doses of SP. Oedema formation and leukocyte accumulation induced by 10^{-9} mol per site SP were unaffected by either antagonist.

4 SP-elicited responses were not significantly affected by the platelet activating factor (PAF) receptor antagonist, UK-74,505 (2.5 mg kg^{-1}, i.v.) or the H_1 histamine receptor antagonist, chlorpheniramine (10^{-8} mol per site, i.d.). However, the ^{111}In-eosinophil accumulation, but not the ^{111}In-neutrophil accumulation or oedema formation, induced by SP was significantly inhibited by the specific 5-lipoxygenase (5-LO) inhibitor, ZM-230,487 (10^{-8} mol per site, i.d.).

5 The accumulation of both ^{111}In-neutrophils and ^{111}In-eosinophils induced by SP was abolished in guinea-pigs treated i.v. with an anti-CD18 monoclonal antibody 6.5E F(ab')$_2$ (2.5 mg kg^{-1}). The oedema response was unaffected in these animals.

6 These results suggest that SP-induced inflammatory events may be mediated via two mechanisms involving NK_1 receptor-dependent and independent pathways. Oedema formation induced by the lower doses of SP may be mediated via the direct activation of NK_1 receptors whilst, at higher doses, oedema formation and leukocyte accumulation may be mediated via the release of secondary mediators, possibly mast cell derived, with 5-LO products playing an important role in the leukocyte infiltration. The leukocyte accumulation, but not the oedema induced by SP, is dependent on the expression of the CD18 antigen on leukocytes.

Keywords: Substance P; eosinophils; neutrophils; oedema formation; inflammation; CP-96,345; RP-67,580; chlorpheniramine; UK-74,505; ZM-230,487

Introduction

Substance P (SP), an 11 amino acid neurotachykinin present in sensory nerve fibres, is believed to be a mediator of neurogenic inflammation (Lembeck & Holzer, 1979). This response, which can be induced by electrical or chemical stimulation of sensory neurones, involves a number of inflammatory events such as local vasodilatation, oedema formation and leukocyte accumulation. These components of the inflammatory response can be elicited by administration of SP. In man, SP causes a classic wheal and flare response (Hagermark *et al.*, 1978; Foreman & Jordan, 1983; Foreman *et al.* 1983) as well as eliciting granulocyte infiltration (Smith *et al.*, 1993) and modulating the activity of another neuropeptide calcitonin gene-related peptide (CGRP) (Brain & Williams, 1988). In addition, SP-induced oedema formation and leukocyte accumulation has been studied in a number of experimental animals (Matsuda *et al.*, 1989; Yano *et al.*, 1989; Lembeck *et al.*, 1992; Wilsoncroft *et al.*, 1994).

The inflammatory properties of SP appear to be primarily mediated via its interaction with the neurokinin$_1$ (NK_1) receptor (Guard & Watson, 1991) which is a member of the G-protein-coupled receptor superfamily (Hershey & Krause, 1990; Takeda *et al.*, 1991). The availability of specific, non-peptide, NK_1 receptor antagonists has allowed direct investigations into the role of endogenous SP in different inflammatory reactions. The NK_1 receptor antagonist, CP-96,345 (Snider *et al.*, 1991) has been shown to inhibit SP-induced plasma extravasation in guinea-pig dorsal skin (Nagahisa *et al.*, 1992) and neurogenic inflammation in rats (Lembeck *et al.*, 1992). Similarly, the NK_1 receptor antagonist, RP-67,580 (Garret *et al.*, 1991) inhibits oedema formation induced by SP and in response to a passive cutaneous anaphylaxis reaction in guinea-pig skin (Wilsoncroft *et al.*, 1994).

A number of mediators have been implicated in the inflammatory responses elicited by SP. In particular, it has been suggested that mast cell-derived mediators such as histamine, play an important role in the induction of SP-induced responses (Hagermark *et al.*, 1978; Foreman *et al.*, 1983; Foreman & Jordan, 1983). In addition, leukotriene B_4, (LTB_4) mediates in part the SP-induced leukocyte infiltration into mouse skin (Iwamoto *et al.*, 1993) and SP selectively induces the expression of messenger RNA for tumour necrosis factor-α (TNF-α) in a mast cell line (Ansel *et al.*, 1993).

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The effects of SP on cutaneous vascular permeability have previously been described in the guinea-pig and a role for NK_1 receptors identified (Iwamoto & Nadel, 1989; Wilsoncroft *et al.*, 1994). The aim of the present study was to extend these findings by addressing the role of NK_1 receptors in SP-induced neutrophil and eosinophil accumulation in guinea-pig skin. In addition, we have investigated the contributions of endogenously formed histamine, PAF and 5-lipoxygenase (5-LO) products in these reactions. Since these responses involve a close interaction between leukocytes and endothelial cells, we have also investigated the role of the leukocyte adhesion molecule, CD18.

Methods

Animals

Female ex-breeder Dunkin-Hartley guinea-pigs (800–900 g) and recipient guinea-pigs of either sex (300–400 g) were purchased from Harlen Olac, Bicester, Oxon, U.K.

Preparation of ^{111}In-labelled guinea-pig neutrophils and eosinophils

Guinea-pig peritoneal neutrophils and eosinophils were purified and radiolabelled with ^{111}In chelated to 2-mercaptopyridine-*N*-oxide as previously described (Faccioli *et al.*, 1991; Sanz *et al.*, 1994). Briefly, to elicit neutrophils, ex-breeder guinea-pigs were injected i.p. with 15 ml of 5% casein solution 18 h before they were killed. To elicit eosinophils, ex-breeder guinea-pigs were injected i.p. with 1 ml horse serum every second day, at least 5 times. The final injection was given 18 h before they were killed. Animals were killed by asphyxiation with CO_2 and the peritoneal cavity was lavaged with 30 ml of heparinized saline (10 u ml^{-1}). Both eosinophils and neutrophils were purified over discontinuous percoll gradients (1.080, 1.085, 1.090, 1.095, 1.100 g ml^{-1}). Cell populations were used only when the purity of the eosinophil preparation was above 95% and the purity of the neutrophil preparation was above 99%. The cells were then incubated with ^{111}In (100 μCi in 10 μl) chelated to 2-mercaptopyridine-*N*-oxide (40 μg in 0.1 ml of 50 mM PBS) for 15 min, washed twice in Ca^{2+}- and Mg^{2+}-free HBSS containing 10 mM HEPES and 10% platelet poor plasma (pH 7.3) and resuspended at a final cell concentration of 10^7 cells ml^{-1}.

Measurement of ^{111}In-leukocyte accumulation and oedema formation

Recipient guinea-pigs (300–400 g) were anaesthetized with 0.1 ml Hypnorm and their dorsal skin shaved. Either radiolabelled neutrophils or eosinophils (5×10^6) were mixed with ^{125}I-HSA (^{125}I-albumin, 5 μCi kg^{-1}) and injected i.v. via an ear vein in a volume of 0.5 ml. After 5 min, test agents were injected i.d. in volumes of 0.1 ml. At the end of the 2 h *in vivo* test period, animals were anaesthetized with i.p. sodium pentobarbitone and a 3 ml cardiac blood sample collected. The animals were then killed by an overdose of anaesthetic, the dorsal skin removed and the injection sites punched out. Skin, blood and plasma samples were counted in a gamma counter. Eosinophil and neutrophil accumulation was expressed as the number of labelled leukocytes per site and oedema formation, as measured by the local accumulation of ^{125}I-albumin, was expressed as μl of plasma per site (Faccioli *et al.*, 1991; Sanz *et al.*, 1994).

The kinetics of responses induced by SP were investigated using a cumulative time-course protocol. The animals were injected with ^{111}In-cells and ^{125}I-albumin as described above. Over a test period of 2 h, SP (10^{-9} mol per site) was injected i.d. at 2, 1, 0.5 and 0 h before the animals were killed. The inflammatory responses induced by i.d. SP (10^{-13}–10^{-9} mol per site) were investigated using 2 specific non-peptide NK_1 receptor antagonists, CP-96,345 (1 mg kg^{-1}, i.v.) and RP-67,580 (10 μg per site, equivalent to 2.0×10^{-8} mol per site, i.d.). In addition, the effects of the specific H_1 receptor antagonist, chlorpheniramine (10 μg per site, equivalent to 2.5×10^{-8} mol per site, i.d.), the PAF receptor antagonist, UK-74,505 (2.5 mg kg^{-1}, i.v.), the 5-lipoxygenase inhibitor, ZM-230,487 (10 μg per site, equivalent to 2.4×10^{-8} mol per site, i.d.) and the anti-CD18 monoclonal antibody 6.5E $F(ab')_2$ (2.5 mg kg^{-1}, i.v.) on the SP-induced responses were investigated.

Materials

Substance P, histamine, 2-mercaptopyridine-N-oxide, BSA were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Horse serum, sterile Hanks Balanced Salt Solution (HBSS, without Ca^{2+} and Mg^{2+} 10 ×) and HEPES (1 M) were obtained from Gibco Limited, Paisley, Renfrewshire, U.K. ^{125}I-human serum albumin (^{125}I-albumin, 20 mg ml^{-1} of sterile saline, 50 μCi ml^{-1}), ^{111}Indium chloride ($^{111}InCl_3$; 10 mCi ml^{-1} sterile pyrogen-free 0.04 N hydrochloric acid) were obtained from Amersham International, Amersham, Buckinghamshire, U.K. Percoll was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Chlorpheniramine (10 mg ml^{-1}) was obtained from Allen and Hanburys Ltd., London, U.K. LTB_4 was obtained from Cascade Biochem Ltd., Reading, Berkshire, U.K. PAF was obtained from Bachem Ltd., Saffron Waldon, Essex, U.K.

The following substances were kind gifts: RP-67,580 (2-[1-imino-2-(methoxy phenyl) ethyl]-7,7 diphenyl-4 perhydro-isoindolone (3aR, 7aR)) was from C. Garret, Rhone-Poulenc Rorer, Vitry, France. CP-96,345 (the dihydrochloride salt of (2S,3S)-*cis*-2-(diphenylmethyl)-*N*-((2-methoxyphenyl)methyl)-1-azabicyclo[2.2.2]octan-3-amine) and its 2R,3R enantiomer CP-96,344 were from Pfizer Incorporation, Groton, U.S.A. and UK-74,505 (4,-(2-chlorophenyl)-1,4-dihydro-3-ethoxy-carbonyl-6-methyl-2-[4-(2-methylimidazol[4,5-c]pyrid-1-yl) phenyl]-5-[N-(2-pyridylcarbamoyl] pyridine) was from Dr John Parry, Pfizer Central Research, Sandwich, U.K. Monoclonal antibody 6.5E $F(ab')_2$ was from Dr Martyn Robinson, Celltech, Slough, U.K. ZM-230,487 (1-ethyl-6-[fluoro-5-(4methoxy-3,4,5,6-tetrahydro-2H-pyran-4-yl)phenoxy] methyl-quinol-2-one) was from Dr Roger McMillan, Zeneca Pharmaceuticals, Macclesfield, Cheshire, U.K.

Data analysis

Results are expressed as the means ± s.e.mean for *n* animals where each datum unit is the average of responses in duplicate sites. Data were analysed by two way analysis of variance (ANOVA) of log transformed data and statistical significance determined with the Neuman-Keuls procedure for repeated comparisons (Snedecor & Cochran, 1967).

Results

Substance P-induced inflammatory responses in guinea-pig skin

The i.d. injection of SP caused a dose-dependent increase in ^{111}In-neutrophil (Figure 1a), ^{111}In-eosinophil (Figure 1b) and oedema formation (Figure 1c) above levels detected in saline injected sites in guinea-pig skin. Oedema formation was significant at 10^{-13} mol per site SP, the lowest dose tested, and increased in a dose-dependent manner. However, significant ^{111}In-neutrophil and ^{111}In-eosinophil accumulation occurred only at the top two doses of SP, namely 10^{-10} and 10^{-9} mol per site ($P < 0.05$).

Cumulative time-course experiments indicated that following the i.d. administration of SP, leukocyte accumulation

(Figure 2a,b) and oedema formation (Figure 2c) reached a maximal level within the first hour.

Effect of NK_1 receptor antagonists on SP-induced responses

The effects of two specific NK_1 receptor antagonists, CP-96,345 and RP-67,580, on the inflammatory responses elicited by SP were examined. Injection of CP-96,345 at 1 mg kg^{-1}, i.v. 30 min prior to injection of labelled cells and ^{125}I-albumin significantly attenuated the oedema formation induced by the lower doses of SP, (10^{-13}–10^{-10} mol per site), but had no significant effect on the response to the highest dose (10^{-9} mol per site; Figure 3a). This antagonist had no significant effect on ^{111}In-neutrophil or ^{111}In-eosinophil accumulation induced by SP; 10^{-9} mol per site SP induced 1404 ± 88 and 1720 ± 195 ^{111}In-neutrophils per site in control and CP-96,345-treated animals respectively, $n = 7$ animal pairs, and 10^{-9} mol per site SP induced 4246 ± 1112 and 5580 ± 1557 ^{111}In-eosinophils per site in control and CP-96,345-treated animals respectively, $n = 8$ animal pairs. SP, 10^{-10} mol per site, induced 1293 ± 269 and 837 ± 147 ^{111}In-neutrophils per site in control and CP-96,345-treated animals respectively, $n = 7$ animal pairs, and 1430 ± 200 and 1805 ± 264 ^{111}In-eosinophils per site in control and CP-96,345-treated animals respectively, $n = 8$ animal pairs. The inactive isomer of CP-96,345, CP-96,344, also given at 1 mg kg^{-1} i.v., had no significant effect on oedema formation (Figure 3a) or cell accumulation in response to SP (data not shown) at any of the doses tested.

Similarly, a second, structurally different NK_1 receptor antagonist, RP-67,580, this time when co-injected i.d.

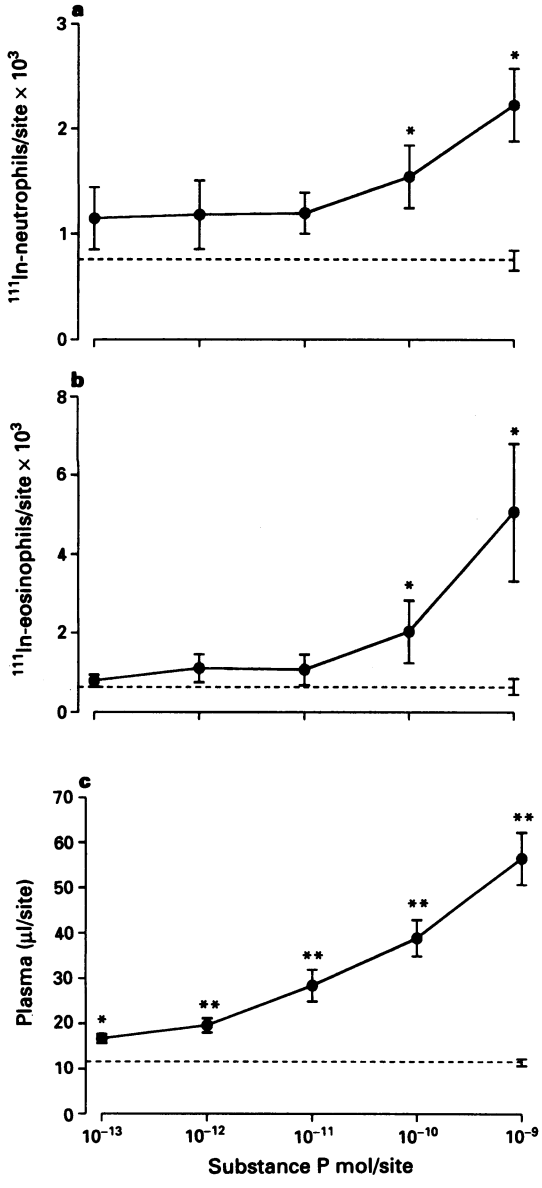

Figure 1 Dose-response relationship of substance P (SP)-induced (a) ^{111}In-neutrophil accumulation, (b) ^{111}In-eosinophil accumulation and (c) oedema formation in guinea-pig skin. Responses were measured over 2 h following i.d. SP (10^{-13}–10^{-9} mol per site, 100 µl per site). The dashed line represents levels detected in sites injected with saline/BSA (0.1% w/v). Results are expressed as the mean ± s.e.mean for $n = 6$ (for neutrophils) and $n = 8$ (for eosinophils) experiments. Asterisks indicate a significant difference from levels in sites injected with saline/BSA: $^*P < 0.05$; $^{**}P < 0.01$.

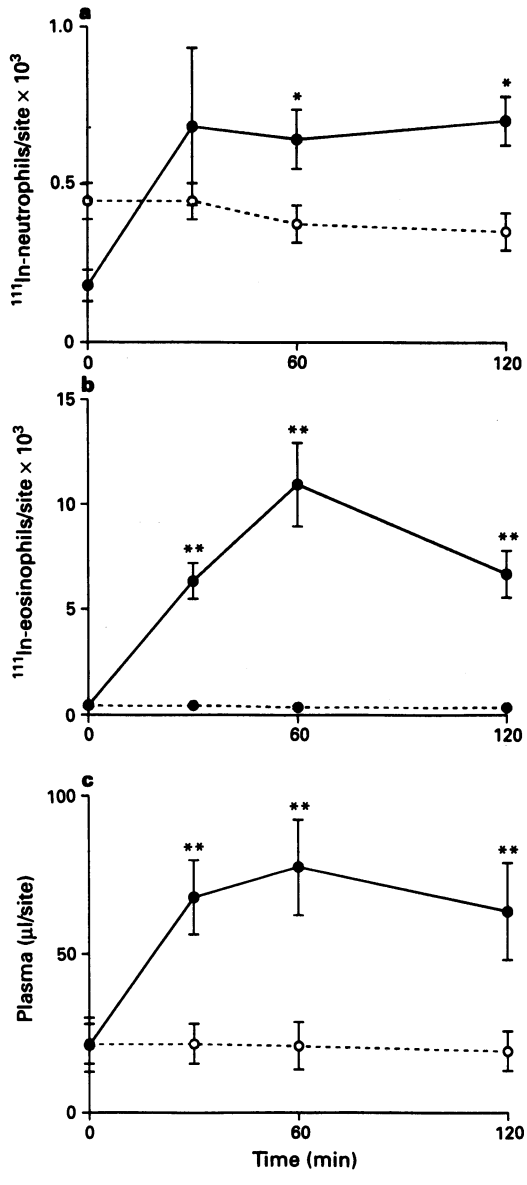

Figure 2 Time course of substance P (SP)-induced (a) ^{111}In-neutrophil accumulation, (b) ^{111}In-eosinophil accumulation and (c) oedema formation in guinea-pig skin. Labelled cells and ^{125}I-albumin were injected i.v. into recipient guinea-pigs and SP (10^{-9} mol per site) was injected i.d. (100 µl per site) at different time points after injection of cells as indicated on the axis. Closed symbols represent the responses to SP 10^{-9} mol per site while open symbols show the effect of saline/BSA injections. Results are the mean ± s.e.mean for $n = 6$ experiments. Asterisks indicate a significant difference from levels in sites injected with saline/BSA: $^*P < 0.05$, $^{**}P < 0.01$.

(2.5×10^{-8} mol per site) with SP, significantly suppressed the oedema formation induced by the lower doses of SP (10^{-13} and 10^{-11} mol per site, Figure 3b). As found with CP-96,345, RP-67,580 had no effect on SP-induced ^{111}In-neutrophil accumulation (10^{-9} mol per site induced 1601 ± 196 cells per site and 1601 ± 364 cells per site in SP- and SP + RP − 67,580-treated sites respectively, 10^{-10} mol per site induced 1295 ± 269 and 1118 ± 233 cells per site in SP- and SP + RP − 67,580 sites respectively, $n = 7$ animals) or ^{111}In-eosinophil accumulation (10^{-9} mol per site induced 5372 ± 1359 cells per site and 3658 ± 1157 in SP- and SP + RP − 67,580-treated sites respectively while 10^{-10} mol per site induced 2401 ± 591 and 2219 ± 590 cells per site in SP- and SP + RP − 67,580 treated sites respectively, $n = 6$ animals). Neither antagonist affected inflammatory responses elicited by other test agents such as 30% zymosan-activated plasma (ZAP), used as a source of C5a des arg, LTB_4 (10^{-10} mol per site) or PAF (10^{-9} mol per site) (data not shown).

Effects of chlorpheniramine, UK-74,505 and ZM-230,487 on responses induced by SP

The roles of histamine, PAF and 5-LO products in SP-induced responses were investigated by use of specific antagonists and inhibitors.

Chlorpheniramine (10 μg per site), co-injected locally with SP (10^{-9} mol per site), had no significant effect on the oedema formation or the leukocyte accumulation induced by the neuropeptide (Table 1). The antihistamine did, however, significantly inhibit the oedema formation induced by histamine (Table 1) and ZAP (30%). The response to ZAP was reduced from 41.9 ± 8.1 to 25.9 ± 5.6 μl plasma ($P < 0.05$, $n = 7$). Chlorpheniramine did not affect the cell accumulation or oedema formation induced by PAF or LTB_4 (data not shown).

The specific and long acting PAF antagonist, UK-74,505 (Alabaster *et al.*, 1991; Parry *et al.*, 1994), when given i.v. at 2.5 mg kg^{-1}, abolished the leukocyte accumulation and oedema formation induced by i.d. PAF (10^{-9} mol per site; Table 1). The antagonist did not, however, inhibit the responses elicited by i.d. SP (10^{-9} mol per site). UK-74,505 had no effect on the responses elicited by LTB_4 (10^{-10} mol per site) or 30% ZAP (data not shown).

To investigate the role of 5-LO products in the inflammatory responses elicited by SP, the effect of the non-redox 5-LO inhibitor, ZM-230,487 (Crawley *et al.*, 1993) was

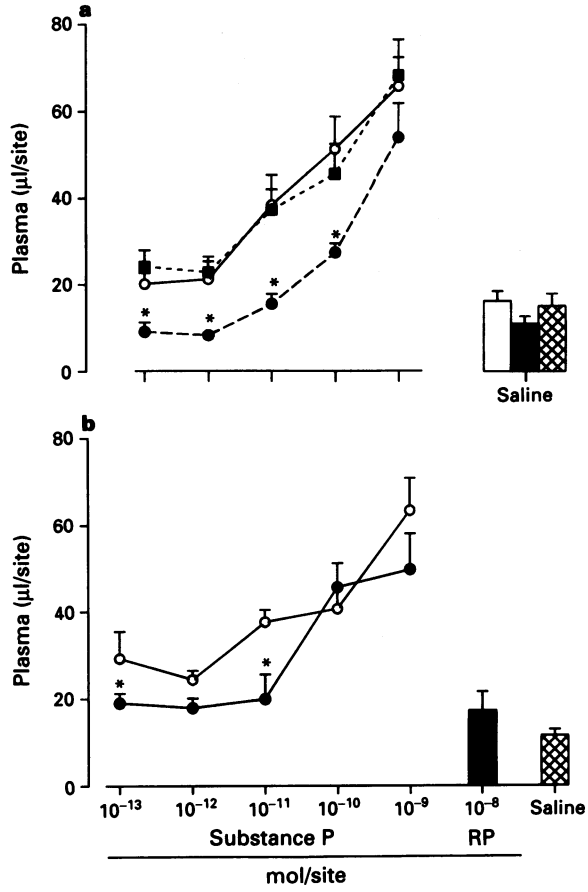

Figure 3 The effect of the NK_1 receptor antagonists, CP-96,345 and RP-67,580, on oedema formation induced by substance P (SP) after a 2 h period. (a) Shows the effects of CP-96,345 and its inactive isomer CP-96,344. Both drugs were given i.v. at 1 mg kg^{-1}, 30 min prior to injection of labelled cells and ^{125}I-albumin; (○) control responses to SP alone: (●) effect of CP-96,345; (■) effect of CP-96,344. Levels detected in saline-injected sites are shown by the columns: open column for control; solid column for CP-96,345; hatched column for CP-96,344-treated animals. (b) Shows the effect of RP-67,580, 10 μg per site co-injected with SP: (●) effect of the drug on SP-induced oedema; solid column effect of RP-67,580 when injected alone. Results are the mean ± s.e.mean for $n = 8$ (a) and $n = 7$ (b). Asterisks indicate a significant difference from SP injected sites: $^*P < 0.05$.

Table 1 The effect of chlorpheniramine and UK-74,505 on substance P-induced inflammatory responses in guinea-pig skin

	Plasma (μl per site)	^{111}*In-neutrophils* (per site)	^{111}*In-eosinophils* (per site)
Substance P (10^{-9} mol per site)	29.3 ± 4.8 (7)	1914 ± 366 (4)	1536 ± 687 (7)
Substance P + chlorpheniramine (10 μg per site)	20.0 ± 2.3 (7)	1767 ± 384 (4)	1487 ± 415 (7)
Histamine (10^{-8} mol per site)	38.7 ± 4.3 (7)	164 ± 76 (4)	91 ± 31 (7)
Histamine + chlorpheniramine (10 μg per site)	7.3 ± 1.9* (7)	192 ± 116 (4)	71 ± 27 (7)
Substance P (10^{-9} mol per site)	50.2 ± 8.7 (6)	1412 ± 312 (6)	2763 ± 730 (6)
Substance P + UK-74,505 (2.5 mg kg^{-1})	49.7 ± 7.5 (6)	1379 ± 292 (6)	4077 ± 1252 (6)
PAF (10^{-9} mol per site)	51.1 ± 5.8 (6)	2217 ± 561 (6)	3045 ± 570 (6)
PAF + UK-74,505 (2.5 mg kg^{-1})	0.0 ± 1.9* (6)	500 ± 245* (6)	70 ± 7* (6)

The effect of the H_1 histamine receptor antagonist, chlorpheniramine (10 μg per site), and the PAF antagonist, UK-74,505 (2.5 mg kg^{-1}, i.v.) on substance P-induced ^{111}In-neutrophil, ^{111}In-eosinophil and ^{125}I-albumin accumulation in the guinea-pig skin. Values are expressed as mean ± s.e.mean after subtraction of saline responses. Asterisks indicate a significant difference from PAF and histamine treated sites: $^*P < 0.05$. Number of experiments in parentheses.

studied. This compound, when co-injected i.d. at 10^{-8} mol per site with SP, significantly inhibited the accumulation of ^{111}In-eosinophils (Figure 4b) but had no effect on the SP-induced oedema formation (Figure 4c). Whilst in these experiments the SP-induced ^{111}In-neutrophil accumulation did not reach statistical significance, ZM-230,487 did not appear to affect this response (Figure 4a). At the dose used, as previously reported (Teixeira & Hellewell, 1994), ZM-230,487 inhibited the leukocyte accumulation, but not the oedema formation, induced by arachidonic acid (3×10^{-8} mol per site; Figure 4).

Effect of the anti-CD18 mAb 6.5E

The role of the CD18 adhesion molecule in SP-induced inflammatory responses was investigated by using the anti-CD18 monoclonal antibody 6.5E F(ab')$_2$ (Andrew *et al.*, 1993). This antibody has previously been shown to block both neutrophil and eosinophil accumulation in the guinea-pig without affecting the number of circulating leukocytes (Teixeira *et al.*, 1994). When given i.v. at 2.5 mg kg^{-1}, 6.5E F(ab')$_2$ greatly attenuated both ^{111}In-neutrophil (Figure 5a) and ^{111}In-eosinophil (Figure 5b) accumulation in response to

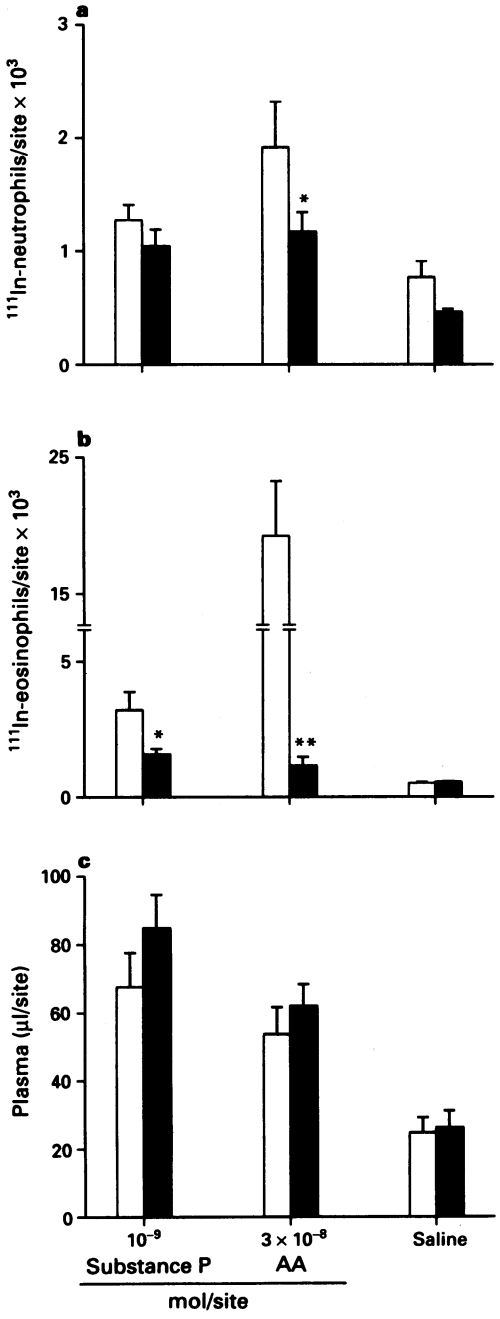

Figure 4 The effect of ZM-230,487 on substance P (SP, 10^{-9} mol per site)- and arachidonic acid (AA, 3×10^{-8} mol per site)-induced (a) ^{111}In-neutrophil accumulation, (b) ^{111}In-eosinophil accumulation and (c) oedema formation after a 2 h period. Open columns show the control response while the solid columns show the effect of ZM-230,487 when co-injected at 10^{-8} mol per site with SP, AA or saline. All data are mean ± s.e.mean for $n = 7$ experiments. Asterisks indicate a significant difference from control responses: $^*P < 0.05$, $^{**}P < 0.01$.

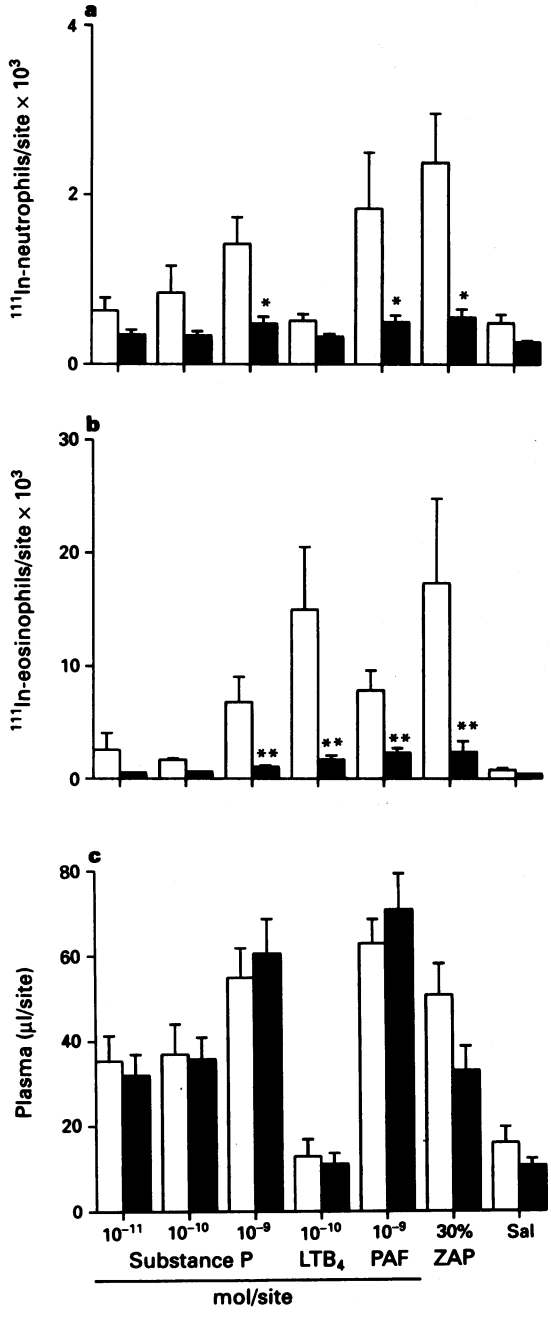

Figure 5 The effect of the anti-CD18 monoclonal antibody 6.5E F(ab')$_2$ on substance P (SP)-induced (a) ^{111}In-neutrophil accumulation, (b) ^{111}In-eosinophil accumulation and (c) oedema formation after a 2 h period. The antibody (solid columns) was given i.v. at 2.5 mg kg^{-1}, 5 min prior to the injection of labelled cells and ^{125}I-albumin. Open columns represent the control responses following the i.d. injections of different mediators. Data presented are mean ± s.e.mean for $n = 6$ experiments. Asterisks indicate a significant difference from untreated controls: $^*P < 0.05$, $^{**}P < 0.01$.

SP and other preformed mediators such as LTB_4, ZAP and PAF. The antibody did not, however, affect the oedema formation induced by any of the mediators tested (Figure 5c). Two control monoclonal antibodies, 1E6 and MOPC 21, have previously been shown not to affect leukocyte accumulation in the present model (Weg *et al.*, 1993; Teixeira *et al.*, 1994).

Discussion

The aim of the present study was to investigate and characterize the ability of SP to induce leukocyte accumulation and oedema formation *in vivo*. We have shown that the intradermal administration of SP caused the accumulation of ^{111}In-neutrophils, ^{111}In-eosinophils and local oedema formation in guinea-pig skin. Interestingly, the dose-range over which these effects occurred differed. Leukocyte accumulation was significant only at the highest doses tested, 10^{-10} and 10^{-9} mol per site whilst oedema formation was significant over the entire dose-range of 10^{-13}–10^{-9} mol per site. In addition, it appeared that SP was more effective in stimulating oedema formation than leukocyte accumulation. The leukocyte accumulation induced by SP was relatively small as compared to the leukocyte accumulation induced by other stimuli, e.g. ZAP, tested in our model. A similar pattern of results has been reported by Yano and colleagues in mice (1989). In our study, the dissociation between the dose-response relationship for leukocyte accumulation and oedema formation suggests differences in the regulatory mechanisms associated with these responses. Cumulative time-course studies indicated that the maximal level of leukocyte accumulation and oedema formation induced by SP was achieved within the first 30–60 min after administration of the peptide.

To investigate the role of NK_1 receptors in the induction of SP-induced inflammatory events in the present study, we investigated the effects of two structurally different non-peptide NK_1 receptor antagonists, CP-96,345 (Snider *et al.*, 1991) and RP-67,580 (Garret *et al.*, 1991). Both CP-96,345 and RP-67,580 significantly inhibited oedema formation induced by the lower doses of SP (10^{-13}–10^{-11} mol per site), but at the higher doses tested, there was no significant reduction. In addition, the antagonists had no significant effect on ^{111}In-neutrophil or ^{111}In-eosinophil accumulation induced by SP. A similar profile of effects was obtained when CP-96,345 was tested at higher doses of 5–10 mg kg^{-1} (data not shown). These results suggest that the oedema formation induced by low doses of SP ($<10^{-10}$ mol per site) is mediated via the activation of NK_1 receptors, the preferred receptor type for this neuropeptide. At higher doses however, when in addition to oedema formation, SP can induce leukocyte accumulation, the responses do not appear to be entirely NK_1 receptor-mediated. At high doses, the SP elicited responses may be partly mediated via the activation of NK_2 receptors. Such a possibility may be investigated by the use of specific NK_2 receptor agonists or antagonists.

Hence, our results suggest that oedema formation induced by SP may be primarily mediated directly via the activation of specific SP receptors. This conclusion was also reached by Iwamoto & Nadel (1989) who demonstrated that the NK_1 receptor is stimulated by its preferred agonist, SP to cause an increase in vascular permeability in guinea-pig skin. Indeed, functional NK_1 receptors, mediating SP-induced rapid elevations in cytoplasmic levels of Ca^{2+}, have been detected on human venular endothelial cells (Greeno *et al.*, 1993). Whilst there is much evidence showing that SP is an activator of cutaneous mast cells (Hagermark *et al.*, 1978; Foreman & Jordan, 1983; Foreman *et al.*, 1983), the SP-induced oedema formation does not appear to be mast cell-dependent (Kowalski & Kaliner, 1988; Baraniuk *et al.*, 1990; Kowalski *et al.*, 1990). This is clearly indicated in the study of Kowalski & Kaliner (1988) where, using a model of cutaneous neurogenic inflammation induced by electrical nerve stimulation in rats and mice, a temporal dissociation was found between increased vascular permeability and mast cell degranulation. Interestingly, the dependency of the SP-induced oedema formation on mast cells may be governed by its route of administration. Kowalski *et al.* (1990) found that mast cell-deficient mice exhibited normal changes in vascular permeability in response to i.v. SP, whilst in a similar study, Yano *et al.* (1989) showed that i.d. administered SP did not induce oedema formation in mast cell-deficient mice. In our study, however, we found that whilst the H_1 receptor antagonist, chlorpheniramine, had a small inhibitory effect on SP-induced oedema, this effect did not reach statistical significance, indicating that this response is not dependent on mast cell-derived histamine. Such discrepancies may be related to animal species. However, in contrast to the oedema formation in our study, the SP-induced leukocyte accumulation which was not inhibited by NK_1 receptor antagonists, may be mediated by mast cell-derived factors as reported in other models (Matsuda *et al.*, 1989; Yano *et al.*, 1989; Iwamoto *et al.*, 1992).

Mast cells are a source of numerous inflammatory mediators including histamine and PAF. To investigate the contribution of these mediators in the SP-induced leukocyte accumulation, we studied the effects of the H_1 receptor antagonist, chlorpheniramine and the PAF antagonist, UK-74,505 (Alabaster *et al.*, 1991; Parry *et al.*, 1994). These drugs had no effect on responses elicited by SP, suggesting that histamine and PAF do not play major roles in the induction of the inflammatory events induced by the neuropeptide in the present model. Another PAF-antagonist, CV-6209, has been reported to be without effect on granulocyte accumulation into mouse skin induced by SP (Iwamoto *et al.*, 1993). However, the potent and specific 5-LO inhibitor, ZM-230,487, did significantly reduce the eosinophil accumulation response, induced by SP. The most likely 5-LO product involved in this response is LTB_4, which is an effective inducer of eosinophil accumulation in guinea-pig skin (see Figure 5 and Faccioli *et al.*, 1991). In this context, it has recently been found that LTB_4 is an important mediator of SP-induced granulocyte accumulation in mice (Iwamoto *et al.*, 1993). The lack of effect of ZM-230,487 on ^{111}In-neutrophil accumulation may be due to the fact that LTB_4 is not a very effective inducer of neutrophil accumulation in the guinea-pig (see Figure 5). Hence, as the 5-LO inhibitor had no effect on neutrophil accumulation and only partially suppressed eosinophil accumulation, the results suggest that in addition to leukotrienes, other inflammatory mediators are generated or released in response to SP. Whilst mast cells contain an array of inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and IL-8 (Gordon *et al.*, 1990; Möller *et al.*, 1993), SP appears to stimulate selectively the induction of tumour necrosis factor-α (TNF-α) gene expression in a murine mast cell line (Ansel *et al.*, 1993). The mRNA for MIP-1α and MIP-1β has also been demonstrated in mast cells (Schall, 1991) and the possible involvement of these and other chemokines such as RANTES (Schall, 1991) in the inflammatory responses elicited by neuropeptides needs to be investigated.

The process of leukocyte accumulation at sites of inflammation is mediated by a cascade of adhesive events involving a number of adhesion molecules (Springer, 1994). The well characterized adhesion molecules on leukocytes include L-selectin and the integrins CD11a/CD18, CD11b/CD18, CD11c/CD18 and VLA-4. On the endothelium, the important adhesion molecules are intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and P-selectin and E-selectin. The adhesive mechanisms involved in the recruitment of leukocytes in response to SP are not yet fully understood. In particular, it is not yet clear whether SP acts on the leukocytes and/or endothelial cells to trigger this inflammatory response. *In vitro*, SP induces mast cell-dependent expression of E-selectin

on human microvascular endothelium in organ cultures of neonatal human foreskins (Matis *et al.*, 1990). These findings are in agreement with the *in vivo* results of Smith *et al.* (1993), where intradermal administration of SP was found to induce infiltration of neutrophils and eosinophils in human skin as well as increasing the endothelial cell surface expressions of E- and P-selectin. Interestingly, in the same study, it was noted that there was no detectable change in the expression of the endothelial cell adhesion molecules ICAM-1 and VCAM-1. SP has also been reported to act directly on neutrophils to stimulate adhesion and to enhance the leukocytes cell surface expression of CD11b (Shipp *et al.*, 1991; DeRose *et al.*, 1994). In contrast to the above, Zimmerman *et al.* (1992) reported that whilst SP enhanced the adhesion of human neutrophils to cultured endothelial cells, this response was not affected by monoclonal antibodies to CD18, L-selectin, ICAM-1 or E-selectin. Our results, however, clearly demonstrate that an anti-CD18 antibody inhibits the neutrophil and eosinophil accumulation induced by SP and other inflammatory stimuli in guinea-pig skin. The discrepancies between our results and the findings of the above studies could be due to difference in species, assay systems and/or concentrations of SP employed. The results of the present investigation are in agreement with previous findings showing that anti-CD18 antibodies can very effectively suppress neutrophil accumulation in a number of inflammatory models (reviewed in Nourshargh, 1992). *In vitro* studies have implicated CD18 in the stimulated adhesion of neutrophils and eosinophils to endothelial cells and their transendothelial cell migration across endothelial cell barriers (Lamas *et al.*, 1988; Smith *et al.*, 1988; 1989; Ebisawa *et al.*, 1992). However, it is not clear from our studies at what stage of their accumulation *in vivo* the anti-CD18 antibody is acting. Interestingly, blockade of the CD18 antigen had no effect on the oedema formation induced by SP, clearly differentiating the mechanisms involved in cell accumulation and oedema formation elicited by the neuropeptide in this species.

In summary, we have demonstrated that SP can induce a dose- and time-dependent accumulation of granulocytes and oedema formation in an *in vivo* model system and that these two effects can be separated by specific NK_1 receptor antagonists. Our results suggest that SP may act through two independent mechanisms, (1) a direct NK_1 receptor-dependent event to cause oedema formation and (2) an NK_1 receptor-independent event, involving 5-LO products with respect to eosinophils, to induce a CD18-dependent accumulation of granulocytes and to stimulate a component of the oedema response.

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