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Effects and interactions of sensory neuropeptides on airway microvascular leakage in guinea-pigs

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1 We have studied the effect of the sensory neuropeptides substance P (SP), neurokinin A (NKA), neurokinin B (NKB) and calcitonin gene-related peptide (CGRP) on microvascular permeability in guinea-pig airways *in vivo* and investigated whether CGRP would potentiate the effect of SP. We used the extravasation of intravenously-injected Evans blue dye as an index of permeability.

2 The tachykinins SP, NKA and NKB (0.025–5.0 nmol kg^{-1}, i.v.) significantly ($P < 0.05$) increased extravasation of dye in a dose-related manner and with a similar pattern of distribution; they were most potent in the trachea and main bronchi, less potent in the larynx and intrapulmonary airways, and had little significant effect in the bladder.

3 SP was significantly more potent in causing extravasation of dye than NKA or NKB with ED_{50} values (nmol kg^{-1}) in the range 0.04–0.1, depending on the airway level, compared with values in the range 0.3–0.7 for the neurokinins.

4 CGRP (0.0025–2.5 nmol kg^{-1}, i.v.) had no significant effect on microvascular permeability and did not potentiate SP-induced extravasation of dye.

5 Each neuropeptide decreased mean arterial blood pressure, indicating vasodilatation, in a dose-related manner. Co-injection of CGRP and SP produced additive decreases in arterial pressure.

6 We conclude that, in guinea-pig airways, tachykinins increase microvascular permeability via tachykinin receptors of the NK-1 sub-type (indicated by an order of potency of SP > NKA = NKB) on endothelial cells. The response appears to be related to mechanisms in addition to vasodilatation. The relevance of the responses to the tachykinins in asthma is discussed.

Introduction

Electrical stimulation of the cervical vagus nerves in rat and guinea-pig induces a number of physiological responses including vasodilatation and increased permeability of the microvasculature of the trachea to macromolecules (Lundberg & Saria, 1982; Lundberg *et al.*, 1983). Both responses are preserved in the presence of atropine, hexamethonium or antihistaminic drugs, but are absent in animals pretreated with capsaicin. Capsaicin is the major pungent principle of hot peppers of the plant genus *Capsicum* and has been shown to deplete primary afferent C-fibres of stored neuropeptides (Buck & Burks, 1986). Capsaicin itself causes a depolarization of afferent C-fibres which induces local effects including vasodilatation and plasma extravasation in guinea-pigs (Lundberg *et al.*, 1984a). The physiological responses may, therefore, be due to release of neuropeptides localized to capsaicin-sensitive nerves. For example, the tachykinin substance P (SP) has been localized to sensory nerves in the airways of several species, including man (Lundberg *et al.*, 1984b). SP and two newer members of the tachykinin family, neurokinin A (NKA) and neurokinin B (NKB) have been shown to increase vascular permeability in guinea-pig trachea (Lundberg *et al.*, 1985) although, of the two neurokinins, only NKA has been localized to capsaisin-sensitive nerves in the guinea-pig (Hua *et al.*, 1985a). Recently, calcitonin gene-related peptide (CGRP) has been localized to airway nerves in rat (Cadieux *et al.*, 1986; Lauweryns & Van Rast, 1987) and man (Palmer *et al.*, 1987). CGRP probably co-exists with tachykinins in capsaicin-sensitive

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nerves and they may be released together (Brain & Williams, 1985; Lundberg *et al.*, 1985; Gulbenkian *et al.*, 1986). CGRP exhibits potent vasodilator activity (Brain *et al.*, 1985) but, unlike the tachykinins, does not increase vascular permeability in guinea-pig trachea (Lundberg *et al.*, 1985). However, CGRP has been shown to potentiate increased permeability induced by SP in rat skin (Brain & Williams, 1985; Gamse & Saria, 1985). It was, therefore, the purpose of the present study to determine, in guinea-pig airways *in vivo*, the order of potency of SP, NKA and NKB in causing microvascular leak and whether or not CGRP would potentiate the effect of SP.

Methods

Male Dunkin-Hartley outbred guinea-pigs (Charles River U.K. Ltd., Kent) 250–600 g body weight were housed in a temperature-controlled room (21°C) in steel-bar cages with food (Special Diet Services Ltd., Essex) and water freely available. They were premedicated by intraperitoneal injection of diazepam (6.5 mg kg^{-1}), anaesthetized 10 min later by intramuscular injection of 0.5 ml Hypnorm (2.4 ml kg^{-1} containing 0.315 mg fentanyl citrate and 10 mg fluanisone per ml) into the medial aspect of each thigh and placed under a lamp to maintain body temperature (34°C). All drugs subsequently were administered via the jugular veins. Evans blue dye, 30 mg kg^{-1}, was injected and allowed to circulate for 1 min before injection of drugs or control vehicles. Five minutes later the animal was perfused to remove intravascular dye in a manner similar to that described previously (Evans *et al.*, 1987). The thorax was opened and a blunt-ended, 13-gauge needle passed through a left ventriculotomy into the aorta. The ventricles were cross-clamped and blood expelled through an incision in the right atrium at 100 mmHg pressure with approximately 100 ml saline (pH 5.5, 21°C). The larynx, trachea, main bronchi, lungs and bladder were removed and the intrapulmonary airways exposed by scraping away the parenchyma. The tissues were 'blotted' dry, placed in pre-weighed tubes and re-weighed, and their dye content extracted in formamide at 37°C for 18 h. The concentration of extractable Evans blue dye was quantified from light absorbance at 620 nm (Pye Unicam SP1750 spectrophotometer) by interpolation on a standard curve of dye concentrations in the range 0.5–10 $\mu g\,ml^{-1}$.

To establish drug activity, blood pressure was measured in selected animals. Pressure in the left carotid artery was recorded on a two-channel recorder (Devices, Ormed Ltd., Welwyn Garden City) via an indwelling Portex cannula filled with heparin-saline (10 u ml^{-1}) linked to a pressure transducer (Bell and Howell, Basingstoke). The cannula was cleared of blood by injecting 0.2 ml heparin-saline before injection of Evans blue dye.

Protocol

The effect of the peptides on permeability was studied in two parts: (1) SP, NKA, NKB or CGRP alone, (2) SP and CGRP co-injected. Each drug or control vehicle was freshly prepared from stock solutions on each day of experimentation and injected intravenously (i.v.) in a volume of 1 ml kg^{-1}, 1 min after Evans blue dye and 5 min before perfusion (see above). In order to minimize the number of animals used, saline (at equivalent volume to that for CGRP, i.e. 1 ml kg^{-1}) was injected simultaneously into the other jugular vein of animals given SP, NKA or NKB which allowed data for SP to be used in both parts of the study. Six groups of animals were studied with saline or drugs (nmol kg^{-1}) given simultaneously: (1) saline plus saline; (2) saline plus SP (0.025–5.0); (3) saline plus CGRP (0.025 and 2.5); (4) saline plus NKA (0.1–5.0); (5) saline plus NKB (0.1–5.0); (6) CGRP (0.0025–2.5) plus SP (0.025–5.0). The effect on vascular leakage of the vehicle for NKB (see below) was also determined. No significant difference in tissue dye content has been found previously between animals given saline and those given BSA-saline (Evans *et al.*, 1987).

Drugs and chemicals

Evans blue dye, formamide, bovine serum albumin (BSA), SP and NKA were obtained from Sigma Chemical Co., Ltd., Poole, Dorset; human alpha-CGRP and NKB from Bachem Feinchemikalien AG, Bubendorf, Switzerland; diazepam from Phoenix Pharmaceuticals Ltd., Gloucester; Hypnorm from Janssen Pharmaceuticals Ltd., Oxford; and sulpholane (tetrahydrothiophen-1,1-dioxide) from BDH Chemicals Ltd., Dagenham, Essex. Saline (Travenol Laboratories, Thetford, UK.), 0.9% sodium chloride intravenous infusion BP, was used for drug dilution and animal perfusion. Evans blue was dissolved in saline (30 mg ml^{-1}) and filtered through a 5.0 μm Millipore membrane. CGRP (1 mg in 200 μl 0.1 N acetic acid), NKA and SP were dissolved in distilled water containing 2.5% w/v BSA and aliquots freeze-dried (Edwards Modulyo freeze-drier). NKB was dissolved in distilled water containing 20% v/v sulpholane, BSA added to a final concentration of 25% (w/v) and aliquots freeze-dried. All aliquots were stored at −80°C before use and were re-constituted in saline.

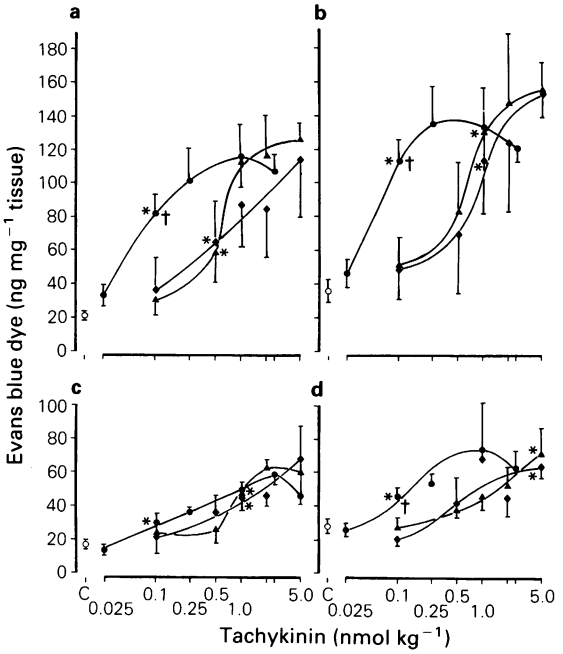

Figure 1 Effect of tachykinins on microvascular permeability in guinea-pig airways: (a) trachea, (b) main bronchi, (c) larynx and (d) intrapulmonary airways. Drugs were injected intravenously and the concentration of Evans blue dye extractable from the tissues was used as an index of plasma leakage: (●) = substance P; (▲) = neurokinin A (NKA); (◆) = neurokinin B (NKB); C = control (i.e. two volumes of saline, i.v.). Each point represents the mean for 3–6 animals with s.e. shown by vertical bars. * denotes the minimum concentration of tachykinin which is significantly different ($P < 0.05$) from control. † $P < 0.05$ compared with values for NKA and NKB at same dose (0.1 nmol kg^{-1}). No significant differences were found between NKA and NKB.

Statistical analyses

Data for the concentration of extractable Evans blue dye approximated a Gaussian distribution and were analysed by one-way analysis of variance to determine whether there were differences between group means (Armitage, 1971). Student's t test for unpaired data (two-tailed) was used to compare mean values using the Bonferroni correction (Wallenstein *et al.*, 1980) to allow for multiple comparison where appropriate. The dose of drug required to produce 50% of the maximal increase in tissue content of Evans blue dye (ED_{50}) was read directly from curves constructed using % maximal responses as a function of the dose of peptide. Mean arterial blood pressure (BP) was calculated from recorded traces as: diastolic pressure + 0.33 (systolic pressure–diastolic pressure). Data for mean BP approximated a Gaussian distribution and Student's t test for unpaired data (two-tailed) was used to compare mean values. Data have been presented as means ± one standard error (s.e.) with the Null hypothesis rejected at values of P less than 0.05.

Results

Effect of drugs on vascular permeability

Intravenous injection of SP, NKA, NKB (Figure 1), but not CGRP (Table 1), increased microvascular leakage of Evans blue dye throughout the airways studied in a dose-related manner and with a similar pattern of distribution. The effect of the tachykinins was greatest in the trachea and main bronchi. Reconstituted vehicle for NKB had no significant effect on concentration of Evans blue dye when compared with saline (Table 1). Inspection of the dose-response

Table 1 Effect of control vehicles and calcitonin gene-related peptide on microvascular permeability

Administration	*Tissue*				
	Larynx	*Trachea*	*Main bronchi*	*IPA*	*Bladder*
Saline	17.0	21.3	35.1	27.9	8.4
	(2.2)	(0.7)	(6.6)	(4.2)	(1.7)
NKB vehicle	15.3	15.9	24.2	25.8	5.4
	(1.8)	(4.0)	(6.8)	(5.4)	(2.0)
CGRP, nmol kg^{-1}					
0.025	21.9	29.5	43.1	25.5	3.9
	(4.3)	(9.9)	(19.1)	(1.9)	(2.5)
2.5	10.0*	20.1	37.6	18.9	6.9
	(1.4)	(3.0)	(10.2)	(5.9)	(2.7)

Values are mean (s.e.) concentration of Evans blue dye extractable from tissues (ng mg^{-1}) in response to intravenous injection of control vehicles (NKB = neurokinin B: for composition of vehicle, see Methods) or calcitonin gene-related peptide (CGRP). n = 4–6 animals. IPA = intrapulmonary airways. There were no significant differences between NKB vehicle or CGRP and saline except * $P < 0.05$ compared with saline.

Table 2 Comparison of effect of neuropeptides on microvascular permeability

Neuropeptide	*Airway*			
	Larynx	*Trachea*	*Main bronchi*	*IPA*
Substance P	0.10	0.05	0.04	0.05
Neurokinin A	0.57	0.71	0.35	0.37
Neurokinin B	0.39	0.32	0.54	0.30
SP + CGRP, nmol kg^{-1}				
0.025	0.14	0.06	0.05	0.06
2.5	0.11	0.08	0.10	0.03

Values are approximate ED_{50} values (nmol kg^{-1}) inducing extravasation of Evans blue dye into airway tissues. CGRP = calcitonin gene-related peptide; IPA = intrapulmonary airways; SP = substance P.

curves reveals that for each airway tissue the order of potency of the tachykinins in increasing vascular leakage was SP > NKA = NKB. This order is reflected in the ED_{50} values where SP was 4–14 times more potent, depending on the neurokinin and the airway level (Table 2). In the trachea, main bronchi and intrapulmonary airways, values for dye content at 0.1 nmol kg^{-1} SP (approximating the ED_{50} value) were significantly ($P < 0.05$) different from those with the neurokinins at the same dose. There were no significant differences between NKA and NKB in the airways. In the larynx there were no significant differences between SP, NKA or NKB. In the bladder SP and NKB, but not NKA, caused significant ($P < 0.05$) leakage of dye at the highest dose used (i.e. 5 nmol kg^{-1}): mean dye content, ng mg^{-1}, (s.e.) was SP 48.8 (12.2, $n = 5$), NKA 21.9 (9.8, $n = 4$), NKB 36.0 (7.0, $n = 4$).

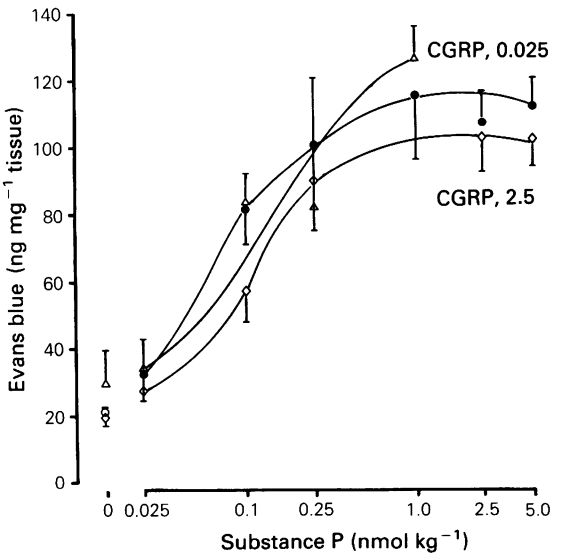

Figure 2 Effect of calcitonin gene-related peptide (CGRP) on substance P (SP)-induced microvascular permeability in guinea-pig trachea. Each drug was injected simultaneously intravenously and tissue content of dye used to quantify plasma leakage: (○) = control (i.e. two volumes of saline, i.v.); (●) = SP + saline; SP in combination with CGRP at doses of 0.025 nmol kg^{-1} (△) or 2.5 nmol kg^{-1} (◇). Each point represents the mean for 3–6 animals with s.e. shown by vertical bars. No significant differences were found between values at any dose of SP.

Calcitonin gene-related peptide alone had no significant effect on leakage of dye compared with saline except in the larynx where there was a reduction with 2.5 nmol kg^{-1} (Table 1). CGRP did not significantly potentiate the vascular leakage produced by SP in any tissue studied. Figure 2 shows, as an example, the effect in the trachea at two doses of CGRP over the range of SP doses. Table 2 gives the ED_{50} values in the airways. The values for dye content with 0.0025 nmol kg^{-1} CGRP at two doses overlay the curves in Figure 2 and are shown in Table 3.

Effect of drugs on blood pressure

Each neuropeptide decreased mean arterial pressure in a dose-related manner (Figure 3). Additive decreases in blood pressure were observed after simultaneous injection of CGRP and SP (Figure 4).

Discussion

The results of the present study show that the neuropeptides SP, NKA and NKB, but not CGRP, increase airway microvascular permeability to Evans blue dye. Evans blue dye has been shown to combine quantitatively with plasma proteins both *in vitro* (Rawson, 1943; LeVeen & Fishman, 1947; Allen & Orahovats, 1948) and *in vivo* (Steinwall & Klatzo,

Table 3 Effect of substance P and calcitonin gene-related peptide on microvascular permeability

Administration	*Tissue*				
	Larynx	*Trachea*	*Main bronchi*	*IPA*	*Bladder*
CGRP + SP:					
0.1	34.8	49.6	62.4	32.2	5.9
	(5.1)	(5.8)	(7.1)	(4.4)	(1.5)
0.25	38.6	87.5	113.8	54.8	13.2
	(6.2)	(8.8)	(21.5)	(11.5)	(2.9)

Values are mean tissue content of Evans blue dye (ng mg^{-1}) after simultaneous intravenous injection of calcitonin gene-related peptide (CGRP) 0.0025 nmol kg^{-1} and substance P (SP) at two doses (nmol kg^{-1}). n = 4 or 5 animals. IPA = intrapulmonary airways.

1966). This property has been used to quantify plasma leakage as a marker of increased vascular permeability in a number of tissues including the airways (for example, see Lundberg & Saria, 1982). In skin, the concentration of extractable dye correlates with extravasation of radiolabelled albumin (Udaka *et al.*, 1970). Thus, in our present study, it is likely that the tachykinins are causing an increase in the permeability of airway vasculature to relatively high molecular weight plasma proteins. SP and NKA have been shown previously to cause increased plasma leakage in guinea-pig trachea (Hua *et al.*, 1984). In the latter study, NKB had no effect on leakage, possibly due to its low solubility in aqueous solution (Hua *et al.*, 1985b). When properly dissolved, NKB has since been shown to cause plasma leakage in guinea-pig trachea (Lundberg *et al.*, 1985). In the present study we have extended these observations to include a number of airway levels and have directly compared the relative potency of each tachykinin.

The site of plasma leakage in systemic vascular beds has been shown by ultrastructural, pathophysiological and pharmacological studies to be post-capillary venules (diameter 10–50 μm) (Persson, 1988), where, it has been suggested (Persson, 1986), the endothelial cells contract and separate by an active process under physiological and pharmacological control. Presumably there are receptors on the surface of endothelial cells which, when coupled with the appropriate agonist, lead to contraction. The results of the present study indicate the presence of tachykinin receptors on the endothelial cells of

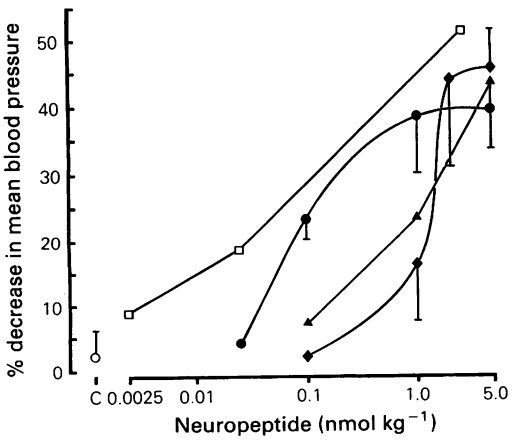

Figure 3 Effect of neuropeptides on arterial blood pressure in guinea-pigs: (□) = calcitonin gene-related peptide; (●) = substance P, (▲) = neurokinin A; (◆) = neurokinin B; C = control injections of saline, n = 5. Other points are the average of two animals or mean of three animals (s.e. shown by vertical bars).

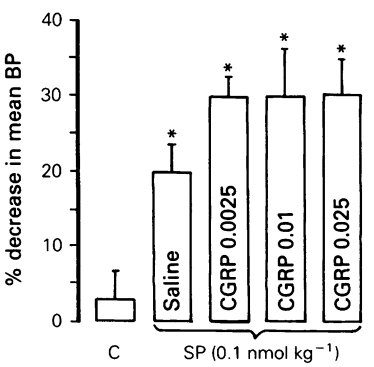

Figure 4 Effect of intravenous substance P (SP) alone (i.e. saline) or in combination with calcitonin gene-related peptide (CGRP; doses in nmol kg^{-1}) on arterial blood pressure in anaesthetized guinea-pigs. C = control (i.e. two volumes of saline, i.v.). Each column represents the mean decrease in blood pressure (BP) for 3 or 4 animals with s.e. shown by vertical bars. * $P < 0.05$ compared with control.

bronchial post-capillary venules whose activation leads to cellular contraction. Currently, three classes of tachykinin receptor are recognised, each of which exhibits a preferential affinity for one of the three mammalian tachykinins (Buck & Burcher, 1986). In our present study, SP was more potent than NKA or NKB in increasing microvascular permeability which indicates that the receptor on endothelial cells of post-capillary venules which leads to cell contraction is of the NK-1 sub-type (previously designated SP-P sub-type). It has been suggested that the same receptor sub-type mediates microvascular leakage in human skin (Barnes *et al.*, 1986). In contrast, the tachykinin receptor mediating bronchial smooth muscle contraction both *in vivo* (Lundberg *et al.*, 1985; Evans *et al.*, 1988) and *in vitro* (Karlsson *et al.*, 1984) appears to be of the NK-2 sub-type as NKA is more potent than SP. However, it should be noted that classification of receptor types on the basis of the relative potency of agonists involves a number of assumptions which may limit conclusions on the nature of the receptor and the biological response (Triggle, 1978). Carstairs & Barnes (1986) have studied the distribution of tachykinin receptors in human and guinea-pig lung and, while receptors were clearly localized to airway smooth muscle, labelling of bronchial vasculature was not so clearly seen. The distribution of tachykinin receptor subtypes has not yet been determined.

In the present study, CGRP did not cause plasma exudation nor did it potentiate the leakage caused by SP. When given alone, only high doses of CGRP have been found to induce plasma leakage in guinea-pig trachea (Lundberg *et al.*, 1985; Haass & Skofitsch, 1985). Previous studies have demonstrated species differences in regard to potentiation by CGRP of SP-induced microvascular leakage when both are injected intradermally. In rabbit (Brain & Williams, 1985) and man (Fuller *et al.*, 1987), CGRP does not potentiate SP-induced wheal. In contrast, there is potentiation in rat skin (Brain & Williams, 1985; Gamse & Saria, 1985). The lack of potentiation in guinea-pig airways in the present study is unlikely to be due to use of inactive CGRP as it caused the expected decrease in blood pressure. Another explanation for the lack of potentiation is that intravenous injection allows degradation of the CGRP which intradermal injection avoids. However, the vasodilator activity of CGRP is retained after incubation with blood (Brain & Williams, 1985). Brain & Williams (1985) have suggested that in vascular beds, such as in rat skin, where SP is active in causing plasma extravasation, CGRP potentiates oedema formation. In vascular beds where SP lacks activity, such as rabbit skin, there is no potentiation. The hypothesis, however, is not consistent with the lack of potentiation in human skin, in which SP is active (Fuller *et al.*, 1987). The potentiation in rat skin has been suggested as being due to vasodilatation (Gamse & Saria, 1985). Certainly, the tachykinins are vasodilators (Lundberg *et al.*, 1985) and their order of potency in reducing blood pressure is reflected in the order in which they cause leakage in the present study. However, CGRP is a potent vasodilator (Brain *et al.*, 1985) but does not induce leakage. Similarly, in the present study, the additive effect of SP and CGRP in reducing blood pressure did not result in potentiation of leakage. One explanation for the lack of potentiation in the airways may be their relatively greater blood flow compared with skin: potentiation is not possible where flow is adequate, but may only be demonstrable where flow is low. Another explanation may be differences in the biochemistry between lung and skin. For example, CGRP has been found *in vitro* to inhibit degradation of SP by an endopeptidase extracted from human cerebro-spinal fluid, possibly by interaction with a common metabolic step (LeGreves *et al.*, 1985). Whether or not similar inhibition occurs in animal airways and skin *in vivo* and whether species and organ differences exist remains to be elucidated.

Airway inflammation is a feature of a number of bronchial diseases in man including chronic bronchitis, cystic fibrosis and asthma. Increased microvascular permeability with exudation of plasma leading to tissue oedema are important aspects of the acute inflammatory response. The importance of plasma exudation into the airways in the pathogenesis of bronchial disease has been emphasised recently by Persson (1986). Plasma proteins have been found in fluid sampled from the airways of asthmatics (Dunnill, 1960; Ryley & Brogan, 1968), and are at concentrations greater than those found in patients with emphysema (Guirgis & Townley, 1973), bronchitis or cystic fibrosis (Brogan *et al.*, 1975). Release of neuropeptides via an axon reflex has been suggested as a mechanism underlying the pathogenesis of asthma (Barnes, 1986). In the present study in guinea-pig we have shown that tachykinins increase microvascular leakage throughout the airways, being most potent in the main bronchi. If they exhibit similar effects in man they may contribute to airway inflammation and precipitate bronchial disease.

In conclusion, we have shown that the sensory neuropeptides substance P, neurokinin A, neurokinin B and calcitonin gene-related peptide reduce arterial pressure (suggestive of vasodilatation) but only SP, NKA and NKB increase plasma exudation in guinea-pig airways. The permeability effect appears to be mediated via specific tachykinin receptors of the NK-1 sub-type. If released from sensory nerve endings, tachykinins may play an important role in the airway inflammatory response in bronchial diseases including asthma.

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