Br. J. Pharmacol. (1989), **98**, 1197–1206

Receptors mediating the effects of substance P and neurokinin A on mucus secretion and smooth muscle tone of the ferret trachea: potentiation by an enkephalinase inhibitor

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1 The effects of substance P (SP) and neurokinin A (NKA) were examined on tracheal smooth muscle tone, mucus volume output, lysozyme output and albumin transport across the ferret *in vitro* whole trachea in the presence and absence of the enkephalinase inhibitor, thiorphan.

2 SP (0.001–3 μM) and NKA (0.01–10 μM) contracted the tracheal smooth muscle and increased mucus volume, lysozyme and albumin outputs into the tracheal lumen. The EC_{50} values for SP and NKA for all of the variables measured were significantly reduced, and all of the maximum responses were significantly enhanced by thiorphan (10 μM).

3 In the presence of thiorphan, SP (1 μM) and NKA (10 μM) produced albumin concentrations in the secreted mucus (8.9 and 7.2 $\mu g \mu l^{-1}$) which were greater than those in the submucosal buffer (4.2 $\mu g \mu l^{-1}$).

4 In the presence of thiorphan, NKA was approximately 5 times more potent than SP at contracting the tracheal smooth muscle. Conversely SP was 23, 15 and 22 times more potent than NKA at stimulating mucus volume, lysozyme and albumin outputs respectively.

5 Thus, there is neutral endopeptidase in the ferret trachea *in vitro* which cleaves exogenously applied SP and NKA, thereby reducing the magnitude and potency of their actions. SP and NKA contract the ferret tracheal muscle probably by an action at NK_2 (or NK_3)-receptors but stimulate mucus volume output, lysozyme output and albumin transport across the tracheal wall probably by an action on NK_1 receptors.

Introduction

The tachykinins are small peptides which are likely to play important roles in the regulation of airway function. Included in these tachykinins are substance P (SP) and neurokinin A (NKA) which are localised to C-fibre nerve endings in the airways of many species including man (Lundberg *et al.*, 1984). Immunohistological studies suggest that SP- and NKA-immunoreactivity is localised to sensory nerves in airway smooth muscle, around blood vessels and ganglia and within and under the epithelium (Lundberg *et al.*, 1985; Ghatei *et al.*, 1987). There is a sparse innervation of SP and NKA immunoreactive nerves around submucosal glands (Lundberg *et al.*, 1984); however receptors for SP are present on guinea-pig (Hoover & Hancock, 1987) and human (Carstairs & Barnes, 1986) bronchial submucosal glands.

SP and NKA have potent effects on the airways of several species. These peptides contract airway smooth muscle (Lundberg & Saria, 1987), are vasodilators of the tracheobronchial circulation (Laitinen *et al.*, 1987; Salonen *et al.*, 1988) and increase microvascular permeability (Rogers *et al.*, 1988). In addition SP is a potent stimulant of macromolecule output from canine and human submucosal glands (Coles *et al.*, 1984; Barnes *et al.*, 1986). Little is known about the effects of NKA on submucosal gland secretion.

SP and NKA are degraded by several peptidases including neutral endopeptidase (NEP), which is also called enkephalinase (Hudgin *et al.*, 1981). This peptidase is a cell membrane-bound enzyme that is present in airway epithelium, glands and smooth muscle from many species including man (Llorens &

Schwartz, 1981; Johnson *et al.*, 1985). Recently inhibitors of NEP, thiorphan and phosphoramidon, have been shown to increase both the maximum responses and potency of SP and NKA on human (Black *et al.*, 1988) and ferret (Sekizawa *et al.*, 1987) airway smooth muscle *in vitro*, and on guinea-pig airways *in vivo* (Dusser *et al.*, 1988). Thiorphan also potentiates the SP-induced secretion of macromolecules from the ferret trachea (Borson *et al.*, 1987).

In the present study we have used the ferret *in vitro* trachea (Webber & Widdicombe, 1987) to examine the effect of SP and NKA on the volume of mucus produced, the lysozyme content of the secreted mucus (as an indicator of serous cell output) and tracheal smooth muscle tone in the presence and absence of thiorphan. Recently we have demonstrated that albumin is actively transported across the ferret tracheal wall (Webber & Widdicombe, 1989) and the effect of SP and NKA on this transport process has also been investigated.

Methods

The ferret in vitro *trachea*

Ferrets of either sex, weighing 0.5–1.5 kg, were anaesthetized by an intraperitoneal injection of sodium pentobarbitone (Sagatal, May & Baker, 50 mg kg^{-1}). The trachea was exposed and cannulated about 5 mm below the larynx with a perspex cannula containing a conical collecting well (Webber & Widdicombe, 1987). The ferret was then killed with an overdose of anaesthetic injected into the heart. The chest was opened along the midline and the trachea exposed to the carina, cleared of adjacent tissue, removed and cannulated just above the carina. The trachea was mounted, laryngeal end down, in a jacketed organ bath with Krebs-Henseleit buffer restricted to the submucosal side. The composition of the Krebs-Henseleit solution was (mM): NaCl 120.8, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 7H_2O$ 1.2, $NaHCO_3$ 24.9, $CaCl_2$ 2.4, glucose 5.6. The buffer was maintained at 37°C and gassed with 95% O_2/5% CO_2. The lumen of the trachea remained air-filled. Secretions were carried by gravity and mucociliary transport to the lower cannula, where they pooled and could be withdrawn periodically into a polyethylene catheter which was inserted into the lower cannula to form an airtight seal. The catheters containing the secretions were sealed at both ends with bone wax, numbered and stored frozen until required.

After defrosting, the secretions were washed out of the catheters into labelled plastic vials with 0.5 ml distilled H_2O. The vials were frozen and stored for use in the albumin and lysozyme assays. Preliminary experiments had shown that frozen storage for up to 6 months does not affect the enzymatic activity of lysozyme or the albumin content. Secretion volumes were estimated by the differences in weights of the catheters with secretions and dried without secretions, and the secretion rates were expressed as $\mu l\,min^{-1}$ (assuming 1 g of secretion is equivalent to 1 ml).

During experiments the carinal cannula was attached to a pressure transducer which was connected to a pen recorder. Changes in smooth muscle tone produced changes in tracheal pressure which were registered by the pressure transducer and recorded on the pen recorder, thus enabling assessment of changes in smooth muscle tone of the trachea during mucus collection.

Before the start of an experiment each trachea was allowed to equilibrate for 20 min, and during this time changes of bathing medium were made every 5 min.

Addition of peptides

Five different concentrations of SP or NKA, covering at least a tenfold concentration range, were added to the submucosal buffer bathing the trachea singly and in a random sequence. Each concentration was left in contact with the trachea for 30 min and during this time any increase in smooth muscle tone was recorded. After 30 min the secretion produced was withdrawn and processed as described above. The trachea was then washed twice and fresh buffer containing no peptide was placed in the organ bath. One or two control periods of 30 min were allowed between each drug addition, depending on how quickly the secretion rate returned to its basal level.

After five concentrations of peptide had been added to the trachea, the buffer surrounding the trachea was replaced with buffer containing thiorphan (10 μM). This buffer was left in contact with the trachea for 1 h and was replenished every 30 min. Any change in smooth muscle tone during this period was measured and any mucus produced was withdrawn at the end of 1 h. The same five concentrations of peptide were then added to the trachea in the same sequence as described above. However, this time the peptide was in a buffer containing thiorphan (10 μM). All mucus samples obtained were assayed for lysozyme and albumin.

It is possible that any change in response of the trachea to the peptides after thiorphan may be due to changes in tissue sensitivity which are independent of any effect on enkephalinase. This was tested in separate experiments by using the same procedure as described above except that thiorphan was not

added to the buffer for the second determination of peptide concentration-response curves.

Assay for lysozyme

The lysozyme concentrations of the mucus samples obtained were measured by a turbidimetric assay which relies on the ability of lysozyme to break down the cell wall of the bacterium *Micrococcus lysodeikticus*. Addition of lysozyme to a solution of the bacteria reduces the turbidity of the solution, thereby leading to a fall in optical density (OD) measured at 450 nm.

A stock suspension of *M. lysodeikticus* of $3\,mg\,ml^{-1}$ was prepared. When diluted 10 fold (the dilution in the assay) this suspension gives an OD of approximately 0.6 at 450 mm. To produce a standard curve, various concentrations of hen egg white lysozyme (0.5 to $100\,ng\,ml^{-1}$) were incubated in duplicate in 1.5 ml potassium phosphate buffer (50 mM, pH 7.4) containing *M. lysodeikticus* ($0.3\,mg\,ml^{-1}$), sodium azide ($1\,mg\,ml^{-1}$) and bovine serum albumin (BSA, $1\,mg\,ml^{-1}$). The BSA was included in the assay for its protein stabilizing effects and the sodium azide was added to prevent the growth of bacteria in the incubating solutions. The reaction mixtures were incubated for 18 h at 37°C. After incubation the OD of each solution was measured at a wavelength of 450 nm with potassium phosphate buffer pH 7.4 containing BSA ($1\,mg\,ml^{-1}$) as a blank. The standard curve was constructed by plotting the fall in OD (reduction in turbidity) against the concentration of lysozyme in the solution.

To estimate the concentration of lysozyme in a mucus sample, 20 μl of sample was incubated in 1.5 ml potassium phosphate buffer (50 mM), pH 7.4), exactly as described above for the known concentrations of lysozyme used in the preparation of the standard curve. The lysozyme concentrations (equivalent to hen egg white lysozyme) of the 20 μl samples and hence of the original mucus samples were estimated from the standard curve. The rate of output of lysozyme was then calculated by dividing the total amount of lysozyme in a mucus sample by the time over which the sample was accumulated.

Albumin transport

To examine the effect of SP and NKA on the transport of bovine serum albumin (BSA) across the ferret trachea, BSA was added to the buffer bathing the submucosal surface of the trachea in a concentration of $4\,mg\,ml^{-1}$. Fluorescent BSA ($0.02–0.03\,mg\,ml^{-1}$) was also added to the buffer as a marker and enabled an estimate to be made of the total amount of albumin which appeared in the mucus samples.

The fluorescence of the mucus samples was measured with a fluorimeter, using an excitation wavelength of 550 nm and an emission wavelength of 490 nm. The fluorescent albumin concentration of the mucus samples was estimated from a standard curve relating fluorescence (arbitrary units) to the concentration of fluorescent albumin (range $25\,ng\,ml^{-1}$ to $3\,\mu g\,ml^{-1}$). The total concentration of albumin in the mucus samples was obtained by multiplying the fluorescent albumin concentration (estimated from the standard curve) by the ratio of non-fluorescent to fluorescent albumin used in the experiment. The rate of output of albumin was determined by dividing the total amount of albumin in a mucus sample by the time over which that sample accumulated.

Intraluminal fluorescent albumin concentration might not reflect that of fluorescent albumin in the organ bath since the fluorescent label might be split off or transferred. Therefore, as described elsewhere (Webber & Widdicombe, 1989), nine mucus samples selected randomly were analysed for albumin using affinity chromatography. Linear regression analysis showed a highly significant correlation between the concentration of albumin using this method and the concentration calculated for the same samples by fluorescence measurements (slope = 1.1, $r = 0.97$, $P < 0.01$). This suggested that the fluorescent label was still attached to the albumin and that the fluorescence of the mucus samples provided an accurate indication of the total albumin concentration.

Analysis of results

The concentration-response curves presented in Results were obtained by pooling the results from experiments with a single peptide. The EC_{50} values and maximum responses (±95% confidence limits) were estimated from fitting concentration-response curves to the data points by a computerised, non-linear, least squares estimate. EC_{50} is the concentration of peptide which produced a response equal to 50% of the maximum obtained response to that peptide.

Differences between EC_{50} values or maximum responses obtained in the absence and presence of thiorphan were analysed for statistical significance using Student's unpaired *t* test.

Results

Effects of thiorphan

Thiorphan (10 μM) had no significant effect on mucus volume output ($0.12 \pm 0.06\,\mu l\,min^{-1}$ compared to control immediately before thiorphan of

$0.09 \pm 0.05\,\mu l\,min^{-1}$, $n = 12$), lysozyme output ($28 \pm 6\,ng\,min^{-1}$ compared to $19 \pm 9\,ng\,min^{-1}$, $n = 12$), albumin output ($2.9 \pm 0.9\,\mu g\,min^{-1}$ compared to $2.7 \pm 0.6\,\mu g\,min^{-1}$, $n = 12$), albumin concentration ($3.6 \pm 0.8\,\mu g\,\mu l^{-1}$ compared to $3.7 \pm 0.4\,\mu g\,\mu l^{-1}$, $n = 12$) or intraluminal tracheal pressure ($+2.7 \pm 2.2\,mmH_2O$, $n = 12$).

Effects of substance P and neurokinin A

Intraluminal pressure SP (0.01 to 3 μM) and NKA (0.01 to 3 μM) produced concentration-dependent increases in intraluminal tracheal pressure. These concentration-response curves were shifted to the left by thiorphan (10 μM) (Figure 1a and b) with significantly increased maximum responses (47 and 94% for SP and NKA respectively) and decreased EC_{50} values (10 fold for SP and 15 fold for NKA, Table 1).

Mucus volume output The mean mucus volume output in control periods before addition of SP was $0.08 \pm 0.05\,\mu l\,min^{-1}$ ($n = 28$) and in control periods before NKA was $0.07 \pm 0.04\,\mu l\,min^{-1}$ ($n = 28$). In the absence of the enkephalinase inhibitor thiorphan, SP (0.01 to 3 μM) and NKA (0.03 to 10 μM) produced small concentration-dependent increases in mucus volume output (Figure 2a and b). The concentration-response curves for SP and NKA-induced mucus volume output were shifted to the left by thiorphan (Figure 2a and b) with significantly increased maximum responses (133 and 240% for SP and NKA respectively) and decreased EC_{50} values (13 fold for SP and 2 fold for NKA, Table 1).

Lysozyme output The mean rate of output of lysozyme in control periods before SP was $32 \pm 5\,ng\,min^{-1}$ ($n = 28$) and in controls before NKA was $27 \pm 6\,ng\,min^{-1}$ ($n = 28$). SP (0.01 to 3 μM) and NKA (0.1 to 10 μM) produced small

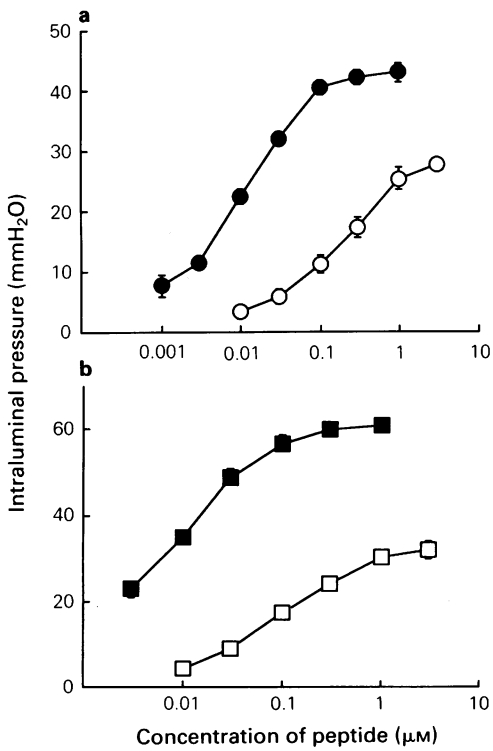

Figure 1 The effect of thiorphan (10 μM) on substance P (SP) and neurokinin A (NKA)-induced contractions of the ferret *in vitro* trachea (intraluminal pressure). Concentration-response curves in (a) are for SP alone (○) and SP in the presence of thiorphan (●), and in (b) are for NKA alone (□) and NKA in the presence of thiorphan (■). All points are means of six determinations with s.e.means shown as vertical bars.

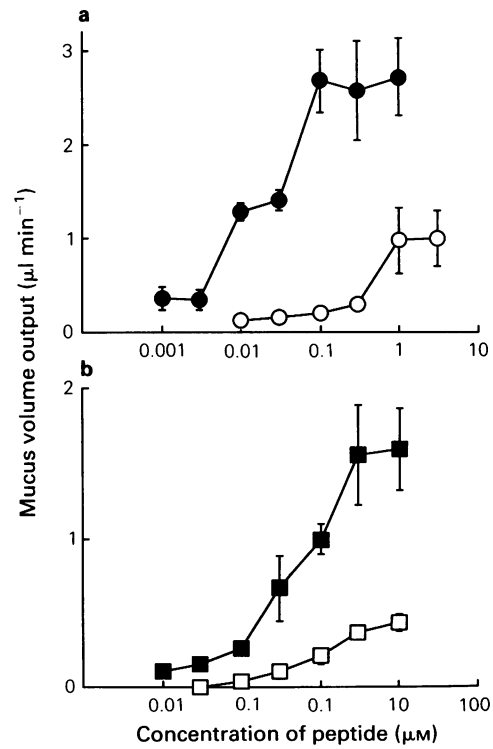

Figure 2 The effect of thiorphan (10 μM) on the mucus volume output from the ferret trachea produced by substance P (SP) and neurokinin A (NKA). Concentration-response curves in (a) are for SP alone (○) and SP in the presence of thiorphan (●), and in (b) are for NKA alone (□) and NKA in the presence of thiorphan (■). All points are means of six determinations with s.e.means shown as vertical bars.

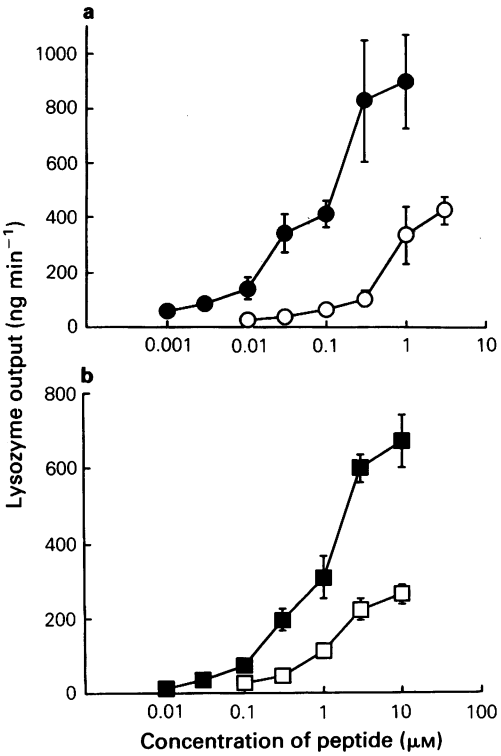

Figure 3 The effect of thiorphan (10 μM) on the rate of output of lysozyme from the ferret trachea produced by substance P (SP) and neurokinin A (NKA). Concentration-response curves in (a) are for SP alone (○) and SP in the presence of thiorphan (●), and in (b) are for NKA alone (□) and NKA in the presence of thiorphan (■). All points are the means of six determinations with s.e.means shown as vertical bars.

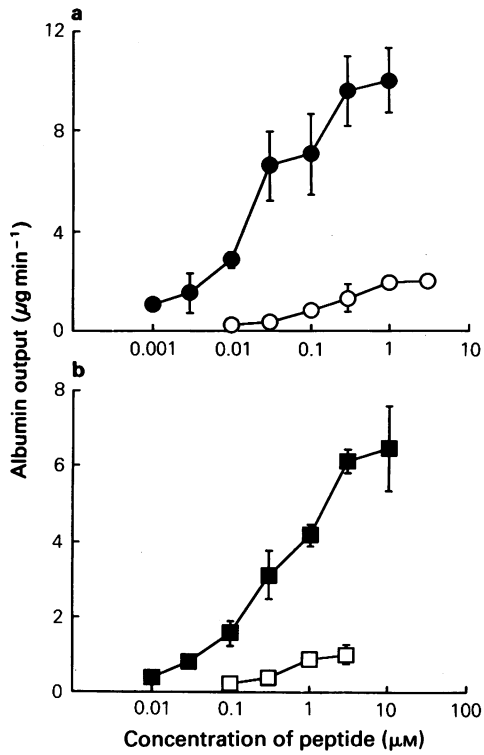

Figure 4 The effect of thiorphan (10 μM) on the rate of output of albumin from the ferret trachea produced by substance P (SP) and neurokinin A (NKA). Concentration-response curves in (a) are for SP alone (○) and SP in the presence of thiorphan (●), and in (b) are for NKA alone (□) and NKA in the presence of thiorphan (■). All points are the means of six determinations with s.e.means shown as vertical bars.

concentration-dependent increases in lysozyme output. The concentration-response curves for SP and NKA-induced lysozyme outputs were shifted to the left by thiorphan (Figure 3a and b) with significantly increased maximum responses (89 and 120% for SP and NKA respectively) and decreased EC_{50} values (eight fold for SP and three fold for NKA, Table 1).

Albumin output and concentration The mean rate of output of albumin in controls before SP was $0.23 \pm 0.06\,\mu g\,min^{-1}$ ($n = 28$) and in controls before NKA was $0.27 \pm 0.09\,\mu g\,min^{-1}$ ($n = 28$). In the absence of thiorphan, SP (0.01 to 3 μM) and NKA (0.1 to 3 μM) produced small, concentration-dependent increases in albumin output (Figure 4a and b). The concentration-response curves for SP and NKA-induced albumin output were shifted to the left by thiorphan (10 μM) (Figure 4a and b) with significantly increased maximum responses (368 and 318% for SP and NKA respectively) and decreased EC_{50} values (ten fold for SP and three fold for NKA, Table 1).

In the absence of thiorphan, the mean concentration of albumin in the mucus collected during control periods before addition of SP (3 μM) was $2.8 \pm 0.6\,\mu g\,\mu l^{-1}$ ($n = 4$). This albumin concentration was not significantly changed ($3.1 \pm 0.5\,\mu g\,\mu l^{-1}$) by addition of SP (3 μM). Similarly in controls before NKA (3 μM) the concentration of albumin was $3.4 \pm 0.7\,\mu g\,\mu l^{-1}$ ($n = 4$), which was not significantly changed ($3.7 \pm 0.4\,\mu g\,\mu l$) by NKA (3 μM).

In contrast, in the presence of thiorphan (10 μM) the concentration of albumin in control periods before SP (1 μM) was $3.2 \pm 0.6\,\mu g\,\mu l^{-1}$; this albumin concentration was significantly increased to

Table 1 EC_{50} values and maximum responses for the effects of substance P (SP) and neurokinin A (NKA) in the presence and absence of thiorphan (10 μM), on intraluminal pressure and mucus volume, lysozyme and albumin outputs from the ferret trachea

	Substance P				*Neurokinin A*			
	EC_{50} (μM)		*Maximum*		*EC_{50} (μM)*		*Maximum*	
	Control	*+ Thiorphan*	*Control*	*+ Thiorphan*	*Control*	*+ Thiorphan*	*Control*	*+ Thiorphan*
Mucus volume (μl min^{-1})	0.4 (0.3–0.6)	0.03 (0.01–0.05)*	1.2 (1.0–1.4)	2.8 (2.7–2.9)*	1.0 (0.7–1.3)	0.5 (0.3–0.8)*	0.5 (0.4–0.6)	1.7 (1.5–1.9)*
Lysozyme (ng min^{-1})	0.6 (0.4–1.0)	0.08 (0.06–0.1)*	530 (500–560)	1000 (850–1150)*	2.0 (1.6–2.4)	0.8 (0.6–1.0)*	350 (310–390)	770 (745–795)*
Albumin (μg min^{-1}	0.2 (0.1–0.4)	0.02 (0.008–0.05)*	2.2 (2.0–2.4)	10.3 (10.1–10.5)*	1.3 (1.0–1.6)	0.5 (0.3–0.7)*	1.7 (1.6–1.8)	7.1 (6.8–7.4)*
Luminal pressure (mmH_2O)	0.2 (0.08–0.5)	0.02 (0.008–0.05)*	30 (26–34)	44 (40–48)*	0.06 (0.04–0.1)	0.004 (0.002–0.008)*	32 (28–36)	62 (57–67)*

The 95% confidence limits for the EC_{50} values and maximum responses are shown in parentheses. Control EC_{50} values and maximum responses are those obtained in the absence of thiorphan.

* Significantly different ($P < 0.05$) from the control EC_{50} or maximum response.

Table 2 EC_{50} values and maximum responses for consecutive concentration-response curves for substance P (SP) and neurokinin A (NKA)-induced increases in intraluminal pressure, and mucus volume, lysozyme and albumin outputs from the ferret trachea

	Substance P				*Neurokinin A*			
	EC_{50}^1 (μM)	*EC_{50}^2 (μM)*	*Maximum^1*	*Maximum^2*	*EC_{50}^1 (μM)*	*EC_{50}^2 (μM)*	*Maximum^1*	*Maximum^2*
Mucus volume (μl min^{-1})	0.4 (0.2–0.6)	0.4 (0.3–0.4)	1.5 (1.2–1.8)	1.6 (1.4–1.8)	1.2 (0.9–1.5)	1.3 (1.0–1.6)	0.4 (0.3–0.5)	0.4 (0.3–0.5)
Lysozyme (ng min^{-1})	0.7 (0.4–1.0)	0.6 (0.4–0.8)	510 (500–520)	550 (520–580)	1.7 (1.4–2.0)	1.6 (1.4–1.8)	300 (280–320)	330 (300–360)
Albumin (μg min^{-1})	0.4 (0.2–0.6)	0.5 (0.3–0.7)	1.7 (1.2–2.2)	1.9 (1.6–2.2)	1.8 (1.6–2.0)	1.9 (1.7–2.1)	1.9 (1.7–2.1)	1.6 (1.3–1.9)
Luminal pressure (mmH_2O)	0.3 (0.1–0.5)	0.4 (0.2–0.6)	35 (28–42)	33 (30–36)	0.08 (0.06–1.0)	0.08 (0.06–1.0)	27 (24–30)	29 (24–39)

EC_{50}^1 and maximum^1 refer to the first curve and EC_{50}^2 and maximum^2 to the second curve. The 95% confidence limits for EC_{50} values and maximum responses are shown in parentheses.

$8.9 \pm 2.1\,\mu g\,\mu l^{-1}$ by SP (1 μM). Similarly, the albumin concentration in controls before NKA (10 μM) was $2.9 \pm 0.7\,\mu g\,\mu l^{-1}$, and this was increased to $7.2 \pm 2.1\,\mu g\,\mu l^{-1}$ by NKA (10 μM).

The concentration of albumin in the submucosal buffer was $4.2 \pm 0.6\,\mu g\,\mu l^{-1}$ ($n = 8$).

Comparison of potencies of substance P and neurokinin A

The EC_{50} values and maximum responses for SP and NKA-induced mucus volume, lysozyme and albumin output and for smooth muscle contraction in the presence and absence of thiorphan (10 μM) are shown in Table 1. The EC_{50} for NKA-induced smooth muscle contraction (0.004 μM) in the presence of thiorphan is significantly lower than that for SP (0.02 μM). In contrast the EC_{50} values for the NKA-induced mucus volume (0.5 μM), lysozyme (0.8 μM) and albumin outputs (0.5 μM) in the presence of thiorphan are significantly higher than those for SP (0.03, 0.08 and 0.02 μM respectively).

Consecutive concentration-response curves for substance P and neurokinin A Table 2 shows the EC_{50} values and maximum responses obtained for SP and NKA-induced mucus volume, lysozyme and albumin outputs, and for smooth muscle contraction when two concentration-response curves were constructed for a single peptide consecutively. There are no significant differences between the EC_{50} values or maximum responses for SP or NKA, obtained from the first or second concentration-response curves (Table 2).

Discussion

SP and NKA dose-dependently increased intraluminal tracheal pressure suggesting contraction of the tracheal smooth muscle. These tachykinins contract tracheal and bronchial smooth muscle *in vitro* and *in vivo* in a variety of other species including man (Lundberg & Saria, 1987). The contractile effects of SP and NKA on the ferret trachea were greatly potentiated by the enkephalinase inhibitor thiorphan with increases in potency and maximum response. Previously, thiorphan was shown to increase the potency and maximum responses of SP and NKA on human (Black *et al.*, 1988) and ferret (Sekizawa *et al.*, 1987) airway smooth muscle *in vitro*, and on guinea-pig airways *in vivo* (Dusser *et al.*, 1988). Since the tachykinins were administered to the submucosal side of the trachea in the present study, it seems likely that there are enkephalinases present within the smooth muscle which, in the absence of thiorphan, break down the tachykinins thereby reducing their effect. Indeed, enkephalinase activity has been identified in ferret tracheal smooth muscle (Sekizawa *et al.*, 1987).

In the absence of thiorphan, SP and NKA produced small increases in mucus volume from the ferret trachea. In contrast, SP is a potent stimulant of glycoprotein secretion from canine tracheal explants (Coles *et al.*, 1984) and of fucose (a marker for mucus glycoprotein) output from isolated human bronchi (Barnes *et al.*, 1986), both effects being obtained in the absence of enkephalinase inhibitors. It is not clear why, in the absence of thiorphan, SP is a weak secretagogue in the ferret but a potent secretagogue in dog and human airways. The most likely explanation is a species variation in the amount of enkephalinase present in *in vitro* airway preparations. Both the potency and maximum responses for SP and NKA-induced secretion of mucus from the ferret trachea were enhanced by addition of thiorphan. Similarly, thiorphan potentiated the SP-induced release of macromolecules from explants of ferret trachea (Borson *et al.*, 1987). Enkephalinases have been identified in the submucosal glands of the ferret trachea by immunocytochemical (Sekizawa *et al.*, 1987) and biochemical (Borson *et al.*, 1986) methods.

SP and NKA may increase mucus output by acting on specific receptors on the myoepithelial cells of the submucosal glands. Contraction of these cells would squeeze existing mucus out of the secretory tubules and gland ducts onto the airway surface (Shimura *et al.*, 1987). Alternatively SP and NKA may interact with receptors on the submucosal glands to promote cellular secretion by an exocytotic mechanism (Gashi *et al.*, 1986). We cannot exclude either of these mechanisms in the present study and both may be occurring. If the tachykinins were working only by contracting myoepithelial cells there should be a rapid tachyphylaxis of SP and NKA-induced mucus volume output, however this was not observed in the present study.

At least part of the mucus volume increase produced by SP and NKA in the present study must come from serous cells in the submucosal glands, since the tachykinins also stimulated the output of lysozyme, which is a specific marker for serous cell secretion (Tom-Moy *et al.*, 1983). Like mucus volume, SP-, and NKA-induced lysozyme output was weak in the absence of thiorphan but was greatly potentiated by pretreatment with the enkephalinase inhibitor. In contrast, SP potently degranulates serous cells in submucosal glands of ferret tracheal explants (Gashi *et al.*, 1986). As with mucus volume this difference probably occurs because of varying amounts of enkephalinase in the two preparations.

Albumin is actively transported across the ferret

trachea *in vitro* from the submucosal side into the lumen (Webber & Widdicombe, 1989). Further evidence suggests this active transport occurs across the tracheal epithelium (Price *et al.*, 1989). In the present study SP and NKA produced small increases in albumin output from the trachea in the absence of thiorphan. However, there was no change in the albumin concentration of the secreted mucus compared to control values, even at the highest concentrations of SP and NKA. After thiorphan pretreatment SP and NKA were potent stimulators of albumin output into the tracheal lumen. Furthermore, the peptides also increased the albumin concentration of the secreted mucus to a level which exceeded that in the submucosal buffer. Thus SP and NKA are not only potent stimulants of albumin transport across the ferret tracheal epithelium but can stimulate this transport to occur against a concentration gradient. Previously β-agonists (Webber & Widdicombe, 1989) and prostaglandin E_1 (Deffebach *et al.*, 1989) have been shown to have a similar action. The increase in responsiveness of albumin transport to SP and NKA after thiorphan suggests that tachykinin breakdown by enkephalinase is being prevented whilst the peptides cross the tracheal mucosa from the submucosal buffer to the epithelium. This breakdown may be prevented at any site within the trachea since enkephalinases are not only present in ferret tracheal smooth muscle and submucosal glands but are also in the epithelium (Sekizawa *et al.*, 1987). SP and NKA also stimulate chloride transport across the canine tracheal epithelium (Al-Bazzaz *et al.*, 1985; Tamaoki *et al.*, 1988) and this transport may be linked with albumin transport, although this has not been investigated in the present study.

It is possible that the changes in the response of the trachea to SP and NKA with thiorphan may be due to changes in tissue sensitivity with time and not due to any effect of thiorphan. However, this seems unlikely since concentration-response curves to SP or NKA constructed consecutively with no thiorphan present produced near-identical EC_{50} values and maximum responses (Table 2).

There are at least three different receptors mediating the actions of the tachykinins. These receptors were characterised using biological (Regoli *et al.*, 1987) and binding assays (Lee *et al.*, 1986) and the classification is based on agonist potency ratios. Thus, NK_1-receptors are found in tissues where SP is more potent than NKA or NKB. In contrast NK_2-receptors occur where the potency order is NKA > NKB > SP and NK_3-receptors are found when the potency order is NKB > NKA > SP.

In the present study NKA was approximately 5 times more potent than SP at contracting the ferret tracheal smooth muscle in the presence of thiorphan (Table 1). Similarly, NKA is more potent than SP at contracting guinea-pig trachea (Uchida *et al.*, 1987; Advenier *et al.*, 1987) and human bronchi (Martling *et al.*, 1987; Advenier *et al.*, 1987) *in vitro* and at contracting guinea-pig airways *in vivo* (Manzini *et al.*, 1988). Therefore, it is probable that NKA and SP contract both tracheal and bronchial smooth muscle from several species by an action on the NK_2-receptor, although since NKB was not used in several of these studies, including the present one, NK_3-receptors cannot be ruled out.

In contrast to the action of the tachykinins on airway smooth muscle, SP was approximately 20 and 10 times more potent than NKA at stimulating mucus volume and lysozyme output from the ferret trachea (Table 1). These results are consistent with the tachykinins stimulating NK_1-receptors on the submucosal glands to produce an increase in the volume of secreted mucus part of which is coming from serous cells. There is little other information concerning the receptors mediating the effects of tachykinins on mucus secretion in the airways. However, SP is more potent than NKA at increasing microvascular leakage within guinea-pig airways (Rogers *et al.*, 1988) and is more potent than NKA at increasing conductance across canine airway epithelium (Rangachari & McWade, 1985). Therefore, effects of tachykinins on the airways apart from smooth muscle contraction but including mucus secretion are probably mediated through NK_1-receptors. Furthermore, SP is more potent than NKA at stimulating salivation in the anaesthetized guinea-pig (Manzini *et al.*, 1988), so secretory actions of the tachykinins other than mucus secretion are also probably NK_1-receptor mediated. In addition, in the present study, SP was 25 times more potent than NKA at stimulating albumin transport across the epithelium. This is consistent with the NK_1-receptor mediated action of the tachykinins on ion-transport across canine tracheal epithelium (Rangachari & McWade, 1985).

In summary, SP and NKA contract ferret tracheal smooth muscle and increase mucus volume, lysozyme output and albumin transport across the epithelium. All of the actions of SP and NKA are greatly potentiated by thiorphan suggesting inhibition of tachykinin breakdown by enkephalinase enzymes within the trachea. The SP- and NKA-induced muscle contractions are probably mediated by NK_2-receptors whereas the other actions of the tachykinins on the trachea are probably mediated by NK_1-receptors.

I would like to thank Professor J.G. Widdicombe for helpful discussion of the manuscript.

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(Received April 10, 1989
Revised August 2, 1989
Accepted August 21, 1989)