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Facilitation by tachykinins of neurotransmission in guinea-pig pulmonary parasympathetic nerves

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1 The effect of tachykinins on cholinergic neurotransmission was studied in an innervated tracheal tube preparation isolated from guinea-pigs anaeshetized with urethane. The tracheal tube was bathed in Krebs-Henseleit solution containing 5 μM indomethacin.

2 Neurokinin A (NKA), eledoisin (El) and substance P (SP) caused concentration-dependent increases in intraluminal pressure (ILP), with an order of potency NKA > El $\gg$ SP.

3 Low concentrations of tachykinins, that had little effect on ILP, caused an increase in the contractions elicited by stimulation of the preganglionic vagal nerve fibres and by postganglionic (transmural) stimulation. The order of potency was NKA $\geqslant$ El > SP. Contractions induced by exogenous acetylcholine (ACh) were not increased by the tachykinins.

4 The magnitude of the tachykinin-induced augmentation of responses to nerve stimulation was inversely related to stimulation voltage and frequency.

5 These results suggest that tachykinins act on NK_2 receptors, both on the trachealis muscle and on postganglionic pulmonary parasympathetic nerve terminals. Activation of the neuronal receptors may increase the probability of transmitter release from the nerve terminals.

Introduction

Substance P (SP) and neurokinin A (NKA) are endogenous tachykinins which are encoded by two genes and expressed in neurones. SP is present in afferent nerves in the airways of several species, including guinea-pigs and man (Nilsson *et al.*, 1977; Lundberg *et al.*, 1984; Polak & Bloom, 1986) and SP-immunoreactive neurones have been identified in the vagus nerve (Gamse *et al.*, 1979). SP and other tachykinins have been shown to cause contraction of human and guinea-pig airways *in vitro* (Mizrahi *et al.*, 1982; Lundberg *et al.*, 1983) and *in vivo* (Andersson & Persson, 1977; Goel & Biggs, 1986). These responses are unaffected by atropine or mepyramine, and are probably due to a direct effect of the peptides on a specific sub-population of tachykinin receptors on the airway smooth muscle cells. This notion is supported by the demonstration by means of an autoradiographic technique that a high density of SP-receptors exists on guinea-pig airways smooth muscle (Carstairs & Barnes, 1986).

In the guinea-pig myenteric plexus, SP stimulates the release of acetylcholine (ACh) from cholinergic nerves (Yau & Youther, 1982) and in the rabbit trachea, SP enhances the cholinergic contractions induced by electrical field stimulation (Tanaka & Grunstein, 1986). This suggests that SP may have a neuromodulatory role and may facilitate neurotransmission at postganglionic cholinergic nerve terminals. SP-immunoreactive neurones have also been identified in airway ganglia (Lundberg *et al.*, 1984; Polak & Bloom, 1986) and it is therefore possible that SP may modulate airway ganglionic neurotransmission.

The rank orders of potency of SP and related peptides on different tissues suggests the presence of at least three distinct sub-populations of tachykinin receptor (Lee *et al.*, 1986; Buck & Burcher, 1986). In guinea-pig trachea, NKA and eledoisin are more potent than SP in causing smooth muscle contraction *in vitro* (Karlsson *et al.*, 1984; Uchida *et al.*, 1987; Grandordy *et al.*, 1988) and bronchoconstriction *in vivo* (Hua *et al.*, 1984) suggesting the presence of NK_2 (SP-E) receptors on airway smooth muscle cells. It is not known, however, whether the putative tachykinin receptors on airway cholinergic nerve terminals are of the same sub-type as those on the smooth muscle.

We have now investigated the effects of three tachykinins on cholinergic neurotransmission at postganglionic nerve terminals, using an innervated

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guinea-pig tracheal tube preparation in which preganglionic stimulation of the vagus nerve and postganglionic (transmural) stimulation were possible (Blackman & McCaig, 1983). Using rank orders of potency of NKA, eledoisin and SP, we have been able to classify the tachykinin receptor subtype involved in the effects. A preliminary study has been communicated to the British Pharmacological Society (Barnes *et al.*, 1987).

Methods

Innervated tracheal tube preparation

Guinea-pigs (350–450 g) of the Dunkin-Hartley strain (Graystoke, Hampshire) were anaesthetized with urethane (1.5 g kg^{-1} i.p.). The trachea was removed with the right vagus and recurrent laryngeal nerves attached, as described by Blackman & McCaig (1983). The trachea was tied onto an electrode holder to form a sealed tube filled with Krebs-Henseleit solution (KHS; composition mM: NaCl 118.4, KCl 4.7, $NaHCO_3$ 2.50, glucose 11.1, KH_2PO_4 1.16, $MgSO_4 \cdot 7H_2O$ 1.19 and $CaCl_2$ 2.6).

The tracheal tube was cannulated and connected to a Statham transducer (P23AC) for measurement of intraluminal pressure (ILP). Trachea and electrode were suspended in a 40 ml bath of KHS, containing 5 μM indomethacin to abolish spontaneous tone (as described by Farmer & Coleman, 1970). Both reservoir and bath were bubbled with a 95% O_2/5% CO_2 mix and the bath temperature was maintained at 37°C.

The vagus nerve was drawn through a silver–silver chloride ring electrode to enable preganglionic nerve stimulation (30 Hz, 0.2 ms for 5 s at 45 s intervals). Transmural stimulation (using the same parameters) was performed using a separate pair of electrodes, one positioned inside the tube and the other in the organ bath. Stimuli were delivered using a Grass S44 stimulator. In experiments using transmural stimulation, propranolol (1 μM) was added to the KHS to abolish relaxant responses due to simultaneous excitation of sympathetic nerve fibres (Farmer & Coleman, 1970). Both types of electrical stimulation, or addition of acetylcholine (ACh), caused an atropine-sensitive increase in ILP due to contraction of the trachealis muscle.

In order to check the pre- and post-ganglionic nature of the two types of electrical stimuli, hexamethonium (25 μM) was added to the organ bath (at the end of each experiment) to abolish the response to preganglionic vagal nerve stimulation, followed by atropine (0.5 μM) to abolish the response to transmural stimulation.

Experimental protocol

After a 1.5 h equilibration time in the presence of 5 μM indomethacin, a stable base-line ILP and constant responses to nerve stimulation were obtained (control values). Tachykinins were added directly to the organ bath in volumes of not more than 0.4 ml and left in contact with the tissue for 8 min. The effects of tachykinins, both on ILP and on responses to nerve stimulation had been previously shown to equilibrate within this time period. After each dose, the tissue was washed and all readings allowed to return to control values before subsequent additions.

In those experiments involving nerve stimulation, the voltage-response relationship was determined at the beginning and checked at the end of each such experiment. The term V_{50} describes the voltage which, in any given preparation, elicited 50% of the maximal response to nerve stimulation. Where stimulation frequency was varied, the number of impulses in the train was kept constant (at 150) by increasing the train length as the frequency decreased.

Drugs and chemicals

Drugs used were as follows: urethane and indomethacin (Sigma), propranolol hydrochloride (ICI), histamine acid phosphate, acetylcholine bromide and atropine sulphate (BDH), hexamethonium bromide (Koch-Light), neurokinin A, eledoisin and substance P (Peninsula Laboratories).

ACh, hexamethonium, histamine and atropine were dissolved in physiological saline. Indomethacin (1 mg ml^{-1}) was made up in buffer (KH_2PO_4 19.76 mM and Na_2HPO_4 118.34 mM; pH adjusted to 7.8 with NaOH) before addition to the KHS. The precautions for handling peptides outlined by Stewart (1983) were followed throughout. Where possible, polyethylene or polypropylene tubes were used, and all glass tubes and the organ bath were treated before use with dimethyldichlorosilane (Sigma).

Concentrated solutions of the peptides were prepared in distilled water, divided into aliquots of 100 μl each and stored at −70°C until used. Freshly made solutions of peptides were diluted in saline and stored on ice throughout the experiments. Peptide concentrations were calculated from the manufacturer's data for purity and molecular weight.

Results

Effect of tachykinins on trachealis muscle contraction

Addition of tachykinins to the organ bath caused slowly-developing, concentration-dependent in-

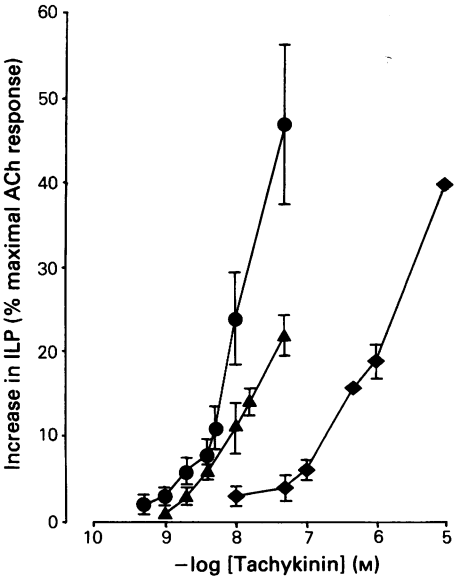

Figure 1 Effects of neurokinin A (●), eledoisin (▲) and substance P (◆) on intraluminal pressure (ILP) of guinea-pig isolated tracheal tube preparation. Increases in ILP are expressed as a % of maximal response to acetylcholine (ACh). Points represent means of 5–9 experiments (except SP 10 μM; $n = 4$); vertical lines show s.e.mean. Indomethacin (5 μM) was present throughout all experiments.

creases in the ILP, reflecting contraction of the trachealis muscle. There was no evidence of tachyphylaxis in our experiments. The effect of the tachykinins on the ILP was not reduced by 0.5 μM atropine, a concentration which totally inhibited the response to preganglionic nerve stimulation, and these results therefore confirm the findings of other workers (Mizrahi *et al.*, 1982; Lundberg *et al.*, 1983).

In each tissue, the contractions elicited by the tachykinins were expressed as a % of the maximal increase in ILP caused by ACh. NKA was more potent than eledoisin, which was more potent than SP (Figure 1). Consideration of expense became a limiting factor at high concentrations of tachykinins, and maximal contractile responses were therefore not measured for the peptides.

Effect of tachykinins on cholinergic neural responses

Preganglionic vagal nerve stimulation, or transmural stimulation in the presence of 1 μM propranolol, caused a rapid, brief increase in ILP due to contraction of the trachealis muscle. The response to vagal stimulation could be abolished by hexamethonium

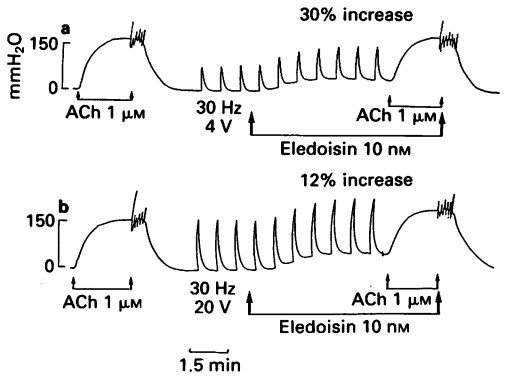

Figure 2 Effect of 10 nM eledoisin on responses of the guinea-pig isolated tracheal tube preparation to preganglionic vagal nerve stimulation at two different voltages (30 Hz, 0.2 ms for 5 s at 45 s intervals). Eledoisin caused a 30% increase in the response to stimulation at 4 V (a) and a 12% increase in response at 20 V (b). The rise in intraluminal pressure produced by 10 nM eledoisin was similar after both challenges, and the pressor responses to 1 μM acetylcholine (ACh) were unaltered by the presence of the tachykinin. Maximal response to ACh was 450 mmH_2O. Indomethacin (5 μM) was present throughout.

(25 μM), indicating that stimulation was preganglionic. Atropine (0.5 μM) abolished the responses to both vagal nerve stimulation and transmural stimulation, indicating that only cholinergic pathways were involved. Responses to both types of electrical stimulation were increased by addition of tachykinins to the organ bath. However, no direct comparison between pre- and post-ganglionic effects was made. The augmenting effect of the tachykinins could be demonstrated at low concentrations, which had little effect on ILP. This is illustrated in Figure 2, which shows the results of an experiment using eledoisin. Eledoisin (10 nM) augmented the responses to nerve stimulation, but caused a rise in ILP equivalent to only 10% of the maximal response to ACh. This figure also shows that the response to 1 μM ACh was unaltered by the presence of the tachykinin, suggesting that the augmenting effect of the eledoisin on neurally-mediated responses involves a prejunctional mechanism. This lack of effect of the peptides on responses to exogeneous ACh was subsequently confirmed for NKA and SP.

The degree of augmentation of neurally-mediated responses was dependent on the stimulation voltage used. This is also illustrated in Figure 2a, which shows that responses to preganglionic vagal nerve stimulation at 4 V were increased by 30% on addition of 10 nM eledoisin to the organ bath. However, at 20 V the same concentration of eledoisin increased

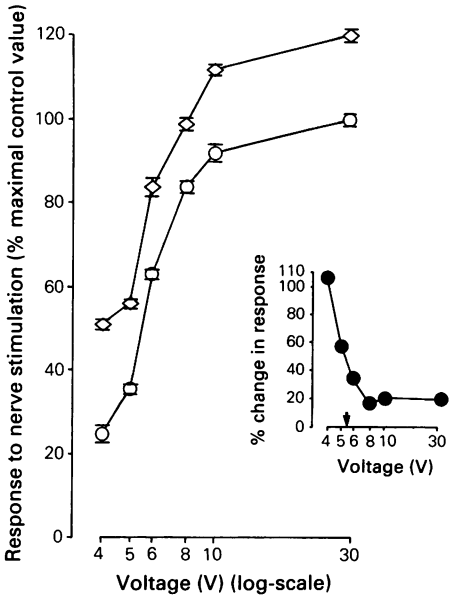

Figure 3 Effect of 5 nM neurokinin A (NKA, ◇) on responses of the guinea-pig isolated tracheal tube preparation to preganglionic vagal nerve stimulation at different voltages (30 Hz, 0.2 ms for 5 s at 45 s intervals). Controls (○) in absence of NKA. Each point represents mean of 5 measurements; vertical lines show s.e.mean. The % change in response at different voltages, calculated from these data, is shown in the inset. Indomethacin (5 μM) was present throughout.

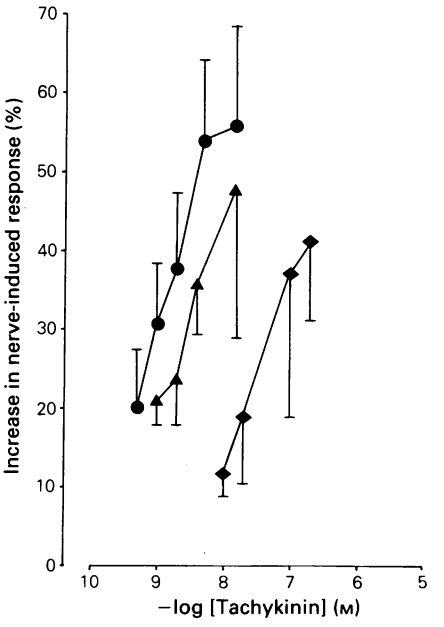

Figure 4 Effect of neurokinin A (●), eledoisin (▲) and substance P (◆) on responses of the guinea-pig isolated tracheal tube preparation to preganglionic vagal stimulation (V_{50}, 30 Hz, 0.2 ms for 5 s at 45 s intervals). Effect is expressed as a % increase in response with respect to control values before addition of the tachykinin. Points represent means of 5–7 experiments; vertical lines indicate s.e.mean. Indomethacin (5 μM) was present throughout all experiments.

the responses by only 12% (Figure 2b), although the small rise in ILP produced by 10 nM eledoisin was similar for both applications. A similar effect of voltage was subsequently confirmed for augmentation by NKA and SP.

Augmentation of responses to vagal stimulation produced by NKA at different voltages is shown graphically in Figure 3. The control curve shows the effect of voltage on the responses elicited by preganglionic vagal nerve stimulation. NKA (5 nM) caused an upward shift of the voltage-response curve, but the % change in response in the presence of the tachykinin was greater at submaximal voltages. Stimulation at very low voltages produced inconsistent responses, and subsequent experiments were performed at V_{50}, which was a voltage low enough to allow a measurable effect of the tachykinins to be observed, yet high enough to produce relatively consistent responses to nerve stimulation.

All 3 tachykinins tested augmented the responses to preganglionic vagal nerve stimulation at V_{50} in a concentration-dependent manner (Figure 4). In any one experiment, the order of potency was NKA > eledoisin > SP. However, the % increase caused by a given concentration of tachykinin varied considerably between preparations. It is probable that this was due to the steep voltage-effect relationship (Figure 3), which meant that any slight deviation from the V_{50} would produce a large difference in % increase between preparations. A comparison of Figures 1 and 4 again illustrates that the augmentation caused by the tachykinins could be seen at concentrations that produced very little rise in ILP.

The magnitude of the augmenting effect of the tachykinins was inversely related to stimulation frequency. Figure 5 illustrates this for SP. Addition of 1 M SP to the organ bath caused a 31% increase in the response to preganglionic vagal stimulation at 30 Hz (Figure 5a), but the same concentration of SP produced a 76% increase in response to stimulation at 5 Hz (Figure 5b). Both SP challenges caused comparable small rises in ILP.

The effect of stimulation frequency on the response to nerve stimulation in the presence and absence of NKA is shown graphically in Figure 6. The control frequency–response curve was obtained

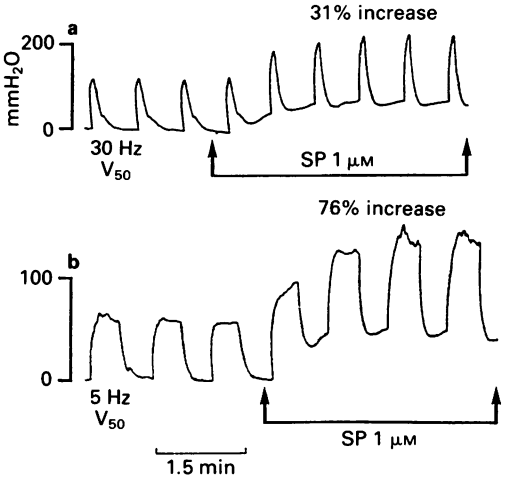

Figure 5 Effect of 1 μM substance P (SP) on responses of the guinea-pig isolated tracheal tube preparation to preganglionic vagal nerve stimulation at two different frequencies (V_{50}; 0.2 ms pulse width). SP caused a 31% increase in the response to stimulation at 30 Hz (a) and a 76% increase in response at 5 Hz (b). The rise in intraluminal pressure produced by 1 μM SP was similar after both challenges. The number of impulses in the train was kept constant (at 150) by increasing the train length as the frequency was decreased. Indomethacin (5 μM) was present throughout.

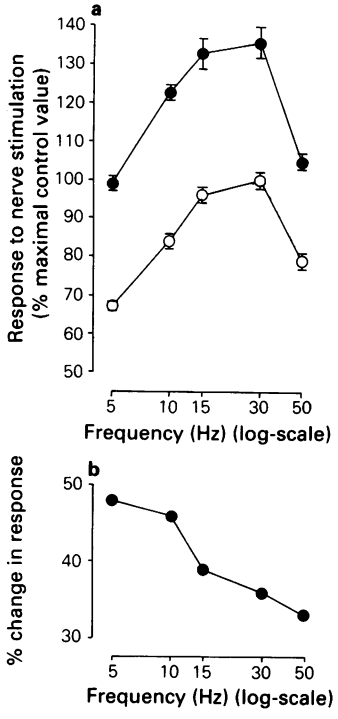

Figure 6 (a) Effect of 5 nM neurokinin A (NKA, ●) on responses of the guinea-pig isolated tracheal tube preparation to preganglionic vagal nerve stimulation at different frequencies (V_{50}; 0.2 ms pulse width). Controls (○) in the absence of NKA. Each point represents the mean of 5 measurements; vertical lines indicate s.e.mean. (b) The % change in response at different frequencies, calculated from the data in (a). Indomethacin (5 μM) was present throughout.

by making step-wise increases in stimulation frequency in the absence of NKA. NKA 5 nM caused an upward shift of the frequency–response curve, but the % change in response in the presence of the tachykinin was greatest at low frequencies.

Discussion

The present results demonstrate that tachykinins cause concentration-dependent contractions of the guinea-pig isolated tracheal tube preparation. Previous studies (mostly with SP) have generally indicated that this effect is due to a direct action of the peptide(s) through receptors located on the trachealis muscle cells (Nilsson *et al.*, 1977; Pernow, 1983). There is good evidence for this hypothesis, since the SP-induced contractions in human and guinea-pig isolated airway preparations are unaltered by atropine or mepyramine (Mizrahi *et al.*, 1982; Lundberg *et al.*, 1983), histamine antagonists, α-adrenoceptor antagonists or β-adrenoceptor antagonists (Malo *et al.*, 1986). However, there have also been at least two accounts of an effect of atropine on SP-induced contractions of airway-smooth muscle (Tanaka & Grunstein, 1984; Malo *et al.*, 1986). In the experiments described in this paper, we found no effect of atropine on the tachykinin-induced contractions, which suggests a direct action of the peptides on the tracheal smooth muscle. The order of potency NKA > eledoisin $\gg$ SP, observed in our experiments, is indicative of an NK_2 receptor, and these findings are consistent with previous studies of guinea-pig tracheal rings *in vitro* (Karlsson *et al.*, 1984; Uchida *et al.*, 1987; Grandordy *et al.*, 1988).

Airway epithelium may attenuate the contractile response to tachykinins and with intact epithelium, as in our tracheal tube preparations, there may be a marked blunting of the response (Tschirhart & Landry, 1986; Grandordy *et al.*, 1988). This may be due to the release of an epithelial relaxant factor (Cuss & Barnes, 1987), but a more likely explanation is that the major metabolising enzyme for tachykinins, neutral metallo-endopeptidase, or enkephalinase, is present in airway epithelial cells (Sekizawa *et al.*, 1987; Frossard *et al.*, 1988).

The major finding in our study was the concentration-dependent enhancement by tachykinins of contractile responses of the tracheal tube preparation to electrical stimulation of cholinergic nerves. This effect was observed even at very low concentrations of trachykinins, where there was little or no direct effect on ILP. The responses both to preganglionic vagal stimulation and to transmural stimulation (in the presence of propranolol) were increased by the tachykinins, but no direct comparison between pre- and post-ganglionic effects was made. On the basis of our study, it is therefore not possible to speculate on a neuromodulatory role for the peptides in the parasympathetic ganglia.

Our experiments showed no effect of tachykinins on the contractile response to exogenous ACh, endorsing the findings of Tanaka & Grunstein (1986), who showed that SP did not affect the cumulative methacholine dose-response curve in the rabbit. This suggests that the augmentation of neurally-mediated responses by the tachykinins involves a prejunctional rather than a postjunctional mechanism, and is probably due to facilitation of ACh release from the nerve terminals. The rank order of potency for the augmentation of responses was NKA $\geqslant$ eledoisin > SP, and this indicates that the neural effect is also mediated via an NK_2 receptor. This correlates with findings in the guinea-pig gastrointestinal tract, in which an NK_2 receptor is involved in modulating ACh release from the mesenteric plexus (Featherstone *et al.*, 1986).

We have also shown that stimulation frequency and voltage affect the magnitude of augmentation by tachykinins of neurally-mediated contractions. A greater enhancement was observed at low stimulation frequencies, and this is consistent with the findings of Tanaka & Grunstein (1986) for SP in the rabbit trachea. The stimulation voltage had a very marked effect on the degree of augmentation observed. When supramaximal voltages were used, as is conventional in these studies, augmentation by tachykinins was small, but increased sharply as voltage was reduced. It may be relevant that, in the study of rabbit trachea (Tanaka & Grunstein, 1986) in which a marked augmentation by SP on responses to electrical field stimulation was observed, the stimulation voltage was probably submaximal. The mechanism for greater enhancement at submaximal voltages is not clear, but one possible explanation may be that tachykinins facilitate the spread of impulses and increase the probability of neurotransmitter release from cholinergic nerve varicosities. In sympathetic nerves, facilitation of transmitter release appears to be due to an increase in the probability of release without any change in the shape or propagation of the nerve action potential, suggesting that it may be due to alteration in stimulus-secretion coupling mechanisms in the invaded varicosity (Brock & Cunnane, 1987). It could be postulated that tachykinins, by activating NK_2 receptors on cholinergic nerve terminals, lead to some intracellular biochemical change in the nerve terminal which increases the probability of ACh secretion when the membrane is depolarized. Several neurotransmitters and neuropeptides influence cholinergic neurotransmission in guinea-pig airways, and therefore may contribute to the regulation of bronchomotor tone (Grundstrom *et al.*, 1981; Fryer & Maclagan, 1984; Maclagan, 1987; Stretton & Barnes, 1988).

The effect of tachykinins on cholinergic neurotransmission in guinea-pig airways may have physiological relevance. The release of NKA from afferent nerves in guinea-pig airways (Uchida *et al.*, 1987) may enhance bronchial tone and may facilitate cholinergic reflex bronchoconstriction, thereby increasing the 'gain' of these reflexes. Whether our findings are relevant to human airways is not yet certain. Axon reflex mechanisms have been implicated in asthma (Barnes, 1986) and, if tachykinins are released, this may lead to enhanced neurally-mediated bronchoconstriction.

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