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Facilitatory effects of selective agonists for tachykinin receptors on cholinergic neurotransmission: evidence for species differences

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1 Exogenous tachykinins modulate cholinergic neurotransmission in rabbit and guinea-pig airways. We have investigated the effect of selective tachykinin receptor agonists and antagonists on cholinergic neurotransmission evoked by electrical field stimulation (EFS) of bronchial rings in rabbit, guinea-pig and human airways *in vitro* to assess which type of tachykinin receptor is mediating this facilitatory effect.

2 Bronchial rings were set up for isometric tension recording. Contractile responses to EFS (60 V, 0.4 ms, 2 Hz for 10 s every min) and exogenous acetylcholine (ACh) were obtained and the effects of selective tachykinin agonists and antagonists were investigated.

3 In rabbit bronchi the endogenous tachykinins, substance P (SP) and neurokinin A (NKA) (10 nM) potentiated cholinergic responses to EFS (by 287.6 ± 121%, $P<0.01$ and 181.4 ± 56.5%, $P<0.001$ respectively).

4 The NK_1 receptor selective agonist, [Sar^9]SP sulphone (10 nM) evoked a maximal facilitatory action on cholinergic responses of 334.9 ± 63% ($P<0.01$) ($pD_2 = 8.5 \pm 0.06$) an effect which was blocked by the selective NK_1-receptor antagonist, CP 96,345 (100 nM) ($P<0.05$) but not by the NK_2 receptor antagonist, MEN 10,376 (100 nM). The NK_2 receptor selective agonist, [βAla^8]NKA(4-10) (10 nM), produced a maximum enhancement of 278 ± 83.5% ($P<0.01$) ($pD_2 = 8.7 \pm 0.1$) an effect which was blocked by MEN 10,376 (100 nM) ($P<0.05$) and not by CP 96,345. [MePhe^7]NKB, an NK_3 receptor selective agonist was without effect.

5 The rank order of potency of NK_2 receptor antagonists against enhancement of cholinergic responses by [βAla^8]NKA(4-10) was MEN 10,376 > L 659,877 > R 396. This pattern together with the observation of the full agonist activity of MDL 28,564 indicates that the NK_2 receptors in the rabbit bronchus are similar to those which are present in the rabbit pulmonary artery.

6 Neither [Sar^9]SP sulphone (5 nM) nor [βAla^8]NKA(4-10) (1 nM) had any effect on contractile responses to ACh (10 μM) suggesting a pre-junctional mechanism of action.

7 By contrast, in guinea-pig bronchi only the NK_1-receptor agonist [Sar^9]SP sulphone (3 nM) was effective in enhancing cholinergic neurotransmission but the effect was relatively small (maximal enhancement 25.7 ± 5.5%, $P<0.01$). In human bronchial rings all the selective neurokinin agonists were without effect on cholinergic neurotransmission.

8 These results suggest that tachykinins may play an important role in modulating cholinergic neurotransmission in rabbit (via NK_1 and NK_2 receptors) and guinea-pig airways (via NK_1 receptor) but have no demonstrable effect on human airways

Keywords: Tachykinins; cholinergic neurotransmission; bronchial smooth muscle; contraction

Introduction

The tachykinins, substance P (SP) and neurokinin A (NKA) are localized to sensory nerves in the airways of several species, including man (Lundberg *et al.*, 1984; Martling *et al.*, 1987; Uddman *et al.*, 1987; Takeda *et al.*, 1990). Tachykinins have been shown to increase mucus secretion (Rogers *et al.*, 1989), vascular permeability (Rogers *et al.*, 1988), increase airway blood flow (Salonen *et al.*, 1988) and produce bronchoconstriction (Advenier *et al.*, 1987).

A neuromodulatory role has also been proposed for neuropeptides in animal airways. In rabbit trachea, SP-induced bronchoconstriction is significantly reduced by atropine suggesting that SP releases acetylcholine (ACh) from cholinergic nerve terminals (Tanaka *et al.*, 1986). In addition, SP potentiates cholinergic nerve-induced contractions in rabbit trachea *in vitro* via a postganglionic, prejunctional mechanism (Armour *et al.*, 1991). Exogenous tachykinins also potentiate cholinergic neurotransmission in guinea-pig trachea (Hall *et al.*, 1989; Watson *et al.*, 1993).

Endogenous tachykinins may also facilitate cholinergic neurotransmission because capsaicin pretreatment, which depletes sensory nerves of tachykinins, results in a significant reduction in cholinergic responses both *in vitro* and *in vivo* in guinea-pig airways (Stretton *et al.*, 1992). Capsaicin, at a sub-threshold concentration, acutely releases tachykinins which enhance cholinergic neurotransmission in guinea-pig trachea *in vitro* (Aizawa *et al.*, 1990). In addition, the inhibitor of neutral endopeptidase (NEP), phosphoramidon, increased the amplitude of contractions produced by preganglionic vagal stimulation in guinea-pig trachea and this effect is blocked by capsaicin pretreatment (Watson *et al.*, 1993). However, Aizawa *et al.* (1990) reported that phosphoramidon also enhanced contractions to electrical field stimulation (EFS) in guinea-pig trachea without changing responses to exogenous ACh. In addition, Sekizawa and colleagues have demonstrated that NEP inhibitors increase

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the vagally-mediated contractions in ferret trachea (Sekizawa *et al.*, 1987). These results suggest that endogenous tachykinins released from afferent sensory nerves may normally facilitate cholinergic neurotransmission pre- and post-ganglionically.

Finally, in human bronchus, NKA potentiates cholinergic neural responses but only in the presence of K^+-channel blockade and this modulation occurs prejunctionally (Black *et al.*, 1990).

The aim of this study was to investigate the effect of receptor-selective tachykinin agonists and antagonists for NK_1 or NK_2 receptors on cholinergic neurotransmission evoked by electrical field stimulation of bronchial rings in rabbit, guinea-pig and human airway smooth muscle in order to define more clearly the nature of the prejunctional tachykinin receptor on airway cholinergic nerves.

Methods

Animal studies

Male albino rabbits (3.0–3.5 kg) and male albino guinea-pigs (240–280 g) were stunned and bled. The lungs, trachea and main bronchi were removed and placed in standard Krebs solution containing indomethacin (5 μM) of the following composition (mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.5, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11; it was gassed continuously with a mixture of 95% O_2/5% CO_2 to give pH 7.4.

Indomethacin was present throughout to prevent fading of neural responses due to endogenous prostaglandin production. Under these experimental conditions reproducible contractile responses to electrical field stimualtion could be achieved for several hours. The main bronchi were isolated and gently rubbed several times on their internal surface by means of a cotton-tipped applicator in order to remove the epithelium, as described previously (Maggi *et al.*, 1990b). The epithelium was removed as it is known that in animal and human airways removal of this layer increases the *in vitro* responsiveness of smooth muscle stimulation by numerous bronchoconstrictor agents including tachykinins (Devillier *et al.*, 1988; Naline *et al.*, 1989). Several theories have been postulated to explain this inhibitory effect exerted by the epithelium on smooth muscle contraction, such as the release of an epithelial derived ralaxant factor (Barnes *et al.*, 1985). In addition, the epithelial inhibitory effect can be, at least in part, due to the degradation of peptides by enkephalinase (endopeptidase 24.11) (Devillier *et al.*, 1988). Therefore, in these experiments we chose to remove the epithelial layer and to conduct the experiments in the presence of peptidase inhibitors (as described below). Each bronchial ring was suspended in a 5 ml organ bath containing Krebs solution and maintained at 37°C. The tissues were allowed to equilibrate for 1 h with frequent washing, under a resting tension of 0.5 g, which was found to be optimal for measuring changes in tension. Contractile responses were measured by means of an isometric transducer connected to a Basile 7050 pen recorder.

Human studies

We studied airways from 6 patients (between 20–47 years, 5 male) obtained from heart or single lung donor patients with brain death. There was no evidence of heart or lung disease in these donors.

Lung tissue was immediately placed in oxygenated Krebs solution and cooled to 4°C and transported to the laboratory. Bronchi (from the level of distal lobar to segmental with internal diameters of 7–9 mm and 3–5 mm respectively) from normal donors were dissected free from adjacent tissue. The bronchi were macroscopically removed and bronchial preparations were then suspended in 10 ml organ baths containing Krebs solution at 37°C with 95% O_2/5% CO_2 (pH 7.4). Indomethacin (5 μM) was present throughout. Bronchial rings were connected via silk threads to Grass FT.03 force-displacement transducers and recorded on a polygraph for the measurement of isometric changes in tension. The tissues were allowed to equilibrate for 1 h with frequent washing under a resting tension of 2 g which was found to be optimal for measuring changes in tension.

In additional experiments, strips of smooth muscle were taken from the major bronchus of three non-smoking donor patients (between 25–42 years, one male) for heart-lung, heart or single lung transplantation. The epithelium was removed by careful dissection, minimizing damage to the smooth muscle; this was confirmed later by macroscopic histology. These experiments were performed so that the studies in human tissue could be compared with those in animal tissue in terms of the airway level studied and the epithelial removal.

Electrical field stimulation

Electrical field stimulation was performed by means of two wire platinum electrodes placed at the top and the bottom of the organ bath for animal tissue and parallel to each other for human tissue, connected to a Grass S88 stimulator (Grass Instruments, Quincy, MA, U.S.A.). Trains of stimuli (2 Hz; 60 V; 0.4 ms pulse width for 10 s) were delivered at 1 min intervals until reproducible responses were obtained. Control experiments showed that there was no significant fading of the response to field stimulation during the experimental period. Contractile responses to field stimulation were inhibited by both tetrodotoxin (0.3 μM) and atropine (1 μM) in all species suggesting that the contractile response was due to stimulation of cholinergic nerves. Experiments were carried out in the presence of peptidase inhibitors (bestatin, captopril and thiorphan, 1 μM each, to inhibit aminopeptidase, angiotensin converting enzyme and endopeptidase 24.11, respectively). Peptidase inhibitors were added 15 min prior to the start of the following experiments. In each experiment, the response to KCl (80 mM added to the bath) was used as an internal standard.

Capsaicin treatment

In some experiments, to avoid any modulation of cholinergic responses to EFS by endogenous tachykinins, guinea-pig and rabbit bronchi were pretreated with capsaicin to deplete neuropeptides. *In vitro* capsaicin desensitization was achieved by prolonged (30 min) exposure of bronchial rings to 10 μM capsaicin, followed by washing and re-equilibration (Geppetti *et al.*, 1990).

Effects of agonists

In these experiments concentrations of SP, NKA, [Sar^9]SP sulphone, [βAla^8]NKA(4–10), [$MePhe^7$]NKB (each between 0.1 nM–30 nM) and MDL 28,564 (0.1 nM–1 μM) were added in a randomized fashion and the effect on responses to subsequent electrical field stimulation investigated. Tissues were washed between concentrations. If the agonist produced a contractile response which resulted in a rise in baseline, EFS was continued and the measurement taken when the contractile response had subsided. Only one agonist was tested per tissue. [Sar^9]SP sulphone is a potent and selective NK_1 receptor agonist (Drapeau *et al.*, 1987) while [βAla^8]NKA(4–10) is a selective NK_2 receptor agonist (Patacchini *et al.*, 1989). MDL 26,564 is a highly selective NK_2 receptor agonist which behaves as a full agonist at one NK_2 receptor subtype while being a competitive antagonist at the other (Buck *et al.*, 1990). [$MePhe^7$]NKB is a selective NK_3 receptor agonist (Drapeau *et al.*, 1987).

In a separate series of experiments the effects of the selective agonists, which were effective at enhancing contractile

responses to EFS, were investigated on responses to the postjunctional effects of acetylcholine (ACh). The effects of the selective neurokinin agonists were studied on the contractile response evoked by a concentration of ACh (10 μM) that produced a response similar in magnitude as that evoked by EFS at the above parameters.

Effect of antagonists

In the next series of experiments the effects of tachykinin antagonists ((±)-CP-96,345 for NK_1 receptors, MEN 10,376 for NK_2 receptors) against the enhancement of cholinergic contractile responses produced by a NK_1-selective ([Sar^9]SP sulphone) (5 nM) or a NK_2-selective ([βAla$_8$]NKA(4–10) (1 nM) agonist was investigated at concentrations that approximated the EC_{50} values for the agonists. The contact time for the antagonists was 15 min. Two reproducible responses to the agonist were obtained prior to administration of the antagonist.

In another series of experiments the effects of other competitive antagonists (0.3 μM–1 μM) for NK_2 receptors were investigated on the enhancement of cholinergic responses produced by the NK_2 agonist, [βAla^8]NKA(4–10) (1 nM). L659,877 is a cyclic peptide introduced by Williams *et al.* (1988) which is selective for NK_2 receptors. MEN 10,376 and R396 are two linear peptides endowed with marked selectivity for NK_2 over NK_1 or NK_3 tachykinin receptors (Maggi *et al.*, 1990a; 1991a; Dion *et al.*, 1990) which also discriminate between different NK_2 receptor subtypes (Maggi *et al.*, 1990a; Van Giesbergen *et al.*, 1991).

Drugs

Drugs used were: acetylcholine chloride, capsaicin, indomethacin, bestatin, thiorphan, (Sigma, St Louis, MO, U.S.A.), captopril (Squibb), tetrodotoxin (Sankyo, Tokyo, Japan), atropine (Serva, Heidelberg, Germany). All drugs were dissolved in distilled water and stored in aliquots at 20°C. Capsaicin was dissolved in absolute ethanol and diluted in distilled water.

Peptides

Subtance P (SP), neurokinin A (NKA), [βAla^8]NKA(4–10), [Tyr^5, D-Trp^{6,8,9}, Lys^{11}]-NKA(4–10) (MEN 10,376) and [MePhe^7]neurokinin B were synthesized at Menarini Laboratories (Florence, Italy) by conventional solid-phase methods. L659,877 was obtained from Cambridge R.B. (Cambridge, U.K.). MDL 28,564 and R 396 were kind gifts from Dr S.H. Buck, Marion Merrell Dow Research Institute, and Prof. D. Regoli, Department of Physiology and Pharmacology, University of Sherbrooke, Canada, respectively. The selective NK_1 receptor agonist, [Sar^9]SP sulphone, was purchased from Peninsula (San Carlos, U.S.A.). The selective NK_1 receptor antagonist ([(2S, 3S)-*cis*-2-(diphenylmethyl)-N-[methoxyphenyl)-methyl]-1-azabicyclo(2,2,2]octan3-amine]) ((±)-CP-96,345) was synthesized as previously described (Lecci *et al.*, 1991). The amino acid sequence of the peptide agonist and antagonists used in this study is shown in Table 1.

Aliquots of SP, NKA, [MePhe^7]NKB, [Sar^9]SP sulphone were dissolved in distilled water and stored at −20°C. Stock solutions (1–10 mM) of all the other peptides were made in dimethyl sulphoxide (DMSO) then diluted in water.

Analysis of results

Contractile responses were expressed as absolute changes in tension, and then transformed to a mean response for 3 control stimulations obtained to EFS in each tissue. The effect of the tachykinin agonists and antagonists on the mean responses was then expressed as a percentage increase. A mean value for 2 successive contractile responses to an ACh bolus was calculated in mg tension and the effect of tachykinins on the mean response analysed. The effects of exogenous drug additions on contractile responses evoked by EFS and acetylcholine in each tissue were assessed by use of the Student's *t* test (one-tailed) for paired data. Probability values of <0.05 were considered significant. EC_{50} values were calculated by using a non-linear iterative curve fitting programme, Inplot (Graphpad Inc. CA, U.S.A.).

Results

Effect of the endogenous tachykinins SP and NKA on cholinergic neurotransmission in rabbit bronchi

SP (0.1–30 nM) significantly potentiated cholinergic contractile responses evoked by EFS in a concentration-dependent manner (maximum enhancement evoked by SP (10 nM) 287 ± 121%, $P<0.01$, $n=6$) (Figure 1). This amounted to an increase from 204 ± 67.2 mg to 445 ± 47.6 mg of evoked contraction in absolute values. At this concentration, SP

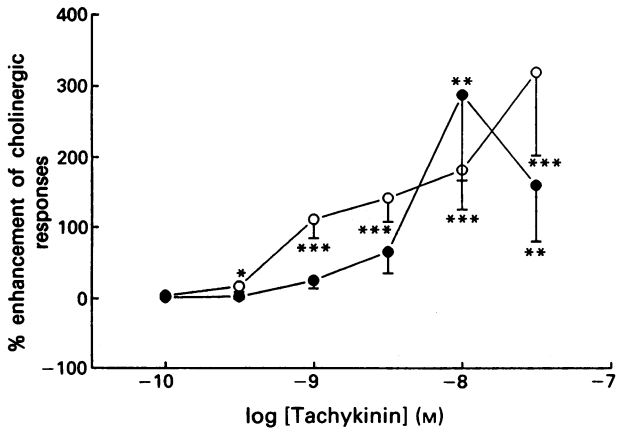


Figure 1 Concentration-dependent facilitation of cholinergic responses to electrical field stimulation (EFS: 60 V, 0.4 ms, 2 Hz for 10 s every min) in rabbit bronchi by neurokinin A (0.1–30 nM, ○) and substance P (0.1–30 nM, ●). Values are mean ($n=4$–9 observations) ± s.e.mean; significance of enhancement: $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$.

Table 1 Amino acid sequences of peptides used in this study

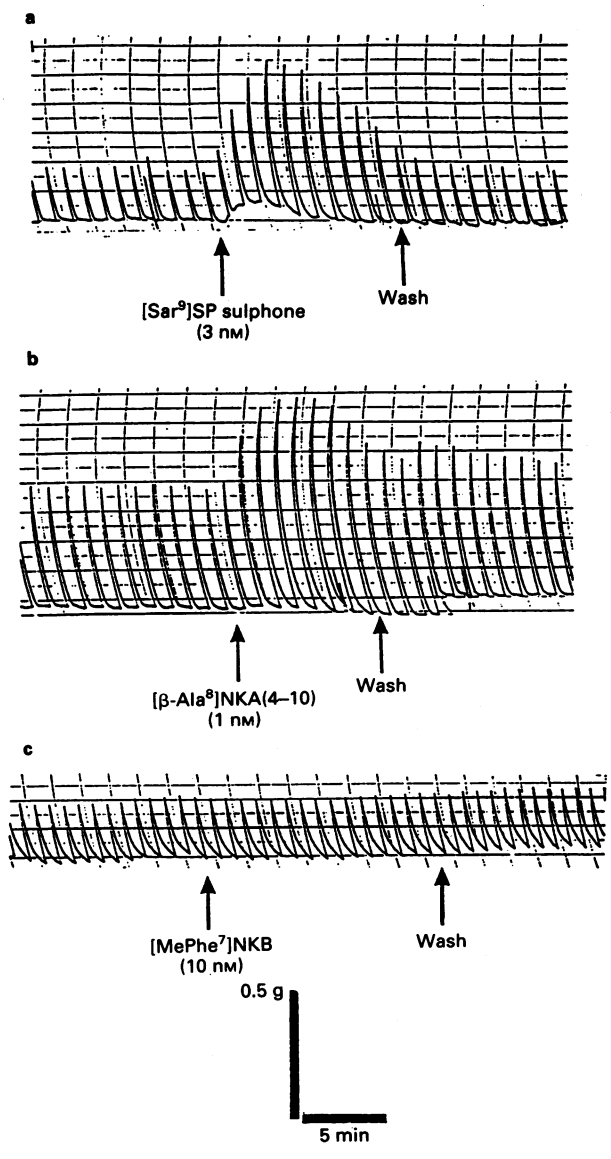
Agonists	
[Sar^9,Met(O$_2$)^{11}]SP	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Sar-Leu-Met(O$_2$)-NH$_2$
[βAla^8]NKA(4–10)	H-Asp-Ser-Phe-Val-βAla-Leu-Met-NH$_2$
[MePhe^7]NKB	H-Asp-Met-His-Asp-Phe-Phe-MePhe-Gly-Leu-Met-NH$_2$
MDL 28,564	H-Asp-Ser-Phe-Val-Gly-Leu (CH$_2$NH)Leu-NH$_2$
Antagonists	
MEN10,376	H-Asp-Tyr-D-Trp-val-D-Trp-D-Trp-Lys-NH$_2$
L659,877	cyclo(Leu-Met-Gln-Trp-Phe-Gly)
R 396	Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH$_2$

produced contraction of rabbit bronchi (222.5 ± 50.4 mg, $n = 5$). However, this increase in tension cannot account for the enhancement in the cholinergic contractile response as this was measured when the initial contractile response had returned to baseline values. In fact, other workers have previously shown that the rise in baseline alone cannot account for the potentiation of EFS-induced contraction in rabbit airway smooth muscle (Armour *et al.*, 1988).

NKA (0.1–30 nM) also significantly enhanced cholinergic neurotransmission in a concentration-dependent manner (maximum enhancement evoked by NKA (30 nM) 319.2 ± 117%, $P < 0.001$, $n = 9$) (Figure 1b). This corresponds to an increase from 156 ± 52.7 mg to 406 ± 75.3 mg of evoked contraction in absolute values. At this concentration NKA evoked a contractile response in rabbit bronchi (942.4 ± 127.5 mg). However, NKA (0.3 nM) enhanced contractile responses to EFS by 16.9 ± 7.8% ($P < 0.05$, $n = 9$) and at this concentration produced no increase in baseline tone.

Effect of selective agonists for tachykinin receptors on cholinergic responses to EFS in rabbit bronchi

The NK_1 receptor-selective agonist, [Sar^9]SP sulphone (0.1–30 nM) evoked a concentration-dependent increase in cholinergic responses to EFS (Figure 2a and Figure 3). [Sar^9]SP sulphone (10 nM) produced a maximal increase in cholinergic responses of 334.9 ± 63% ($P < 0.01$, $n = 8$) with a pD_2 value of 8.5 ± 0.06. In further experiments involving the use of selective tachykinin receptor antagonists a concentration of [Sar^9]SP sulphone was chosen (5 nM) which approximated the pD_2 value. A concentration of 10 nM [Sar^9]SP sulphone produced contraction of rabbit bronchi (378.6 ± 90.8 mg, $n = 7$). However, [Sar^9]SP sulphone (3 nM) still produced considerable enhancement of cholinergic responses to EFS (186.2 ± 62.9% enhancement, $P < 0.05$, $n = 7$) at concentrations that produced negligible bronchoconstriction (27.1 ± 14.6 mg, $n = 6$).



The NK_2 receptor-selective agonist, [βAla^8]NKA(4–10) (0.1–30 nM), also evoked a concentration-dependent increase in cholinergic responses to EFS (Figure 2b and Figure 3). [βAla^8]NKA(4–10) (10 nM) produced a maximum enhancement of 278 ± 83.5% ($P < 0.01$, $n = 4$) with a pD_2 value of 8.7 ± 0.1. In the next set of experiments involving the use of selective tachykinin antagonists, a concentration of [βAla^8]NKA(4–10) was chosen (1 nM) which approximated the pD_2 value. In addition to producing a maximum enhancement of the cholinergic response, [βAla^8]NKA(4–10) (10 nM) also produced contraction of rabbit bronchi (340 ± 41.7 mg, $n = 5$). However, [βAla^8]NKA(4–10) (1 nM) evoked no change in the baseline tone (6.25 ± 6.25 mg, $n = 6$) while the enhancement of cholinergic contractile responses evoked by EFS was considerable (125.6 ± 39.6%, $P < 0.05$, $n = 7$).

[MePhe^7]NKB (0.1–30 nM), an NK_3 receptor-selective agonist, produced no direct contractile response and no enhancement of cholinergic responses to EFS in rabbit bronchi (Figure 2c and Figure 3).

Finally, in the rabbit, MDL 28,564 (10 nM), a highly selective NK_2 receptor ligand, enhanced cholinergic responses by 12.5 ± 4.7% ($P < 0.05$) ($n = 5$) ($pD_2 = 6.7 \pm 0.22$, maximum enhancement 441.3 ± 159% at 1 μM, $P < 0.05$, $n = 6$) (Figure 3). In further experiments involving the use of selective tachykinin receptor antagonists, a concentration of MDL 28,564 was chosen (0.1 μM) which approximated the pD_2 value.

All cholinergic contractile responses evoked to EFS were abolished by atropine (1 μM) or tetrodotoxin (0.3 μM).

Effect of NK_1 and NK_2 receptor agonists on responses to ACh in rabbit bronchi

Both the NK_1 receptor-selective agonist, [Sar^9]SP sulphone (5 nM) and the NK_2 receptor-selective agonist [βAla^8]NKA (4–10) (1 nM), had no effect on contractile responses to ACh (10 μM) in rabbit bronchi ($n = 6$). This suggests that the augmentation of neurally-mediated responses to EFS by agonists selective for the NK_1 and NK_2 receptor involves a prejunctional mechanism.

Effect of NK_1 and NK_2 receptor antagonists on rabbit bronchi

[Sar^9]SP sulphone (5 nM), the NK_1-selective agonist, produced 111.3 ± 37.5% ($n = 7$) enhancement of the cholinergic response, an effect which was not affected by the NK_2-selective antagonist, MEN 10,376 (0.1 μM) (114.9 ± 44.7% enhancement, $n = 7$, NS) and completely blocked by the selective NK_1 receptor antagonist, CP 96,345 (0.1 μM) (4.61 ± 3.2% enhancement, $n = 6$, $P < 0.05$) (Figure 4a).

Figure 2 Trace illustrating the effect of selective tachykinin receptor agonists on cholinergic responses to EFS (60 V, 0.4 ms, 2 Hz for 10 s every 1 min) in rabbit bronchi. (a) Effect of NK_1-receptor agonist, [Sar^9]SP sulphone (3 nM) at a concentration that approximated the EC_{50} value for enhancement of responses. (b) Effect of NK_2-receptor agonist, [βAla^8]NKA(4–10) (1 nM) at a concentration that approximated the EC_{50} value for enhancement of responses. (c) Effect of a maximal concentration of the NK_3-receptor agonist, [MePhe^7]NKB (10 nM).

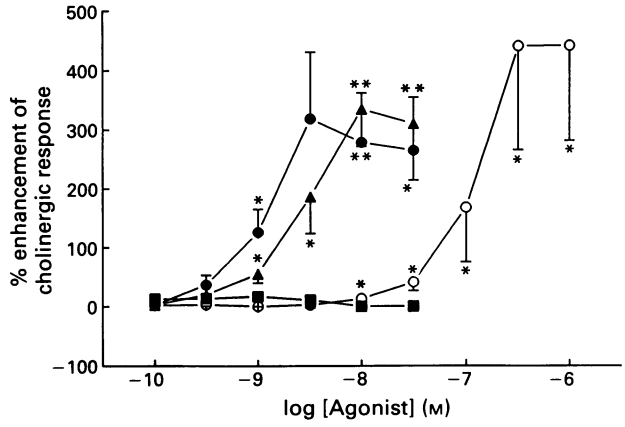


Figure 3 Concentration-dependent facilitation of cholinergic responses to electrical field stimulation (EFS: 60 V, 0.4 ms, 2 Hz for 10 s every min) in rabbit bronchi by [βAla^8]NKA(4–10) (0.1–30 nM) (●), [Sar^9]SP sulphone (0.1–30 nM) (▲), [MePhe^7]NKB (0.1–30 nM) (■) and MDL 28,564 (0.1 nM–1 μM) (○). Values are mean (n = 3–7 observations) ± s.e.mean; significance of enhancement: $^{**}P<0.01$, $^{*}P<0.05$.

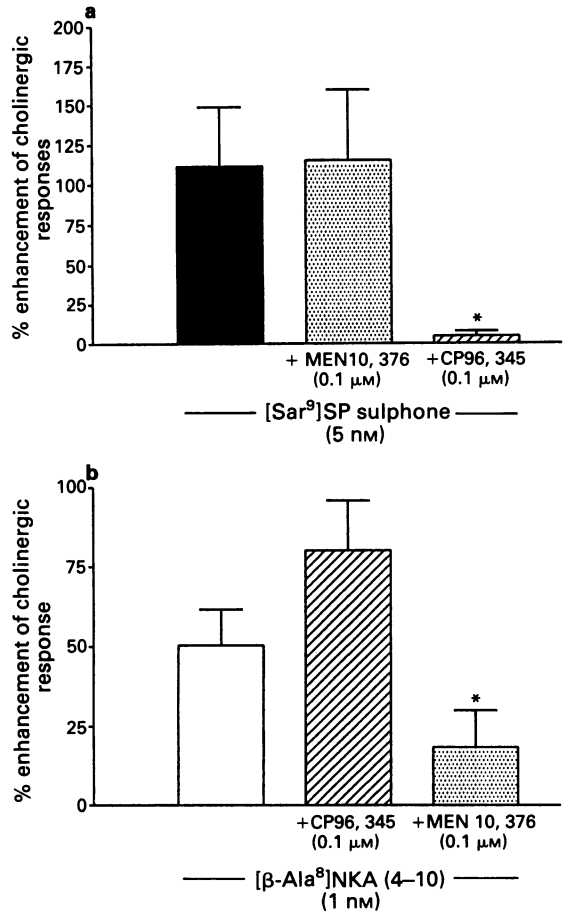


Figure 4 Histogram demonstrating the inhibitory effects of tachykinin antagonists on facilitation of cholinergic responses evoked by EFS produced by selective NK_1 and NK_2 agonists. (a) Enhancement of cholinergic response produced by [Sar^9]SP sulphone (5 nM) (solid column) and the effect of the NK_2 antagonist, MEN 10,376 (0.1 μM) (stippled column) and the NK_1 antagonist CP 96,345 (0.1 μM) (hatched column). (b) Enhancement of cholinergic response produced by [βAla^8]NKA(4–10) (1 nM) (open column) and the effect of the NK_1 antagonist, CP 96,345 (0.1 μM) (hatched column) and the NK_2 antagonist, MEN 10,376 (0.1 μM) (stippled column). Values are mean (n = 6 or 7), with s.e. of mean; significance of inhibition: $^{*}P<0.05$.

[βAla^8]NKA(4–10) (1 nM), the NK_2 receptor-selective agonist, produced 50.4 ± 11.3% (n = 7) enhancement of cholinergic responses evoked by EFS, an effect which was not affected by the NK_1-selective antagonist, CP 96,345 (0.1 μM) (80.2 ± 15.8% enhancement, n = 7, NS) and significantly inhibited by the NK_2 receptor antagonist, MEN 10,376 (0.1 μM) (18.4 ± 11.6% enhancement, n = 7, $P<0.05$) (Figure 4b) and completely inhibited by MEN 10,376 (0.3 μM) (0% enhancement, n = 4, $P<0.05$) (Figure 6a).

MDL 28,564 (0.1 μM), a selective NK_2 receptor agonist, produced 193.2 ± 108% (n = 5) enhancement of cholinergic responses evoked by EFS an effect which was not blocked by the NK_1 receptor-selective antagonist, CP 96,345 (0.1 μM) (256.9 ± 183.2% enhancement, n = 5, NS). However, the NK_2 antagonist, MEN 10,376 (0.1 μM) inhibited this response (83.8 ± 50% enhancement, n = 5, NS) and MEN 10, 376 (0.3 μM) completely abolished this enhancement (0% enhancement, n = 4, $P<0.05$) (Figure 5).

[βAla^8]NKA(4–10) (1 nM) evoked a 264.9 ± 133.2% enhancement of cholinergic responses to EFS, an effect which was completely antagonized by, MEN 10,376 (0.3 μM) (0% enhancement, n = 4, $P<0.05$) (Figure 6a). However, L659,877 (0.3 μM) was without effect on enhancement of responses produced by the same concentration of [βAla^8]NKA(4–10) (1 nM) (98.7 ± 16.2% enhancement in the absence and 100.1 ± 16.3% enhancement in the presence of L659,877, n = 5, NS) (Figure 6a). Finally, R396 (0.3 μM) (another NK_2 antagonist) was also without effect (81.1 ± 22.1% enhancement by [βAla^8]NKA(4–10) in the absence and 148.7 ± 66.6% enhancement in the presence of R 396, n = 5, NS) (Figure 6a). The response to [βAla^8]NKA(4–10) (1 nM) was variable (the range of potentiation evoked being between 81 to 264%). However, the variation was probably due to the fact that in the first group (the data with MEN 10,376) the maximum potentiation of the cholinergic responses evoked was 550% and the minimum 21.4% leading to a large standard error in this group. However, as each tissue was used as its own control this does not influence the results obtained with the antagonists as the inhibition achieved was not dependent upon the magnitude of the initial facilitation of the cholinergic contractile response.

L659,877 (1 μM) antagonized the enhancement of the response to [βAla^8]NKA(4–10) (1 nM) (147.5 ± 68.6% enhancement in the absence and 8.4 ± 5.0 in the presence of L659, 877, n = 4, $P<0.05$) (Figure 6b). R 396 was inactive at concentrations up to 1 μM; [βAla^8]NKA(4–10) (1 nM) evoked a 69.2 ± 23% increase in cholinergic responses to EFS in the absence and 87.1 ± 41.6% increase in the presence of R 396 (1 μM) (Figure 6b).

The antagonists used in this study, had no effect on cholinergic responses to EFS at the concentrations stated.

Effect of selective agonists on neurally-evoked, atropine-sensitive contractions in guinea-pig bronchi

In guinea-pig bronchi, there is a prominent excitatory non-adrenergic, non-cholinergic contraction which is due to the release of tachykinins (NKA and SP) (Maggi *et al.*, 1991a,b). Tissues were pretreated with capsaicin (10 μM) to deplete tachykinins so that any results obtained were not affected by the release of endogenous tachykinins. In guinea-pig bronchi. only the NK_1 receptor agonist [Sar^9]SP sulphone (3 nM) was effective at enhancing cholinergic neurotransmission but the effect was relatively small (maximal enhancement 27.5 ± 5.5%, n = 4, $P<0.01$) (Figure 7).

Effect of selective agonists on neurally-evoked, atropine-sensitive contractions of human airways

In human bronchial rings all the selective tachykinin receptor agonists (0.1–30 nM) ([Sar^9]SP sulphone, [βAla^8]NKA(4–10), [MePhe^7]NKB) were without effect on cholinergic neural re-

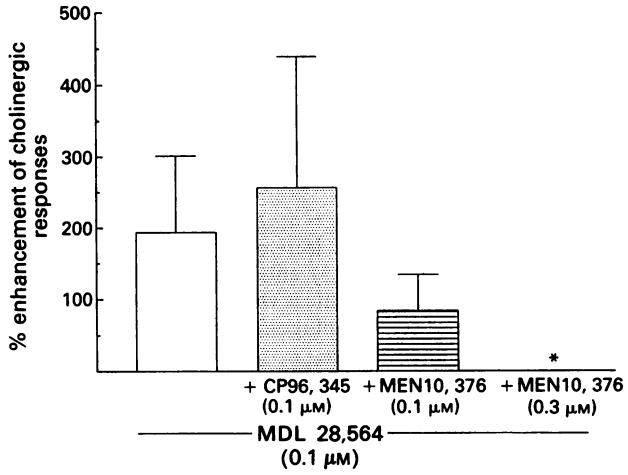


Figure 5 Histogram demonstrating the facilitatory effect of the selective NK_2 agonist MDL 28,564 (0.1 μM) (open column) on cholinergic responses in rabbit bronchi in the presence of the selective NK_1 antagonist, CP 96,345 (0.1 μM) (stippled column), and the NK_2 antagonist, MEN 10,376 (0.1 μM) (horizontal lined column) and (0.3 μM) (complete inhibition). Values are mean ($n = 5$ or 4), with s.e.mean; significance of inhibition: $^*P < 0.05$.

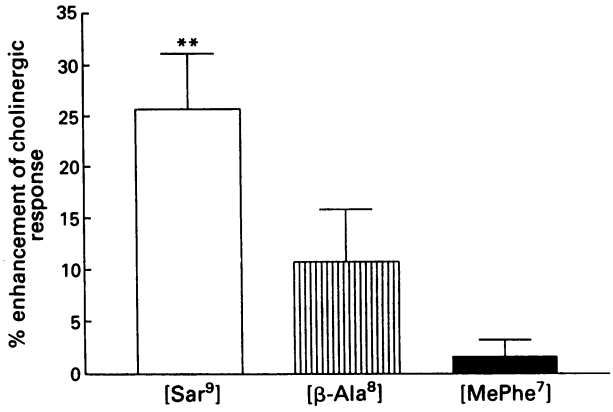


Figure 7 Histogram illustrating the effects of selective tachykinin agonists (each at 3 nM) for the NK_1 receptor ([Sar^9]SP sulphone, open column), the NK_2 receptor ([βAla^8]NKA(4–10, vertical lined column) and the NK_3 receptor ([MePhe^7]NKB, solid column) on cholinergic responses to EFS (EFS: 60 V, 0.4 ms, 2 Hz for 10 s every 10 min) in guinea-pig bronchi. Values are mean ($n = 4$ or 5 observations) with s.e.mean; significance of inhibition: $^*P < 0.01$.

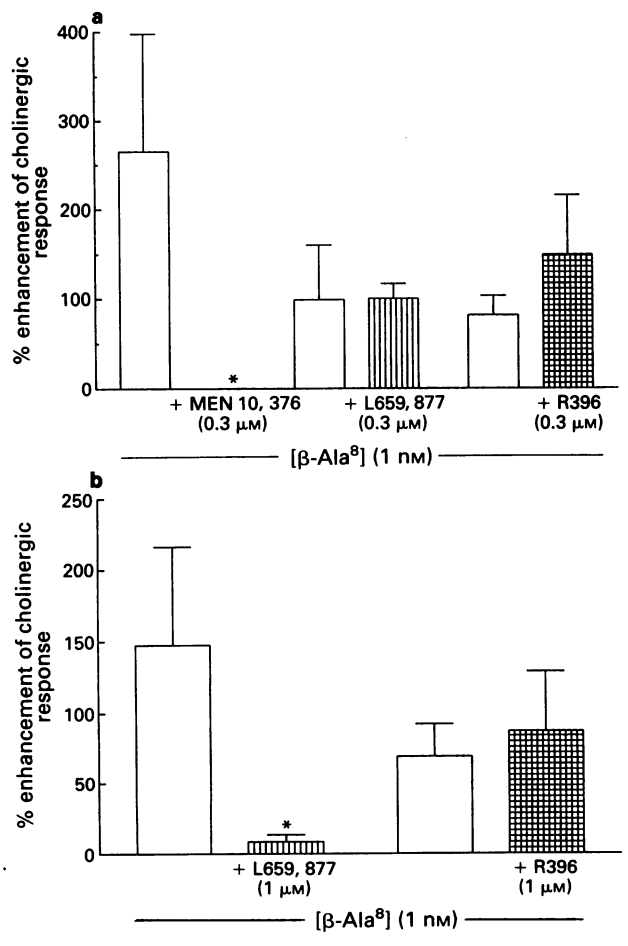


Figure 6 Histogram demonstrating the facilitatory effects of the NK_2 agonist [βAla^8]NKA(4–10) (1 nM) (open column) on cholinergic responses in rabbit bronchi in the presence of (a) the NK_2 antagonist L 659,877 (0.3 μM) (vertical lined column) and another NK_2 antagonist R 396 (0.3 μM) (checked column). (b) L 659,877 (1 μM) (vertical lined column) and R 396 (1 μM) (checked column). Values are mean ($n = 4$ or 5 observations), with s.e.mean; significance of inhibition: $^*P < 0.05$.

sponses at any of the concentrations studied ($n = 6$ patients).

In strips of human major bronchus with the epithelium removed all the above selective tachykinin receptor agonists, at the same concentrations as were used above, were still ineffective on cholinergic neural contractile responses ($n = 3$ patients).

Discussion

The first report that suggested that tachykinins may have a neuromodulatory role in the peripheral nervous system was in the guinea-pig myenteric plexus where SP was found to evoke the release of ACh in a concentration-dependent manner (Yau & Youther, 1982). More recently, it has been suggested that tachykinins may play an important role in modulating cholinergic neurotransmission in airway smooth muscle on the basis of immunohistochemical (Dey *et al.*, 1991) and functional studies.

Exogenous tachykinins have been previously shown to facilitate cholinergic neurotransmission in airway smooth muscle. In rabbit isolated trachea, SP potentiated, in a concentration-dependent manner, contractile responses evoked by cholinergic nerve stimulation via a postganglionic, prejunctional mechanism (Tanaka *et al.*, 1986; Armour *et al.*, 1991). However, in rabbit trachea the tachykinin receptors mediating this effect were not investigated. Exogenous tachykinins also potentiate cholinergic neurotransmission at pre- and postganglionic nerve terminals in guinea-pig trachea (Hall *et al.*, 1989; Watson *et al.*, 1993). The tachykinin receptor mediating these effects appeared to be of the NK_1 receptor subtype (Watson *et al.*, 1993). However, in addition, NKA, which preferentially stimulates NK_2 receptors facilitated contractions evoked by pre- and postganglionic nerve stimulation (Hall *et al.*, 1989; Watson *et al.*, 1993). Therefore, these data do not exclude the involvement of NK_2 receptors. However, since a range of selective agonists and antagonists for NK_2 receptors were not investigated, the receptor classification in these two studies was not conclusive. In human bronchus NKA in the presence of K^+-channel blockade potentiates cholinergic neural responses and this modulation occurs prejunctionally (Black *et al.*, 1990).

The present results demonstrate that the tachykinins NKA and SP can produce concentration-dependent enhancement of contractile responses evoked by EFS of rabbit trachea *in*

vitro, confirming previous data on SP-evoked facilitatory effects (Tanaka *et al.*, 1986; Armour *et al.*, 1991). Further experiments were performed to assess the effects of receptor-selective neurokinin agonists and antagonists on cholinergic neurotransmission to investigate which tachykinin receptors were involved. The selective NK_1 tachykinin receptor agonist, [Sar^9]SP sulphone, was very effective at enhancing responses to EFS, an effect which was blocked by the selective NK_1 receptor antagonist CP 96,345 but not by the NK_2 receptor antagonist, MEN 10,376 suggesting that NK_1 receptor activation may be important in this response. However, the NK_2 receptor-selective agonist [βAla^8]NKA(4–10), also enhanced cholinergic responses to EFS in rabbit bronchi and this effect was blocked by MEN 10,376 but not by CP 96,345 suggesting involvement of NK_2 receptors. NK_3 receptor activation does not seem to be involved in the enhancement of cholinergic neurotransmission by tachykinins as the selective NK_3-receptor agonist, [MePhe^7] NKB, was without effect. Augmented contractile responses evoked by EFS in the presence of the tachykinins e.g. SP and NKA or the selective tachykinin receptor agonists. e.g. [Sar^9]SP sulphone or [βAla^8]NKA(4–10), were completely inhibited by tetrodotoxin and atropine indicating that the augmented airway contractile response was neural in origin and cholinergic in nature. In addition, the potentiating effects of tachykinins (SP and NKA) or the selective agonists for NK_1 and NK_2 receptors were observed even at very low concentrations where there was very little or no change in the contractile state of the tissue.

Neither [Sar^9]SP sulphone nor [βAla^8]NKA(4–10) had any effect on contractile responses to exogenous ACh. This is in agreement with an earlier study in which contractile responses to methacholine in rabbit trachea were unaltered by SP (Tanaka *et al.*, 1986). This implies that the potentiation of contractile responses to EFS by tachykinins is not related to a postjunctional change in airway smooth muscle function, such as changes in the rate of ACh degradation, enhanced muscarinic receptor binding, potentiation of intrinsic contractile processes. Therefore, these results indicate that the enhancement of the cholinergic response produced by tachykinins is likely to be due to an increased prejunctional release of ACh.

The results obtained in the rabbit bronchi suggest the presence of both NK_1 and NK_2 receptors on cholinergic nerves in rabbit bronchi. The full agonist activity of MDL 28,564 and the rank order of potency of the NK_2 receptor antagonists, MEN 10,376, L 659,877 and R 396, indicates that the NK_2 receptors mediating facilitation of ACh release in rabbit bronchi belong to the same subtype that mediates contraction of the endothelium-deprived rabbit pulmonary artery (termed NK_{2A}) (Maggi *et al.*, 1990a).

In guinea-pig bronchi only the NK_1 receptor agonist, [Sar^9]SP sulphone, was effective in enhancing cholinergic neurotransmission but the effect was relatively small. These data are in agreement with Watson *et al.* (1993) who demonstrated that the NK_1 agonist, GR73632, facilitated preganglionic and postganglionic contractile responses to electrical stimulation in guinea-pig trachea. However, the ineffectiveness of the selective NK_2 agonist, [βAla^8]NKA(4–10), seems to suggest that NK_2 receptors are not involved in the facilitation of cholinergic constrictor responses in the guinea-pig bronchi in contrast to the suggestions made in previous reports (Hall *et al.*, 1989; Watson *et al.*, 1993).

In human bronchial rings, none of the selective tachykinin agonists had any effect on cholinergic neurotransmission. In fact, it has been shown previously that NKA produces potentiation of the response to EFS in human bronchi, but only in the presence of K^+ channel blockade. This points to a neuromodulatory role for NKA in human airways, only in situations where the K^+ channel activity is decreased (Black *et al.*, 1990).

Facilitatory effects of tachykinins on cholinergic neurotransmission may have physiological relevance as there has been some suggestion that endogenous tachykinins facilitate cholinergic contractile responses in airway smooth muscle. The metallopeptidase neutral endopeptidase 24.11 is a major enzyme involved in the breakdown of tachykinins (Erdos & Skidgel, 1989). Inhibition of this enzyme by phosphoramidon (an inhibitor of neutral endopeptidase) would be expected to augment the actions of endogenously released tachykinins. In guinea-pig trachea phosphoramidon facilitates contractile responses evoked by preganglionic vagal nerve stimualtion but not transmural stimulation in a concentration-dependent manner and this effect is blocked by capsaicin pretreatment (Watson *et al.*, 1993). These results indicate that there is release of endogenous tachykinins during pre- but not postganglionic nerve stimulation in guinea-pig trachea suggsting that there are facilitatory tachykinin receptors (probably of the NK_1 receptor subtype) at the level of the parasympathetic ganglia (Watson *et al.*, 1993). However, Aizawa *et al.* (1990) have reported that phosphoramidon also enhances contractions to EFS in guinea-pig trachea without changing responses to exogenous ACh. In addition, Sekizawa *et al.* (1987) have demonstrated that NEP inhibitors increase the contractions of the ferret trachea induced by EFS. Some of these discrepancies may be due to species differences. Capsaicin pretreatment, which depletes sensory nerves of tachykinins, results in a significant reduction in cholinergic responses both *in vivo* and *in vitro* in guinea-pig airways (Stretton *et al.*, 1992) suggesting a role for endogenous tachykinins in the facilitation of cholinergic neurotransmission. In addition, capsaicin, at a sub-threshold concentration, acutely releases tachykinins which enhance cholinergic responses in guinea-pig trachea *in vitro* again indicative of endogenous tachykinin-induced modulation of cholinergic responses (Aizawa *et al.*, 1990). In this study endogenous tachykinins failed to facilitate cholinergic responses to field stimulation in rabbit bronchi as there was no effect of the selective tachykinin antagonists in the absence of exogenous agonist. These data are in agreement with Watson *et al.* (1993) who demonstrated that endogenous tachykinins facilitate cholinergic nerve-induced contractions at the level of the parasympathetic ganglia in guinea-pig airways and, in addition, that facilitatory tachykinin receptors on postganglionic nerve terminals can only be demonstrated by exogenous agonists. Other workers (mentioned above) have demonstrated an effect of endogenous tachykinins on facilitation of cholinergic contractile responses but they have demonstrated these effects with tools such as phosphoramidon and capsaicin which may have actions other than those on endogenous tachykinins.

These results suggest that tachykinins may play an important role in modulating cholinergic neurotransmission in rabbit (via NK_1 and NK_2 receptors) and guinea-pig (via NK_1 receptors) airways with no demonstrable effects on human airways. However, this does not rule out a role for endogenous tachykinins in the modulation of cholinergic neurotransmission in human airways. Another consideration is that while this may not be important under normal conditions, the system may be active in disease. For example, if K^+ channels were impaired in disease then modulatory effects of tachykinins on cholinergic neurotransmission may become evident.

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