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Effect of non-peptide tachykinin NK_1 receptor antagonists on non-adrenergic, non-cholinergic neurogenic mucus secretion in ferret trachea

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Abstract

We investigated, in ferret trachea in vitro, the binding characteristics and the inhibition of non-adrenergic, non-cholinergic (NANC) neural mucus secretion of four tachykinin receptor antagonists: the non-peptide tachykinin NK_1 receptor antagonists CGP 49823 ((2*R*,4*S*)-2-benzyl-1-(3,5-dimethylbenzoyl)-4-(quinolin-μ-ylmethyl amino) piperidine), CGP 55000 ((2*R*,4*S*)-2-benzyl-1-(3,5-bistrifluoromethyl-benzoyl)-4-(quinolinyl-methylamino)piperidine) and CP 99,994 ((+)-(2*S*,3*S*)-3-methoxybenzyl amino)-2-phenylpiperidine), and the peptide tachykinin NK_2 receptor antagonist MEN 10,627 (cyclo(Met-Asp-Trp-Phe-Dap-Leu)cyclo(2β–5β)). CGP 49823, CGP 55000 and CP 99,994 concentration-dependently displaced [^{125}I]Bolton–Hunter substance P binding in tracheal membranes with Hill coefficients not different from unity and IC_{50} values of 1.4, 1.7 and 1.3 nM, respectively. In contrast, MEN 10,627 displaced binding according to a two-site model, with IC_{50}s of 0.2 nM and 1.3 μM. Electrical stimulation of tracheal segments with adrenoceptor and cholinoceptor blockade increased output of the mucus marker $^{35}SO_4$ by 59% above baseline (representing the NANC neural secretory response). CGP 49823, CGP 55000 or CP 99,994 concentration-dependently inhibited NANC neural secretion with IC_{50} values of 30, 8 and 120 nM, respectively. In contrast, MEN 10,627 (3 μM) did not inhibit secretion. The NK_1 antagonists, but not the NK_2 antagonist, inhibited [Sar^9]substance P-induced secretion, while none of the antagonists affected acetylcholine-induced secretion. We conclude that NANC neural secretion in ferret trachea in vitro is a useful test system for tachykinin NK_1 receptor antagonists with therapeutic potential in conditions of the airways in which tachykininergic mechanisms and mucus hypersecretion are implicated in pathophysiology, for example asthma and chronic bronchitis.

Keywords: CGP 49823; CGP 55000; CP 99,994; MEN 10,627; Mucus; Tachykinin; Tachykinin receptor; Tachykinin receptor antagonist

1. Introduction

Mucus secretion in the airways is a protective response against inhaled irritants, and is under humoral and neuronal control. In mammalian airways, the dominant neural control is cholinergic (Rogers, 1997a). Capsaicin-sensitive 'sensory-efferent' nerves also contribute to control of secretion, although the extent of their contribution varies with species (Ramnarine and Rogers, 1994). Inhibition of airway neurogenic mucus secretion is of interest because neural mechanisms and mucus hypersecretion are implicated in the pathophysiology of certain severe respiratory conditions, most notably asthma (Liu et al., 1998) and chronic obstructive pulmonary disease (Wells and Richardson, 1997). Experimental systems have, therefore, been developed to assess compounds that will inhibit neurogenic mucus secretion.

We have characterised neurogenic mucus secretion in the ferret trachea in vitro (Ramnarine et al., 1994). Capsaicin-induced mucus output was inhibited by the peptide tachykinin NK_1 receptor antagonist FK 888, indicating that activation of sensory-efferent nerves stimulates mucus output in this system, and that tachykinin NK_1 receptors mediate the secretory response. However, the response to capsaicin was variable. More consistent was the response to electrical stimulation. Elimination of cholinergic and adrenergic neural pathways with cholinoceptor and adreno-

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ceptor antagonists left a non-adrenergic, non-cholinergic (NANC) neurogenic secretory response which comprised ~40% of the total secretory response. FK 888 and the peptide dual tachykinin NK_1/NK_2 receptor antagonist FK 224 inhibited the remaining response, whereas a tachykinin NK_2 receptor antagonist had no inhibitory effect. In addition, selective agonist studies find no role for tachykinin NK_3 receptors in mediating ferret tracheal mucus secretion (Geppetti et al., 1993; Meini et al., 1993). These combined observations indicate that electrically evoked mucus output in ferret trachea in vitro is a useful test system for preclinical evaluation of tachykinin NK_1 receptor antagonists as inhibitors of NANC neurogenic mucus secretion. However, the above study (Ramnarine et al., 1994) utilized peptide tachykinin NK_1 receptor antagonists. Recently, non-peptide antagonists have become available, and these may have advantages over peptide compounds (Maggi et al., 1993; Khawaja and Rogers, 1996). It was, therefore, desirable to determine whether the ferret trachea retained its potential as a mucus secretory test preparation using non-peptide tachykinin NK_1 receptor antagonists.

In the present study, we investigated in ferret trachea in vitro the effect on NANC neurogenic mucus output of the non-peptide NK_1 tachykinin receptor antagonists CGP 49823 ((2*R*,4*S*)-2-benzyl-1-(3,5-dimethylbenzoyl)-4-(quinolin-μ-ylmethylamino) piperidine)) (Hauser et al., 1993), a related compound CGP 55000 ((2*R*,4*S*)-2-benzyl-1-(3,5-bistrifluoromethyl-benzoyl)-4-(quinolinyl-methyl-amino)piperidine) (originally termed compound 18k: Ofner et al., 1996), and CP 99,994 ((+)-(2*S*,3*S*)-3-methoxybenzylamino)-2-phenylpiperidine) (McLean et al., 1993). In our previous study, we investigated the activity of a non-peptide tachykinin NK_2 receptor antagonist (SR 48968). Consequently, in the present study, we utilized a peptide NK_2 antagonist, MEN 10,627 (cyclo(Met-Asp-Trp-Phe-Dap-Leu)cyclo(2β–5β)) (Maggi et al., 1994). We also characterised the antagonists in radioligand binding studies.

2. Materials and methods

Male ferrets (Regal Rabbits, Great Bookham, Surrey, UK) weighing 1.0–2.0 kg were used throughout and were kept 4–5 in a room. They were allowed 1 week to acclimatize after delivery and had free access to food and water. They were terminally anaesthetized with pentobarbitone sodium (Sagatal: 60 mg kg^{-1}, i.p.), bled by incising the left ventricle, and the tracheae were removed. For the binding studies, tracheae were immediately frozen in liquid nitrogen and stored at −80°C until required. For the secretory studies, tracheae were bathed in Krebs–Henseleit solution, aerated (95% O_2 + 5% CO_2) to pH 7.4, of the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.5 and glucose 5.05, until required (~10 min).

2.1. Binding characteristics of tachykinin receptor antagonists in ferret tracheal membranes

Frozen tracheae were ground in liquid nitrogen and then suspended in 10 vol. of 50 mM Tris–HCl buffer (pH 7.4 at 4°C) containing 0.32 M sucrose, 4 μg/ml leupeptin, 4 μg/ml chymostatin, 4 μg/ml bacitracin. The suspension was homogenized (Polytron homogeniser, Kinematica, Basel, Switzerland) for two 30 s bursts. Large organelles and cellular debris were removed from the homogenate by high-speed centrifugation (Model RC5C, Rotor SS34, Sorvall Instruments, Dupont, USA) at 1000 × *g* at 4°C for 10 min. The supernatant was re-centrifuged at 40,000 × *g* at 4°C for 20 min. The resulting pellet was washed with 50 mM Tris–HCl buffer (pH 7.4 at 4°C, no sucrose) and re-centrifuged at the same speed. After a repeat washing, the final pellet was suspended in 50 mM Tris–HCl buffer (pH 7.4 at 25°C) containing 10 μM phosphoramidon, 4 μg/ml leupeptin, 4 μg/ml chymostatin, 4 μg/ml bacitracin, 3 mM $MnCl_2$ and 0.2% (w/v) bovine serum albumin, and kept on ice (4°C) until required. Binding experiments were performed on the same day as membrane preparation. The protein concentration of the membrane suspension was determined using the Lowry method (Lowry et al., 1951), with bovine serum albumin as the standard.

To determine the optimal concentrations of radiolabel for use in the competition studies, saturation experiments were carried out. Twenty μg membrane protein was incubated with increasing concentrations of [^{125}I]Bolton Hunter substance P for 30 min at 25°C (final incubation volume of 250 μl). Non-specific binding was defined as total binding in the presence of excess (1 μM) unlabelled substance P.

For the competition experiments, 20 μg tracheal membrane protein (optimal concentration from above experiments) was incubated with 0.1 nM [^{125}I]Bolton–Hunter substance P at 25°C for 30 min (optimal, determined previously), either in the absence (total binding) or presence of 10^{-13}–10^{-5} M competing ligands (CGP 49823, CGP 55000, CP 99,994 or MEN 10,627). The final volume of the incubation buffer was 250 μl. Assays were terminated by rapid filtration under pressure (Brandel cell harvester, model M24, MD, USA) over Whatman GF/B glass fibre filters which had been pre-soaked for at least 3 h in 0.01% polyethylenimine. Filters (with bound membranes) were washed three times with 50 mM Tris–HCl buffer (pH 7.4 at 4°C). Non-specific binding was defined in the presence of 1 μM unlabelled substance P. Each filter was counted in a gamma-scintillation counter (Packard Cobra II Autogamma, model-D5003).

2.2. Tracheal preparation for measurement of mucus secretion

Our methodology for measurement of mucus secretion has been described in detail previously (Meini et al., 1993;

Ramnarine et al., 1994). Tracheae were cut longitudinally through the dorsal membrane, opened flat and cut transversely to give four segments. Each segment was pinned and clamped across the aperture separating the two halves of perspex Ussing-type chambers so that the tissue divided the chambers into 'luminal' (i.e., mucus-producing) and 'submucosal' sides. The chambers were rectangular in cross-section to ensure optimal use of the tissue with an exposed surface area for each segment of 1.12 cm^2. Each side of the tissue was bathed with 5 ml warmed (37°C) Krebs–Henseleit solution which was oxygenated (95% O_2:5% CO_2) and circulated using gas-lift pumps.

2.3. Radiolabelling of newly synthesized mucus

At time 0 h, $Na_2{}^{35}SO_4$ (0.1 mCi) was added to the submucosal half-chambers, to label newly synthesized intracellular mucus, where it remained throughout the experiment. At unit time intervals, the fluid in the luminal side of the chamber (containing secretions) was collected and replaced with fresh Krebs–Henseleit solution. Baseline stability of spontaneous output of $^{35}SO_4$-labelled macromolecules was reached after taking four 30-min collections followed by two 15-min collections (i.e., over 2.5 h following addition of radiolabel). After stabilization, drugs or control solutions were added and the tissues were electrically stimulated (see Section 2.6).

2.4. Electrical stimulation

The tracheal segments were subjected to an electrical current to stimulate excitable tissues (e.g., nerves). Two pins piercing the tissue on either side were connected via outlet wires through the chambers to a Grass stimulator (model S88; Grass Instruments, Quincy, USA). Tissues were stimulated at 10 Hz, 50 V, 0.5 ms for the first 5 min of a 15-min incubation period.

2.5. Measurement of $^{35}SO_4$ output

Luminal fluid, approximately 4 ml and comprising secretions in Krebs–Henseleit solution, was drained into tubes containing 5 g guanidine hydrochloride to dissolve the mucus. The final concentration of guanidine hydrochloride in the fluid was 6 M. Following this, each sample was exhaustively dialyzed against distilled water containing excess Na_2SO_4 and sodium azide (10 mg/l) using cellulose tubing (Medicell International, London) which allowed molecules of < 14 kDa to pass through. The sodium azide was present to inhibit bacterial growth. The samples were recovered after at least six changes of distilled water when the radioactive count of the dialysis water was the same after dialysis as before dialysis (~ 20 disintegrations per minute [d.p.m.]). The recovered samples were weighed and the remaining radioactivity in 1 ml duplicates of each sample mixed with 2 ml scintillant (Ultima Gold XR, Canberra Packard, Pangbourne, Berks., UK) was determined by scintillation spectrometry (model 1900CA Spectrophotometer, Canberra Packard). The total radioactivity of each sample was estimated by multiplying the radioactivity present in a 1 ml aliquot of sample by the total weight of the sample (assuming a 1 ml sample weighs 1 g).

2.6. Protocols for investigation of the effect of tachykinin receptor antagonists on NANC neurogenic secretion

The effects of CGP 49823, CGP 55000, CP 99,994 and MEN 10,627 were determined on $^{35}SO_4$ output induced by electrical stimulation of the tissue. Drugs were added to the luminal half-chamber 30 min before stimulation (i.e., for two incubation periods). CGP 49823, CGP 55000 and MEN 10,627 were dissolved in dimethylsulphoxide (DMSO) and CP 99,994 was dissolved in distilled water. All drug solutions were stored as 10^{-3} M aliquots at -20°C until required. Controls were carried out using the appropriate vehicles. NANC neurogenic (tachykininergic) was isolated by incubating the tissue in the presence of atropine, phentolamine and propranolol (10 μM each) to remove respectively cholinergic, α-adrenergic and β-adrenergic neural influences. Autonomic antagonists were added one hour prior to stimulation, and were present throughout the stimulation period.

The treatment groups were: (1) sham stimulation; i.e., collections in the absence of electrical stimulation at the same time-point where the tissue would normally be stimulated; (2) tachykininergic stimulation in the presence of antagonist vehicle (i.e., DMSO or distilled water); (3) tachykinergic stimulation; (4) tachykininergic stimulation in the presence of one concentration of tachykinin antagonist per chamber: CGP 49823 (1 nM, 10 nM, 30 nM or 3 μM), CGP 55000 (1 nM, 10 nM, 30 nM or 3 μM), CP 99,994 (10 nM, 100 nM, 1 μM or 3 μM) and MEN 10,627 (3 μM), incubated for 30 min prior to, and during, electrical stimulation.

2.7. Protocol for investigation of effect of tachykinin receptor antagonists on [Sar^9]substance P- or acetylcholine-induced mucus secretion

Tissue was stimulated at 3 h with either the selective tachykinin NK_1 receptor agonist [Sar^9]-SP or acetylcholine (both at 1 μM), instead of electrical stimulation. The concentration of 1 μM [Sar^9]substance P and acetylcholine is submaximal for inducing $^{35}SO_4$ output in ferret trachea in vitro (Meini et al., 1993; Ramnarine et al., 1994). Both agonists were dissolved in distilled water, were added to the luminal half-chamber, and left in contact with the tissue for the full 15 min incubation period. Treatment groups were: (1) vehicle for antagonist (see above) and

distilled water vehicle for [Sar^9]substance P and acetylcholine; (2) [Sar^9]substance P or acetylcholine stimulation in the presence of antagonist vehicle; 3) [Sar^9]substance P or acetylcholine stimulation in the presence of tachykinin receptor antagonists: CGP 49823, CGP 55000, CP 99,994 and MEN 10,627 (all at 3 μM), incubated for 30 min prior to, and during, addition of [Sar^9]substance P or acetylcholine.

2.8. Data analysis

In Section 3, data are the arithmetic mean and one standard error (S.E.M.). Data for the saturation and competitive binding studies were analysed using non-linear regression analysis (Munson and Rodbard, 1980: LIGAND program) to yield equilibrium dissociation constants (K_D) and receptor density (B_{max}). The precision of fit to a one- or two-site model was determined by *F*-test, comparing the residual sum of squares for fitting data to either model. For $^{35}SO_4$ output, because baseline d.p.m. displayed variability between tracheal segments, responses obtained from individual segments were calculated as percentage changes in radiolabel output for the difference between response to drug or electrical stimulation and the proceeding collection. The concentration of tachykinin antagonist causing a 50% inhibition (IC_{50}) of neurogenic secretion was calculated by nonlinear regression using GraphPad Prism software (Microsoft, San Diego, USA). Significance of changes in $^{35}SO_4$ output, pre- and post-drug or electrical stimulation, were assessed using the Mann–Whitney *U*-test between two groups, or the Kruskal–Wallis test followed by Dunns multiple comparison test for multiple groups. The null hypothesis was rejected at $P < 0.05$ (two-tail).

2.9. Drugs and chemicals

The following drugs and chemicals were used (Sigma, Poole, Dorset, UK, except where stated): acetylcholine chloride, dimethylsulphoxide (DMSO), atropine sulphate (Phoenix Pharmaceuticals, Pharma Hameln, Germany); pentobarbitone sodium B.P. (Sagatal; RMB Animal Health, Dagenham, Essex, UK), phentolamine mesylate (Ciba Laboratories, Horsham, West Sussex, UK), propranolol hydrochloride (Imperial Chemical Industries, Macclesfield, Cheshire, UK), saline (0.9% sodium chloride BP for intravenous infusion, Travenol Laboratories, Thetford; Norfolk), [Sar^9,Met(O$_2$)^{11}]substance P ([Sar^9]substance P) (Bachem (UK), Saffron Walden, Essex, UK), $Na_2{}^{35}SO_4$ and [^{125}I]-Bolton–Hunter SP (Amersham Imternational, Bucks., UK). CGP 49823 and CGP 55000 were supplied by Novartis, Basle, Switzerland, CP 99,994 was a gift from Pfizer, Groton, CT, USA (courtesy Dr. R.M. Snider), and MEN 10,627 was a gift from A. Menarini Pharmaceuticals, Florence, Italy (courtesy Dr. C.A. Maggi).

3. Results

3.1. Binding characteristics of tachykinin receptor antagonists in ferret tracheal membranes

From saturation curves, [^{125}I]Bolton–Hunter substance P bound to the membrane receptors with a K_D of 0.12 nM and a B_{max} of 80 fmol/mg protein. Binding showed a homologous population of receptors. Specific binding represented 63 ± 1% ($n = 6$) of total binding. A concentration of 0.1 nM [^{125}I]Bolton–Hunter substance P (approximating its K_D) was used in the following antagonist binding experiments.

CGP 49823 (10^{-13}–10^{-6} M) concentration-dependently inhibited [^{125}I]Bolton–Hunter substance P binding with an IC_{50} of 1.4 nM and a Hill coefficient (−0.93 ± 0.34) not significantly different from unity (Fig. 1A). CGP 55000 (10^{-13}–10^{-6} M) displaced [^{125}I]Bolton–Hunter substance P binding with a similar profile to that of CGP 49823, with an IC_{50} of 1.7 nM and a Hill coefficient (−0.94 ± 0.3) not significantly different from unity (Fig. 1B). CP 99,994 (10^{-13}–10^{-6} M) displaced [^{125}I]Bolton–Hunter substance P binding with an IC_{50} of 1.3 nM (Fig. 1C). The Hill coefficient of −0.67 ± 0.2 did not differ significantly from unity, and computer analysis showed its curve fitted best to a single-site model. The tachykinin

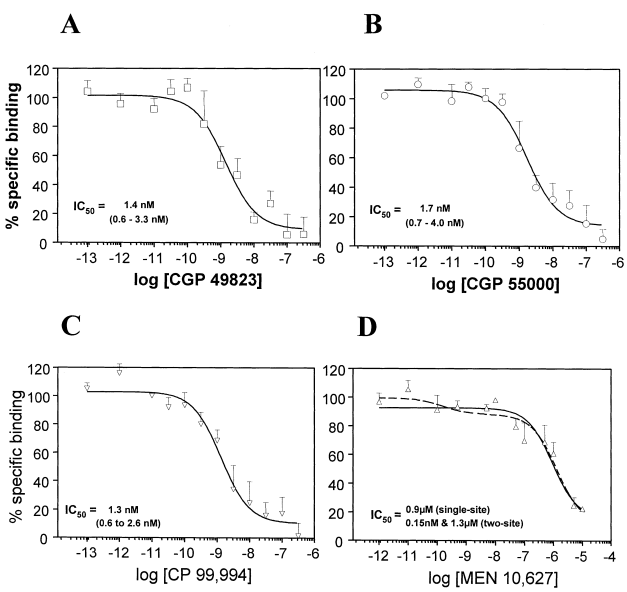

Fig. 1. Tachykinin NK_1 receptor antagonist displacement of [^{125}I]Bolton–Hunter substance P binding in ferret tracheal membranes in vitro. Membranes were incubated with 0.1 nM [^{125}I]Bolton–Hunter substance P and competition curves to the tachykinin NK_1 receptor antagonists CGP 49823 (panel A), CGP 55000 (panel B) and CP 99,994 (panel C), and the tachykinin NK_2 antagonist MEN 10,627 (panel D), were constructed. Single site and two site models decided from Hill coefficients. IC_{50}, concentration of antagonist causing 50% inhibition of [^{125}I]Bolton–Hunter substance P binding (95% confidence intervals).

NK_2 receptor antagonist MEN 10,627 (10^{-12}–10^{-5} M) also inhibited [^{125}I]Bolton–Hunter substance P binding, although only at concentrations $> 10^{-8}$ M, with an IC_{50} of 0.93 μM (Fig. 1D). The Hill coefficient for MEN 10,627 (-0.58 ± 0.11) was significantly different from unity, which suggested a binding profile with more than one binding site. Fitting the curve to a two-site model revealed two distinct binding sites with IC_{50}s of 0.15 nM and 1.3 μM (Fig. 1D).

3.2. Contribution of NANC-mediated secretion to total neurogenic secretion

In seven representative tracheal segments mean (±S.E.M.) baseline d.p.m. was 605 ± 63 (range 319–955 d.p.m.), which was increased to 1453 ± 184 d.p.m. (range 698–2166 d.p.m.) after electrical stimulation: an overall increase in $^{35}SO_4$ output of 140%. Because of the variability in baseline and stimulated d.p.m., $^{35}SO_4$ output is expressed below as percentage change between the stimulated response and the preceding collection (see Section 2.8). Electrical stimulation of ferret trachea for 5 min (50 V, 10 Hz, 0.5 ms) increased $^{35}SO_4$ output by 133 ± 31% ($n = 8$) above baseline, compared with sham stimulation which caused a 5 ± 2% ($n = 6$) decrease in output ($P < 0.01$). Atropine, propranolol and phentolamine had no significant effect on sham stimulated $^{35}SO_4$ output (increase of 4 ± 4% above baseline, $n = 6$), but stimulated output was now 59 ± 9% above baseline ($P < 0.01$ compared with sham stimulation, $n = 8$). Thus, the NANC portion of the secretory response comprised 44% of the total neurogenic secretory response.

3.3. Effect of tachykinin receptor antagonists on NANC neurogenic secretion

Baseline radioactivity of collected secretions was of the order of 600 d.p.m., and did not differ significantly between treatment groups. There was, however variability in the electrically stimulated response between groups, with tracheal segments from ferrets in the first set of experiments giving a greater secretory response than those in the second set of experiments (compare Fig. 2A and B with Fig. 2C). CGP 49823 (3 μM) had no significant effect on baseline secretion and concentration-dependently (1 nM–3 μM) inhibited NANC neurogenic secretion with an approximate IC_{50} of 10 nM and a maximal inhibition of 84% at 30 nM (Fig. 2A): secretion at the latter concentration was not significantly different to the value with sham stimulation. CGP 55000 (3μM) also had no effect on baseline secretion rate and concentration-dependently (1 nM–3 μM) inhibited secretion with an approximate IC_{50} of 8 nM, with complete inhibition at 3 μM (Fig. 2B). CP 99,994 concentration-dependently inhibited secretion with an approximate IC_{50} of 120 nM and a maximal inhibition of 76% at 3 μM (Fig. 2C): secretion at the latter concentration was not significantly different to the value with sham stimulation. In contrast to the above three compounds, MEN 10,627 did not significantly inhibit NANC neurogenic $^{35}SO_4$ output: even at 3 μM MEN 10,627, stimulated secretion was 61% above baseline (not significantly different to the 59% increase with NANC neurogenic stimulation in the absence of MEN 10,627).

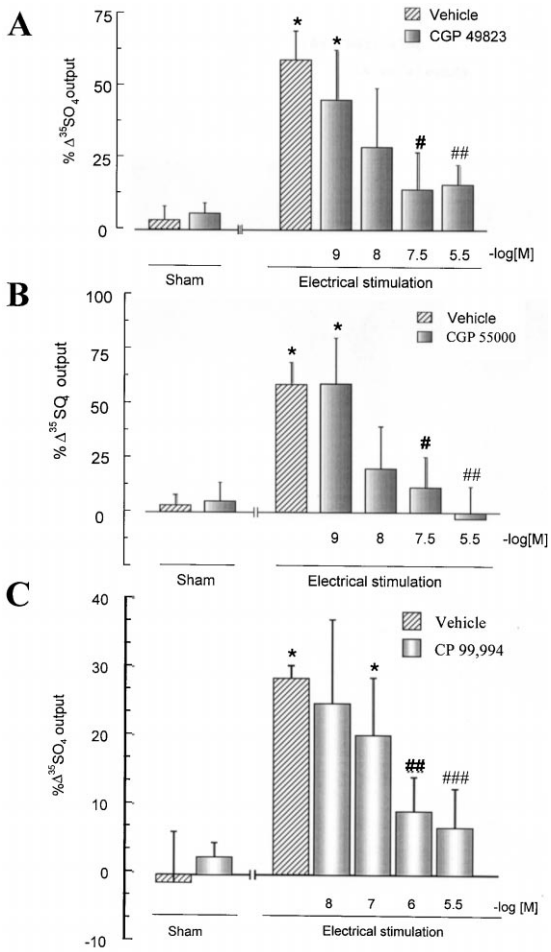

Fig. 2. Effect of tachykinin NK_1 receptor antagonists on non-adrenergic, non-cholinergic (NANC) neurogenic mucus secretion in ferret trachea in vitro. Atropine, phentolamine and propranolol (APP, 10 μM each) were used to exclude cholinergic and adrenergic neural influences at stimulation parameters of 10 Hz, 50 V, 0.5 ms for 5 min. Tracheal segments were incubated with tachykinin antagonists for 30 min prior to electrical stimulation. 'Sham': same time-point as stimulated secretion, but without electrical stimulation. Data are mean percent change (vertical bars = S.E.M.) in output of macromolecules labelled in situ with $^{35}SO_4$ (a marker for mucus) for 6–8 animals per group. $^{*}P < 0.05$ compared with vehicle + sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with vehicle + stimulation group (not significantly different to vehicle + sham group).

3.4. Effect of tachykinin receptor antagonists on [Sar^9]substance P and acetylcholine-induced secretion

Baseline radioactivity of collected secretions was of the order of 600 d.p.m., and did not differ significantly be-

tween treatment groups. However, similar to the sets of experiments above using electrical stimulation, there was variability in the secretory response to [Sar^9]substance P. In the experiments with CGP 49823 and CGP 55000, [Sar^9]substance P (1 μM) increased $^{35}SO_4$ output by 94% above baseline (Fig. 3A): there was a 5% decrease in output with sham stimulation. In contrast, in the experiments with CP 99,994 and MEN 10,627, [Sar^9]substance P (1 μM) only increased output by 63% above baseline (Fig. 3B). [Sar^9]substance P-induced secretion was inhibited by CGP 49823 by 80%, CGP 55000 by 82%, and by CP 99,994 by 66% (all at 3 μM) (Fig. 3): in each case, inhibited secretion was not significantly different to the baseline vehicle control value. In contrast, MEN 10,627 (3 μM) did not inhibit [Sar^9]substance P-induced secretion (Fig. 3B).

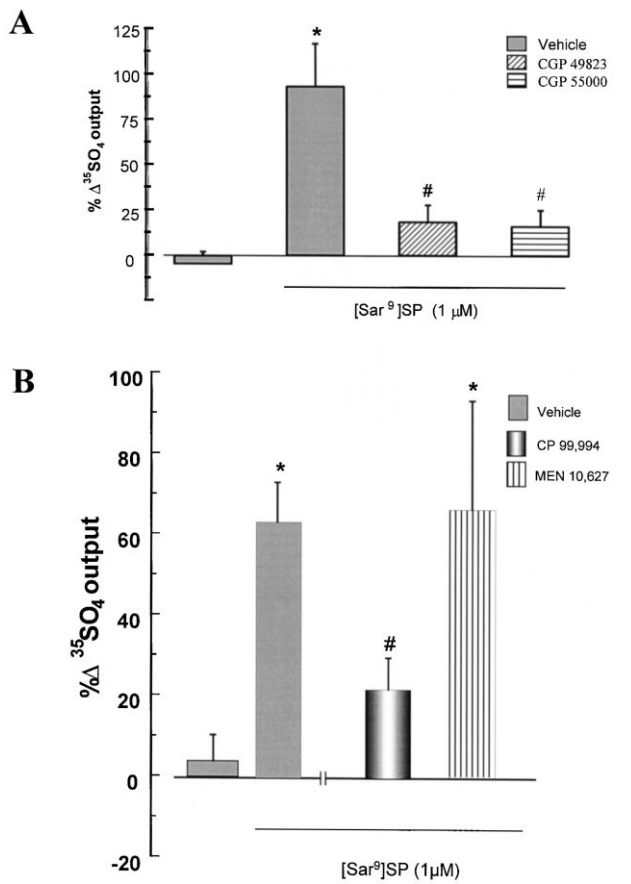

Fig. 3. Effect of tachykinin receptor antagonists on [Sar^9]substance P ([Sar^9]SP; 1 μM)-induced mucus secretion in ferret trachea in vitro: panel A, the tachykinin NK_1 receptor antagonists CGP 49823 and CGP 55000; panel B, the tachykinin NK_1 receptor antagonist CP 99,994 and the tachykinin NK_2 receptor antagonist MEN 10,627 (all at 3 μM). Data are mean percent change (vertical bars = S.E.M.) in output of macromolecules labelled in situ with $^{35}SO_4$ (a marker for mucus) for 3–5 animals per group. $^*P < 0.05$ compared with baseline vehicle group; $^\#P < 0.05$ compared with vehicle + stimulation group (not significantly different to baseline vehicle group).

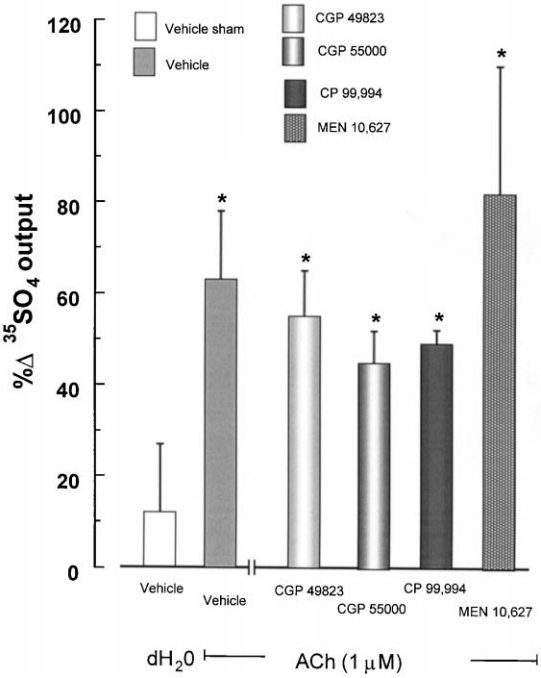

Fig. 4. Effect of tachykinin receptor antagonists on acetylcholine (ACh: 1 μM)- induced mucus secretion in ferret trachea in vitro. The tachykinin NK_1 receptor antagonists CGP 49823, CGP 55000 and CP 99,994, and the tachykinin NK_2 antagonist MEN 10,627 (all at 3 μM) were used. Data are mean percent change (vertical bars = S.E.M.) in output of macromolecules labelled in situ with $^{35}SO_4$ (a marker for mucus) for 3–5 animals per group. $^*P < 0.05$ compared with baseline vehicle (distilled water; dH_2O) group (no significant differences between any of the acetylcholine-stimulated groups).

Acetylcholine (1 μM) caused a 63% increase in $^{35}SO_4$ output, which was not affected by any of the tachykinin antagonists (all at 3 μM) (Fig. 4).

4. Discussion

In the present study, binding characteristics and inhibitory effects on mucus secretion of four tachykinin receptor antagonists were examined in ferret trachea in vitro. In tracheal membranes, [^{125}I]Bolton–Hunter substance P interacted with a homogenous population of binding sites with an equilibrium dissociation constant (K_D) of ~0.1 nM, which is in the range (0.06–0.4 nM) for a number of different tissue types (Appell et al., 1992). [^{125}I]Bolton–Hunter substance P binding is to sites characteristic of the tachykinin NK_1 receptor (Cascieri et al., 1992), and is equally selective as [^{125}I]Sar^{9}substance P for the tachykinin NK_1 receptor (Lew et al., 1990). By autoradiography, [^{125}I]Bolton–Hunter substance P is localized, in ferret trachea, to epithelium, submucosal glands and to a lesser extent the smooth muscle (Meini et al., 1993). This is a similar profile to that in human lung (Carstairs and Barnes, 1983), and is consistent with the secretory rather than contractile nature of the tachykinin NK_1 receptor in these species. Thus, in the present study, [^{125}I]Bolton–

Hunter substance P binding is consistent with binding to tachykinin NK_1 receptors. Consequently, displacement of [^{125}I]Bolton–Hunter substance P binding by the tachykinin receptor antagonists used herein indicates that they are interacting with the tachykinin NK_1 receptor.

The tachykinin NK_1 receptor antagonists CGP 49823, CGP 55000 and CP 99,994 used herein competed avidly with [^{125}I]Bolton–Hunter substance P binding, with IC_{50}s of 1.3–1.7 nM. The IC_{50} for CGP 49823 (1.4 nM) herein is of a similar order to that of 12 nM in displacement of [^{3}H]substance P in bovine retinal membranes (Hauser et al., 1993). The related compound, CGP 55000 (Ofner et al., 1996), shared similar activity, with an IC_{50} herein of 1.7 nM. The IC_{50} value for CP 99,994 (1.3 nM) is in the range reported previously for this compound in a variety of different preparations including cell lines and tissues from laboratory animals (McLean et al., 1993; Longmore et al., 1994). In contrast to the tachykinin NK_1 receptor antagonists, the tachykinin NK_2 receptor antagonist MEN 10,627 (Maggi et al., 1994) was weak in displacing [^{125}I]Bolton–Hunter substance P from ferret tracheal membranes, with a Hill coefficient suggesting that binding conformed to a two-site model. When the curve was fitted to this model, two distinct IC_{50}s were observed: a high affinity (IC_{50} 0.15 nM) site accounting for 13% of binding, and a low affinity site (IC_{50} 1.3 μM) accounting for 87% of binding. The high affinity site may represent a small proportion of tachykinin NK_2 sites labelled by [^{125}I]Bolton–Hunter substance P, as MEN 10,627 has been shown to have affinity for this receptor in a number of tissues with IC_{50}s of 0.08–2 nM (Maggi et al., 1994). Also, at high concentrations (3–30 μM), MEN 10,627 has been shown to antagonize tachykinin NK_1 and NK_3 receptors in guinea-pig ileum (Maggi et al., 1994). This could represent the remaining low affinity displacement of the radiolabel in this study.

In the present study, the three tachykinin NK_1 receptor antagonists, but not the NK_2 antagonist, inhibited NANC neurogenic $^{35}SO_4$ output. The $^{35}SO_4$ output is consistent with mucus secretion from submucosal glands, because there are few goblet cells but numerous submucosal glands in ferret trachea (Robinson et al., 1986; Meini et al., 1993). By autoradiography, there is uptake of $^{35}SO_4$ by ferret tracheal submucosal glands rather than epithelium (Gashi et al., 1987). Stimulation of ferret trachea in vitro increased radioactivity in the incubation medium, with associated loss of autoradiographic grains from the glands (Gashi et al., 1987). In cats, $^{35}SO_4$-labelled tracheal washings have a molecular weight and buoyant density characteristic of a mucin molecule (Davies et al., 1990). Currently, a number of mucin macromolecules comprise airway mucus. MUC5AC and MUC5B are considered the major respiratory mucins in humans, and antibodies for these have been developed (Thornton et al., 1997). To our knowledge, direct antibody/$^{35}SO_4$ label association studies have not been reported, but would be of interest in determining whether $^{35}SO_4$-labelled high molecular weight glycoconjugates from ferret trachea correspond to either of these MUC5 mucins, or to any other respiratory mucin.

In the present study, mucus output in response to electrical stimulation of ferret trachea in vitro was reduced by 56% in the presence of atropine, propranolol and phentolamine. Thus, NANC neural pathways accounted for ~44% of total neurogenic secretion, which is a similar proportion to that found previously (Borson et al., 1984; Ramnarine et al., 1994). The NANC response was concentration-dependently attenuated by the tachykinin NK_1 receptor antagonists, CGP 49823, CGP 55000 and CP 99,994, while the tachykinin NK_2 receptor antagonist, MEN 10,627, did not alter the response. In addition, the tachykinin NK_1 receptor antagonists, but not the tachykinin NK_2 receptor antagonist, inhibited [Sar^9]substance P-induced mucus output. These findings are consistent with previous studies (Geppeti et al., 1993; Ramnarine et al., 1994). We found previously that the dual tachykinin NK_1/NK_2 receptor antagonist FK224 inhibited NANC neurogenic mucus output in ferret trachea by 73% at 10 μM (Ramnarine et al., 1994). Similar potency was found herein and in our previous study (Ramnarine et al., 1994) for tachykinin NK_1 receptor antagonists. Our present data and previous data (Ramnarine et al., 1994) showing that tachykinin NK_1 receptor antagonists but not tachykinin NK_2 receptor antagonists inhibit NANC neurogenic mucus secretion indicate that the inhibitory effect of FK224 lay in its tachykinin NK_1 receptor antagonist capacity rather than its NK_2 receptor antagonist capacity. Taken together the above observations do not indicate that there is synergy between tachykinin NK_1 and NK_2 receptor antagonists in the present experimental system. However, formal combination studies of tachykinin NK_1 and NK_2 receptor antagonists would be of interest. None of the antagonists used herein inhibited acetylcholine-induced mucus output. This indicates that the inhibition by the NK_1 antagonists was due to a selective effect rather than to non-selective inhibitory actions. The above observations demonstrate that the NANC neural pathway responsible for stimulation of mucus output in ferret trachea is tachykininergic, with end-organ regulation principally via NK_1 receptors.

The NK_1 antagonists used herein inhibited NANC neurogenic mucus secretion with IC_{50}s in the nanomolar range. Our IC_{50} value for CGP 49823 (10 nM) is similar to that of 13 nM for inhibition of substance P-induced inositol phosphate production by U373MG human astrocytoma cells and inhibition of substance P-induced contraction of rabbit vena cava (Hauser et al., 1993). The IC_{50} for CGP 55000 (8 nM), a new putative NK_1 antagonist, was similar to CGP 49823. In contrast, IC_{50}s for CGP 49823 inhibition of septide-induced depolarizations in rat and gerbil motoneurones were 8 μM and 0.2 μM, respectively, in the two species (Pozza et al., 1998). Similarly low potencies were found for other NK_1 antagonists. Low-potency for NK_1 receptor antagonists appears to be a feature of prepa-

rations in the central nervous system of gerbil and rat (Lepre et al., 1993). Interestingly, the IC_{50} of 120 nM (i.e. 0.12 μM) found herein for CP 99,994 is similar to that of 0.38 μM for its inhibition of gerbil motoneurone depolarization (Pozza et al., 1998). In other systems CP 99,994 is a more potent NK_1 receptor antagonist. For example, for guinea-pig tracheal contraction, CP 99,994 caused a parallel shift in the concentration response curve to [Sar^9]-SP with a pK_B of 7.7 (IC_{50}: ~20 nM) (Longmore et al, 1994). In guinea-pig locus coeruleus neurones, CP 99,994 inhibited SP-induced firing with an IC_{50} of 50 nM (Desai et al, 1992).

In the present study, we found variability in secretory response between different sets of experiments. We have noted similar variability previously (Ramnarine et al., 1994). Variability in mucus output in the present study (investigating tachykininergic neurogenic secretion) is related to efficiency of $^{35}SO_4$ uptake and its incorporation into mucus, to the amount of submucosal gland present, to the tenacity of mucus which affects its release from the luminal surface and its dispersion into the bathing medium, to the amount and distribution of tachykininergic nerves, and to the number and distribution of tachykinin NK_1 receptors. The precise reasons for the variability in the present study are unclear. Baseline secretion was similar between sets of experiments, whereas stimulated secretion was different. There was a greater difference between sets of electrically stimulated tissue than between tissue incubated with [Sar^9]substance P. The latter observation indicates a difference more in tachykininergic nerves (their amount or distribution) than in tachykinin NK_1 receptors, mucus or submucosal glands.

Inhibition of tachykininergic mucus secretion has clinical implications. For example, tachykinins have been implicated in the pathophysiology of asthma and bronchial hyperresponsiveness (Rogers, 1997b; Spina et al., 1998), and in chronic obstructive pulmonary disease (Rogers, 1997b; Barnes, 1998). In both asthma and chronic obstructive pulmonary disease, hypersecretion of mucus in the airways contributes markedly to morbidity and mortality. Inhibition of neurogenic mucus secretion is, therefore, a valid therapeutic target. Inhalation of the tachykinin NK_1/NK_2 receptor antagonist FK 224 reduces cough and sputum production in patients with chronic bronchitis (Ichinose et al., 1993). Because FK 224 does not block NKA-induced bronchoconstriction in asthmatic subjects (Joos et al., 1996), its inhibitory effects on sputum production may be due to activity at tachykinin NK_1 receptors. Formal clinical studies of the effects of tachykinin receptor antagonists on mucus secretion in asthma and chronic obstructive pulmonary disease are therefore indicated. Deciding upon an appropriate end-point marker for inhibition of production of mucus, as opposed to sputum, will be a significant challenge in the design of such trials.

In summary, the present study has shown that radioligand binding and NANC neurogenic mucus secretion in ferret trachea in vitro is a suitable test system to characterize non-peptide tachykinin NK_1 receptor antagonist drugs. The system can identify compounds with dissimilar characteristics in terms of IC_{50} values (8–120 nM) and maximal inhibitions (76%–100%). The preparation is also of use in evaluating the selectivity of putative NK_2 receptor antagonists, which consistently do not block neurogenic secretion.

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References

Appell, K.C., Fragale, B.J., Loscig, J., Singh, S., Tomczak, B.E., 1992. Antagonists that demonstrate species differences in neurokinin-1 receptors. Mol. Pharmacol. 41, 772–778.

Barnes, P.J., 1998. Chronic obstructive pulmonary disease: new opportunities for drug development. Trends Pharmacol. Sci. 19, 415–423.

Borson, D.B., Charlin, M., Gold, B.D., Nadel, J.A., 1984. Neural regulation of $^{35}SO_4$-macromolecule secretion from glands of ferrets. J. Appl. Physiol. 57, 457–466.

Carstairs, J., Barnes, P.J., 1983. Autoradiographic mapping of substance P receptors in the lung. Eur. J. Pharmacol. 127, 295–296.

Cascieri, M.A., Huang, R.R., Fong, T.M., Cheung, A.H., Sadowski, S., Ber, E., Strader, C.D., 1992. Determination of the amino acid residues in substance P conferring selectivity and specificity for the rat neurokinin receptors. Mol. Pharmacol. 41, 1096–1099.

Davies, J.R., Corbishley, C.M., Richardson, P.S., 1990. The uptake of radiolabelled precursors of mucus glycoconjugates by secretory tissues in the feline trachea. J. Physiol. 420, 19–30.

Desai, M.C., Lefkowitz, S.L., Thadeio, P.F., Longo, K.P., Snider, R.M., 1992. Discovery of a potent substance P antagonist: recognition of the key molecular determinant. J. Med. Chem. 35, 4911–4913.

Gashi, A.A., Nadel, J.A., Basbaum, C.B., 1987. Autoradiographic studies of the distribution of ^{35}sulphate label in ferret trachea: effects of stimulation. Exp. Lung Res. 12, 83–96.

Geppetti, P., Bertrand, C., Bacci, E., Huber, O., Nadel, J., 1993. Characterisation of tachykinin receptors in ferret trachea by peptide agonists and nonpeptide antagonists. Am. J. Physiol. 265, L164–L169.

Hauser, K., Heid, J., Criscione, L., Brugger, F., Ofner, S., Veenstra, S., Schilling, W., 1993. CGP 49823, a novel, non-peptidic NK-1 receptor antagonist: in vitro pharmacology. Neuropeptides P7/8, 37, (April suppl.).

Ichinose, M., Katsumata, U., Kikuchi, R., Fukuhima, T., Ishii, M., Inoue, C., Shirato, K., Takishima, T., 1993. Effect of tachykinin antagonist on chronic bronchitis patients. Am. Rev. Respir. Dis. A318, (suppl.).

Joos, G.F., Van Schoor, J., Kips, J.C., Pauwels, R.A., 1996. The effect of inhaled FK224, a tachykinin NK-1 and NK-2 receptor antagonist, on neurokinin A-induced bronchoconstriction in asthmatics. Am. J. Respir. Crit. Care Med. 153, 1781–1784.

Khawaja, A.M., Rogers, D.F., 1996. Tachykinins: receptor to effector. Int. J. Biochem. Cell Biol. 28, 721–738.

Lepre, M., Evans, R.H., Olpe, H.-R., Brugger, F., 1993. The modulation of the monosynaptic reflex by substance P in the hemisected spinal cord preparation in the rat and gerbil. Neuroscience 55, 727–735.

Lew, R., Geraghty, D.P., Drapeau, G., Regoli, D., Burcher, E., 1990.

Binding characteristics of [^{125}I] Bolton Hunter [Sar^{9},Met(O_2)^{11}] substance P, a new selective radioligand for the NK_1 receptor. Eur. J. Pharmacol. 184, 97–108.

Liu, Y.-C., Khawaja, A.M., Rogers, D.F., 1998. Pathophysiology of airway mucus secretion in asthma. In: Barnes, P.J., Rodger, I.W., Thomson, N.C. (Eds.), Asthma: Basic Mechanisms and Clinical Management. 3rd edn. Academic Press, London, UK, pp. 205–227.

Longmore, J., Razzaque, Z., Shaw, D., Hill, R.G., 1994. Differences in the effects of NK_1-receptor antagonists, CP-96,345 and CP-99,994 on agonist-induced responses in guinea-pig trachea. Br. J. Pharmacol. 112, 176–178.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Maggi, C.A., Patacchini, R., Rovero, P., Giachetti, A., 1993. Tachykinin receptors and tachykinin receptor antagonists. J. Auton. Pharmacol. 13, 23–93.

Maggi, C.A., Astolfi, M., Giuliani, S., Goso, C., Manzini, S., Meini, S., Patacchini, R., Pavone, V., Pedone, C., Quartara, S., Renzetti, A.R., Giachetti, A., 1994. MEN 10,627, a novel polycyclic peptide antagonist of tachykinin NK_2 receptors. J. Pharmacol. Exp. Ther. 271, 1489–1500.

McLean, S., Ganong, A., Seymour, P.A., Snider, R.M., Desai, M.C., Rosen, T., Bryce, D.K., Longo, K.P., Reynolds, L.S., Robinson, G., Schmidt, A.W., Siok, C., Heym, J., 1993. Pharmacology of CP-99,994; a non-peptide antagonist of the tachykinin neurokinin-1 receptor. J. Pharmacol. Exp. Ther. 267, 472–482.

Meini, S., Mak, J.C.W., Rohde, J.A.L., Rogers, D.F., 1993. Tachykinin control of ferret airways: mucus secretion, bronchoconstriction and receptor mapping. Neuropeptides 24, 81–89.

Munson, J., Rodbard, D., 1980. Ligand: a versatile computerised approach to characterization of ligand-binding systems. Anal. Biochem. 107, 220–239.

Ofner, S., Hauser, K., Schilling, W., Vassout, A., Veenstra, S.J., 1996. SAR of 2-benzyl-4-aminopiperidines: CGP 49823, an orally and centarlly active non-peptide NK_1 antagonist. Bioorg. Med. Chem. Lett. 6, 1623–1628.

Pozza, M., Olpe, H.-R., Ofner, S., Schoeffel, C., Evans, R., 1998. The potency of the novel tachykinin receptor antagonist CGP 49823 at rat and gerbil motoneurones in vitro. Eur. J. Pharmacol. 342, 203–208.

Ramnarine, S.I., Rogers, D.F., 1994. Non-adrenergic, non-cholinergic neural control of mucus secretion in the airways. Pulm. Pharmacol. 7, 19–33.

Ramnarine, S.I., Hirayama, Y., Barnes, P.J., Rogers, D.F., 1994. 'Sensory-efferent' neural control of mucus secretion: characterisation using tachykinin receptor antagonists in ferret trachea in vitro. Br. J. Pharmacol. 113, 1183–1190.

Robinson, N.P., Venning, L., Kyle, H., Widdicombe, J.G., 1986. Quantitation of the secretory cells of the ferret tracheobronchial tree. J. Anat. 145, 173–188.

Rogers, D.F., 1997a. Neural control of airway secretions. In: Barnes, P.J. (Ed.), Autonomic Control of the Respiratory System. Harwood Academic Publishers, Amsterdam, pp. 201–227.

Rogers, D.F., 1997b. Neurogenic inflammation in lung disease: burnt out? Inflammopharmacol. 5, 319–329.

Spina, D., Shah, S., Harrison, S., 1998. Modulation of sensory nerve function in the airways. Trends Pharmacol. Sci. 19, 460–466.

Thornton, D.J., Davies, J.R., Carlstedt, I., Sheehan, J.K., 1997. Structure and biochemistry of human respiratory mucins. In: Rogers, D.F., Lethem, M.I. (Eds.), Airway Mucus: Basic Mechanisms and Clinical Perspectives. Birkhäuser Verlag, Basel, Switzerland, pp. 19–39.

Wells, U.M., Richardson, P.S., 1997. Mucus hypersecretion and its role in the airway obstruction of asthma and chronic obstructive pulmonary disease. In: Rogers, D.F., Lethem, M.I. (Eds.), Airway Mucus: Basic Mechanisms and Clinical Perspectives. Birkhäuser Verlag, Basel, Switzerland, pp. 275–300.