Neuroregulation by vasoactive intestinal peptide (VIP) of mucus secretion in ferret trachea: activation of BK_{Ca} channels and inhibition of neurotransmitter release

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1 The aims of this study were to determine: (1) whether vasoactive intestinal peptide (VIP) regulates cholinergic and 'sensory-efferent' (tachykinergic) ³⁵SO₄ labelled mucus output in ferret trachea *in vitro*, using a VIP antibody, (2) the class of potassium (K⁺) channel involved in VIP-regulation of cholinergic neural secretion using glibenclamide (an ATP-sensitive K⁺ (K_{ATP}) channel inhibitor), iberiotoxin (a large conductance calcium activated K⁺ (BK_{Ca}) channel blocker), and apamin (a small conductance K_{Ca} (SK_{Ca}) channel blocker), and (3) the effect of VIP on cholinergic neurotransmission using [³H]-choline overflow as a marker for acetylcholine (ACh) release.

2 Exogenous VIP (1 and 10 μM) alone increased ³⁵SO₄ output by up to 53% above baseline, but suppressed (by up to 80% at 1 μM) cholinergic and tachykinergic neural secretion without altering secretion induced by ACh or substance P (1 μM each). Endogenous VIP accounted for the minor increase in non-adrenergic, non-cholinergic (NANC), non-tachykinergic neural secretion, which was compatible with the secretory response of exogenous VIP.

3 Iberiotoxin (3 μM), but not apamin (1 μM) or glibenclamide (0.1 μM), reversed the inhibition by VIP (10 nM) of cholinergic neural secretion.

4 Both endogenous VIP (by use of the VIP antibody; 1 : 500 dilution) and exogenous VIP (0.1 μM), the latter by 34%, inhibited ACh release from cholinergic nerve terminals and this suppression was completely reversed by iberiotoxin (0.1 μM).

5 We conclude that, in ferret trachea *in vitro*, endogenous VIP has dual activity whereby its small direct stimulatory action on mucus secretion is secondary to its marked regulation of cholinergic and tachykinergic neurogenic mucus secretion. Regulation is *via* inhibition of neurotransmitter release, consequent upon opening of BK_{Ca} channels. In the context of neurogenic mucus secretion, we propose that VIP joins NO as a neurotransmitter of i-NANC nerves in ferret trachea.

Keywords: Acetylcholine release; airways; cholinergic nerve; ferret trachea; iberiotoxin; mucus secretion; potassium channel; vasoactive intestinal peptide

Abbreviations: ACh, acetylcholine; ATP, adenosine triphosphate; BK_{Ca} (channel), large conductance, calcium-activated potassium (channel); CGRP, calcitonin gene-related peptide; DMSO, dimethyl sulphoxide; i-NANC, inhibitory non-adrenergic, non-cholinergic; K⁺ (channel), potassium (channel); K_{ATP} (channel), adenosine triphosphate sensitive potassium channel; NANC, non-adrenergic, non-cholinergic; NKA, neurokinin A; NO, nitric oxide; NOS, nitric oxide synthase; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase-activating peptide; PHM, peptide histidine methionine; SK_{Ca} (channel), small conductance calcium-activated potassium channel; SP, substance P; VIP, vasoactive intestinal peptide; VIP-Ab, vasoactive intestinal peptide antibody

Introduction

Airway neural pathways are classified currently as adrenergic, cholinergic and non-adrenergic non-cholinergic (NANC): they are anatomically interlinked and interact functionally. The excitatory and inhibitory neural mechanisms regulating airway smooth muscle function are relatively well defined (Barnes, 1996; Widdicombe, 1996). In contrast, for neurogenic airway mucus secretion, although excitatory pathways are comparatively well characterized (Rogers 1997), inhibitory pathways are poorly defined. In mammalian airways, the predominant neural control for mucus secretion is cholinergic with a minor adrenergic component. There is also a 'sensory-efferent' neural pathway controlling mucus secretion. The nerves comprising the latter pathway are C-fibres sensitive to capsaicin and whose

neurotransmitters are calcitonin gene related peptide (CGRP) and the tachykinins substance P (SP) and neurokinin A (NKA), collectively termed sensory neuropeptides (Ramnarine & Rogers, 1994). In ferret trachea *in vitro*, sensory-efferent pathways account for up to 40% of the total neurogenic secretory response, with the functional response (i.e. mucus secretion) mediated *via* tachykinin NK₁ receptors on the secretory cells (Ramnarine *et al.*, 1994). Nitric oxide (NO) and vasoactive intestinal peptide (VIP) are neurotransmitters of inhibitory NANC (i-NANC) neural pathways regulating the magnitude of neurogenic bronchoconstriction, although their relative involvement varies with species (Lammers *et al.*, 1992). We found that endogenous NO regulated the magnitude of neurogenic mucus secretion in ferret trachea *in vitro* (Ramnarine *et al.*, 1996). The role of VIP as a regulator of neurogenic mucus secretion is unexplored.

Immunohistochemical studies localize VIP-containing airway nerves to intrinsic ganglia, smooth muscle, submucosal

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glands and blood vessels in human (Dey *et al.*, 1981; Laitinen *et al.*, 1985), dog and cat (Dey *et al.*, 1988). VIP coexists with SP and galanin in cat airways (Dey & Zhu, 1994; Dey *et al.*, 1988), nitric oxide synthase (NOS) and acetylcholine (ACh) in human airways (Fischer & Hoffman, 1996; Laitinen *et al.*, 1985), neuropeptide Y (NPY) in guinea-pig trachea (Bowden & Gibbins, 1992), and with SP and NOS in ferret trachea (Dey *et al.*, 1996). By autoradiography, VIP receptors are localized to submucosal glands, epithelium and smooth muscle in airways of pig (Lazarus *et al.*, 1986), rat (Leroux *et al.*, 1984) and human (Fischer *et al.*, 1992). These anatomical studies imply that VIP has a role in modulating airway mucus secretion and may interact with other neurotransmitters.

In the present study, we explored the role of VIP on neural and non-neural control of mucus secretion in ferret trachea *in vitro* in Ussing chambers using $^{35}\text{SO}_4$ as a mucus marker. Firstly, we determined the effect of VIP itself on non-neural mucus secretion. Secondly, we investigated the effect of VIP on cholinergic, tachykinergic, and non-NANC non-tachykinergic neural mucus secretion, using exogenous VIP, a selective VIP antibody (VIP-Ab) (Shimura *et al.*, 1988), the peptidase α -chymotrypsin, or a putative VIP₁ receptor antagonist [D-p-Cl-Phe⁶,Leu¹⁷]-VIP (Usdin *et al.*, 1994) to investigate the role of endogenous VIP. Thirdly, we determined the class of potassium (K^+) channel involved in VIP inhibition using the ATP sensitive K^+ (K_{ATP}) channel inhibitor glibenclamide (Niki *et al.*, 1989; Schmid-Antomarchi *et al.*, 1990), the blocker of calcium activated K^+ (K_{Ca}) channels of small conductance (SK_{Ca} channels) apamin (Banks *et al.*, 1979), and the large conductance K_{Ca} (BK_{Ca}) channel blocker iberiotoxin (Garcia *et al.*, 1991). Finally, to more directly confirm a neuromodulatory role for VIP on cholinergic neurotransmission, we determined the effect of VIP, α -chymotrypsin and VIP-Ab on ACh release from ferret trachea *in vitro* using [^3H]-choline as a marker for ACh (Alberts *et al.*, 1982; Wessler *et al.*, 1991).

Methods

Male ferrets (Regal Rabbits, Great Bookham, Surrey, U.K.) weighing 1.0–2.0 kg were used throughout and were kept four to five in a room. They were allowed 1 week to recuperate 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 and bathed in aerated (95% O_2 + 5% CO_2) Krebs-Henseleit solution 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) for study of either mucus secretion or acetylcholine release.

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 a 'luminal' (i.e. mucus-producing) and 'submucosal' side. 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 cm². 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.

Radiolabelling of newly synthesized mucus

At time 0 h, $\text{Na}_2^{35}\text{SO}_4$ (0.1 mCi) was added to the submucosal half-chambers, in order 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 $^{35}\text{SO}_4$ -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 *Protocols for secretory studies* below).

Electrical stimulation

The tracheal segments could be 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, U.S.A.) (Borson *et al.*, 1984). Tissues were stimulated at 2.5 or 10 Hz, 50 V, 0.5 ms for the first 5 min of a 15 min incubation period.

Measurement of $^{35}\text{SO}_4$ 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⁻¹) using cellulose tubing (Medicell International Ltd., London, U.K.) which allowed molecules of 12–14 kDa or less 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 min [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 Ltd., Pangbourne, Berkshire, U.K.) was determined by scintillation spectrometry (model 1900CA Spectrophotometer, Canberra Packard Ltd.). 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).

Protocols for secretory studies

To investigate the effect of exogenous VIP on non-neural mucus secretion, concentration-response and time course studies of VIP were carried out. The first collection at 2.25 h was the baseline control after which VIP (1 nM–10 μM) or vehicle (distilled water) were added to the submucosal chamber where they remained for the duration of the experiment.

To validate the effectiveness of VIP-Ab in neutralizing the secretory response induced by exogenous VIP, VIP-Ab (final dilution 1:500; Shimura *et al.*, 1988) or control serum was added to the submucosal chamber 30 min prior to the administration of VIP (10 nM–10 μM).

To examine the effects of endogenous VIP in regulating cholinergic neural secretion, VIP-Ab (1 : 500) or control serum, the putative VIP₁ receptor antagonist, [D-p-Cl-Phe⁶,Leu¹⁷]-VIP (0.5 or 5 μ M) (Usdin *et al.*, 1994), or α -chymotrypsin (2 u ml⁻¹) were added to the submucosal chamber 30 min before and during electrical stimulation (2.5 or 10 Hz, 50 V, 0.5 ms for 5 min) in the presence of phentolamine, propranolol (10 μ M for each), and the tachykinin NK₁ receptor antagonist CP-99,994 (3 μ M; McLean *et al.*, 1993) to remove α -adrenergic, β -adrenergic and tachykinergic neural influences respectively.

To examine the effects of exogenous VIP on cholinergic neural secretion, VIP or control vehicle was added to the submucosal chamber 30 min before and during 2.5 or 10 Hz stimulation in the presence of phentolamine, propranolol and CP-99,994 (as above). In addition, in the studies investigating the effect of VIP on ACh release, epithelially-scraped tissues were used in the presence of indomethacin (see below). To assess the influence of this treatment on secretion, the effect of 10 Hz cholinergic stimulation (in the presence of adrenoceptor and NK₁ receptor blockade; as above) was compared between tissues not scraped and without indomethacin, and tissues scraped in the presence of indomethacin (10 μ M).

To determine the effects of endogenous VIP on tachykinergic neural secretion, VIP-Ab (1 : 500) or control serum was added to the submucosal chamber 30 min prior to and during 2.5 or 5 Hz stimulation in the presence of phentolamine, propranolol and atropine (10 μ M each) to eliminate adrenergic and cholinergic neural influences.

To study the effects of exogenous VIP in modulating tachykinergic neural secretion, VIP (1 μ M) was added to the submucosal chamber 30 min before and during 10 Hz stimulation in the presence of phentolamine, propranolol and atropine (10 μ M each).

To assess the contribution of endogenous VIP to NANC non-tachykinergic neural secretion, VIP-Ab was added to the submucosal side for 30 min before and during stimulation at 2.5 or 10 Hz in the presence of atropine, phentolamine, propranolol (10 μ M each) and CP-99,994 (3 μ M).

To test whether or not the post-junctional secretory responses induced by ACh or SP were affected by VIP or VIP-Ab, ACh (1 μ M) or SP (1 μ M) was added to the luminal chamber in the presence of VIP (1 μ M), VIP-Ab or control serum for 30 min.

To investigate the involvement of, and determine the class of, K⁺ channels in VIP-regulation of cholinergic neural secretion (i.e. electrical stimulation in the presence of phentolamine, propranolol and CP-99,994; as above), vehicle solution, glibenclamide (0.1 μ M), apamin (1 μ M) or iberiotoxin (3 μ M) were added to the luminal half-chamber 15 min before VIP (10 nM), which in turn was added 30 min before 10 Hz stimulation.

Tracheal preparation for measurement of ACh release

Release of ACh from cholinergic nerves was measured as described previously (Ward *et al.*, 1993; Patel *et al.*, 1995). Tracheae were bathed in Krebs-Henseleit solution, opened longitudinally by cutting anteriorly through the cartilage, and the epithelium was carefully peeled off. Strips of smooth muscle were dissected from the tracheae and as much cartilage as possible was removed. Indomethacin (10 μ M) was present in the Krebs-Henseleit solution throughout to prevent the formation of endogenous prostanoids which have been demonstrated previously to affect cholinergic neurotransmission and ACh release (Walters *et al.*, 1984; Deckers *et al.*, 1989;

Wessler *et al.*, 1990; Belvisi *et al.*, 1996). The tracheal segments (~1 × 5 mm) were suspended top and bottom with silver wire (Goodfellow Metals, Cambridge, Cambridgeshire, U.K.), mounted in perfusion chambers, and superfused with Krebs-

Figure 1 Effect of exogenous vasoactive intestinal peptide (VIP) on mucus secretion in ferret trachea *in vitro*. (a) Concentration-response and inhibition by VIP antibody (VIP-Ab), (b) time course. Data are mean per cent change in output of macromolecules labelled *in situ* with ³⁵SO₄ (a marker for mucus) for 5–8 animals per group; vertical bars are one s.e.mean. **P* < 0.05, ***P* < 0.01 compared with baseline control (a), or with corresponding vehicle control time point (b); #*P* < 0.05 compared with VIP group (a).

Figure 2 Effect of endogenous vasoactive intestinal peptide (VIP) on neurogenic mucus secretion in ferret trachea *in vitro*. (a) Effect on cholinergic secretion. A VIP antibody (VIP-Ab, 1:500 dilution) was used to exclude VIP, and phentolamine, propranolol (10 μ M each) and the tachykinin NK₁ receptor antagonist CP-99,994 (3 μ M) were used to exclude adrenergic and tachykinergic neural influences at stimulation parameters of 2.5 or 10 Hz, 50 V, 0.5 msec for 5 min. ACh, acetylcholine (1 μ M). (b) Effect on tachykinergic neural secretion. VIP antibody (1:500 dilution) was used to exclude VIP, and phentolamine, propranolol and atropine (10 μ M each) were used

Henseleit solution, bubbled with 95% O₂, 5% CO₂ at 37°C at 1 ml min⁻¹ via a perfusion pump (model 503S; Smith and Nephew Watson-Marlow, Falmouth, Cornwall, U.K.).

Loading with [³H]-choline

The tracheal strips were allowed to equilibrate during a 30-min period of continuous superfusion with Krebs-Henseleit solution, and electrical stimulation (4 Hz, 40 V, 0.5 ms) was applied for the last 10 min of this period (model S88 stimulator; Grass Instrument Co.) via the silver wires. Tissues were then immersed in 1.5 ml oxygenated Krebs-Henseleit solution containing [methyl-³H]-choline chloride (67 nmol liter⁻¹; specific radioactivity 2.78 TBq mmol⁻¹) and electrically stimulated for another 45 min to allow uptake of [³H]-choline into the tissues. At the end of this period, superfusion was continued with Krebs-Henseleit solution containing hemicholinium-3 (10 μ M) to prevent further uptake of [³H]-choline into the tissues. Tissues were then washed for 2.5 h to reach a stable baseline of [³H] release before beginning collections for the experiment (see *Protocols* below). The single term '[³H]' is used herein to acknowledge that the precise identity of the molecule to which the released [³H] is bound is unknown (choline, ACh or phosphorylcholine; see Wessler *et al.*, 1990, 1991). Radioactivity in 1 ml aliquoted sample mixed with 4 ml scintillant (Pico-Fluor 40, Canberra Packard Ltd., Pangbourne, Berkshire, U.K.) was determined by scintillation spectrometry (Model 1900CA Spectrophotometer, Canberra Packard Ltd.).

Protocols for [³H] release studies

Each tracheal tissue segment was stimulated twice (once at 19 min and once at 56 min after equilibration) at 2.5 or 10 Hz, 50 V, 0.5 ms for 1 min, to provoke [³H] overflow. VIP (10 nM and 0.1 μ M), VIP-Ab (1:500), α -chymotrypsin (2 u ml⁻¹) or control solutions were administered 30 min before the second stimulation. In certain experiments, ibuprofen (0.1 μ M) was added 5 min before the administration of VIP. Samples were collected every minute for 3 min before stimulation, for the 1 min of stimulation, every minute for 3 min after stimulation, and at 5 min intervals outside these times.

Drugs and chemicals. The following drugs were used: VIP (porcine; synthetic), substance P, [D-p-Cl-Phe⁶,Leu¹⁷]-VIP (human, porcine, rat; synthetic), α -chymotrypsin, acetylcholine, dimethylsulphoxide (DMSO), indomethacin, tetrodotoxin (TTX) and hemicholinium-3 (Sigma Chemical Company Ltd., Poole, Dorset, U.K.); atropine sulphate (Phoenix Pharmaceuticals Ltd., Pharma Hameln, G.m.b.H., Germany); phentolamine mesylate (Ciba Laboratories, Horsham, West Sussex, U.K.); propranolol hydrochloride (Imperial Chemical Industries Ltd., Macclesfield, Cheshire, U.K.); Na₂³⁵SO₄ and [methyl-³H]-choline chloride (Amersham International plc, Amersham, Berkshire, U.K.); and pentobarbitone sodium B.P. (Sagatal; RMB Animal Health Ltd., Dagenham, Essex,

to exclude adrenergic and cholinergic influences at stimulation parameters of 2.5 or 5 Hz, 50 V, 0.5 msec for 5 min. SP, substance P (1 μ M). Data are mean per cent change in output of macromolecules labelled *in situ* with ³⁵SO₄ (a marker for mucus) for 5–7 animals per group; vertical bars are one s.e.mean. **P* < 0.05 compared with serum control group; #*P* < 0.05, ##*P* < 0.01 compared with sham stimulation group.

U.K.). CP-99,994 (2*S*,3*S*)-3-(2-methoxybenzyl)-amino-2-phenyl-piperidine was a kind gift from Pfizer, Groton, U.S.A. (courtesy Dr R. Michael Snider). VIP-Ab was a rabbit anti-VIP (human, porcine, rat) anti-serum, without cross-reactivity to VIP 1-12 (human, porcine, rat), peptide histidine methionine (PHM)-27 (human), substance P, endothelin-1 (human, porcine, canine, rat, mouse, bovine), secretin (porcine), glucagon (human), galanin (rat), somatostatin, pituitary adenylate cyclase-activating peptide (PACAP)-38 (human, bovine, rat) or PACAP-38 (16-38) (human, bovine, rat), and with little cross-reactivity to VIP 10-28 (human, porcine, rat) (4.4%) or PACAP-27 (human, bovine, rat) (0.02%) (Pennisula Laboratories, Inc., Belmont, U.S.A.).

Data analysis

In Results, data for mucus secretion are the arithmetic mean and one standard error of the mean (s.e.mean). 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. Data for ACh release are rate changes for the second stimulation compared with the first stimulation, with the rate coefficient the fractional release of initial [^3H] content over time. The concentration of VIP causing a 50% increase (EC_{50}) in secretion or inhibition (IC_{50})

Figure 3 Effect of exogenous vasoactive intestinal peptide (VIP) on neurogenic mucus secretion in ferret trachea *in vitro*. (a and b) Effect on cholinergic neural secretion. Phentolamine, propranolol ($10\mu\text{M}$ each) and the tachykinin NK_1 receptor antagonist CP-99,994 ($3\mu\text{M}$) were used to exclude adrenergic and tachykinergic influences at stimulation parameters of 2.5 (a) or 10 Hz (b), 50 V, 0.5 msec for 5 min. (c) Effect on tachykinergic neural secretion. Phentolamine, propranolol and atropine ($10\mu\text{M}$ each) were used to exclude adrenergic and cholinergic influences at stimulation parameters of 10 Hz, 50 V, 0.5 msec for 5 min. SP, substance P ($1\mu\text{M}$). Data are mean per cent change in output of macromolecules labelled *in situ* with $^{35}\text{SO}_4$ (a marker for mucus) for 5-7 animals per group; vertical bars are one s.e.mean. * $P < 0.05$ compared with stimulation control group (a and b), or with serum control group (c).

Figure 4 Inhibition by exogenous vasoactive intestinal peptide (VIP) of cholinergic neural mucus secretion in ferret trachea *in vitro*, and the effect of potassium (K^+) channel blockers. Phentolamine, propranolol ($10 \mu M$ each) and the tachykinin NK_1 receptor antagonist CP-99,994 ($3 \mu M$) were used to exclude adrenergic and tachykinergic influences at stimulation parameters of 10 Hz, 50 V, 0.5 msec for 5 min. Gli: glibenclamide, ATP-sensitive K^+ (K_{ATP}) channel inhibitor; apamin, small conductance calcium-activated K^+ (SK_{Ca}) channel blocker; IbTx: iberiotoxin, large conductance calcium-activated K^+ (BK_{Ca}) channel blocker. Data are mean per cent change in output of macromolecules labelled *in situ* with $^{35}SO_4$ (a marker for mucus) for 5–6 animals per group; vertical bars are one s.e.mean. * $P < 0.05$, ** $P < 0.01$ compared with stimulation control group; # $P < 0.05$ compared with VIP + vehicle group.

of neurogenic secretion was calculated by non-linear regression using GraphPad Prism software (Microsoft, San Diego, U.S.A.). Significance of changes in $^{35}SO_4$ output and [3H]-choline overflow, 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).

Results

Median baseline radioactivity in the secretion studies was of the order of 740 d.p.m. (range 387–1272 d.p.m., depending upon the specific experiment). There were no significant differences between treatment groups.

Effect of exogenous VIP on mucus secretion

VIP (1 nM – $10 \mu M$) caused an increase in $^{35}SO_4$ output in ferret tracheal segments during the 15 min incubation period after addition. The increase was concentration-dependent with an

Figure 5 Endogenous vasoactive intestinal peptide (VIP) as mediator of NANC, non-tachykinergic neural mucus secretion in ferret trachea *in vitro*. A VIP antibody (VIP-Ab, 1:500 dilution) was used to exclude VIP, and phentolamine, propranolol, atropine ($10 \mu M$) and the tachykinin NK_1 receptor antagonist CP-99,994 ($3 \mu M$) were used to exclude adrenergic, cholinergic and tachykinergic influences at stimulation parameters of 2.5 or 10 Hz, 50 V, 0.5 msec for 5 min. Data are mean per cent change in output of macromolecules labelled *in situ* with $^{35}SO_4$ (a marker for mucus) for six animals per group; vertical bars are one s.e.mean. * $P < 0.05$ compared with serum control group.

approximate EC_{50} of $0.9 \mu M$ and a maximal increase of 53% above control at $10 \mu M$ (Figure 1a). In the continued presence of VIP ($1 \mu M$) in the submucosal side, $^{35}SO_4$ output peaked during the first 15 min incubation and, although showing a reduced response, was increased significantly above controls for at least 60 min (Figure 1b).

Effects of VIP-Ab on VIP-induced secretion, and cholinergic and tachykinergic neural mucus secretion

Preincubation for 30 min with VIP-Ab caused a rightward shift in the VIP concentration-response curve with significant inhibitions of 90 and 70% respectively at 0.1 and $1 \mu M$ VIP (Figure 1a). Control serum had no significant inhibitory effect on VIP-induced $^{35}SO_4$ output (Figure 1a).

In the presence of phentolamine, propranolol ($10 \mu M$ each) and CP-99,994 ($3 \mu M$), both 2.5 and 10 Hz (50 V, 0.5 ms for 5 min) stimulation significantly induced cholinergic $^{35}SO_4$ output by 21 and 73% respectively above pre-stimulation controls (Figure 2a). VIP-Ab significantly potentiated cholinergic $^{35}SO_4$ output by 219% at 2.5 Hz, but had no significant effect at 10 Hz (an increase of 25% above the stimulated value). VIP-Ab did not affect $^{35}SO_4$ output elicited by exogenous ACh ($1 \mu M$), of an equivalent magnitude to that elicited by 2.5 Hz electrical stimulation (Figure 2a). The putative VIP_1 receptor antagonist, [D-p-Cl-Phe⁶,Leu¹⁷]-VIP (0.5 or $5 \mu M$), did not alter the magnitude of cholinergic $^{35}SO_4$

Figure 6 Effect of 10 Hz stimulation (50 V, 0.5 msec, 5 min) on acetylcholine (ACh) release by ferret tracheal strips *in vitro*. Tissues were pre-incubated with [3 H]-choline chloride (a marker for ACh) and stimulated electrically (S). Each panel gives the rate of [3 H] overflow for an individual tracheal strip. VIP, vasoactive intestinal peptide (0.1 μ M); TTX, tetrodotoxin (0.1 μ M); IbTx, iberiotoxin (0.1 μ M).

output at 2.5 or 10 Hz stimulation, nor did it affect exogenous VIP-elicited $^{35}\text{SO}_4$ output. α -Chymotrypsin (2 u ml $^{-1}$) itself caused a significant increase in $^{35}\text{SO}_4$ output of 40% above control ($n=6$, $P<0.05$). Neither the VIP receptor antagonist nor α -chymotrypsin were utilized further to evaluate the role of endogenous VIP in neurogenic secretion.

In the presence of atropine, phentolamine and propanolol (10 μ M each), VIP-Ab significantly enhanced tachykinergic $^{35}\text{SO}_4$ output by 237% above serum controls at 5 Hz stimulation (Figure 2b). VIP-Ab did not affect $^{35}\text{SO}_4$ output induced by SP (1 μ M) (Figure 2b).

Effect of exogenous VIP on cholinergic and tachykinergic neural mucus secretion

In the presence of phentolamine, propanolol (10 μ M each) and CP-99,994 (3 μ M), exogenous VIP inhibited cholinergic $^{35}\text{SO}_4$ output: at 2.5 Hz stimulation with complete inhibition at 1 μ M (Figure 3a), and at 10 Hz with a maximal inhibition of 80% at 10 nM (Figure 3b). In contrast, exogenous VIP (1 μ M) had an additive effect with ACh in increasing $^{35}\text{SO}_4$ output (41 $\pm$ 3% increase with ACh alone vs 78 $\pm$ 9% increase with ACh + VIP, $P<0.05$). Epithelial scraping and indomethacin had no significant effect on cholinergic $^{35}\text{SO}_4$ output compared with non-scraped tissues without indomethacin: respectively 13 $\pm$ 10% vs 8 $\pm$ 9% increase in output in sham stimulated

tissues, 227 $\pm$ 30% vs 245 $\pm$ 25% in stimulated tissues, 62 $\pm$ 18% vs 96 $\pm$ 20% in stimulated tissues in the presence of VIP (10 nM) ($n=4$ for each group).

In the presence of atropine, phentolamine and propanolol (10 μ M each), 10 Hz stimulation caused an increase in tachykinergic $^{35}\text{SO}_4$ output of 42% above pre-stimulation values, which was inhibited by exogenous VIP (1 μ M) by 59% (Figure 3c). In contrast, SP (1 μ M)-induced increase in $^{35}\text{SO}_4$ output was not significantly changed by pre-treatment with VIP (1 μ M) (Figure 3c).

Effect of K^+ channel blockers on cholinergic neural secretion

In the presence of phentolamine, propanolol (10 μ M each) and CP-99,994 (3 μ M), exogenous VIP (1 μ M) significantly diminished 10 Hz cholinergic secretion by 79% compared with stimulation control, and this effect was reversed (by 60%) by pre-incubation with iberiotoxin (3 μ M), but not by apamin (1 μ M) or glibenclamide (0.1 μ M) (Figure 4).

Effect of VIP-Ab on NANC, non-tachykinergic neural mucus secretion

In the presence of atropine, phentolamine, propanolol (10 μ M each) and CP-99,994 (3 μ M), 10 Hz stimulation significantly

Figure 7 Involvement of large-conductance K^+ (BK_{Ca}) channels in vasoactive intestinal peptide (VIP)-inhibition of acetylcholine (ACh) release in ferret tracheal strips *in vitro*. IbTx, iberiotoxin ($0.1 \mu M$); BK_{Ca} channel blocker. Tissues were pre-incubated with [3H]-choline chloride (a marker for ACh) and stimulated electrically at 10 Hz, 50 V, 5 msec for 5 min. Data are mean per cent change in rate of [3H] overflow at a second stimulation compared with the first stimulation for 5–6 animals per group; vertical bars are one s.e.mean. * $P < 0.05$ compared with Krebs's solution alone; ## $P < 0.01$ compared with VIP + IbTx group.

increased $^{35}SO_4$ output by 25% above pre-stimulation control, whereas stimulation at 2.5 Hz did not significantly increase $^{35}SO_4$ output (Figure 5). The 10 Hz stimulation-induced $^{35}SO_4$ output was eliminated by VIP-Ab (Figure 5).

Effects of VIP and iberiotoxin on cholinergic neural [3H] output

Exogenous VIP ($0.1 \mu M$) significantly reduced [3H] overflow at 10 Hz stimulation by 34% compared with control (Figures 6 and 7). This effect was completely reversed by pre-administration of iberiotoxin ($0.1 \mu M$) (Figures 6 and 7). Iberiotoxin alone had no significant effect on stimulated [3H] output (Figure 7). Tetrodotoxin ($3 \mu M$) markedly inhibited (by ~90%) the [3H] overflow evoked by 10 Hz stimulation (Figure 6): mean 10% ($\pm 12\%$) change in [3H] overflow compared with first stimulation ($n = 5$, $P < 0.01$).

Effects of VIP-Ab and α -chymotrypsin on cholinergic neural [3H] output

Pre-treatment with VIP-Ab ($1:500$) or α -chymotrypsin (2 u ml^{-1}) potentiated neural [3H] output at 2.5 Hz by 44 and 40%, respectively (Figures 8 and 9). Tetrodotoxin ($3 \mu M$) markedly inhibited (by ~95%) the [3H] overflow evoked by 2.5 Hz stimulation (Figure 8): mean 5% ($\pm 10\%$) change in [3H] overflow compared with first stimulation ($n = 5$, $P < 0.01$).

Discussion

In the present study in ferret trachea *in vitro*, exogenous VIP increased $^{35}SO_4$ -labelled mucus output whereas endogenous VIP regulated the magnitude of neurogenic secretion. The increase in $^{35}SO_4$ output is consistent with an increase in mucus secretion by submucosal glands, because there are few surface epithelial goblet cells but large numbers of submucosal glands in ferret trachea (Robinson *et al.*, 1986; Meini *et al.*, 1993). By autoradiography, under basal conditions there is selective uptake and retention of $^{35}SO_4$ by tracheal submucosal glands, rather than epithelium, in cat (Davies *et al.*, 1990) and ferret (Gashi *et al.*, 1987), with some labelling of cartilage. Stimulation of ferret trachea *in vitro* increased radioactive counts in the incubation medium, with concomitant loss of autoradiographic grains from the glands (Gashi, 1987): neither cartilage nor epithelium showed loss of grains. In cats, tracheal washings labelled with $^{35}SO_4$ have a molecular weight and buoyant density typical of a mucin molecule (Davies *et al.*, 1990). Thus, the increased output of $^{35}SO_4$ observed herein is indicative of an increase in tracheal submucosal gland mucus secretion.

We demonstrated herein that exogenous VIP caused a small increase in secretion in a concentration-dependent fashion with an increase of 32% above control at $1 \mu M$. This observation is consistent with previous reports showing that VIP induces release of a variety of mucus markers with similar potency (maximal/near maximal effects at $\sim 1 \mu M$) in a number of different *in vitro* airway preparations: ferret trachea (Peatfield *et al.*, 1983; Gashi *et al.*, 1986), cat isolated tracheal submucosal glands (Shimura *et al.*, 1988), rat trachea (Wagner *et al.*, 1998) and human nasal mucosal explants (Baraniuk *et al.*, 1990; Mullol *et al.*, 1992). VIP also stimulated rhinorrhea *in vivo* in human volunteers (Chatelain *et al.*, 1995). With secretory activity apparent at $\sim 1 \mu M$, it has to be concluded that VIP is not a potent secretagogue. However, it would appear from the present study that the activity of VIP is related primarily to neuroregulation of airway secretion rather than to induction of secretion. The VIP-induced increase in mucus secretion found herein was inhibited by a VIP-Ab. VIP-Ab has previously been shown to inhibit the potentiation by VIP of methacholine-elicited [3H]-glycoconjugate release by cat isolated submucosal glands (Shimura *et al.*, 1988), and to inhibit smooth muscle relaxation induced by i-NANC electrical stimulation in cat and guinea-pig trachea, a response mediated by VIP (Li & Rand, 1991). Taken together, airway responses to exogenous and endogenous VIP are effectively suppressed by VIP-Ab, making it a useful tool for investigating the biological role of endogenous VIP. α -Chymotrypsin is a peptidase which has also been used to investigate the effect of endogenous VIP. For example, α -chymotrypsin greatly reduced the relaxant response to exogenous VIP and to NANC electrical stimulation in guinea-pig tracheal smooth muscle (Ellis & Farmer, 1989a; Tucker *et al.*, 1990; Li & Rand, 1991), which gave support to the notion that VIP is a neurotransmitter of the i-NANC nervous system in guinea-pig airways. However, in the present study, we found that α -chymotrypsin itself caused an increase in $^{35}SO_4$ output which precluded its use in characterizing the role of exogenous VIP on secretion, and of endogenous VIP on neurogenic secretion. In the present study, a putative VIP₁ antagonist, [D-p-Cl-Phe⁶,Leu¹⁷]-VIP, did not alter the magnitude of cholinergic neural secretion nor did it diminish exogenous VIP-induced mucus secretion. This antagonist has variable effects depending upon species and preparation (Ellis & Farmer, 1989b; Aizawa *et al.*, 1994; Shvilkin *et al.*, 1994), but is not suitable for

Figure 8 Effect of 2.5 Hz stimulation (50 V, 0.5 msec, 5 min) on acetylcholine (ACh) release in ferret tracheal strips *in vitro*. Tissues were pre-incubated with [3 H]-choline chloride (a marker for ACh) and stimulated electrically (S). Each panel gives the rate of [3 H] overflow for an individual tracheal strip. VIP-Ab, antibody to vasocative intestinal peptide (1:500 dilution); TTX, tetrodotoxin (0.1 μ M); α -chymotrypsin (2 u ml $^{-1}$).

investigating the role of endogenous VIP in neurogenic mucus secretion in ferret trachea.

We found herein that exogenous VIP markedly inhibited cholinergic neural secretion at both 2.5 and 10 Hz, and that VIP-Ab potentiated cholinergic neural secretion (at 2.5 Hz). In contrast, exogenous VIP (1 μ M) and ACh combined had an additive effect on secretion, whilst VIP-Ab had no significant effect on ACh-induced secretion. These results suggest that VIP regulates prejunctional cholinergic neuro-transmission to mucus secretion in ferret trachea rather than affecting the post-junctional activity of ACh. There is evidence to suggest that VIP regulates cholinergic neurotransmission in guinea-pig and ferret tracheal smooth muscle prejunctionally, post-junctionally or ganglionically. For example, VIP inhibited both ganglionic and post-ganglionic cholinergic contractile responses in an isolated innervated guinea-pig tracheal preparation in response to vagal stimulation or electrical field stimulation (EFS) (Martin *et al.*, 1990). Similarly, VIP inhibited guinea-pig tracheal smooth muscle contraction evoked by EFS, and also decreased ACh-induced contraction (Shigyo *et al.*, 1997). Depending upon concentration, VIP may augment (at 1 pM–1 nM) or suppress (at 10 nM–0.2 μ M) cholinergic neurotransmission in ferret tracheal smooth muscle (Sekizawa *et al.*, 1988). VIP also has variable effects on secretion, depending upon stimulus. VIP inhibited maintained methacholine-stimulated mucous gland cell liquid secretion *in vitro* in ferret trachea, but potentiated maintained phenylephr-

ine-stimulated serous gland cell liquid secretion (Webber & Widdicombe, 1987). In contrast, VIP augmented methacholine-induced [3 H]-glycoconjugate secretion from isolated cat tracheal submucosal glands (Shimura *et al.*, 1988). Thus, the conflicting data of the latter two studies, combined with our present data of no inhibitory or potentiating effect for VIP on ACh-induced secretion, indicate that the effect of VIP on cholinomimetic-induced secretion is complex and varies with animal species, study design, and degree of isolation of the glands. It is possible that isolation of the cat gland from the epithelium removes an inhibitory influence (Sasaki *et al.*, 1989). However, in the present study, epithelial scraping did not alter the secretory response to cholinergic nerve stimulation.

In both our present study and the latter study using the isolated cat gland, cyclooxygenase inhibition did not affect stimulated mucus output, which suggests that endogenous prostaglandins do not regulate secretion. Interestingly, depending upon the prostaglandin, exogenous prostaglandins can increase, decrease or have no effect on different parameters of secretion in ferret trachea *in vitro* (Deffebach *et al.*, 1990). Thus, the data of the latter study combined with the lack of effect of cyclooxygenase discussed above indicate either that prostaglandins are not generated endogenously under the above experimental conditions, or that the opposing effects of different generated prostaglandins leads to an overall lack of secretory response.

Figure 9 Effect of endogenous vasoactive intestinal peptide (VIP) on acetylcholine (ACh) release in ferret tracheal strips *in vitro*. A VIP antibody (VIP-Ab, 1:500 dilution; a) or α -chymotrypsin (2 μM ; b) were used to exclude VIP. Tissues were pre-incubated with $[^3\text{H}]$ -choline chloride (a marker for ACh) and stimulated electrically at 2.5 Hz, 50 V, 5 msec for 5 min. Data are mean per cent change in rate of $[^3\text{H}]$ overflow at a second stimulation compared with the first stimulation for five animals per group; vertical bars are one s.e.mean. * $P < 0.05$ compared with control group.

We found herein that exogenous VIP significantly suppressed tachykininergic neural secretion at 10 Hz stimulation and VIP-Ab markedly potentiated tachykininergic neural secretion at 5 Hz stimulation. Neither VIP nor VIP-Ab altered the secretory response to exogenous SP. These results suggest that VIP modulates the release of tachykinins *via* a prejunctional mechanism. A similar conclusion has been reached for VIP-inhibition of tachykininergic contractility of guinea-pig smooth muscle (Stretton *et al.*, 1991). VIP also inhibited SP-induced ACh release in rabbit tracheal smooth muscle strips (Giuseppe *et al.*, 1995).

In the presence of atropine, propranolol, phentolamine and CP-99,994 (i.e. NANC, non-tachykininergic), 10 Hz stimulation evoked a significant increase in mucus secretion of 25% above control, which was abolished by VIP-Ab indicating that VIP was the mediator of the response. It is interesting that the magnitude of the response was equivalent to that induced by exogenous VIP (1 μM). The concentration of VIP in the tissues, after release from nerves, is unknown, although local concentrations may be high. However, whatever the concentration near the secretory cells, the small increases in secretion elicited by either exogenous or endogenous VIP do not indicate that VIP is an important airway secretagogue. Rather, neuronal VIP elicits two responses: one is a small stimulatory

effect upon mucus secretion, but this is offset by its effect in curbing prejunctional cholinergic and tachykininergic neural secretion.

Incubation with $[^3\text{H}]$ -choline and subsequent overflow of $[^3\text{H}]$ can substitute for bioassay methods in the measurement of ACh release from guinea-pig ileum (Alberts *et al.*, 1982) and has been applied to a variety of airway smooth muscle preparations (Wessler *et al.*, 1990, 1991; Ward *et al.*, 1993; Patel *et al.*, 1995). In the present study, tetrodotoxin virtually abolished $[^3\text{H}]$ overflow evoked by electrical stimulation of ferret tracheal smooth muscle strips which supports the concept that $[^3\text{H}]$ overflow in our ACh release preparation is of neural origin. We found that exogenous VIP significantly inhibited $[^3\text{H}]$ overflow, whereas VIP-Ab and α -chymotrypsin augmented $[^3\text{H}]$ overflow. These data support the contention that endogenous VIP regulates the magnitude of neurogenic tracheal mucus secretion in ferret *via* inhibition of ACh release.

In this study, we found that the BK_{Ca} channel inhibitor, iberiotoxin, but not the SK_{Ca} channel inhibitor, apamin, or the K_{ATP} channel inhibitor, glibenclamide, reversed the VIP-inhibition of cholinergic neural secretion. Similarly, iberiotoxin reversed the suppression of ACh release by exogenous VIP. Reversal in both instances was unlikely to be due to any possible potentiating effect of iberiotoxin on neurotransmission because we have shown previously that iberiotoxin has no significant effect on neurogenic mucus output (Ramnarine *et al.*, 1998), and herein did not affect stimulated $[^3\text{H}]$ overflow. These data suggest that VIP-inhibition of cholinergic neurotransmission is mediated *via* opening of BK_{Ca} channels. We have found previously that selective opioid OP_1 and OP_3 receptor agonists inhibit neurogenic mucus secretion in ferret trachea at a prejunctional site *via* opening of BK_{Ca} channels (Ramnarine *et al.*, 1998).

VIP inhibited glycoconjugate secretion and lysozyme release from normal human tracheal explants. In contrast, in explants with 'bronchitic' changes, VIP did not inhibit baseline or methacholine-stimulated glycoconjugate release, or mucous or serous cell discharge (Cole *et al.*, 1981). In severe asthma, there is evidence of an imbalance in NANC nerves within the airways; specifically a deficiency of VIP- and an excess of SP-immunoreactive nerves (Ollerenshaw *et al.*, 1989, 1991), but not in symptomatic mild to moderate asthmatics (Howarth *et al.*, 1995). In studies where plasma levels of VIP and SP were correlated with airway function, it was concluded that an over-excitation of excitatory-NANC and a deficiency in i-NANC activity were closely related to asthma attacks and bronchial hyperresponsiveness (Liu *et al.*, 1993a,b). Rapid degradation of VIP by a variety of enzymes in asthmatic airways, for example mast cell tryptase (Caughey *et al.*, 1988), and increased plasma levels and affinity of catalytic anti-VIP antibodies in mild to moderate asthmatics (Paul *et al.*, 1995) might contribute to the deficiency in VIP. This might be one explanation for the ineffectiveness of inhaled VIP as a bronchodilator in asthmatics (Barnes & Dixon, 1984).

In conclusion, in ferret trachea *in vitro*, endogenous VIP regulates cholinergic and tachykininergic neural secretion at a prejunctional site *via* opening of BK_{Ca} channels. In the context of mucus secretion, we propose that VIP joins NO as a neurotransmitter of i-NANC neural pathways in ferret trachea.

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