British Journal of Pharmacology (1999) 128, 1353–1359
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Effect of vasoactive intestinal peptide (VIP)-related peptides on cholinergic neurogenic and direct mucus secretion in ferret trachea *in vitro*

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1 We investigated whether vasoactive intestinal peptide (VIP) and its related peptides, pituitary adenylate cyclase activating peptide (PACAP) and secretin, regulate cholinergic neural mucus secretion in ferret trachea *in vitro*, using $^{35}SO_4$ as a mucus marker. We also studied the interaction between VIP and secretin on cholinergic mucus output.

2 VIP (1 and 10 μM) increased secretion, whereas neither $PACAP_{1-27}$, $PACAP_{1-38}$ nor secretin (up to 10 μM) increased mucus output. In contrast, VIP, $PACAP_{1-27}$ and $PACAP_{1-38}$ concentration-dependently inhibited cholinergic neural secretion, with an order of potency of VIP > $PACAP_{1-38}$ > $PACAP_{1-27}$. Neither $PACAP_{1-27}$ nor $PACAP_{1-38}$ altered the secretion induced by acetylcholine (ACh).

3 Secretin increased cholinergic neural secretion with a maximal increase of 190% at 1 μM. This potentiation was blocked by VIP or atropine. Similarly, secretin (1 μM) potentiated VIP (1 μM)-induced mucus output by 160%. Secretin did not alter exogenous ACh-induced secretion. VIP *vs* secretin competition curves suggested these two peptides were competing reversibly for the same receptor.

4 We conclude that, in ferret trachea *in vitro*, VIP and PACAPs inhibit cholinergic neural secretion *via* pre-junctional modulation of cholinergic neurotransmission. VIP and secretin compete for the same receptor, possibly a VIP_1 receptor, at which secretin may be a receptor antagonist.

Keywords: Airways; cholinergic nerve; mucus; mucus secretion; nerves; pituitary adenylate cyclase activating peptide; secretin; vasoactive intestinal peptide

Abbreviations: ACh, acetylcholine; DMSO, dimethylsulphoxide; PACAP, pituitary adenylate cyclase activating peptide; VIP, vasoactive intestinal peptide

Introduction

Mucus secretion onto the internal surface of the airways protects the mucosa from inhaled insult, and is under humoral and neuronal control. In all species studied, the predominant neural pathway is cholinergic (Rogers, 1997). In ferret trachea, the magnitude of cholinergic neural secretion is regulated endogenously by both nitric oxide (NO) (Ramnarine *et al*., 1996) and vasoactive intestinal peptide (VIP) (Liu *et al*., 1999). The identity of the VIP receptor mediating the regulation, and whether other VIP-related peptides show similar activity, is unexplored.

VIP, pituitary adenylate cyclase activating peptide (PACAP) and secretin comprise a family of structurally-related regulatory peptides that includes glucagon, gastric inhibitory peptide and growth hormone releasing factor (Harmar & Lutz, 1994; Segre & Goldring, 1993). VIP is a 28-amino acid peptide with 68% homology with the N-terminal 1–28 sequence of PACAP (Kimura *et al*., 1990). PACAP exhibits two forms physiologically: a 38-amino acid form ($PACAP_{1-38}$), and a form corresponding to the first 27 amino acids of $PACAP_{1-38}$ ($PACAP_{1-27}$). Human secretin is a 27 amino acid peptide, having 48% homology with human VIP (Couvineau *et al*, 1994).

VIP and PACAPs are widely distributed in the central nervous system and in peripheral tissues, including the lung, where they exert diverse biological effects (Christophe, 1993; Arimura & Shioda, 1995). In the airways of human, rat, guinea-pig and ferret, PACAP immunoreactive fibres are located beneath the epithelium, around blood vessels and mucous glands, and among bundles of smooth muscle (Uddman *et al*., 1991). In human and ferret airways, PACAPs are colocalized with VIP. Secretin is distributed primarily in the gastrointestinal tract and central nervous system.

Several types of receptor that interact with VIP and PACAPs have been described, and it is possible that PACAPs are the mediators of the biological activity ascribed to VIP (Harmar & Lutz, 1994). A class of receptor which binds VIP, $PACAP_{1-27}$ and $PACAP_{1-38}$ with similar affinity, and with similar potency in increasing intracellular cyclic AMP, is found in lung, liver and spleen. This class of receptor was divided into VIP_1 and VIP_2 types. Secretin is a selective VIP_1 receptor agonist, albeit with low affinity for these receptors (Ishihara *et al*., 1992; Sreedharan *et al*., 1993; Usdin *et al*., 1994). A second class of receptor, highly specific for PACAPs, is divided into two types on the basis of binding characteristics: $PACAP_{1A}$ receptors bind $PACAP_{1-27}$ with slightly higher affinity than $PACAP_{1-38}$, whilst $PACAP_{1B}$ receptors bind $PACAP_{1-38}$ with high affinity and $PACAP_{1-27}$ with low affinity.

Recently, PACAPs, in particular $PACAP_{1-27}$, have been found to exert airway effects. For example, $PACAP_{1-27}$ stimulated mucus secretion from isolated rat trachea (Wagner *et al*., 1998), relaxed basal tone of guinea-pig airways and inhibited bronchoconstriction induced by thromboxane B_2 (Conroy *et al*., 1995), and reduced guinea-pig tracheal

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contraction to histamine or ovalbumin (Linden *et al*., 1995). Similarly, $PACAP_{1-38}$ prolongs airway smooth muscle relaxation in guinea-pig tracheal strips (Foda *et al*., 1995).

The aims of the present study were to investigate in ferret trachea *in vitro*: (1) the effect of PACAPs and secretin on secretion; (2) the role of PACAPs and secretin in modulating cholinergic neural secretion; and (3) the interaction between secretin (as a VIP_1 receptor agonist) and VIP in non-neural secretion, in an attempt to determine the receptor type involved in mediating the secretory and regulatory responses. We used $^{35}SO_4$ as a marker for mucus (Gashi *et al*., 1987; Davies *et al*., 1990).

Methods

Male ferrets (Regal Rabbits, Great Bookham, Surrey) weighing 1–2 kg were kept 4–5 in a room with free access to food and water, and were allowed 1 week to acclimatize after delivery. 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).

Tracheal preparation for measurement of mucus secretion

Our method for determination of mucus secretion has been detailed previously (Ramnarine *et al*., 1994; Meini *et al*., 1993). Tracheae were cut longitudinally through the dorsal membrane, opened flat and cut transversely into four equal pieces. Each piece 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 (exposed surface area for each tissue piece 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.

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. 2.5 h following addition of radiolabel). After stabilization, drugs or control solutions were added and the tissues were electrically stimulated (see *Protocols*).

Electrical stimulation

Tracheal segments were subjected to an electrical current to stimulate 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.). Tissues were stimulated at 2.5 Hz (threshold, for determination of stimulatory effects of drugs) or 10 Hz (near maximal), 50 V, 0.5 ms for the first 5 min of a 15 min incubation period.

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 (final concentration of guanidine hydrochloride ~6 M). 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 14 kDa or less to pass through. Sodium azide was present to inhibit bacterial growth. 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.]). Recovered samples were weighed, and the radioactivity in 1 ml duplicates in 2 ml scintillant (Ultima Gold XR, Canberra Packard Ltd., Pangbourne, Berks, 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 of a 1 ml aliquot by the total weight of the sample (assuming a 1 ml sample weighs 1 g).

Protocols

To investigate the effects of exogenous VIP, $PACAP_{1-27}$, $PACAP_{1-38}$ or secretin on mucus secretion, concentration-response studies were carried out. Samples were collected 15 min before and after addition of peptide (1 nM – 10 μM) or vehicle (distilled water) to the submucosal half-chamber. To determine whether or not the effect of VIP on secretion was mediated via cholinergic mechanisms, tissues were incubated for 30 min with atropine (10 μM) before addition of 1 μM VIP.

To investigate the effect of exogenous VIP, $PACAP_{1-27}$ or $PACAP_{1-38}$ in regulating cholinergic neural secretion, peptide (0.1 nM – 1 μM) or control vehicle was added to the submucosal half-chamber 30 min before electrical stimulation (10 Hz, 50 V, 0.5 ms for 5 min) in the presence of phentolamine, propranolol (10 μM each) and the tachykinin NK_1 receptor antagonist CP-99,994 (3 μM; McLean *et al*., 1993) to remove α-adrenergic, β-adrenergic and tachykininergic neural influences respectively.

To examine the effects of secretin on cholinergic neural secretion, secretin (10 nM – 10 μM) or control vehicle was added to the submucosal half-chamber 30 min before 2.5 Hz stimulation (50 V, 0.5 ms, 5 min) in the presence of phentolamine, propanolol and CP-99,994 (as above). Samples were collected 15 min before and after electrical stimulation.

To investigate the effect of secretin on VIP-induced secretion, tissues were pre-incubated for 30 min with secretin (1 μM) or vehicle before VIP (10 nM – 1 μM) was added to the submucosal half-chamber.

To investigate whether secretin was a reversible antagonist for VIP, competition curves of VIP *vs* secretin were constructed. Tissue was pre-incubated with secretin (0.1 μM) or vehicle for 30 min prior to the addition of VIP (1 pM – 0.1 μM). Tissue was then subjected to electrical stimulation at 10 Hz, 50 V, 5 ms, for 5 min in the presence of atropine, phentolamine, propranolol and CP-99,994 (as above).

To study the effect of VIP or atropine on secretin-potentiated cholinergic neural secretion, VIP (1 μM) or atropine (10 μM) was added 30 min prior to the addition of secretin (1 μM). Tissue was then subjected to electrical

stimulation (2.5 Hz, 50 V, 5 ms, for 5 min) 30 min after the administration of secretin. Samples were collected every 15 min starting from 15 min before treatment with VIP or atropine.

To test whether or not the post-junctional secretory response to exogenous ACh was affected by $PACAP_{1-27}$, $PACAP_{1-38}$ or secretin, peptide (1 μM each) was added to the submucosal half-chamber 30 min before the administration of ACh (1 μM).

Drugs and chemicals

The following drugs were used: VIP (porcine; synthetic), acetylcholine, $PACAP_{1-38}$, $PACAP_{1-27}$, secretin and dimethylsulphoxide (DMSO) (Sigma Chemical Co. 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.); $Na_2{}^{35}SO_4$ (Amersham International plc, Amersham, Berks., U.K.); and pentobarbitone sodium B.P. (Sagatal; RMB Animal Health Ltd., Dagenham, Essex, U.K.). CP-99,994 (2S,3S)-3-(2-methoxybenzyl)-amino-2-phenyl-piperidine) was a kind gift from Pfizer, Groton, U.S.A. (courtesy Dr R. Michael Snider).

Data analysis

In Results, data are the arithmetic mean and one standard error of the mean (s.e.mean). Because baseline d.p.m. varied between tracheal segments, responses obtained from individual segments were calculated as percentage changes in $^{35}SO_4$ output for the difference between response to drug or electrical stimulation and the proceeding collection. The concentration of peptide causing a 50% increase in secretion (EC_{50}) or inhibition of neurogenic secretion (IC_{50}) was calculated by non-linear regression using GraphPad Prism software (Microsoft, San Diego, U.S.A.). The negative logarithm to base 10 of the dissociation equilibrium constant of a reversible competitive antagonist (pK_B) (Jenkinson *et al*., 1995) was derived from Schild plots using GraphPad Prism. 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).

Results

Median baseline radioactivity in the studies was of the order of 630 d.p.m. (range 401 – 1324 d.p.m., depending upon the specific experiment). There were no significant differences between treatment groups. VIP (1 and 10 μM) significantly increased $^{35}SO_4$ output from the tracheal segments, whereas neither $PACAP_{1-27}$, $PACAP_{1-38}$ nor secretin (1 nM – 10 μM) had any significant effect on $^{35}SO_4$ output (Figure 1). The stimulatory effect of VIP on $^{35}SO_4$ output was not inhibited by

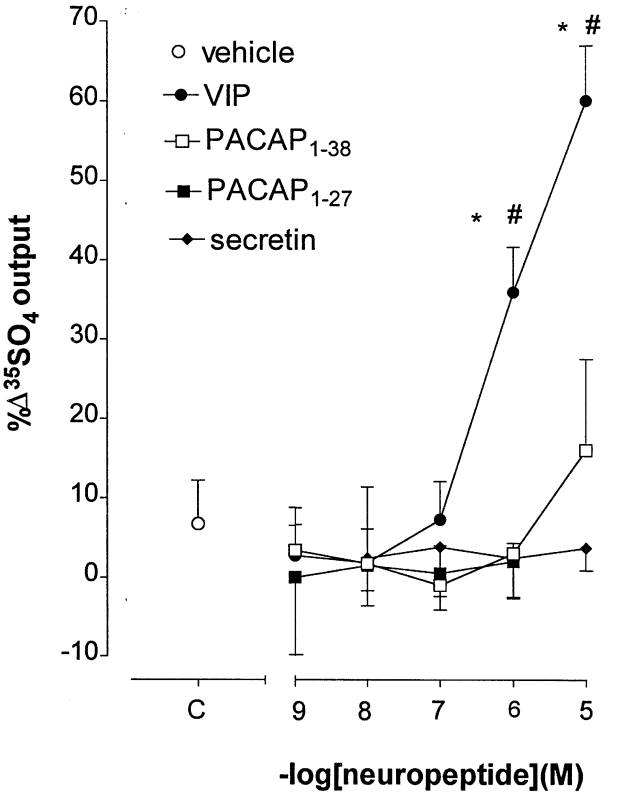

Figure 1 Effect of vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide $(PACAP)_{1-27}$, $PACAP_{1-38}$ or secretin on mucus secretion in ferret trachea *in vitro*. Data are mean per cent change in output of macromolecules labelled *in situ* with $^{35}SO_4$ (representing mucus) for 5 – 8 animals per group; vertical bars are one s.e.mean. $*P<0.05$ compared with control (C); $\#P<0.05$ compared with corresponding concentration of $PACAP_{1-27}$, $PACAP_{1-38}$ or secretin.

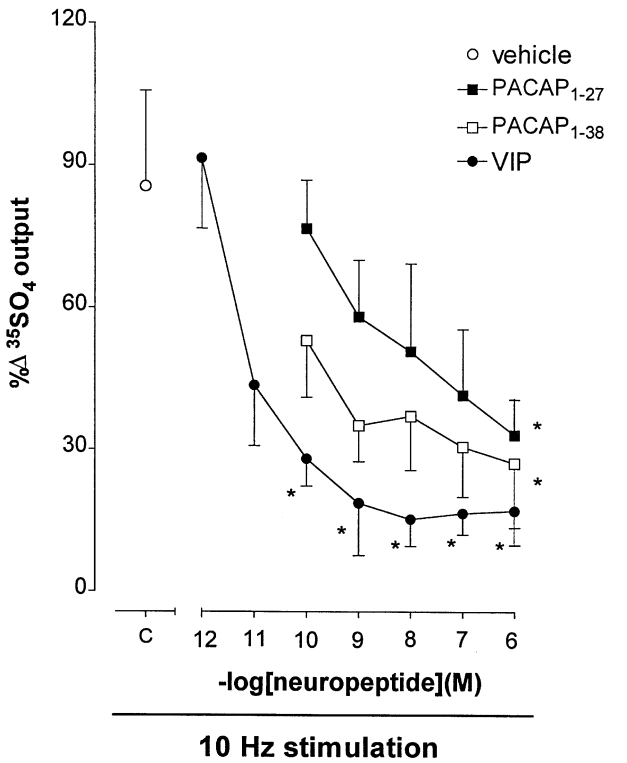

Figure 2 Effect of VIP, $PACAP_{1-27}$ or $PACAP_{1-38}$ on 10 Hz cholinergic neural mucus secretion in ferret trachea *in vitro*. Phentolamine (10 μM), propanolol (10 μM) and the tachykinin receptor antagonist CP-99,994 (3 μM) were used to exclude adrenergic and tachykininergic neural influences at stimulation parameters of 10 Hz, 50 V, 0.5 ms for 5 min. Data are mean per cent change in output of macromolecules labelled *in situ* with $^{35}SO_4$ (representing mucus) for 6 – 7 animals per group; vertical bars are one s.e.mean. $*P<0.05$ compared with stimulation control (C).

10 μM atropine (mean $\pm$ s.e.mean per cent change in output: baseline control, -6.4 ± 1.3; VIP, 22.4 ± 13.9; atropine + VIP, 20.4 ± 11.8, $n=3$ per group).

Effect of VIP, $PACAP_{1-27}$ or $PACAP_{1-38}$ on cholinergic neural mucus secretion

In the presence of phentolamine, propanolol and CP-99,994, at 10 Hz stimulation, VIP, $PACAP_{1-27}$ or $PACAP_{1-38}$ significantly suppressed cholinergic $^{35}SO_4$ output in a concentration-dependent fashion with IC_{50}s of 4.1 pM, 6.7 nM and 0.2 nM, respectively, and maximal inhibitions at 10 nM VIP and >1 μM for $PACAP_{1-27}$ and $PACAP_{1-38}$ (Figure 2). Neither $PACAP_{1-27}$ nor $PACAP_{1-38}$ (1 μM each) significantly affected the increased $^{35}SO_4$ output induced by exogenous ACh (1 μM) (Figure 3).

Effect of secretin on cholinergic neural mucus secretion

In the presence of phentolamine, propanolol and CP-99,994, secretin (10 nM – 10 μM) potentiated 2.5 Hz cholinergic $^{35}SO_4$ output, with a maximal potentiation of 190% at 1 μM (Figure 4). Secretin (1 μM) had no effect on ACh-induced $^{35}SO_4$ output (Figure 4).

Effect of secretin on exogenous VIP-induced mucus secretion

After pre-incubation with secretin (1 μM), exogenous VIP (10 nM – 1 μM) significantly increased $^{35}SO_4$ output compared with secretin vehicle, with an increase of 210% at 1 μM, the highest concentration tested (Figure 5).

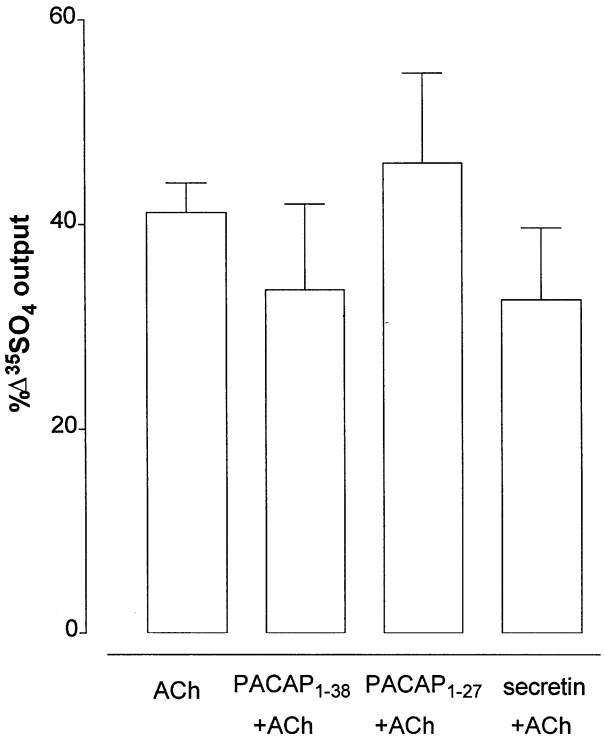

Figure 3 Effect of $PACAP_{1-38}$, $PACAP_{1-27}$ or secretin (1 μM for all) on exogenous acetylcholine (ACh; 1 μM)-induced mucus secretion in ferret *in vitro*. Data are mean per cent change in output of macromolecules labelled *in situ* with $^{35}SO_4$ (representing mucus) for 5 – 6 animals per group; vertical bars are one s.e.mean.

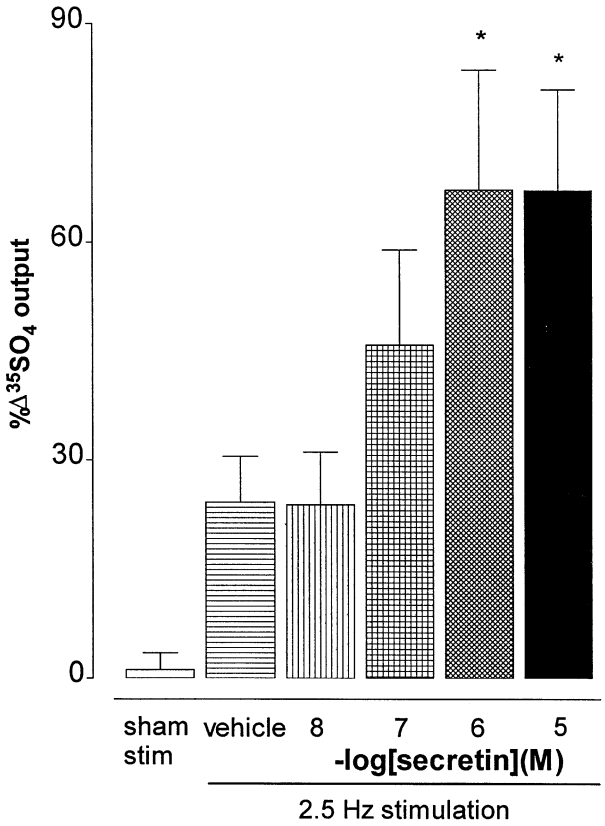

Figure 4 Effect of secretin on cholinergic neural mucus secretion in ferret trachea *in vitro*. Phentolamine (10 μM), propanolol (10 μM) and the tachykinin receptor antagonist CP-99,994 (3 μM) were used to exclude adrenergic and tachykininergic neural influences at stimulation parameters of 2.5 Hz, 50 V, 0.5 ms for 5 min. Data are mean per cent change in output of macromolecules labelled *in situ* with $^{35}SO_4$ (representing mucus) for 6 – 7 animals per group; vertical bars are one s.e.mean. *$P>0.05$ compared with stimulation vehicle control.

VIP versus secretin competition curves on cholinergic neural mucus secretion

In the presence of phentolamine, propanolol and CP-99,994, secretin (0.1 μM) caused a parallel rightward shift of the cholinergic VIP-inhibition curve with a pK_B of 8.1 (Figure 6).

Effect of VIP or atropine on secretin-potentiated cholinergic neural mucus secretion

In the presence of phentolamine, propanolol and CP-99,994, pre-treatment with atropine (10 μM) or VIP (1 μM) significantly inhibited the potentiation of cholinergic neural $^{35}SO_4$ output by secretin by 90 and 94%, respectively (Figure 7).

Discussion

In the present study in ferret trachea *in vitro*, VIP, PACAPs and secretin altered the magnitude of cholinergic neural $^{35}SO_4$ output. The change in $^{35}SO_4$ output is consistent with a change in mucus secretion by 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 selective uptake of $^{35}SO_4$ by ferret tracheal submucosal glands, rather than epithelium (Gashi *et al.*, 1987). Stimulation of ferret trachea *in vitro* increased

radioactive counts in the incubation medium, with concomitant loss of autoradiographic grains from the glands (Gashi, 1987). In cats, $^{35}SO_4$-labelled tracheal washings have a size and density characteristic of a mucin molecule (Davies *et al*., 1990). Thus, the output of $^{35}SO_4$ observed herein is indicative of an increase in tracheal submucosal gland mucus secretion.

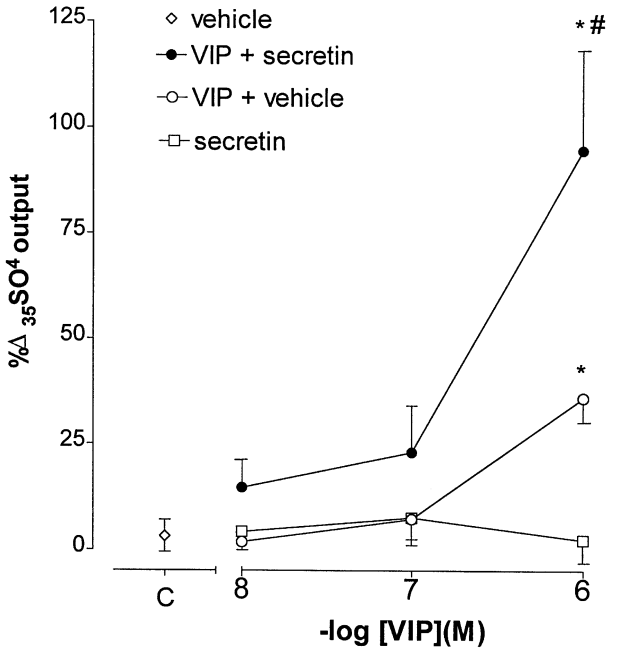

Figure 5 Effect of secretin (1 μM) on exogenous VIP-induced mucus secretion in ferret trachea *in vitro*. Data are mean per cent change in output of macromolecules labelled *in situ* with $^{35}SO_4$ (representing mucus) for 5–6 animals per group; vertical bars are one s.e.mean. $^*P<0.05$ compared with vehicle control; $\#P<0.05$ compared with VIP + vehicle.

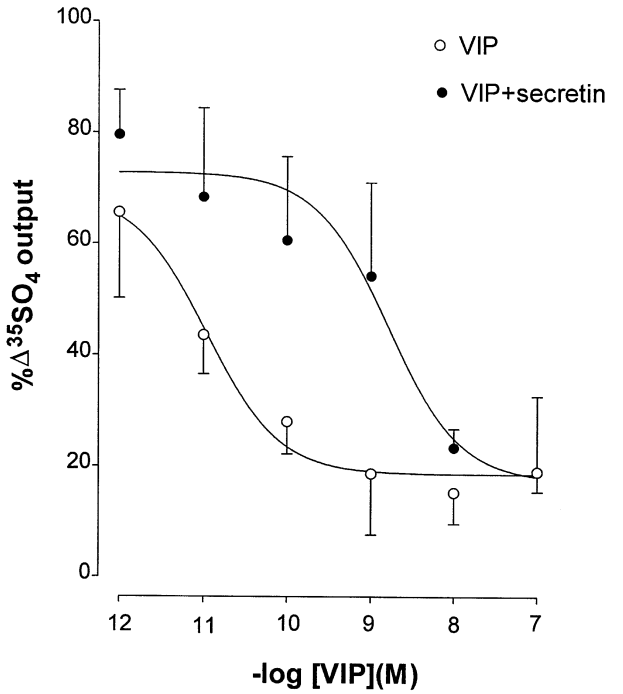

Figure 6 VIP *vs* secretin competition curves on cholinergic neural mucus secretion in ferret trachea *in vitro*. Phentolamine (10 μM), propanolol (10 μM) and the tachykinin receptor antagonist CP-99,994 (3 μM) were used to exclude adrenergic and tachykininergic neural influences at stimulation parameters of 10 Hz, 50 V, 0.5 ms for 5 min. Secretin (0.1 μM) competes with VIP. Data are mean per cent change in output of macromolecules labelled *in situ* with $^{35}SO_4$ (representing mucus) for 4–5 animals per group; vertical bars are one s.e.mean.

In the present study, VIP caused a modest increase in mucus secretion, whereas $PACAP_{1-27}$, $PACAP_{1-38}$ and secretin did not significantly increase secretion. This result is in contrast to data in rat trachea *in vitro* where $PACAP_{1-27}$, at 10 and 100 nM, but not 1 μM, gave a greater increase in $^{35}SO_4$ output than did VIP (Wagner *et al*., 1998). The reason for the discrepant observations is unclear but might be due to different study designs, for example the use of a short $Na_2{}^{35}SO_4$ incubation period and long peptide contact time in the latter study, and to a species difference: the principle source of mucus in rat trachea is the goblet cells (Rogers & Jeffery, 1986), compared with the submucosal glands in ferret trachea. Whether different secretory structures respond differently to these peptides remains to be determined. We found herein that atropine did not inhibit VIP-induced secretion. This indicates that VIP has direct post-junctional secretory activity, rather than an indirect effect *via* cholinergic nerve activation. This is consistent with previous observations that mixtures of autonomic antagonist drugs, including atropine, do not inhibit VIP-induced secretion in the isolated cat tracheal submucosal gland (Shimura *et al*., 1988) or in ferret trachea (Peatfield *et al*., 1983).

In the present study, we found that VIP, $PACAP_{1-27}$ and $PACAP_{1-38}$ suppressed cholinergic neural mucus secretion in a concentration-dependent fashion, with an order of potency of

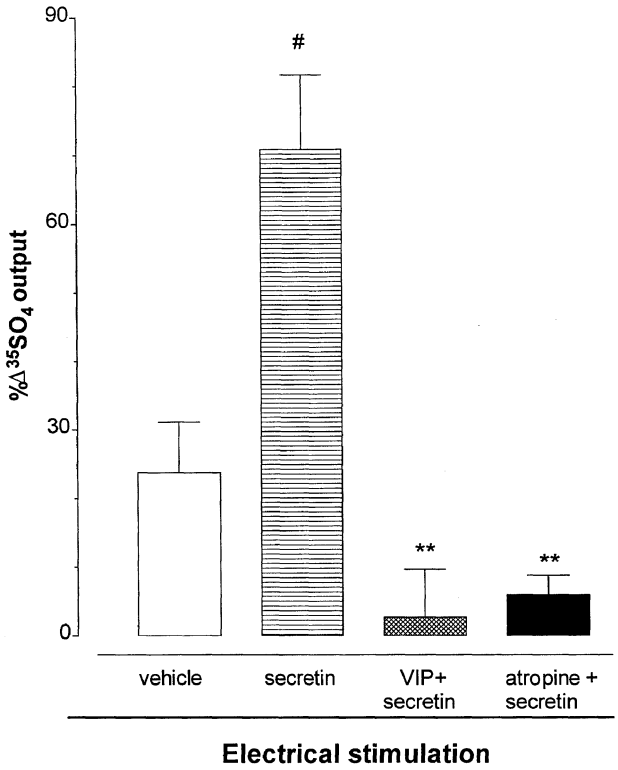

Figure 7 Effect of VIP (1 μM) or atropine (10 μM) on secretin-potentiated cholinergic neural mucus secretion in ferret trachea *in vitro*. Phentolamine (10 μM), propanolol (10 μM) and the tachykinin receptor antagonist CP-99,994 (3 μM) were used to exclude adrenergic and tachykininergic neural influences at stimulation parameters of 2.5 Hz, 50 V, 0.5 ms for 5 min. Data are mean per cent change in output of macromolecules labelled *in situ* with $^{35}SO_4$ (representing mucus) for 5–6 animals per group; vertical bars are one s.e.mean. $\#P<0.05$ compared with vehicle stimulation; $^{**}P<0.01$ compared with secretin alone.

VIP > $PACAP_{1-38}$ > $PACAP_{1-27}$. None of these peptides changed ACh-induced mucus secretion. These results suggest pre-junctional regulation of cholinergic neural secretion by VIP, $PACAP_{1-27}$ and $PACAP_{1-38}$ in ferret trachea. VIP and $PACAP_{1-27}$ significantly reduced basal tone and inhibited thromboxane B_2-evoked airway constriction in guinea-pig airways (Conroy *et al*., 1995). $PACAP_{1-38}$ relaxed guinea-pig and rabbit airways *in vitro*, pre-contracted by histamine, ACh or methacholine (Cardell *et al*., 1991; Foda *et al*., 1995). In binding studies, VIP, $PACAP_{1-27}$ and $PACAP_{1-38}$ have similar binding affinity at VIP receptors (Ishihara *et al*., 1992; Sreedharan *et al*., 1993; Lutz *et al*., 1993). $PACAP_{1-38}$ was found to induce a greater cyclic AMP accumulation in guinea-pig airways (Foda *et al*., 1995), in rat anterior pituitary cells and neurons (Miyata *et al*., 1989), or had comparable potency in activating cyclic AMP (Ishihara *et al*., 1992; Sreedharan *et al*., 1993). However, VIP was more potent than $PACAP_{1-27}$ or $PACAP_{1-38}$ in causing mucus secretion and modulating cholinergic neural secretion in ferret trachea herein, and also in relaxing guinea-pig airways (Conroy *et al*., 1995; Foda *et al*., 1995). This suggests that factors apart from accumulation of cyclic AMP activity play a role in controlling mucus secretion and smooth muscle relaxation in the airways.

In the present study, it was shown that secretin potentiated cholinergic neural secretion (at 2.5 Hz), whereas alone it did not induce mucus secretion, nor did it alter secretion elicited by exogenous ACh. This result indicated that the potentiation was due to the facilitation of release of ACh from nerve endings. This suggestion gains support from the observation herein that atropine abrogated the cholinergic neural secretion potentiated by secretin. Furthermore, VIP also abolished the cholinergic neural secretion potentiated by secretin. In our previous study, VIP inhibited ACh release (Liu *et al*., 1999). This suggests that secretin may compete for the same receptors as VIP, or that they both simply antagonize ACh release from cholinergic nerves. In this study, secretin shifted the VIP inhibitory curve to the right, which implies that VIP and secretin were competing for the same prejunctional receptor, rather than antagonizing functionally the release of ACh. To potentiate electrically-induced cholinergic secretion, secretin would have to antagonize the inhibitory effect of VIP released by electrical stimulation at 2.5 Hz. We have shown previously in ferret trachea *in vitro* that 2.5 Hz stimulation induces VIP release from cholinergic nerves, as evidenced by potentiation of [^{3}H]-choline overflow by a VIP antibody or by α-chymotrypsin, both of which interfere with the actions of VIP (Liu *et al*., 1999). Similarly, in human airways *in vitro*, VIP receptor antagonists potentiate electrically-induced contractions at 5 Hz (and also at higher frequencies) (Aizawa *et al*., 1994). Thus, our own data and those of others indicate that VIP is released at comparatively low stimulation frequencies. VIP_1 receptor mRNA is highly expressed in human and rat lung (Ishihara *et al*., 1992; Sreedharan *et al*., 1993, Usdin *et al*., 1994), and is found in nerves in rat lung sections (Ichikawa *et al*, 1995). In contrast, secretin was shown to have no activity in lung tissue, and secretin receptor mRNA was found to be lowly expressed in human and rat lungs (Chow, 1995; Ishihara *et al*., 1991). Herein, secretin potentiated cholinergic neural secretion. In human secretin receptor-transfected COS-7 cells, the EC_{50} in inducing cyclic AMP is 0.5 nM (Jiang & Ulrich, 1995). The EC_{50} for secretin at the VIP_1 receptor in accumulating cyclic AMP is about 100 nM (Sreedharan *et al*., 1993). Taken together, the prejunctional receptors for which VIP and secretin compete are more likely to be VIP_1 receptors than secretin receptors.

In this study, secretin enhanced VIP-induced mucus secretion. At the concentrations used (up to 1 μM VIP), although there was an apparent leftward shift of the VIP concentration-response curve, it was not clear whether secretin also increased the maximal effect of VIP (Figure 5). The mechanism for secretin enhancement of VIP-induced secretion is unclear from the present study. It is possible that secretin acts as a peptidase inhibitor, thereby enhancing VIP-induced secretion by inhibiting endogenous degradation of VIP. To the knowledge of the authors, there are no reports that secretin is a peptidase inhibitor. In addition, tracheal mucus secretion induced by VIP does not appear to be regulated by peptidases. For example, *in vitro* in rat trachea, the peptidase inhibitor thiorphan did not enhance the secretory effect of VIP (Wagner *et al*., 1998). In cat tracheal explants and isolated submucosal glands, neither bacitracin, phosphoramidon, pepstatin nor bestatin enhanced VIP-induced secretion (Shimura *et al*., 1988). These observations do not support a role for secretin in influencing VIP-induced secretion by acting as a peptidase inhibitor. Secretin might disclose an excitatory effect of PACAPs on secretion, *via* a similar action to that on VIP-induced secretion, although this aspect was not investigated in the present study. Herein, secretin alone did not induce mucus secretion. There are no reports on the effect of secretin on airway mucus secretion, although secretin does increase exocrine gland and insulin secretion in rat pancreas (for review see Daniel, 1990). It is uncertain whether ferret airways have secretin receptors, but it is unlikely that secretin receptors are responsible for mucus secretion in this preparation, or maybe there are insignificant numbers of receptors on submucosal glands in ferret airways. Secretin is a weak VIP_1 receptor agonist with a K_D of 0.1 μM in transfected cell lines, and an EC_{50} of 0.1 μM in accumulating cyclic AMP (Ishihara *et al*., 1992; Sreedharan *et al*., 1993, Usdin *et al*., 1994). High concentrations of secretin might be utilized to determine the functions of different VIP receptors. However, secretin (up to 10 μM) herein had no activity in inducing mucus secretion in ferret trachea, which suggests secretin might act as an antagonist at the VIP_1 receptor in this preparation. This might explain why secretin and VIP were competing for the same receptor pre-junctionally in modulating cholinergic neural secretion. Also, high concentrations of secretin might bind VIP_1 receptors in the synaptic cleft to allow VIP access to VIP_2 receptor at a post-junctional site and, hence, elicit mucus release. In our previous study, a putative VIP_1 antagonist, [D-p-Cl-Phe^6, Leu^{17}]-VIP, did not alter the magnitude of cholinergic neural secretion, nor did it diminish exogenous VIP-induced mucus secretion (Liu *et al*., 1998). Similarly, neither [D-p-Cl-Phe^6, Leu^{17}]-VIP nor [Ac-Try^1, D-Phe^2]-GRF(1-29)-NH_2 antagonized VIP-induced relaxation elicited by electrical stimulation in guinea-pig trachea (Ellis & Farmer, 1989). Currently, there are no selective secretin antagonists available to test this theory.

In conclusion, in ferret trachea *in vitro*, VIP, $PACAP_{1-27}$ and $PACAP_{1-38}$ regulate cholinergic neural mucus secretion pre-junctionally. Secretin, in contrast, increases cholinergic neural mucus secretion pre-junctionally, and VIP-induced secretion post-junctionally, probably by competing for VIP_1 receptors.

We thank Chang Gung Memorial Hospital, Taipei, Taiwan (CMRP520) for financial support. Y.-C. Liu is a recipient of a Biomedicine Scholarship from Chang Gung Memorial Hospital.

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(Received January 8, 1999
Revised July 21, 1999
Accepted September 15, 1999)