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The effects of peptide histidine isoleucine and neuropeptide Y on mucus volume output from the ferret trachea

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1 The effects of peptide histidine isoleucine (PHI) and neuropeptide Y (NPY) were examined on the mucus volume output produced by methacholine and phenylephrine in the ferret whole trachea *in vitro*.

2 Sustained application of methacholine (5 μM) or phenylephrine (20 μM) produced a maintained volume output of mucus from the trachea. Both these agonists also increased the output of lysozyme (a marker for serous cell secretion).

3 PHI inhibited the maintained mucus volume output produced by methacholine but had no effect on that due to phenylephrine. The output of lysozyme produced by methacholine or phenylephrine was not significantly changed by PHI.

4 NPY enhanced the volume output of mucus produced by methacholine or phenylephrine; however, the rate of output of lysozyme in mucus produced by both agonists was reduced by NPY.

5 We suggest that PHI has no effect on serous cell secretion but inhibits secretion from another source, possibly mucous cells. NPY inhibits serous cell secretion but has a stronger stimulant action on secretion from another source, again possibly mucous cells.

6 PHI and NPY may be important physiological modulators of mucus volume output in the ferret trachea.

Introduction

Peptide with N-terminal histidine and C-terminal isoleucine amide (PHI) is a 27 amino acid peptide with marked structural similarities to vasoactive intestinal peptide (VIP). Immunohistological studies have shown that PHI has an identical distribution to that of VIP in the airways of several species; in human airway nerves, peptide histidine methionine (PHM), a peptide closely related to PHI, has been isolated and co-exists with VIP (Lundberg *et al.*, 1984; Ghatei *et al.*, 1987).

Neuropeptide Y (NPY), a 36 amino acid peptide, occurs in the lungs of several species with a distribution similar to that of sympathetic nerves. It is localised primarily near blood vessels (Uddman *et al.*, 1984; Sheppard *et al.*, 1984), with few NPY-immunoreactive nerve fibres in airway smooth muscle. In a recent study, nerve fibres immunoreactive to PHM and NPY were found localized abundantly around the acini of seromucous glands in the submucosa of the trachea and bronchi of the cynologus monkey, *Macaca fasacularis* (Ghatei *et al.*, 1987).

Histological examination has shown that the ferret trachea has very few goblet cells in the surface epithelium (Robinson *et al.*, 1986). Therefore the secretion of mucus is almost entirely from serous and mucous cells in acini of the submucosal glands. A small amount of secretion may come from ciliated and other cells in the epithelium but this is unlikely to represent more than 1% of the total volume of mucus secreted (Robinson *et al.*, 1986).

Mucus volume output from the ferret trachea is potently stimulated by drugs acting on muscarinic cholinoceptors and α-adrenoceptors (Basbaum *et al.*, 1981; Tom-Moy *et al.*, 1983; Gashi *et al.*, 1984; Webber & Widdicombe, 1987a,b). Immunohistochemical as well as functional studies (Gashi *et al.*, 1984; Basbaum, 1984; Webber & Widdicombe, 1987a,b) suggest that methacholine (a muscarinic agonist) can stimulate secretion from both mucous

and serous cells. In contrast, phenylephrine (a relatively specific α_1-adrenoceptor agonist) stimulates mainly serous cells (Basbaum *et al.*, 1981; Tom-Moy *et al.*, 1983; Webber & Widdicombe, 1987a,b) with little or no effect on mucous cells.

The ferret *in vitro* whole trachea preparation (Webber & Widdicombe, 1987a,b) allows measurement of the volume output of tracheal mucus. Recently it has been shown that mucus volume output from the ferret trachea can be modulated by VIP (Webber & Widdicombe, 1987a). The present paper describes similar experiments with PHI and NPY with lysozyme determinations as an indicator of serous cell secretion. Preliminary observations showed that PHI and NPY had no effect on the basal rate of mucus volume output from the ferret trachea (see Results). However, any inhibitory action of these peptides would not be apparent since the resting secretion rate is close to zero. Therefore the peptides were tested on maintained secretion due to methacholine or phenylephrine.

Methods

Ferrets of either sex, weighing 0.5–1.5 kg, were anaesthetized by an intraperitoneal injection of sodium pentobarbitone (Sagatal, May & Baker, 50 mg kg^{-1}). The trachea was exposed and cannulated about 5 mm below the larynx with a perspex cannula containing a conical collecting well (Webber & Widdicombe, 1987a). The ferret was then killed with an overdose of anaesthetic injected into the heart. The chest was opened along the midline and the trachea was exposed to the carina, cleared of adjacent tissue, removed and cannulated just above the carina.

The trachea was mounted laryngeal-end-down in a jacketed organ bath with Krebs-Henseleit buffer bathing the adventitial surface (Figure 1). The composition of the Krebs-Henseleit solution was (mM): NaCl 120.8, KCl 4.7, KH_2PO_4 1.2, $MgSO_47H_2O$ 1.2, $NaHCO_3$ 24.9, $CaCl_2$ 2.4, glucose 5.6. The buffer was maintained at 37°C and gassed with 95% O_2/5% CO_2. The lumen of the trachea remained air-filled. Secretions were carried by gravity and mucociliary transport to the lower cannula, where they collected and could be withdrawn periodically into a polyethylene catheter inserted into the lower cannula to form an airtight seal. The catheters containing the secretions were sealed at both ends with bone wax, the length of the secretion in the catheter was measured, and the volume of secretion calculated from the length. The samples were numbered and stored frozen until required.

After defrosting, the secretions were washed out of the catheters into labelled plastic vials with 0.5 ml distilled H_2O. The vials were frozen and stored for use in the lysozyme assay. Preliminary experiments had shown that frozen storage for up to 6 months did not affect the amount of lysozyme in the mucus samples. Mucus secretion volumes were estimated by the difference in weights of the catheters with secretions and dried without secretions. There was no significant difference between the volumes of the samples obtained by this method and the volumes calculated by measuring the length of the samples in the catheters. Therefore no sample was lost during collection or frozen storage. Values based on weighing are used in the text. The mucus secretion rate is expressed as $\mu l\,min^{-1}$ (assuming 1 g of mucus to be equivalent to 1 ml).

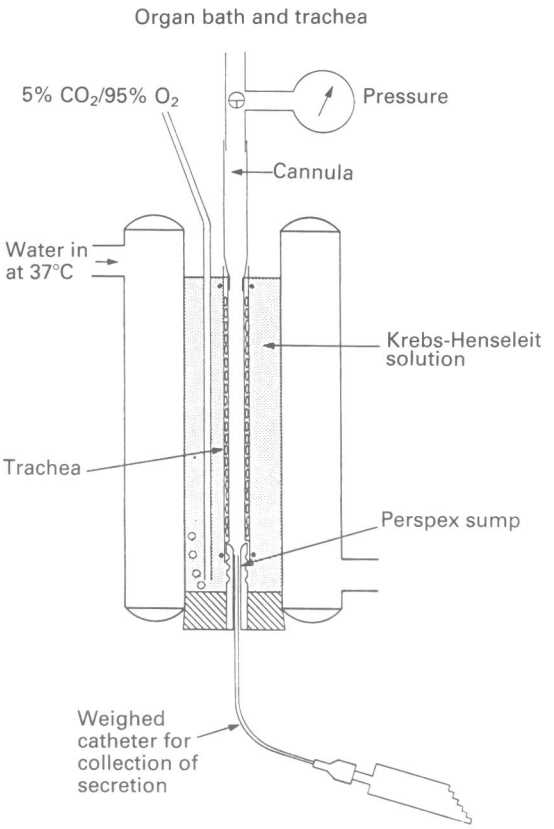

Figure 1 The *in vitro* ferret whole trachea preparation.

Before the start of an experiment each trachea was allowed to equilibrate for 20 min. During this time changes of bathing medium were made every 5 min and any secretion produced was removed after 20 min.

Previous work has shown that methacholine and phenylephrine produce concentration-dependent increases in both mucus volume output and lysozyme output. The potencies of each agonist on

mucus output and lysozyme output were similar (Webber & Widdicombe, 1987a). After a 30 min control period, either methacholine (5 μM) or phenylephrine (20 μM) was added to the buffer bathing the trachea. These concentrations of methacholine and phenylephrine produce approximately 70–80% of the maximum mucus volume output and lysozyme output for each agonist (Webber & Widdicombe 1987a). Mucus was withdrawn every 15 min for the first 30 min and at 30 min intervals thereafter until a steady rate of mucus volume output had been obtained. After each 30 min period the buffer surrounding the trachea was replaced with fresh buffer containing either metacholine or phenylephrine.

When a steady mucus volume output had been obtained (maintained mucus volume output), either NPY or PHI (each 1–100 nM) was added to the secretagogue in the buffer surrounding the trachea. The peptide was allowed to equilibrate with the trachea for 15 min and any mucus collected during this period was withdrawn and discarded. The mucus volume output was determined for a further 30 min period and then the organ bath was drained and replenished with buffer containing the secretagogue but no further peptide. The mucus volume output was then measured for two further periods of 30 min. The change in mucus volume output produced by PHI or NPY was calculated as the difference in the mucus volume output obtained between the period immediately before the peptide was added and the period when the peptide was in the organ bath, expressed as a percentage. All the mucus samples obtained in these experiments were assayed for lysozyme.

The lysozyme output was determined by a turbidimetric assay which relies on the ability of lysozyme to break down the cell wall of the bacterium *Micrococcus lysodeikticus*. Addition of lysozyme to a solution of the bacteria reduces the turbidity of the solution, thereby leading to a fall in optical density (OD) measured at 450 nm (Selsted & Martinez, 1980).

A stock suspension of *M. lysodeikticus* of 3 mg ml^{-1} was prepared. When diluted 10 fold (the dilution in the assay) this suspension gives an OD of approximately 0.6 at 450 nm. To produce a standard curve, various concentrations of hen egg-white lysozyme (0.5 to 100 ng ml^{-1}) were incubated in duplicate in 1.5 ml potassium phosphate buffer (50 mM, pH 7.4) containing *M. lysodeikticus* (0.3 mg ml^{-1}), sodium azide (1 mg ml^{-1}) and bovine serum albumin (BSA, 1 mg ml^{-1}). The BSA was included in the assay for its protein-stabilizing effects and the sodium azide was added to prevent the growth of bacteria in the incubating solutions. The reaction mixtures were incubated for 18 h at 37°C. After incubation, the OD of each solution was measured at a wavelength of 450 nm with potassium phosphate buffer, pH 7.4, containing BSA (1 mg ml^{-1}) as a blank. The standard curve was constructed by plotting the fall in OD (reduction in turbidity) against the concentration of lysozyme in the solution.

To estimate the concentration of lysozyme in a mucus sample, 20 μl of sample were incubated in 1.5 ml potassium phosphate buffer (50 mM, pH 7.4), exactly as described above for the known concentrations of lysozyme used in the preparation of the standard curve. The lysozyme concentrations (equivalent to hen egg-white lysozyme) of the 20 μl samples, and hence of the original mucus samples, were estimated from the standard curve. The rate of output of lysozyme was obtained by dividing the total amount of lysozyme in a mucus sample by the time over which the sample had accumulated.

Materials

The materials used were acetyl-β-methylcholine chloride (methacholine), phenylephrine hydrochloride, peptide histidine isoleucine, neuropeptide Y, bovine serum albumin, lysozyme (hen egg white) and *Micrococcus lysodeikticus* (all from Sigma), and sodium azide (BDH).

Analysis of results

The effects of the peptides on the maintained mucus volume outputs and lysozyme output produced by methacholine and phenylephrine were analysed for statistical significance by one-way analysis of variance followed by Student's paired t tests. Significance was accepted for $P < 0.05$. Values given are means $\pm$ s.e.mean.

Results

The mean mucus volume output during all control periods was 0.04 $\pm$ 0.03 μl min^{-1} ($n = 48$) which was not significantly different from zero. PHI and NPY (1–100 nM each) produced no significant change ($P > 0.05$) in the control output of mucus (0.03 $\pm$ 0.02, $n = 6$ and 0.05 $\pm$ 0.05 μl min^{-1}, $n = 6$ for PHI and NPY respectively).

The mean lysozyme output during all control periods was 8 $\pm$ 2 ng min^{-1} ($n = 48$). PHI and NPY produced no significant change in the control output of lysozyme (11 $\pm$ 6, $n = 6$ and 7 $\pm$ 4 ng min^{-1}, $n = 6$ for PHI and NPY respectively).

Effects of methacholine and phenylephrine on mucus volume output

Addition of methacholine (5 μM) or phenylephrine (20 μM) to the trachea significantly increased

($P < 0.05$) the rate of mucus volume output to 3.5 ± 0.3 ($n = 24$) and $2.3 \pm 0.2\,\mu l\,min^{-1}$ ($n = 28$) respectively.

On continued application of either methacholine or phenylephrine the mucus volume output declined but reached a steady level after 2.5–3 h (maintained mucus volume output). The mucus volume output at this time was significantly higher than that before the drugs had been added. The mean volume outputs of mucus in the 30 min period before addition of PHI or NPY were 0.45 ± 0.04 ($n = 24$) and $0.53 \pm 0.07\,\mu l\,min^{-1}$ ($n = 28$) for methacholine and phenylephrine respectively.

Effects of methacholine and phenylephrine on the output of lysozyme

In the 30 min period immediately after addition of methacholine (5 μM) or phenylephrine (20 μM) the rate of output of lysozyme increased significantly from the preceding control period to $620 \pm 32\,ng\,min^{-1}$ ($n = 24$) and $830 \pm 40\,ng\,min^{-1}$ ($n = 28$) respectively.

On continued application of methacholine or phenylephrine the rate of output of lysozyme declined but reached a steady level at the same time as the mucus volume output (i.e. 2.5–3.5 h after addition of secretagogue). The rates of output of lysozyme in the 30 min period before addition of PHI or NPY were 170 ± 23 ($n = 24$) and $169 \pm 10\,ng\,min^{-1}$ ($n = 28$) for methacholine and phenylephrine respectively.

Effect of PHI on mucus volume output and lysozyme output

PHI (1–100 nM) inhibited the maintained methacholine-induced mucus volume output (Figure 2) in a concentration-dependent manner. The inhibition was significant ($P < 0.05$) at each concentration of PHI. However, PHI had no significant effect ($P > 0.05$) on the output of lysozyme obtained during maintained methacholine-induced secretion (Figure 3).

The maintained mucus volume output and the output of lysozyme produced by phenylephrine were not significantly ($P > 0.05$) changed by addition of PHI (Figures 2, 3).

Effect of NPY on mucus volume output and lysozyme output

NPY (1–100 nM) increased the maintained mucus volume output produced by methacholine and phenylephrine in a concentration-dependent manner (Figure 4). At each concentration of NPY the mean

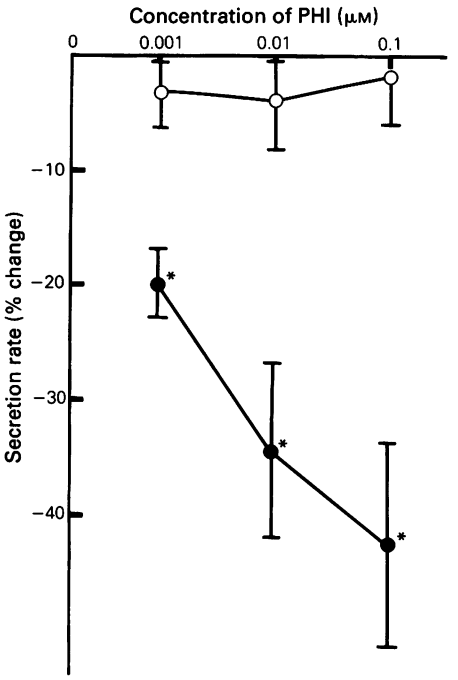

Figure 2 Concentration-response curves showing the effect of peptide histidine isoleucine (PHI) on maintained mucus volume output from the ferret trachea produced by methacholine (5 μM, ●) and phenylephrine, (20 μM, ○). PHI concentration is on a log scale. Points are means of six determinations and vertical bars represent s.e.means. *Significant ($P < 0.05$, paired t test) inhibition of maintained secretion rate.

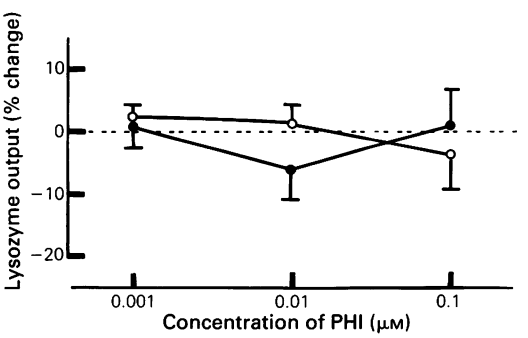

Figure 3 The effect of PHI on the rate of output of lysozyme during maintained mucus volume output produced by methacholine (5 μM, ●) and phenylephrine (20 μM, ○). PHI concentration is on a log scale. Points are means of six determinations and vertical bars represent s.e.means.

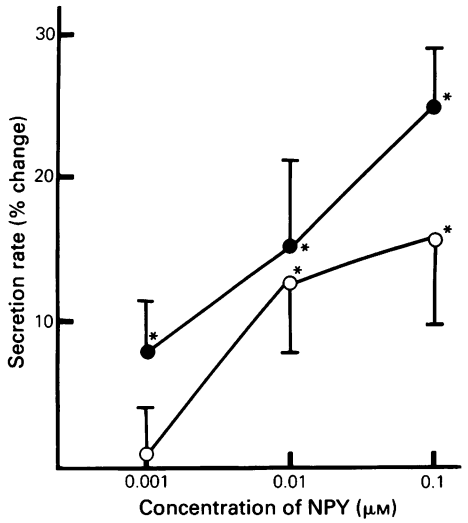

Figure 4 Concentration-response curves showing the effect of neuropeptide Y (NPY) on maintained mucus volume output from the ferret trachea produced by methacholine (5 μM, ●) and phenylephrine (20 μM, ○). NPY concentration is on a log scale. Points are means of six determinations and vertical bars represent s.e.means. *Significant ($P < 0.05$, paired t test) increase of maintained secretion rate.

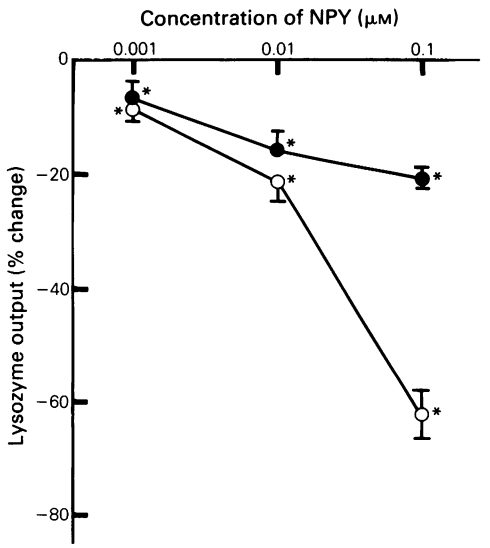

Figure 5 The effect of neuropeptide Y (NPY) on the rate of output of lysozyme obtained during maintained mucus volume output produced by methacholine (5 μM, ●) and phenylephrine (20 μM, ○). NPY concentration is on a log scale. Points are means of six determinations and vertical bars represent s.e.means. *Significant ($P < 0.05$, paired t test) inhibition of lysozyme output.

increase in methacholine-induced mucus volume output was greater than that due to phenylephrine.

In contrast, NPY inhibited in a concentration-dependent manner the output of lysozyme during the maintained mucus volume output produced by both methacholine and phenylephrine (Figure 5). At each concentration of NPY the inhibition was greater for phenylephrine than for methacholine-induced lysozyme output.

Discussion

There is now considerable evidence that PHI may be a co-transmitter with VIP in cholinergic nerves to the airways (Lundberg *et al.*, 1984; Palmer *et al.*, 1986). In the present study, PHI inhibited the maintained mucus volume output produced by methacholine. A similar inhibitory action on methacholine-induced mucus volume output has previously been reported for VIP (Webber & Widdicombe, 1987a). However, PHI had no significant effect on the maintained mucus volume output produced by phenylephrine, whereas VIP enhanced the mucus volume output in the ferret due to this secretagogue (Webber & Widdicombe, 1987a).

Lysozyme is a bactericidal agent found in respiratory tract fluid (Konstan *et al.*, 1981). Immunohistochemistry has shown that lysozyme is located in the secretory granules of serous but not mucous cells in the submucosal glands of the human (Mason & Taylor, 1975; Bowes & Corrin, 1977) and ferret trachea (Tom-Moy *et al.*, 1983) and is therefore a useful marker for serous cell secretion.

PHI had no significant effect on the output of lysozyme produced by methacholine or phenylephrine suggesting that this peptide has no action on the serous cell secretion produced by these agonists. In contrast, VIP enhances the secretory action of phenylephrine on serous cells in the ferret (Webber & Widdicombe, 1987a) and inhibits serous cell secretion in human airways *in vitro* (Coles *et al.*, 1981). Since PHI decreased the volume output of mucus produced by methacholine but had no effect on serous cell secretion, it must inhibit secretion from another source, possibly mucous cells. A similar inhibitory action on the mucous cell secretion produced by methacholine has previously been observed with VIP (Webber & Widdicombe, 1987a). The lack of effect of PHI on phenylephrine-induced secretion could be because phenylephrine is a poor stimulant of mucous cell secretion (Basbaum, 1984; Basbaum *et al.*, 1981); alternatively PHI may modify secretion produced only through stimulation of muscarinic cholinoceptors.

NPY is probably a co-transmitter with noradrenaline in adrenergic nerves to the airways and in this

study it enhanced the secretory action of phenylephrine on the ferret trachea. However, NPY also increased the mucus volume output due to methacholine and *in vivo* this peptide may modulate secretion due to stimulation of muscarinic cholinoceptors as well as of α-adrenoceptors.

In contrast to its stimulation of the volume output of mucus, NPY inhibited the output of lysozyme produced by both methacholine and phenylephrine, which suggests that this peptide inhibits serous cell secretion. However, since the total volume of mucus was increased by NPY, this peptide must have a stronger stimulatory action on secretion from another cell type. The source of this other secretion is not known but could be mucous cells. This is supported by the fact that NPY caused a larger increase in the volume output of mucus produced by methacholine (a stimulant of both mucous and serous cells) compared to phenylephrine (a stimulant of mainly serous cells).

In conclusion, both PHI and NPY can modulate mucus volume output in the ferret trachea but this effect depends on the source of the secretion and the receptor stimulated to produce the secretion. PHI probably inhibits the mucous cell secretion produced by methacholine, but has no effect on serous cell secretion. NPY inhibits the serous cell secretion due to methacholine and phenylephrine, but produces an overall increase in the volume output of mucus due to these agents by a stimulatory action on a mucus source other than serous cells. The receptor mechanisms underlying the effects of these peptides on mucus volume output are not known.

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