Vasoactive intestinal peptide stimulates mucus secretion, but nitric oxide has no effect on mucus secretion in the ferret trachea

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Vasoactive intestinal peptide (VIP) is the most abundant neuropeptide isolated from lung tissue and is a dilator of vascular and airway smooth muscle (2, 35). VIP has been localized to the airway submucosal glands of many species, including humans and ferrets (4, 8, 10, 22, 32). The density of VIP-positive nerves is significantly higher in the glands of bronchitic than in nonbronchitic subjects (23). Thus we speculated that VIP may contribute to the control of airway mucus secretion.

The neural control of mucus secretion in the airway has been very well reviewed by Rogers (25, 28). Published studies of the effects of VIP on mucus secretion have yielded conflicting results. VIP has been reported to stimulate mucus secretion in a dose-dependent fashion. VIP-induced mucus secretion was partially blocked by a VIP receptor antagonist (a chimeric VIP-pituitary adenylate cyclase-activating peptide analog, VIP receptor antagonist) at a 10-fold excess concentration. At all concentrations tested, neither N\textsuperscript{G},N\textsuperscript{6}-dinitro-arginine methyl ester, an inhibitor of NO synthase, nor S-nitroso-N-acetyl-penicillamine, an NO donor, had any significant effect on constitutive or VIP-induced mucus secretion. We conclude that VIP-stimulated mucin and lysozyme secretion was both time dependent and dose dependent and that NO neither stimulates nor inhibits mucus secretion in the ferret trachea.

mucin; lysozyme; neuropeptides; nonadrenergic noncholinergic nervous system

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J Appl Physiol 101: 486–491, 2006. First published April 27, 2006; doi:10.1152/japplphysiol.01264.2005.—Vasoactive intestinal peptide (VIP) and nitric oxide (NO) are neurotransmitters involved in the regulation of bronchial and pulmonary vascular tone. Published studies of the effects of VIP on airway mucus secretion have yielded conflicting results. The purpose of this study was to determine the effect of VIP on mucus secretion in the ferret trachea and if this effect was influenced by NO. We used a sandwich enzyme-linked lectin assay to measure mucin secretion and a turbidimetric assay to measure lysozyme (serous cell) secretion from ferret tracheal segments. VIP (10\textsuperscript{-5} M) increased mucin secretion over 2 h. VIP (10\textsuperscript{-5} to 10\textsuperscript{-9} M) stimulated mucin secretion in a dose-dependent manner. VIP-induced mucin secretion was partially blocked by a VIP receptor antagonist (a chimeric VIP-pituitary adenylate cyclase-activating peptide analog, VIP receptor antagonist) at a 10-fold excess concentration. At all concentrations tested, neither N\textsuperscript{G},N\textsuperscript{6}-dinitro-arginine methyl ester, an inhibitor of NO synthase, nor S-nitroso-N-acetyl-penicillamine, an NO donor, had any significant effect on constitutive or VIP-induced mucus secretion. We conclude that VIP-stimulated mucin and lysozyme secretion was both time dependent and dose dependent and that NO neither stimulates nor inhibits mucus secretion in the ferret trachea.

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incubated for another 30 min with test agents: MCh as a positive control, VIP, L-NAME with and without VIP, S-nitroso-N-acetylpenicillamine (SNAP), an NO donor, with and without VIP, or KHS as a negative control (period 2).

The relative contribution by mucous and serous cells to the secretion was evaluated by measuring the amount of mucin glycoconjugates, a marker for mucous cell secretion (16, 17), and lysozyme as a marker of serous cell secretion (31). A secretory index (SI) expressing the relative increase in secretion under experimental conditions for each tracheal segment was calculated as the mucin or lysozyme concentration after 30-min exposure to the test agent (period 2) divided by the concentration measured after 30-min exposure to KHS alone (period 1) in each segment. The relative change in the SI (RSI) was calculated using the SI of stimulated mucin or lysozyme secretion divided by that of unstimulated (KHS) secretion in each animal. This calculation made the RSI of the control group (KHS alone) equal to 1. The effects of agents were determined by comparing the RSI of the treated samples with those of matched control samples.

Mucin analysis by sandwich enzyme-linked lectin assay. Mucin is a complex secretion composed of water, mucin, ions, secreted proteins, phospholipids, etc. Mucin is secreted from mucous cells and glands and lysozyme from the serous glands. We have shown that, in the ferret, mucin can be used as a marker of mucous cell secretion and lysozyme as a marker of serous gland secretion. Ferret tracheal mucins have an abundance of galactose-N-acetylα-1–3 (fucose-α1–2) galactose-R (20). These antigens can be detected by Dolichos biflorus agglutinin (DBA). This binds specifically to goblet cells and submucosal glands in the ferret trachea. DBA does not identify membrane bound mucins or mucin that has been deglycosylated (17).

A sandwich enzyme-linked lectin assay was used to measure DBA-associated mucous glycoconjugates (16). A 96-well microtiter plate was coated with 60 μl of DBA (8 μg/ml in PBS) and incubated at room temperature overnight. After rinsing four times with PBS with 0.05% Tween 20 (PBS-Tween), the plate was exposed to sample buffer and incubated at 37°C for 2 h. It was then incubated with 50 μl of DBA conjugated with horseradish peroxidase (0.25 μg/ml) in PBS containing 1% BSA. Before and after this step, the plate was washed four times with PBS-Tween. One hundred and fifty microliters of tetramethylbenzidine (0.42 mM) in citrate-acetate buffer (pH 6.0) were then added to each well and incubated for 10 min. The reaction was stopped by adding 50 μl of 4.7 N H2SO4. Color development was read as the difference in absorbance at 450 and 650 nm in an ELISA reader. The concentration of mucin was calculated by comparison with asialo bovine submaxillary mucin: 20–200 ng/ml. Concentration curves were also developed for type II porcine gastric mucin and were nearly identical.

Lysozyme assay. Lysozyme is a bacteriolytic enzyme found in airway fluid. Its only source in the ferret airway is reported to be the serous cells of submucosal glands (31). The lysozyme concentration of the mucous samples was measured by a turbidimetric assay, which relies on the ability of lysozyme to break down the cell wall of the bacterium Micrococcus lysodeikticus. A 0.1-ml volume of sample was added to 0.9 ml of PBS at pH 6.0 containing M. lysodeikticus (0.3 mg/ml), sodium azide (1 mg/ml), and BSA (1 mg/ml). The reaction mixtures were then incubated in 24-well culture plates for 4 h at 37°C. After incubation, 300 μl of the reaction mixture were moved to a 96-well microtiter plate, and the optical density of each solution was measured at a wavelength of 450 nm in an ELISA reader. The standard curve was constructed by plotting the reduction in optical density against a known concentration of hen egg white lysozyme (1.6–100 ng/ml).

Data analysis. Statistical analysis of data was performed using the Stat View 5.0 statistics software (SAS Institute, Cary, NC). After verifying that data were normally distributed about the mean, these data were analyzed by ANOVA to assess secretory dose response and the effect of agents used. A probability of <0.05 was taken as significant. Results are presented as means ± SE.

RESULTS

Kinetics of VIP-induced mucin secretion. Tracheal segments were incubated in KHS with VIP (10⁻⁸ to 10⁻⁶ M) or KHS alone, and the incubation solution was collected after 5, 10, 20, and 30 min. The amount of mucin released from control tissue and tissues exposed to VIP increased over a 30-min period (Fig. 1A). Over a 2-h period, mucin secretion continued to increase in response to 10⁻⁷ M VIP and 10⁻⁵ M MCh (Fig. 1B).

Mucin and lysozyme secretion induced by VIP. VIP (10⁻⁹ to 10⁻⁵ M) increased mucin (Fig. 2A) and lysozyme (Fig. 2B) secretion over control (RSI) in a dose-dependent fashion.

A chimeric VIP-pituitary adenylate cyclase-activating peptide analog [VIP receptor antagonist (VRA) from Sigma] is reported to be a specific VRA with no reported cross-reactivity (11). We added 10⁻⁷ M and 10⁻⁶ M VRA to 10⁻⁷ M VIP and compared lysozyme and mucin secretion with that secreted in response to 10⁻⁷ M VIP or to KHS alone. VRA (10⁻⁷ M) had no effect on 10⁻⁷ M VIP-induced mucin or lysozyme secretion, but 10⁻⁶ M VRA decreased mucin (61.5%, Fig. 3A) and lysozyme (88.4%, Fig. 3B) secretion stimulated by 10⁻⁷ M VIP (P < 0.05 each). VRA had no direct effect on secretion.

Effect of L-NAME (NOS inhibitor) on constitutive and VIP-induced mucin secretion. VIP (10⁻⁶ M) significantly induced mucin and lysozyme secretion. L-NAME (10⁻⁶ to 10⁻⁴ M), an inhibitor of NOS, had no significant effect on constitutive or...
VIP-induced mucin or lysozyme secretion (Fig. 4, A for mucin and B for lysozyme). A 1-μmol concentration of VIP was chosen to ensure that there would be a large enough response to VIP to pick up a weaker signal of inhibition. This concentration of VIP has been used by other investigators evaluating the regulation of mucous secretion (12, 22).

Effects of SNAP (NO donor) on constitutive and VIP-induced mucous secretion. VIP (10⁻⁶ M) significantly increased mucin and lysozyme secretion over control, but SNAP (10⁻⁶ to 10⁻⁴ M), an NO donor, had no significant effect on constitutive or VIP-induced mucin or lysozyme secretion (Fig. 5, A for mucin and B for lysozyme).

DISCUSSION

We have shown that VIP stimulates mucin and lysozyme secretion in the ferret trachea in a dose-dependent manner, and VIP continued to induce secretion over a 2-h exposure period. A VRA at a 10-fold excess concentration partially blocked both mucin and lysozyme secretion. These data are similar to those of Fishbein and colleagues (11), who showed that this same antagonist had no effect on VIP-mediated pancreatic secretion in guinea pigs, but there was a 40% inhibition of secretion when administered in a 10-fold excess concentration. Inhibition of NO or NO donation had no significant effect on mucin secretion alone or in combination with VIP.

The reported effects of VIP on airway mucus secretion are conflicting. VIP has been reported to inhibit MCh-induced secretion in the ferret trachea (34). However, in that study, the total secretion volume was measured rather than measuring only mucin secretion. A common method to measure mucin secretion is to incubate cells or tissues with radioactive precursors of mucins and then to evaluate the release of radiolabeled mucin (6, 12, 22, 24, 29). Using this method, VIP has been reported to stimulate mucous glycoconjugate release from ferret trachea (24), gland exocytosis in the ferret trachea (12, 22), and mucin secretion from isolated feline tracheal submu-
cosal glands (29). We have demonstrated that the secretion of mucin and lysozyme after 10^{-5} \text{M} VIP was higher than that after 10^{-6} \text{M} VIP and did not appear to reach a maximum as might be expected (Fig. 2). Using radioisotope translocation across an Ussing chamber, Peatfield et al. (24) showed that the VIP response was near maximal at 10^{-6} \text{M}, but they did not test 10^{-5} \text{M} of VIP. A potential disadvantage of this approach is poor temporal resolution, because sampling periods must be long enough to obtain sufficient counts in the sample to determine changes between successive periods. Thus, if stimulation of mucin release is transient and of shorter duration than the collecting period, the maximal increase in the rate of mucin release will be underestimated (9). Recognizing this rapid response, we used a 30-min time for both period 1 and period 2 to calculate the RSI. Had we used 2-h stimulation for each period, the experiment would have taken at least 4 h, and the rapid secretory response may have been masked.

VIP at 1 and 10 \text{µM} stimulated secretion from the ferret trachea in vitro, but VIP also inhibited cholinergic neural secretion (22). The stimulatory action of VIP alone on mucus output is thought to be compensated for by its inhibition of cholinergic stimulated mucus secretion (10).

Our laboratory has previously shown that DBA binds to blood group antigens and that, in the ferret trachea, this substantively recognizes only mucin (16), that mucin secretagogues will increase the secretion of mucin-like glycoconjugates, and that this induced secretion can be blocked by specific agonists of secretion (17). In the study reported here, we temporally measured VIP and MCh-stimulated mucin glycoconjugate secretion from 5 min to 2 h and found a constant increase in mucin secreted over the full 2 h. This suggests that VIP and MCh do not transiently increase mucin secretion. Therefore, we do not believe that differences in sampling times explain the difference in reported results, and, indeed, these results are consistent with studies that measured mucin secretion in response to VIP.

![Figure 4](https://via.placeholder.com/150)

**Fig. 4.** Effect of N^G^-nitro-L-arginine methyl ester (L-NAME) on constitutive and VIP-induced mucus secretion. VIP (10^{-6} \text{M}) significantly increased mucin and lysozyme secretion over control (KHS alone). L-NAME (10^{-6} to 10^{-4} \text{M}), an inhibitor of nitric oxide synthase, does not have a significant effect on constitutive or VIP-induced mucin (A) or lysozyme (B) secretion. Data are expressed as means \pm SE. Numbers in parentheses represent the number of experiments. Significant increase over constitutive secretion: *\text{P} < 0.05 and **\text{P} < 0.01.

![Figure 5](https://via.placeholder.com/150)

**Fig. 5.** Effect of S-nitroso-N-acetyl-penicillamine (SNAP) on constitutive and VIP-induced mucus secretion. VIP (10^{-6} \text{M}) significantly increased mucin and lysozyme secretion over control (KHS alone). SNAP (10^{-6} to 10^{-4} \text{M}), a nitric oxide donor, has no significant effect on constitutive or VIP-induced mucin (A) or lysozyme (B) secretion. Data are expressed as means \pm SE. Numbers in parentheses represent the number of experiments. Significant increase over constitutive secretion: *\text{P} < 0.05 and **\text{P} < 0.01.
VIP-stimulated mucin secretion is not inhibited by a mixture of tetradoxin, atropine, 1-propranolol, and phenotolamine, suggesting that VIP may act on submucosal glands via specific VIP receptors (24). We have shown that the mucin secretagogue effect of VIP can be largely inhibited by a VRA, confirming that VIP-stimulated secretion is, at least in part, mediated by receptor stimulation.

There are few reported studies evaluating the effect of NO on airway mucus secretion. NO and VIP are thought to be released with cholinergic neurotransmitters and to regulate their effects (8). NO is reported to inhibit both basal and neurogenic stimulation of mucus secretion (26). One study using guinea pig tracheal epithelium showed that the stimulatory effect of inflammatory mediators, including histamine, platelet-activating factor, TNF-α, and xanthine oxidase, was inhibited by L-NAME but that L-NMMA alone did not directly affect mucin secretion (1). In contrast to this, it has also been reported that NO donor, reduced basal secretion in the ferret trachea (26). In the rat, nasal perfusion with L-NAME blocks albumin leakage but not mucin secretion in response to histamine (19). We found that neither L-NMMA nor SNAP had a significant effect on constitutive or VIP-induced mucus secretion. Explanations for the difference in the effect of NO on airway mucous secretion may be due to the species studied, incubation methods, or materials used (18, 28). Comparing the amount of mucin released from whole tissue before and after drug treatment can produce misleading results. By evaluating mucin secretion as the RSI (normalized to control cells), we eliminate variations due to differences in absolute mucin and lysozyme secretion in different size tissue sections.

The published literature regarding the effect of VIP on mucus secretion is contradictory. Although there are studies that suggest that VIP is a secretagogue, there are others that suggest that VIP is anti-inflammatory and inhibits mucus secretion. After demonstrating that VIP is a potent secretagogue in the ferret trachea, an accepted model for submucosal glands (24), we have shown that the mucin secretagogue effect of VIP was not inhibited at a 10-fold excess of receptor antagonist with results nearly identical to previously reported studies of VIP-mediated pancreatic secretion (11). These results suggest that VIP stimulates mucus secretion in the ferret airway independently of NO but mediated, at least in part, by specific receptor activation. Furthermore, NO does not appear to play a role in constitutive or VIP-induced mucous glycoconjugate secretion in the ferret trachea. Finally, these data suggest that the secretion of mucin glycoconjugates from mucous cells and lysozyme from serous cells occurs together with VIP stimulation in the ferret trachea, an accepted model for submucosal gland secretion.

In experiments to investigate the effect of L-NMMA on the response to VIP, no pretreatment with L-NMMA was included before exposure to VIP. As a result, there is a possibility that the VIP could stimulate secretion before the concentration of L-NMMA inside the cell reached a level high enough to inhibit NO synthesis. In Fig. 1A, this concentration of VIP begins to increase mucin secretion within 5 min, a time period that may be less than the time that takes L-NMMA to inhibit NO. However, at all concentrations tested from 10^{-6} to 10^{-2} M, neither SNAP nor L-NMMA used alone had any measurable effect on mucin or lysozyme secretion. This was true over the full observation period of 30 min while our studies showed a time-dependent increase in mucin secretion with 1 μmol VIP. Although this does not confirm a lack of involvement of NO, this lack of an effect with no VIP stimulation is supportive of this and consistent with previously published literature.

It was noted that there are significant differences in the size of the response to 1 μmol VIP in Figs. 2, 4, and 5. There are several possible explanations for this degree of variability. Tissues systems are intrinsically variable. We have shown differences not only between the response comparing different animals or on different days, but even at different levels of the trachea in the same animal (16, 17). The degree of variability comparing Fig. 2 with Figs. 4 and 5 is greater than we usually observe with this system, but, because the experiments were conducted by the same investigator under seemingly identical conditions, this variability is inexplicable.

We showed that VIP-induced secretion was not inhibited at equimolar concentrations by a specific antagonist, VRA, but was partially inhibited at a 10-fold excess of receptor antagonist with results nearly identical to previously reported studies of VIP-mediated pancreatic secretion (11). These results suggest that VIP stimulates mucus secretion in the ferret airway independently of NO but mediated, at least in part, by specific receptor activation. Furthermore, NO does not appear to play a role in constitutive or VIP-induced mucous glycoconjugate secretion in the ferret trachea. Finally, these data suggest that the secretion of mucin glycoconjugates from mucous cells and lysozyme from serous cells occurs together with VIP stimulation in the ferret trachea, an accepted model for submucosal gland secretion.

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