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Effects of epithelium removal on relaxation of airway smooth muscle induced by vasoactive intestinal peptide and electrical field stimulation

Stephen G. Farmer & James Togo

Nova Pharmaceutical Corporation, 6200 Freeport Centre, Baltimore, Maryland 21224-2788, U.S.A.

1 We have studied the effect of epithelium removal on relaxation of guinea-pig isolated tracheal smooth muscle induced by vasoactive intestinal peptide (VIP) or stimulation of non-adrenergic, non-cholinergic (NANC) inhibitory nerves. Also examined were the effects of inhibitors of neutral endopeptidase (NEP) and angiotensin-converting enzyme (ACE).

2 Epithelium removal produced a 3.6 ± 0.4 fold leftward shift in the VIP concentration-response curve. The supersensitivity to VIP, following epithelium removal was abolished by phosphoramidon or thiorphan (NEP inhibitors), but unaffected by captopril (an ACE inhibitor). In intact trachea, the NEP inhibitors produced leftward shifts in the VIP curves similar to those produced by epithelium removal.

3 In contrast to responses to exogenous VIP, neurogenic NANC inhibitory responses to electrical field stimulation were affected neither by epithelial denudation nor by the peptidase inhibitors.

4 As in previous studies, epithelium removal increased tracheal sensitivity to isoprenaline. This was not altered by pretreatment with a cocktail of peptidase inhibitors. Thus, the effect of the NEP inhibitors on responses to VIP appears to be relatively specific.

5 These data indicate that exogenous VIP is a substrate for airway NEP, since inhibition of the enzyme potentiates the peptide. This is further evidence that the airway epithelium provides a source for the metabolism of mediators.

6 In guinea-pig trachea the NEP responsible for cleaving VIP may be located largely in the epithelial layer, since NEP inhibition was without effect on sensitivity to VIP in epithelium-denuded preparations. If VIP is a NANC inhibitory neurotransmitter in this tissue, its degradation endogenously does not appear to involve epithelial NEP.

Introduction

Epithelium removal modulates airway smooth muscle responsiveness to a variety of spasmogenic and relaxant agonists (see reviews by Fedan *et al.*, 1988; Goldie *et al.*, 1990), and there is evidence for an epithelium-derived inhibitory factor (EpDIF) that inhibits vascular and airway smooth muscle (Hay *et al.*, 1987; Fernandes *et al.*, 1989). In contrast, augmentation of tracheal responses to some agents may be due to loss of epithelial sites of uptake and/or enzymatic degradation for those particular agents. For example, increased sensitivity of guinea-pig trachea to the relaxant effect of isoprenaline, following epithelium removal, appears to be due solely to loss of catecholamine uptake and degradation (Farmer *et al.*, 1986). This is suggested by the observation that an inhibitor of extraneuronal uptake abolished supersensitivity to isoprenaline induced by removal of the epithelium. Moreover, epithelium removal had no effect on sensitivity to salbutamol, which is not a substrate for extraneuronal uptake (Farmer *et al.*, 1986).

Epithelium removal increases tracheal sensitivity to adenosine-induced relaxation (Farmer *et al.*, 1986; Advenier *et al.*, 1988). This, too, is due to loss of epithelial sites of adenosine degradation, as the effect is abolished by drugs which inhibit adenosine uptake and metabolism (Advenier *et al.*, 1988). The epithelium also converts exogenous arachidonic acid into relaxant products, and denudation transforms arachidonate-induced relaxation into contraction in guinea-pig trachea (Nijkamp & Folkerts, 1986; Farmer *et al.*, 1987). In addition, the increase in responsiveness to contractile effects of substance P may be due largely to loss of epithelial neutral endopeptidase (NEP; EC 3.4.24.11), which degrades this peptide (Devillier *et al.*, 1988; Frossard *et al.*, 1989).

There is much evidence to suggest that vasoactive intestinal peptide (VIP) is a non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmitter in airway smooth muscle (Matsuzaki *et al.*, 1980; Sundler *et al.*, 1988; Ellis & Farmer, 1989a,b). Although the mechanisms underlying VIP degradation in the airway are unclear, it has been demonstrated that certain mast cell-derived proteases will degrade VIP (Caughey *et al.*, 1988) and that these same enzymes reverse VIP-induced tracheal relaxation *in vitro* (Franconi *et al.*, 1989). In addition, a metalloendopeptidase cleaves VIP in rat spinal tissue *in vitro* (Barbato *et al.*, 1988).

Recently, the functions of airway NEP have been the subject of much interest (Thompson & Sheppard, 1988; Djokic *et al.*, 1989; Dusser *et al.*, 1989). This enzyme is present in the plasma membrane of epithelial cells in various organs (Matsas *et al.*, 1984; Erdös & Skidgel, 1989; Ryan, 1989) and human recombinant NEP cleaves VIP into several peptide fragments (Goetzl *et al.*, 1989). The purpose of the present investigation was to assess the effects of epithelium removal on guinea-pig tracheal sensitivity to VIP and the influence of inhibitors of NEP and angiotensin-converting enzyme (ACE). We also examined the effect of epithelium removal and peptidase inhibitors on NANC inhibitory responses to electrical field stimulation (EFS). Some of these data have been presented to the British Pharmacological Society (Farmer & Togo, 1989).

Methods

Tissue preparation

Male, Dunkin-Hartley guinea-pigs (350–500 g: Hazelton, Denver, Pennsylvania) were stunned, exsanguinated and the trachea removed. This was placed in modified Krebs-Henseleit solution (composition mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 10.0). Extraneous tissue was dissected free and transverse strips, consisting of two adjacent cartilage rings, were suspended in organ chambers containing Krebs solution maintained at 37°C and

gassed with 95% O_2/5% CO_2. Alternate tracheal strips were denuded of epithelium with a cotton-tipped applicator. Tissues were equilibrated for 60 min at an initial resting tension of 1.5 g and washed with Krebs solution every 15 min.

Experimental protocol

At the end of equilibration, each preparation was exposed to an equieffective concentration of methacholine (MCh EC_{60} + epithelium 2 μM; −epithelium 1 μM; Hay *et al.*, 1986) to assess tissue viability. VIP was added to the bath in increasing concentrations cumulatively (1 nM–0.3 μM). In some experiments, VIP concentration-response curves were obtained in tissues precontracted with MCh, at the same concentrations used to assess viability. Responses to VIP are expressed as a % of the maximal relaxation to sodium nitroprusside (SNP; 30 μM) added at the end of the experiment.

Where appropriate, EFS was delivered to platinum electrodes from a Grass S-88 stimulator whose output was passed through a Stimu-Splitter II (Med-Lab Instruments, Loveland, Colorado) for signal amplification. Frequency-response curves were generated by applying stimuli (20 V, 0.2 ms, 1–20 Hz) for 30 s. All EFS experiments were conducted in the presence of atropine (1 μM) and propranolol (1 μM) to abolish cholinergic and noradrenergic responses, respectively. Both the peak magnitude of NANC relaxations, and the time taken for 50% recovery of prestimulation tone were determined. Each NANC response was expressed as a percentage of the maximum relaxation induced by 20 Hz stimulation.

The effects of phosphoramidon and DL-thiorphan, NEP inhibitors, and captopril, an ACE inhibitor (each at 10 μM) were examined after they had been added 20 min before the application of VIP or EFS. We also determined the effect of epithelium removal and the peptidase inhibitors on tracheal sensitivity to isoprenaline (0.1 nM–0.1 μM), which was also added to the bath in a cumulative manner. Each response to isoprenaline was expressed as a percentage of the maximum relaxation to this agent. All experiments with peptidase inhibitors were conducted in preparations with basal tone.

The pD_2 values for VIP or isoprenaline were determined from regression analyses of logit-transformed concentration-response curves. Responses to EFS, VIP or isoprenaline are expressed as mean ± s.e.mean. The effects of epithelium removal and peptidase inhibitors were compared with their respective controls by Student's two-tailed *t* test for paired observations. Probability values of ≤ 0.05 were considered significant.

Drugs

Methacholine Cl, atropine H_2SO_4, (±)-isoprenaline HCl, propranolol HCl, SNP, DL-thiorphan and tetrodotoxin (TTX), were obtained from the Sigma Chemical Co. (St. Louis, Missouri). Porcine VIP was purchased either from Sigma or from Bachem Inc. (Philadelphia, Pennsylvania). Phosphoramidon was obtained from Peninsula Laboratories Inc. (Belmont, California). Captopril was purchased from Squibb Pharmaceuticals Inc. (Princeton, New Jersey).

Atropine, captopril, MCh, propranolol, SNP and TTX were each dissolved in 0.9% w/v NaCl solution (saline). Isoprenaline was prepared extemporaneously, as a 10 mM solution, in saline containing 0.25% w/v ascorbic acid, and thiorphan and phosphoramidon were dissolved in dimethylsulphoxide and distilled water, respectively.

Results

Effects of epithelium removal

Epithelium removal produced an increase in potency and rate of relaxation to VIP in tracheal strips with basal or MCh-induced tone (Figure 1). In intact and denuded trachea with basal tone, the pD_2 values for VIP of 7.74 ± 0.05 and 8.25 ± 0.06 respectively, were significantly different. Epithelium removal produced a 3.6 ± 0.4 fold leftward shift in the VIP concentration-response curve ($n = 14$, Figure 2). In the presence of an EC_{60} of MCh, the VIP concentration-response curves were shifted in a dextral manner, to a similar extent in intact and denuded tissues (Figure 2). In MCh-treated trachea, the pD_2 value for VIP in intact tissues (7.02 ± 0.08, $n = 9$) was significantly lower than in denuded preparations (7.42 ± 0.10). Epithelium removal caused a 2.8 ± 0.3 fold increase in sensitivity to VIP in the presence of MCh.

The pD_2 values for VIP in intact tissues treated and not treated with atropine and propranolol were 7.82 ± 0.27 and 7.79 ± 0.21, respectively ($n = 3$). The corresponding values for

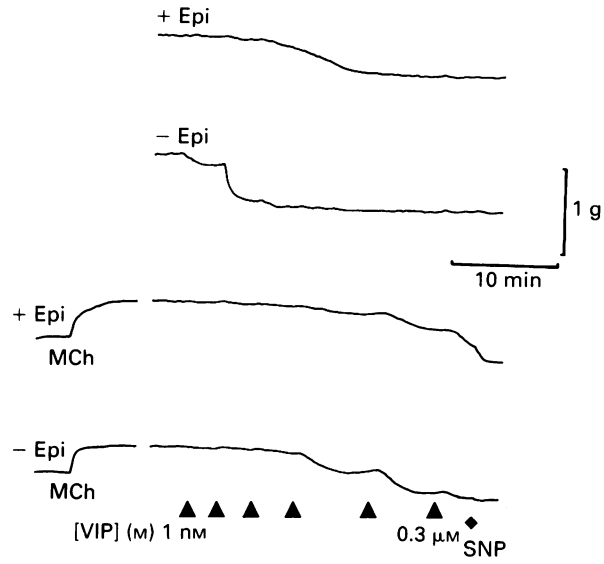

Figure 1 Relaxations of guinea-pig trachea to vasoactive intestinal peptide (VIP). The top two parts of the figure show responses in preparations with basal, spontaneous tone, and the lower two, in preparations precontracted with an EC_{60} methacholine (+ epithelium 2 μM; − epithelium 1 μM). The tissues represented by the second and fourth tracings were denuded of their epithelium. For each preparation, VIP (1 nM–0.3 μM) was added at the arrows and sodium nitroprusside (SNP, 30 μM), applied at the end of the experiment, was used to determine the maximum relaxation (i.e. zero active tension). MCh = methacholine.

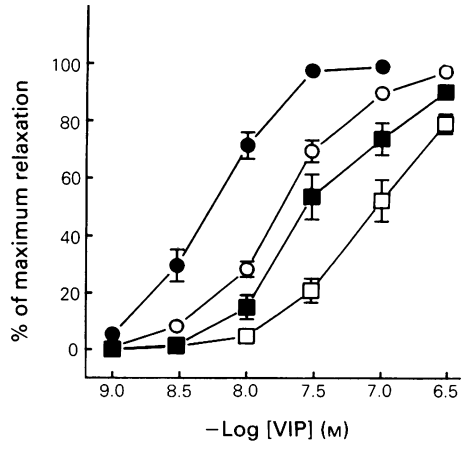

Figure 2 Concentration-response curves for vasoactive intestinal peptide (VIP), showing the effect of epithelium removal, in guinea-pig trachealis. Responses to VIP were expressed as a % of the maximum relaxation induced by sodium nitroprusside (30 μM). (○) Epithelium-intact controls with basal tone; (●) epithelium-denuded tissues with basal tone; (□) intact tissues precontracted with methacholine (MCh, 2 μM); (■) denuded tissues precontracted with MCh (1 μM). Each point represents the mean of 14 (basal tone) or 9 (precontracted) observations; vertical lines show s.e.mean.

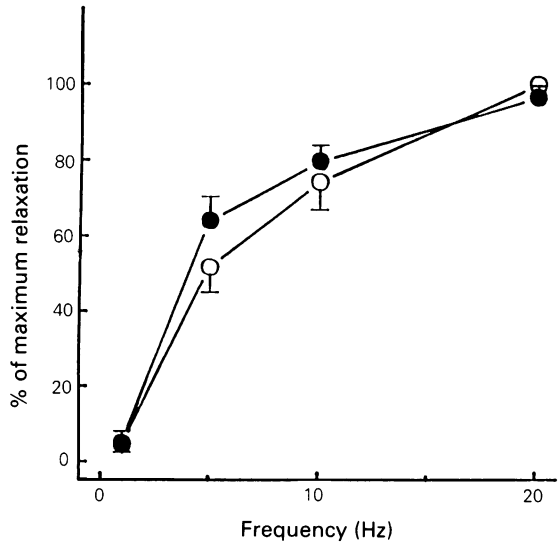

Figure 3 Frequency-dependent, non-adrenergic, non-cholinergic inhibitory responses of guinea-pig trachea to electrical field stimulation and the effect of epithelium removal. Responses were determined as a percentage of the maximum relaxation induced by stimulation at 20 Hz. Experiments were carried out in the presence of atropine and propranolol (each at 1 μM). (○) + Epithelium; (●) − epithelium. Each point represents the mean of 13 observations; vertical lines show s.e.mean.

epithelium-denuded tissues were 8.60 ± 0.41 and 8.34 ± 0.12, respectively (n = 3). Thus, VIP had similar relaxant activity in trachea treated with atropine and propranolol and, in such tissues, epithelium removal caused a similar potentiation (4.4 ± 1.3 fold, $P \leqslant 0.05$) to VIP.

In the presence of atropine (1 μM) and propranolol (1 μM), EFS induced frequency-dependent relaxations which returned slowly to baseline upon cessation of stimulation. These NANC inhibitory responses were abolished by TTX (0.1 μM) confirming their neurogenic origin. Epithelium removal did not alter the magnitude of relaxation at any frequency (Figure 3). Similarly, the maximum NANC relaxation of 658 ± 67 mg (n = 13) in intact trachea was not different from 691 ± 89 mg in denuded tissues. Epithelium removal did not influence the time taken for preparations to recover 50% of their pre-stimulation level of tone. In intact tissues at 20 Hz, for example, this time was 5.67 ± 0.42 min compared with 6.02 ± 0.82 min in denuded trachea. Thus, epithelium removal had no discernible effect on NANC responses.

Effects of peptidase inhibitors

The pD_2 values for VIP in intact tissues treated and not treated with captopril (10 μM) were 7.74 ± 0.05 and 7.64 ± 0.12 respectively. The corresponding values for epithelium-denuded tissues were 8.25 ± 0.06 and 8.10 ± 0.12 respectively. These data suggest that captopril neither modifies the action of VIP on intact trachea nor modifies the effects of epithelium removal on the action of VIP (Figure 4).

In contrast to the ACE inhibitor, both phosphoramidon and thiorphan caused leftward shifts in the VIP concentration-response curve and abolished the effect of epithelium removal (Figures 5 and 6). Phosphoramidon increased

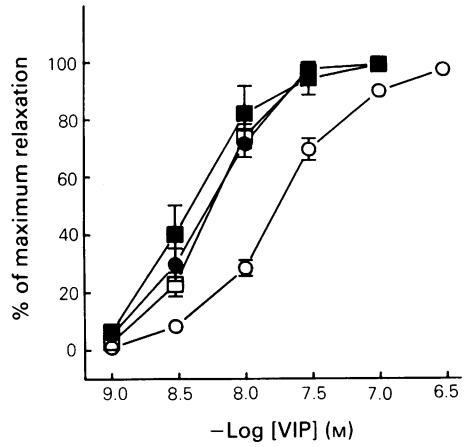

Figure 5 Concentration-response curves for vasoactive intestinal peptide (VIP), showing the effect of epithelium removal and phosphoramidon (10 μM), in guinea-pig trachealis with basal tone. Responses to VIP were expressed as a % of the maximum relaxation induced by sodium nitroprusside (30 μM). (○) Epithelium-intact controls; (●) epithelium-denuded tissues; (□) intact tissues in the presence of phosphoramidon; (■) denuded tissues in the presence of phosphoramidon. Each point represents the mean of 8 observations; vertical lines show s.e.mean.

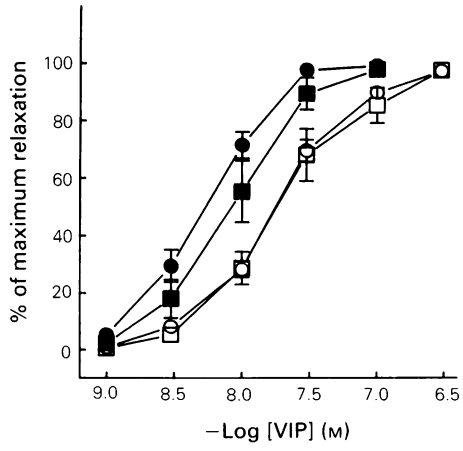

Figure 4 Concentration-response curves for vasoactive intestinal peptide (VIP), showing the effect of epithelium removal and captopril (10 μM), in guinea-pig trachealis with basal tone. Responses to VIP were expressed as a % of the maximum relaxation induced by sodium nitroprusside (30 μM). (○) Epithelium-intact controls; (●) epithelium-denuded tissues; (□) intact tissues in the presence of captopril; (■) denuded tissues in the presence of captopril. Each point represents the mean of 9 observations; vertical lines show s.e.mean.

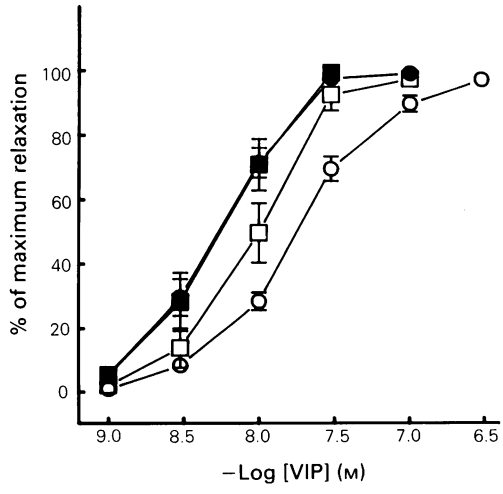

Figure 6 Concentration-response curves for vasoactive intestinal peptide (VIP), showing the effect of epithelium removal and thiorphan (10 μM), in guinea-pig trachealis with basal tone. Responses to VIP were expressed as a % of the maximum relaxation induced by sodium nitroprusside (30 μM). (○) Epithelium-intact controls; (●) epithelium-denuded tissues; (□) intact tissues in the presence of thiorphan; (■) denuded tissues in the presence of thiorphan. Each point represents the mean of 7 observations; vertical lines show s.e.mean.

Table 1 Effects of epithelium removal and peptidase inhibitors[1] on sensitivity of guinea-pig tracheal smooth muscle to the relaxant effect of isoprenaline

Treatment	pD_2 values for isoprenaline + Epithelium	− Epithelium	Mean shift
Control	8.35 ± 0.06	8.68 ± 0.14*	2.5 ± 0.6
Plus inhibitors[1]	8.38 ± 0.06	8.94 ± 0.20*	6.4 ± 3.2

[1] The cocktail of inhibitors comprised phosphoramidon and captopril, each at 10 μM. * Denotes significantly different from epithelium-intact control. Data are expressed as the mean ± s.e.mean of seven observations.

sensitivity to VIP in intact trachea only. In the presence of the NEP inhibitor, the pD_2 value in intact tissues (8.24 ± 0.06; $n = 8$), was significantly different from control and represented a leftward shift of 3.2 fold. Phosphoramidon abolished the effect of epithelium removal on sensitivity to VIP (mean shift, 1.6 ± 0.3, Figure 5). Similarly, thiorphan increased sensitivity to VIP in preparations with intact epithelium, but had no effect in denuded trachea (Figure 6). The pD_2 value for intact tissues, in the presence of thiorphan, was 8.07 ± 0.10 and this was not different from the value of 8.30 ± 0.08 in denuded trachea (shift, 1.80 ± 2.3, $n = 7$).

Phosphoramidon was without effect on the magnitude or time-course of NANC inhibitory responses to EFS, irrespective of epithelial integrity. The maximum magnitude of relaxation in intact tissues, pretreated with phosphoramidon, was 777 ± 76 mg and this was not different from 746 ± 83 mg in denuded tissues. Further, in the presence of phosphoramidon, epithelium removal had no effect on the time taken to recover 50% of initial tone (+ epithelium 6.09 ± 0.39 min; − epithelium 5.78 ± 0.55 min; 20 Hz).

Isoprenaline

Epithelium removal caused an approximately 3 fold increase in sensitivity to isoprenaline (Table 1). In the presence of the peptidase inhibitors, epithelium removal caused an apparently larger shift in the isoprenaline curve, but this was not significantly different from the shift in the absence of inhibitors.

Discussion

Removal of guinea-pig tracheal epithelium increases responsiveness of the underlying smooth muscle to tachykinin-induced contraction (Fine *et al.*, 1989; Frossard *et al.*, 1989; Tschirhart *et al.*, 1989), as well as to adenosine- (Farmer *et al.*, 1986; Advenier *et al.*, 1988) and isoprenaline-induced relaxations (Farmer *et al.*, 1986). For substance P, the effect of epithelium removal may be due partly to elimination of epithelial NEP (and thus, substance P degradation) and partly to loss of prostanoid and non-prostanoid inhibitory factors (Fine *et al.*, 1989; Frossard *et al.*, 1989). Conversely, enhancement of responses to adenosine and isoprenaline, following epithelium denudation, appears to result solely from removal of uptake and/or catabolic processes for these agents (Farmer *et al.*, 1986; Advenier *et al.*, 1988).

The present study demonstrates that epithelium removal increases tracheal sensitivity to VIP and that this potentiation is abolished by inhibitors of NEP. That the effect of the inhibitors did not influence potentiation of responses to isoprenaline suggests their action exhibits selectivity. These data provide further evidence that the airway epithelium may be an important source of metabolism for several substances. It is also suggested that increased sensitivity to VIP following epithelium removal is due to loss of NEP. Since the NEP inhibitors had no effect in epithelium-denuded tissues, the principal source of degradation of VIP by NEP probably exists in the epithelial layer. These observations concur with another study wherein it was found that substance P is degraded by guinea-pig tracheal NEP, located mainly in the epithelium (Devillier *et al.*, 1988).

VIP is the principal candidate for the NANC inhibitory neurotransmitter in the airways of guinea-pigs (Matsuzaki *et al.*, 1980; Carstairs & Barnes, 1986; Ellis & Farmer, 1989a,b,c) and cats (Ito & Takeda, 1982; Diamond *et al.*, 1988). In addition, VIP-immunoreactive nerves and VIP receptors have been localized in human airways (Dey *et al.*, 1981; Lundberg *et al.*, 1984; Carstairs & Barnes, 1986). VIP is a very potent relaxant of airway smooth muscle *in vitro* (Altiere & Diamond, 1985; Ellis & Farmer, 1989b) and inhibits bronchoconstriction in animals (Said, 1982; Diamond *et al.*, 1983). Conversely, it has proven disappointing as a bronchodilator in man (see references in Barnes, 1986; 1988), probably due to enzymatic destruction in the lungs. Indeed, one study demonstrates that inhaled VIP is ineffective as a bronchodilator, in rats, due to metabolism during its passage through the airway epithelial layer (Barrowcliffe *et al.*, 1986). This study also found that neither the serine protease inhibitor aprotinin, nor the ACE inhibitor captopril had any effect on pulmonary destruction of VIP. Further, in feline airways *in vitro* aprotinin and captopril have no effect on VIP-induced relaxation (Altiere & Diamond, 1984). Previous studies in our laboratory also showed that aprotinin had no effect on responses of guinea-pig trachea, either to exogenous VIP or to NANC inhibitory responses to EFS (Ellis & Farmer, 1989b). However, the nature of endogenous pathways for the degradation of VIP is unclear at present. The failure of captopril to potentiate airway effects of VIP in rat (Barrowcliffe *et al.*, 1986) and cat (Altiere & Diamond, 1984) is confirmed in the present study with guinea-pig trachea. Furthermore, the ability of epithelium removal to enhance sensitivity to VIP was not affected by the ACE inhibitor. Therefore, the effect of epithelium removal probably does not involve loss of ACE.

NANC inhibitory responses were not altered by removal of the epithelium, confirming previous findings in guinea-pig (Holroyde, 1986) and cat (Thompson *et al.*, 1988b) airways. That epithelium removal increased sensitivity to exogenous VIP and yet had no effect on NANC inhibitory responses, should be commented upon in view of the putative role for this peptide in neurotransmission. Furthermore, although the present study indicates that exogenous VIP is degraded by NEP located in the tracheal epithelium, inhibition of this peptidase was without noticeable effect on the NANC response. This may be interpreted as negating a transmitter role for VIP. As alluded to above, however, there is much evidence in support of VIP as a NANC transmitter. In particular, desensitization of cat or guinea-pig trachea to VIP attenuates NANC inhibitory responses to EFS (Ito & Takeda, 1982; Ellis & Farmer, 1989a). In addition, incubation of guinea-pig trachea with antiserum specific for VIP markedly reduces the NANC response (Matsuzaki *et al.*, 1980; Ellis & Farmer, 1989a). Only when specific antagonists for airway VIP receptors become available will its role in neurotransmission be confirmed. Unfortunately, known VIP antagonists are without effect, either on responses to VIP or to EFS, in guinea-pig or feline airway smooth muscle (Thompson *et al.*, 1988a; Ellis & Farmer, 1989a).

If VIP is indeed involved in NANC neurotransmission in the trachea, then the lack of effect of epithelium removal and NEP inhibition on responses to EFS also requires comment. Epithelial NEP may not be important in regulating airway levels of endogenous VIP. In fact, although myriad peptides are known to be cleaved by NEP *in vitro*, only a few (and VIP is not among them) have been shown to be cleaved *in vivo* (Erdös & Skidgel, 1989). In human airways NEP is most concentrated in the luminal membranes of epithelial cells, in addition to being present in fibroblasts (Johnson *et al.*, 1985). As mentioned earlier, human recombinant NEP cleaves VIP *in vitro* into several fragments (Goetzl *et al.*, 1989), which have little or no effect on guinea-pig trachea (Bodansky *et al.*, 1973). Nevertheless, nerve endings involved in the control of airway tone lie predominantly in the smooth muscle layer (Laitinen, 1985; Barnes, 1986; Gabella, 1987). Further, in human airways, VIP-like immunoreactive nerves are found predomi-

nantly in the smooth muscle layer and bronchial glands (Laitinen, 1985; Sundler *et al.*, 1988), whereas the epithelium does not receive VIP-like immunoreactive nerves (Laitinen, 1985). Conversely, VIP-containing nerves are present in the airway lamina propria, in close proximity to the epithelial basement membrane (Said, 1988). It has also been demonstrated that VIP receptors are widely distributed in human and guinea-pig airway tissues, including smooth muscle and the epithelium (Carstairs & Barnes, 1986). Therefore, it is conceivable that under 'normal' conditions, neuronal VIP does not reach the epithelial sites where NEP is localized, and that the ability of NEP to cleave VIP has trivial physiological significance in large airways.

In summary, the present study indicates that increased sensitivity of guinea-pig trachea to VIP following epithelium removal, is not due to loss of epithelial diffusion barriers or epithelium-derived factors. Rather, the epithelium may contain NEP which can degrade exogenous VIP, thereby decreasing its availability to its smooth muscle receptors and limiting tissue sensitivity to the relaxant action. That epithelium removal and NEP inhibition had no effect on NANC inhibitory responses to EFS suggests that epithelial NEP may not be an important regulator of endogenous VIP. The mechanisms whereby neuronal VIP are removed and/or degraded in the airway await clarification.

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