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Relaxation of cat tracheobronchial and pulmonary arterial smooth muscle by vasoactive intestinal peptide: lack of influence by peptidase inhibitors

Ralph J. Altiere & Louis Diamond

Pharmacology and Toxicology Division, College of Pharmacy, University of Kentucky, Lexington, KY 40536, U.S.A.

1 Vasoactive intestinal peptide (VIP) caused concentration-dependent relaxation in precontracted segments of trachea, hilar bronchus, intrapulmonary bronchus and intrapulmonary artery (IPA) isolated from cat lungs.

2 VIP-induced relaxation responses were abolished by preincubation of tissues with the proteolytic enzyme, α-chymotrypsin (2 units ml^{-1}). At the concentration employed, α-chymotrypsin treatment did not adversely affect tissue viability as isoprenaline and bethanechol continued to relax airways and IPA, respectively.

3 Aprotinin prevented enzymatic degradation of VIP by α-chymotrypsin as demonstrated by the ability of VIP to relax tissues incubated with both the peptidase inhibitor and α-chymotrypsin.

4 A spectrum of peptidase inhibitors, including aprotinin, leupeptin, bestatin, bacitracin, β-phenylpropionic acid and captopril, individually or in combination, did not augment the relaxant effects of VIP in isolated pulmonary tissues.

5 These results suggest that local enzymatic degradation may not be a primary route for inactivation of VIP in cat isolated airways and IPA. If VIP acts as a neurotransmitter in these tissues, a mechanism other than enzymatic proteolysis may be responsible for terminating its action.

Introduction

Vasoactive intestinal peptide (VIP) is considered to be a neural peptide that acts locally within a tissue as a neurotransmitter or neuromodulator (Said, 1980). In recent studies, we have shown that VIP is a potent relaxant of cat isolated tracheal and bronchial smooth muscle (Altiere & Diamond, 1984) and that it causes bronchodilatation in the cat *in vivo* (Diamond *et al.*, 1983). We also have demonstrated the existence of a non-adrenergic non-cholinergic (NANC) inhibitory nervous system mediating bronchodilatation in the intact, anaesthetized cat (Diamond & O'Donnell, 1980) and smooth muscle relaxation in isolated tracheal and bronchial smooth muscle of the cat (Szarek *et al.*, 1983).

The chemical mediators associated with the lung NANC inhibitory nervous system have yet to be identified, but recent studies have suggested that purines are unlikely neurotransmitter candidates (Irvin *et al.*, 1982). Additional evidence suggests that peptides, in particular VIP, may serve as the neurotransmitter of the lung NANC nervous system. Studies with immunofluorescence techniques have demonstrated the presence of VIP-containing neurones within glands and smooth muscle of airways and within the walls of pulmonary and bronchial blood vessels in the lungs of dogs, cats and humans (Dey *et al.*, 1981). VIP also has been shown to be released from guinea-pig isolated trachea upon electrical field stimulation and the amount released correlated with the degree of relaxation (Matsuzaki *et al.*, 1980). Furthermore, in the *in situ* guinea-pig tracheal pouch preparation, prolonged exposure to VIP caused desensitization to the relaxant effects of exogenous VIP and a corresponding decrease in response to NANC nerve stimulation, suggesting a neurotransmitter role for VIP (Venugopalan *et al.*, 1981).

The question of whether VIP is the neurotransmitter of the NANC inhibitory nervous system in cat airways has not been answered. However, if VIP does serve as a neurotransmitter in airway smooth muscle, a mechanism for its inactivation should exist within these tissues. One such mechanism might be rapid enzymatic degradation. If so, inhibition of peptidases

should potentiate responses to VIP in isolated airway smooth muscle, just as peptidase inhibition increases the potency of the natural opioid peptide, enkephalin, in various isolated tissues (McKnight *et al.*, 1983). To date, there have been no investigations of the influence of enzymatic metabolism on the activity of VIP in airway smooth muscle.

In the present study, we have assessed the effects of a spectrum of peptidase inhibitors on VIP-induced relaxation responses in isolated tracheal and bronchial smooth muscle of the cat. In view of the relaxant action of VIP in cat pulmonary arteries (Altiere & Diamond, 1983; Hamasaki *et al.*, 1983), the presence of VIP-containing neurones around pulmonary blood vessels (Dey *et al.*, 1981) and the postulated role of VIP as a neurotransmitter in other blood vessels (Duckles & Said, 1982; Said, 1982), we have also investigated the effects of peptidase inhibitors on VIP-induced relaxation responses in isolated segments of cat intrapulmonary artery.

Methods

Adult cats (2–4 kg) of either sex were anaesthetized with an intraperitoneal injection of allobarbitone (100 mg kg^{-1}) and urethane (400 mg kg^{-1}). Trachea and lungs were removed and placed in room-temperature Krebs Henseleit solution (KHS) containing (mM): NaCl 118.2, KCl 4.74, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 26.2, dextrose 11.1 and $CaNa_2EDTA$ 0.027, aerated with 95% O_2–5% CO_2. Airway and vessel segments were prepared as previously described (Altiere & Diamond, 1983; 1984). Four tissue segments were dissected from the lung: thoracic trachea just proximal to the carina, right main stem bronchus as it entered the diaphragmatic lobe (hilar bronchus), intrapulmonary bronchus (IPB) 2 cm into the right diaphragmatic lobe and intrapulmonary artery (IPA) immediately proximal to the IPB segment. Tissues were cleaned and set up as cylindrical segments between stainless steel hooks in 10 ml isolated tissue baths containg KHS aerated with 95% O_2–5% CO_2 and mantained at 37°C. Tissues were equilibrated for 2 h after application of optimal passive load (trachea, 3 g; hilar bronchus, 4 g; IPB, 1.5 g; IPA, 5 g) and washed with fresh KHS by overflow every 10 min. Changes in isometric force development were measured with Grass FTO3C force displacement transducers and recorded on a Hewlett-Packard Model 7404A recorder. Tissue baths and glass support rods were siliconized periodically to reduce peptide binding to glass surfaces.

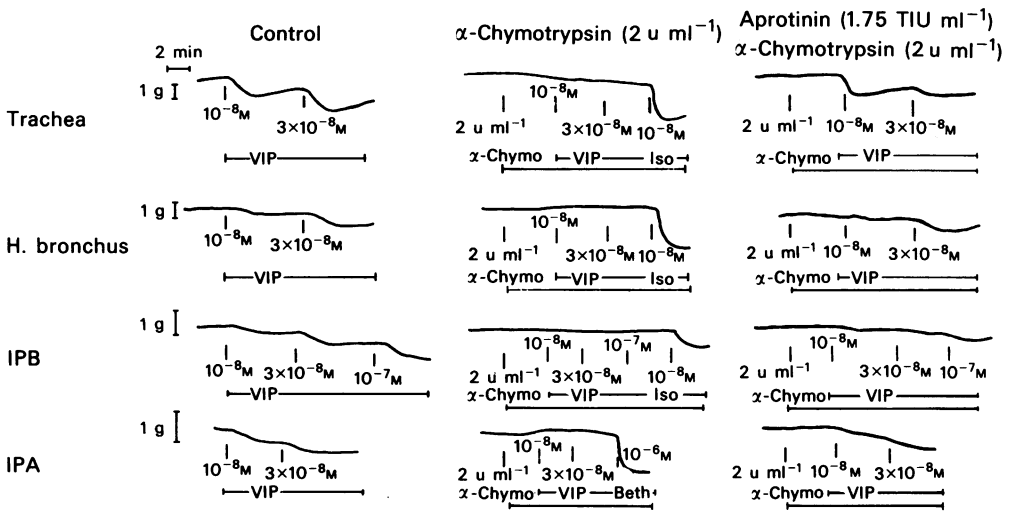

Figure 1 Effects of aprotinin and α-chymotrypsin on vasoactive intestinal peptide (VIP)-induced relaxation of cat trachea, hilar (H.) bronchus, intrapulmonary bronchus (IPB) and intrapulmonary artery (IPA). Smooth muscle tone was increased by 5-hydroxytryptamine (5-HT, 1 μM) in airways and phenylephrine (100 μM) in IPA. Recorder tracings in the left panel illustrate concentration-dependent relaxation induced by VIP. In the centre panel, tissues were incubated with α-chymotrypsin (2 units ml^{-1} of bath fluid) for 4 min before addition of VIP; α-chymotrypsin abolished the relaxant actions of VIP, but did not alter tissue response to isoprenaline (Iso, 10 nM) in airways or bethanechol (Beth, 1 μM) in IPA. In the right panel, tissues were incubated with 1.75 trypsin inhibitor units (TIU) ml^{-1} of bath fluid of the peptidase inhibitor, aprotinin, for 30 min before addition of α-chymotrypsin (2 units ml^{-1}) to the tissue baths. Aprotinin prevented degradation of VIP by α-chymotrypsin as evidenced by the return of concentration-dependent relaxation responses to VIP in all tissues.

Since cat isolated airways and IPA have little or no intrinsic tone, airways were precontracted with 5-hydroxytryptamine (5-HT) (1 μM) and IPA with phenylephrine (100 μM) to facilitate measurement of relaxation responses. Cumulative concentration-response curves to VIP were determined in the precontracted tissue segments and compared to relaxation responses to isoprenaline in airways and to bethanechol in IPA. Effects of the peptidase inhibitors, either singly or in combination, were examined on VIP concentration-response curves determined in the same tissue segment. Tissues were incubated with peptidase inhibitor for 20 or 30 min (leupeptin for 1 h) before determination of second VIP concentration-response curves. The protocol for drug additions in experiments with α-chymotrypsin and aprotinin is described in Results.

Relaxation responses, expressed as a percentage of induced precontraction, were plotted against the negative logarithm of VIP molar concentration. For comparison of concentration-response curves, the concentration of VIP producing 50% reversal of precontraction (EC_{50} value) was determined graphically from log concentration-response curves and reported as $-\log [EC_{50}]$ M values. Data were analyzed by analysis of variance and means compared by least squares difference. Probability values of $P < 0.05$ were taken to indicate significance.

The following drugs and chemicals were used: 5-hydroxytryptamine creatinine sulphate, isoprenaline hydrochloride, carbamyl-β-methylcholine chloride (bethanechol), vasoactive intestinal peptide, α-chymotrypsin (type 1-S), aprotinin, bacitracin, bestatin, and β-phenylpropionic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.); phenylephrine hydrochloride (Amend Drug & Chemical Co., New York, NY, U.S.A.); leupeptin (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.); and captopril (E.R. Squibb and Sons, Princeton, NJ, U.S.A.). All drugs were prepared in KHS immediately before use; dilutions of VIP were prepared from stock solution stored at −20°C.

Results

We have shown previously that VIP causes concentration-dependent relaxation of cat tracheal, bronchial and IPA smooth muscle (Altiere & Diamond, 1983; 1984). In these studies, it was also demonstrated that sequential concentration-response curves to VIP were reproducible and that effects of pharmacological agents on VIP responses could be compared to control curves determined in the same tissue segment. In the present study, a similar protocol was used to investigate the effects of peptidase inhibitors on VIP concentration-response curves.

In order to confirm the efficacy of peptidase inhibition by aprotinin in our isolated tissue preparations, we tested the inhibitor against the endopeptidase, α-chymotrypsin. Results from these experiments are illustrated in the representative tracings shown in Figure 1. Pretreatment of the tissues with α-chymotrypsin completely inhibited the relaxant activity of exogenous VIP. Tissue viability during α-chymotrypsin treatment was confirmed by eliciting a rapid reversal of precontraction with isoprenaline in airways and with bethanechol in IPA. The slight decrease in precontraction tone observed during these experiments was the result of α-chymotrypsin treatment alone, as confirmed in separate experi-

Table 1 Effects of various peptidase inhibitors on EC_{50} values of vasoactive intestinal peptide (VIP) in cat trachea, hilar bronchus, intrapulmonary bronchus (IPB) and intrapulmonary artery (IPA)

Trachea	*Hilar bronchus*	*IPB*	*IPA*
	$-\log [EC_{50}]$ M		
Aprotinin (1.75 TIU ml^{-1})			
7.70±0.33	7.40±0.40	6.99±0.30	8.43±0.18
N.D.	6.97±0.12	6.66±0.26	8.27±0.34
(3)	(3)	(4)	(3)
Leupeptin (50 μM)			
7.61±0.36	7.08	7.01±0.23	8.13±0.34
7.86±0.23	7.28	7.24±0.32	7.97±0.52
(3)	(2)	(3)	(3)
Bestatin (30 μM)			
8.15±0.15	8.00±0.20	7.73±0.22	8.52±0.24
8.36±0.11	7.97±0.15	7.89±0.12	7.83±0.60
(3)	(3)	(3)	(3)
Bacitracin (60 μM)			
8.77±0.21	7.45±0.21	7.25±0.25	8.59±0.40
8.60±0.28	8.25±0.27*	7.65±0.19	7.93±0.16
(4)	(5)	(5)	(3)
β-Phenylpropionic acid (1 mM)			
8.55±0.20	7.91±0.04	7.76±0.10	8.52±0.19
8.75±0.08	7.53±0.31	7.78±0.20	8.27±0.51
(3)	(3)	(3)	(3)
Captopril (10 μM)			
8.49±0.08	7.57±0.36	7.61±0.21	8.38±0.43
8.67±0.07	7.82±0.22	7.48±0.12	8.42±0.26
(4)	(4)	(4)	(4)
Aprotinin + bestatin + β-phenylpropionic acid + captopril			
8.44±0.22	7.48±0.18	7.62±0.16	n.d.
8.19±0.36	7.95±0.52	7.44±0.35	n.d.
(6)	(6)	(6)	

Values are mean ± s.e.mean. Number of observations shown in parentheses. First value of each pair represents control response and second value represents response in the presence of the peptidase inhibitor. The concentration of each peptidase inhibitor in the mixture was the same as that used individually. ND = not determined. * $P = 0.05$ compared to control value.

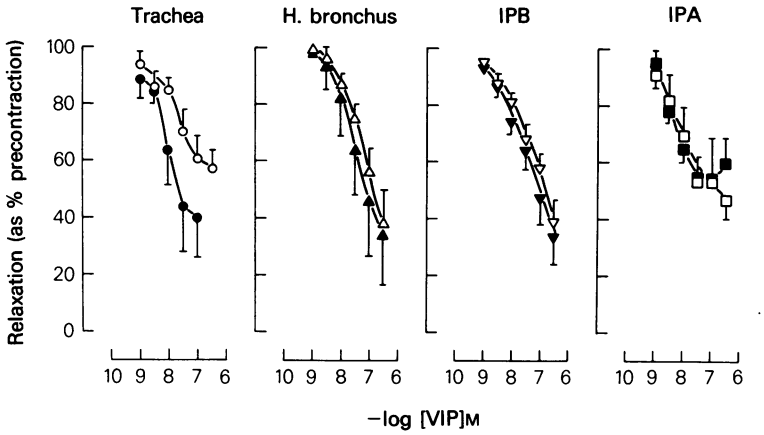

Figure 2 Concentration-response curves to vasoactive intestinal peptide (VIP) in cat isolated trachea, hilar (H.) bronchus, intrapulmonary bronchus (IPB) and intrapulmonary artery (IPA). Airways were precontracted with 5-hydroxytryptamine (1 μM) and IPA was precontracted with phenylephrine (100 μM). Solid symbols (●, ▲, ▼, ■) represent first control curves to VIP in the absence of drug treatment. Open symbols (○, △, ▽, □) represent second curves to VIP after 30 min incubation with aprotinin (1.75 TIU ml^{-1}). Each point is the mean of 3 to 4 experiments; vertical lines show s.e.mean.

ments. The inhibitory effects of α-chymotrypsin on VIP-induced responses were completely reversible as demonstrated by the return of VIP-induced relaxation responses after washout and re-equilibration of the tissues. As also shown in Figure 1, aprotinin completely prevented the inhibition of VIP activity by α-chymotrypsin. Having established the efficacy of peptidase inhibition by aprotinin in these isolated tissues, we next examined the effects of aprotinin alone on VIP concentration-response curves.

Incubation of airways and IPA for 30 min with aprotinin at the same concentration that inhibited the proteolytic activity of α-chymotrypsin had no effect on VIP concentration-relaxation curves, as shown in Figure 2 and in the calculated $-\log[EC_{50}]$ M values (Table 1). The effects of a second endopeptidase inhibitor, leupeptin, also were investigated on VIP-induced relaxation responses. At a concentration previously shown to inhibit proteolysis in skeletal and cardiac muscle strips *in vitro* (Libby & Goldberg, 1978), leupeptin did not affect VIP concentration-response curves (Table 1). Further, there were no observable changes in the duration of response to VIP in the presence of either aprotinin or leupeptin.

Incubation of isolated airways and IPA with the aminopeptidase inhibitor, bestatin, or the carboxy-

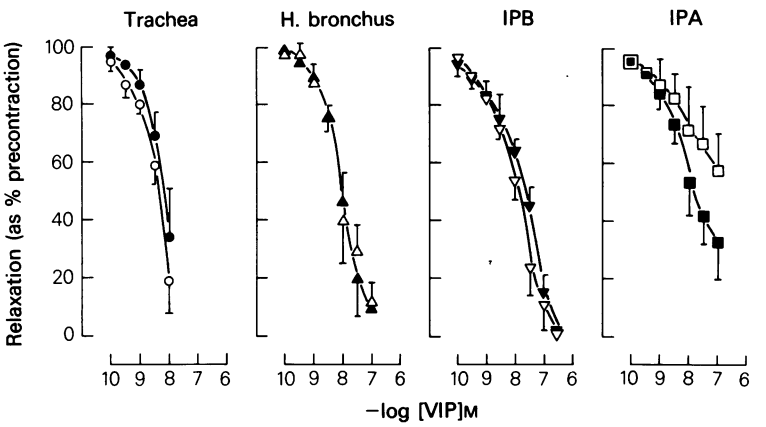

Figure 3 Concentration-relaxation curves to vasoactive intestinal peptide (VIP) in isolated, precontracted airways and IPA of cat. First control curves are shown by closed symbols (●, ▲, ▼, ■) and second curves after 20 min incubation with the aminopeptidase inhibitor, bestatin (30 μM), are shown by open symbols (○, △, ▽, □). Each point is the mean of 3 experiments; vertical lines show s.e.mean.

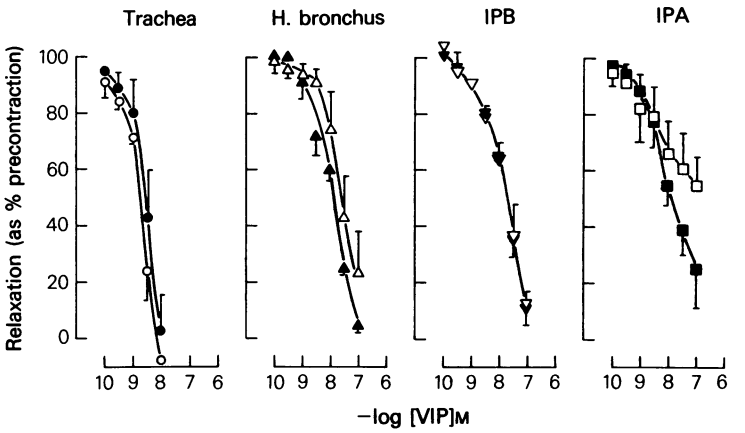

Figure 4 Concentration-relaxation curves to vasoactive intestinal peptide (VIP) in isolated, precontracted cat airways and IPA of cat. First control curves are shown by closed symbols (●, ▲, ▼, ■) and second curves after 20 min incubation with the carboxypeptidase inhibitor, β-phenylpropionic acid (1 mM), are shown by open symbols (○, △, ▽, □). Each point is the mean of 3 experiments; vertical lines show s.e.mean.

peptidase inhibitor, β-phenylpropionic acid, also failed to potentiate the relaxant effects of VIP (Figures 3 and 4, respectively; Table 1). Another aminopeptidase inhibitor, bacitracin, the dipeptidyl carboxypeptidase inhibitor, captopril, or a combination of inhibitors of each type (aprotinin, bestatin, β-phenylpropionic acid and captopril) (Figure 5) did not enhance VIP-induced relaxation responses. As shown in Table 1, there were no significant differences in $-\log [EC_{50}]$ M values for VIP in the presence of the inhibitors as compared to control values.

Discussion

The experiments described here confirm that VIP is a potent relaxant of cat tracheobronchial and pulmonry vascular smooth muscle. To determine whether rapid enzymatic metabolism is an important mechanism in terminating the action of this peptide, we evaluated the effects of peptidase inhibition on VIP-induced relaxation responses in isolated lung tissues. Because relatively little is known about specific enzymes that degrade VIP, peptidase in-

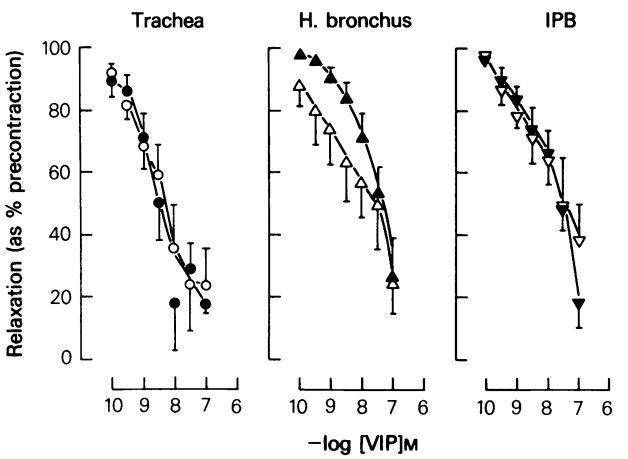

Figure 5 Concentration-relaxation curves to vasoactive intestinal peptide (VIP) in isolated, precontracted airways of cat. First control curves are shown by closed symbols (●, ▲, ▼) and second curves after 30 min incubation with a mixture of peptidase inhibitors (aprotinin, 1.75 TIU ml^{-1}; bestatin, 30 μM; β-phenylpropionic acid, 1 mM; captopril, 10 μM) are shown by open symbols (○, △, ▽). Each point is the mean of 6 experiments; vertical lines show s.e.mean. No data are shown for IPA which failed to precontract in the presence of the mixture of peptidase inhibitors.

hibitors from several different classes were used, individually and in combination, in an attempt to potentiate the action of VIP.

Aprotinin inhibits the proteolytic activity of several different enzymes by reversibly binding to their active sites. Numerous enzymes, including trypsin, chymotrypsin, plasmin and kallikrein, as well as intracellular peptidases of various tissues, are inhibited by aprotinin. The inhibitor is synthesized within mast cells and relatively large amounts of it are normally present in lung tissue. Aprotinin recently has attained widespread use as an effective antiproteolytic agent in radioimmunoassay procedures for many different peptide hormones (Zyznar, 1981). Protection by aprotinin against enzymatic degradation of VIP has been demonstrated for plasma samples and for radioimmunoassay mixtures (Fahrenkrug & Schaffalitzky de Muckadell, 1977; Pandian *et al.*, 1982). Therefore, aprotinin appeared to be a reasonable choice as a peptidase inhibitor for protecting against local enzymatic degradation of VIP in isolated tissue preparations.

Leupeptin also is an inhibitor of several endopeptidases and it has been shown to decrease protein degradation in isolated strips of skeletal and cardiac muscle (Libby & Goldberg, 1978). In the latter report, evidence was presented that leupeptin entered intact cells and inhibited intracellular proteolytic enzymes that were resistant to another peptidase inhibitor that did not gain access to the intracellular space. Because leupeptin is effective in intact muscle preparations and apparently is able to cross cellular membranes, it was selected, along with aprotinin, as an endopeptidase inhibitor for use in these experiments.

In the present study we demonstrated enzymatic degradation of exogenous VIP by the endopeptidase, α-chymotrypsin. The relaxant action of VIP in cat airways and IPA was completely abolished by prior incubation with the enzyme, thus extending the observation made by MacKenzie & Burnstock (1980) in which α-chymotrypsin prevented VIP-induced relaxation of guinea-pig taenia coli. Moreover, we were able to demonstrate effective peptidase inhibition in isolated pulmonary tissue preparations; aprotinin prevented degradation of VIP by α-chymotrypsin. However, neither aprotinin nor leupeptin potentiated VIP-induced relaxation responses, suggesting that VIP is not rapidly metabolized by endopeptidases within cat isolated airways or IPA.

It is possible that VIP may be degraded by other types of peptidases, such as amino- or carboxypeptidases. Indeed, effects of the natural opioid, enkephalin, in guinea-pig ileum, rat and mouse vas deferens and in rat brain were potentiated by aminopeptidase inhibition (Chaillet *et al.*, 1983; Cohen *et al.*, 1983; McKnight *et al.*, 1983). In contrast, results of the present study show that the smooth muscle relaxant action of VIP is unaffected by aminopeptidase (bestatin, bacitracin), carboxypeptidase (β-phenylpropionic acid) or dipeptidyl carboxypeptidase (captopril) inhibition. It may be that more than one type of peptidase metabolizes VIP such that selective inhibition of a single type does not suffice to bring about detectable changes in tissue responsiveness to VIP. Accordingly, we used a mixture of endo-, amino- and carboxypeptidase inhibitors but still were unable to potentiate the effects of VIP. It appears from these results that, unlike the effects of endogenous opioid peptides, the actions of VIP are not readily enhanced by peptidase inhibition within isolated airways and IPA.

Several factors may contribute to the lack of potentiation of VIP responses in the presence of these peptidase inhibitors. It may be that enzymes which degrade VIP were inaccessible to these agents although this seems unlikely for leupeptin (see above). Alternatively, VIP may be degraded by enzymes, such as specific VIPases, that are not susceptible to the peptidase inhibitors used in this study, however aprotinin and bacitracin have been used as inhibitors of VIP proteolysis in plasma samples and radioimmunoassay mixtures (Pandian *et al.*, 1982). Enzymatic degradation of VIP may occur at such a rapid rate, i.e. enzyme levels may be so high or the affinity of the enzyme for VIP may be so great, that the concentrations of inhibitors used were ineffective. However, VIP responses were relatively slow to develop and also were sustained (except for trachea) so the former possibility appears remote. A VIP peptidase present in several tissues other than lung (see below) has a relatively low affinity for the peptide, suggesting that the latter factor also may be of little consequence. Furthermore, several of the inhibitors employed were present at concentrations previously shown to be effective in potentiating effects of enkephalin in isolated tissues (McKnight *et al.*, 1983). It is possible that more than one mechanism for inactivation of VIP exists such that two or more routes of inactivation must be inhibited in order to observe potentiation of VIP-induced responses, a circumstance analogous to the potentiation of noradrenaline responses, i.e. enhancement of noradrenaline responses by extraneuronal uptake inhibitors generally is observed only when neuronal uptake is blocked simultaneously. Since there is little knowledge at the moment of the mechanisms by which VIP is inactivated locally within tissues, each of the factors mentioned above remains a viable alternative.

The half-life of VIP *in vivo* is extremely short, averaging 1 to 2 min in man (Domschke *et al.*, 1978), pig and dog (Mitchell *et al.*, 1982), indicating that VIP is rapidly metabolized or quickly removed from effector sites in various organs. These observations

are consistent with the postulated neurotransmitter role of VIP. Furthermore, extracts of rabbit liver, kidney and brain have been shown to contain a peptidase that specifically degrades VIP (Keltz *et al.*, 1980). In intact animal preparations, a considerable amount of exogenously administered VIP is cleared by the liver (Kitamura *et al.*, 1975; Mitchell *et al.*, 1982); however, lung does not appear to metabolize VIP (Kitamura *et al.*, 1975). Our results also suggest that enzymatic degradation may not be a major route of inactivation of VIP in lung tissues and impart an element of uncertainty concerning the role of VIP as a neurotransmitter in the lung. In this regard, other investigators recently have suggested that VIP may not be the neurotransmitter associated with the lung NANC nervous system in cat and human airways (Davis *et al.*, 1982; Ito & Takeda, 1982; Karlsson & Persson, 1983). Further studies are needed to provide direct evidence for or against the neurotransmitter role of VIP in the lung. The question of whether or not enzymatic metabolism of VIP is an important means for its inactivation in other tissues in which it is postulated to be a neurotransmitter also requires further investigation.

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