Br. J. Pharmacol. (1989), **96**, 521–526

Effects of peptidases on non-adrenergic, non-cholinergic inhibitory responses of tracheal smooth muscle: a comparison with effects on VIP- and PHI-induced relaxation

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1 The effects of peptidase enzymes on non-adrenergic, non-cholinergic (NANC) inhibitory responses of guinea-pig trachea to electrical field stimulation (EFS), and on relaxations induced by vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI) have been examined.

2 α-Chymotrypsin reduced both the magnitude and, particularly, the duration of the inhibitory response to EFS, whereas papain reduced only the magnitude. Aprotinin, a peptidase inhibitor prevented the effects of α-chymotrypsin but was without effect on papain.

3 α-Chymotrypsin and papain both abolished relaxant responses to exogenous VIP and PHI. The action of α-chymotrypsin was prevented by aprotinin, whereas that of papain was not affected.

4 The peptidases were without effect on concentration-response curves to methacholine or to isoprenaline. It was also observed that, in the absence of the peptidases, aprotinin had no effect on inhibitory responses either to EFS or to exogenous VIP and PHI.

5 It is suggested that neuropeptides, possibly VIP and PHI, released during EFS of guinea-pig trachea, partly mediate NANC relaxations, and that their action may be inhibited by peptidases. However, the lack of effect of aprotonin alone, on responses to EFS, suggests that, if endogenous peptidases are important in terminating the action of neuropeptides, they are resistant to the effect of this particular peptidase inhibitor. It is further suggested that neurogenic relaxation of guinea-pig trachea is also partly mediated by a substance, possibly non-peptide, other than VIP or PHI.

Introduction

It is now recognized that, in addition to the classical neurotransmitters, acetylcholine and noradrenaline, postganglionic nerve endings in most smooth muscle tissues release non-adrenergic, non-cholinergic (NANC) mediators (Daniel, 1985; Burnstock, 1986). NANC inhibitory responses to electrical field stimulation (EFS) in airway smooth muscle from a number of species, including man, have also been demonstrated (Barnes, 1986). The nature of the airway NANC neurotransmitter(s) has not been conclusively elucidated, and probably varies with species studied. In feline (Ito & Takeda, 1982) and guinea-pig (Matsuzaki *et al.*, 1980) airway smooth muscle, a putative candidate is vasoactive intestinal peptide (VIP).

VIP is a potent relaxant of guinea-pig (Venugopalan *et al.*, 1986) and feline (Altiere & Diamond, 1984) airway smooth muscle *in vitro*. The latter study demonstrated that preincubation of cat trachea with the peptidase enzyme α-chymotrypsin abolished the effect of exogenous VIP. Conversely, they also showed that α-chymotrypsin was without effect on the NANC relaxation of this preparation (Altiere & Diamond, 1985), and it was proposed that VIP or related peptides may not act as neurotransmitters in cat airways. It was also considered that peptides resistant to degradation by α-chymotrypsin

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may be responsible for the NANC relaxation in feline trachea. Conversely, canine lung appears not to metabolize VIP (Kitamura *et al.*, 1975), and this may also be the case in the cat.

It has been suggested (Karlsson, 1986) that VIP released during nerve stimulation may act in conjunction with another inhibitory substance. For example, it is known that VIP and peptide histidine isoleucine (PHI), another inhibitory peptide, have identical distribution in the airways (Lundberg *et al.*, 1984). It has been proposed that these peptides, released as cotransmitters, may mediate the NANC inhibitory response of airway smooth muscle (Barnes, 1986).

The present study set out to determine the effects of the peptidases, α-chymotrypsin and papain, on NANC responses of guinea-pig isolated trachea to EFS, and to compare them with effects on responses to exogenous VIP and PHI. In addition, the effect of the peptidase inhibitor aprotinin was examined.

Methods

Male, Duncan-Hartley guinea-pigs (350–500 g: Hazelton, Denver, PA) were used. Preparation of and measurement of isometric tension responses from transverse tracheal strips is described elsewhere (Ellis & Farmer, 1989). Strips were suspended between two platinum ring electrodes in organ baths containing oxygenated, modified Krebs-Henseleit solution at 37°C, and equilibrated for 60 min at an initial resting tension of 1.5 g.

For EFS, rectangular pulses were delivered to the electrodes from a HSE Type 215 stimulator, and frequency-response curves were generated by applying stimuli (50 V, 0.1 ms, 0.1–100 Hz) for 30 s. Control frequency-response curves were obtained in each preparation. Drugs were added to the bath 10 min before the construction of a second curve. Concentration-response curves for VIP or PHI were obtained following their addition cumulatively to the bath. Peptidases were added to the bath 10 min before obtaining a second curve. Where appropriate, aprotinin was added 20 min before the peptidases. Responses are expressed as the mean ± s.e.mean.

Drugs

Atropine H_2SO_4, propranolol HCl, acetyl-β-methylcholine Cl (MCh), isoprenaline HCl, porcine VIP, papain (Type 1V), α-chymotrypsin (Type 1S) and aprotinin were obtained from the Sigma Chemical Co., Missouri, U.S.A. Porcine PHI was obtained from Bachem Inc, California, U.S.A. Isoprenaline was freshly dissolved as a 1 mM stock solution in 0.9% w/v NaCl solution (saline) containing ascorbic acid (0.25%), and dilutions were made in saline. All other agents used were freshly dissolved in saline. Atropine (1 μM) and propranolol (1 μM) were present except where stated.

Results

In the presence of atropine and propranolol, frequency-dependent relaxation responses to EFS were elicited, with the maximum response occurring at 50 Hz. At this frequency, the magnitude of the response was 608 ± 32 mg ($n = 32$). Following cessation of stimulation, the tension returned slowly to baseline. After stimulation at 50 Hz, the time taken for 50% recovery was 317 ± 34 s ($n = 10$). Relaxation responses to both VIP and PHI were qualitatively similar, although VIP was more potent (Figure 1).

α-Chymotrypsin (2 units ml^{-1}) had two effects on the inhibitory response to EFS (Figures 2 and 3). Firstly, it decreased the magnitude of the response to about 65% of control (Figure 3a), and, secondly, it markedly reduced the duration of the NANC response (Figure 3b). For example, at a frequency of 50 Hz, the time taken to attain 50% prestimulation baseline was decreased from over 5 min in controls, to approximately 1 min in the presence of the peptidase (Figure 3b). Effects of α-chymotrypsin were prevented completely by preincubation with aprotinin (1.75 u ml^{-1}; Figures 2 and 3). In the presence of α-chymotrypsin, responses to both VIP and PHI, even

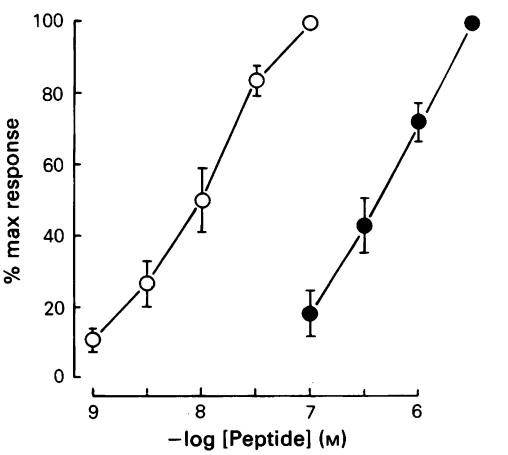

Figure 1 Concentration-response curves to vasoactive intestinal peptide (VIP, ○) and peptide histidine isoleucine (PHI, ●) in guinea-pig trachea. Each point is the mean of 8 observations and vertical lines indicate s.e.mean.

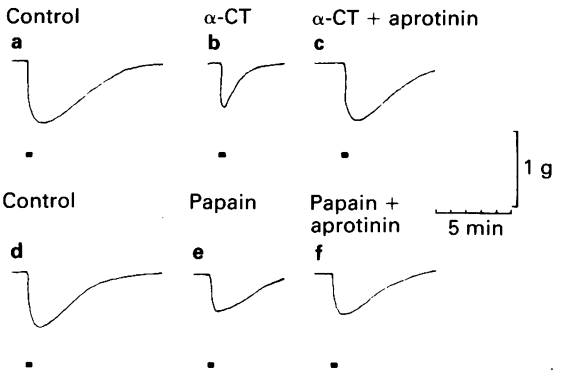

Figure 2 Typical non-adrenergic, non-cholinergic (NANC) relaxations of guinea-pig trachea to electrical field stimulation. Stimulation (50 Hz, 0.1 ms) was applied for 30 s, as indicated by the bars. Atropine (1 μM) and propranolol (1 μM) were present throughout. Responses (a), (b) and (c) were obtained from the same tissue and show, (a) control; (b) the effect of α-chymotrypsin (α-CT, 2 units ml^{-1}) added 10 min before stimulation; (c) inhibition of the effect of α-chymotrypsin by preincubation with aprotinin (1.75 u ml^{-1}, 20 min). Responses (d), (e) and (f) were obtained from the same tissue and show, (d) control; (e) the effect of papain (2 units ml^{-1}) added 10 min before stimulation; (f) inability of aprotinin to inhibit papain.

at high concentrations, were completely abolished, effects which were prevented by preincubation with aprotinin.

Papain (2 units ml^{-1}) decreased the magnitude of the NANC inhibitory response to about 65% of control (Figures 2 and 4a), without affecting its time course (Figure 4b). Aprotonin did not inhibit papain (Figure 4). In addition, papain completely abolished responses to exogenous VIP and PHI, and like its effect on the NANC response, this action of papain was also resistant to prior incubation with aprotinin. Aprotinin alone had no effect on either the magnitude or the duration of the NANC inhibitory responses, or on tracheal responsiveness to VIP or PHI.

In order to ascertain whether the effects of the peptidases on responses to EFS are non-specific, we obtained concentration-response curves to MCh and isoprenaline in the presence and absence of α-chymotrypsin or papain. Atropine and propranolol were absent during these experiments. As can be seen (Figure 5) α-chymotrypsin did not affect responsiveness to either agonist. Similar data were obtained with papain (data not shown). In addition, the inhibitory effects of either of the peptidases on responses to EFS were completely reversible by washing.

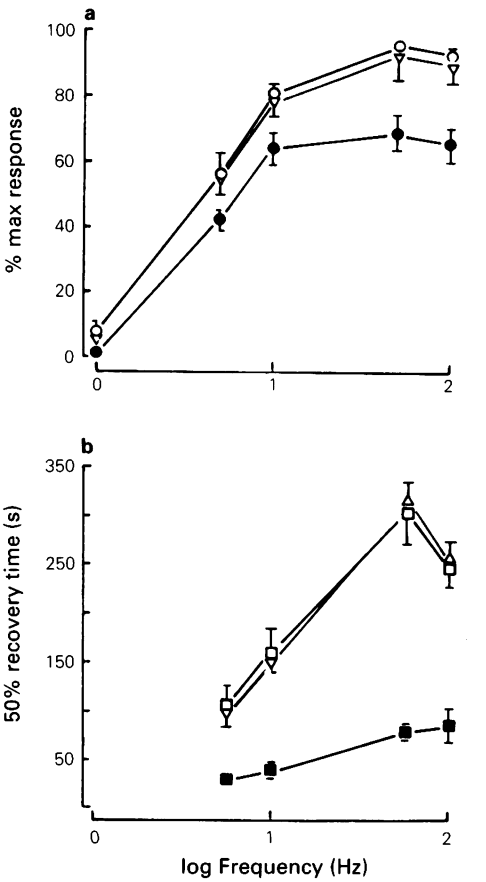

Figure 3 Effects of α-chymotrypsin (2 units ml^{-1}) on NANC relaxations of guinea-pig trachea to electrical field stimulation (50 Hz, 0.1 ms, 30 s), and their reversal by aprotinin (1.75 u ml^{-1}). α-Chymotrypsin was added 10 min before stimulation, and aprotinin, 20 min before α-chymotrypsin. Atropine (1 μM) and propranolol (1 μM) were present throughout. (a) Effect of α-chymotrypsin on magnitude of relaxation: (○) Controls; (●) in presence of α-chymotrypsin; (▽) in presence of α-chymotrypsin and aprotinin. (b) Effect of α-chymotrypsin on time taken for 50% recovery to baseline: (□) Controls; (■) in presence of α-chymotrypsin; (△) in presence of α-chymotrypsin and aprotinin. Each point is the mean of at least 8 observations and vertical lines indicate s.e.mean.

Discussion

In feline trachea, whereas responses to VIP are abolished by α-chymotrypsin (Altiere & Diamond, 1984), the NANC inhibitory response is unaffected (Altiere & Diamond, 1985). This suggested firstly, that VIP,

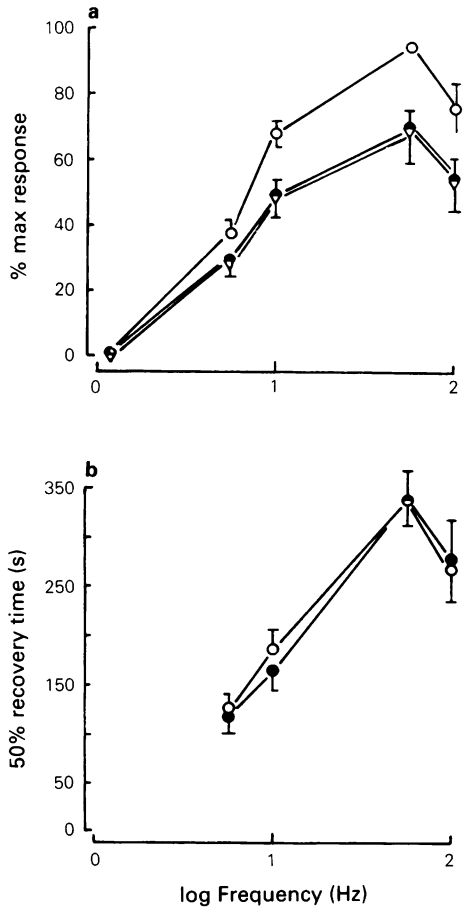

Figure 4 Effects of papain (2 units ml^{-1}) on non-adrenergic, non-cholinergic (NANC) relaxations of guinea-pig trachea to electrical field stimulation (50 Hz, 0.1 ms, 30 s). Papain was added 10 min before stimulation, and aprotinin (1.75 u ml^{-1}), 20 min before papain. Atropine (1 μM) and propranolol (1 μM) were present throughout. (a) Effect of papain on magnitude of relaxation: (○) Controls; (●) in presence of papain; (▽) in presence of papain and aprotinin. (b) Effect of papain on time taken for 50% recovery to baseline: (○) Controls: (●) in presence of papain. Each point is the mean of at least 6 observations and vertical lines indicate s.e.mean.

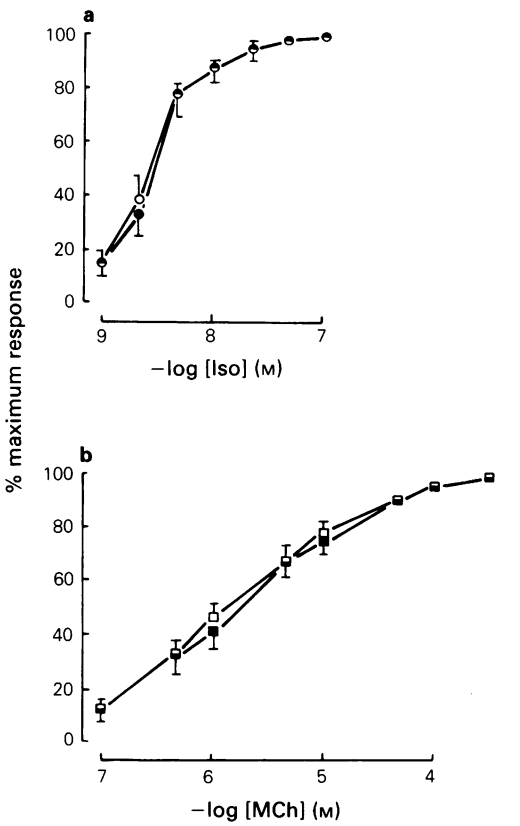

Figure 5 Effect of α-chymotrypsin (2 units ml^{-1}) on guinea-pig tracheal relaxations to isoprenaline (Iso, a) and contractions to methacholine (MCh, b). (a) (○) Control responses to Iso; (●) responses to Iso in presence of α-chymotrypsin; (b) (□) control responses to MCh; (■) responses to MCh in presence of α-chymotrypsin. Each point is the mean of 4 observations and vertical lines indicate s.e.mean.

or related peptides, may not mediate the NANC response or, secondly, if peptides are involved, they may not be susceptible to degradation by α-chymotrypsin. Altiere & Diamond (1984) also showed that a variety of peptidase inhibitors, including aprotinin, did not potentiate cat tracheal responsiveness to VIP, indicating that local enzymatic degradation is not important in inactivation of VIP in this preparation.

Using guinea-pig trachea, we have examined the effect of α-chymotrypsin on neurogenic NANC relaxations and on responses to exogenous VIP, a putative NANC transmitter in this tissue (Matsuzaki *et al.*, 1980; Ellis & Farmer, 1989). In contrast to its action in feline trachea (Altiere & Diamond, 1985), α-chymotrypsin reduced the magnitude and, more pronouncedly, the duration of the NANC response. These effects of α-chymotrypsin were prevented completely by preincubation with the peptidase inhibitor aprotinin. α-Chymotrypsin, which acts at aromatic amino acyl bonds, (Dixon *et al.*, 1979a), also abolished relaxations to VIP and PHI, its effect being prevented by aprotinin. These data suggest that the

NANC inhibitory response in guinea-pig trachea is, at least partly, peptidergic and that it may be mediated by VIP and/or PHI. This is supported further by the observation that papain, another peptidase, also inhibited the NANC response. Moreover, the effects of these peptidases are unlikely to be on effector proteins, since they are completely reversible on washout. In addition, neither peptidase affected tracheal contractions to methacholine or relaxations to isoprenaline.

Aprotinin alone did not influence responses to EFS or to exogenous VIP or PHI. This suggests that neither the NANC transmitter nor the two peptides are inactivated to any appreciable extent by endogenous peptidases such as α-chymotrypsin, trypsin, plasmin or kallikrein, each of which is inhibited by aprotinin (see references in Zynar, 1981). Conversely, papain, which acts at arginine, lysine and glycine residues (Dixon *et al.*, 1979b), also abolished responses to VIP or PHI, an action which was unaffected by aprotinin. Therefore, it is foreseeable that these peptides may be inactivated by airway enzymes that are resistant to the inhibitory effect of aprotinin.

Currently, there is relatively little known concerning the metabolic inactivation of VIP and PHI in the lungs (Bunnett, 1987; Said, 1987). It is noteworthy, however, that relaxations of trachea by VIP can be maintained for several hours (Ellis & Farmer, 1989), indicating that enzymatic degradation, at least *in vitro*, is not a major inactivating pathway for VIP. In canine lung also, there is evidence that VIP is not metabolized (Kitamura *et al.*, 1975). Conversely, relaxation of guinea-pig trachea by VIP is potentiated by phosphoramidon, a neutral peptidase ('enkephalinase') inhibitor (Liu *et al.*, 1987), and it would be interesting to determine the effect of this drug on the tracheal NANC inhibitory response. However, it is unlikely that an opioid peptide is involved, since the NANC response is unaffected by naloxone (Ellis & Farmer, 1989). Our results agree with those obtained with cat trachea (Altiere & Diamond, 1984), in that, in guinea-pig trachea, enzymatic inactivation of VIP does not seem to be an important mechanism. If VIP or PHI do play a role as NANC transmitters in the lung, they may be removed by the circulation and metabolized by blood-borne peptidases.

It is interesting that in rat gastric smooth muscle, α-chymotrypsin produces effects qualitatively similar to those found in guinea-pig trachea in the present study. DeBeurme & Lefebvre (1987) demonstrated recently that while α-chymotrypsin abolished VIP-induced gastric relaxation, it had little effect on the magnitude of the NANC response to EFS, but reduced the duration of the neurogenic response. These authors also showed that, as in the present study, α-chymotrypsin did not affect responsiveness to another mediator, in this case, noradrenaline.

The residual component of the NANC response, in the presence of the peptidases (and propranolol), is unlikely to be mediated by degradation fragments of VIP or PHI, since the activity of the exogenous peptides was abolished, even at high concentrations, by the peptidases. To our knowledge, there are no data on the nature of the degradation products of the action of α-chymotrypsin on VIP, or the biological activity of such peptide fragments. Recently, however, it was demonstrated that α-chymotrypsin-like mast cell chymases cleave VIP, at Tyr^{22}, into two fragments (Caughey *et al.*, 1988). Previous studies on the biological activity, including tracheal relaxation, of various VIP fragments suggests that neither of the resulting fragments produced by chymases would have significant biological activity (Bodanszky *et al.*, 1973; Staun-Olsen *et al.*, 1986).

Another consideration concerning the peptidase-resistant component of the NANC inhibitory response is the possibility that the enzymes, due to their size, may be partially excluded from the synaptic cleft. This is, however, unlikely for the following reasons. Firstly, α-chymotrypsin completely abolishes NANC responses, believed to be mediated by VIP, in dog gastric muscularis mucosa (Angel *et al.*, 1983). Secondly, papain and α-chymotrypsin are similar in size: papain, a single polypeptide chain consisting of 212 amino acid residues, has a molecular weight of 20,700, and α-chymotrypsin, which has 254 amino acid residues in three polypeptide chains, has a molecular weight of 23,000. Yet, these enzymes had qualitatively different effects on the NANC response. Whereas papain and α-chymotrypsin both decreased to the same extent, the maximum magnitude of the NANC response, the latter also markedly reduced the duration of the response. It seems unlikely that these differences could be explained by differential access of the enzymes to the synaptic space. The observation that α-chymotrypsin, but not papain reduced the time course of the NANC response is an interesting one for which we have no explanation at present. The observation that in the presence of α-chymotrypsin, the duration of the response is much reduced suggests that α-chymotrypsin is removing the peptidergic component more rapidly, or more completely, than papain.

If degradation products of VIP or PHI are not responsible for the peptidase-resistant component of the NANC inhibitory response, and if the peptidases employed in the present study diffuse freely to the synaptic cleft, it is possible that another, perhaps non-peptide, substance is released from NANC nerve endings. The nature of this mediator is, at present, unknown but is unlikely to be an opioid peptide, adenosine or ATP (Ellis & Farmer, 1989). Further

studies are therefore necessary to elucidate the nature of the NANC transmitters in the airway.

The authors are grateful to Dr Ron Burch for his helpful advice during this study, and in preparation of this manuscript.

References

ALTIERE, R.J. & DIAMOND, L. (1984). Relaxation of cat tracheobronchial and pulmonary arterial smooth muscle by vasoactive intestinal peptide: lack of influence of peptidase inhibitors. *Br. J. Pharmacol.*, **82**, 321–328.

ALTIERE, R.J. & DIAMOND, L. (1985). Effect of α-chymotrypsin on the non-adrenergic noncholinergic inhibitory system in cat airways. *Eur. J. Pharmacol.*, **114**, 75–78.

ANGEL, F., GO, V.L.W., SCHMALZ, P.F. & SZURSZEWSKI, J.H. (1983). Vasoactive intestinal polypeptide: a putative transmitter in the canine muscularis mucosa. *J. Physiol.*, **341**, 641–654.

BARNES, P.J. (1986). Neural control of human airways in health and disease. *Am. Rev. Respir. Dis.*, **134**, 1289–1314.

BODANSZKY, M., KLAUSNER, Y.S. & SAID, S.I. (1973). Biological activities of synthetic peptides corresponding to fragments of and to the entire sequence of the vasoactive intestinal peptide. *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 382–384.

BUNNETT, N.W. (1987). Postsecretory metabolism of peptides. *Am. Rev. Respir. Dis.*, **136**, Suppl. S27–S34.

BURNSTOCK, G. (1986). The non-adrenergic noncholinergic nervous system. *Arch. Int. Pharmacodyn.*, **280**, Suppl., 1–15.

CAUGHEY, G.H., LEIDIG, F., VIRO, N.F. & NADEL, J.A. (1988). Substance P and vasoactive intestinal peptide degradation by mast cell tryptase and chymase. *J. Pharmacol. Exp. Ther.*, **244**, 133–137.

DANIEL, E.E. (1985). Nonadrenergic, noncholinergic (NANC) neuronal interactions with smooth muscle. In *Calcium and Contractility*, ed. Grover, A.K. & Daniel, E.E., pp. 385–426. Clifton, New Jersey: Humana Press.

DE BEURME, F.A. & LEFEBVRE, R.A. (1987). Influence of α-chymotrypsin and trypsin on the non-adrenergic noncholinergic relaxation in the rat gastric fundus. *Br. J. Pharmacol.*, **91**, 171–177.

DIXON, M., WEBB, E.C., THORNE, C.J.R. & TIPTON, K.F. (1979a). In *Enzymes*, pp. 259–263. New York, San Francisco: Academic Press.

DIXON, M., WEBB, E.C., THORNE, C.J.R. & TIPTON, K.F. (1979b). In *Enzymes*, p. 306. New York, San Francisco: Academic Press.

ELLIS, J.L. & FARMER, S.G. (1989). The effects of vasoactive intestinal peptide (VIP) antagonists, and VIP and peptide histidine isoleucine antisera on non-adrenergic, non-cholinergic relaxations of tracheal smooth muscle. *Br. J. Pharmacol.*, **96**, 521–528.

ITO, Y. & TAKEDA, K. (1982). Non-adrenergic inhibitory nerves and putative transmitters in the smooth muscle of cat trachea. *J. Physiol.*, **330**, 497–511.

KARLSSON, J.-A. (1986). *In vivo* and *in vitro* studies of the non-adrenergic non-cholinergic nervous system of the guinea-pig airways. *Arch. Int. Pharmacodyn. Ther.*, **280**, Suppl., 191–207.

KITAMURA, S., YOSHIDA, D. & SAID, S.I. (1975). Vasoactive intestinal polypeptide: inactivation in liver and potentiation in lung of anesthetized dogs. *Proc. Soc. Exp. Biol. Med.*, **148**, 25–29.

LIU, L.W., SATA, T., KUBOTA, E., PAUL, S. & SAID, S.I. (1987). Airway relaxant effect of vasoactive intestinal peptide (VIP): selective potentiation by phosphoramidon, an enkephalinase inhibitor. *Am. Rev. Respir. Dis.*, **135**, A86.

LUNDBERG, J.M., FAHRENKRUG, J., HOKFELT, T., MARTLING, C.-R., LARSSON, O., TATEMOTO, K. & ANGGARD, A. (1984). Coexistence of peptide HI (PHI) and VIP in nerves regulating blood flow and bronchial smooth muscle tone in various mammals including man. *Peptides*, **5**, 593–606.

MATSUZAKI, Y., HAMASAKI, Y. & SAID, S.I. (1980). Vasoactive intestinal peptide: a possible transmitter of non-adrenergic relaxation of guinea-pig trachea. *Science*, **210**, 1252–1253.

SAID, S.I. (1987). Influence of neuropeptides on airway smooth muscle. *Am. Rev. Respir. Dis.*, **136**, Suppl. S52–S58.

STAUN-OLSEN, P., OTTESEN, B., GAMMELTOFT, S. & FAHRENKRUG, J. (1986). VIP binding sites on synaptosomes from rat cerebral cortex: structure-binding relationship. *Peptides*, **7**, Suppl. 1, 181–186.

VENUGOPALAN, C.S., O'MALLEY, N.A. & SRIKANDAKUMAR, A. (1986). Autonomic interplay in VIP-induced tracheal smooth muscle relaxation. *J. Auton. Pharmacol.*, **5**, 269–273.

ZYNAR, E.S. (1981). A rationale for the application of Trasylol as a protease inhibitor in radioimmunoassay. *Life Sci.*, **28**, 1861–1866.

(Received March 15, 1988
Revised August 14, 1988
Accepted October 19, 1988)