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MODULATION OF CHOLINERGIC NEUROTRANSMISSION BY THE PEPTIDE VIP, VIP ANTISERUM AND VIP ANTAGONISTS IN DOG AND CAT TRACHEA

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SUMMARY

1. Comparative studies on the effects of vasoactive intestinal polypeptide (VIP), commercially available VIP antiserum or VIP antagonists [Ac-Tyr^1, D-Phe^2]-GRF(1–29)-NH_2 and [4-Cl-D-Phe^6, Leu^{17}]-VIP on excitatory neuroeffector transmission in the dog and cat trachea were performed with microelectrode, double sucrose-gap, and tension recording methods.

2. VIP (10^{-11}–10^{-9} M) had no effect on the resting membrane potential or on the input resistance of the smooth muscle cells of dog and cat trachea. However, with increased concentrations ($> 10^{-8}$ M) VIP hyperpolarized the membrane and decreased the input resistance of the membrane in both tissues.

3. VIP (10^{-10}–10^{-7} M) dose-dependently reduced the amplitude of the contractions evoked through the nervous structure excited by field stimulation in the combined presence of indomethacin (10^{-5} M) and guanethidine (10^{-6} M) in the dog, and in the presence of guanethidine (10^{-6} M) in cat trachea. In parallel with actions on twitch contractions, VIP (10^{-11} –10^{-7} M) reduced the amplitude of the excitatory junction potentials (EJPs) evoked through the nervous structure excited by single pulse field stimulation in both tissues.

4. VIP (10^{-9} M) had no effect on the post-junctional response of smooth muscle cells to exogenous acetylcholine (ACh) (10^{-9}–10^{-5} M).

5. During repetitive field stimulation at the stimulus frequency of 0·033–0·1 Hz, the amplitude of the EJPs was gradually reduced, and VIP (10^{-9} M) enhanced this depression phenomenon in the dog and cat trachea.

6. EJPs also showed summation when repetitive field stimulation was applied at high frequency (20 Hz) in the dog trachea. The slope of the relationship between the relative amplitude of the EJP and number of stimuli at 20 Hz was $2{\cdot}2 \pm 0{\cdot}4$ mV/stimulation ($n = 4$) in the dog trachea. However, in the cat trachea, summation of EJPs was not prominent, giving a mean slope of $0{\cdot}6 \pm 0{\cdot}2$ mV/stimulation ($n = 6$) measured by the microelectrode method. VIP (10^{-9} M) shifted downward the relationship between the relative amplitude of the EJP and the number of stimuli at 20 Hz in both tissues.

7. Overnight incubation with VIP antiserum (10^{-6} g/ml) had little effect on the depression of the EJP in the dog and cat trachea, or the summation of the EJP

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observed in the dog trachea. However, this procedure enhanced the summation of EJPs observed in the cat trachea, and the mean slope of the relationship between relative amplitude of the EJP and number of stimuli at 20 Hz was increased from $0{\cdot}6 \pm 0{\cdot}2$ mV/stimulation ($n = 6$) to $1{\cdot}5 \pm 0{\cdot}1$ mV/stimulation ($n = 5$) when the microelectrode method was employed.

8. Overnight incubation of the cat tracheal tissue with VIP antiserum markedly reduced the amplitude of the muscle relaxation evoked by electrical field stimulation during contraction induced by 5-hydroxytryptamine (10^{-5} M) in the presence of atropine (10^{-6} M) and guanethidine (10^{-6} M).

9. The VIP antagonists [Ac-Tyr^1, D-Phe^2]-GRF(1–29)-NH_2 ($> 10^{-9}$ M) and [4-Cl-D-Phe^6, Leu^{17}]-VIP ($> 10^{-8}$ M), dose-dependently increased the amplitude of the EJP in the cat trachea when a single stimulus was applied with no change in the membrane potential and input membrane resistance. Overnight incubation of the cat tracheal tissue with [4-Cl-D-Phe^6, Leu^{17}]-VIP (10^{-7} M) enhanced the summation of EJPs evoked by repetitive field stimulation at 20 Hz. In the control cat trachea treated with Krebs solution overnight, repetitive stimulation only slightly enhanced the amplitude of the EJP but a linear relationship between the number of stimuli and the amplitude of the EJP was not observed with the double sucrose-gap method. However, after treatment of the tissue with the VIP antagonist, a linear relationship was observed, and the slope was $0{\cdot}7 \pm 0{\cdot}1$ mV/stimulation ($n = 6$).

10. The VIP antagonists, [Ac-Tyr^1, D-Phe^2]-GRF(1–29)-NH_2 (10^{-7} M) and [4-Cl-D-Phe^6, Leu^{17}]-VIP (5×10^{-7} M), showed no effect on the amplitude of the muscle relaxation evoked by electrical field stimulation during contraction induced by 5-hydroxytryptamine (5×10^{-6} M) in the presence of atropine (10^{-6} M) and guanethidine (10^{-6} M).

11. These results indicate that VIP in low concentrations has a prejunctional action inhibiting the excitatory neuroeffector transmission in addition to a direct action on the smooth muscle cells, presumably by suppressing transmitter release from the vagus nerves in dog and cat trachea. Furthermore in the cat trachea, the effects of VIP antiserum and VIP antagonists suggest that VIP may contribute to non-adrenergic non-cholinergic inhibitory responses and the endogenous VIP also inhibits ACh release from the vagus nerve when a single stimulus or repetitive stimuli are applied.

INTRODUCTION

It is generally considered that the parasympathetic nervous system in the airways involves inhibitory neurotransmitters, in addition to ACh. These are neither adrenergic nor cholinergic (NANC) in nature (see for example Barnes, 1986), but neuropeptides such as vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine (PHI) or peptide histidine methionine (PHM) are candidates as possible neurotransmitters (Matsuzaki, Hamasaki & Said, 1980; Ito & Takeda, 1982; Ellis & Farmer, 1989*a*, *b*).

VIP-immunoreactive nerves have been identified in human and animal airway smooth muscle layers (Dey, Shannon & Said, 1981; Polak & Bloom, 1982; Hakanson, Sundler, Moghimzadeh & Leander, 1983). Recently, VIP-immunoreactivity was localized to cholinergic nerves, and ultrastructural studies have demonstrated the

presence of peptidergic granules within cholinergic nerves in the airway smooth muscle (Latinen, Partanen, Hervonen & Latinen, 1985*a*). Immunocytochemical studies also showed that many VIP-immunoreactive fibres appear to be present in cholinergic nerves of the tracheal and bronchial smooth muscle layer (Lundberg, 1981; Latinen, Partanen, Hervonen, Pelto-Huikko & Latinen, 1985*b*).

VIP is known to facilitate or attenuate the releasing mechanisms of various neurohormones, neurotransmitters or glandular secretions. For example VIP facilitates the release of prolactin (Frawley & Neill, 1981) and adrenocorticotrophic hormone (Oliva, Nicosia, Spada & Giannattasio, 1982), and inhibits the release of somatostatin (Epelbaum, Tapia-Arancibia, Besson, Rotsztejn & Korden, 1979) in the central nervous system. In peripheral tissues, VIP enhances secretion from salivary glands induced by ACh (Lundberg, Hedlund & Bartfai, 1982) and inhibits neurally evoked smooth muscle contraction of rat uterus and ferret trachea, indicating presynaptic inhibitory actions of VIP on the neuroeffector transmission (Stjernquist & Owman, 1984; Sekizawa, Tamaoki, Graf & Nadel, 1988).

Thus, it seems reasonable to inquire into the possible role of VIP on excitatory cholinergic neuroeffector transmission, in addition to the direct inhibitory action on the smooth muscle cells (Ito & Takeda, 1982), since it may co-exist with ACh in the same nerves (Lundberg, 1981; Latinen *et al.* 1985*b*) and may be released with ACh (Barnes, 1986).

For this purpose, we analysed the effects of VIP, VIP antiserum and VIP antagonists [Ac-Tyr^1, D-Phe^2]-GRF(1–29)-NH_2 (Waelbroeck, Robberecht, Coy, Camus, De Neef & Christophe, 1985) and [4-Cl-D-Phe^6, Leu^{17}]-VIP (Pandol, Dharmsathaphorn, Schoeffield, Vale & Rivier, 1986) on the contractions and excitatory junction potentials (EJPs) recorded from cat and dog trachea. These two different animal tissues were used because NANC inhibitory responses to electrical field stimulation have been demonstrated in the former (Ito & Takeda, 1982), but not in the latter (Suzuki, Morita & Kuriyama, 1976; Ito & Tajima, 1981*a*).

METHODS

Adult mongrel dogs and cats of either sex, weighing 10–13 kg and 2–3 kg respectively, were anaesthetized with I.V. administration of pentobarbitone (30 mg/kg). Segments of cervical trachea were excised, and a dorsal strip of transversely running smooth muscles was separated from the cartilage. The mucosa and adventitial areolar tissue were carefully removed. The tracheal smooth muscle was cut at a width of 2·0–2·5 mm and a length of about 15 mm for the double sucrose-gap method. The preparation was bathed in a modified Krebs solution with the following ionic concentrations (mM): Na^+ 137·4, K^+ 5·9, Mg^{2+} 1·2, Ca^{2+} 2·5, Cl^- 134·0, $H_2PO_4^-$ 1·2, HCO_3^- 15·5 and glucose 11·5. The solution was aerated with 97% O_2 and 3% CO_2 and the pH was 7·3–7·4.

The double sucrose-gap method was used to record the membrane potential and tension development in the tissue. The chamber used has been described elsewhere (Ito & Tajima, 1981*a*). To produce neurogenic responses, electrical field stimulation was applied by a ring electrode, placed in the centre pool of the apparatus, using an electronic stimulator (Nihon Kohden Ltd, SEN-7103). Single and repetitive stimulation was applied with a current pulse of 50 μs in duration and about 10–20 V in strength. The voltage of the current pulse was adjusted so that an EJP of a defined amplitude was evoked by a single pulse. Drugs were dissolved in Krebs solution and applied to the tissue through the centre pool of the double sucrose-gap apparatus using a multiway tap (dead time approximately 30 s).

For intracellular recording of the membrane potential from a single cell, thin strips of tissue 10–15 mm in length, 4–5 mm in width and 0·3–0·4 mm thick were used. A conventional

microelectrode filled with 3 M-KCl (30–50 MΩ) was inserted from the outer surface of the preparation. Field stimulation was applied to the nerves through a pair of Ag–AgCl wires 3–5 mm apart and placed so that a current pulse would pass transversely across the tissue. Single and repetitive stimuli at 20 Hz were applied, with a pulse of 30–100 μs duration and 30–50 V strength

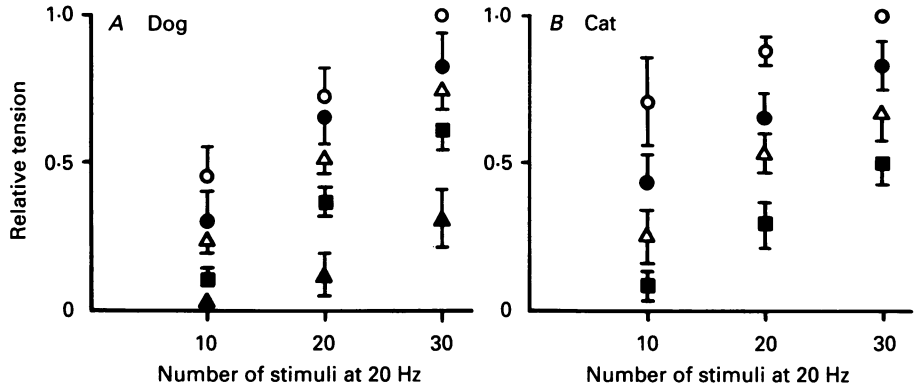

Fig. 1. Effects of various concentrations of VIP (10^{-10}–10^{-7} M) on contractions evoked by field stimulation (10, 20 and 30 stimuli at 20 Hz) of dog (*A*) and cat (*B*) tracheal tissues. The amplitude of twitch contractions evoked by 30 stimuli at 20 Hz in normal Krebs solution was defined as relative tension of 1·0. ○, control; ●, VIP 10^{-10} M; △, 10^{-9} M; ■, 10^{-8} M; ▲, 10^{-7} M. Each point is the mean value of five to nine experiments, and vertical bars represent 2 × S.D. The absolute values of tension developments ranged between 0·2 and 1·3 g.

using an electronic stimulator (Nihon Kohden Ltd, SEN-7103). The chamber in which the strips were mounted had a volume of 2 ml, and was superfused at a rate of 3 ml/min at a temperature of 35–36 °C. To avoid recording artifacts due to twitch-like contraction of the muscle tissue, the preparation was well pinned on the rubber plate in the chamber using insect pins with 100 μm diameter.

To measure the mechanical changes, the tissue was mounted in a 1 ml organ bath through which the test solution at a temperature of 35–36 °C flowed continuously at a rate of 3 ml/min. The preparation was placed vertically and one end of the strips was tied to a mechanotransducer (Nihon Kohden Ltd, RCA-5734) and the other end to a hook at the bottom of the bath by fine silk thread. The strips were set up with an initial tension of 0·1–0·2 g and mechanical activity was recorded with a pen recorder.

The following drugs were used: isoprenaline, (−)-isoprenaline, (+)-bitartrate (Nakai Chemicals)), indomethacin, 5-hydroxytryptamine (5-HT) and acetylcholine hydrochloride (Sigma), guanethidine (Tokyo Kasei), vasoactive intestinal polypeptide (VIP; Peptide Institute, Osaka), rabbit antiporcine VIP antiserum, non-immune serum from a non-immunized rabbit (Funakoshi Pharmaceuticals, Tokyo), VIP antagonists [Ac-Tyr^1, D-Phe^2]-GRF(1–29)-NH_2 and [4-Cl-D-Phe^6, Leu^{17}]-VIP (Cosmo Bio., Co. Ltd, Tokyo) and atropine sulphate (Daiichi).

Rabbit antiporcine VIP antiserum showed no cross-reactivity to gastrin, cholecystokinin or secretin (Cosmo Bio., Co. Ltd, Tokyo). Results (amplitude of contractions or EJPs) are expressed as mean ± S.D. and were analysed for statistical significance by Student's *t* test.

RESULTS

Effects of VIP on contraction of dog and cat trachea evoked by electrical field stimulation

The effects of VIP on the contractions evoked by electrical field stimulation of cholinergic nerve fibres were studied in dog and cat tracheal tissues. The dog and cat airway smooth muscle cells are innervated by both cholinergic and adrenergic nerves

(Russel, 1980) and noradrenaline released from sympathetic nerves can activate prejunctional β-adrenoceptors to inhibit cholinergic transmission (Danser, van den Ende, Lorenz, Flavahan & Vanhoutte, 1987; Ito, 1988). In the dog trachea, contractions evoked by nerve stimulation decreased progressively in amplitude,

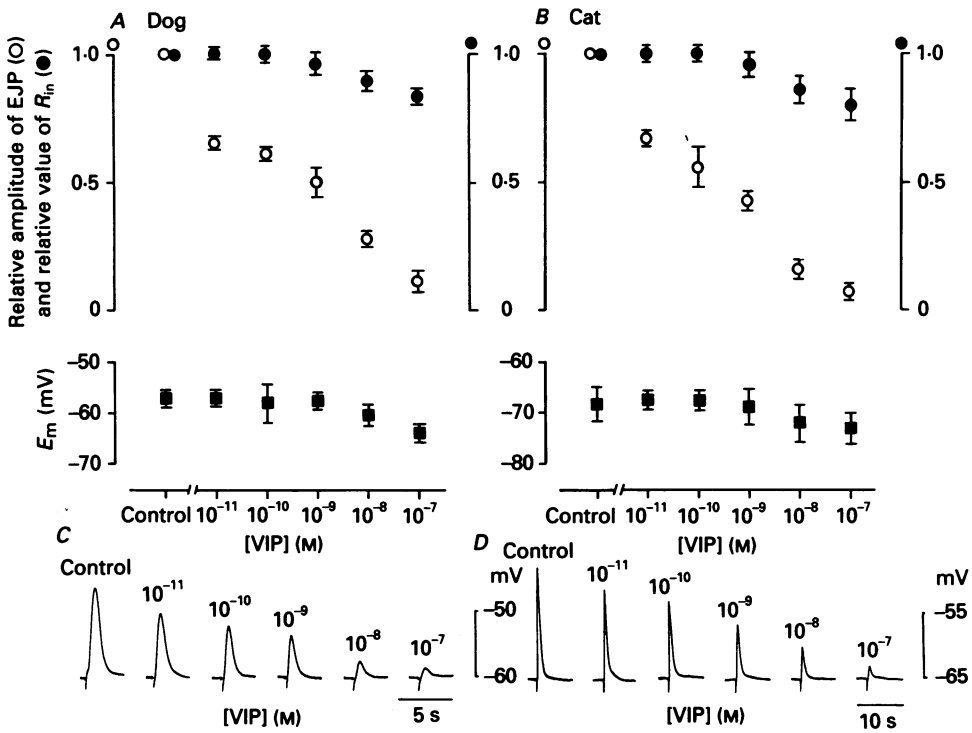

Fig. 2. Relationship between the concentration of VIP (10^{-11}–10^{-7} M) and the relative amplitude of EJP (○), relative value of input membrane resistance (R_{in}, ●) and the resting membrane potential (E_m, ■) of the smooth muscle cells in the dog (*A*) and cat (*B*) trachea. The amplitude of EJP evoked by single field stimulation in normal Krebs solution, and the amplitude of electrotonic potentials produced by square pulses in normal Krebs solution are defined as 1·0. Each point is the mean value derived from five to twenty-five experiments, vertical bars indicate 2 × S.D. Microelectrode recording of EJP and resting membrane potential was used, and electrotonic potentials were recorded by the double sucrose-gap method. Absolute values of EJP amplitude ranged between 2 and 15 mV.

because endogenous prostaglandin E compounds inhibit transmitter release from the vagus nerves (Ito & Tajima, 1981*a*, *b*; Inoue, Ito & Takeda, 1984; Walters, O'Byrne, Fabbir, Gof, Holtzman & Nadel, 1984; Inoue & Ito, 1986). Therefore the experiments on the dog trachea were carried out in the combined presence of guanethidine (10^{-6} M) and indomethacin (10^{-5} M), and on the cat trachea in the presence of guanethidine (10^{-6} M). These drugs were used to suppress the release of noradrenaline from the sympathetic nerve terminals and the synthesis of prostaglandins in the muscle tissues in response to the electrical field stimulations.

Figure 1 shows the effects of VIP (10^{-10}–10^{-7} M) on the amplitude of contractions evoked by repetitive field stimulation (10, 20 and 30 stimuli at 20 Hz), in the dog and

cat trachea. VIP (10^{-10} M) had no effects on the resting tension but did suppress the amplitude of contractions in both tissues to 0·6–0·8 times the control value. With increased concentrations, this drug dose-dependently suppressed the amplitude of contractions evoked by nerve stimulation at 20 Hz.

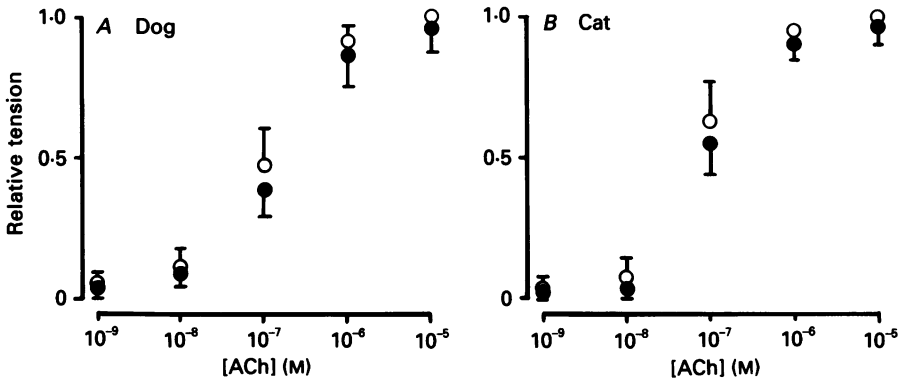

Fig. 3. Effects of VIP (10^{-9} M) on dose–response curves (ACh against tension development) of the dog (*A*) and cat (*B*) tracheal tissues. Ordinate: relative tension development where the amplitude of contracture evoked by 10^{-5} M-ACh was registered as a relative tension of 1·0. Abscissa: dose of ACh. Circles with bars indicate mean values and S.D. of four to six preparations of control (○) and after the treatment of the tissue with 10^{-9} M-VIP (●). Absolute values of tension development induced by various concentrations of ACh in the presence or absence of VIP ranged between 0·1 and 0·9 g.

Effects of VIP on the amplitude of the excitatory junction potential (EJP)

The mechanisms involved in the inhibitory effects of VIP on twitch contractions were investigated with the microelectrode and double sucrose-gap methods. With the microelectrode method, single field stimulation evoked an EJP and VIP ($> 10^{-11}$ M) significantly suppressed the EJP amplitude to $0{\cdot}65 \pm 0{\cdot}03$ times the control ($n = 6$, $P < 0{\cdot}01$) in the dog and to $0{\cdot}67 \pm 0{\cdot}02$ ($n = 7$, $P < 0{\cdot}01$) in the cat trachea with no change in the membrane potential. The double sucrose-gap method was also used to record EJPs and the following contractions, and confirmed that VIP (10^{-11} M) similarly suppressed the EJP amplitude to $0{\cdot}76 \pm 0{\cdot}10$ times the control ($n = 5$, $P < 0{\cdot}01$) in the dog and to $0{\cdot}69 \pm 0{\cdot}08$ ($n = 4$, $P < 0{\cdot}001$) in the cat trachea when a single stimulus was applied with no change in the membrane potential and the input membrane resistance (Fig. 2*C* and *D*). At higher concentrations (10^{-8}–10^{-7} M) VIP increased the resting membrane potential and reduced input membrane resistance in a dose-dependent manner in both tissues. Figure 2*A* and *B* summarizes the effects of

Fig. 4. Effects of repeated single stimuli every 30 s (*A* and *C*) and 10 s (*B* and *D*) on the amplitude of EJPs of the cat trachea in the absence (*A* and *B*) or presence (*C* and *D*) of VIP (10^{-9} M). *E* and *F* show relative changes in amplitude of the EJP during repetitive stimulation measured in dog (*E*) and cat (*F*) trachea. The double sucrose-gap method was used to record EJP. Circles and squares indicate stimulation every 30 and 10 s respectively. Filled circles and squares indicate the presence of VIP (10^{-9} M). Each point is the mean value obtained from four to six experiments. Absolute values of EJP amplitude ranged between 2 and 20 mV. Vertical bars show 2 × S.D.

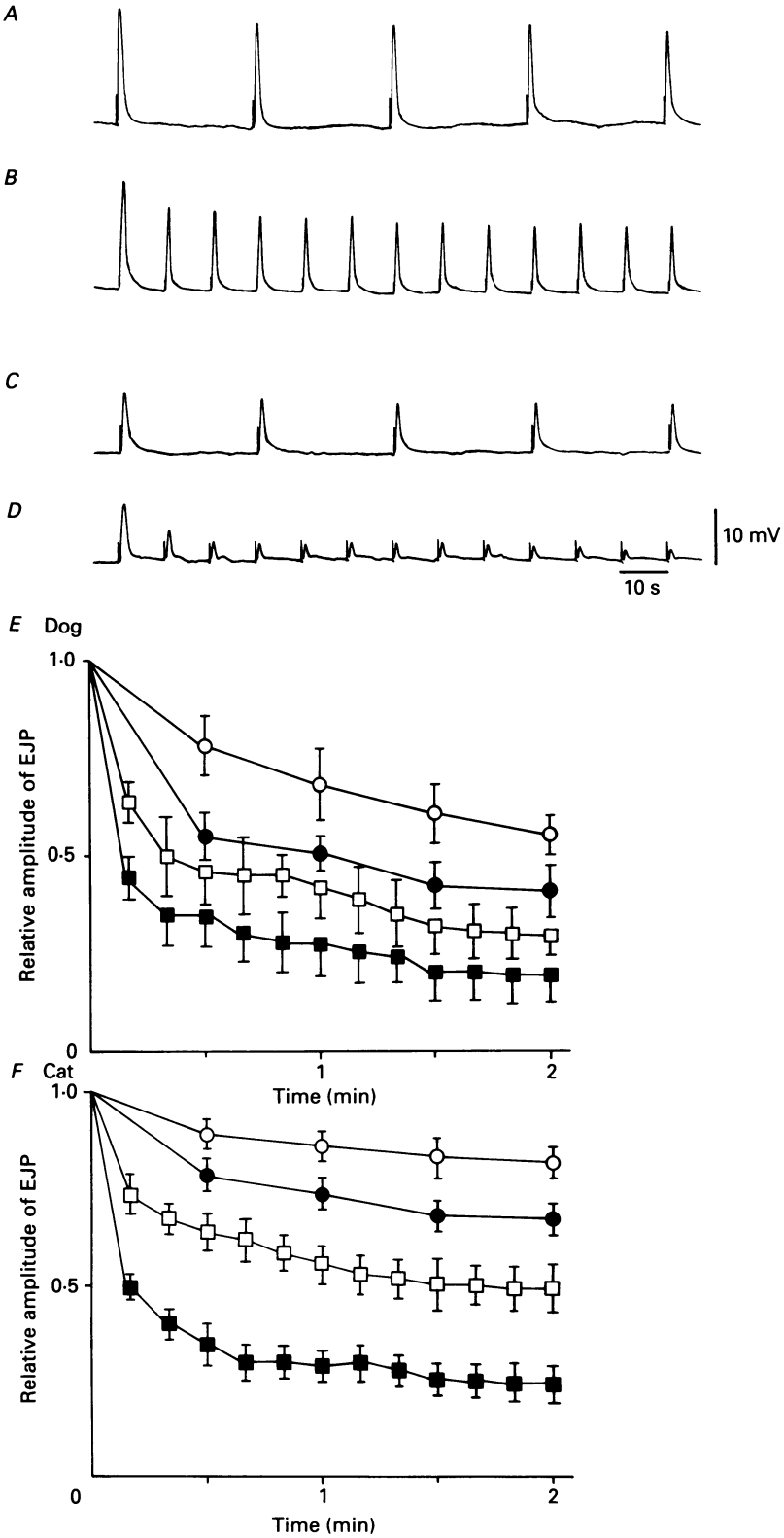

Fig. 4. For legend see facing page.

VIP on the relative amplitude of the EJP and the input membrane resistance and on the resting membrane potential of the smooth muscle cells in dog and cat tracheal tissues.

Effects of VIP on acetylcholine-induced contraction

As low concentrations of VIP (10^{-11}–10^{-9} M) reduced the amplitude of the EJP and contractions evoked by field stimulation with no change in the membrane potential or input membrane resistance, it was of interest to observe the effects of VIP on the sensitivity of smooth muscle cells to ACh. For this purpose, the tension development induced by various concentrations of ACh (10^{-9}–10^{-5} M) was examined in the presence of VIP. The relationship between the tension development and the concentration of ACh showed a sigmoidal curve, and the curve was not affected by VIP (10^{-9} M) in the dog or cat trachea, as shown in Fig. 3.

Effects of VIP on the EJP evoked by repetitive stimulation

When repetitive field stimulation at a frequency of 0·033–0·1 Hz was applied to the dog or cat trachea, a marked depression in the amplitude of EJPs was observed. Figure 4*A* and *B* shows an example of the depression of EJPs evoked by repetitive field stimulation at 0·033 and 0·1 Hz in the cat trachea with a pulse duration of 50 μs.

The effects of VIP on the amplitude of EJPs during repetitive stimulation was also observed. As shown in Fig. 4*C* and *D*, VIP (10^{-9} M) reduced the amplitude of EJPs during the field stimulation and enhanced the depression phenomenon. Fig. 4*E* and *F* shows the time course of depression in the amplitude of EJPs measured from dog and cat trachea in the presence or absence of VIP (10^{-9} M) at stimulus frequencies of 0·1 and 0·033 Hz, where the relative amplitude of EJP evoked by the first impulse was defined as 1·0 in each experiment. VIP (10^{-9} M) enhanced the depression of EJPs recorded from both the dog and cat trachea.

To analyse and further to observe the effects of VIP on the depression in the amplitude of EJPs, changes in the amplitude of a test EJP evoked at different intervals after application of a conditioning pulse were observed in the presence and absence of VIP (10^{-9} M). Figure 5*A* and *B* shows the mean value of the relative amplitude of the test EJP to the first EJP measured from several experiments, VIP enhanced the depression phenomenon of EJP amplitude in both tissues. Depression can be expressed as $D = (Y_0 - Y)/Y_0$, where Y = amplitude of the test EJPs and Y_0 = amplitude of the first conditioning EJP. When the depression of test EJPs evoked at different intervals after a conditioning EJP was plotted on a log scale against the time intervals, the depression could be expressed as a single exponential with intervals between 10 and 60 s, i.e. the depression can be expressed by the equation $D = D_0 \exp(-bt)$, where D_0 is the assumed depression where the time interval between the two stimuli is nil (equal to 0·42 and 0·52) and b is the rate constant of the decay curve (equal to 0·022 and 0·063 s^{-1} in the dog and cat, respectively). In the dog trachea, VIP had little effect on the rate constant of decay, but enhanced D_0 from 0·42 to 0·65. In the cat, on the other hand, VIP slightly decreased the rate constant and also enhanced D_0 from 0·52 to 0·74.

When repetitive stimulation at higher stimulus frequency (> 10 Hz) was applied, EJPs showed summation in the dog trachea. A linear relationship between the

number of stimuli and the amplitude of the EJP could be seen when several stimuli at high frequency (20 Hz) were applied (Ito & Tajima, 1981*b*; Ito & Yoshitomi, 1988). Therefore it is of interest to observe the effects of VIP on the relationship between the number of stimuli at 20 Hz and EJP amplitude in dog and cat trachea.

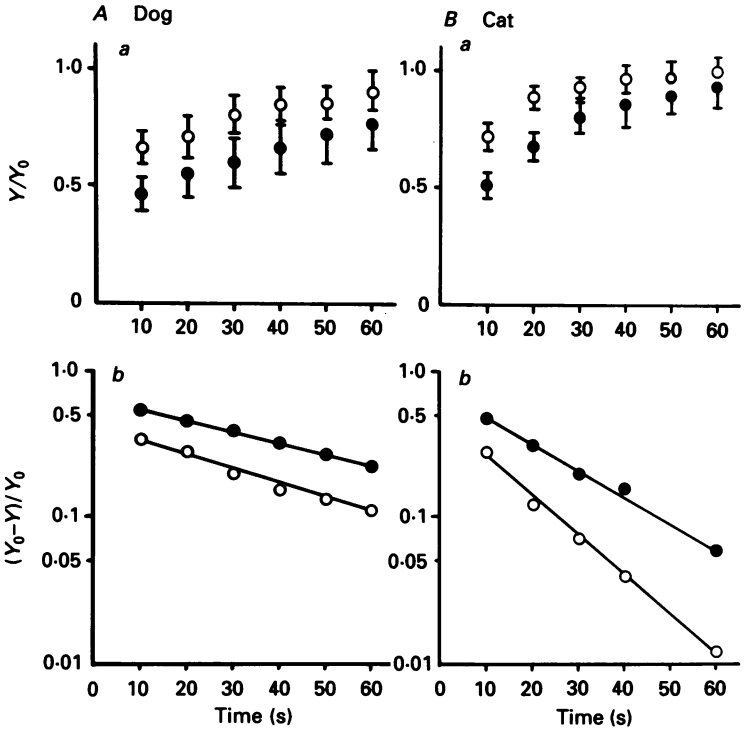

Fig. 5. Effects of a conditioning stimulus on the amplitude of the EJP evoked at various time intervals in the absence (○) or presence (●) of VIP (10^{-9} M) (*Aa* and *Ba*). *Ab* and *Bb*, the relationship between log $(Y_0-Y)/Y_0$ and the time interval between the two stimuli, where Y_0 is the amplitude of the conditioning EJP (mV) and Y is the amplitude of test EJP. Each point gives the mean amplitude from four to six experiments. The double sucrose-gap method was used to record the EJPs.

In the dog trachea the slopes of the linear relationship between the number of stimuli and the amplitude of the EJP ranged between 2·0 and 2·6 mV/stimulation (2·2±0·4 mV/stimulation, $n = 4$), and the relative amplitude of the EJP evoked by ten stimuli at 20 Hz was 22·3±4·2 times the control ($n = 6$), when the amplitude of EJP evoked by a single stimulus was defined as a relative amplitude of 1·0. VIP (10^{-9} M) suppressed the relationship to the lower value of the EJP and the slopes ranged between 1·0 and 1·6 mV/stimulation, giving a mean value of 1·4±0·3 mV/stimulation ($n = 3$).

In the cat trachea, on the other hand, repetitive stimuli at high frequency (20 Hz) only slightly enhanced the amplitude of EJPs, so that the EJP amplitude evoked by 10 stimuli at 20 Hz relative to that evoked by a single stimulus was only 2·2±2·0

times, and a linear relationship was not observed when the double sucrose-gap method was used. VIP (10^{-9} M) shifted downward the relationship between the number of stimuli and the relative amplitude of EJPs evoked by repetitive stimulation at 20 Hz (Fig. 6).

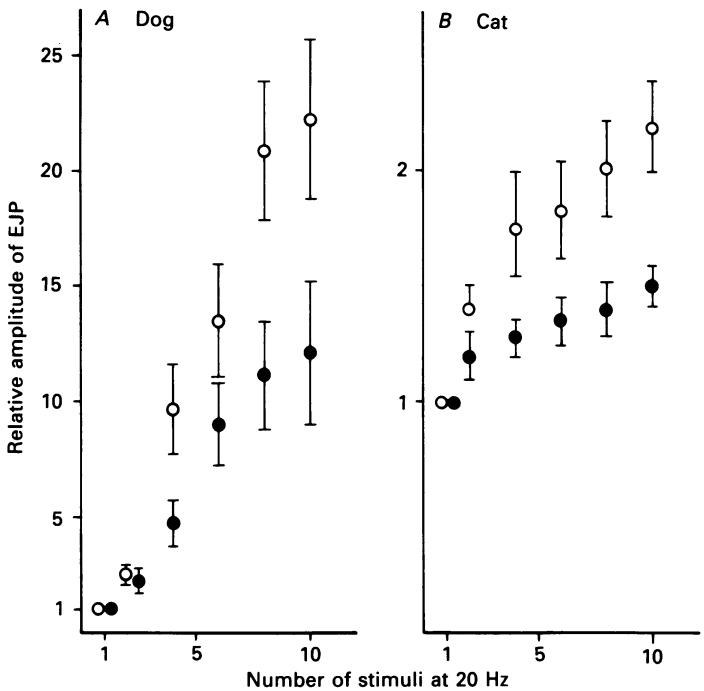

Fig. 6. Relationship between the amplitude of the EJP and the number of stimuli at 20 Hz recorded from dog (*A*) and cat (*B*) trachea in the presence (●) and absence (○) of VIP (10^{-9} M). Each point is the mean value of five to seven experiments, and the vertical bars indicate 2 × S.D. Absolute values of EJP amplitude ranged between 2 and 50 mV.

Effects of VIP antiserum on the amplitude of the EJP evoked by repetitive field stimulation at low and high frequencies

In an attempt to investigate the effects of endogenous VIP on the excitatory neuroeffector transmission in the dog and cat trachea, we used commercially available VIP antiserum and non-immunized rabbit serum as a control.

We observed the effects of repetitive field stimulation on the amplitude of EJPs at low and high frequencies after the treatment of the dog and cat tracheal tissues with VIP antiserum (10^{-6} g/ml) overnight.

Figure 7*Aa* and *Ba* shows relative changes in the amplitude of the test EJPs evoked at different time intervals (10–60 s) after application of conditioning pulses. The conditioning EJP was registered as a relative amplitude of 1·0. Treatment with VIP antiserum did not affect the relationship between the test EJP and time intervals in the dog trachea. In the cat trachea, after treatment with VIP antiserum, D_0 was not affected (before 0·60 and after 0·65) but the rate constant of the depression (b) was increased from 0·024 to 0·038 s^{-1}. Figure 7*Ab* and *Bb* shows the relationship between log $(Y_0 - Y)/Y_0$ and time interval between the two stimuli.

To elucidate differences in the nature of EJPs observed in control and VIP antiserum-treated tracheal muscle tissues, repetitive stimulation at high frequency was applied. Figure 8 shows the relationship between the number of stimuli at 20 Hz and the amplitude of EJPs. Here the amplitude of the EJP evoked by a single

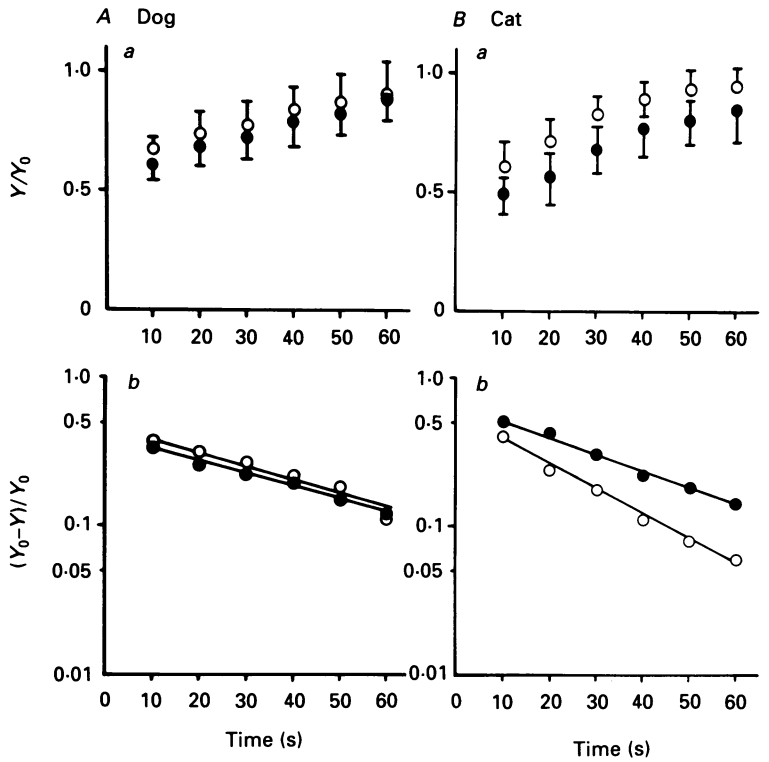

Fig. 7. Effects of incubation with VIP antiserum (10^{-6} g/ml) and non-immune serum (10^{-6} g/ml) on the depression of EJPs observed in the dog and cat trachea with the double sucrose-gap method. *Aa* and *Ba*: mean amplitude of the test EJP at various intervals after a conditioning stimuli measured in the dog and cat trachea. *Ab* and *Bb*: the relationship between log $(Y_0-Y)/Y_0$ and time interval between the two stimuli. ○, the control (non-immune serum); ●, following incubation with VIP antiserum. Each point is the mean value of four to seven experiments.

stimulus was defined as 1·0. In the dog trachea, VIP antiserum showed no effects on the relationship between the amplitude of the EJP and the number of stimuli at 20 Hz, and the slopes of the linear relationship were 2·4 (control) and 2·3 mV/stimulation (Fig. 8*A*). In the cat trachea, on the other hand, VIP antiserum markedly enhanced the amplitude of EJP evoked by repetitive field stimulation. In the control cat trachea or in cat trachea treated with normal rabbit serum (10^{-6} g/ml), repetitive stimulation only slightly enhanced the amplitude of the EJP and a linear relationship between the number of stimuli and the amplitude of the EJP was not observed with the double sucrose-gap method (Fig. 8*B*). However, after treatment of the tissue with VIP antiserum, a linear relationship was observed, and the slope was $1{\cdot}1 \pm 0{\cdot}4$ mV/stimulation ($n = 4$). Thus, the summation was enhanced in cat tracheal tissues treated with VIP antiserum.

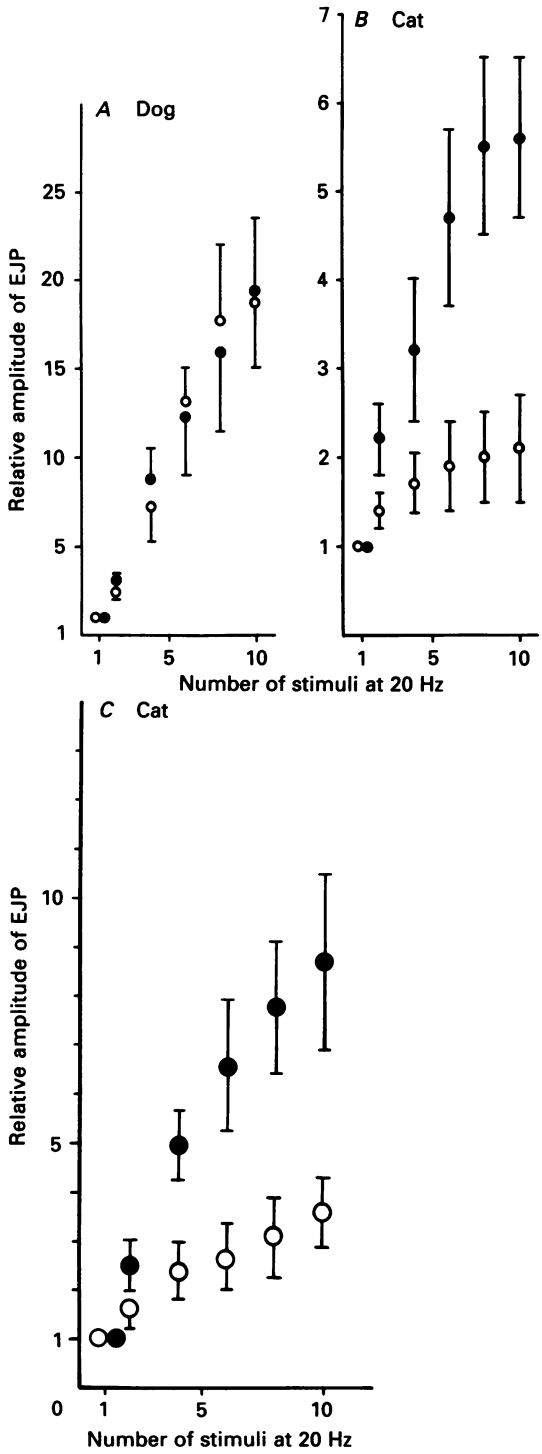

Fig. 8. For legend see facing page.

To confirm the above observation made with the double sucrose-gap method on the cat trachea, we also employed the microelectrode technique. The mean values of the resting membrane potential of smooth muscle cells of the cat trachea after treatment with VIP antiserum or normal rabbit serum overnight were $-54{\cdot}6 \pm 5{\cdot}1$ mV ($n = 15$) and $-58{\cdot}0 \pm 2{\cdot}8$ mV ($n = 25$). Thus the treatment of the tissue depolarized the membrane (in untreated muscle the mean value was $-68{\cdot}5 \pm 4$ mV ($n = 20$)). Figure 8*C* shows the relationship between the amplitude of EJP and number of stimuli at 20 Hz measured by the microelectrode method, using cat tracheal tissue. In the cat trachea the slope of the relationship ranged from 0·4 to 0·7 mV/stimulation in the control (treated with normal rabbit serum) and 1·2 to 1·6 mV/stimulation in the trachea treated with VIP antiserum, giving the mean values of $0{\cdot}6 \pm 0{\cdot}2$ mV/stimulation ($n = 6$) and $1{\cdot}5 \pm 0{\cdot}1$ mV/stimulation ($n = 5$) respectively. This confirms that VIP antiserum enhances the summation process of EJPs in the cat trachea.

Effects of VIP antiserum on the amplitude of muscle relaxation of the cat evoked by field stimulation

In the cat trachea, electrical field stimulation evoked muscle relaxation in the presence of atropine (10^{-6} M) and guanethidine (10^{-6} M). The degree of relaxation was greater, the greater the number of stimuli. However, the amplitude of the relaxation was relatively small and inconsistent (Ito & Takeda, 1982). Electrical field stimulation applied during contraction evoked by 5-hydroxytryptamine (5-HT, 5×10^{-6} M) in the presence of atropine (10^{-6} M) and guanethidine (10^{-6} M) consistently produced phasic relaxation, and when the number of stimuli was increased in a stepwise manner at a constant stimulus intensity and frequency (20 Hz), the amplitude of the relaxation was increased proportionally (Fig. 9*A* and *C*).

Treatment of the cat tracheal tissues with VIP antiserum (10^{-6} g/ml) overnight markedly reduced the amplitude of the muscle relaxation (Fig. 9*B*). Figure 9*C* shows the relative amplitude of the muscle relaxation and number of stimuli at 20 Hz, where the amplitude of muscle relaxation evoked by isoprenaline (10^{-8} M) in the presence of 5-HT (5×10^{-6} M), atropine (10^{-6} M) and guanethidine (10^{-6} M) was taken as a relative relaxation of 1·0 (Fig. 9*A* and *B*). VIP antiserum markedly reduced the amplitude, but did not abolish the muscle relaxation, confirming the previous observations made with guinea-pig trachea (Ellis & Farmer, 1989*a*).

Effects of VIP antagonists on the amplitude of EJPs evoked by single and repetitive field stimulation

Further to investigate the effects of endogenous VIP on the excitatory neuroeffector transmission in the cat trachea, we also used VIP antagonists [Ac-Tyr[1], D-Phe[2]]-GRF(1–29)-NH_2 and [4-Cl-D-Phe[6], Leu[17]]-VIP.

Fig. 8. Effects of incubation with antiserum to VIP (10^{-6} g/ml) on the relationship between the relative amplitude of the EJP and number of stimuli at 20 Hz in the dog (*A*) and cat (*B* and *C*) trachea. The EJPs were recorded with the double sucrose-gap (*A* and *B*) or microelectrode method (*C*). ○, the control (non-immune serum, 10^{-6} g/ml); ●, after treatment with VIP antiserum. Each point is the mean value derived from five to seven experiments. Absolute values of EJP ranged between 2 and 50 mV.

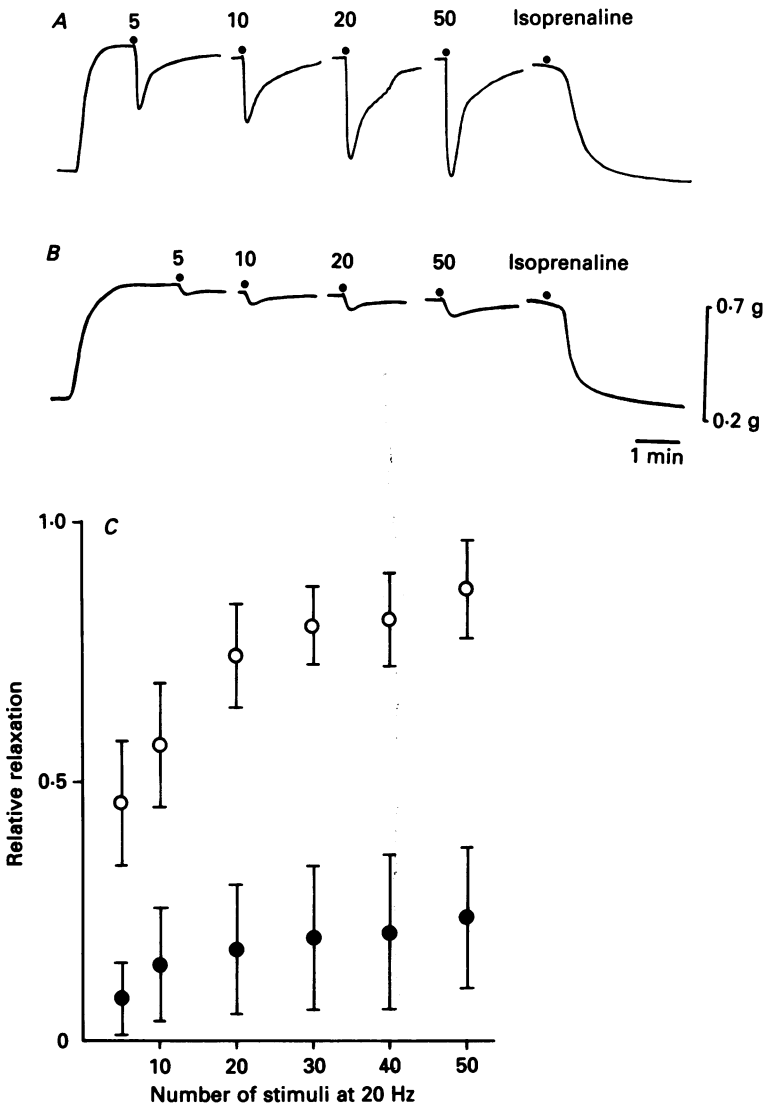

Fig. 9. Effect of incubation with antiserum to VIP (10^{-6} g/ml) and non-immune serum (10^{-6} g/ml) on the muscle relaxation of the cat trachea induced by electrical field stimulation and isoprenaline (10^{-8} M) in the presence of 5-hydroxytryptamine (5×10^{-6} M), atropine (10^{-6} M) and guanethidine (10^{-6} M). *A*: control. *B*: after treatment with VIP antiserum. Repetitive field stimuli (5, 10, 20, 30, 40 and 50 stimuli) at 20 Hz were applied during the contracture evoked by 5-hydroxytryptamine. *C*, relationship between the relative amplitude of the muscle relaxation and number of stimuli at 20 Hz, where the muscle relaxation evoked by 10^{-8} M isoprenaline is taken as a relative relaxation of 1·0. ○, the control; ●, after treatment with VIP antiserum. Each point is the mean value derived from four to six experiments. Absolute values of tension development evoked by 5-HT ranged between 0·5 and 1·1 g and the amplitude of muscle relaxation ranged between 0·07 and 1·0 g. Vertical bars are 2 × S.D.

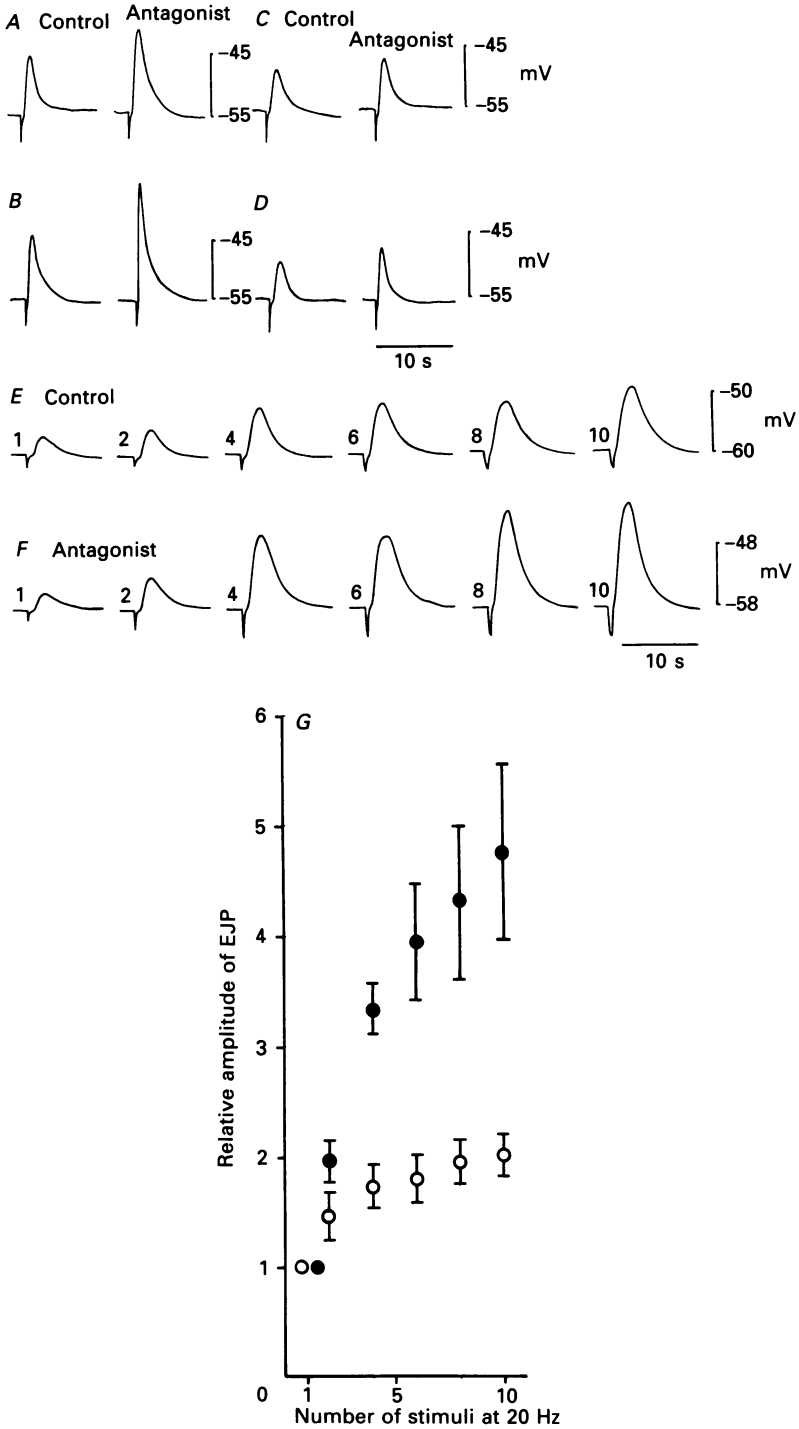

Fig. 10. Effects of VIP antagonists [Ac-Tyr^1, D-Phe^2]-GRF(1–29)-NH_2 and [4-Cl-D-Phe^6, Leu^{17}]-VIP on the amplitude of EJP evoked by a single or repetitive field stimulation and recorded with double sucrose-gap method, in the cat trachea. Effects of [Ac-Tyr^1, D-Phe^2]-GRF(1–29)-NH_2 (10^{-9} and 10^{-8} M, *A* and *B*) and [4-Cl-D-Phe^6, Leu^{17}]-VIP (10^{-8} and

Figure 10*A*, *B*, *C* and *D* shows the effects of both VIP antagonists on the amplitude of the EJP evoked by a single field stimulation. [Ac-Tyr^1, D-Phe^2]-GRF(1–29)-NH_2 (10^{-9} and 10^{-8} M) and [4-Cl-D-Phe^6, Leu^{17}]-VIP (10^{-8} and 10^{-7} M), significantly increased the EJP amplitude to $1{\cdot}61 \pm 0{\cdot}21$ times ($n = 5$, $P < 0{\cdot}01$) and $1{\cdot}86 \pm 0{\cdot}37$ times the control ($n = 5$, $P < 0{\cdot}01$), or to $1{\cdot}34 \pm 0{\cdot}07$ times ($n = 5$, $P < 0{\cdot}01$) and $1{\cdot}56 \pm 0{\cdot}25$ times the control value ($n = 5$, $P < 0{\cdot}01$), respectively. The mean values of the resting membrane potential of smooth muscle cells of the cat trachea after treatment with the two different VIP antagonists (10^{-7} M) were $-66{\cdot}2 \pm 2{\cdot}3$ mV ($n = 20$) and $-65{\cdot}5 \pm 1{\cdot}5$ mV ($n = 20$) (mean values before treatment were $-68{\cdot}2 \pm 3$ mV ($n = 15$) or $-67{\cdot}2 \pm 4$ mV ($n = 10$)), respectively. Thus the treatment of the tissues had no effect on the resting membrane potential. Furthermore the input membrane resistance of the smooth muscle cells measured by the double sucrose-gap method was not affected by these VIP antagonists (10^{-7} M).

One to two hours after the treatment of the cat tracheal tissues, both agents showed no effect on the relationship between the amplitude of the EJP and the number of stimuli at 20 Hz (data not shown).

Overnight incubation of the cat tracheal tissue with [Ac-Tyr^1, D-Phe^2]-GRF(1–29)-NH_2 ($> 10^{-7}$ M), however, markedly enhanced the amplitude of EJP evoked by repetitive field stimulation (Fig. 10*E* and *F*). In the control cat trachea treated with Krebs solution overnight, repetitive stimulation only slightly enhanced the amplitude of the EJP and a linear relationship between the number of stimuli and the amplitude of the EJP was not observed (Fig. 10*G*). After treatment of the tissue with [4-Cl-D-Phe^6,Leu^{17}]-VIP (10^{-7} M), a linear relationship was observed, and the slope was $0{\cdot}7 \pm 0{\cdot}1$ mV/stimulation ($n = 6$), indicating the summation was enhanced by the VIP antagonist as in the case of treatment with VIP antiserum (Fig. 10*G*).

Effects of VIP antagonists on the amplitude of muscle relaxation of the cat evoked by field stimulation

In the cat trachea, as mentioned above, electrical field stimulation applied during contraction evoked by 5-HT (5×10^{-6} M) in the presence of atropine (10^{-6} M) and guanethidine (10^{-6} M) produced a phasic relaxation, and the amplitude of the contraction was increased proportionally to the number of stimuli. Thus, it was of interest to observe the effects of VIP antagonists on the muscle relaxation observed in the cat trachea.

Both VIP antagonists, [Ac-Tyr^1, D-Phe^2]-GRF(1–29)-NH_2 (10^{-7} M) and [4-Cl-D-Phe^6, Leu^{17}]-VIP (5×10^{-7} M), altered neither the magnitude nor the duration of phasic relaxation evoked by field stimulation during the contraction evoked by 5-HT (5×10^{-6} M) (Fig. 11*A* and *B*). Figure 11*C* and *D* shows the relative amplitude of

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10^{-7} M. *C* and *D*) on the amplitude of EJP evoked by a single stimulus. After the application, both antagonists enhanced the amplitude of EJP within 5–10 min. *E* and *F*: effects of incubation with Krebs solution (*E*) or [4-Cl-D-Phe^6, Leu^{17}]-VIP (10^{-7} M, *F*) overnight, on the amplitude of EJP evoked by repetitive field stimulation at 20 Hz. *G*: effects of incubation with [4-Cl-D-Phe^6, Leu^{17}]-VIP (10^{-7} M) on the relationship between the relative amplitude of the EJP and the number of stimuli at 20 Hz in the cat trachea. Each point is the mean value derived from five to six experiments, and vertical bars are $2 \times$ S.D. ○, the control (Krebs solution); ●, after treatment with the VIP antagonist. EJP amplitude ranged between 5 and 30 mV throughout the experiments.

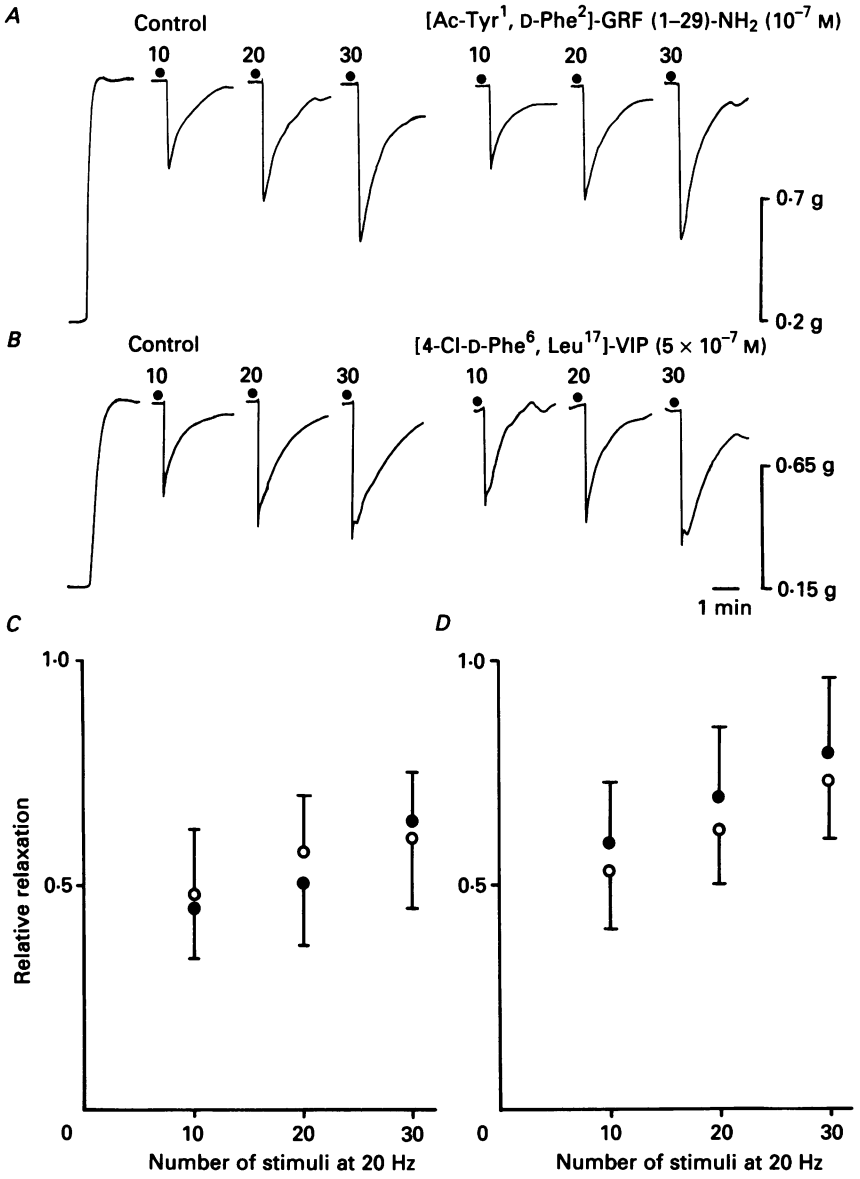

Fig. 11. *A* and *B*: effects of VIP antagonists, [Ac-Tyr¹, D-Phe²]-GRF(1–29)-NH_2 (10^{-7} M, *A*) and [4-Cl-D-Phe⁶, Leu¹⁷]-VIP (5×10^{-7} M, *B*), on the muscle relaxation of the cat trachea induced by electrical field stimulation in the presence of 5-HT (5×10^{-6} M), atropine (10^{-6} M) and guanethidine (10^{-6} M). Repetitive field stimuli (10, 20 and 30 stimuli at 20 Hz) were applied during the contraction evoked by 5-HT. *C* and *D*: relationship between the relative amplitude of muscle relaxation and number of stimuli at 20 Hz, where the muscle relaxation evoked by 10^{-8} M-isoprenaline was taken as a relative relaxation of 1·0. Open and filled circles indicate before and after treatment with [Ac-Tyr¹, D-Phe²]-GRF(1–29)-NH_2 (10^{-7} M, *C*) or [4-Cl-D-Phe⁶, Leu¹⁷]-VIP (5×10^{-7} M), *D*). Vertical bars denote S.D. Each point is the mean value derived from five to six experiments. Absolute value of tension development evoked by 5-HT ranged between 0·7 and 1·9 g and the amplitude of muscle relaxation between 0·05 and 1·6 g.

muscle relaxation and number of stimuli at 20 Hz in the presence or absence of VIP antagonists, where the amplitude of muscle relaxation evoked by isoprenaline (10^{-8} M) in the presence of 5-HT (5×10^{-6} M), atropine (10^{-6} M) and guanethidine (10^{-6} M) was taken as a relative relaxation of 1·0.

DISCUSSION

The results presented here show that low concentrations of VIP (10^{-11}–10^{-9} M) act prejunctionally to inhibit excitatory neuroeffector transmission, presumably by suppressing ACh release from the vagus nerves in the dog and cat trachea, in addition to the well-documented direct inhibitory actions on the airway smooth muscle cells (Coburn & Tomita, 1973; Diamond & O'Donnell, 1980; Ito & Takeda, 1982; Altiere & Diamond, 1984; Ellis & Farmer, 1989*a*, *b*), since low concentrations of VIP significantly suppressed the amplitude of the EJP and contractions evoked by nerve stimulation without changing the resting membrane potential, input membrane resistance or ACh-sensitivity of the smooth muscle cells. In ferret trachea, it was reported that VIP has a dual effect on cholinergic neurotransmission at different concentrations (facilitation up to 10^{-9} M VIP and inhibition at 10^{-8} and 2×10^{-7} M VIP), probably through a specific VIP receptor (Sekizawa *et al.* 1988). The present observations provided further evidence that VIP acts presynaptically. Enhancement of the depression of EJPs by VIP, and inhibitory effects of VIP on the summation of EJPs observed both in the dog and cat tracheal tissues, also support the presynaptic actions of VIP on the cholinergic nerves since low concentrations of VIP showed no effects on the electrical membrane properties of the tracheal smooth muscle cells.

One of the most striking differences in the nature of EJPs observed in the dog and cat tracheal tissues is the summation phenomenon. When several stimuli at 20 Hz were applied, a linear relationship between the number of stimuli and the amplitude of the EJP was observed and slope of the linear relationship between the number of stimuli and the amplitude of EJP was 2·4 mV/stimulation in the dog confirming previous observations (Ito & Tajima, 1981*a*; Ito & Yoshitomi, 1988), but this was not observed in the cat trachea. Concomitantly, the muscle relaxation due to activation of NANC inhibitory nerves was observed in the cat (Ito & Takeda, 1982; Altiere & Diamond, 1984) but not in the dog trachea (Suzuki *et al.* 1976; Ito & Tajima, 1981*a*), although immunoreactive neurones to VIP were found in both tissues (Dey *et al.* 1981). After treatment with VIP antiserum, muscle relaxation due to activation of NANC nerves was markedly reduced and EJPs recorded from the cat trachea showed summation in response to repetitive field stimulation at 20 Hz, giving mean slopes of $1{\cdot}1 \pm 0{\cdot}4$ mV/stimulation ($n = 4$) and $1{\cdot}5 \pm 0{\cdot}1$ mV/stimulation ($n = 5$) measured by double sucrose-gap and microelectrode methods, respectively. Furthermore, the treatment of cat tracheal tissues with VIP antagonists, [Ac-Tyr^1, D-Phe^2]-GRF(1–29)-NH_2 and [4-Cl-D-Phe^6, Leu^{17}]-VIP, increased the amplitude of the EJP within 5–10 min, and overnight incubation with [4-Cl-D-Phe^6, Leu^{17}]-VIP markedly enhanced the summation phenomenon of EJPs of the cat trachea evoked by repetitive field stimulations. These results indicate that the summation of EJPs is partly of prejunctional origin, and that endogenous VIP may inhibit ACh release from the vagus nerve when a single stimulus or repetitive stimuli are applied.

The chemical transmitters associated with lung NANC inhibition have not yet

been identified, but recent studies have suggested that purines are unlikely as neurotransmitter candidates (Ito & Takeda, 1982; Irvin, Martin & Macklem, 1982; Ellis & Farmer, 1989*a*). Evidence suggest that peptides, in particular VIP, may serve as the neurotransmitter for the following reasons: (1) VIP relaxes bronchial smooth muscle of the cat after treatment with adrenergic and cholinergic blocking agents (Altiere & Diamond, 1984), (2) studies with indirect immunofluorescence technique have demonstrated the presence of immunoreactive neurones to VIP within glands and smooth muscle of airways and within the wall of pulmonary and bronchial blood vessels in the lungs of cat and other animals (Dey *et al.* 1981), (3) VIP-like substances have been shown to be released from the isolated guinea-pig trachea in response to electrical field stimulation and the amount released correlated with the degree of relaxation (Matsuzaki *et al.* 1980), (4) release of VIP-like substances is abolished by tetrodotoxin, which also blocks the relaxation (Matsuzaki *et al.* 1980), and (5) preliminary incubation with VIP antiserum reduces tracheal relaxation in response to field stimulation (Ellis & Farmer, 1989*a*). However, the VIP antagonists, [Ac-Tyr^1, D-Phe^2]-GRF(1–29)-NH_2 and [4-Cl-D-Phe^6, Leu^{17}]-VIP, which had been found to have a competitive action against VIP in intestinal and pancreatic cells (Waelbroeck *et al.* 1985; Pandol *et al.* 1986), had no effect on the NANC relaxation in the guinea-pig (Ellis & Farmer, 1989*a*) and cat trachea (present observations). Furthermore, these agents were without effect on the responsiveness of the guinea-pig trachea to exogenous VIP or PHI (Ellis & Farmer, 1989*a*). These observations might indicate that VIP is not a NANC transmitter in the guinea-pig or cat trachea, or that the VIP receptors mediating tracheal relaxation are different from those mediating release of pancreatic amylase or colonic epithelial Cl^- secretion (Pandol *et al.* 1986). In the present experiments, both antagonists enhanced the amplitude of the EJP without affecting the electrical membrane properties of the smooth muscle cells, and [4-Cl-D-Phe^6, Leu^{17}]-VIP enhanced the summation phenomenon of EJPs in the cat trachea, indicating that the VIP receptors distributed in the vagus nerve terminals are different from those mediating the NANC relaxation in the airway smooth muscle cells.

The question of whether VIP is the neurotransmitter of NANC nerves in the cat airways has yet to be answered. However, if VIP does serve as a neurotransmitter, it may play an important role in inhibiting the transmitter release from the vagus nerve terminal in addition to the well-documented direct inhibitory actions on the smooth muscle cells. VIP and ACh may co-exist in the same nerves (Lundberg, 1981; Dey *et al.* 1981) and VIP may be released as a co-transmitter (Barnes, 1986). VIP, when released from the vagus nerve terminals, directly relaxes the airway smooth muscle cells without affecting the electrical membrane properties of the smooth muscle cells (Ito & Takeda, 1982) and probably inhibits the release of ACh from the nerve, thus playing a 'double braking' role in bronchoconstriction. This is in sharp contrast to the observation made in the submandibular glands of cat, where it was shown that VIP-like immunoreactivity is also present in acetylcholinesterase-rich neurones innervating exocrine gland tissues, and that ACh produces mainly secretion by a muscarinic action and VIP causes mainly vasodilatation, thus the co-existing substances co-operate in the salivation process. Thus the physiological significance of co-existing transmitters may be different from one tissue to another.

Treatment of the tissues with VIP antiserum enhanced the summation of EJPs in

the cat trachea, but had no effects on the depression of the EJP observed both in the dog and cat tracheal tissues. This observation indicates that the depression of EJPs in the dog and cat trachea is not due to VIP released from the vagus, but that other substances may contribute to this depression. Co-existence of PHI or PHM and VIP in nerves regulating bronchial smooth muscle has already been demonstrated (Lundberg, Fahrenkrug, Hökfelt, Martling, Larsson, Tatemoto & Anggard, 1984).

Lung tissues obtained at autopsy or lobectomy from subjects, with or without asthma, have been examined for immunoreactivity to VIP. The specimens from asthmatic patients did not show immunoreactivity to VIP, although more than 90% of the preparations examined from the lungs of patients without asthma showed immunoreactivity to VIP (Ollerenshaw, Jarvis, Woolcock, Sullivan & Scheibner, 1989). The absence of nerve fibres immunoreactive to VIP in the group with asthma is unlikely to be related to the persistence of endopeptidase activity after the collection of tissues, since substance P, which would also be subject to peptidase degradation, was present in these specimens. From these observations, it was concluded that in patients with asthma there is a loss of VIP from pulmonary nerve fibres and this loss may diminish neurologically mediated bronchodilatation.

Mechanisms involved in ACh release from the vagus nerve are widely modulated by various agents, for example by ACh, catecholamines, neuropeptides, and autacoids such as histamine, serotonin and prostaglandins (see for example Ito, 1990). Prejunctional inhibition of the release of ACh from vagal nerves has been noted with ACh, noradrenaline, prostaglandins and VIP, whereas mediators such as histamine, serotonin and substance P enhanced the release of ACh by prejunctional mechanisms. ACh is one of the most potent agents causing bronchoconstriction (Ito & Itoh, 1984; Inoue & Ito, 1985; Nadel, Barnes & Holtzman, 1986), and the parasympathetic nerves are the dominant bronchoconstrictor neural pathways. Such being the case, the overactivity of cholinergic mechanisms may contribute to airway obstruction and bronchial hyper-responsiveness (Nadel *et al.* 1986), and modulatory mechanisms may play an important role related to cholinergic bronchoconstriction.

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