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Involvement of vasoactive intestinal polypeptide in nicotine-induced relaxation of the rat gastric fundus

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1 Nicotine-induced relaxation and release of vasoactive intestinal polypeptide (VIP)- and peptide histidine isoleucine (PHI)-like immunoreactivity (LI) were measured in longitudinal muscle strips from the rat gastric fundus.

2 Under non-cholinergic conditions (0.3 μM atropine), nicotine (3–300 μM) produced concentration-dependent relaxations of the 5-hydroxytryptamine (3 μM)-precontracted strips. Under non-adrenergic non-cholinergic (NANC) conditions (0.3 μM atropine + 1 μM phentolamine + 1 μM nadolol), relaxations induced by sub-maximal nicotine concentrations (10 and 30 μM) were significantly smaller, while that produced by the highest concentration used (300 μM) was similar to that seen under non-cholinergic conditions.

3 Re-exposure to the same nicotine concentration 1 h later induced smaller relaxations, indicating desensitization. The reductions seen in the second responses were proportional to the concentration used.

4 Under non-cholinergic conditions, the relaxant response to 30 μM nicotine was abolished by hexamethonium (100 μM) and significantly reduced by tetrodotoxin (TTX, 3 μM). The TTX-resistant component was not observed under NANC conditions.

5 NANC relaxation induced by 30 μM nicotine was significantly reduced by a specific anti-VIP serum (approximately 35% less than that seen with normal rabbit serum).

6 Nicotine (30–300 μM) caused significant, concentration-dependent increases in the outflow of VIP- and PHI-LI from the strips; these effects were also diminished with re-exposure. The increases in both types of immunoreactivity evoked by nicotine (300 μM) were abolished by hexamethonium (300 μM), TTX (3 μM) and a calcium-free medium.

7 These findings indicate that VIP and possibly PHI are involved in NANC relaxation of the rat gastric fundus induced by nicotine.

Keywords: Nicotine; non-adrenergic non-cholinergic (NANC) relaxation; rat gastric fundus; vasoactive intestinal polypeptide (VIP); peptide histidine isoleucine (PHI)

Introduction

Receptive and adaptive relaxations of the proximal third of the stomach are physiological reflexes that occur respectively during and after food intake (Abrahamsson, 1986). They allow the stomach to hold large amounts of food with small increases in intraluminal pressure. Receptive relaxation is stimulated during swallowing by the pressure of the food bolus on mechanoreceptors in the pharynx and oesophagus. The post-ganglionic inhibitory neurones in the efferent chain of this reflex arc are intramural and non-adrenergic. They are activated by acetylcholine released by preganglionic vagal neurones, which binds to nicotinic receptors located on their bodies. Adaptive relaxation, in contrast, occurs when the food reaches the stomach and appears to be elicited by local activation of intramural pathways (Desai *et al*., 1991).

The most probable neurotransmitters of non-adrenergic non-cholinergic (NANC) relaxation of the proximal third of the stomach are vasoactive intestinal polypeptide (VIP) and/or nitric oxide (NO) (or a substance that releases it). The apparent roles of these putative transmitters vary somewhat from species to species and also seem to depend on the characteristics of the stimulus used to elicit the response. NO seems to play a predominant role in the stomachs of guinea-pigs (Lefebvre *et al*., 1992; Meulemans *et al*., 1993; Desai *et al*., 1994), pigs (Lefebvre *et al*., 1995) and mice (Yano *et al*., 1995), while both are involved in the gastric smooth muscle inhibition seen in rats (Li & Rand, 1990; D'Amato *et al*., 1992a), ferrets (Grundy *et al*., 1993) and cats (Barbier & Lefebvre, 1993); conflicting results have been obtained in dogs (Ito *et al*., 1988; Meulemans *et al*., 1995).

In rat gastric fundus strips, NANC relaxation produced by *in vitro* electrical field stimulation (EFS) is attenuated by L-arginine analogues (Li & Rand, 1990; Boeckxstaens *et al*., 1991; 1992; D'Amato *et al*., 1992a; Currò *et al*., 1996), which inhibit NO synthase (the enzyme that converts L-arginine into NO and L-citrulline), peptidases and anti-VIP sera (De Beurme & Lefebvre, 1987; 1988; Kamata *et al*., 1988; Li & Rand, 1990; Boeckxstaens *et al*., 1992; D'Amato *et al*., 1992a). The latter findings indicate that this response includes a peptidergic component that is probably mediated by VIP and possibly peptide histidine isoleucine (PHI), which is co-synthesized and co-released with it. The peptidergic component appears to be restricted to responses induced by high-frequency EFS (D'Amato *et al*., 1992b; Currò *et al*., 1994). The neural activation caused by vagal stimulation or nicotinic receptor agonists is more selective and involves only those neurones with nicotinic receptors (mainly the parasympathetic postganglionic neurones, both excitatory, cholinergic and the inhibitory, NANC). There is evidence that both NO and VIP are involved in rat gastric relaxation elicited in the presence of atropine by *in vitro* vagal stimulation (Takahashi & Owyang, 1995). Moreover, nicotine has been shown to relax rat fundus strips *in vitro* under NANC conditions and this effect was reduced not only by the NO-synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), but also by α-chymotrypsin (McLaren *et al*., 1993), which indicates the involvement of one or more peptides. The objective of our study was to investigate the roles of VIP and PHI in the peptidergic component of this latter response. The study was based on *in vitro* immunoneutralization experiments with an anti-VIP serum and assays of VIP- and PHI-like immunoreactivity (LI) released into the incubation medium.

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Methods

Male and female Wistar rats (180–320 g) were decapitated after an overnight fast and exsanguinated. The gastric fundus was removed and two longitudinal muscle strips (3 × 20 mm) were prepared according to the method of Vane (1957) in a Krebs solution of the following composition (mM): NaCl 118.5, KCl 4.8, $CaCl_2$ 1.9, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 10.1. Each strip was then suspended under a 1 g load inside an organ bath (for functional experiments) or glass tube (for release experiments) containing 5 or 2 ml Krebs solution, respectively. The solution was maintained at 37°C and bubbled with a 95:5 mixture of O_2/CO_2 (pH 7.4).

In the functional experiments, the bath solution also contained 5-hydroxytryptamine (5-HT, 3 μM) to increase strip tone so that relaxation responses could be studied. Relaxation was investigated under both non-cholinergic and NANC conditions. In the former experiments, the bath contained atropine (0.3 μM); in the latter, phentolamine (1 μM) and nadolol (1 μM) were also present. The strips were mounted between parallel platinum electrodes connected to a Palmer Bioscience 6012 Stimulator linked in series with a constant-current unit (Basile Biological Research Apparatus). This system was used to deliver electrical field stimulation (EFS) consisting of rectangular, bipolar pulses of constant duration (1 ms) and amplitude (120 mA). Smooth-muscle activity, magnified 10–20 times, was measured with isotonic transducers (Mod. 7006, Basile Biological Research Apparatus) connected to Rikadenki R-01 or R-02 recorders. Stimuli were delivered after an equilibration period of 60 min; the bath solution was changed every 10 min between stimuli.

After the induction of a control relaxation with EFS at 4 Hz for 2 min, each strip was exposed to a single concentration of nicotine (3–300 μM) for 2 min under non-cholinergic or NANC conditions. One hour later, the strip was again exposed to the same concentration to evaluate responses to repeat stimulation. Nicotine concentration-response curves were plotted for both situations. Studies were then conducted to identify the effects of hexamethonium (100 μM; exposure time: 20 min), tetrodotoxin (TTX, 3 μM, 20 min) and anti-VIP serum (1:100 and 1:50, 1 h) on the sub-maximal response evoked by 30 μM nicotine. The effect of TTX (3 μM) on the response evoked by 300 μM nicotine (i.e., the maximal concentration used) was also studied. Because the previous experiments had revealed that the strips were less responsive to the second nicotine stimulation, these studies were conducted on pairs of strips from the same fundus (one of which served as a non-exposed control). The effect of the anti-VIP serum was compared with that of normal rabbit serum (NRS, 1:100 and 1:50). At the beginning of each experiment, control and test strips were subjected to 4 Hz EFS to exclude differences in their relaxant responses. At the end of each experiment, each strip was exposed for 5 min to papaverine (100 μM), which induced maximal relaxation, and all other relaxations were expressed as percentage of this response.

In release experiments, bovine serum albumin (BSA, 50 mg l^{-1}), bacitracin (30 mg l^{-1}) and aprotinin (100,000 u l^{-1}) were added to the bath solution. After a 60 min equilibration period (during which the bath solution was renewed every 10 min), the strips were subjected to a series of three (or, in some cases, five) consecutive 5 min incubations in different tubes. In the first (pre-stimulation), there was no nicotine in the medium. In the second (during stimulation), nicotine was added to the medium after the first 3 min (exposure time: 2 min). The third (and, in some cases, fourth and fifth) incubations were carried out without nicotine in the medium (post-stimulation). Each strip was exposed to a single concentration of nicotine (10–300 μM). To evaluate responses to repeated nicotine stimulation, some of the strips were also subjected to a second identical series of incubations. During the 1 h interval between the two series, the bath solution was renewed every 10 min. After each 5 min incubation, the bath medium was collected on ice, divided into 0.4 ml aliquots and stored at −80°C. On the day of the assay, the aliquots were lyophilized and subjected to radioimmunoassay (RIA) to measure levels of VIP-LI and PHI-LI.

The PHI-LI RIA was based on R8403 anti-porcine PHI (pPHI) serum, which was kindly supplied by Prof. Dr N. Yanaihara (Yanaihara Institute Inc., Shizuoka, Japan). The RIA used to measure VIP-LI was based on an anti-serum produced in our laboratory by immunization of New Zealand white rabbits with synthetic VIP coupled to bovine serum albumin (BSA) with carbodiimide as previously described (Currò *et al.*, 1994). The chloramine T method used to prepare iodinated VIP and pPHI and the RIA method have been described in detail elsewhere (Currò *et al.*, 1994). Briefly, the lyophilized samples of bath solution were reconstituted with RIA buffer (sodium phosphate 10 mM, NaCl 154 mM, ethylene diamine tetraacetic acid (EDTA) 25 mM, thimerosal 0.01%, BSA 0.5% and Tween 20 0.03%, pH 7.2) and concentrated four fold. The R8403 and anti-VIP sera were used at respective final dilutions of 1:500,000 and 1:1,200,000 to bind approximately 40% of the iodinated peptides (≈ 6,000 c.p.m. per tube). The standard curves ranged from 1.95 to 250 pg/tube of peptide, and the total incubation volume and time were, respectively, 0.4 ml and 72 h. The second antibody method (anti-rabbit IgG goat serum) was used to separate free from bound antigen. Since peptide levels were not related to the wet weight of tissues (Currò *et al.*, 1994), the release findings were expressed as fmol ml^{-1}. Total evoked peptide release was calculated by subtracting basal values from those observed during and after incubations with nicotine, and expressed as an absolute value (fmol).

The influence of hexamethonium, TTX and a calcium-free bath solution on the release of VIP- and PHI-LI induced by nicotine (300 μM) was also investigated. Paired strips from the same fundus (test and control) were also used in these experiments (as described above). The calcium-free medium was prepared by substituting the Ca^{2+} in the bath solution with an equimolar concentration of Mg^{2+} and adding the calcium chelator, ethylene glycol-O, O′-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA, 1 mM). After pre-incubation with hexamethonium (300 μM, 20 min), TTX (3 μM, 20 min) or the calcium-free Krebs solution (30 min), each strip was subjected to 5-min incubations before, during and after exposure to nicotine (300 μM), and assays for VIP-LI and PHI-LI were carried out as described above.

Drugs used

Atropine sulphate, bacitracin, BSA, chloramine T, guanethidine sulphate, hexamethonium chloride, human corticotrophin-releasing factor (CRF), human growth hormone-releasing factor (GRF), 5-HT creatinine sulphate, nadolol, (−)-nicotine hydrogen tartrate salt, phentolamine hydrochloride, porcine gastric inhibitory peptide (GIP), porcine glucagon, pPHI, porcine secretin, sauvagine, tetrodotoxin, thimerosal, urotensin I, VIP, VIP (1–12) (Sigma, St Louis, MO, U.S.A.); aprotinin (Lepetit, Milano, Italy); 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (Calbiochem, La Jolla, CA, U.S.A.); EDTA (Carlo Erba, Milano, Italy); EGTA (Fluka, Buchs, Switzerland); helodermin (Novabiochem, Laufelfingen, Switzerland); $Na^{125}I$ (Amersham, Buckinghamshire, U.K.); papaverine hydrochloride (Merck, Darmstadt, Germany); peptide histidine methionine (PHM), (Pyr^{16})-VIP (16–28), rat PHI (rPHI), VIP (10–28) (Bachem, Bubendorf, Switzerland). VIP (22–28) was kindly supplied by Dr P. Romualdi (Institute of Pharmacology, School of Farmacy, University of Bologna, Bologna, Italy).

Statistical analysis

The results were evaluated by means of unpaired Student's *t* test (functional experiments) and Dunnett's test for multiple comparisons with a control (release experiments). All values are presented as mean ± s.e.mean. $P<0.05$ was considered statistically significant.

Results

The anti-VIP serum produced in our laboratory proved to be highly specific and was not directed to any particular sequence in the VIP molecule. Cross-reactivity with rPHI, PHM, secretin, sauvagine, urotensin I, GRF, CRF, glucagon, GIP and helodermin (each added at concentrations of $\leqslant 1$ μg/tube) was less than 0.01%. The serum displayed no cross-reactivity with VIP (1–12) and VIP (22–28) (each added at concentrations of $\leqslant 3$ ng/tube). (Pyr^{16})-VIP (16–28) and VIP (10–28) partially displaced antigen-antibody binding at the maximal concentration (3 ng/tube) (by 49.5 and 56.6%, respectively), indicating that the entire VIP molecule is necessary for full binding of the anti-serum. The mean IC_{50} of the standard curve was 21.2 ± 2.5 pg/tube, and non-specific binding of the labelled ligand was less than 3%. The intra-assay ($n=4$) and inter-assay ($n=5$) coefficients of variation were respectively ± 1.43% and ± 1.58% at the lowest level of standard (1.95 pg/tube) and ± 4.54% and ± 3.93% at the highest level of standard (250 pg/tube). The lowest concentration that could be measured with 95% confidence (i.e. 2 s.d. at zero) was 1.95 pg/tube. The detection limit was thus 4.87 pg ml^{-1} of the original sample (since each dried 0.4 ml sample was reconstituted in 0.1 ml in the RIA system).

EFS at 4 Hz for 2 min induced similar relaxations under non-cholinergic and NANC conditions. In the experiments performed for calculation of the nicotine concentration-response curves, the mean non-cholinergic and NANC relaxations were, respectively, 83.4 ± 2.2% ($n=36$) and 85.9 ± 3.5% ($n=32$) of those induced by papaverine under the same conditions.

Nicotine (5.5–300 μM) produced concentration-dependent relaxations (Figure 1), that began immediately after the addition of this substance to the bath medium and persisted throughout the 2-min incubation (Figure 2). Their total duration varied and was generally longer with higher nicotine concentrations. The relaxation induced by lower concentrations of nicotine under NANC conditions was less marked than that observed under non-cholinergic conditions: significant differences of 38% and 27% were observed with nicotine at 10 μM and 30 μM, respectively. In contrast, the NANC and non-cholinergic responses to 300 μM nicotine were almost identical (respectively, 69.8 ± 3.3%, $n=8$, and 68.4 ± 5.9%, $n=7$, of the corresponding papaverine-evoked relaxations) (Figures 1 and 2). Re-administration of nicotine 1 h later induced smaller relaxation (Figure 2), indicating the occurrence of desensitization, which appeared to be concentration-dependent (Figure 3). This phenomenon was somewhat more evident under NANC conditions, but this difference was not statistically significant. The greatest desensitization was seen with a concentration of 300 μM: in these experiments the second responses represented 24.0 ± 3.0% (under non-cholinergic conditions) and 15.4 ± 3.6 (NANC conditions) of the first.

The effects of hexamethonium (100 μM), TTX (3 μM) and anti-VIP serum (1:100 and 1:50) on strip relaxation produced by 30 μM nicotine are shown in Table 1 and Figures 4 and 5. In all of these experimental series, there were no significant differences between the initial relaxations induced by EFS at 4 Hz in the test and control strips of each pair. Under non-cholinergic conditions, the strips that had been pre-incubated with hexamethonium responded to nicotine with small contraction

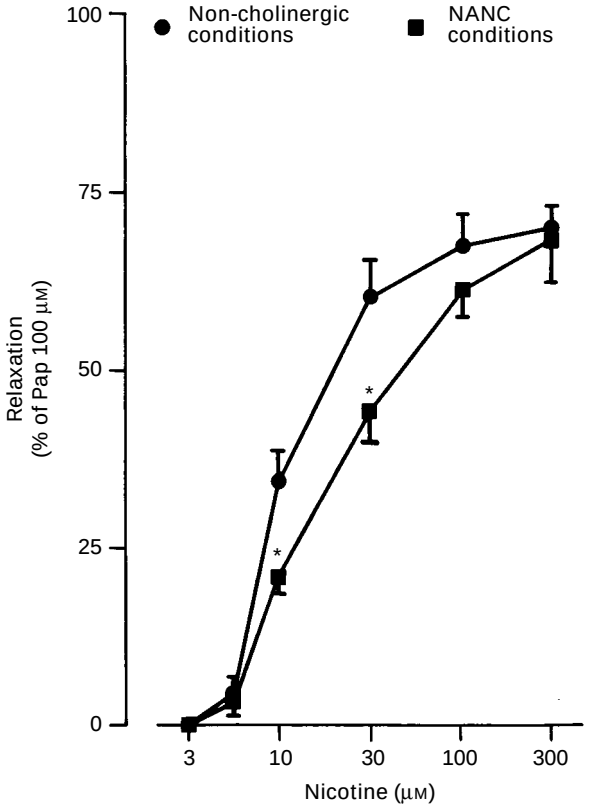

Figure 1 Mean concentration-response curves for nicotine (3–300 μM)-induced relaxation of 5-HT (3 μM)-precontracted longitudinal muscle strips of the rat gastric fundus under non-cholinergic (0.3 μM atropine) and NANC (0.3 μM atropine + 1 μM phentolamine + 1 μM nadolol) conditions. Relaxations were measured as peak amplitudes and expressed as percentages of maximal relaxation of the same strip induced by papaverine (Pap; 100 μM). Each strip was tested with a single concentration of nicotine. Each point represents the mean and vertical lines s.e. mean of responses observed in 6–10 strips. * $P<0.05$ vs non-cholinergic conditions.

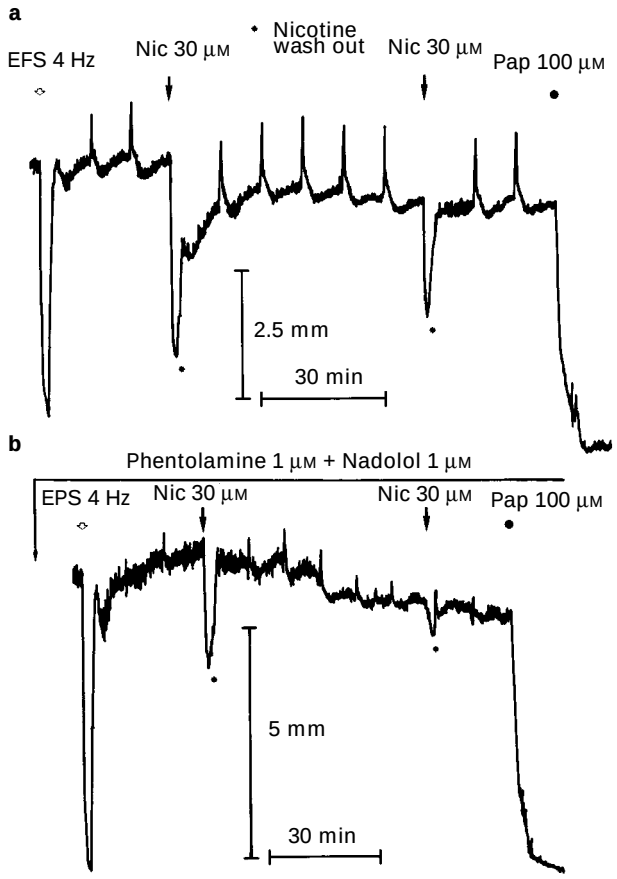

Figure 2 Representative tracings showing the relaxant effects of 30 μM nicotine (Nic) on 5-HT (3 μM)-precontracted longitudinal muscle strips of the rat gastric fundus under non-cholinergic (a) and NANC (b) conditions. At the beginning of each tracing, a control relaxation was evoked with EFS (120 mA, 1 ms, 4 Hz, 2 min). Readministration of nicotine 1 h after the first induced smaller relaxations. At the end of each experiment, maximal relaxation was

(7.9±4.5%, $n=3$). In those exposed to TTX, the nicotine-induced relaxation was 27.9±5.2% ($n=7$) of the control. Under NANC conditions, 30 μM nicotine produced contraction in two of the TTX-treated strips, small relaxation in two others and no effect whatsoever in the remaining two. The average effect was a small contraction (6.4±5.7%, $n=6$) similar to that observed in hexamethonium-treated strips. In the experiments performed to test the effect of TTX (3 μM) on the response induced by 300 μM nicotine under non-cholinergic conditions, the control relaxation was 63.0±3.9% ($n=9$). During the 2-min incubation with 300 μM nicotine, the nine strips exposed to TTX responded as follows: two contracted, three relaxed and the remaining four displayed biphasic responses consisting of relaxation followed by contraction. Since the latter was the most frequently observed response, we decided to express the mean results of these experiments in biphasic (i.e., relaxation/contraction) terms. To this end, the responses of the three strips that displayed relaxation only were added to the relaxant phases of the four biphasic responses, and a value of zero was inserted for each of the two strips that responded with contractions alone. This sum was divided by the total number of strips tested to obtain a mean relaxant phase. The mean contractile phase was calculated in the same manner, with zero values included for each of the three strips that displayed exclusively relaxant responses. Using this method, the mean biphasic response of the TTX-treated strips to 300 μM nicotine was an initial relaxation of 19.1±5.8% followed by a con-

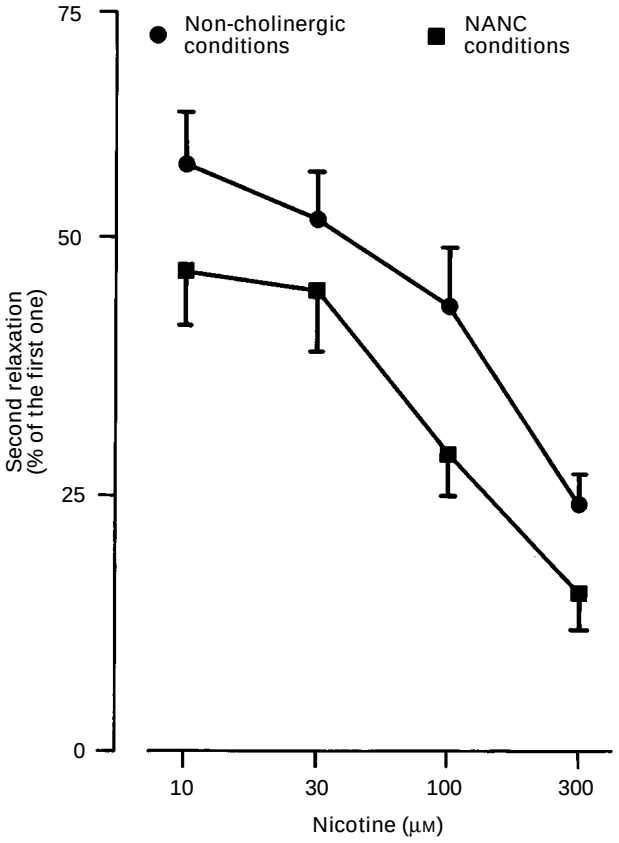

Figure 3 Mean concentration-response curves for relaxation of 5-HT (3 μM)-precontracted longitudinal muscle strips of the rat gastric fundus induced by a second administration of nicotine (10–300 μM) 1 h after the first. Responses observed under non-cholinergic and NANC conditions. Relaxations are expressed as percentages of the first response in the same strip. Each point represents the mean and vertical lines s.e.mean of responses observed in 6–10 strips.

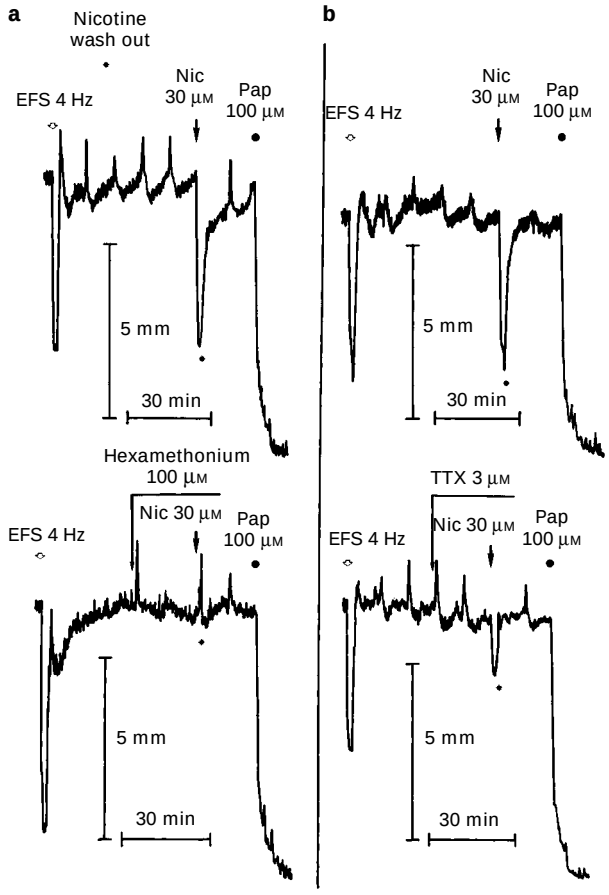

Figure 4 Representative tracings showing the effects of hexamethonium (100 μM) (a) and TTX (3 μM) (b) on the responses of 5-HT (3 μM)-precontracted longitudinal muscle strips of the rat gastric fundus to 30 μM nicotine (Nic). Control tracings without hexamethonium or TTX are shown in the upper parts of each panel. All four tracings were recorded under non-cholinergic conditions. Each strip was exposed only once to nicotine. EFS- and papaverine-induced relaxations were obtained as described in Figure 2.

Table 1 Effects of various test agents on nicotine (30 μM)-induced relaxation of rat gastric fundus strips under non-cholinergic and/or NANC conditions

Test agent	*Conditions*[a]	n	*Control strips*	*Test strips*[b]
Hexamethonium (100 μM)	Non-cholinergic	3	56.8±6.1	−7.9±4.5
TTX (3 μM)	Non-cholinergic	7	50.6±5.8	13.6±2.2*
Anti-VIP serum (1:100)	NANC	10	31.5±4.3[c]	19.8±2.2*
Anti-VIP serum (1:50)	NANC	6	33.8±4.5[c]	20.3±2.5*
TTX (3 μM)	NANC	6	45.2±8.9	−6.4±5.7

Results are expressed as % of relaxation produced by papaverine (100 μM) (means±s.e.mean of n experiments). Control and test strips for each experiment were prepared from the same fundus. [a] Non-cholinergic= in the presence of atropine (0.3 μM). NANC= in the presence of atropine (0.3 μM)+phentolamine (1 μM)+nadolol (1 μM). [b] Negative values indicate contraction. [c] Control strips were incubated with normal rabbit serum (NRS) at identical dilutions. *Significant ($P<0.05$) differences between test and control strips.

traction of 16.5±5.0% ($n=9$). The anti-VIP serum (1:100) abolished the relaxation induced by VIP (0.1 μM) ($n=3$) and caused a significant reduction (33.1±6.8, $n=10$) in that produced by 30 μM nicotine, compared with NRS treated controls. At a lower dilution (1:50), the anti-VIP serum produced a somewhat greater attenuation (37.6±5.9%, $n=6$) of the nicotine (30 μM)-induced response that was not significantly different from that observed with the 1:100 dilution ($P=0.72$). The mean nicotine-induced relaxations observed in strips incubated with NRS (at both 1:100 and 1:50) were smaller than those of the TTX control strips (Table 1) and those of the concentration-response curve (Figure 1). However, when the pooled responses of the latter two experimental series (44.7±5.2%, $n=13$) were compared with those of the NRS-treated strips, the differences were not significant ($P=0.13$ by one-way analysis of variance).

At 30–300 μM, nicotine produced statistically significant, concentration-dependent increases in the outflow of both VIP-LI and PHI-LI (Figure 6). Bath levels of both peptides had generally returned to basal values 5 min after the strips were removed from the bath containing nicotine, with the exception of PHI-LI induced by 300 μM nicotine. Mean ($n=8$) total release was (in fmol): VIP-LI 1.47±0.43/PHI-LI 2.18±0.94 after 30 μM nicotine; VIP-LI 4.7±1.1/PHI-LI 5.3±1.5 after 100 μM; VIP-LI 5.4±1.3/PHI-LI 10.4±2.2 after 300 μM. The VIP-LI/PHI-LI ratios were close to 1:1 with nicotine concentrations of 30 and 100 μM; with 300 μM, the total release of VIP-LI was only 53.1±6.1% of that of PHI-LI ($P<0.05$). Nicotine-induced release of both types of immunoreactivity also appeared to be subject to desensitization: although the second stimulation (1 h after the first) still increased the outflow of both VIP-LI and PHI-LI with respect to basal levels, neither of the increases was statistically significant (data not shown). The increased outflow of VIP-LI and PHI-LI produced by nicotine (300 μM) was abolished by hexamethonium (300 μM) ($n=4$), TTX (3 μM) ($n=4$) and the removal of calcium from the bath solution ($n=4$). Atropine (0.3 μM), phentolamine (1 μM) and nadolol (1 μM) had no effect on nicotine-induced release of either peptide (data not shown).

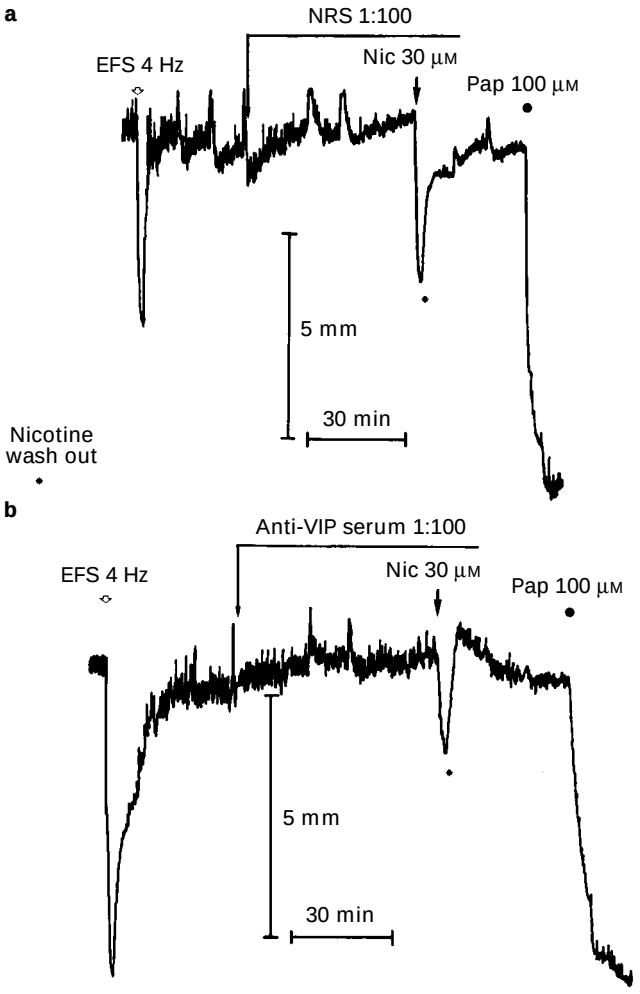

Figure 5 Representative tracings showing the effects of normal rabbit serum (NRS, 1:100) (a) and anti-VIP serum (1:100) (b) on the responses of 5-HT (3 μM)-precontracted longitudinal muscle strips of the rat gastric fundus to nicotine (30 μM) under NANC conditions. Each strip was exposed only once to nicotine. EFS- and papaverine-induced relaxations were obtained as described in Figure 2.

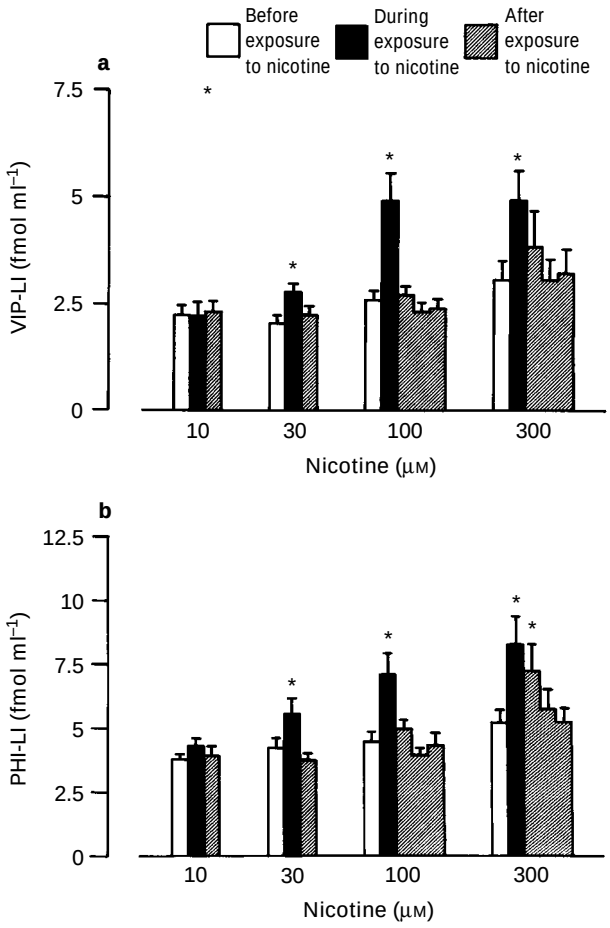

Figure 6 Vasoactive intestinal polypeptide-like (a) and peptide histidine isoleucine-like (b) immunoreactivity (VIP-LI and PHI-LI) released by rat gastric fundus strips before (open columns), during (solid columns) and after (hatched columns) exposure to nicotine (10–300 μM). Each column represents means±s.e.mean measured in 5 min collection fractions of bath medium from 8 strips. Nicotine was added to the medium after the first 3 min of the second 5 min incubation (total exposure time: 2 min). Each strip was exposed only once to nicotine. * $P<0.05$ vs baseline levels.

Discussion

Intramural inhibitory neurones in the proximal third of the stomach are responsible for the NANC smooth muscle relaxation that underlies the receptive and adaptive responses of the organ to food and liquids (Abrahamsson, 1986). Studies of NANC innervation are frequently based on the *in vitro* responses of organ strips to EFS, but this approach does not permit analysis of the inhibitory neurones located in any specific reflex circuit. In the rat gastric fundus, the main neurotransmitters involved in the NANC inhibitory responses to

EFS seem to be NO (or a molecule that releases it) and VIP. These mediators appear to be, respectively, responsible for the immediated and sustained components of the relaxant response (Li & Rand, 1990; D'Amato *et al*., 1992a). There is also evidence that the release of VIP and PHI occurs only in response to high-frequency stimulation (> 4 Hz) (D'Amato *et al*., 1992b; Currò *et al*., 1994). The predominant effect of nicotinic agonists in our experimental model is the global stimulation of postganglionic parasympathetic neurones, including the inhibitory neurones located in the efferent chain of the vago-vagal reflex arc, which is activated during swallowing. The relaxant response observed under non-cholinergic conditions thus simulates physiological gastric receptive relaxation. The attenuating effects of L-NAME and α-chymotrypsin on *in vitro* nicotine-induced NANC relaxation of the rat gastric fundus indicate that this response also includes nitrergic and peptidergic components (McLaren *et al*., 1993). The purpose of our study was to determine if the latter component might be mediated by VIP and PHI, which are putative neurotransmitters of NANC relaxation of this tissue evoked by EFS.

In this study, nicotine induced concentration-dependent relaxation under non-cholinergic conditions, with an EC_{50} in the range of 10–30 μM. The maximal nicotine-induced relaxation was smaller than the near-maximal response to EFS 4 Hz, indicating that not all of the intramural inhibitory neurones have nicotinic receptors. The nicotine-evoked relaxation (like that induced by EFS) began immediately, a feature considered to be indicative of NO involvement. The relaxation was maintained throughout the 2 min period of exposure to nicotine and for variable periods thereafter. Recovery of basal tone was generally slower after exposure to higher concentrations, but it was always more rapid than that observed after high-frequency (> 4 Hz) EFS (Currò *et al*., 1994). These characteristics are believed to be peculiar to the peptidergic component of the response. Antagonists of α- and β-adrenoceptors diminished the relaxation produced by sub-maximal nicotine concentrations, indicating that this effect is partially dependent on the release of noradrenaline. A noradrenergic component has also been demonstrated in nicotine-induced relaxation of the guinea-pig gastric fundus (Kojima *et al*., 1993). The desensitization of the strips to nicotine is also a feature of nicotinic receptors. When the strips were re-exposed to an identical concentration of nicotine one hour later, the responses were significantly less marked and the magnitude of the reduction was proportional to the concentration used. In a previous study of rat gastric fundus strips, no desensitization was noted, even though a longer nicotine incubation (3 min) and a shorter interval between exposures (40 min) were used (McLaren *et al*., 1993). We do not have any adequate explanation of this discrepancy with our findings.

In light of the effects of hexamethonium, nicotine-induced relaxation appears to be caused exclusively by its binding to neuronal type receptors. In hexamethonium-treated strips, nicotine caused small contraction, which was also observed under NANC conditions in strips pre-incubated with TTX. This latter finding indicates that the nicotine-induced contraction does not appear to depend on propagated neural action potentials. It might be due to binding of receptors unaffected by the hexamethonium and located on muscle cells or on the peripheral terminals of primary, capsaicin-sensitive afferent neurones. Activation of these terminals, which can also be achieved with nicotine (Jinno *et al*., 1994), is, in fact, believed to depend on a local mechanism that does not involve action-potential propagation (Holzer, 1991). In addition, they are known to contain and release tachykinins that excite smooth muscle. Further investigation of this contractile effect was beyond the scope of the present study. Pretreatment with TTX merely attenuated the relaxation induced by nicotine under non-cholinergic conditions, but, as noted above, it reversed the NANC response. It is thus possible that the noradrenergic component of the relaxant response is due to activation of nicotinic receptors located on terminals, rather than on the soma or dendrites, of noradrenergic neurones. These receptors might induce noradrenaline release by means of a spatially limited local depolarization that does not involve the opening of voltage-dependent Na^+ channels. Kojima *et al*. (1993) described a TTX-resistant component in nicotine-induced relaxation of guinea-pig gastric fundus, and it has also been observed in other peripheral tissues, including rat duodenum (Irie *et al*., 1994), bovine retractor penis muscle (Klinge *et al*., 1988), guinea-pig urinary bladder (Hisayama *et al*., 1988) and the mainstem bronchi of rabbits (Takayanagi *et al*., 1984). However, no such component was seen by McLaren *et al*. (1993) in their study of the rat gastric fundus. Again, we have no plausible explanation for this discrepancy. As shown in Figure 1, the responses to 100 and 300 μM nicotine were almost identical under non-cholinergic and NANC conditions, suggesting the possible absence of a noradrenergic component, which was evident in the responses to lower concentrations (10 and 30 μM) of nicotine. To investigate this possibility, we evaluated the response of TTX-treated strips to 300 μM nicotine under non-cholinergic conditions. However, in these experiments, the mean response of the toxin-treated strips was an initial relaxation (similar to that seen when they were stimulated with 30 μM nicotine) followed by a contraction of roughly the same magnitude. This observation indicates that the response to higher concentrations of nicotine does indeed include a noradrenergic component. The fact that no significant reduction was observed with blockade of the latter component (Figure 1) can be explained as follows: in the response to higher nicotine concentrations, the nitrergic and peptidergic components play more substantial roles, and they are thus able to compensate for the abolished contribution of noradrenaline. The contractile component of the response of the TTX-treated strips can probably be attributed to the higher nicotine concentration, which would be expected to produce more contraction than that seen with 30 μM nicotine under NANC conditions in the presence of TTX or under non-cholinergic conditions in the presence of hexamethonium.

A number of studies have suggested that VIP is a mediator of the NANC relaxation of the rat gastric fundus induced by various stimuli (Kamata *et al*., 1988; De Beurme & Lefebvre, 1988; Li & Rand, 1990; D'Amato *et al*., 1992b; Takahashi & Owyang, 1995). Our findings clearly demonstrate that this peptide fulfills the criteria for a mediator of the neural inhibitory response to nicotine. In 1993, McLaren *et al*. showed that α-chymotrypsin reduced the nicotine-induced response by up to 40%, suggesting the involvement of one or more peptide mediators. In the present study, we achieved a reduction of approximately 35% with anti-VIP serum. The fact that this attenuation is quite similar to that produced with α-chymotrypsin in the McLaren *et al*. (1993) study would tend to exclude the role of other peptides sensitive to α-chymotrypsin in this response. However, it is also possible that the peptidase-induced inhibition would have been greater than that produced by our anti-VIP serum if a higher concentration of α-chymotrypsin and/or a longer incubation had been used. Our observation of a calcium-dependent, TTX-sensitive release of both VIP- and PHI-LI by nicotine-stimulated strips suggests that there may indeed be a PHI-mediated component in this relaxant response. Both dilutions of NRS (1:100 and 1:50) caused similar attenuation of the nicotine (30 μM)-induced relaxation, but neither of these reductions was significant. At present, we have no explanation for the inhibitory effects of this serum. However, other authors (De Beurme & Lefebvre, 1988) have shown that NRS also inhibits EFS-induced NANC relaxation of the rat gastric fundus. The EFS frequency used by these investigators (5 Hz) should not provoke co-release of VIP- and PHI-LI (D'Amato *et al*., 1992b; Currò *et al*., 1994). In addition, De Beurme & Lefebvre (1988) found that the NRS did not have any significant effect on submaximal relaxation induced by VIP or isoprenaline. In light of these findings, it seems improbable that NRS exerts a pre- or post-junctional effect on the VIP-mediated component or an aspecific post-junctional effect. Instead, it might be that one or more factors

present in the serum partially trap the released NO, or interfere with the NO synthase or guanylate cyclase system.

At lower concentrations of nicotine, VIP- and PHI-LI were released in equimolar quantities, but the amount of PHI-LI released in response to 300 μM nicotine was about twice as much as that of VIP-LI. The PHI-LI release observed under the latter conditions may partially reflect release of peptide histidine valine (PHV), a C-terminal-extended form of PHI that is also fully recognized by the R8403 serum we used (Currò *et al*., 1994), and this release is probably independent of that of VIP-LI. The total evoked release of PHI-LI observed after high-frequency EFS of the same tissue is much greater than that observed after nicotine stimulation (Currò *et al*., 1994). It is possible that only a small percentage of the neurones that release PHI-LI have nicotinic receptors, and that PHI and PHV play more important roles in locally triggered accomodative relaxation of the stomach, rather than in the receptive response. The release of both VIP- and PHI-LI was blocked by hexamethonium, indicating that this effect is mediated by the activity of nicotine at the level of neuronal type receptors. Like the functional response, the release of the two peptides induced by a second administration of nicotine was not as great as that seen with the first administration, and, on the basis of the statistical analysis, it would seem that this desensitization was complete. However, the fact that the relaxant response to readministration of nicotine was partially preserved is not surprising, since various mediators are believed to be involved in this response.

In conclusion, our findings support the hypothesis that VIP and probably PHI, as well, are mediators of nicotine-induced NANC relaxation of the rat gastric fundus. Since reflex receptive relaxation of the stomach during food intake involves the activation of nicotinic receptors located on parasympathetic postganglionic neurones, we can conclude that this physiological response is partially mediated by VIP.

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