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Study of NO and VIP as non-adrenergic non-cholinergic neurotransmitters in the pig gastric fundus

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1 The contribution of nitric oxide (NO) and vasoactive intestinal polypeptide (VIP) to non-adrenergic non-cholinergic (NANC) relaxations in the pig gastric fundus was investigated.

2 Circular and longitudinal muscle strips were mounted for isotonic registration in the presence of atropine and guanethidine; tone was raised with 5-hydroxytryptamine. Electrical field stimulation with 10 s trains at 5 min intervals induced short-lasting, frequency-dependent relaxations while continuous stimulation with cumulative increase of the stimulation frequency induced sustained frequency-dependent relaxations in both types of strips; the electrically induced responses were abolished by tetrodotoxin.

3 The short-lasting as well as the sustained electrically induced NANC relaxations were significantly reduced by N^G-nitro-L-arginine methyl ester (L-NAME). Pretreatment with L-arginine but not D-arginine, prevented the inhibitory effect of L-NAME except for sustained relaxations in the longitudinal muscle strips.

4 Sodium nitroprusside, forskolin, zaprinast and 3-isobutyl-1-methylxanthine induced concentration-dependent relaxations. Exogenous NO mimicked the short-lasting electrically induced relaxations, while endogenous VIP evoked sustained relaxations. The responses to exogenous NO and VIP were not influenced by tetrodotoxin and L-NAME.

5 α-Chymotrypsin abolished the responses to exogenous VIP but only moderately reduced NANC relaxations induced by continuous electrical stimulation. Zaprinast potentiated the relaxant responses to sodium nitroprusside and increased the duration of the NANC relaxations induced by electrical stimulation with 10 s trains in circular muscle strips but not in longitudinal muscle strips.

6 The cyclic GMP and cyclic AMP response to electrical stimulation, NO and VIP was measured in circular muscle strips. Short-lasting as well as sustained electrical field stimulation induced an approximately 1.5 fold increase in cyclic GMP content, while NO induced nearly a 40 fold increase. An increase in cyclic AMP content was obtained only with sustained electrical field stimulation.

7 Immunocytochemistry for NO synthase (NOS) revealed immunoreactive neuronal cell bodies in the submucous and myenteric plexuses and nerve fibres in both the circular and longitudinal muscle layer; double-labelling for NOS and VIP showed that VIP coexists in a major part of the intrinsic neurones. NADPH diaphorase-histochemistry showed the same pattern of nitrergic neurones and nerves as NOS-immunocytochemistry.

8 It is concluded that a cyclic GMP- and a cyclic AMP-dependent pathway for relaxation is present in both the circular and longitudinal muscle layer of the pig gastric fundus. NO appears to contribute to short-lasting as well as sustained NANC relaxations. A peptide, possibly VIP, may be involved during sustained stimulation at lower frequencies of stimulation.

Keywords: Pig gastric fundus; NANC relaxation; NO; VIP; cyclic GMP; cyclic AMP; NOS immunocytochemistry

Introduction

The inhibitory non-adrenergic non-cholinergic (NANC) neurones of the proximal part of the stomach mediate gastric relaxation during food intake (receptive relaxation) and during vomiting (Abrahamsson, 1986). With regard to the neurotransmitters involved, most evidence favours a role of vasoactive intestinal polypeptide (VIP) and nitric oxide (NO; Lefebvre, 1993). *In vitro* experiments in the rat and cat gastric fundus suggest that NO and VIP might be cotransmitters, with NO being released mainly during short-lasting electrical field stimulation whereas VIP contributes to sustained relaxation induced by prolonged electrical field stimulation (Li & Rand, 1990; Boeckxstaens *et al.*, 1992; Barbier & Lefebvre, 1993). In anaesthetized ferrets too, the gastric relaxation in response to vagal stimulation appeared to consist of an initial NO-linked component and a second VIP-mediated component (Grundy *et al.*, 1993). In the guinea-pig gastric fundus, VIP was proposed as the primary neurotransmitter, inducing relaxation partially via activation of adenylate cyclase and partially via stimulation of NO production in the smooth muscle cells (Grider *et al.*, 1992; Jin *et al.*, 1993); this sequential link between VIP and NO, whereby neuronal VIP induces the synthesis of muscular NO, clearly contrasts with the above-mentioned cotransmission model, in which both VIP and NO are released from neurones and act in parallel to induce relaxation. Desai *et al.* (1994) reported that NO is the principal neurotransmitter of vagally induced relaxation of the guinea-pig stomach.

Among the non-primate mammalian species, the pig has been proposed as one of the best models for the study of nutritional issues in man, due to the similarity of the morphology and physiology of the gastrointestinal tracts (Miller & Ullrey, 1987). NANC relaxations can be induced by electrical field stimulation in longitudinal and circular smooth muscle strips of the pig gastric fundus (Miyazaki *et al.*, 1991); arguments against ATP as the neurotransmitter involved were provided in the longitudinal muscle tissues (Ohga & Taneike, 1977). Mandrek & Milenov (1991) reported that VIP induces relaxation of porcine fundus strips, but its role as well as that of NO in neuronally induced NANC relaxation was not ex-

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amined. The aim of the present study was to investigate the response to NO, VIP and electrical stimulation under NANC conditions in the porcine upper stomach. Functional and cyclic nucleotide responses were measured and the distribution of VIP and NO synthase was examined by immunocytochemistry.

Methods

Tissue preparation for functional and cyclic nucleotide measurements

The stomach was removed from healthy 6 months old male castrated pigs, slaughtered at a local abattoir and transported to the laboratory in ice-chilled physiological salt solution. The mucosa was removed and strips (15 × 3 mm) were cut in the direction of the circular ($n=4$) and longitudinal ($n=4$) muscle layer from the gastric fundus. Usually, all strips were used immediately except for measurement of the cyclic nucleotide content, when half of the strips were kept in carbogen (95% O_2, 5% CO_2) gassed ice-chilled physiological salt solution for 2 h.

Strips were mounted between 2 platinum plate electrodes under a load of 2 g in 5 or 20 ml organ baths, containing physiological salt solution at 37°C and gassed with carbogen. The physiological salt solution was composed of (mM): Na^+ 137, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 124.1, HCO_3^- 25, $H_2PO_4^-$ 1.2 and glucose 11.5 (Mandrek & Milenov, 1991). Atropine (10^{-6} M) and guanethidine (4×10^{-6} M) were continuously present. Changes in length were recorded isotonically via Hugo Sachs B40 Lever transducers type 373 on a Graphtec Linearcorder 8 WR 3500 in 5 ml baths and via Palmer Bioscience T3 transducers on a Kipp & Zonen BD 112 recorder or a Graphtec Linearcorder WR 3701 F. Electrical field stimulation was performed by means of a Grass S88 stimulator. The tissues were equilibrated for 1 h. All experiments ended with relaxation of 5-hydroxytryptamine-induced tone by 10^{-5} M sodium nitroprusside.

Protocols

All strips were first contracted with 3×10^{-7} M 5-hydroxytryptamine and subsequently relaxed by 10^{-5} M sodium nitroprusside. After an interval of at least 1 h with regular rinsing, 3×10^{-7} M 5-hydroxytryptamine was again administered and once a stable contraction was obtained, electrical field stimulation was performed or relaxant agents were administered. Frequency-response curves to electrical field stimulation (40 V, 0.1 ms, 0.25–8 Hz) were obtained by stimulating the tissues either with 10 s trains at 5 min intervals (train stimulation) or continuously with stepwise increases of the frequency, once the response to a lower frequency had become maximal (cumulative stimulation). VIP, sodium nitroprusside (SNP), zaprinast, 3-isobutyl-1-methylxanthine (IBMX) and forskolin were administered in a cumulative way. As the relaxant effect of NO was not sustained, increasing concentrations were administered at 5 min intervals.

To study the influence of tetrodotoxin (TTX), N^G-nitro-L-arginine methyl ester (L-NAME), α-chymotrypsin, zaprinast and methylene blue on the relaxation induced by electrical field stimulation or NO, these drugs were added (see Results for the exact incubation period) at the end of the interval before a third 5-hydroxytryptamine-induced contraction and left in contact with the tissue during this contraction, when the relaxant stimulus was studied again; in parallel control strips, only the solvent was incubated. To study their influence on the relaxation induced by VIP, only one concentration-response curve to VIP was constructed in their presence or in the presence of their solvent in a parallel control strip. The response to the third 5-hydroxytryptamine administration was in general somewhat more pronounced than that to the second administration.

Cyclic nucleotide assay

Circular muscle strips were mounted in 5 ml baths and after equilibration, they were contracted with 3×10^{-7} M 5-hydroxytryptamine and then relaxed with 10^{-5} M sodium nitroprusside (SNP). After an interval of at least 50 min with regular rinsing, tone was again raised with 5-hydroxytryptamine and one relaxant stimulus per strip was then applied: electrical stimulation at 4 Hz for 10, 30, 120 or 300 s, 3×10^{-7} M VIP or 10^{-4} M NO until maximal relaxation was reached. The tissue was then quickly clamped between 2 liquid nitrogen cooled plates. For comparison, some tissues were also clamped in the non-contracted basal condition or after contraction with 5-hydroxytryptamine without relaxing the tissue (controls). After freezing, the tissue was homogenized with a membrane dismembrator (B. Braun A.G., Melsungen, Germany) for 60 s. Four ml of 6% trichloro acetic acid (TCA) was then added and the material was then homogenized on ice with an ultrasonic probe (B. Braun A.G. 300 s, Melsungen, Germany) 4 times for 7 s at 15 s intervals. The homogenate was then centrifuged for 20 min at 2600 *g*. The supernatant was separated and the TCA was extracted 4 times with 5 volumes of water-saturated ether. The guanosine 3′:5′-cyclic monophosphate (cyclic GMP) content was determined by radioimmunoassay, the adenosine 3′:5′-cyclic monophosphate (cyclic AMP) content by a binding assay with commercially available kits. The protein content of the pellet was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Immunocytochemistry and NADPHd-histochemistry

For immunocytochemistry and NADPH diaphorase (NADPHd)-histochemistry, tissue of the gastric fundus was obtained from 6 week-old domestic pigs, which were injected intraperitoneally with colchicine (10 mg kg^{-1} body weight) 5 h prior to dissection as in our previous studies in the same animal (Barbiers *et al.*, 1993; Timmermans *et al.*, 1994b) as colchicine treatment optimalizes visualization of VIP in the perikarya (Costa *et al.*, 1980; Furness *et al.*, 1981). A fundic area (±4 cm^2) was dissected out along the major curvature and fixed for 2 h at room temperature in 4% paraformaldehyde in 0.1 M sodium phosphate buffer. When only immunocytochemistry was performed, 0.2% picric acid was added to the fixative. After thorough rinsing in phosphate-buffered saline (PBS), 5–10 μm thick cryosections were made with a SLEE-cryostat. For double immunostaining for NO synthase (NOS) and VIP, the cryosections were preincubated for 30 min in 10% normal serum in 0.01 M PBS containing 1% Triton X-100. They were then simultaneously incubated in mouse monoclonal NOS antiserum raised against human bNOS (diluted 1:200; M31020/L2; Transduction Laboratories, Lexington, U.S.A.) and a rabbit VIP antiserum (diluted 1:400; VA1285; Affiniti Research, Nottingham, U.K.) overnight at room temperature. Visualization of the primary antisera was achieved by immersion in DTAF-conjugated goat anti-rabbit IgG (diluted 1:100;111-015-003; Jackson ImmunoRes., West Grove, U.S.A.) and biotinylated sheep anti-mouse IgG (diluted 1:50; RPN.1001; Amersham, Brussels, Belgium) for 6 h at room temperature followed by an overnight incubation in a streptavidin-Texas Red complex (diluted 1:50; RPN.1233; Amersham).

For NADPHd-staining, cryosections were incubated in a solution containing 0.25 mg ml^{-1} nitroblue tetrazolium, 1 mg ml^{-1} β-NADPH and 0.5% Triton X-100 in 0.1 M TRIS-HCl buffer (pH 7.6) for 10–20 min at 37°C (Scherer-Singler *et al.*, 1983).

All sections were viewed under a Zeiss Axiophot equipped for conventional and fluorescence microscopy.

Drugs used

D-Arginine hydrochloride, L-arginine hydrochloride, atropine sulphate, α-chymotrypsin, forskolin, guanethidine sulphate, 3-

isobutyl-1-methylxanthine, methylene blue, N^G-nitro-L-arginine methyl ester and sodium nitroprusside were obtained from Sigma (St.-Louis, U.S.A.), 5-hydroxytryptamine creatinine monosulphate and tetrodotoxin from Janssen Chimica (Beerse, Belgium) and vasoactive intestinal polypeptide from CRB (Northwich, U.K.). Zaprinast was a gift from Rhone-Poulenc (Dagenham, U.K.). The cyclic GMP[^{125}I] RIA-kit was from DuPont Canada Inc (Ontario, Canada), the cyclic AMP[^{3}H] binding assay was from Amersham (Little Chalfont, U.K.). All drugs were dissolved in physiological salt solution except for IBMX which was dissolved in 50 vol % ethanol up to 5×10^{-2} M, forskolin which was dissolved in pure ethanol up to 10^{-2} M and zaprinast which was dissolved in 2 vol % triethanolamine up to 10^{-2} M; further dilutions were made in physiological salt solution. The solvents had no effect *per se* on the tone of the strips. Stock solutions of tetrodotoxin (10^{-3} M) and VIP (10^{-4} M) were prepared in distilled water and stored at −70°C; all other solutions were prepared on the day of the experiment. A saturated NO solution was prepared as described by Kelm & Schrader (1990), by bubbling argon gas and then NO gas through 3 consecutive in-line connected gas-tight vials, the first 2 containing KOH-solutions, the latter distilled water. The concentration of NO in the saturated solution in vial 3 was taken as 2×10^{-3} M.

Data analysis

Relaxations were expressed as percentage of the relaxation induced by 10^{-5} M SNP at the end of the experiment, except for the strips used for cyclic nucleotide assay, where relaxations were expressed as percentage of the relaxation induced by 10^{-5} M SNP at the beginning of the experiment. When constructing a concentration-response curve with SNP, relaxation at 10^{-4} M was taken as 100%. Results are given as means ± s.e.mean and n refers to strips from different animals unless otherwise stated. Results within tissues were compared by the paired t test and results between tissues with an unpaired t test. A difference was considered statistically significant at $P < 0.05$.

Results

Responses to electrical field stimulation, NO and VIP

Although the tone of the tissues tended to increase during the course of the experiment (more markedly in longitudinal than in circular muscle strips), it had to be raised to obtain reproducible relaxant responses. 5-Hydroxytryptamine (3×10^{-7} M) was used to increase the tone; it induced stable plateau contractions. In concentration-response curves for 5-hydroxytryptamine, obtained in preliminary experiments, the contractile response to 10^{-7} and 10^{-6} M 5-hydroxytryptamine was 59 ± 7 and 93 ± 3% of maximum in circular muscle strips ($n = 12$ from 6 animals) and 46 ± 5 and 84 ± 4% in longitudinal muscle strips ($n = 12$ from 6 animals).

Results were similar in circular and longitudinal muscle strips unless otherwise indicated. Train stimulation with 10 s trains at 5 min intervals induced short-lasting frequency-dependent (0.25–8 Hz) relaxations, while cumulative increase of the stimulation frequency induced sustained and more pronounced relaxations, that were also frequency-dependent (Figure 1a and b). Both types of NANC relaxations were abolished by 3×10^{-6} M tetrodotoxin ($n = 3$–4 per type of stimulation and type of strips), while the responses in parallel control strips were reproducible. NO (2×10^{-6}–10^{-4} M) induced concentration-dependent short-lasting relaxations (Figure 1c); once the maximal amplitude of relaxation was reached for a given concentration, tone returned and often overshot the level of contraction present before administration of NO. The relaxation induced by 10^{-4} M NO was more pronounced than that induced by EFS at 8 Hz for 10 s in parallel tissues obtained from the same animals (Figure 2). VIP induced relaxation from 10^{-8} M upwards; the VIP-induced relaxations

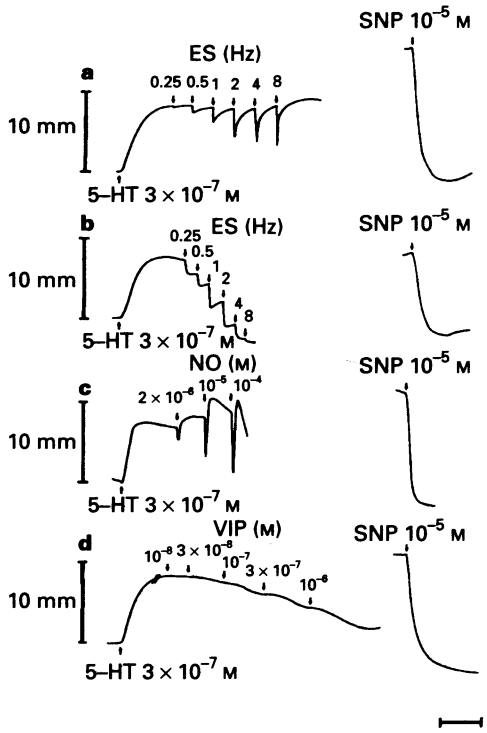

Figure 1 Representative traces showing the responses to electrical field stimulation (40 V, 0.1 ms) with 10 s trains (a) or with cumulative increase of the frequency (b), NO (c) and vasoactive intestinal polypeptide (VIP) (d) in circular muscle strips of the pig gastric fundus. In each trace, the reference relaxation to 10^{-5} M sodium nitroprusside (SNP) at the end of the experiment is also shown.

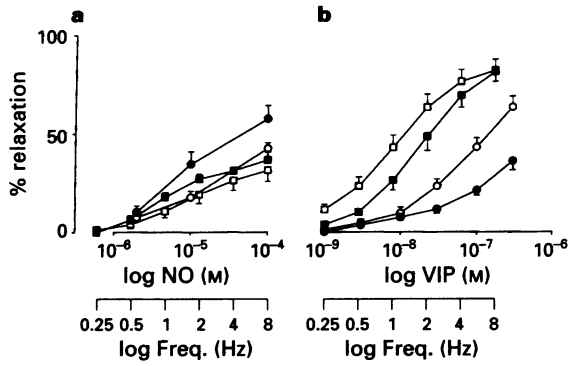

Figure 2 Mean frequency-response curves to electrical field stimulation (40 V, 0.1 ms) with 10 s trains (a) and with cumulative increase of the frequency (b) in circular (□) and longitudinal (■) muscle strips and mean concentration-response curves to NO (a) and vasoactive intestinal polypeptide (VIP) (b) in circular (○) and longitudinal (●) muscle strips. Mean ± s.e.mean of $n = 4$–6 in (a) and 7–9 in (b). The responses to electrical field stimulation and to NO or VIP were obtained in parallel tissues from the same animals.

developed at a slow rate and were sustained (Figure 1d). The VIP-induced responses were not potentiated in the presence of 50 mg l^{-1} bovine serum albumin and 30 mg l^{-1} bacitracin ($n = 2$). The relaxation obtained with the highest concentration of VIP tested (3×10^{-7} M) was less pronounced than that induced by cumulative stimulation at the highest frequency (8 Hz; Figure 2). Tetrodotoxin did not influence the effect of NO and VIP ($n = 4$ for NO and VIP in both types of strips). The administration of TTX did not influence the tone of the tissues. IBMX, forskolin, SNP and zaprinast all caused concentration-dependent relaxations (Figure 3). The amplitude of the relaxations was similar in both types of strips for SNP and zaprinast whereas the responses to forskolin and IBMX were less pronounced in longitudinal muscle strips, reaching significance at 10^{-5} M forskolin ($P < 0.01$, unpaired t test) and

3×10^{-5} M IBMX ($P < 0.05$, unpaired t test). The relaxations developed at a slow rate, the time to peak relaxation being the shortest for SNP (5–10 min) and the longest for forskolin (> 15 min).

Influence of L-NAME, α-chymotrypsin, zaprinast and methylene blue

L-NAME (3×10^{-4} M, incubated for 15 min before inducing contraction with 5-hydroxytryptamine) clearly reduced the relaxant responses to electrical field stimulation, either with 10 s trains or with cumulative increase of the frequency in circular muscle strips (Figure 4b). At the higher frequencies of stimulation, the reduction was more pronounced versus responses to train stimulation than versus those to cumulative stimulation. (The % reduction at 2, 4 and 8 Hz was 84±2, 70±5 and 55±6 for the short-lasting relaxations and 67±6, 51±9 and 40±11 for sustained relaxations, $n=8$). When 10^{-3} M L-arginine was administered 15 min before L-NAME, the inhibitory effect of L-NAME versus the responses to train stimulation was nearly abolished, while that versus the responses to cumulative stimulation was reduced (Figure 4c). At a stimulation frequency of 0.5 and 1 Hz, the response to cumulative stimulation in the presence of L-arginine plus L-NAME was still significantly decreased (Figure 4c) but when comparing this decrease with that in the presence of L-NAME alone (Figure 4b), it was significantly smaller ($P < 0.05$, unpaired t test), illustrating the effect of L-arginine. The incubation of 10^{-3} M D-arginine before L-NAME did not influence its effect (Figure 4d). The results with L-NAME and L- and D-arginine ($n=4$–8 for each series) in the longitudinal muscle strips were the same with regard to train stimulation. With regard to relaxations in response to cumulative stimulation, the inhibitory effect of L-NAME was less pronounced than in circular muscle strips. (The % reduction of the stimulation-induced relaxation by L-NAME at 2, 4 and 8 Hz e.g. was 36±9, 24±11 and 14±16% respectively, $n=8$). Neither L-arginine ($n=5$) nor D-arginine ($n=4$) influenced the inhibitory effect of L-NAME. L-NAME (3×10^{-4} M) did not influence the relaxant responses to 2×10^{-6}–10^{-4} M NO in circular ($n=8$) and longitudinal ($n=7$) muscle strips, nor did it influence the relaxation induced by 10^{-8}–3×10^{-7} M VIP. In circular muscle strips e.g., the VIP-induced relaxation was 7±2, 16±5, 32±8 and 52±7% at 10^{-8}, 3×10^{-8}, 10^{-7} and 3×10^{-7} M respectively, in the presence of L-NAME ($n=6$), while the results in the presence of the solvent in parallel control strips were 11±2, 22±4, 41±8 and 63±9%, respectively ($n=6$). The administration of L-NAME consistently induced an increase in resting tone of the tissues. When testing it versus the relaxations induced by NO e.g., the increase in tone was 24±6% in circular muscle strips ($n=8$) and 26±6% in longitudinal muscle strips ($n=7$), expressed as a percentage of the preceding 5-hydroxytryptamine-induced contraction. In circular but not in longitudinal muscle strips, the contractile effect of L-NAME was smaller after previous administration of L-arginine. This reached significance in the series, where these agents were tested versus the responses to cumulative stimulation (25±4%, $n=7$, for L-NAME; 10±2%, $n=5$, for L-NAME in the presence of L-arginine; $P < 0.05$).

α-Chymotrypsin was tested versus the relaxation induced by VIP and cumulative stimulation. When incubated for 30 min in a concentration of 2 u ml^{-1}, it completely prevented the relaxant effect of VIP ($n=3$ in both types of strips), but it had no significant influence on the relaxations induced by cumulative stimulation ($n=8$ for both types of strips). In a concentration of 10 u ml^{-1}, α-chymotrypsin had some influence on the responses to cumulative stimulation. In circular muscle strips, the reduction of the relaxant responses to cumulative stimulation in the presence of 10 u ml^{-1} α-chymotrypsin (Figure 5b) was more pronounced than in the presence of its solvent at 0.5, 1 and 2 Hz ($P < 0.05$, unpaired t test). In the longitudinal control muscle strips, the frequency-response

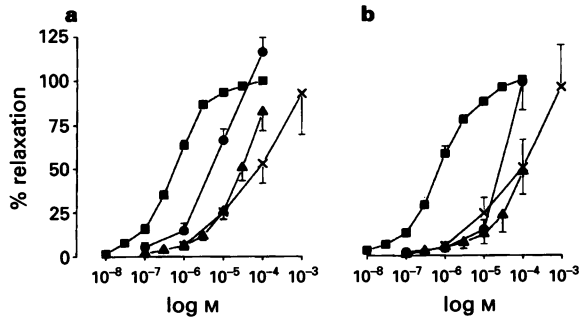

Figure 3 Mean concentration-response curves in circular (a) and longitudinal (b) muscle strips for the relaxant effect of sodium nitroprusside (■, $n=5$ in both types of strips), forskolin (●, $n=7$ circular and 5 longitudinal muscle strips), 3-isobutyl-1-methylxanthine (IBMX, ▲, $n=8$ circular and 4 longitudinal muscle strips) and zaprinast (x, $n=5$ circular and 3 longitudinal muscle strips). Mean±s.e.mean.

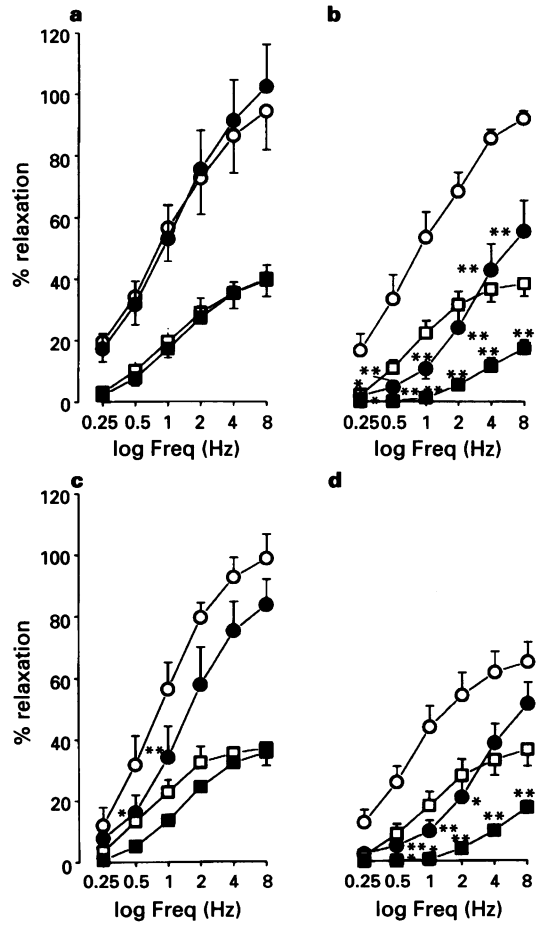

Figure 4 Mean frequency-response curves in circular muscle strips for the relaxant response to electrical field stimulation (40 V, 0.1 ms) with 10 s trains (□, ■) or with cumulative increase of the frequency (○, ●). (a) In control tissues, 2 frequency-response curves were constructed before (open symbols) and after (closed symbols) addition of the solvent of N^G-nitro-L-arginine methyl ester (L-NAME) ($n=7$–8). (b) Responses in the absence (open symbols) and presence (closed symbols) of 3×10^{-4} M L-NAME ($n=8$). (c,d) As for (b) but tissues were incubated with 10^{-3} M L-arginine (c, $n=5$–6) or 10^{-3} M D-arginine (d, $n=5$–6) before L-NAME was administered. Mean±s.e.mean.
*$P < 0.05$, **$P < 0.01$: significantly different from the response before addition of L-NAME and L- or D-arginine.

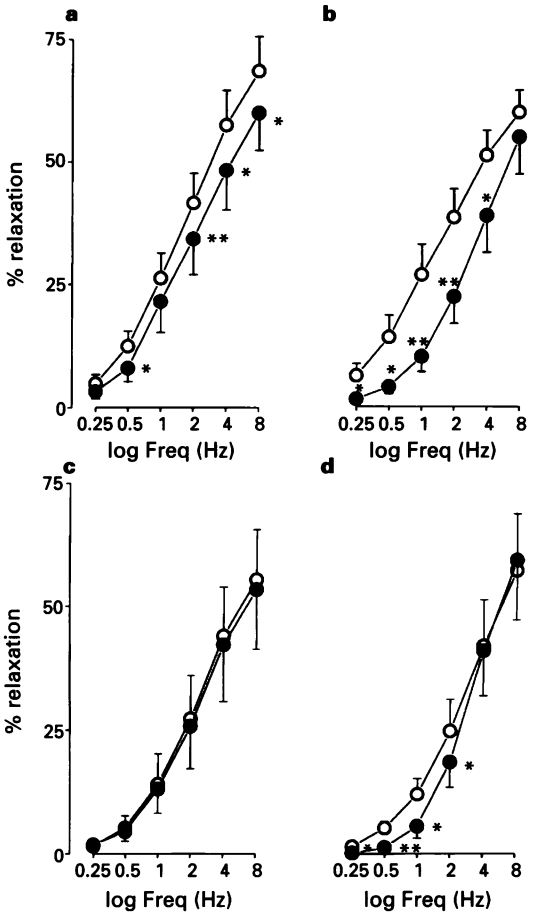

Figure 5 Mean frequency-response curves in circular (upper panel) and longitudinal (lower panel) muscle strips for the relaxant response to electrical field stimulation (40 V, 0.1 ms) with cumulative increase of the frequency. (a,c) Responses in the absence (○) and presence (●) of the solvent of α-chymotrypsin. (b,d) Responses in the absence (○) and presence (●) of 10 u ml^{-1} α-chymotrypsin. Mean ± s.e.mean of $n = 7$–8 in each series. $*P < 0.05$; $**P < 0.01$: significantly different from the response before addition of α-chymotrypsin or its solvent.

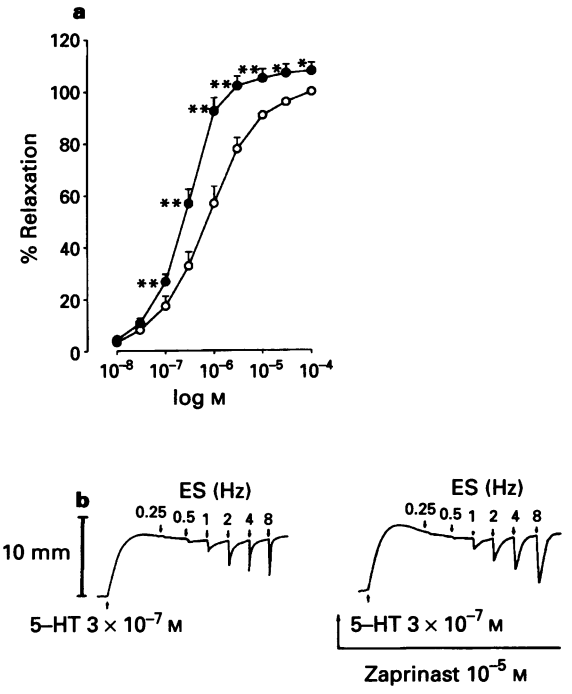

Figure 6 Influence of zaprinast in circular muscle strips. (a) Mean concentration-response curves for the relaxant effect of sodium nitroprusside (SNP) before (○) and in the presence (●) of zaprinast. Mean ± s.e.mean of $n = 8$. $*P < 0.05$; $**P < 0.01$: Significantly different from the response before addition of zaprinast. (b) Representative trace showing the responses to electrical stimulation (ES) with 10 s trains at increasing frequencies before and in the presence of 10^{-5} M zaprinast.

curve to cumulative stimulation was perfectly reproducible; α-chymotrypsin at 10 u ml^{-1} moderately reduced the responses at 0.25 to 2 Hz. The concentration of 10 u ml^{-1} α-chymotrypsin markedly increased resting tone by 82 ± 3% in circular ($n = 8$) and 103 ± 23% in longitudinal ($n = 8$) muscle strips, expressed as a percentage of the preceding 5-hydroxytryptamine-induced contraction. To have a comparable plateau contraction as in the parallel control strips, incubated with the solvent of α-chymotrypsin, the concentration of 5-hydroxytryptamine administered was lowered or no 5-hydroxytryptamine was administered.

The influence of zaprinast was tested versus the relaxation induced by SNP and train stimulation. When incubated for 30 min at a concentration of 3×10^{-6} M before induction of tone with 5-hydroxytryptamine, zaprinast did not significantly influence the relaxations to SNP and train stimulation (only tested in circular muscle strips). Zaprinast, 10^{-5} M, was then studied in circular and longitudinal muscle strips. During the incubation with 10^{-5} M zaprinast, tone tended to decrease but the 5-hydroxytryptamine-induced contraction was similar to that in the control strips. In circular muscle control strips, the concentration-response curve to SNP was reproducible, except for a small though significant decrease at 3×10^{-7} and 10^{-6} M and increase at 3×10^{-5} and 10^{-4} M ($n = 8$). In tissues incubated with zaprinast, the response to SNP was significantly increased from 10^{-7} to 10^{-4} M (Figure 6a). Although zaprinast did not change the amplitude of the relaxant responses to electrical train stimulation, it clearly took more time in the presence of zaprinast to return to the preceding tone level once the maximal amplitude of relaxation was reached (Figure 6b). When considering the time from the start of relaxation until the return to the existing tone, the ratio between this parameter in the presence of zaprinast compared to that before its addition was significantly higher at 2 Hz (2.4, $P < 0.05$), 4 Hz (3.6, $P < 0.01$) and 8 Hz (4.5, $P < 0.01$) of stimulation than in control tissues (corresponding values 1.1, 1.2 and 1, $n = 6$). In longitudinal muscle control strips, the concentration-response curve to SNP was reproducible ($n = 5$) while zaprinast significantly enhanced the relaxations to SNP at 10^{-7} to 10^{-5} M ($n = 5$). Zaprinast did not influence the amplitude or the duration of the relaxant responses to the electrical train stimulation ($n = 5$).

Methylene blue at 3×10^{-5} M (incubated for 30 min) did not influence the relaxations induced by train stimulation ($n = 4$ for both circular and longitudinal muscle strips). At 10^{-4} M, methylene blue clearly inhibited the responses to stimulation at 2, 4 and 8 Hz. In circular muscle strips, the relaxation induced by stimulation at 8 Hz for 10 s e.g. was significantly reduced from 44 ± 4% to 22 ± 7% ($n = 4$), while the response was well maintained in control strips (42 ± 5% and 43 ± 4%, $n = 3$).

Cyclic nucleotide measurements

The cyclic GMP and cyclic AMP content in circular muscle strips in basal conditions and upon relaxation is given in Table 1. The administration of 3×10^{-7} M 5-hydroxytryptamine did not influence the cyclic GMP content of the tissues but it significantly lowered the cyclic AMP content. The responses after application of a relaxant stimulus were therefore compared with that after administration of 5-hydroxytryptamine. Electrical train stimulation at 4 Hz induced a relaxation of 37 ± 7 (10 s), 76 ± 11 (30 s), 104 ± 15 (2 min) and 115 ± 22 (5 min) %, while 3×10^{-7} M VIP in this series induced a pronounced relaxation of 134 ± 13%; 10^{-4} M NO relaxed the tissues for

Table 1 The cyclic AMP and cyclic GMP content (pmol mg^{-1} protein) in basal conditions, after administration of 3×10^{-7} M 5-hydroxytryptamine (5-HT) and after administration of different relaxant stimuli in addition to 5-HT in circular muscle strips

	Cyclic GMP (pmol mg^{-1} protein)	*Cyclic AMP* (pmol mg^{-1} protein)
Basal	0.68 ± 0.10 $n=7$	8.70 ± 0.47** $n=7$
5-HT, 3×10^{-7} M	0.66 ± 0.07 $n=13$	6.62 ± 0.61 $n=13$
ES, 4 Hz, 10 s	0.99 ± 0.11* $n=9$	5.80 ± 0.43 $n=8$
ES, 4 Hz, 30 s	1.16 ± 0.15* $n=9$	7.67 ± 0.74 $n=9$
ES, 4 Hz, 2 min	1.07 ± 0.15* $n=9$	8.41 ± 1.27 $n=8$
ES, 4 Hz, 5 min	1.04 ± 0.11** $n=9$	8.56 ± 0.54* $n=9$
VIP, 3×10^{-7} M	0.71 ± 0.06 $n=14$	7.32 ± 0.34 $n=14$
NO, 10^{-4} M	24.37 ± 5.76** $n=10$	9.67 ± 2.14 $n=10$

ES indicates electrical stimulation. Mean ± s.e.mean. *$P<0.05$, **$P<0.01$, significantly different from the results after administration of 3×10^{-7} M 5-HT.

$77 \pm 17\%$. Electrical stimulation at 4 Hz induced a modest though significant increase in cyclic GMP content, that was similar for the different periods of stimulation. VIP did not change the cyclic GMP content but NO caused a nearly 40 fold increase in cyclic GMP content. None of the relaxant stimuli manifestly changed the cyclic AMP content, although the small increase in cyclic AMP content during electrical stimulation at 4 Hz for 5 min reached the level of significance.

Immunocytochemistry and NADPHd-histochemistry

Both NOS-immunocytochemistry and NADPHd-histochemistry revealed an identical pattern of nitrergic innervation in the fundic wall. NOS-immunoreactivity was mainly found within neuronal cell bodies of the external submucous and myenteric plexuses (Figure 7). As in other mammals, the internal submucous plexus is poorly developed in the pig stomach and harbours only occasionally an NOS-immunoreactive neurone. Double-labelling for NOS and VIP illustrated that both substances coexist in a major part of the intrinsic nitrergic neurones of the external submucous and myenteric plexuses (Figure 9). In the circular as well as the longitudinal muscle, a similar fibre pattern of relative density was seen after NOS/NADPHd-staining and VIP-labelling (Figures 8,9). Colocalization of NOS and VIP was also found in the majority of the nitrergic fibres innervating the circular and longitudinal muscle (Figure 9).

Discussion

The aim of this study was to investigate the role of NO and VIP as inhibitory NANC neurotransmitters in the pig gastric fundus. The criterion for a putative neurotransmitter, that it or its synthesizing system should be present in neurones and nerve terminals, is fulfilled for both NO and VIP. NOS immunocytochemistry was found within the neuronal cell bodies of the submucous and myenteric plexuses and in nerve fibres within both the circular and longitudinal muscle layer. The same pattern was revealed with NADPHd-histochemistry suggesting that the NADPHd-activity is due to the presence of NOS (Dawson *et al.*, 1991; Hope *et al.*, 1991). Although discrepant staining with NADPHd-histochemistry and NOS-immunochemistry has been reported for the central nervous system (Vizzard *et al.*, 1994), complete overlap for the constitutive neuronal isoform of NOS and NADPHd has been found in the enteric nervous system of different species (Belai *et al.*, 1992; Saffrey *et al.*, 1992; Ward *et al.*, 1992; Young *et al.*, 1992; Barbiers *et al.*, 1993; Timmermans *et al.*, 1994a,b). In the majority of nitrergic neurones and nerve fibres, VIP was co-

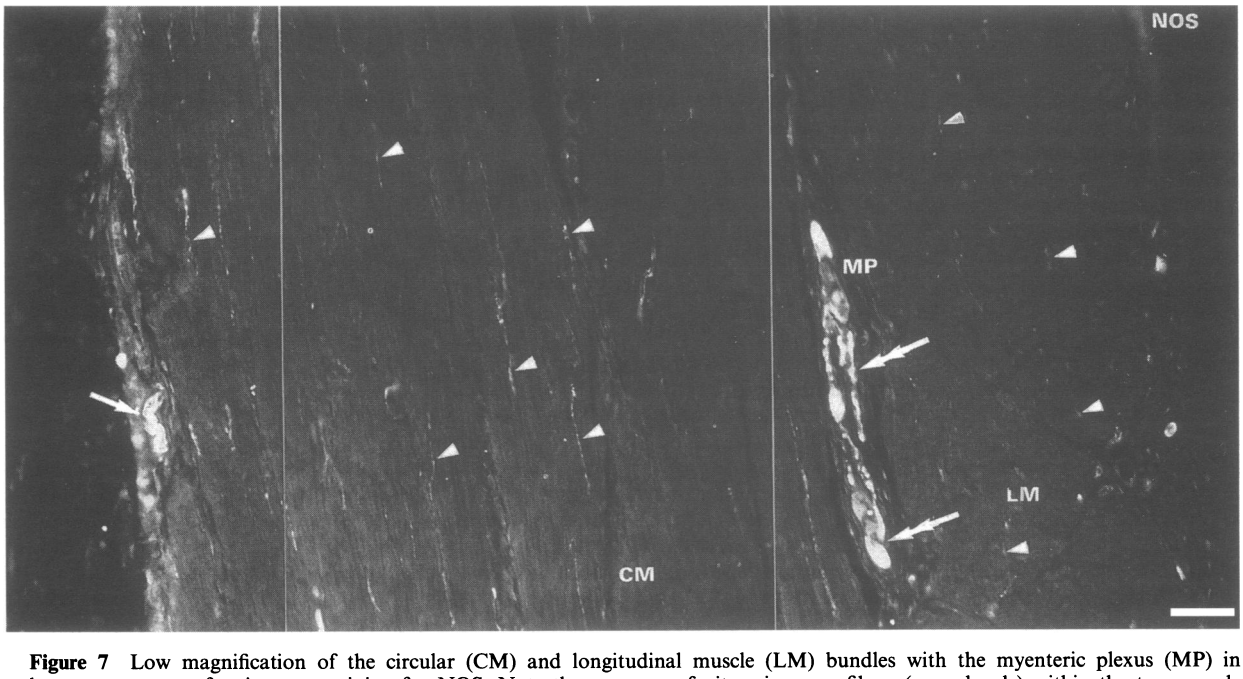

Figure 7 Low magnification of the circular (CM) and longitudinal muscle (LM) bundles with the myenteric plexus (MP) in between, as seen after immunostaining for NOS. Note the presence of nitrergic nerve fibres (arrowheads) within the two muscle layers as well as immunoreactive perikarya in ganglia of the myenteric (double-headed arrows) and outer submucous plexus (arrow). Scale bar = 100 μm (×95).

localized, which contrasts with previous findings in the porcine small and large intestine, where only a minor part of the nitrergic neurones stained for VIP (Barbiers *et al.*, 1993; Timmermans *et al.*, 1994b). Also in other species, the degree of colocalization of NOS and VIP differs between different parts of the gastrointestinal tract. In the duodenum of the rat, virtually all VIP-positive neurones showed NADPHd-activity (Aimi *et al.*, 1993), while VIP-immunostained neurones in the rat gastric corpus were not NADPHd-positive (Forster & Southam, 1993). The colocalization of NOS and VIP in the neurones and nerve fibres of the pig gastric fundus suggests that NO and VIP might be inhibitory cotransmitters, relaxing the circular and longitudinal muscle layer, but it does not necessarily mean a parallel release at all levels of stimulation. Release of classical neurotransmitters such as acetylcholine and noradrenaline in general occurs at lower frequencies of stimulation than release of the colocalized peptide neurotransmitters (Bartfai *et al.*, 1988). In the postganglionic parasympathetic neurones of the cat submandibular gland e.g., VIP is stored together with acetylcholine but mainly released at high stimulation frequencies (Lundberg, 1981; Burnstock, 1990).

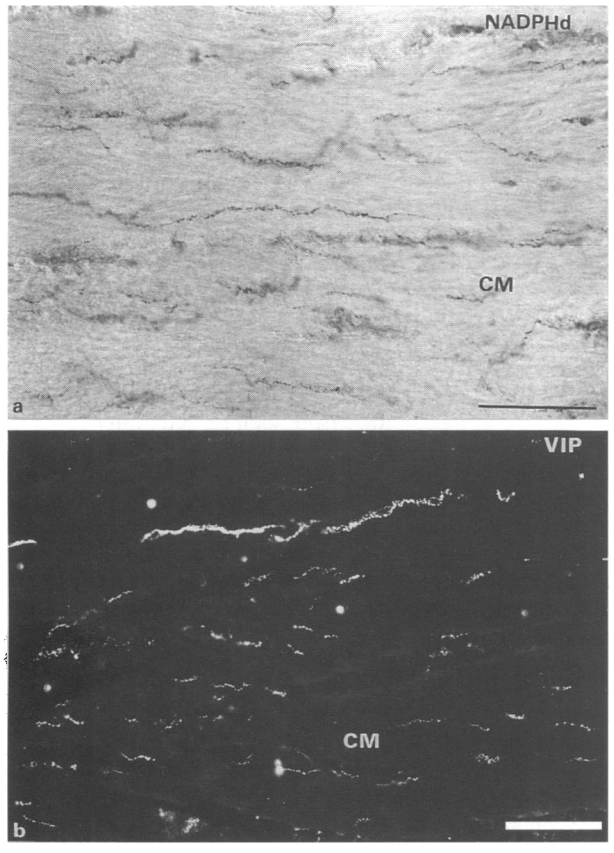

Figure 8 (a) NADPHd-staining shows the presence of a relative dense nitrergic innervation pattern of the outer muscle layers of the fundus. (b) A similar pattern of fibre density can be seen after immunolabelling for vasoactive intestinal polypeptide (VIP). CM: Circular muscle layer. Scale bar = 100 μm (a, ×190; b, ×170).

The concentration-dependent effect of forskolin, a direct activator of the catalytic subunit of adenylate cyclase (Seamon & Daly, 1981) and of zaprinast, a selective cyclic GMP-specific phosphodiesterase inhibitor (Beavo & Reifsnyder, 1990) illustrates that a cyclic AMP- and a cyclic GMP-dependent pathway for relaxation are present in the pig gastric fundus. Also the exogenous NO-donor, SNP, and IBMX induced concentration-dependent relaxations. IBMX is a non-selective phosphodiesterase inhibitor (Beavo & Reifsynder, 1990) but some preference for cyclic AMP-degrading phosphodiesterases has been reported (Fujimoto & Matsudo, 1990; Barbier & Lefebvre, 1992). The cyclic AMP- and cyclic GMP-dependent relaxant pathways are present in both circular and longitudinal muscle strips, although the efficacy of the cyclic AMP-dependent pathway seems smaller in the longitudinal strips.

As NO seems mainly to be released during short-lasting electrical field stimulation and at the beginning of a more sustained stimulation, while VIP contributes to sustained relaxation in the rat, cat and ferret stomach (Boeckxstaens *et al.*, 1992; Barbier & Lefebvre, 1993; Grundy *et al.*, 1993), 2 types of electrically induced NANC relaxations were studied: short-lasting relaxation induced by 10 s trains of pulses concentrating on a possible role of NO and sustained relaxation by continuous electrical field stimulation concentrating on a possible role of VIP. Our results suggest that NO, via the cyclic GMP-dependent pathway of relaxation, is involved in short-

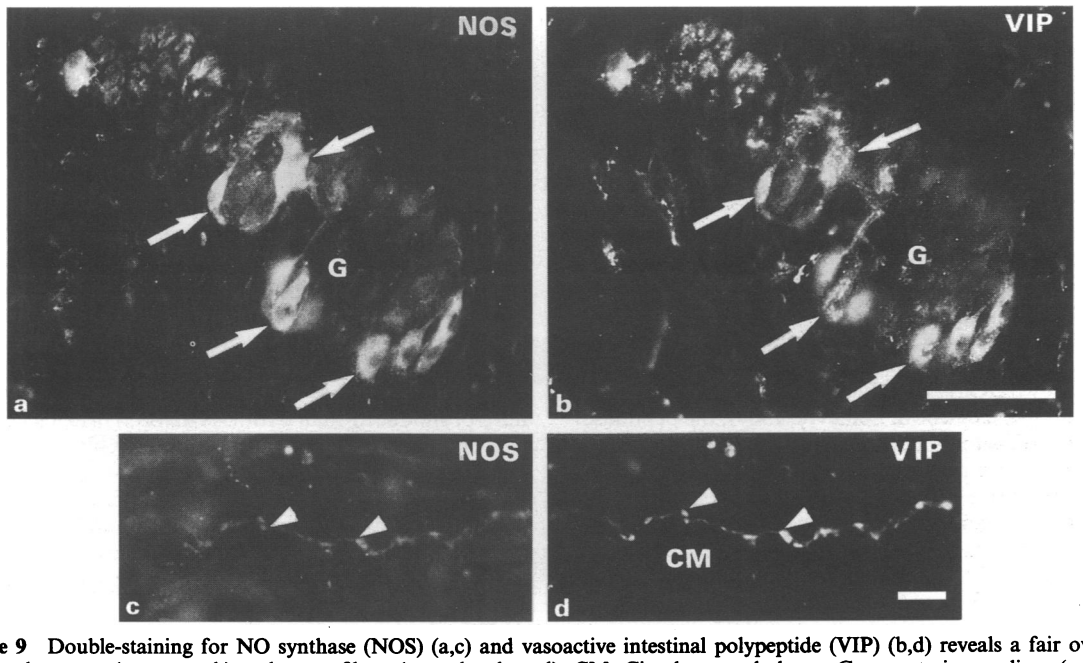

Figure 9 Double-staining for NO synthase (NOS) (a,c) and vasoactive intestinal polypeptide (VIP) (b,d) reveals a fair overlap in neuronal somata (arrows; a,b) and nerve fibres (arrowheads; c,d). CM: Circular muscle layer; G: myenteric ganglion. (a,b). Scale bar = 100 μm (×220); (c,d) Scale bar = 10 μm (×670).

lasting NANC relaxation for the following reasons: (1) The shape of the relaxation induced by exogenous NO was similar to that induced by train stimulation. The somewhat faster return to the original level of contraction after exogenous NO is probably related to the bolus administration whereby NO breakdown immediately starts without any further addition of NO, whereas the NO release probably continues for 10 s during train stimulation. (2) The short-lasting NANC relaxations were inhibited by the NO synthase inhibitor L-NAME and this inhibition was stereospecifically prevented by L-arginine. (3) The short-lasting NANC relaxations were potentiated by zaprinast and reduced by methylene blue, suggesting the involvement of cyclic GMP. Zaprinast increased the amplitude of the SNP-induced contractions but only retarded the return to the pre-existing level of contraction for the short-lasting NANC relaxations. This might be related to the length of the relaxation and the speed of development. Also in the rat and cat gastric fundus, we previously observed that a cyclic GMP-mediated relaxation should be prolonged and accompanied by a sustained accumulation of cyclic GMP to observe an increase in amplitude by zaprinast (Barbier & Lefebvre, 1992; 1995). We have no explanation as to why zaprinast did not influence the short-lasting NANC relaxations in the longitudinal muscle strips of the pig gastric fundus. Methylene blue is generally considered a guanylate cyclase inhibitor but inhibition of NO synthesis (Mayer *et al.*, 1993) and generation of superoxide anions (Marczin *et al.*, 1992) have also been proposed as mechanisms for its inhibitory effect on endothelium-dependent vascular smooth muscle relaxation. In a concentration (3×10^{-5} M) that selectively reduced the relaxations induced by SNP, in the opossum lower oesophageal sphincter and internal anal sphincter (Moummi & Rattan, 1988; Rattan & Moummi, 1989), methylene blue did not reduce short-lasting NANC relaxations in the pig gastric fundus. We cannot exclude the possibility that the inhibitory effect seen with 10^{-4} M methylene blue is due to non-specific actions. (4) The involvement of cyclic GMP in the short-lasting relaxations is confirmed by the cyclic nucleotide measurements, that were limited to circular muscle strips. The 1.5 fold rise in cyclic GMP induced by a 10 s train at 4 Hz was less pronounced that we observed for train stimulation in the rat gastric fundus (Smits & Lefebvre, 1995) but similar to that observed in the rat ileum (Kanada *et al.*, 1992). A nearly 40 fold increase in cyclic GMP content was observed with 10^{-4} M NO, although the amplitude of relaxation was only twice as pronounced than with the 10 s train stimulation at 4 Hz. A similar differentiation in the cyclic GMP increase between electrical field stimulation and NO was observed in sheep urethral muscle (Garcia-Pascual & Triguero, 1994) but not in the rat gastric fundus (Smits & Lefebvre, 1995). NO released from neurones by electrical stimulation in some tissues might evoke a more localized cyclic GMP increase than exogenous NO, that is in contact with the whole tissue.

Less evidence was obtained for a role of VIP or a peptide in sustained NANC relaxation. Exogenous administration of VIP induced prolonged relaxation but except for the strips used for cyclic nucleotide measurements, the amplitude of relaxation was always less pronounced than that obtained with continuous electrical stimulation. Peptidases have been shown to reduce preferentially the NANC relaxation, induced by sustained stimulation, in the rat gastric fundus (Boeckxstaens *et al.*, 1992; D'Amato *et al.*, 1992). In a concentration of 10 u ml^{-1}, that was shown clearly to reduce NANC relaxation in the rat gastric fundus (De Beurme & Lefebvre, 1987), the peptidase α-chymotrypsin only moderately reduced the relaxation amplitude at lower frequencies of stimulation. The fact that a higher concentration of α-chymotrypsin was needed to see any effect on electrically induced NANC relaxations than was required to antagonize exogenous VIP, might be related to the difficult penetration of the enzyme in the synaptic cleft. These penetration problems might also explain the modest inhibitory effect of α-chymotrypsin and leave the possibility of a more pronounced contribution of peptides. Still, the clear antagonizing effect of sustained NANC relaxations by L-NAME suggest that NO is also an important neurotransmitter for this type of relaxation. The inhibitory action of L-NAME on sustained relaxation was stereospecifically prevented by L-arginine in circular but not in longitudinal muscle strips. Yet a non-specific effect of L-NAME in the latter strips seems unlikely as relaxations induced by NO and VIP were not influenced. The antagonizing effect of L-NAME on sustained NANC relaxations was most pronounced at the lower frequencies of stimulation, where the moderate effect of α-chymotrypsin was observed. No arguments for a link between VIP and NO were found. In the guinea-pig gastric fundus and colon, and in the rabbit stomach, VIP has been shown to induce relaxation partially by stimulation of NO production from the smooth muscle cells (Grider *et al.*, 1992; Grider, 1993; Murthy *et al.*, 1993). Although the axonal conductance blocker, tetrodotoxin, did not influence the relaxation induced by exogenous VIP in the pig gastric fundus, suggesting a direct muscular effect of VIP, the VIP-induced relaxation was not influenced by L-NAME, excluding the possibility that VIP activates a NO synthase within the experimental conditions. Tone had to be raised to obtain consistent relaxations in the pig gastric fundus. In the rabbit stomach, increasing tone by activators of protein kinase C inhibits VIP-stimulated NO synthase activity in the gastric smooth muscle cells (Murthy *et al.*, 1994). Although, to our knowledge, the 5-hydroxytryptamine receptor mediating contraction in the pig gastric fundus has not been characterized, it is likely that this receptor belongs to the classical 5-hydroxytryptamine$_2$ receptor family, mediating contraction by stimulation of phosphoinositide metabolism and protein kinase C (Hoyer *et al.*, 1994). Thus we cannot exclude the possibility that the increase in tone induced by 5-hydroxytryptamine might mask stimulation of a muscular NOS by VIP. On the other hand, *in vivo* gastric tone is partially cholinergic and the acetylcholine-induced stimulation of protein kinase C should then also mask the stimulation of the muscular NOS.

We have previously shown in the guinea-pig gastric fundus that sustained relaxation can also be obtained with exogenous NO provided it is continuously infused into the organ bath (Lefebvre *et al.*, 1992). The involvement of NO in sustained NANC relaxations in the pig gastric fundus is also corroborated by the maintained increase in cyclic GMP when stimulating the tissues for 30 s, 2 min and 5 min. The cyclic AMP content was increased significantly only after 5 min of stimulation, and no increase of the cyclic AMP content was observed upon maximal relaxation with 3×10^{-7} M VIP. This is in contrast to the rat gastric fundus (Ito *et al.*, 1990) and might be related to compartmentalization of the cyclic AMP increase due to VIP (Murray, 1990) or to a high level of cyclic AMP-directed phosphodiesterase activity (Bitar & Makhlouf, 1982). Alternatively, the relaxant effect of VIP can be mediated by additional transduction mechanisms (Gozes & Brenneman, 1989).

L-NAME consistently induced contraction and at least in circular muscle strips, this effect was partially prevented by L-arginine suggesting that it is, at least in part, related to suppression of NO synthesis and thus of a tonic nitrergic inhibition. As tetrodotoxin did not influence the tone of the tissues, tonic release of NO via action potential conduction seems excluded but some leakage of NO out of the nitrergic nerve endings might occur (Boeckxstaens *et al.*, 1991). The peptidase α-chymotrypsin produced an increase in tone, that was clearly more pronounced than with L-NAME. This effect has already been described in the rat gastric fundus (Gilfoil & Kelly, 1966) and most probably reflects a non-specific action and not cleavage of a continuously leaking inhibitory peptide.

It is concluded that a cyclic GMP- and a cyclic AMP-dependent pathway for relaxation are present in both the circular and longitudinal muscle layer of the pig gastric fundus. NO appears to contribute to short-lasting as well as sustained NANC relaxations. A peptide, possibly VIP, might be involved during sustained stimulation at lower frequencies of stimulation.

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