British Journal of Pharmacology (2000) 129, 12–20

www.nature.com/bjp

Role of nitric oxide- and vasoactive intestinal polypeptide-containing neurones in human gastric fundus strip relaxations

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1 The morphological pattern and motor correlates of nitric oxide (NO) and vasoactive intestinal polypeptide (VIP) innervation in the human isolated gastric fundus was explored.

2 By using the nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)-diaphorase and specific rabbit polyclonal NO-synthase (NOS) and VIP antisera, NOS- and VIP-containing varicose nerve fibres were identified throughout the muscle layer or wrapping ganglion cell bodies of the myenteric plexus. NOS-immunoreactive (IR) neural cell bodies were more abundant than those positive for VIP-IR. The majority of myenteric neurones containing VIP coexpressed NADPH-diaphorase.

3 Electrical stimulation of fundus strips caused frequency-dependent NANC relaxations. N^G-nitro-L-arginine (L-NOARG: 300 μM) enhanced the basal tone, abolished relaxations to 0.3–3 Hz (5 s) and those to 1 Hz (5 min), markedly reduced ($\sim$50%) those elicited by 10–50 Hz, and unmasked or potentiated excitatory cholinergic responses at frequencies $\geqslant$1 Hz. L-NOARG-resistant relaxations were virtually abolished by VIP (100 nM) desensitization at all frequencies.

4 Relaxations to graded low mechanical distension ($\leqslant$1 g) were insensitive to tetrodotoxin (TTX: 1 μM) and L-NOARG (300 μM), while those to higher distensions (2 g) were slightly inhibited by both agents to the same extent ($\sim$25%).

5 In the human gastric fundus, NOS- and VIP immunoreactivities are colocalized in the majority of myenteric neurones. NO and VIP mediate electrically evoked relaxations: low frequency stimulation, irrespective of the duration, caused NO release only, whereas shortlasting stimulation at high frequencies induced NO and VIP release. Relaxations to graded mechanical distension were mostly due to passive viscoelastic properties, with a slight NO-mediated neurogenic component at 2 g distension. The difference between NO and VIP release suggests that in human fundus accommodation is initiated by NO.

British Journal of Pharmacology (2000) **129,** 12–20

Keywords: Human gastric fundus; VIP- and NOS-immunohistochemistry; NADPH-diaphorase histochemistry; NANC inhibitory nerves; electrically-induced relaxations; mechanically-induced relaxations

Abbreviations: IR, immunoreactive; L-NOARG, N^G-nitro-L-arginine; NADPH-diaphorase, nicotinamide adenine dinucleotide phosphate hydrogen-diaphorase; NANC, non-adrenergic, non-cholinergic; NO, nitric oxide; NOS, nitric oxide synthase; TTX, tetrodotoxin; VIP, vasoactive intestinal polypeptide

Introduction

Non-adrenergic, non-cholinergic (NANC) inhibitory neurones in the myenteric plexus are present throughout the gastrointestinal tract of several animal species, including humans (Furness *et al*., 1988; Wattchow *et al*., 1995). These neurones are involved in the relaxation of sphincter regions (Allescher *et al*., 1992; Rattan & Chakder, 1992; Preiksaitis *et al*., 1994), in the accommodation processes of the circular muscle in the small (Waterman *et al*., 1994) and large intestine (Ciccocioppo *et al*., 1994), as well as in the descending reflex relaxation in response to localized gut wall distension (Tonini *et al*., 1982; Grider & Makhlouf, 1986) and peristalsis (Ciccocioppo *et al*., 1994). Adenosine 5′-triphosphate (ATP) (Hoyle & Burnstock, 1989), vasoactive intestinal polypeptide (VIP) (Fahrenkrug, 1989), and nitric oxide (NO) (Sanders & Ward, 1992) are currently considered the main transmitters responsible for neurogenic NANC relaxations. Although each of these transmitters may possess an important role as an inhibitory mediator (depending on the enteric segment, the type of stimulation, and the animal species under investigation), it appears now clear that most neurogenic relaxations are mediated by the release of more than one transmitter (Costa *et al*., 1986; Crist *et al*., 1992; Maggi & Giuliani, 1993; 1996).

In the stomach, NANC inhibitory neurones are responsible for receptive relaxation, a response through which the gastric fundus adapts to food and fluids, which is characterized by graded wall distension with a low increase in intragastric pressure (Abrahamsson, 1973; Desai *et al*., 1991). Evidence from rat, cat and pig isolated gastric fundus specimens suggests that neurogenic relaxations evoked by electrical field stimulation depend on a combination of NO and VIP release (Li & Rand, 1990; Boeckxstaens *et al*., 1992; D'Amato *et al*., 1992;

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Barbier & Lefebvre, 1993; Lefebvre *et al*., 1995), which may act as co-transmitters. Other findings suggest that NO is the predominant transmitter mediating accommodation to fluids and relaxation to short and sustained electrical field stimulation in the guinea-pig isolated stomach (Desai *et al*., 1991; Lefebvre *et al*., 1992). Furthermore, NO plays a pivotal role in gastric fundus relaxations to electrical vagal stimulation in intact dogs (Meulemans *et al*., 1995), and in isolated stomach preparations from the guinea-pig (Desai *et al*., 1994; Meulemans *et al*., 1995) and mouse (Yano *et al*., 1995).

In the human isolated gastric fundus, the nature of neurogenic NANC relaxations elicited either electrically or mechanically has never been explored. This study was therefore designed to examine the distribution of NO synthesizing and VIP containing neurones in human gastric fundus specimens, and to establish their role in the control of fundus strip relaxations.

Methods

Tissue collection and preparation

Fresh specimens of human gastric fundus were obtained from ten male and three female patients (age range, 47–82 years) undergoing total gastrectomy due to cancer. The use of human tissue was approved by the Ethics Committees of the Universities of Pavia and Bologna. As soon as the organ was removed, a portion of apparently normal gastric fundus was placed in oxygenated (95% O_2 and 5% CO_2) modified Krebs solution at 4°C and transported to the laboratory within 30–45 min. All specimens were used for functional studies; four of these specimens were also used for morphological studies. In this case, gastric fundus specimens were thoroughly rinsed with saline, stretched slightly and pinned on wax plates. Specimens were fixed by immersion-fixation with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, overnight at 4°C and subsequently placed in 25% sucrose in 0.1 M PB for cryoprotection until sectioning. Tissues were cut with a cryostat at 10–12 μm, mounted onto chrome-alum gelatin coated slides, and stored at −20°C until processing.

To evaluate the general morphology and the quality of the collected tissues, some sections from each gastric fundus specimen were stained with haematoxylin-eosin. No obvious abnormalities were detected in the mucosa, submucosa and muscle layers.

Immunohistochemistry

Tissue sections were processed with the indirect immunofluorescence method (De Giorgio *et al*., 1992). Briefly, sections were washed in 0.1 M PB, pretreated for 30 min at room temperature with 10% normal goat serum, and incubated in rabbit VIP (VIP7913, 1 : 500) (Furness *et al*., 1981) or neuronal NOS polyclonal antisera (1 : 100) (Schmidt *et al*., 1992) overnight at 4°C in a humid chamber. Sections were then washed in PB and incubated for 2 h at room temperature in affinity purified goat anti-rabbit IgG (diluted 1 : 50) conjugated to either fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate (Sigma Immunochemicals, St. Louis, MO, U.S.A.). Sections were washed again in 0.1 M PB and coverslipped with 9 : 1 glycerol/PB. Both primary and secondary antibodies were diluted in 0.5% Triton X-100 in 0.1 M PB. Sections were analysed with a Leitz Dialux microscope using a Ploem epi-illumination system with 'I2' or 'L2' and 'N2' filter cubes to visualize fluorescein isothiocyanate and tetramethyl rhodamine isothiocyanate fluorescence, respectively.

Specificity studies included the following control experiments: (a) omission of the primary antibodies; (b) substitution of neuronal NOS and VIP antibodies with commercially available normal rabbit serum used at a dilution of 1 : 50; and (c) incubation with the primary antibody preadsorbed for 12–16 h at 4°C with 10 μM synthetic VIP peptide (Bachem, Torrance, CA, U.S.A.).

NADPH-diaphorase histochemistry

The histochemical technique was performed as previously described (De Giorgio *et al*., 1994). Cryostat sections were washed in 0.1 M PB (three times, 10 min each), preincubated in 0.3% Triton X-100 in 0.1 M PB for 20 min at room temperature, and then incubated in a mixture containing 1 mg ml^{-1} reduced β-nicotinamide adenine phosphate dinucleotide (NADPH) and 0.25 mg ml^{-1} nitro blue tetrazolium (Sigma, St. Louis, MO, U.S.A.) in 0.1 M PB in the dark at 37°C for 1 h. To stop the reaction, sections were thoroughly washed in 0.1 M PB, dehydrated through graded ethanols, and finally coverslipped with mounting medium. The results obtained were evaluated with a Leitz Dialux microscope using bright field optics.

Control experiments were performed by incubating sections with solutions in which the β-NADPH reagent was omitted.

Double labelling

A sequential staining procedure was used for colocalization experiments (De Giorgio *et al*., 1994). Sections processed for immunofluorescence were analysed and photographed, and the coordinates of the photographic images were recorded for subsequent examination following NADPH-diaphorase staining. After photography, coverslips were removed and sections were thoroughly washed in 0.1 M PB, and then processed for NADPH-diaphorase histochemistry as described above. Preparations were examined, and the same areas previously photographed for VIP-immunoreactivity (IR) were identified by the coordinates with brightfield microscope and photographed again for NADPH-diaphorase staining.

Functional studies

Six to nine circular muscular strips (25 mm long, 3 mm wide) were prepared by removing the serosal and mucosal layers from each surgical segment and were mounted isometrically (tension: 20 mN) in 5 ml organ baths containing modified Krebs solution maintained at 37°C, and gassed with a mixture of 95% O_2 and 5% CO_2. Each strip served for a distinct pharmacological procedure (see below). A minimum initial equilibration period of 90 min was allowed before the experiments were started, during which time the solution was changed every 15 min. The tension was recorded by means of an isometric transducer connected to a paper chart recorder (Servocorder SR6221, Graphtec). Electrical field stimulation was applied *via* two platinum electrodes placed at the top and the bottom of the chamber, connected to a MARB ST 87 stimulator. Isometric motor responses to electrical field stimulation were evoked using trains of pulses at 0.3–50 Hz and 5 s in duration, delivered at 5 min intervals, at 0.5 ms pulse width and 60 V. In some experiments, to assess the nature of the relaxation during sustained stimulation, electrical field stimulation was applied at 1 Hz for 5 min. To investigate the nature of inhibitory

motor responses the following drugs were used: 1 μM tetrodotoxin (TTX), 1 μM phentolamine and 1 μM propranolol (α- and β-adrenoceptor blockers), 300 μM N^G-nitro-L-arginine (L-NOARG: a NO-synthase inhibitor), and 300 μM suramin (a non selective P_2 purinoceptor antagonist). For the same purpose, some tissues were desensitized by high (100 nM) VIP concentrations. Under this condition, the effectiveness of a second VIP administration (100 nM) was markedly reduced after the inhibitory effect of the priming VIP concentration had declined. TTX (1 μM) and hyoscine (3 μM) were used to investigate the type of excitatory motor responses.

In separate experiments, strips were mounted isotonically (basal load: 0.2–0.4 g) in 20 ml organ baths. Isotonic relaxations to graded smooth muscle distension were evoked by enhancing strip load (range 0.25–2 g) for 3 min at 10 min intervals, in the absence and in the presence of TTX (1 μM) or L-NOARG (300 μM).

Data analysis

Electrically-induced motor responses in the absence or in the presence of drug treatment (10–20 min incubation for antagonists and ionic channel blockers; 30 min for L-NOARG) were expressed as a percentage of the relaxation obtained at 50 Hz (100% response). To assess the inhibitory effect of hyoscine or TTX, the electrically-induced contractions in the presence of L-NOARG were expressed as a percentage of the contraction obtained at 50 Hz. Relaxations to graded distension were expressed as a percentage of the relaxation induced by 2 g. Data were calculated as means $\pm$ s.e. mean and n refers to the number of the tissues from different subjects. Statistical significance of mean differences was assessed by one way analysis of variance with Scheffé F test for multiple comparisons. P values <0.05 were considered significant.

Solutions and drugs

The modified Krebs solution (pH 7.4) had the following composition (mM): NaCl 120, KCl 4.7, $MgSO_4 7H_2O$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.0 and glucose 10. Suramin was a kind gift of Dr A. Faggiotto (Bayer S.p.A., Italy). N^G-nitro-L-arginine (L-NOARG) was donated by Janssen Chimica (Geel, Belgium); tetrodotoxin (TTX) was obtained from Sankyo (Kyoto, Japan); vasoactive intestinal polypeptide (VIP), hyoscine hydrobromide, phentolamine hydrochloride, propranolol hydrochloride, isoprenaline hydrochloride, acetylcholine chloride were obtained from Sigma Chimica (Milan, Italy).

Results

Immunohistochemistry and NADPH-diaphorase histochemistry

Specificity for VIP and NOS immunostaining was confirmed by the absence of immunoreaction in sections incubated with normal rabbit serum or with VIP antibody preadsorbed with the homologous peptide. Specificity for NADPH-diaphorase staining was demonstrated by the absence of labelling in sections in which NADPH was omitted.

VIP containing fibres were found, running singly or in small fascicles (Figure 1a,b), in the muscle layer as well as in myenteric plexus encircling stained (Figure 1c) or unstained perikarya. VIP-IR varicose fibres were occasionally observed in the submucosa. VIP-IR could not be identified in the submucosal plexus. In the mucosa, some VIP positive nerve processes were seen in the muscularis mucosae and in association with the gastric glands.

NOS-IR was observed in nerve fibres (either single beaded processes or bundles) distributed to the muscle layer (Figure 2a) and to the myenteric ganglia where they formed a dense network surrounding enteric neurones (Figure 2b). NOS-IR

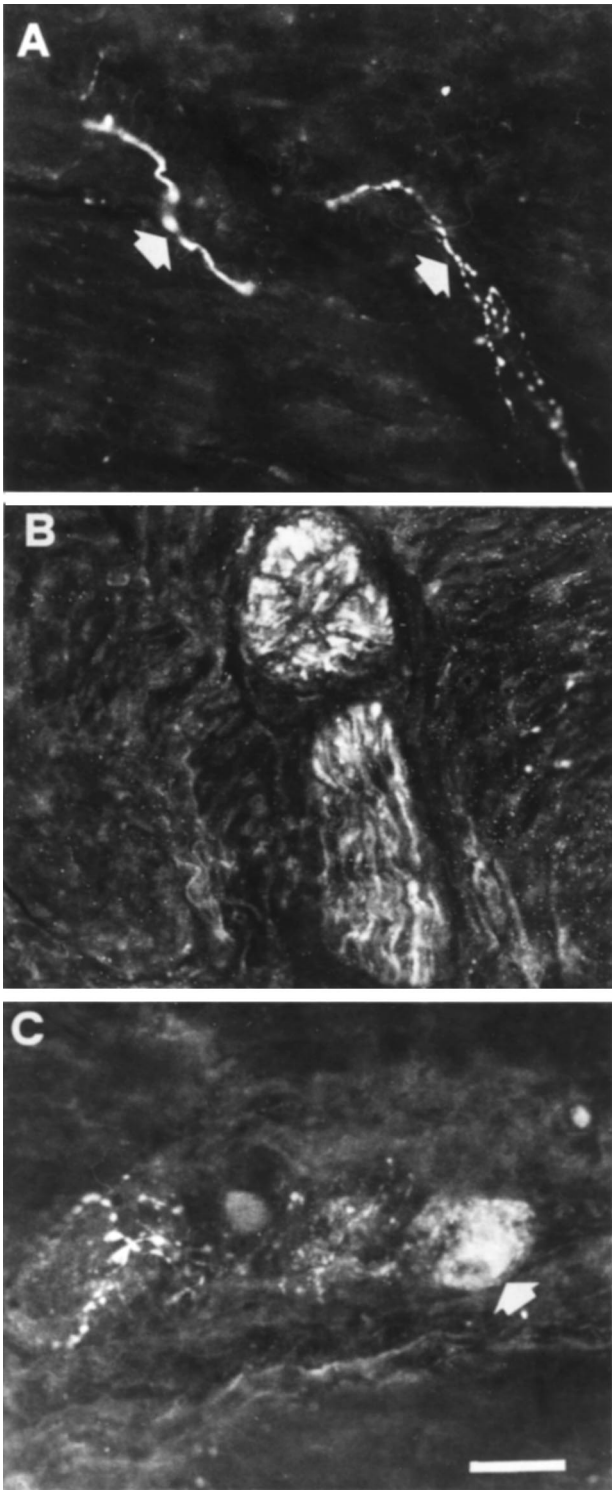

Figure 1 Representative examples of the VIP innervation in the human gastric fundus. (a) VIP-IR varicose processes and thin bundles of fibres (arrows) and (b) thick bundles of processes throughout the muscle layer. (c) VIP-IR in a ganglion cell body (arrow) and fibres in the myenteric plexus. Calibration bar = 25 μm.

was also found in the majority of myenteric neurones (Figure 2b). In the submucosa, few NOS-IR fibres were seen in association with the vasculature, mostly in paravascular position. NOS positive ganglion cells were not seen in the submucosal plexus. The muscularis mucosae was richly supplied by NOS-IR nerve fibres. The mucosa showed occasional NOS positive processes mainly confined at the base of the gastric glands. The pattern observed with NADPH-diaphorase histochemistry was comparable to that described with NOS immunohistochemistry. Overall, the NOS/NADPH labelled innervation was denser than that displaying VIP-IR.

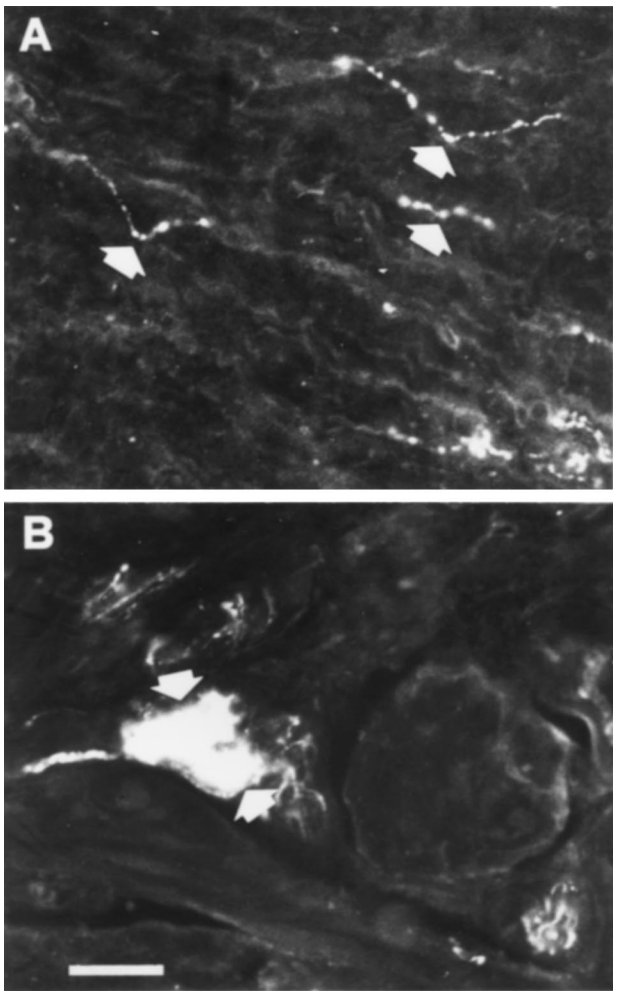

Figure 2 NOS-IR in the human gastric fundus. Similarly to the VIP innervation, NOS labelled varicose nerve fibres (arrows) were densely distributed to the muscle layer of the gastric fundus (a), as well as in the myenteric plexus where they surrounded immunostained perikarya (arrows) (b). Calibration bar = 25 μm.

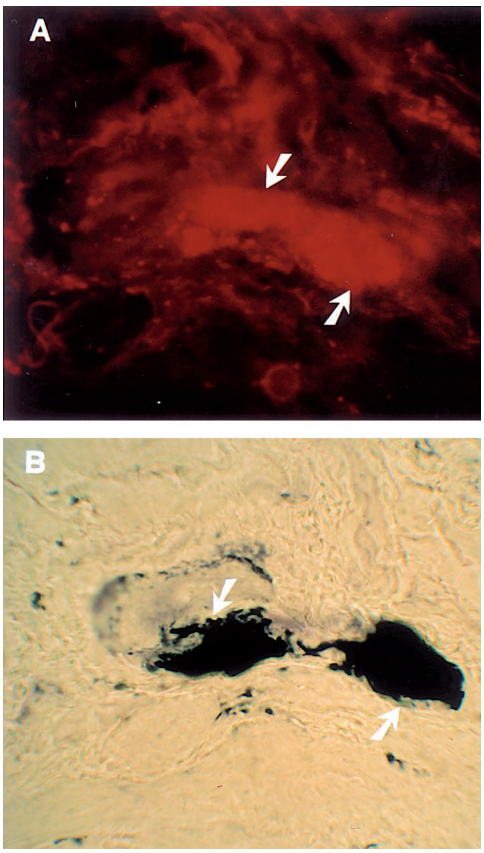

Figure 3 Representative photomicrographs showing colocalization of VIP-IR (a) and NADPH-diaphorase histochemistry (b) in neurons (arrows) of the myenteric plexus of the human gastric fundus. Calibration bar = 25 μm.

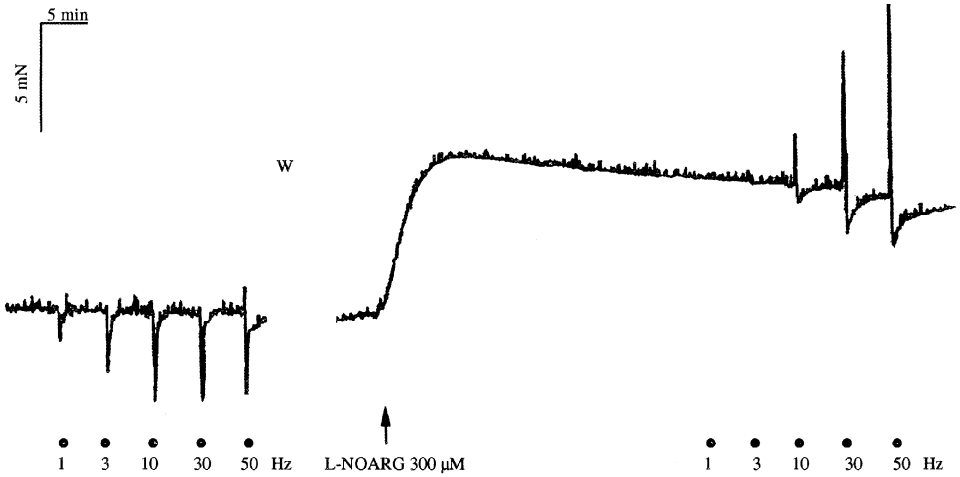

Figure 4 A representative tracing illustrating the motor response of a human isolated gastric fundus strip to electrical stimulation under control conditions or in the presence of 300 μM L-NOARG. Repetitive trains of electrical pulses at 1–50 Hz and 5 s in duration were delivered at 5 min intervals at 0.5 ms pulse width and 60 V. W indicates washing. Note the increase in tone, the reduction of NANC relaxations and the appearance of contractions induced by L-NOARG.

Double labelling

The majority of the VIP containing myenteric ganglion cell bodies and nerve processes coexpressed NADPH-diaphorase (Figure 3) confirming and extending previous findings in the mammalian gastrointestinal tract (Costa *et al*., 1992; Singaram *et al*., 1994; Lefebvre *et al*., 1995).

Motor responses to electrical stimulation

During the equilibration period ($\geqslant$90 min), a slowly developing increase in the basal tone (range 5–12 mN) was observed in most (87%) of human isolated gastric fundus strips. The minority of preparations showing no tone was not included in this study. Electrical field stimulation produced frequency-dependent (0.3–50 Hz) relaxations, which were preceeded by a brief small contraction at the highest (30, 50 Hz) frequencies (Figure 4). These results somewhat differ from those of Sanger (1985), who found that electrical stimulation primarily evoked nerve-mediated contractions in the human isolated stomach. However, he used longitudinal muscle of any part of the stomach and high strength electrical stimulation (80–120 V cm^{-1}).

The electrically-induced relaxations were unaffected by a combination of hyoscine, propranolol and phentolamine (each at 1 μM, $n=4$) and abolished by TTX (1 μM, $n=4$), indicating that they were mediated by NANC inhibitory nerves. Therefore, hyoscine, propranolol and phentolamine were not used in the subsequent experiments.

Administration of 300 μ M L-NOARG induced the following effects. Firstly, it induced a tonic contraction which was 60.6$\pm$12.7% ($n=3$) of that induced by 10 μM acetylcholine, and was reduced to 41.0$\pm$5.8% ($n=3$) and 26.1$\pm$6.6% ($n=3$) in the presence of hyoscine (1 μM) or TTX (1 μM), respectively. Hyoscine *per se* had no effect on basal tone, while TTX caused a transient increase in basal tone with superimposed phasic contractions. Secondly, it abolished the relaxation to short (5 s up to 3 Hz) (Figures 4 and 5) and sustained (5 min at 1 Hz, $n=3$) trains of stimulation (Figure 6). Thirdly, it significantly reduced relaxation evoked by 10–50 Hz (Figures 4 and 5), and concomitantly unmasked or potentiated a contractile response, whose amplitude was directly related to the frequency of stimulation (Figures 4 and 5). Contractile responses that were unmasked or potentiated by L-NOARG were markedly inhibited by hyoscine (3 μM, $n=6$) or a combination of hyoscine and TTX (1 μM, $n=6$) by approximately the same extent (Figure 7).

Administration of VIP (100 nM) produced a slowly developing relaxation, which was followed by a slow decline in the response up to recovery of the initial tone (Figure 8). The peak relaxation was reached after 20.1$\pm$1.3 min ($n=8$) of VIP exposure, while recovery of the basal tone was obtained after 54.0$\pm$4.4 min ($n=5$). VIP-induced relaxations, which were 52.7$\pm$5.4% ($n=5$) of those induced by 1 μM isoprenaline (peak response: 2.5 min), were unaffected by TTX (1 μM: $n=3$)

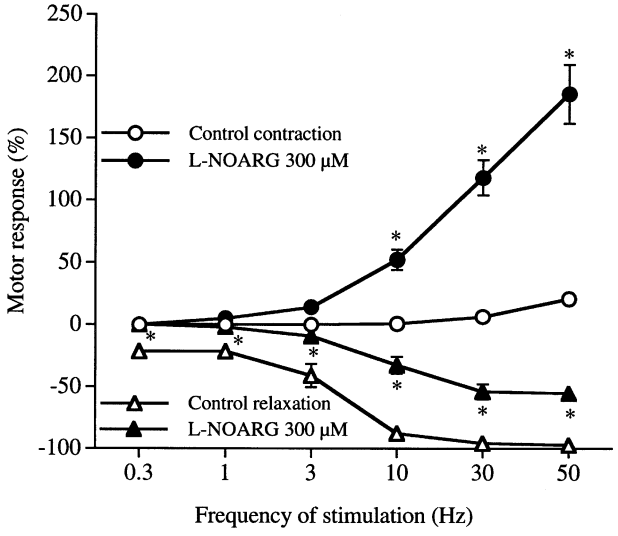

Figure 5 Frequency-dependent relaxant and contractile responses of gastric fundus strips to electrical stimulation, under control conditions or in the presence of L-NOARG. Values are expressed as per cent of the absolute value of the control inhibitory response obtained at 50 Hz and represent the means$\pm$s.e.mean of ten preparations. *$P<0.05$ versus control responses.

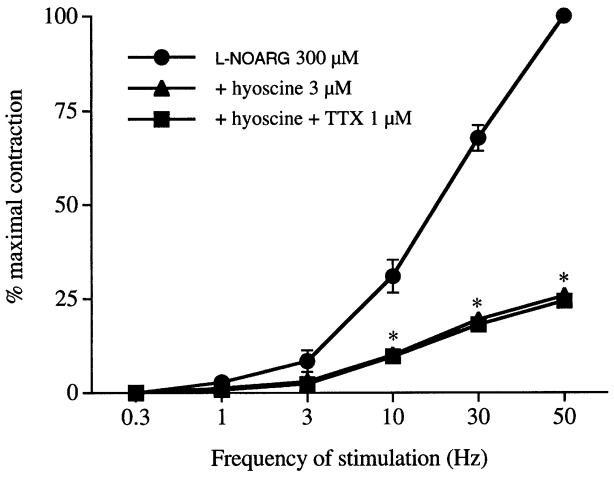

Figure 7 Frequency-dependent contractile responses of gastric fundus strips to electrical stimulation in the presence of L-NOARG alone, in combination with hyoscine, or in combination with hyoscine plus TTX. Values are expressed as per cent of the maximal contraction obtained at 50 Hz in the presence of L-NOARG and represent the means of six preparations. For clarity, error bars ($\pm$s.e.mean) are included in the L-NOARG curve only. *$P<0.05$ versus L-NOARG alone.

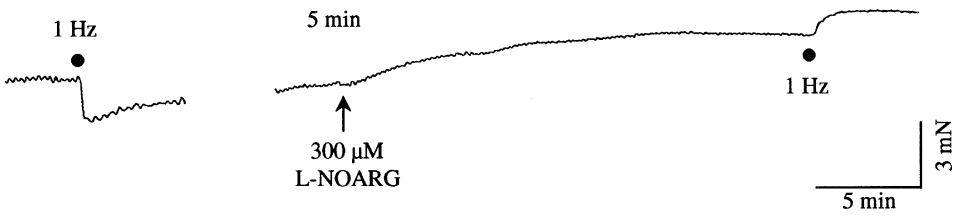

Figure 6 Tracing illustrating the effect of L-NOARG on electrically-evoked relaxations induced by stimulation at 1 Hz for 5 min. Note that L-NOARG (300 μM) increased the basal tone and turned the relaxant response into a contractile one.

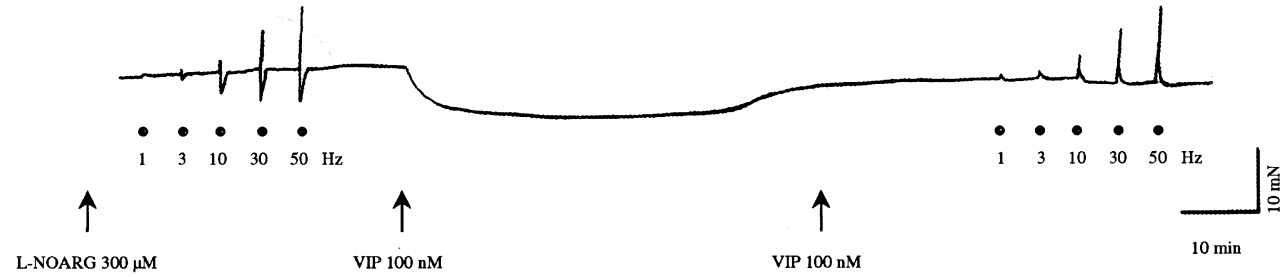

Figure 8 Tracing illustrating the effect of VIP desensitization on L-NOARG (300 μM)-resistant electrically-induced NANC relaxations. Note the relaxation induced by the priming VIP (100 nM) administration, the lack of response following the second VIP administration, and the disappearance of NANC relaxations.

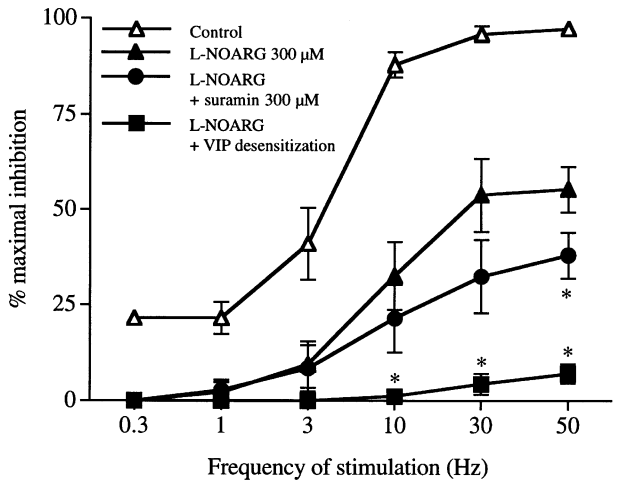

Figure 9 Frequency-dependent relaxations of gastric fundus strips to electrical stimulation under control conditions or after pharmacological treatment. Relaxations in control and following L-NOARG alone are data from Figure 5 (refer to this figure for statistical comparisons). The data with L-NOARG plus suramin or L-NOARG plus VIP desensitization are derived from five preparations each. Values are expressed as per cent of the relaxation obtained at 50 Hz. $^*P<0.05$ versus L-NOARG alone.

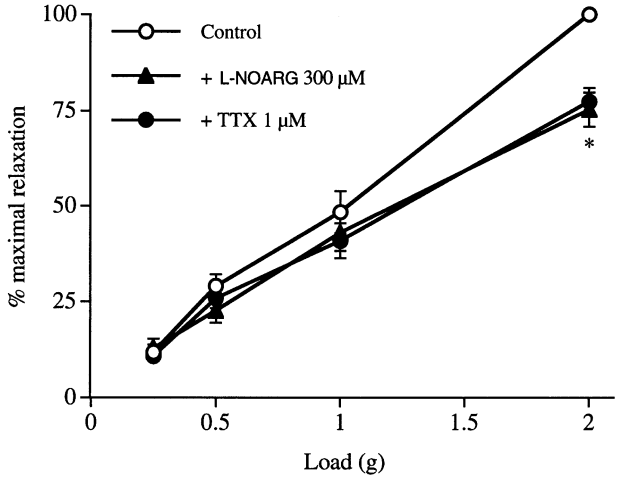

Figure 10 Relaxations of gastric fundus strips to graded distensions under control conditions or in the presence of L-NOARG or TTX. Values are expressed as per cent of the maximal relaxation induced by 2 g load and represent the means $\pm$ s.e.mean of 4–9 preparations. $^*P<0.05$ versus control.

and L-NOARG (300 μM: $n=3$) pretreatment, but reduced to $23.3\pm5.2\%$ ($n=3$) of isoprenaline-induced relaxation in the presence of 300 μM suramin. When a strip was treated with a priming (100 nM) VIP concentration, a second 100 nM VIP administration (carried out as soon as the effect of the priming dose had vanished) was ineffective in three strips (Figure 8) or caused a slight response (12% of the original one) in two additional preparations, indicating the occurrence of VIP desensitization. By contrast, relaxations caused by 1 μM isoprenaline were unaffected by VIP desensitization procedure ($n=3$). In tissues desensitized by 100 nM VIP, the L-NOARG-resistant component of the electrically-induced relaxations was virtually abolished (Figure 9).

Suramin (300 μM) had no effect *per se* on the frequency-dependent inhibitory curve and, unlike L-NOARG, did not unmask or potentiate contractile responses at any frequency of stimulation ($n=4$, data not shown). Conversely, combination of suramin (300 μM) plus L-NOARG (300 μM) was slightly, but significantly more effective than L-NOARG alone in inhibiting relaxations to 50 Hz (Figure 9).

Relaxations to mechanical distension

Graded relaxations were obtained in gastric fundus strips by increasing isotonic load within the range 0.25–2 g. In four out of seven tissues, treatment with TTX (1 μM) had no effect on the relaxations evoked by $\leqslant 1$ g distension, but caused a slight (20%) albeit significant reduction of the responses obtained with 2 g load (Figure 10). In the remaining three strips, TTX was ineffective at any distension tested. In five out of seven tissues, treatment with L-NOARG (300 μM), like TTX, caused a small but significant reduction of the relaxations evoked by 2 g distension only. The two preparations which were insensitive to L-NOARG were also insensitive to TTX.

Discussion

The present study characterized the morphological pattern and functional motor correlates of NO and VIP innervation in the human isolated gastric fundus and provided evidence on the role of these transmitters in mediating relaxations to electrical field stimulation and mechanical distension.

Identification of NOS- and VIP-containing neurones

We demonstrated that the human gastric fundus possesses prominent nitrergic and VIPergic innervation, which is comparable to the one observed in other regions of the digestive system (De Giorgio *et al*., 1994; Singaram *et al*., 1994; Matini *et al*., 1995). We also provided the first evidence for the colocalization of these two transmitters in a subpopulation of myenteric neurons of the human gastric fundus. These findings

expand previous observations of NO and VIP distribution in the enteric nervous system of the mammalian stomach (Belai *et al*., 1992; Timmermans *et al*., 1994; Lefebvre *et al*., 1995), thus providing a structural basis to interpret the functional responses obtained by electrical field stimulation and mechanical distension.

Characterization of transmitters involved in electrically-evoked NANC relaxations

The presence of NO and VIP in myenteric neurones and fibres supplying smooth muscle cells suggests the possibility that both inhibitory transmitters participate in neurogenic NANC relaxations, accounting for the reservoir function of this stomach region. Nevertheless, the colocalization of different transmitters within a neural structure does not necessarily imply their concomitant release at any level of electrical or mechanical stimulation. NO-mediated relaxations are preferentially activated by shorter trains or lower frequencies of electrical field stimulation in both gastric (Li & Rand, 1990) and extra-gastric isolated preparations (Maggi & Giuliani, 1996). Conversely, VIP is released either by brief stimuli at high frequency or by sustained stimuli at low frequency (Lefebvre *et al*., 1995; Currò & Preziosi, 1998). In the human isolated gastric fundus strips, our results with L-NOARG indicate that electrically-induced NANC relaxations are mediated by NO in the frequency range of 0.3–3 Hz with trains lasting 5 s. Even with longer trains (5 min) at low frequency (1 Hz) NO seems the only inhibitory transmitter involved. In this respect, the characteristics of stimulation for NO release parallel those of other established transmitters, such as acetylcholine or noradrenaline, which require a lower level of stimulation than any other accompanying peptide transmitter (Bartfai *et al*., 1988). By contrast, L-NOARG only significantly reduced (without suppressing) relaxations obtained in the range of 10–50 Hz, suggesting the participation of other inhibitory transmitter(s) in addition to NO.

A VIP desensitization procedure was used to assess whether VIP participates in the L-NOARG-resistant component of NANC relaxation. The attenuation of receptor function by means of agonist-induced receptor desensitization is considered a reliable alternative to the use of antagonists, especially when the latter lack potency and selectivity, such as the C-terminal fragment of VIP, $VIP_{(10-28)}$, which may (Crist *et al*., 1992; Jin *et al*., 1994) or may not antagonize relaxations to applied VIP (Morris & Murphy, 1989; Maggi & Giuliani, 1993). When desensitization was induced to the relaxant effect of VIP, it also markedly reduced the relaxant effects of electrical field stimulation (range 10–50 Hz) in the presence of L-NOARG. Compared to control, the relevant inhibition of NANC relaxations observed under these conditions ($\geqslant$90%) suggests that at frequencies $\geqslant$10 Hz, relaxations are mostly mediated by the concomitant release of NO and VIP, which are costored in neurones innervating smooth muscle cells, as observed in our immunohistochemical experiments. In our hands, VIP desensitization probably involved VIP receptors specifically (and not other receptor types or post-receptor mechanisms), since isoprenaline-induced relaxation, which is mediated *via* cyclic AMP formation like that of VIP (Bitar & Makhlouf, 1982), was not affected by this procedure. Furthermore, the finding that the relaxation induced by VIP was insensitive to L-NOARG could be taken as evidence that in human gastric fundus strips, like in pig or canine fundus strips (Lefebvre *et al*., 1995; Bayguinov *et al*., 1999) or guinea-pig isolated whole stomach (Desai *et al*., 1994), VIP does not seem to produce part of its relaxant effect by releasing NO from neurones or directly from isolated smooth muscle cells (Grider *et al*., 1992; Murthy *et al*., 1993; Chakder & Rattan, 1996; Jin *et al*., 1996). However, since human intestinal and rabbit gastric smooth muscle cells were recently found to express a constitutive endothelial NOS (Teng *et al*., 1998), experiments with human dispersed smooth muscle cells from fundus are required to establish whether VIP is able to promote NO release under these conditions.

With regard to the L-NOARG-resistant component of NANC relaxations evoked at the highest frequencies (50 Hz), it was slightly, but significantly reduced by suramin, a non-selective P_2 purinoceptor antagonist (Hoyle *et al*., 1990). Although this could be taken as evidence for the involvement of ATP as an additional mediator of NANC relaxations, we believe that ATP plays no role in our experimental conditions for several reasons. Firstly, combined L-NOARG and VIP desensitization virtually abolished NANC relaxations; secondly, in our hands suramin antagonized VIP-induced relaxations, as observed by Briejer *et al*. (1995) in the guinea-pig proximal colon. Therefore, the suramin-sensitive component of NANC relaxation could be tentatively ascribed to partial VIP inhibition. In our hands, however, this minor inhibition by suramin was detectable only after suppression of the nitrergic function, since suramin was ineffective when the nitrergic innervation was fully operating.

At frequencies $\geqslant$3 Hz, L-NOARG unmasked an early contractile response that was substantially reduced by TTX and hyoscine. This suggests that endogenous NO may tonically inhibit the release of excitatory transmitters. Prejunctional modulation of cholinergic transmission by endogenous NO has been reported in the guinea-pig and dog intestine and in rabbit gastric corpus (Knudsen & Tøttrup, 1992; Baccari *et al*., 1993; Hryhorenko *et al*., 1994), while evidence for a postjunctional mechanism was obtained in the guinea-pig (Milenov & Kalfin, 1996) and pig gastric fundus (Leclere & Lefebvre, 1998). In addition, as observed in isolated intestinal preparations from several mammalian species (Li & Rand, 1990; Meulemans *et al*., 1993; Ciccocioppo *et al*., 1994), L-NOARG enhanced the basal tone of human fundus strips through a mechanism that was partially sensitive to TTX and hyoscine. This suggests that L-NOARG acts by removing a tonic nitrergic inhibition both on smooth muscle cells and excitatory cholinergic neurones.

Relaxations to graded mechanical distension

Electrical field stimulation is generally regarded as a reliable procedure to evoke transmitter release from enteric nerve terminals, although its physiological relevance is still debated. In our study, we also resorted to mechanical distension to assess the role of the neural mechanisms underlying NANC relaxation.

Although relaxations of gastric fundus strips to graded distension were mainly due to passive elongation of smooth muscle cells, a neurogenic component ($\sim$25%) apparently mediated only by NO was observed at 2 g distension. Based on our results, NO is released upon mild electrical or mechanical stimulation. Therefore, NO could be viewed as the primary inhibitory transmitter initiating accommodation in the human gastric fundus, as already observed in other species (Desai *et al*., 1991) and in other parts of the gut (Waterman *et al*., 1994; Ciccocioppo *et al*., 1994).

Conclusions

In conclusion, NO, VIP and NO/VIP were identified in ganglion cells of the myenteric plexus and in nerve fibres supplying smooth muscle cells of the human gastric fundus.

NO is responsible for NANC relaxations evoked by low frequency electrical stimulation or by mild mechanical distension, whereas VIP release occurs at high frequency stimulation. In addition, NO was found to exert an inhibitory influence both on cholinergic innervation and basal tone.

This work was supported in part by the Italian Ministry for University and Scientific Research (MURST) and FAR 1998 Funds to M. Tonini, and by NIH grants DK54155 and DK41301 (Morphology/Imaging Core) to C. Sternini. The authors wish to thank Ms Helen C. Wong and Dr John H. Walsh of the Antibody Core (subsection of DK 41301 CURE: Digestive Diseases Research Center Grant) for the rabbit polyclonal VIP7913, and Dr Harald H.H.W. Schmidt (Würzburg University, Germany) for the rabbit polyclonal NOS antiserum. The authors wish also to thank Drs E. Messori, M. Tagliani and S.M. Candura for their critical and helpful comments on the manuscript.

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(Received August 8, 1999
Revised September 13, 1999
Accepted October 1, 1999)