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Nitric oxide, and not vasoactive intestinal peptide, as the main neurotransmitter of vagally induced relaxation of the guinea pig stomach

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1 Nitric oxide synthase (NOS) was localized in the guinea pig stomach by immunocytochemistry. *In vitro* experiments were carried out on the isolated stomach of the guinea pig to study any possible links between nitric oxide (NO) and vasoactive intestinal peptide (VIP) in mediating relaxations induced by vagal stimulation.

2 NOS was localized to nerve cell bodies and nerve fibre varicosities of the myenteric plexus in wholemounts of the longitudinal muscle–myenteric plexus of the stomach fundus. The NOS-positive cells had a Dogiel type I morphology characteristic of motor neurones.

3 The cross-sections of the stomach wall showed NOS-positive neurones mainly in the myenteric plexus ganglia and NOS-positive nerve fibre varicosities in the circular muscle layer.

4 Relaxations induced by vagal stimulation were almost completely prevented by L-NAME with an IC_{50} value of 5.5×10^{-6}M. This inhibition was reversed by L-arginine (2 mM).

5 VIP (100 nM) induced reproducible relaxations of the stomach. These were unaffected by tetrodotoxin (2 μM) or N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM).

6 Desensitization to the relaxant effect of VIP partially reduced relaxations induced by vagal stimulation, glyceryl trinitrate or sodium nitroprusside but not noradrenaline.

7 These results show that NO has a neuronal origin in the guinea pig stomach, and support NO, and not VIP, as the major neurotranmitter of vagally induced gastric relaxation in the guinea pig.

Keywords: Nitric oxide; vasoactive intestinal peptide; myenteric plexus; NANC mediator; guinea pig stomach

Introduction

Nitric oxide (NO) is an inhibitory non-adrenergic, non-cholinergic (NANC) mediator at various sites in the gastrointestinal (GI) tract and is widely believed to be released from neurones (Gillespie *et al.*, 1989; Gibson *et al.*, 1990; Li & Rand, 1990; Desai *et al.*, 1991a,b; Boeckxstaens *et al.*, 1992; Meulemans *et al.*, 1993). This has been confirmed in a number of studies by immunocytochemical localization of nitric oxide synthase (NOS), the enzyme catalysing the formation of NO from endogenous L-arginine, to neurones of the myenteric plexus in the guinea pig and rat ileum, rat stomach, duodenum, canine proximal colon and the human gut (Bredt *et al.*, 1990; Schmidt *et al.*, 1992; Springall *et al.*, 1992; Ward *et al.*, 1992; Forster & Southam, 1993). In the guinea pig ileum, NOS-immunoreactive neurones in the myenteric plexus synapse with other neurones and innervate the circular muscle (Llewellyn-Smith *et al.*, 1992). In the rat duodenum and canine proximal colon the NOS-positive neurones have a Dogiel type I morphology and axons going towards the muscle, which is characteristic of motor neurones (Ward *et al.*, 1992; Aimi *et al.*, 1993). In addition, NOS has been localized to the vagal efferent fibres innervating the rat stomach (Forster & Southam, 1993). Histochemistry also suggests that NO is a neurotransmitter with paraneuronal actions in the brain (Schmidt *et al.*, 1992), and recently NO release has been shown from isolated ganglia of the myenteric plexus of the guinea pig ileum (Grider & Jin, 1993). Thus, NO is now established as a neurotransmitter in the brain and the periphery. Vasoactive intestinal peptide (VIP) has also been proposed as a mediator of gastric relaxation in the guinea pig, rat, cat and ferret (Grider *et al.*, 1985; De Beurme & Lefebvre, 1988; D'Amato *et al.*, 1988, 1992; Kamata *et al.*, 1988; Grundy *et al.*, 1993). For instance, electrical field stimulation of muscle strips from the guinea-pig and rat gastric fundus causes release of VIP-like immunoreactivity (Grider & Makhlouf, 1987; D'Amato *et al.*, 1992). It has also been suggested that VIP and NO are co-transmitters of gastric relaxation in the guinea pig, rat and ferret (Li & Rand, 1990; Boeckxstaens *et al.*, 1992; Grider *et al.*, 1992; Said, 1992; Grider & Jin, 1993; Grundy *et al.*, 1993) and internal anal sphincter in the opossum (Chakder & Rattan, 1993). One possibility is that VIP acts partly through stimulation of NO formation, as has been suggested in the smooth muscle cells of the guinea pig stomach (Grider *et al.*, 1992). To examine this possibility we have localized NOS by immunocytochemistry to confirm that it is present in neurones and determined if there is any involvement of VIP in mediating vagally induced relaxation of the guinea pig stomach.

Some of the results of this study were reported earlier to the British Pharmacological Society as an abstract (Desai *et al.*, 1993).

Methods

Immunocytochemical studies

These studies were carried out on wholemount preparations of the longitudinal muscle–myenteric plexus (LM–MP) and

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on cross-sections of the stomach fundus of the guinea pig. Male Hartley guinea pigs (300–350 g) were killed by cervical dislocation and exsanguination. The stomach was removed, cut open along the lesser curvature and the contents washed out with Krebs' solution NaCl, 118 mM; KCl, 4.7 mM; $NaHCO_3$, 25 mM; KH_2PO_4, 1.17 mM; $MgSO_4$, 2.5 mM; $CaCl_2$, 2.5 mM; glucose, 5.6 mM; pH 7.4). For wholemounts (Costa *et al.*, 1980) the fundal portion was cut as a flat sheet and pinned, slightly stretched, on to a thin board with the mucosal surface facing down. It was then fixed by immersion for 16–18 h in Zamboni's solution (2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, plus saturated picric acid solution in a ratio of 85:15). The tissue was removed from the board and washed in 80% ethanol. It was dehydrated through 95% and 100% ethanol, cleared in xylol and rehydrated through 100%, 80% and 50% ethanol (30 min each) and stored in phosphate-buffered saline (PBS, 0.1 M, pH 7.2) at 4°C until use. The LM–MP was separated as a sheet by separating the mucosal and circular muscle layers with watchmakers' forceps under a dissecting microscope, and incubated for 16–18 h at room temperature with rabbit antibody to NOS, diluted 1:5000 and 1:10,000 in 0.01 M PBS (pH 7.2) with 0.1% bovine serum albumin (BSA) and 0.01% sodium azide. It was then washed in PBS (3 × 5 min) and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit serum diluted 1:40 for 1 h at room temperature and washed in PBS (3 × 5 min). The preparation was then mounted in PBS–glycerine (1:9, v/v) and examined under a confocal microscope. Photographs were taken using FP4 black and white film (speed 100 ASA) (Ilford).

For cross-sections of the stomach wall, the stomach fundus was fixed as before and washed in PBS containing 15% sucrose and 0.01% sodium azide. Cryostat blocks were prepared from small pieces of tissue (2 × 1 cm) and 7–8 μm sections were cut at −20°C, transferred to poly-L-lysine-coated slides and allowed to dry for 1 h. They were immunostained for NOS using the indirect avidin–biotin complex (ABC) immunoperoxidase method (Polak, 1988). The sections were immersed in 0.03% (v/v) hydrogen peroxide in PBS for 30 min to remove the endogenous peroxidase activity. Possible background staining was reduced by incubation for 30 min at room temperature with normal goat serum, diluted 1:30. The sections were then incubated with the primary rabbit antibody to NOS, diluted 1:5000 and 1:10,000, for 16–18 h at room temperature in a humid chamber. For the second layer, biotinylated goat anti-rabbit antibody, diluted 1:100, was applied for 30 min at room temperature. The final layer consisted of Elite Vector reagents A and B (5 μl each of A and B diluted in 90 μl of diluent) incubated for 1 h at room temperature. The reaction was visualized by the nickel-enhanced diaminobenzidine method (Graham & Karnovsky, 1976). When developed, the sections were counterstained with neutral red, dehydrated with absolute alcohol and Inhibisol and mounted. The sections were observed under a transmitted light microscope (Reichert-Jung).

The rabbit polyclonal antibody against the rat brain constitutive NOS had been characterized for specificity (Springall *et al.*, 1992). For negative controls only the diluent was used for first-layer incubation. Antibody against the general neuronal marker protein gene product 9.5 (PGP 9.5, human, 1:4000) (Gulbenkian *et al.*, 1987) was used to confirm neuronal staining with NOS antibody.

In vitro *functional studies on the isolated stomach*

The isolated stomach of the guinea pig was prepared as described previously (Desai *et al.*, 1991b). Briefly, the guinea pig stomach with the vagus nerves was isolated and immersed in oxygenated (95% O_2, 5% CO_2) Krebs' solution and the pyloric end was cannulated. The oesophagus was ligated and the contents of the stomach were flushed out. It was then placed in an isolated organ bath in 100 ml of gassed (95% O_2 + 5% CO_2) Krebs' (37°C) solution containing atropine (3 μM) and guanethidine (5 μM) to study the non-adrenergic, non-cholinergic (NANC) inhibitory effects of vagal stimulation. Bacitracin (3 μg ml^{-1}), thiorphan (1 μM) and captopril (1 μM) were also added to the Krebs' solution to inhibit the endogenous peptidases and angiotensin-converting enzyme and prevent metabolism of VIP. The pyloric cannula was connected to a reservoir (2 l) containing 1 l of Krebs' solution. The reservoir was placed on a movable rack so that it could be moved up or down to increase or decrease the pressure of fluid entering the stomach (Paton & Vane, 1963). Intragastric pressure was measured with a Statham pressure transducer. The reservoir was sealed and connected to a float recorder to measure intragastric volume changes with a Harvard isotonic transducer. The volume and pressure changes were recorded on a Graphtec WR 3310 recorder. The vagus was stimulated with a pair of ring electrodes connected to a Grass S 88 stimulator. Experiments were started after a 45–60 min equilibration period. The threshold pressure for a NANC adaptive response (Paton & Vane, 1963; Desai *et al.*, 1991a) was determined first and the intragastric pressure was elevated to 1–2 cmH_2O below it, usually to 3 cmH_2O, to give an elevated intragastric volume from which to measure subsequent relaxant responses to drugs or vagal stimulation (supramaximal voltage, 10–16 Hz, 1 ms, 50–60 s). After a response the fluid in the stomach was emptied via a side cannula and the next response was recorded after 10 min. Under these conditions the vagally induced responses were reproducible. Since the electrodes were immersed in Krebs' solution surrounding the vagus nerves, supramaximal voltage was used to eliminate variations in the electrical current reaching the nerves for stimulation. In previous experiments, a stimulation frequency of 10–16 Hz gave optimal reproducible monophasic relaxant responses in the presence of atropine (3 μM) and guanethidine (5 μM), and these frequencies were used here for stimulation. Stimulation frequencies between 0.1 and 8 and between 32 and 256 Hz gave monophasic relaxation responses smaller than with 10 and 16 Hz. In those experiments vagal responses at all frequencies from 0.1 to 256 Hz were completely prevented by inhibition of NOS.

To study the effects of tetrodotoxin (TTX), N^{ω}-nitro-L-arginine methyl ester (L-NAME) or L-arginine (L-Arg) on the relaxant responses, the stomach was incubated, both intra- and extraluminally, with the drug(s) for the required time before eliciting a response.

Statistical analysis

The volume changes due to relaxation induced by vagal/drug stimulation are expressed as a percentage of the total gastric volume. All results are expressed as the mean ± s.e. mean of *n* observations. The data were analysed for statistical significance by analysis of variance and post-hoc Bonferroni test or Student's unpaired two-tailed *t*-test, with a *P*-value <0.05 taken as significant.

Drugs used

Noradrenaline bitartrate, atropine sulphate, bacitracin, bovine serum albumin, captopril, glucose oxidase (type III), guanethidine sulphate, L-arginine hydrochloride, N^{ω}-nitro-L-arginine methyl ester, sodium nitroprusside (sodium ferricyanide), *dl*-thiorphan and vasoactive intestinal peptide (VIP) were purchased from Sigma Chemical Co. Glyceryl trinitrate (GTN) (Nitronal) was purchased from Lipha Pharmaceuticals, U.K. The NOS antibody was kindly provided by The Wellcome Research Laboratories, Beckenham, Kent, U.K. All other reagents used were of the highest commercially available purity and were purchased from either BDH or Sigma. The drug solutions were made in distilled water. The stock solution of VIP was made in 0.1% BSA in distilled water and stored at −20°C until used.

Results

Immunohistochemical localization of NOS in wholemounts of LM–MP

Wholemounts of the LM–MP of the stomach fundus showed NOS localized to neuronal cell bodies (confirmed by PGP 9.5 staining) of the myenteric plexus and to nerve fibre varicosities of internodal strands and fibres going to the muscle. The NOS-positive cells were grouped towards the periphery of the ganglia (Figure 1) and had a Dogiel type I morphology (Figure 2) with multistellate shape, 4–5 short broad lamellar processes and a long axon, which is characteristic of motor neurones innervating the muscle. There was no staining of the muscle fibres.

Cross-sections of the stomach fundus wall

The cross-sections of the stomach fundus showed NOS localized mainly to nerve cell bodies in the myenteric plexus ganglia with occasional NOS-positive nerve cells in the submucosa. NOS-positive nerve fibres were seen running parallel to circular smooth muscle fibres and very few fibres in the longitudinal muscle layer. There was no immunostaining of smooth muscle cells or mucosal cells.

Tetrodotoxin prevents vagus-but not VIP-induced gastric relaxation

Vagal stimulation induced reproducible monophasic gastric relaxations. Exogenous VIP in lower concentrations (1, 3, 10 and 30 nM, 4–5 min) failed to induce consistent and reproducible relaxations. However, VIP in a concentration of 100 nM (3–4 min) induced at least three reproducible relaxations (VIP first exposure, 24.2 ± 6%; second exposure, 21.3 ± 1.3%; third exposure, 22.9 ± 2.2%; $n = 3$, $P > 0.05$ between each group). A higher concentration of 200 nM VIP (3–4 min) did not increase the magnitude of relaxation and was subsequently not used to avoid desensitization. Incubation of the stomach with tetrodotoxin (TTX, 2 μM, 20 min) completely prevented gastric relaxation induced by vagal stimulation (control, 36.1 ± 3.3%; + TTX, 0.1 ± 0%; $n = 3$, $P < 0.001$), but did not affect that induced by VIP (100 nM; control, 32.7 ± 5.0%; + TTX, 33.4 ± 3.5%; $n = 3$, $P > 0.05$); or by GTN (2 μM, 1–1.5 min; control, 41.6 ± 4.0%; + TTX, 34.5 ± 6.1%; $n = 3$, $P > 0.05$).

L-NAME inhibits relaxations of the stomach induced by vagal stimulation

A dose–response curve (Figure 3) was obtained for the inhibitory effect of L-NAME (0.1, 1, 3, 10, 30, 100 μM, 20 min each, $n = 4$) on gastric relaxations induced by vagal stimulation in the presence of atropine (3 μM) and guanethidine (5 μM). After control reproducible responses to vagal stimulation the lowest concentration of L-NAME was incubated, intra- and extraluminally simultaneously, for the specified time. After recording a response to vagal stimulation the stomach was emptied and washed. The next higher concentration of L-NAME was then incubated for the same time and a response was recorded. This sequence was repeated. L-NAME had an IC_{50} value of 5.5×10^{-6} M. In subsequent experiments gastric relaxation induced by vagal stimulation was almost completely inhibited by L-NAME (100 μM, 20 min). This inhibition was partially reversed after washout of L-NAME and incubation with L-arginine (L-Arg, 2 mM, 30 min; Figure 4) (control, 46.4 ± 1.4%; + L-NAME, 5.7 ± 1.0%; + L-Arg, 36.8 ± 3.5%; $n = 4$, $P < 0.001$). Washout of L-NAME with L-Arg was attempted since co-incubation of L-NAME (100 μM) with L-Arg (0.5–2 mM) does not reverse the inhibitory effect of L-NAME (Desai *et al.*, 1991a), which could be because L-NAME is an irreversible inhibitor of NOS (Dwyer *et al.*, 1991).

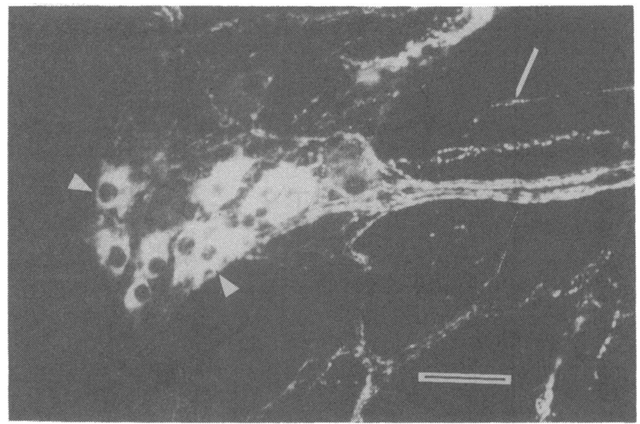

Figure 1 Immunocytochemical localization of nitric oxide synthase (NOS) in a wholemount of the longitudinal muscle–myenteric plexus of the guinea pig stomach fundus. Immunostaining for NOS was by the indirect immunofluorescence method. This figure shows a myenteric plexus ganglion with NOS-positive nerve cells (arrowheads) grouped towards the periphery and surface of the ganglion. NOS-positive nerve fibre varicosities can also be seen (arrow) in internodal fibres and tertiary fibres going to muscle cells. Scale bar = 50 μm.

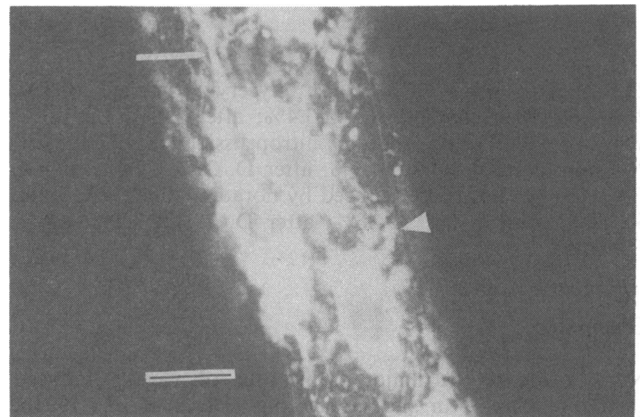

Figure 2 A wholemount preparation of the myenteric plexus of the guinea pig stomach fundus immunostained for nitric oxide synthase (NOS) using the indirect immunofluorescence method. NOS-positive nerve cells in a ganglion can be seen. The cells show a Dogiel type I morphology with a multistellate shape, short broad lamellar processes (arrowhead) and a single long axon (arrow), which is characteristic of motor neurones. Scale bar = 25 μm.

L-NAME does not affect relaxation of the stomach induced by exogenous VIP

VIP (100 nM, 3–4 min) induced relaxation of the stomach which was not affected by incubation with L-NAME (100 μM, 20 min) or L-Arg (2 mM, 30 min) (Figure 4) (control, 30.5 ± 1.9%; + L-NAME, 31.0 ± 1.7%; + L-Arg, 27.9 ± 3.2%; $n = 6$, $P > 0.05$).

Desensitization to the relaxant effect of VIP reduces gastric relaxations induced by vagal stimulation, glyceryl trinitrate or sodium nitroprusside

Desensitization to the relaxant effect of VIP was induced by repeatedly (4–5 times) exposing the stomach to VIP (100 nM) for 90–150 min. Desensitization was evident as a reduced response to VIP (100 nM) (VIP first exposure, 41.9 ± 3.5%; VIP after desensitization, 23.6 ± 3.1%; $n = 9$, $P < 0.01$). This desensitization (D) also reduced the relaxations induced by vagal stimulation (VS; control, 39.3 ± 1.1%; after D,

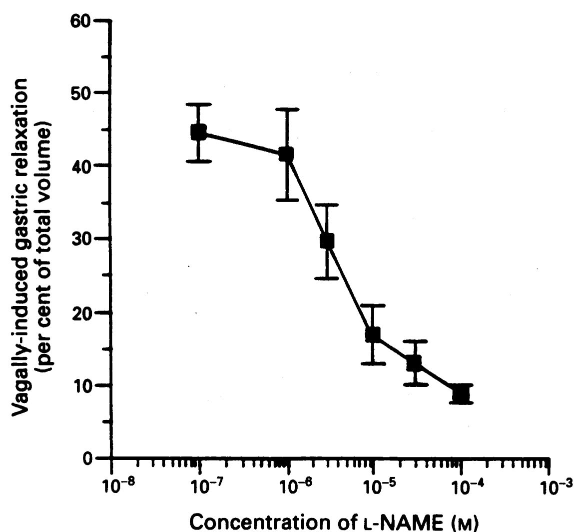

Figure 3 Dose–response relationship of the inhibitory effect of L-NAME. This graph shows the inhibitory effect of increasing concentrations of L-NAME on vagally induced relaxations of the isolated stomach of the guinea pig in the presence of atropine (3 μM) and guanethidine (5 μM). Values are mean ± s.e. mean from four experiments.

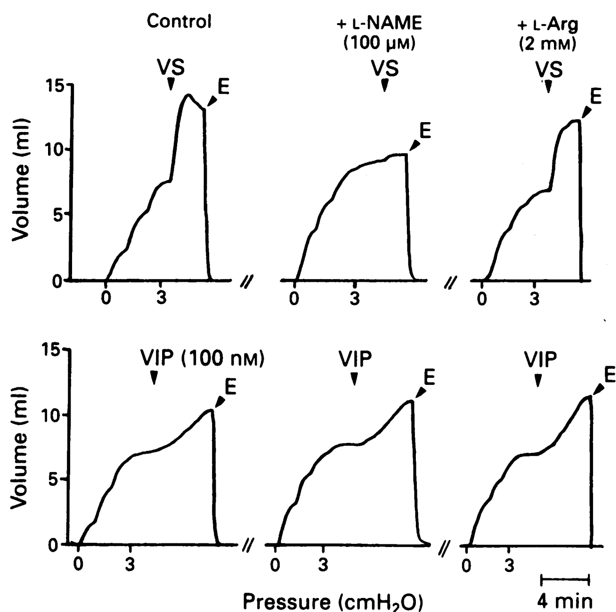

Figure 4 L-NAME inhibits gastric relaxation induced by vagal stimulation but not by VIP. The intragastric pressure was elevated to 3 cmH_2O and the vagus was stimulated (VS, 1 min) (upper panel) or VIP (100 nM lower panel) given to induce relaxation. Incubation with L-NAME (100 μM, 20 min) prevented the relaxation to VS but did not affect that to VIP. Washout of L-NAME with L-Arg (2 mM, 30 min) reversed the inhibition of vagally induced relaxation, whereas VIP-induced relaxation remained unaffected. E denotes emptying of the stomach. This figure is representative of at least five experiments.

23.5 ± 1.9%; $n = 9$, $P<0.001$), glyceryl trinitrate (GTN, 2 μM, 60–90 s; control, 46.7 ± 3.4%; after D, 31.1 ± 1.6%; $n = 6$, $P<0.01$), or sodium nitroprusside (SNP, 0.5 μM, 2–3 min; control, 34.5 ± 4.3%; after D, 21.5 ± 2.4%; $n = 4$, $P<0.05$) but not those induced by noradrenaline (NA, 2 μM, 60–90 s; control, 30.7 ± 4.6%; after D, 29.2 ± 3.2%; $n = 7$, $P>0.05$).

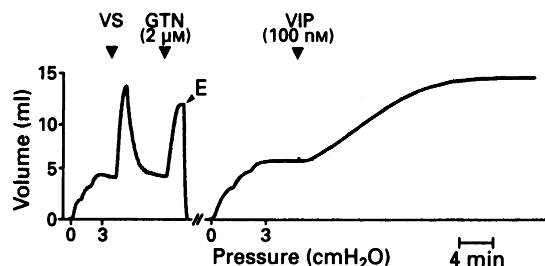

Figure 5 Comparison of the relaxant effects of vagal stimulation (VS), glyceryl trinitrate (GTN) and VIP on the isolated stomach. The intragastric pressure was elevated to 3 cmH_2O and then the relaxant responses to either VS, GTN (2 μM) or VIP (100 nM) were recorded. VS induced a relaxation which developed rapidly to a peak in about 45 s, when stimulation was stopped, and faded rapidly to the baseline within 4–5 min. GTN induced a response which developed rapidly to a peak within 90 s, at which time the stomach was emptied (E). The relaxation induced by VIP developed slowly, reaching a peak in about 3–4 min, and was sustained for 20–30 min. This figure is representative of responses seen in at least four experiments.

Discussion

Nitric oxide is the major transmitter of vagally induced gastric relaxation in the guinea pig (Desai et al., 1991b; Meulemans et al., 1993). Here we have localized NOS, using immunocytochemistry, to nerve cell bodies in the myenteric plexus and nerve fibre varicosities in the circular muscle layer. This strongly supports our view that NO is released from efferent neurones upon vagal stimulation and that NO is responsible for adaptive and receptive relaxation. No NOS was detected in muscle cells by this antibody. Thus any NOS present in muscle cells must be different from neuronal NOS.

VIP has also been proposed as the main NANC neurotransmitter of gastric relaxation in the guinea pig (Grider et al., 1985; 1992). However, in our functional studies we found that vagal stimulation induced a rapid relaxation that faded rapidly when stimulation was stopped (Figure 5). This is consistent with the short half-life of NO (Palmer et al., 1987). Glyceryl trinitrate and sodium nitroprusside, both of which induce relaxation through liberation of NO (Noack & Feelisch, 1991), produced similar responses. VIP, on the other hand, induced a different pattern of relaxation which developed slowly and also faded very slowly (Figure 5). Lefebvre et al. (1992) also observed a more sustained relaxation that developed slowly in response to VIP (10^{-9} to 10^{-7} M) in circular muscle strips of guinea pig gastric fundus.

Experiments using L-NAME also support NO as being the main neurotransmitter mediating these gastric relaxations. Vagally induced relaxation was prevented by L-NAME, an inhibitor of NO formation and reversed by L-arginine, the substrate for NO formation, whereas the relaxation induced by VIP was unaffected by L-NAME or L-arginine. In previous studies L-NAME inhibited relaxations induced by vagal stimulation even at high frequencies between 32 and 256 Hz (unpublished). Meulemans et al. (1993) also obtained a maximal amplitude of relaxation at 20 Hz vagal stimulation in the guinea pig stomach. They reported a virtually complete abolition of this relaxation by N^G-nitro-L-arginine (10^{-4} M). Similarly, Lefebvre et al. (1992) showed that in circular muscle strips of guinea pig gastric fundus N^G-nitro-L-arginine (10^{-5} and 10^{-4} M) inhibited relaxations elicited with short-lasting frequency-dependent as well as continuous electrical stimulation with cumulative increase in frequency from 0.125 to 16 Hz, but did not affect relaxations induced by VIP (10^{-9}

to 10^{-7} M). This is in contrast to other reports which have shown that inhibition of NOS only partially prevents gastric relaxation in the rat and ferret (Li & Rand, 1990; Boeckxstaens *et al.*, 1992; Grundy *et al.*, 1993), findings that could be due variations in the relative importance of NO and VIP as inhibitory transmitters in different species and tissues. For instance, even though NO and VIP have been reported to be co-transmitters in the guinea pig trachea (Tucker *et al.*, 1990; Li & Rand, 1991), NO is the main transmitter in feline and human trachea (Belvisi *et al.*, 1992; Fisher *et al.*, 1993). The lack of effect of tetrodotoxin on relaxations induced by VIP confirms a direct action and shows that the latter is not activating neurones causing release of a mediator such as NO for its relaxant effect. This is because we have localized NOS to neurones and not to smooth muscle cells. The most important evidence that VIP does not act through NO is that L-NAME prevented relaxations induced by vagal stimulation but did not affect those induced by VIP. Thus, even if VIP is released by vagal stimulation, it does not act through stimulation of NO production from neurones or smooth muscle cells, as has been suggested (Grider *et al.*, 1992). This would hold even if these tissues contain isoforms of NOS not detected by our antibody staining.

When densensitization was induced to the relaxant effect of VIP it also reduced the relaxant effects of vagal stimulation, glyceryl trinitrate and sodium nitroprusside, but not that of noradrenaline. Since glyceryl trinitrate and sodium nitroprusside cause release of NO (Noack & Feelisch, 1991), which directly induces relaxation, this suggests that desensitization to the relaxant effect of VIP interferes with the action of NO. Thus, this interference could be at the level of second messengers cGMP and cAMP, which mediate relaxations induced by NO (Arnold *et al.*, 1977) and VIP (Bitar & Makhlouf, 1982) respectively. Interactions between cAMP and cGMP and the protein kinases activated by them have been reported (see Jiang *et al.*, 1992). However, it could be argued that NO is causing relaxation through the release of VIP, but this seems unlikely since the vagally induced relaxation is mimicked by glyceryl trinitrate and sodium nitroprusside but not by VIP. Thus, the effect of desensitization to VIP on NO-induced relaxation requires further study, especially at the second-messenger level.

In conclusion, NO seems to be the major neurotransmitter of gastric relaxation induced by vagal stimulation in the guinea pig, and the role of VIP in this response, if any, seems minor.

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