Vagal control of nitric oxide and vasoactive intestinal polypeptide release in the regulation of gastric relaxation in rat

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1. Gastric motility and neurotransmitter release in response to vagal stimulation were studied using a vascularly isolated perfused rat stomach. Gastric motor responses were recorded by a strain gauge force transducer implanted on the proximal stomach.

2. Electrical stimulation of vagal trunk (0·5–20 Hz) produced a triphasic response which was composed of a rapid transient relaxation (first phase) followed by a phasic contraction (second phase) and a delayed prolonged relaxation (third phase). Maximum responses of the first, second and third phase were observed at 2·5, 5 and 10 Hz, respectively. Intra-arterial infusion of tetrodotoxin (0·1 μM) or hexamethonium (100 μM) completely abolished the triphasic response.

3. The nitric oxide (NO) biosynthesis inhibitor N^G-nitro-L-arginine (L-NNA; 100 μM) significantly antagonized the rapid relaxation but had no effect on the delayed relaxation, while vasoactive intestinal polypeptide (VIP) antagonist (1 μM) significantly reduced the delayed relaxation without affecting the rapid relaxation.

4. In response to vagal stimulation, NO production ([^{3}H]citrulline formation in gastric tissue preloaded with [^{3}H]arginine) was maximum at 2·5 Hz, whereas VIP release into the venous effluent was largest at 10 Hz. Hexamethonium abolished vagal-stimulated NO production and VIP release. L-NNA had no effect on VIP release in response to vagal stimulation.

5. The nicotinic receptor agonist 1,1-dimethyl-4-phenylpiperizinium (DMPP; 100 μM) also caused a triphasic response similar to that observed with vagal stimulation and produced a significant increase in VIP and NO formation. DMPP-evoked VIP release was not affected by L-NNA. Similarly, DMPP-evoked NO production was not antagonized by VIP antagonist.

6. These results suggest that vagus nerve stimulation evokes NO and VIP release via nicotinic synapses which cause different modes of relaxation of the stomach. There is no interaction between NO and VIP release in response to vagal stimulation.

Receptive relaxation allows the stomach to receive large volumes with only minimal increase in pressure during food intake (Canon & Lieb, 1911). The vagus nerve plays an important role in the mediation of receptive relaxation via non-adrenergic, non-cholinergic (NANC) innervation to the stomach (Abrahamsson & Jansson, 1969). However, the nature of the specific neurotransmitter(s) released by these NANC neurons responsible for gastric relaxation remains to be established.

Pharmacological evidence has suggested that at least two different NANC inhibitory systems are present in the gastrointestinal (GI) tract (Costa, Furness & Humpreys, 1986; Manzini, Maggi & Meli, 1986). Vasoactive intestinal polypeptide (VIP) has been proposed as a likely neurotransmitter of NANC neurons, as VIP immunoreactivity is present in intrinsic neurons of the stomach (Larsson, Fahrenkrug, Schaffalitzky, Sundler, Hakanson & Rehfeld, 1976; Fahrenkrug, Haglund, Jordal, Lundgren, Olbe &

Schaffalitzky de Muckadell, 1978). Furthermore, VIP is released upon nerve depolarization by KCl or electrical stimulation (Grider & Makhlouf, 1987; Agoston, Conlon & Whittaker, 1988; Ohta, Itoh & Ohga, 1990) and relaxes gastric muscle cells with a concomitant increase of intracellular cyclic AMP (Bitar & Makhlouf, 1982). Vagal stimulation produces a frequency-dependent increase of VIP released into the portal vein (Yasui et al. 1987; Reid, Shulkes & Titchen, 1988). Trypsin or VIP antiserum inhibits a neurally induced relaxation of the stomach (D'Amato, De Beurme & Lefebvre, 1988; Li & Rand, 1990). These observations support the concept that VIP is an inhibitory NANC transmitter. However, in other studies, the VIP antagonist fails to prevent NANC-induced relaxation (D'Amato et al. 1988; Fisher, Anderson & Waldron, 1993) and there were no similarities between gastric relaxation induced by VIP and that induced by the stimulation of NANC neurons in cat gastric fundus (D'Amato et al. 1988).

Recently, nitric oxide (NO) has been proposed as an inhibitory NANC neurotransmitter in the gastrointestinal tract. Gastric relaxation induced by the stimulation of NANC nerves was significantly antagonized by the NO biosynthesis inhibitor N^G-nitro-L-arginine (L-NNA) or N^G-nitro-L-arginine methyl ester (L-NAME) in the rat stomach (Boeckxstaens et al. 1991; D'Amato, Curro & Montuschi, 1992; Shimamura, Fujisawa, Toda & Sunano, 1993). Exogenously applied NO or sodium nitroprusside, an activator of soluble guanylate cyclase, produces relaxation which mimics NANC-induced relaxation in rat gastric fundus (Boeckxstaens et al. 1991), guinea-pig ileum (Osthaus & Galligan, 1991) and canine ileocolonic junction (Bult, Boeckxstaens, Pelckmans, Jordaens, Van Maercke & Herman, 1990). The presence of NO synthase was detected in the myenteric plexus (Bredt, Hwang & Snyder, 1990; Costa et al. 1992; Aimi, Kimura, Kinoshita, Minami, Fujimura & Vincent, 1993) as well as in the central nervous system (Bredt et al. 1990). A rapid relaxation of the stomach in response to vagal stimulation was inhibited by L-NAME (Lefebvre, De Vriese & Smits, 1992b). The involvement of the NO pathway in adaptive relaxation of the stomach, mediated by the intramural myenteric plexus, was recently shown in the isolated guinea-pig stomach (Desai, Sessa & Vane, 1991). These observations suggest that NO may be involved in mediating gastric relaxation.

Grider, Murthy, Jin & Makhlouf (1992) demonstrated that VIP itself stimulates NO production from isolated guinea-pig gastric muscle cells; however, the interaction between NO and VIP release upon vagal stimulation still remains unclear. The exact roles of NO and VIP in the vagal mediation of gastric motility have not been fully studied. Furthermore, there is no direct evidence showing NO is released from the gastric myenteric plexus upon vagal stimulation.

Therefore, the aims of this study were (1) to characterize the role of NO and VIP in the regulation of gastric relaxation, (2) to investigate the possible mechanism of NO and VIP release in response to vagal stimulation, and (3) to clarify the interaction between NO and VIP release in response to vagal stimulation.

METHODS

Preparation of the isolated perfused stomach

One hundred and twenty-five male Sprague–Dawley rats, weighing 250–300 g, were anaesthetized with an intramuscular injection of ketamine hydrochloride (50 mg kg^{-1}) after a 24 h fast. Isolation and vascular perfusion of the stomach were performed as previously described (Yokotani, Okuma & Osumi, 1991; Schubelt & Makhlouf, 1993). After opening the abdomen with a mid-line incision, the abdominal aorta was exposed retroperitoneally. The coeliac artery was identified and the abdominal aorta was ligated just above the branching of the coeliac artery; a cannula was inserted into the coeliac artery. The stomach was perfused through the coeliac artery with a peristaltic pump (Harvard Apparatus, South Natick, MA, USA) at a constant flow rate of 2 ml min^{-1}. The perfusate was composed of modified Krebs–Henseleit bicarbonate (KHB) buffer containing (mM): 118 NaCl, 4·8 KCl, 2·5 CaCl$_2$, 25 NaHCO$_3$, 1·2 KH$_2$PO$_4$, 1·2 MgSO$_4$, 11·1 glucose; and 0·2% bovine serum albumin and 4% dextran. The perfusate was maintained at pH 7·4 and 37 °C, bubbled with a mixture of 95% O$_2$ and 5% CO$_2$. The oesophagus, duodenum, spleen and pancreas were dissected after ligation of vessels. The gastric venous effluent was recovered through a cannula in the portal vein. Both vagal trunks around the oesophagus were carefully isolated and cut 1 cm above the lower oesophageal sphincter. The vascularly perfused stomach was kept in a chamber prewarmed at 37 °C. Following isolation of the stomach, rats were killed by an overdose of pentobarbitone given i.v. (200 mg kg^{-1}). After washing the gastric contents through a cannula inserted into the stomach lumen via the pylorus ring, the stomach was slightly distended with 2 ml of saline prewarmed to 37 °C. A volume of 2 ml was used because this volume represents 10–30% of the normal feeding capacity of the stomach (Schubelt & Makhlouf, 1993), and preliminary experiments showed that maximal gastric response to a maximal dose of carbachol (10 μM) was observed with this volume.

Measurement of gastric contraction

Since receptive relaxation mainly involves the proximal stomach (Wilbur, Kelly & Code, 1974), this study was designed to record motor responses of the gastric body (proximal stomach) in response to electrical stimulation of the vagus nerve. Gastric motility was monitored by a force transducer implanted on the serosal surface of the mid-portion of the gastric body to detect circular muscle contraction as previously described (Mizumoto, Sano, Matsunaga, Yamamoto, Itoh & Oshima, 1993). The lead wires of transducers were connected to an amplifier (FS-04M; Star Medical, Tokyo), and the signals from the amplifier were recorded on a multi-channel, pen-writing recorder (Gould, Cleveland, OH, USA). Gastric motility was monitored in response to graded electrical stimulation of the vagus nerve and studies were repeated in the presence of various antagonists. For frequency–response studies, different frequencies (0·5–20 Hz) were applied in random order.

Experimental procedures

After an equilibration period of 60 min, bilateral vagus nerves were electrically stimulated with square-wave pulses (150 pulses at 0·5–20 Hz frequency, supramaximal voltage of 5 V and 2 ms duration) using platinum electrodes. The responses to vagal stimulations were quite reproducible up to 6–8 times when applied every 20 min. Stimulation was performed every 20 min and evaluated in triplicate, and the mean value was used to calculate tension change. To examine possible mediators by which vagus nerves mediate gastric motility, the following drugs were used: atropine (muscarinic receptor antagonist), hexamethonium (nicotinic receptor antagonist), phentolamine (α-antagonist), propranolol (β-antagonist), tetrodotoxin (Na^+ channel blocker), N^G-nitro-L-arginine (L-NNA; NO biosynthesis inhibitor), Methylene Blue (a soluble guanylate cyclase inhibitor), a VIP antagonist devised by a hybrid peptide strategy (Gozes, Meltzer, Rubinrout, Brenneman & Fridkin, 1989), and trypsin (type XII-S; peptidase). After intra-arterial infusion of various antagonists for 15 min, the vagus nerve was stimulated again and gastric motor activities with and without pretreatment with antagonists were compared. Only one antagonist was administered in each stomach preparation.

Measurement of VIP

Effluent from the portal vein was collected every 30 s in chilled tubes containing bacitracin (20 μM) and aprotinin (1000 u ml^{-1}) before, during and after the vagal stimulation (5 V, 1–10 Hz, 2 ms, 150 pulses) or following the administration of 1,1-dimethyl-4-phenylpiperizinium (DMPP; 1–100 μM). Samples were stored at −20 °C for subsequent radioimmunoassay (RIA). RIA of VIP was performed with rabbit VIP antiserum (Incstar, Stillwater, MN, USA) as previously described (Wiley, O'Dorisio & Owyang, 1988). Intra-assay and interassay variability were 5 and 8%, respectively. VIP release was expressed as the percentage change from basal levels measured in the absence of test agents.

Measurement of NO production

Production of NO was measured in gastric tissue preloaded with L-[^{3}H]arginine and expressed as amount of L-[^{3}H]citrulline formed in the tissue as described by Bredt & Snyder (1989). L-Citrulline and NO are produced in a 1 : 1 ratio from L-arginine by the action of NO synthase. One hundred and sixty male Sprague–Dawley rats, weighing 250–300 g, were anaesthetized with intramuscular injection of ketamine hydrochloride (50 mg kg^{-1}) after a 24 h fast. Whole stomach with attached vagus nerve was quickly removed and incubated in a 20 ml organ bath with [^{3}H]arginine (3 μCi ml^{-1}) for 5 min at 37 °C. Animals were killed by an overdose of pentobarbitone given I.V. (200 mg kg^{-1}). Immediately following vagal stimulation (5 V, 2 ms, 1–10 Hz, 150 pulses) or DMPP administration (1–100 μM for 1 min), the reaction was stopped by flash freezing gastric tissues in liquid nitrogen. The samples were stored at −70 °C for subsequent measurement of L-[^{3}H]citrulline. After removing the mucosa from the stomach, muscle tissue was homogenized by a 1 ml straight wall grinder (Radnoti Glass Technology, Monrovia, CA, USA). Following the precipitation of protein with 1 M trichloroacetic acid, samples were centrifuged at 1800 g for 15 min. The protein content of the pellets was measured by the Biorad method using bovine serum albumin (Bradford, 1976). L-[^{3}H]citrulline in the supernatants was extracted with diethyl ether and an alliquot applied to a Dowex AG50WX-8 column and eluted with Hepes buffer (pH 6·0). L-[^{3}H]citrulline in the

effluent was measured by liquid scintillation spectroscopy, and the production of L-citrulline (stoichiometrically equivalent to NO) was expressed as c.p.m. per milligram protein.

Materials

Atropine, aprotinin, D-arginine, L-arginine, bacitracin, bovine serum albumin, dextran, 1,1-dimethyl-4-phenylpiperizinium (DMPP), hexamethonium, phentolamine, propranolol, tetrodotoxin and trypsin (type XII-S) were obtained from Sigma Chemical Co. (St Louis, MO, USA); L-[^{3}H]arginine from New England Nuclear (Boston, MA, USA); Dowex AG50W-X8 from Biorad (Richmond, CA, USA); Methylene Blue from Fisher Scientific Company (Springfield, NJ, USA); VIP and VIP antagonist from Bachem (Torrance, CA, USA). NO solutions were made from 99% NO gas (MG Industries, Valley Forge, PA, USA). Oxygen was eliminated by bubbling iced KHB buffer with He for 2 h. A saturated solution of NO (10 μM) was prepared by injection of 250 μl of NO gas into 1 ml of the oxygen-free Krebs–Henseleit buffer as described by Ignarro, Byrns, Buga & Wood (1987) and dilutions were prepared in vacuum tubes.

Analysis of data

Before the experiment each force transducer was calibrated by application of known weights (1, 2, 5, 10, 20 and 50 g), according to a previously described method (Jacoby, Bass & Bennett, 1963). The calibration curve was linear with 1–50 g weights. Every contraction or relaxation in response to vagal stimulation was estimated from the basal (pre-stimulating) levels. The mean amplitude of contraction or relaxation was determined by dividing the sum of the amplitudes of each contraction or relaxation by the number of contractions or relaxations and expressed in grams. Results were expressed as means $\pm$ S.E.M. In the case of the study of the action of antagonists on gastric motor responses, Student's paired t test was used. In the case of the antagonists study on VIP and NO releases, Student's t test was used. $P < 0.05$ was considered significant.

RESULTS

Mechanical responses to NO, VIP, DMPP and vagal stimulation

Strain gauge transducers implanted on the serosal surface of the gastric body enabled us to detect spontaneous phasic contractions during the resting state in a vascularly isolated perfused rat stomach. The system gave reproducible results and responded to known stimulatory agents (carbachol or substance P) or inhibitory agents (VIP or NO). Intra-arterial infusion of VIP (1 μM) or NO (10 μM) for 10 s produced different modes of relaxation. VIP (1 μM) caused a delayed prolonged relaxation, whereas NO (10 μM) provoked a rapid transient relaxation (Fig. 1). VIP-evoked relaxation was not affected by L-NNA (100 μM). NO-induced relaxation was not affected by VIP antagonist (1 μM; data not shown).

As shown in Fig. 2, electrical stimulation (5 V, 2 ms, 5 Hz, 150 pulses) of the vagal trunk produced a triphasic response which was composed of a rapid transient relaxation (first phase) followed by a phasic contraction (second phase) and a delayed prolonged relaxation (third
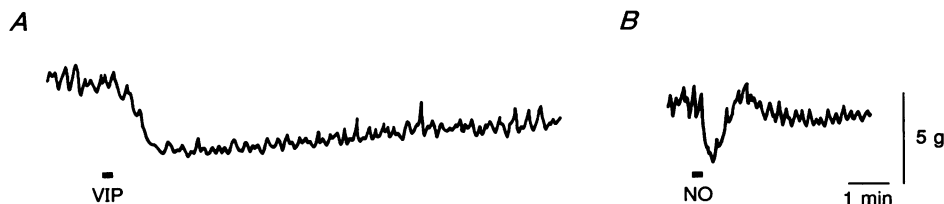
Figure 1

Relaxation of isolated vascularly perfused rat stomach evoked by intra-arterial infusion of 1 μM VIP (A) and 10 μM nitric oxide (NO; B)

phase). Rapid relaxation was observed during vagal stimulation with various frequencies and phasic contraction was observed after the cessation of vagal stimulation ('off contraction'). Following phasic contraction, delayed prolonged relaxation was observed which gradually returned to basal level in 1–3 min. In 125 preparations tested, these triphasic responses to vagal stimulation (5 Hz) were observed in 103 preparations (82·4%). In eighteen preparations (14·4%) we did not observe the third phase and in four preparations (3·2%) the first phase was not observed. The preparations which showed triphasic response were used for further studies with

antagonists. Administration of phentolamine (1 μM) or propranolol (1 μM) had no effect on these triphasic responses (data not shown). Intra-arterial infusion of Ca^{2+}-free medium containing 1 mM EDTA, which slightly decreased the basal tone (Fig. 2A), completely abolished the triphasic response (Fig. 2A). Similarly, intra-arterial infusion of tetrodotoxin (0·1 μM) for 15 min (total dose, 0·96 μg) abolished the triphasic response without affecting the basal tone (Fig. 2B); and administration of hexamethonium (100 μM) for 15 min (total dose, 1·09 mg) also abolished the triphasic response to vagal stimulation without affecting the basal tone (Fig. 2C).
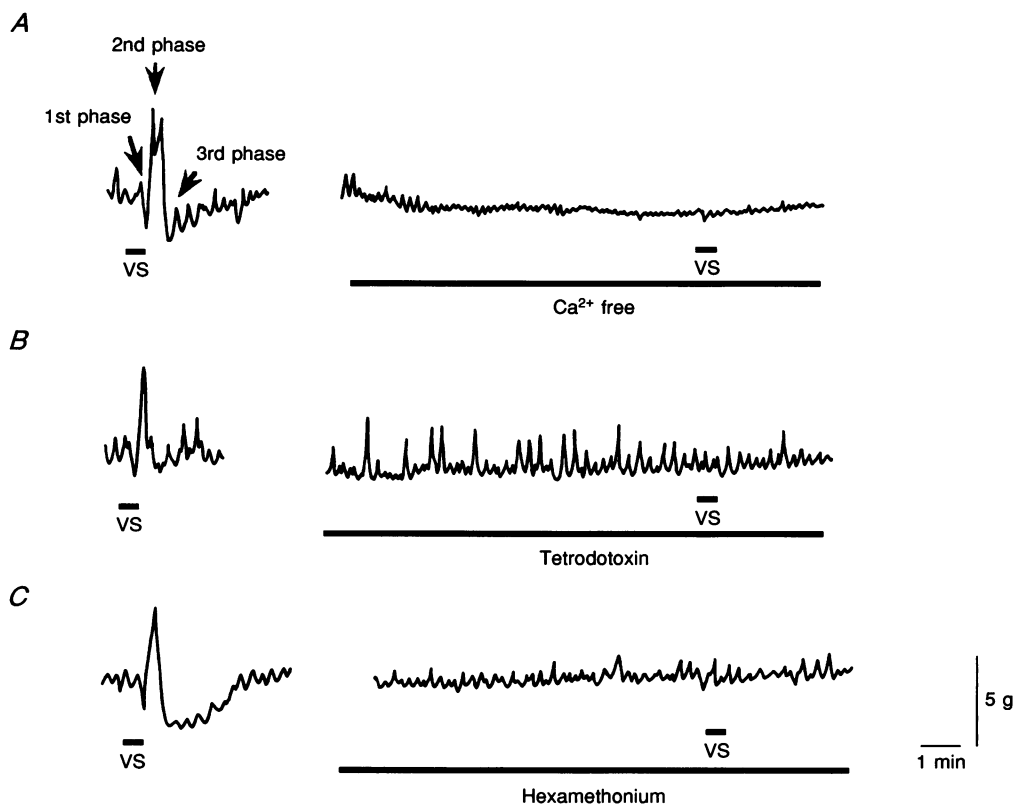
Figure 2. Effects of intra-arterial infusion of Ca^{2+}-free medium, tetrodotoxin, or hexamethonium on the triphasic response to vagal stimulation

Vagal stimulation (VS): 5 V, 5 Hz frequency, 150 pulses, 2 ms duration. Intra-arterial infusion of Ca^{2+}-free medium (A), tetrodotoxin (1 μM; B) or hexamethonium (100 μM; C) completely abolished the triphasic response to vagal stimulation.

The three phases of contractile response to vagal stimulation were frequency dependent. Rapid relaxation was most pronounced during lower frequencies of stimulation (0·5–2·5 Hz), whereas the delayed prolonged relaxation was observed mostly during higher frequencies of stimulation (5–20 Hz). Maximum relaxation of the first phase, estimated from the frequency–response curve, was observed at 2·5 Hz frequency, whereas maximum relaxation of the third phase was observed at 10 Hz. Maximum contraction of the second phase occurred at 5 Hz (Fig. 3).

To investigate the role of NO in the regulation of gastric relaxation, we examined the effects of L-NNA, a NO biosynthesis inhibitor. Boeckxstaens *et al.* (1991) found that L-NMMA and L-NNA increased the basal muscle tension of the canine gastric fundus and suggested that tonic release of NO may regulate basal tone in the proximal stomach. In our experiments using a vascularly perfused rat stomach, a transient, small increase of basal tension (0·35 ± 0·1 g, *n* = 3) was observed following the administration of L-NNA (100 μM) in three of eight preparations tested. A 15 min infusion of L-NNA (100 μM; total dose, 0·66 mg) markedly antagonized the first phase of relaxation (by 95 ± 3%) and enhanced the second phase of contraction (by 55 ± 15%) in response to vagal stimulation (5 Hz; *P* < 0·01, *n* = 8; Fig. 4*A*). In the

presence of L-NNA (100 μM), a rapid relaxation was almost completely abolished and a phasic contraction was observed immediately after the start of vagal stimulation. L-NNA had no effect on the third phase of relaxation (Fig. 4*A*). The effect of L-NNA was prevented by the pre-administration of L-arginine (1 mM; total dose, 5·1 mg; Fig. 5*A*) but not by D-arginine (1 mM; data not shown). Similarly, Methylene Blue (50 μM; total dose, 561 μg), an inhibitor of soluble guanylate cyclase, also completely antagonized the first phase of relaxation and enhanced the second phase of contraction (Fig. 4*B*).

To investigate whether the contractile response to vagal stimulation is mediated via the cholinergic pathway, we examined the effects of atropine. Atropine (0·1 μM; total dose, 2·03 μg), which had no effect on the basal tone, markedly reduced the second phase of contraction in response to vagal stimulation (5 Hz; *P* < 0·01, *n* = 8). However, relaxation of neither the first phase nor the third phase was affected by pretreatment with atropine (0·1 μM; Figs 4*C* and 5*B*).

To investigate the role of VIP in gastric relaxation, we examined the effects of VIP antagonist. VIP antagonist (1 μM; total dose, 103 μg) reduced by 55 ± 7% the amplitude of relaxation evoked by exogenously applied VIP (1 μM; *P* < 0·01, *n* = 8) but had no action on that
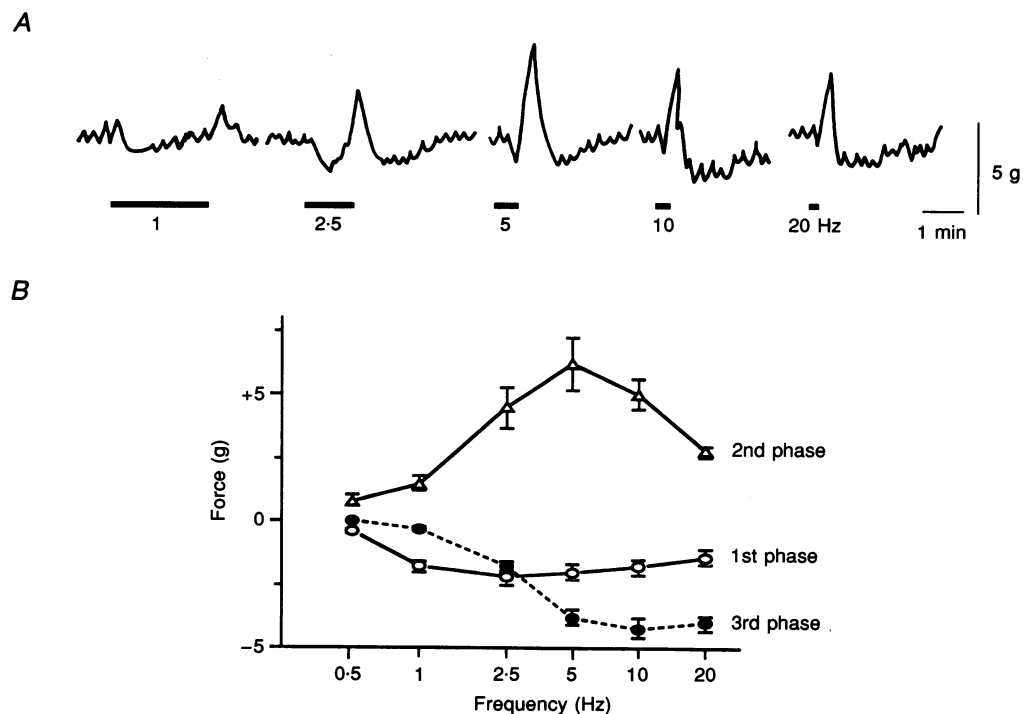
A

B

Figure 3. Gastric motor responses to vagal stimulation

A, typical tracings of gastric motor responses to vagal stimulation (VS; 5 V, 2 ms duration, 150 pulses) at various frequencies (1–20 Hz). *B*, gastric contraction or relaxation in response to different frequencies of vagal stimulation (0·5–20 Hz). A rapid relaxation (1st phase) was observed at lower frequencies (0·5–1 Hz), while a delayed prolonged relaxation (3rd phase) was observed at higher frequencies (5–10 Hz). Maximum contractions of the second phase occured at 5 Hz. Means ± S.E.M., *n* = 8.

evoked by NO (10 μM). VIP antagonist (1 μM) also markedly reduced the amplitude of the third phase of prolonged relaxation in response to vagal stimulation (5 Hz) without affecting the first and second phases ($P < 0.01$, $n = 8$; Figs 4D and 5C). The duration of the third phase of delayed relaxation was also significantly reduced by VIP antagonist from 1.8 ± 0.3 to 1.1 ± 0.2 min ($P < 0.05$, $n = 8$) and as a result the motility index of the third phase was reduced by $76 \pm 12\%$ ($P < 0.05$, $n = 8$). Similar results were obtained by pretreatment with trypsin (3 μM; total dose, 2·16 mg; Fig. 4E).

Intra-arterial infusion of DMPP at 1 μM produced a modest inhibition of the ongoing phasic activity. DMPP at 10 μM produced a biphasic response characterized by a rapid transient relaxation followed by a phasic contraction (Fig. 6). At a higher concentration, DMPP (100 μM) caused a triphasic response, which was composed

of a rapid transient relaxation followed by a phasic contraction and delayed prolonged relaxation, similar to that observed with electrical vagal stimulation (Fig. 6). The delayed prolonged relaxation was only observed with a higher concentration of DMPP (100 μM).

NO production and VIP release in response to vagal stimulation

To confirm the role of NO and VIP in the mediation of gastric relaxation, we examined NO production and VIP release in response to vagal stimulation with different frequencies. Basal level of [^{3}H]citrulline (NO production) was 256 ± 34 c.p.m. (mg protein)$^{-1}$. Vagal stimulation produced a significant increase of tissue [^{3}H]citrulline, with maximum production observed at 2·5 Hz (122 $\pm$ 39% increase over basal). There was no further increase of [^{3}H]citrulline in response to vagal stimulation at 5 or 10 Hz (Fig. 7). Pretreatment with hexamethonium
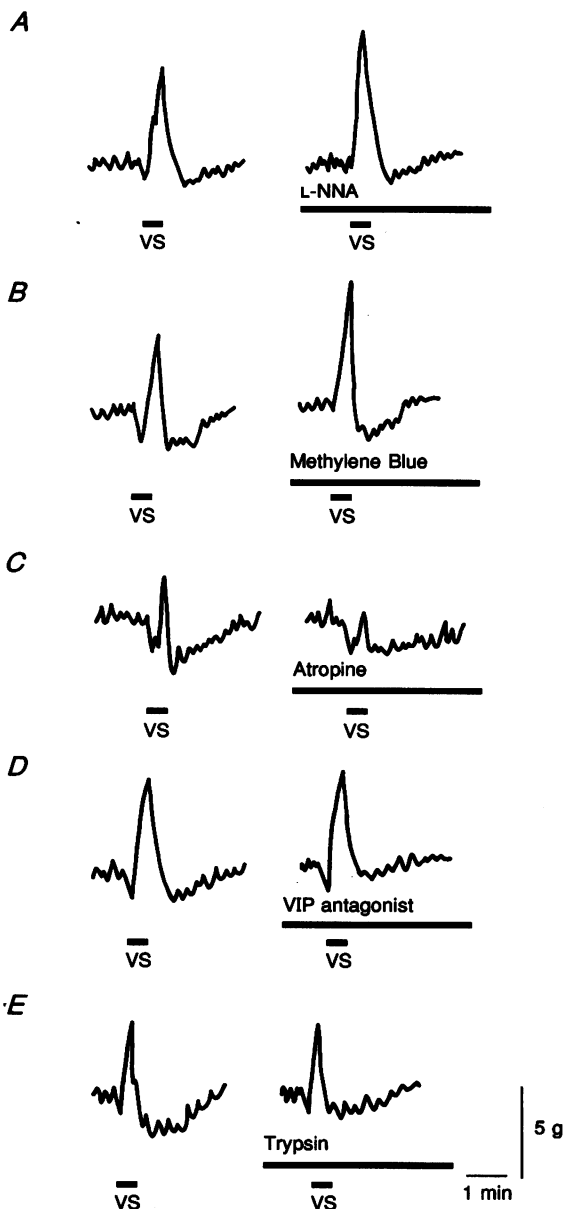

Figure 4. Studies to determine the mediators of the triphasic response to vagal stimulation

Representative tracings to demonstrate the effects of L-NNA (100 μM; A), Methylene Blue (50 μM; B), atropine (0·1 μM; C), VIP antagonist (1 μM; D) and trypsin (3 μM; E) on the triphasic response to vagal stimulation (VS; 5 V, 5 Hz, 150 pulses, 2 ms duration). L-NNA (A) or Methylene Blue (B) significantly antagonized the first phase of relaxation, while VIP antagonist (D) or trypsin (E) significantly reduced the third phase of prolonged relaxation.

Figure 5. Effects of L-NNA, atropine and VIP antagonist on the triphasic response to vagal stimulation (5 V, 2 ms, 5 Hz)

L-NNA (100 μM; *A*), atropine (0·1 μM; *B*) and VIP antagonist (1 μM; *C*) significantly antagonized the first phase of relaxation, the second phase of contraction and the third phase of prolonged relaxation, respectively, in response to vagal stimulation. Means ± S.E.M., $n = 8$, ** $P < 0.01$.
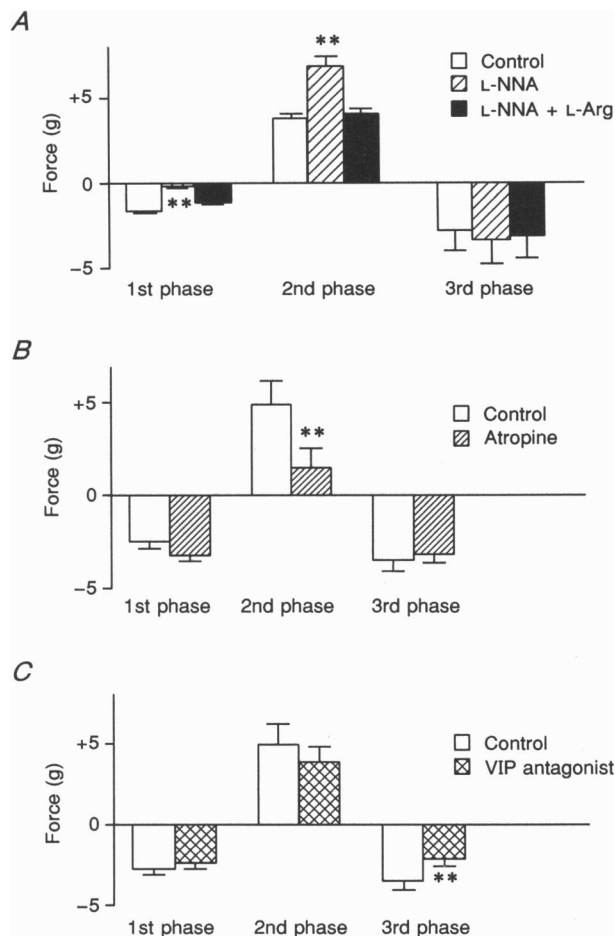
(100 μM) for 15 min completely abolished the NO increase in response to vagal stimulation (2·5 Hz; Table 1). Furthermore, L-NNA (100 μM) and Ca^{2+}-free medium completely abolished the increase of [^{3}H]citrulline in response to vagal stimulation (2·5 Hz; Table 1).

The basal VIP concentration in the venous effluent was 66 ± 12 pg ml^{-1}. Significant increases were observed immediately following vagal stimulation. On terminating the stimulation, VIP levels rapidly decreased to basal levels within 2 min. VIP responses to vagal stimulation increased in a frequency-dependent manner and the largest increase was observed at 10 Hz, which produced a

$74 \pm 16\%$ increase over the basal level (Fig. 7). Pre-treatment with hexamethonium (100 μM) or Ca^{2+}-free medium for 15 min abolished VIP increase in response to vagal stimulation (10 Hz; Table 1). In contrast, L-NNA (100 μM) failed to prevent VIP increase in response to vagal stimulation (10 Hz; Table 1).

VIP release and NO production in response to DMPP

We next studied the effects of DMPP on NO production and VIP release. DMPP (1–100 μM) significantly increased [^{3}H]citrulline and VIP release in a dose-dependent manner. The largest effects were observed at 100 μM
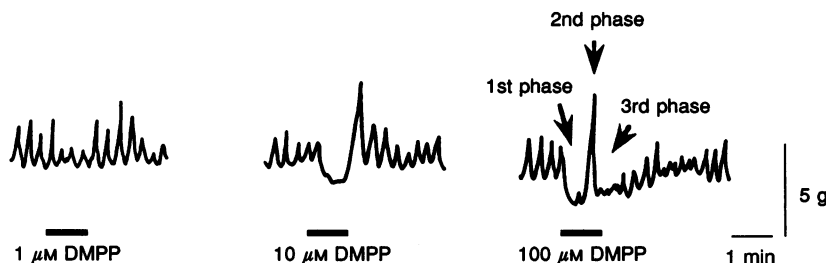

Figure 6. Typical tracings of gastric motor responses to intra-arterial infusion of DMPP, a nicotinic receptor agonist

The motility response to DMPP (1–100 μM) appears to be dose dependent (see text).

Table 1. Vagal stimulation-evoked NO production and VIP release from the rat stomach

	NO production (%)	VIP release (%)
Control	122 ± 39	74 ± 12
Ca^{2+}- free medium	8 ± 5**	4 ± 3**
Hexamethonium (100 μM)	12 ± 8**	7 ± 5**
Tetrodotoxin (0·1 μM)	8 ± 6**	5 ± 4**
L-NNA (100 μM)	6 ± 4**	72 ± 15

Vagal stimulation (5 V, 2 ms, 150 pulses) was applied at 2·5 Hz for NO assay and 10 Hz for VIP assay to obtain maximal responses. Data are expressed as percentage increase over basal levels. Means ± s.e.m., $n = 6$, ** $P < 0.01$.

DMPP, which produced a $175 \pm 32\%$ increase of [^{3}H]citrulline over the basal level and $120 \pm 24\%$ increase of VIP over the basal level, respectively (Fig. 8). The DMPP (100 μM)-evoked increase in [^{3}H]citrulline was completely abolished by L-NNA (100 μM) or tetrodotoxin (0·1 μM). On the other hand, VIP antagonist (1 μM) had no effect on DMPP-evoked [^{3}H]citrulline increase. DMPP-evoked VIP release was not affected by L-NNA (Table 2).

DISCUSSION

In this study, we demonstrated that both nitric oxide and VIP are involved in the vagal regulation of gastric relaxation. Electrical stimulation (5 V, 2 ms, 5 Hz, 150 pulses) of the vagal trunk produced a triphasic response which was composed of a rapid transient relaxation (first phase) followed by a phasic contraction (second phase) and a delayed prolonged relaxation (third phase). Intra-arterial infusion of a Ca^{2+}-free medium containing 1 mm EDTA or tetrodotoxin completely abolished these responses, suggesting the involvement of neural mechanisms. Our studies also demonstrated that electrical vagal stimulation resulted in both gastric relaxation and contraction but optimal responses occurred at different frequencies, suggesting that these responses are mediated via different neural pathways and neurotransmitters. Triphasic responses to electrical nerve stimulation in the rat stomach have been shown both *in vitro* (Ito, Kimura & Ohga, 1988) and *in vivo* (Lefebvre, Baert & Barbier, 1992a; Baccari, Calamai & Staderini, 1992). Ito *et al.*

(1988) showed a triphasic response using circular muscle strips obtained from embryonic rat stomach. Consistent with our results, they showed that the first phase of rapid relaxation, the second phase of contraction and the third phase of delayed relaxation were at a maximum at 2, 5 and 10 Hz, respectively. Lefebvre *et al.* (1992a) reported that vagal stimulation produced a triphasic response which was composed of a rapid relaxation, followed by a rebound contraction and a delayed relaxation. As L-NNA significantly antagonized the rapid relaxation and enhanced the contraction without affecting the delayed relaxation, and as atropine inhibited the rebound contraction, the authors proposed that the first phase of relaxation and the second phase of contraction were mediated by NO and acetylcholine, respectively. However, the mechanism for the third phase of delayed relaxation was not addressed.

To confirm the role of NO in the regulation of gastric relaxation, we examined the effects of L-NNA, a nitric oxide biosynthesis inhibitor. L-NNA (100 μM) significantly antagonized the first phase of relaxation in response to vagal stimulation. The inhibitory effect of L-NNA (100 μM) was significantly prevented by the pre-administration of L-arginine (1 mm) but not by D-arginine (1 mm). These observations indicate that the rapid relaxation in response to vagal stimulation was mediated by the release of NO. Methylene Blue has been reported to be a selective inhibitor of soluble guanylate cyclase, the proposed site of action of NO (Ward *et al.* 1992; Chakder & Rattan, 1993).
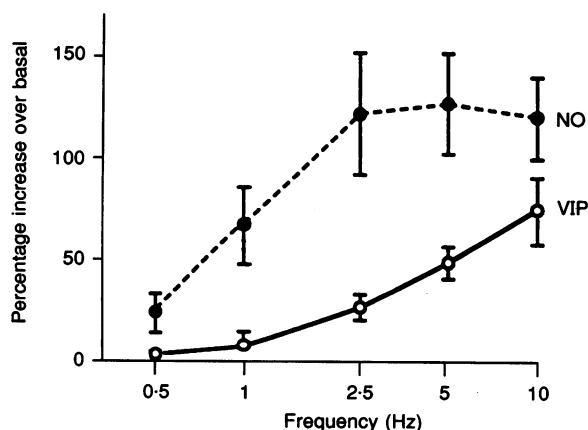

Figure 7. NO production and VIP release in response to vagal stimulation

Vagal stimulation: 5 V, 2 ms duration, 150 pulses, 1–10 Hz. The maximal NO production was observed at 2·5 Hz, while VIP release was the largest at 10 Hz, suggesting differential release of NO and VIP during vagal stimulation. Means ± s.e.m., $n = 6$.

Table 2. DMPP-induced NO production ([³H]citrulline) and VIP release from the rat stomach

	NO production (%)	VIP release (%)
Control	175 ± 32	120 ± 24
Hexamethonium (100 μM)	5 ± 4**	7 ± 5**
Tetrodotoxin (0·1 μM)	11 ± 5**	13 ± 10**
L-NNA (100 μM)	7 ± 4**	126 ± 31
VIP antagonist (1 μM)	156 ± 45	n.t.

Data are expressed as percentage increase over basal levels. n.t., not tested. Means $\pm$ S.E.M., $n = 6$, ** $P < 0.01$.

Similar to L-NNA, Methylene Blue (50 μM) completely antagonized the first phase of relaxation and enhanced the second phase of contraction. This further demonstrated that the observed motor effects of vagal stimulation involved generation of NO in the gastric wall.

The second phase of contraction in response to vagal stimulation was significantly antagonized by atropine, suggesting mediation by acetylcholine release from the gastric myenteric plexus. However, atropine did not cause complete inhibition of the second phase of contraction. This suggests the possible participation of other neurotransmitter(s) besides acetylcholine. Baccari *et al.* (1992) demonstrated that in the atropine- and guanethidine-treated animals, vagally mediated rebound contraction was depressed by prostaglandin synthesis inhibitors. As the PGE₂-evoked excitatory motor responses closely mimicked the vagally induced rebound contraction, these investigators proposed that PGE₂ may be involved in mediating the post-stimulus excitatory contraction.

The second phase of phasic contraction was significantly enhanced by L-NNA, suggesting a neuromodulatory role of NO on excitatory neurotransmission, as previously reported by Gustafsson, Wiklund, Wiklund, Persson & Moncada (1990). They proposed the possibility that the potentiation of excitatory response to transmural stimulation by NO biosynthesis inhibitor may be due to the inhibitory action of endogenous NO on the release of substance P (Gustafsson *et al.* 1990) or acetylcholine (Lefebvre *et al.* 1992*b*).

As shown in Fig. 1, intra-arterial infusion of NO and VIP produced different patterns of relaxation in vascularly isolated perfused rat stomach. VIP caused delayed, prolonged relaxation, whereas NO produced rapid transient relaxation. This observation led us to hypothesize that released NO and VIP during vagal stimulation provoke distinct different modes of gastric relaxation. Thus, we next investigated the role of VIP in gastric relaxation using a novel VIP antagonist. VIP antagonist (1 μM) significantly reduced the third phase of prolonged relaxation without affecting the first and second phase. Similar results were obtained by the pretreatment of trypsin (3 μM). Therefore, the delayed prolonged relaxation was, at least in part, mediated by VIP release. As VIP antagonist or trypsin did not completely abolish the third phase of relaxation, we cannot exclude the possibility that another peptidergic or non-peptidergic neurotransmitter is released upon vagal stimulation to mediate the delayed, prolonged relaxation. Besides NO and VIP, adenosine triphosphate (ATP) has been suggested to be a candidate of the NANC neurotransmitter in the GI tract (Baccari, Calamai & Staderini, 1990; Crist, He & Goyal, 1992; Keef, Du, Ward, McGregor & Sanders, 1993). Further study is needed to clarify the participation of ATP release in the mediation of the delayed relaxation in response to vagal stimulation in rat stomach.

The frequency-dependent release of various neurotransmitters in response to nerve stimulation has been suggested by several authors. Low frequency (2–5 Hz)

Figure 8. Effects of DMPP on NO production ([³H]citrulline) and VIP release from the rat stomach

DMPP (1–100 μM) caused a dose-dependent increase of VIP release and NO production, suggesting that VIP and NO releases were mediated by nicotinic synapses. Means $\pm$ S.E.M., $n = 6$.
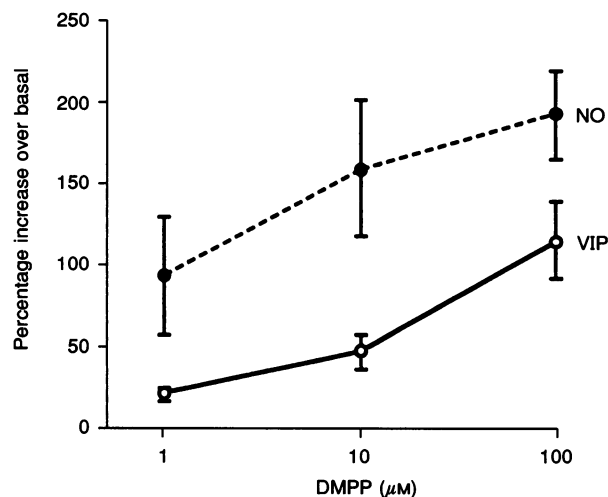
stimulation of the myenteric nerve selectively depleted ACh release, whereas higher frequency (10–50 Hz) mainly stimulated VIP release in the guinea-pig myenteric plexus–longitudinal muscle preparation (Agoston et al. 1988). Yokotani et al. recently demonstrated that maximum release of ACh and noradrenaline in response to vagal stimulation was observed at 5 (Yokotani, Okuma, Nakamura & Osumi, 1993) and 10 Hz (Yokotani et al. 1991), respectively, in the rat stomach. The relaxation of rat fundic strips evoked by transmural stimulation at lower frequencies was completely abolished by L-NMMA, while responses to higher frequencies of stimulation (> 5 Hz) were only partially reduced by L-NMMA (Li & Rand, 1990). In contrast, trypsin only reduced relaxation induced by high frequencies of stimulation, suggesting that the relaxation in the rat gastric fundus in response to low frequencies of stimulation was mediated mainly by NO, whereas peptidergic neurotransmitter(s) was released at higher frequencies (Boeckxstaens, Pelckmans, De Man, Bult, Herman & Van Maercke, 1992). We have confirmed and extended these observations by clearly demonstrating that the vagal release of NO and VIP are frequency dependent. In our present study, vagal stimulation provoked a significant increase of NO production in the stomach, and maximum effect was observed at 2·5 Hz. On the other hand, VIP release in response to vagal stimulation was greatest at 10 Hz.

Grundy, Gharib-Naseri & Hutson (1993) demonstrated that in the anaesthetized ferret, vagal stimulation (1, 2 and 5 Hz) produced two components of relaxation, an initial rapid relaxation followed by a slower relaxation. Similarly to our results, they also showed that administration of L-NAME significantly reduced the initial rapid relaxation without affecting the slower relaxation, whereas VIP immunization only antagonized the slower relaxation in response to vagal stimulation (1 Hz). However, the possible interaction between NO and VIP release in response to vagal stimulation still remains unclear. We found that there was no interaction between the release of NO and VIP following vagal stimulation.

It is not known if NO is the final neurotransmitter mediating rapid phasic relaxation, or if it serves as a neuromodulatory substance that facilitates the release of another NANC neurotransmitter. Previous studies demonstrate that NO can stimulate VIP release from the isolated myenteric plexus of the guinea-pig ileum (Grider & Jin, 1993), suggesting a possible presynaptic stimulatory action of NO on VIP release. However, in our studies, L-NNA had no effect on vagally stimulated VIP release, and NO-induced relaxation was not antagonized by VIP antagonist in a vascularly isolated perfused rat stomach. Therefore, it does not appear that the action of NO is mediated by VIP in the rat stomach. It is also important to determine whether NO is released primarily

or secondarily by another NANC neurotransmitter following vagal stimulation. Grider, Murthy, Jin & Makhlouf (1992) demonstrated that VIP itself is capable of stimulating NO production from isolated guinea-pig gastric muscle cells devoid of neural elements. However, in our studies, VIP-induced gastric relaxation was not antagonized by L-NNA. This suggests that the action of VIP is not mediated by NO in the rat stomach. In addition, we demonstrated that DMPP-induced NO production was not affected by VIP antagonist, further supporting the proposal that NO production is not mediated by VIP.

Bult et al. (1990) reported that DMPP caused tetrodotoxin-sensitive relaxation in the canine ileocolonic junction, and Desai et al. demonstrated that activation of nicotinic receptors produced relaxation of the guinea-pig stomach, which was inhibited by L-NMMA (Desai et al. 1991). These data suggest that stimulation of nicotinic receptors on postganglionic neurons is responsible for the activation of the inhibitory neurons which release NO within the gut wall. The administration of hexamethonium also abolished the triphasic responses to vagal stimulation, indicating that all three phases of gastric responses were mediated by nicotinic synapses. This possibility was confirmed by the observation that exogenously applied DMPP (100 μM), a nicotinic receptor agonist, also caused a triphasic response as shown in Fig. 6. We showed that DMPP (1–100 μM) significantly increased both NO and VIP production in a dose-dependent manner. These observations confirmed that vagal stimulation of NO and VIP release was mediated by nicotinic synapses.

In summary, we have shown that there is differential release of NO and VIP depending on the frequency of electrical vagal stimulation and that there are no interactions between NO and VIP release upon vagal stimulation. Through nicotinic synapses, the preganglionic fibres in the vagal trunk are connected to at least three different types of postganglionic neurons which contain acetylcholine, NO and VIP and these serve as neurotransmitters to mediate gastric contraction and different modes of relaxation.

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