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Parallel pathways mediate inhibitory effects of vasoactive intestinal polypeptide and nitric oxide in canine fundus

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1 The gastric adaptation reflex is activated by the release of non-adrenergic, non-cholinergic (NANC) inhibitory transmitters, including nitric oxide (NO) and vasoactive intestinal polypeptide (VIP). The role of NO in this reflex is not disputed, but some investigators suggest that NO synthesis is stimulated by VIP in post-junctional cells or in nerve terminals. We investigated whether the effects of these transmitters are mediated by independent pathways in the canine gastric fundus.

2 VIP and NO produced concentration-dependent relaxation of the canine fundus. N^{ω}-nitro-L-arginine (L-NNA) reduced relaxation induced by electrical field stimulation (EFS; 0.5–8 Hz), but had no effect on responses to exogenous VIP and sodium nitroprusside (SNP, 10 μM).

3 Oxyhaemoglobin reduced relaxations produced by EFS and SNP. Oxyhaemoglobin also reduced relaxation responses to low concentrations of VIP (<10 nM), but these effects were non-specific and mimicked by methaemoglobin which had no effect on nitrergic responses.

4 A blocker of guanylyl cyclase, 1H-[1,2,4]oxidiazolo [4,3,-a]quinoxalin-1-one, (ODQ) inhibited responses to EFS, SNP and DETA/NONOate (an NO·donor), but had no effect on responses to VIP. *cis*-N-(2-phenylcyclopentil)-azacyclotridec-1en-2-amine monohydrochloride (MDL 12,330A), a blocker of adenylyl cyclase, reduced responses to EFS, VIP and forskolin, but did not affect responses to SNP.

5 Levels of cyclic GMP were enhanced by the NO donor S-nitroso-n-acetylpenicillamine (SNAP) but were unaffected by VIP (1 μM). The increase in cyclic GMP in response to SNAP was blocked by ODQ.

6 The results suggest that at least two transmitters, possibly NO and VIP, mediate relaxation responses in the canine fundus. NO and VIP mediate responses *via* cyclic GMP- and cyclic AMP-dependent mechanisms, respectively. No evidence was found for a serial cascade in which VIP is coupled to NO-dependent responses.

Keywords: Enteric nervous system; non-adrenergic; non-cholinergic (NANC); nerves; gastric motility; gastric adaptive relaxation

Abbreviations: DETA/NONOate, NOC-18, DETA/NO,(Z)-1-[2-aminoethyl)-N-(2-ammonioethyl) amino]diazen-1-ium-1,2-diolate; ODQ, 1H-[1,2,4] oxidiazolo [4,3,-a]quinoxalin-1-one; IBMX, 3-isobutyl-1-methylxanthine; MDL 12,330A, *cis*-N-(2-phenylcyclopentil)-azacyclotridec-1-en-2-amine monohydrochloride; Met-Hb, methaemoglobin; L-NNA, N^{ω}-nitro-L-arginine; NANC, non-adrenergic, non-cholinergic; HbO, oxyhaemoglibin; SNAP, S-nitroso-n-acetylpenicillamine; SNP, sodium nitroprusside; TTX, tetrodotoxin; VIP, vasoactive intestinal polypeptide

Introduction

After ingestion of food, mechanical tone in the proximal stomach is reduced to accommodate the increase in volume without significant effects on gastric pressure (Paton & Vane, 1963). This reflex has been termed adaptive relaxation (Meyer, 1987), and it depends upon release of inhibitory neurotransmitters from non-adrenergic, non-cholinergic enteric inhibitory (NANC) motoneurons. Nitric oxide (NO) has been shown to be a critical mediator of adaptive relaxation (Desai *et al*., 1991), but other inhibitory transmitters, such as vasoactive intestinal polypeptide (VIP) and adenosine triphosphate (ATP), may also contribute to the regulation of gastric tone (e.g. Grider *et al*., 1985; Mashimo *et al*., 1996). Studies of several species have shown that a significant portion of the mechanical responses to enteric inhibitory neural activation is inhibited by antagonists of NO or cyclic GMP synthesis (Li & Rand, 1990; Lefebvre *et al*., 1992; 1995; Desai *et al*., 1994; Bayguinov & Sanders, 1998), and NO is released when NANC neurons are activated (Boeckxstaens *et al*., 1990). In some species the electrical response is partially sensitive to inhibition of NO synthesis (Burns *et al*., 1996), while in others, electrical responses are resistant to blockade of the NO pathway (Bayguinov & Sanders, 1998). Taken together, these findings suggest involvement of multiple neurotransmitters.

The sequence of events that constitute inhibitory neurotransmission in the fundus remains controversial. Some investigators believe that NO is primarily a neurotransmitter, and it is synthesized and released along with other transmitters from NANC neurons (Lefebvre, 1993). In this model the post-junctional actions of these transmitters occur in parallel (e.g. Li & Rand, 1990; Barbier & Lefebvre, 1993). In support of this concept, the dominant and threshold responses to enteric inhibitory neurotransmission have been shown to be due to NO (Li & Rand, 1990; Desai *et al*., 1994; Bayguinov & Sanders, 1998), nitric oxide synthase (NOS) has been localized to enteric motoneurons with immunofluorescence techniques (Desai *et al*., 1994; Lefebvre *et al*., 1995), and motor responses are blocked by inhibitors of guanylyl cyclase (Bayguinov & Sanders, 1998).

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Other authors have suggested that VIP is the predominant enteric inhibitory transmitter and NO is largely produced in post-junctional cells in response to VIP stimulation (Grider *et al*., 1992). For this 'serial cascade' model of enteric inhibitory neurotransmission to be correct, VIP would be expected to activate, at a minimum, the same pathways and mechanisms activated by exogenous NO. Studies on isolated smooth muscle cells have reported that NO donors activate cyclic GMP-dependent mechanisms and VIP activates cyclic AMP-dependent and cyclic GMP-dependent mechanisms (Jin *et al*., 1993; Murthy & Makhlouf, 1995). Many of the studies upon which the serial cascade model is based were performed on isolated gastric smooth muscle cells in which cell length is the main parameter of smooth muscle function. The majority of studies on intact gastric muscle have not supported the serial cascade model of enteric inhibitory neurotransmission (e.g. Desai *et al*., 1994; Lefebvre *et al*., 1992).

In the present study we have investigated enteric neurotransmission and the effects of NO and VIP in the canine fundus and studied the interdependence of these neurotransmitters. We have used drugs to block guanosine 3′,5′-cyclic monophosphate (cyclic GMP) and adenosine 3′,5′-cyclic monophosphate (cyclic AMP) pathways to determine whether independent second messenger pathways produce the effects of enteric inhibitory transmission.

Methods

Preparation of muscle strips from the gastric fundus

Mongrel dogs of either sex (averaging 15 Kg) were obtained from vendors licenced by the United States Department of Agriculture. The use of dogs for these experiments was approved by the Institutional Animal Care and Use Committee. The animals were sacrificed with an overdose of pentobarbitone sodium (I.P. 100 mg kg^{-1}). The abdomen was opened and entire stomach was removed. A section of the fundus was dissected free of the underlying mucosal tissues and pinned out in a dissecting dish containing oxygenated Krebs-Ringer bicarbonate (KRB) solution. Smooth muscle strips ($3 \times 10 \times 1$ mm) were cut parallel to the circular muscle fibres. The muscle strips were mounted in organ bath under a resting tension of 15 mN and allowed to equilibrate for 90–120 min. The muscles were continuously perfused with oxygenated and warmed (37.5°C) Krebs-bicarbonate solution. Contractile force was monitored *via* an isometric force transducer. All muscles gained tone spontaneously in KRB or KRB and 'NANC' conditions (see below) without addition of exogenous agonists. The mechanical signals were digitized and recorded with a computerized data acquisition and analysis system (MP100 from BIOPAC Systems, Inc.). Electrical field stimulation (EFS) was delivered as square wave pulses (0.5 ms duration, supramaximal voltage at frequencies ranging from 0.5–8 Hz; stimulus trains of 30 s from a Grass S88 stimulator coupled *via* stimulus isolation units (Grass SIU5) to platinum ring electrodes, placed around the muscle strips. Responses to EFS were blocked by tetrodotoxin (TTX; 1 μM).

Measurements of cyclic GMP levels

Muscle strips used for measurements of cyclic nucleotides were attached to a force transducer and equilibrated for 2 h during which time KRB solution was exchanged every 10 min. After 2 h TTX, 100 nM, was added to the bath to reduce spontaneous release of neurotransmitters, and IBMX, 100 μM, and Zaprinast, 100 μM, added to block phosphodiesterase activity. IBMX and Zaprinast completely relaxed the muscles, suggesting significant basal levels of cyclic nucleotides were produced. Twenty minutes after addition of the phosphodiesterase inhibitors VIP or SNAP were added to the bath and maintained for an additional 10 min before flash freezing tissues in liquid nitrogen while still attached to the transducer wires. Cyclic GMP was assayed using commercially available reagents (Caymen Chemical Company, Ann Arbor, MI, U.S.A.). Samples were prepared for assay by homogenization in 6% TCA with glass Duall tissue grinders followed by extraction with water-saturated diethyl ether. Aqueous phases were then lyophilized to dryness and resuspended in 1.0 M potassium phosphate buffer (pH 7.4) before addition to duplicate microtiter plate wells. Cyclic GMP levels in samples and standards were detected and competition between cyclic GMP and the acetylcholinesterase-linked cyclic GMP tracer for specific antiserum binding sites. The antiserum complex, linked to acetylcholinesterase, was used to cleave Ellman's reagent (5,5′-dithio-bis-(2-nitrobenzoic acid), and absorbance was measured at 412 nm. Cyclic GMP content of samples was determined from a standard curve constructed from determination of known amounts of cyclic GMP added to the plate. Levels of cyclic GMP are expressed as pmol cyclic GMP mg^{-1} protein (determined by method of Bradford, 1976). Duplicate variation in the cyclic GMP assay was less than 3%.

Solutions and drugs

The standard Krebs solution used in this study contained (in mM) Na^+ 137.4, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+}1.2, Cl^- 134, HCO_3^-15.5, $H_2PO_4^-$ and dextrose 11.5. This solution had a final pH of 7.3–7.4 after equilibration with 97% O_2–3% CO_2. All experiments were conducted in the presence of the sulphate salt of atropine, the hydrochloride salt of propranolol (both from Sigma), and mesylate salt of phentolamine (Ciba Geigy), all at a concentration of 1 μM (non-adrenergic, non-cholinergic ('NANC') solution). N^{ω}-nitro-L-arginine (L-NNA), vasoactive intestinal polypeptide (VIP), methaemoglobin (Met-Hb), sodium nitroprusside (SNP), forskolin, zaprinast, 3-isobutyl-1-methylxanthine (IBMX) and tetrodotoxin (TTX) were obtained from Sigma and dissolved at the desired concentrations in Krebs solution. 1H-[1,2,4] oxidiazolo [4,3,-a] quinoxalin-1-one (ODQ) was obtained from Toris Cookson. *cis*-N-(2-phenylcyclopentil) - azacyclotridec - 1en - 2 -amine monohydrochloride (MDL 12,330A) and S-nitroso-n-acetylpenicillamine (SNAP) was obtained from RBI. Oxyhaemoglobin (HbO) was

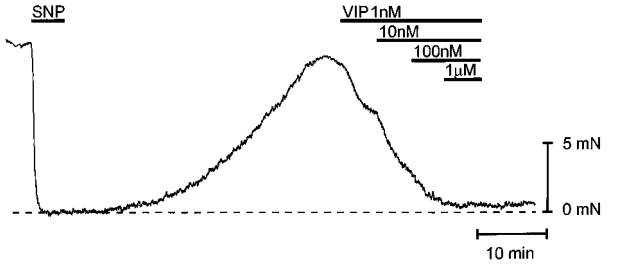

Figure 1 Effects of VIP on tone. Canine fundus develops tone *in vitro* (level of trace at beginning of the record) and in the presence of tetrodotoxin (3 μM). The tone was completely inhibited by SNP (10 μM; black bar). After recovery following washout of SNP, VIP was applied in a cumulative manner (1 nM–1 μM; denoted by black bars). A maximum response to VIP was achieved at 100 nM.

prepared as a haemolysate of canine blood according to the method of Bowman and Gillespie (1982). [NOC-18, DETA/NO, (Z)-1-[2-aminoethyl)-N-(2-ammonioethyl) amino]diazen-1-ium-1,2-diolate] (DETA/NONOate) was obtained from Alexis corporation.

Statistical analysis

Data were tabulated as means ± s.e.mean. Data sets were tested for significance using Student's *t*-test. In the text '*n*' values refer to the number of muscle strips used for each experiment. Data were considered significantly different from control values when $P < 0.05$.

Results

Effect of VIP on tone in the fundus

Canine fundus muscles develop spontaneous tone *in vitro* (Bayguinov & Sanders, 1998). VIP and nitric oxide donors (SNP, SNAP and DETA/NONOate) reduced the tone of fundus muscles bathed with 'NANC solution' containing TTX (30 μM; Figure 1). Maximum relaxation was produced by SNP (10 μM), and this was used as a standard against which responses were normalized. VIP caused concentration-dependent relaxation of tone (Figure 1). At 100 nM, the relaxation caused by VIP was 95 ± 2.3% ($n = 6$) of the relaxation caused

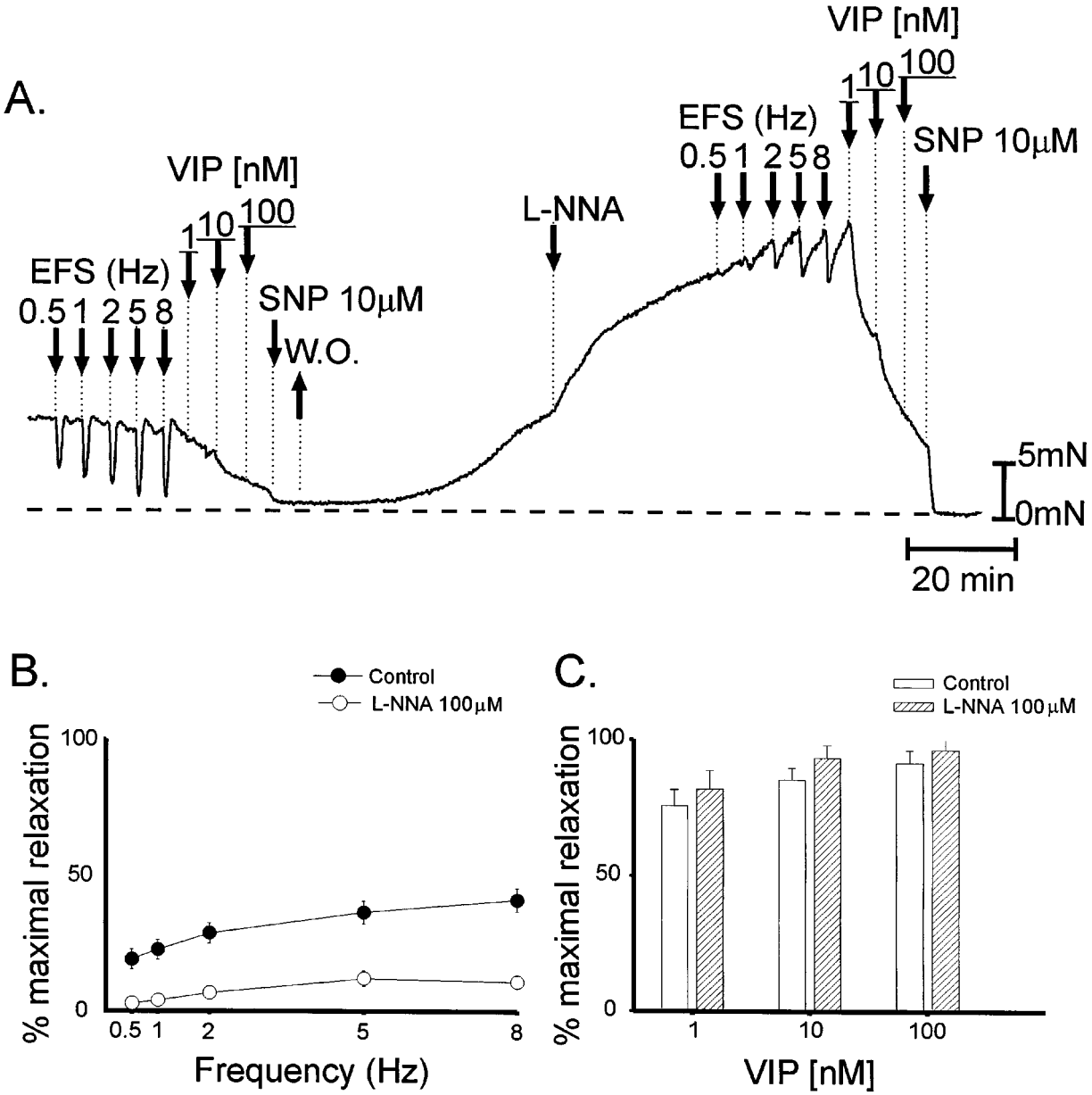

Figure 2 Effects of L-NNA on responses to electrical field stimulation (EFS) and VIP. (A) EFS (0.5–8 Hz; 30 trains beginning at arrows) caused frequency dependent relaxation of tone. VIP (1–100 nM) caused concentration-dependent relaxation. After control responses, L-NNA (100 μM) was added and the responses to EFS and VIP were repeated. L-NNA caused a rise in the tone. After L-NNA the relaxation responses to EFS were greatly diminished, but VIP responses were not significantly affected. (B) shows the average effects of EFS in 18 muscle strips before (●) and after (○) L-NNA. (C) shows the average effects of VIP from the same muscle strips before (□) and after (▨) L-NNA. Data in B and C are means ± s.e.mean.

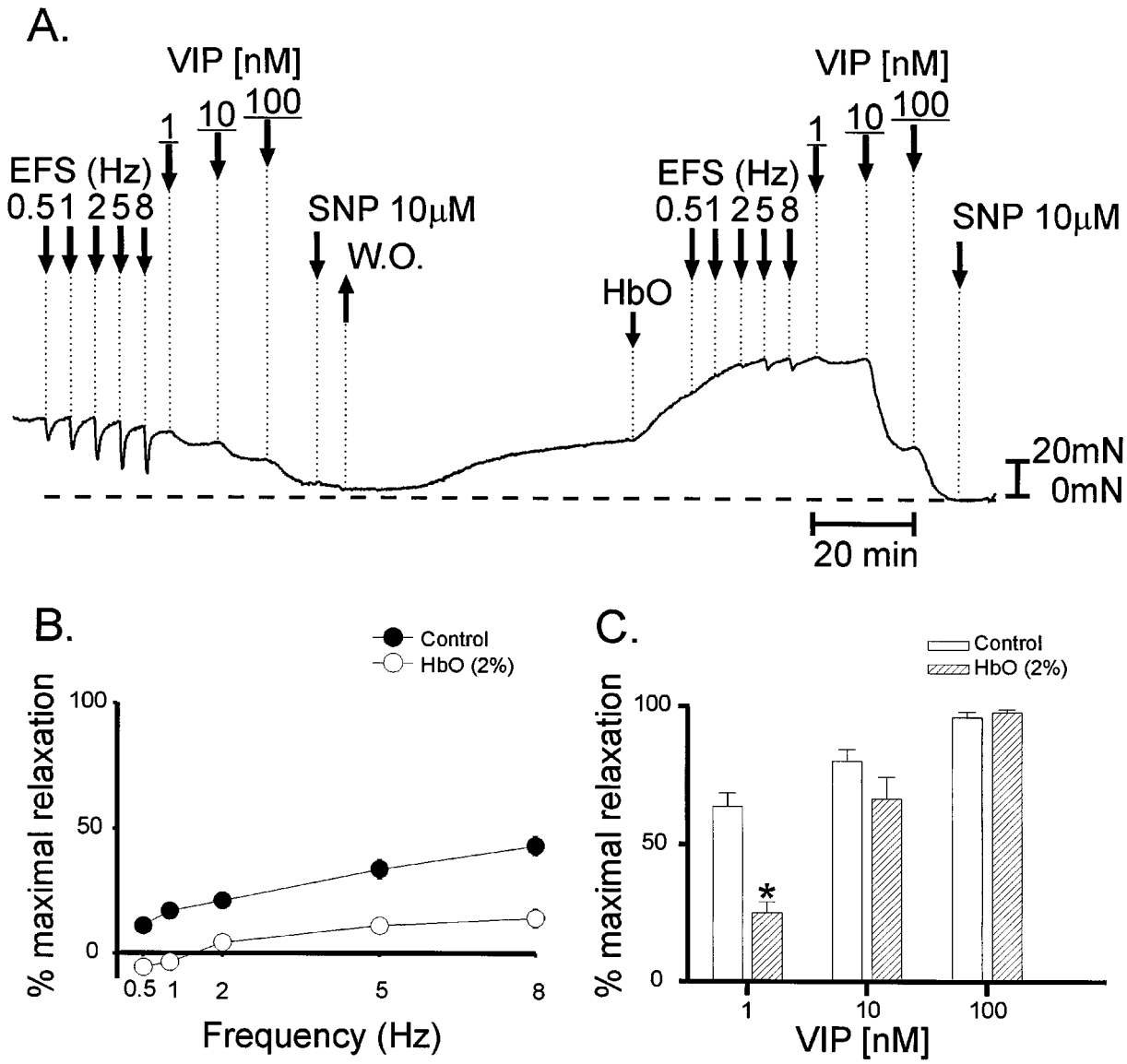

Figure 3 Effects of oxyhaemoglobin on responses to electrical field stimulation (EFS) and VIP. (A) EFS (0.5–8 Hz; 30 trains beginning at arrows) caused frequency dependent relaxation of tone. VIP (1–100 nM) caused concentration-dependent relaxation. After control responses, oxyhaemoglobin (2%) was added, and this raised the tone. After oxyhaemoglobin the relaxation responses to EFS were greatly diminished, but VIP responses were not significantly affected, except at the lowest concentration of VIP tested (1 nM; *denotes $P<0.05$). (B) shows the average effects of EFS in 18 muscle strips before (●) and after (○) oxyhaemoglobin. (C) shows the average effects of VIP from the same muscle strips before (□) and after (▨) oxyhaemoglobin. Data in B and C are means ± s.e.mean.

by SNP (10 μM). Higher concentrations of VIP did not result in additional reduction in tone.

NOS inhibition and NO sequestration do not affect VIP induced relaxations

Since others have reported that the effects of VIP in the gastric fundus are partially mediated by NO (e.g. Grider *et al*., 1992), we tested the effects of L-NNA on responses to electrical field stimulation (EFS) and VIP. EFS caused transient, frequency-dependent reductions in tone (Figure 2A). In 26 of 60 (43%) muscles we noted small rebound contractions following cessation of EFS. Cumulative application of VIP decreased the tone as described in the previous paragraph. Addition of L-NNA (100 μM) increased tone (55 ± 11%) ($n=18$), suggesting that production of NO in the tissue limits the degree of spontaneous tone. After L-NNA, relaxations evoked by EFS were reduced, but L-NNA had no significant effect on the magnitude of relaxations caused by VIP (Figure 2A). Figures 2B and C summarize the effects of L-NNA on responses to EFS and VIP in experiments on 18 muscle strips.

In another series of experiments we tested whether sequestration of NO by oxyhaemoglobin affects responses to EFS and VIP (Figure 3A). When oxyhaemoglobin (2%) was added we noted an increase in tone (96 ± 17% increase; $P<0.001$). Oxyhaemoglobin reduced relaxations evoked by EFS and responses to SNP. Responses to low concentrations of VIP (i.e. 1–10 nM) were reduced by oxyhaemoglobin, but higher concentrations (100 nM) were not significantly affected (Figure 3A). Figures 3B and C summarize the effects of oxyhaemoglobin on EFS and VIP responses in experiments from 18 muscle strips. We wondered whether the effects of oxyhaemoglobin on low concentrations of VIP were non-specific (possibly due to binding of the peptide), so we tested

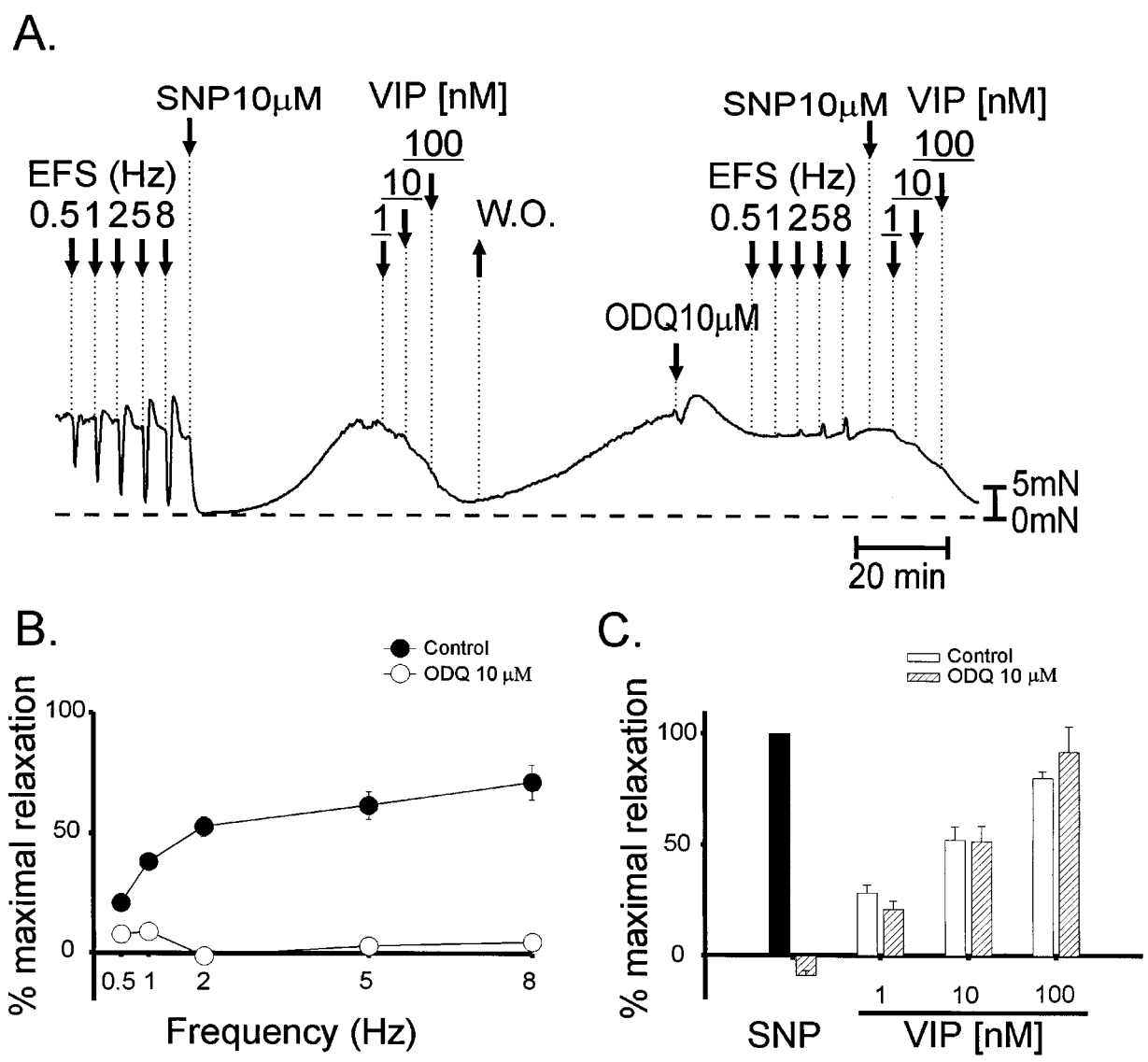

Figure 4 Effects of ODQ on responses to electrical field stimulation (EFS), SNP and VIP. (A) EFS (0.5–8 Hz; 30 trains beginning at arrows) caused frequency dependent relaxation of tone. SNP and VIP (1–100 nM) also relaxed the tone. After control responses, ODQ (10 μM) was added. After ODQ the relaxation responses to EFS and SNP were greatly diminished, but VIP responses were not significantly affected. (B) shows the average effects of EFS in 18 muscle strips before (●) and after (○) ODQ. (C) shows the average effects of SNP (■) and VIP (□) from the same muscle strips before and after (▨) ODQ. Data in B and C are means ± s.e.mean.

methaemoglobin as a negative control. Methaemoglobin had no effect on responses to EFS (0.5–8 Hz; $n=6$), but, similar to oxyhaemoglobin, methaemoglobin reduced the effects of lower concentrations of VIP (i.e. 35 ± 1.8% and 19 ± 4.2% reduction in responses to VIP at 1 and 10 nM, respectively; $n=6$). Thus, the inhibitory effects of oxyhaemoglobin on responses to VIP were likely to be non-specific. Taken together, experiments in which NO synthesis was blocked and NO was sequestered suggest that the effects of VIP in the canine fundus are independent of NO production.

Effect of guanylyl and adenylyl cyclase inhibitors on responses to VIP and NO donors

Considerable evidence suggests the effects of nitrergic neurotransmission in post-junctional cells are mediated *via* activation of soluble guanylyl cyclase and increased levels of cyclic GMP (Bowman & Drummond, 1984; Mirzazadeh *et al*., 1991; Cellek *et al*., 1996). Others have suggested that VIP, in addition to activation of adenylyl cyclase (Bitar & Makhlouf, 1982), is able to activate cyclic GMP because VIP stimulates production of NO in post-junctional cells (Grider *et al*., 1992; Jin *et al*., 1993; Murthy & Makhlouf, 1995). We tested blockers of guanylyl and adenylyl cyclases to investigate the second messenger pathways activated by exogenous NO and VIP.

1H-[1,2,4]oxidiazolo[4,3,-a]quinoxalin-1-one, (ODQ) is a specific blocker of soluble guanylyl cyclase and NO-evoked increases in cyclic GMP in the brain (Garthwaite *et al*., 1995), vascular tissues and platelets (Moro *et al*., 1996), and anococcygeus muscle (Cellek *et al*., 1996). We have previously shown that ODQ (10^{-5} M) reduces changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and tone in response to EFS and SNP (10^{-5} M) in the canine fundus (Bayguinov & Sanders, 1998). Addition of ODQ caused a transient rise in tone, and then tone returned to a stable level that was 113 ± 9% of the control tone. This level was not significantly different from the control tone ($P>0.05$). As in our previous study, we found that ODQ (10 μM), inhibited response to EFS (0.5–8 Hz) and SNP (10 μM).

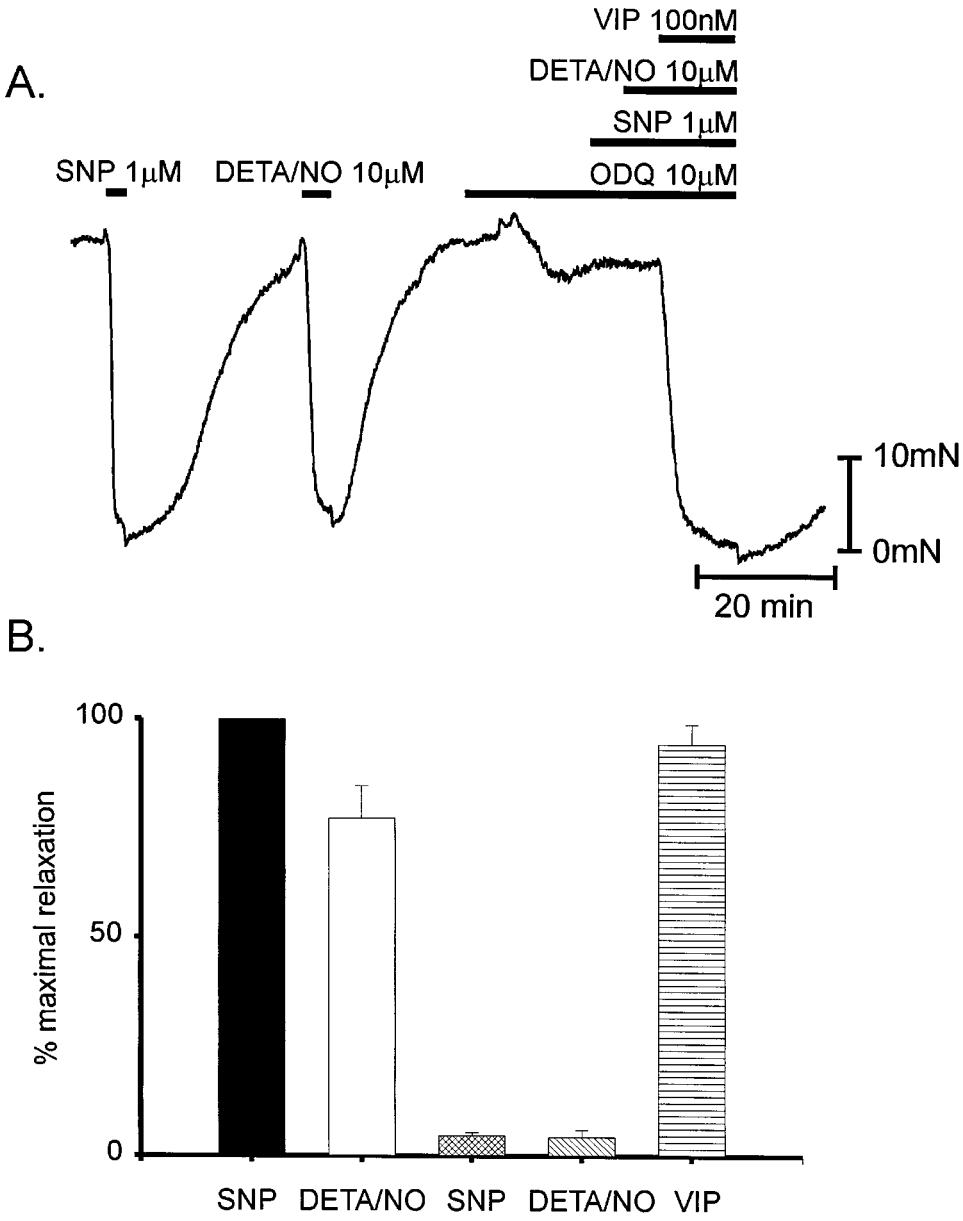

Figure 5 Comparison of the effects of ODQ on responses to SNP and DETA/NONOate. DETA/NONOate is a pure NO·donor. (A) shows that SNP (1 μM) had similar effects on the tone as DETA/NONOate (10 μM). ODQ blocked the relaxation responses to both compounds, but did not affect the relaxation response to VIP (100 nM). (B) shows a summary of six experiments of this type. Data are means$\pm$s.e.mean.

Responses to VIP (1 – 100 nM), however, were not significantly affected by ODQ (Figure 4A). Figure 4C summarizes experiments with ODQ performed on 18 strips of muscle. We also tested the effects of ODQ on relaxation responses caused by a second NO donor which is reported to be a pure NO·donor (DETA/NONOate; see Morley & Keefer, 1993; Li *et al*., 1998). The response to this donor was also blocked by ODQ (Figure 5).

cis - N - (2 - phenylcyclopentil) - azacyclotridec - 1en -2-amine monohydrochloride (MDL 12,330A) has been shown to block adenylyl cyclase in numerous preparations (e.g. Grupp *et al*., 1980; Satake *et al*., 1996). Addition of MDL 12,330A (50 μM) caused an increase in tone (to 235$\pm$34% of the basal level) ($n=18$), suggesting ongoing production of cyclic GMP in these muscles (Figure 6). The relative amplitude of relaxations, provoked by EFS decreased in the presence of MDL 12,330A and this compound also reduced relaxations caused by VIP. MDL 12,330A had no effect on relaxations caused by SNP. The effects of MDL 12,330A on responses to EFS and VIP in 12 experiments are summarized in Figure 6B and C. As a control, we tested that the effects of MDL 12,330A on cyclic AMP-dependent relaxation by testing its effects on relaxations caused by forskolin. MDL 12,330A blocked responses to forskolin (1 μM) by 32$\pm$5%; $n=6$; $P<0.005$). This effect was not significantly different from the block of VIP effects elicited by MDL 12,330A.

Effects of VIP and SNP on tissue cyclic GMP

The pharmacological experiments suggest that NO and VIP are 'parallel' neurotransmitters in canine gastric fundus, utilizing separate second messenger pathways. The effects of NO were dependent upon cyclic GMP, and the effects of VIP appear to be mediated by cyclic AMP. We tested the ability of

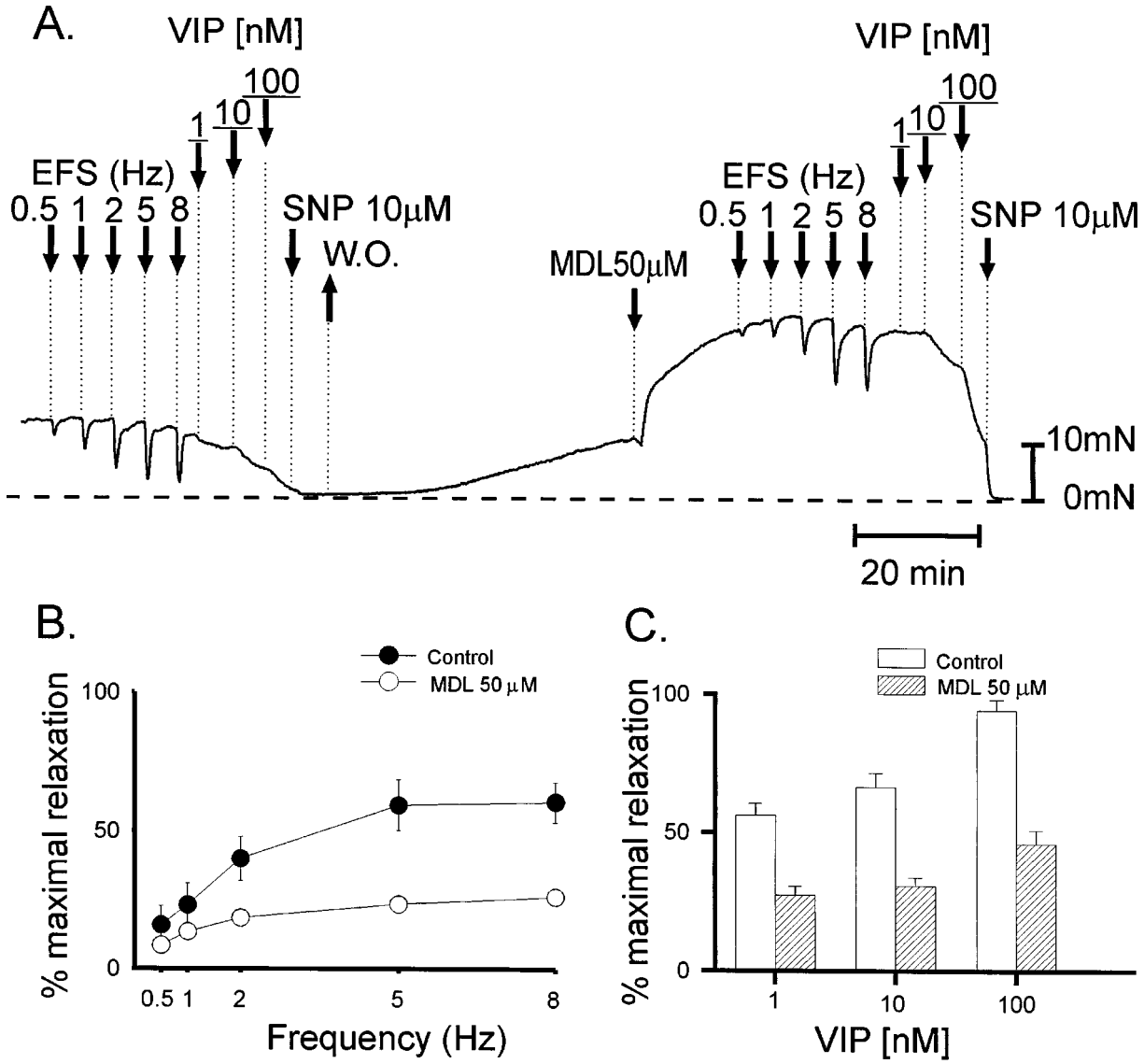

Figure 6 Effects of MDL 12,330A on responses to electrical field stimulation (EFS), SNP and VIP. (A) EFS (0.5–8 Hz; 30 trains beginning at arrows) caused frequency dependent relaxation of tone. SNP (10 μM) and VIP (1–100 nM) also relaxed tone. After control responses, MDL 12,330A (50 μM) was added. This compound increased the tone. After MDL 12,330A the relaxation responses to VIP and EFS were diminished, but responses to SNP were not significantly affected. (B) shows the average effects of EFS in 12 muscle strips before (●) and after (○) MDL 12,330A. (C) shows the average effects of VIP from the same muscle strips before (□) and after (▨) MDL 12,330A. Data in B and C are means ± s.e.mean.

the NO donor SNAP and VIP to generate cyclic GMP in muscles of the canine fundus ($n=6$). SNAP (10 μM) for 2 min), but not VIP (1 μM for 2 min), significantly increased cyclic GMP levels (Figure 7). ODQ prevented the increase in cyclic GMP caused by NO donors.

Discussion

We addressed the question of whether enteric inhibitory neurotransmission in the canine fundus is a parallel process in which NO and VIP are released by neurons and elicit responses *via* discrete second messenger pathways. Our results support the parallel neurotransmitter hypothesis, and argue against 'serial cascade' models in which the synthesis of NO depends upon stimulation of post-junctional cells by VIP (Grider *et al*., 1992), VIP release is stimulated by NO (Grider *et al*., 1992), or NO release by neurons is activated by neural release of VIP (Mashimo *et al*., 1996). In any of the serial cascade models proposed, the effects of exogenous VIP or NO would be mixed, and blocking of pathways utilized by one transmitter would affect responses to the other. In the present study (i) blocking NO synthesis had no effect on responses to VIP; (ii) sequestration of NO had no specific effects on responses to VIP; (iii) blocking guanylyl cyclase had no effect on responses to VIP; and (iv) blocking adenylyl cyclase did not affect responses to NO donors. Responses of the canine fundus to enteric inhibitory neural inputs appear to be primarily mediated by neurally-released NO; up to 75% of the response (depending upon frequency) was inhibited by blockade of NO synthesis or sequestration of NO. Other inhibitory transmitters, such as VIP may contribute to neural responses. These transmitters activate separate pathways, and probably discrete effectors within smooth muscle cells (as in canine colon; see Koh *et al*., 1995; Shuttleworth *et al*., 1996) to elicit integrated responses.

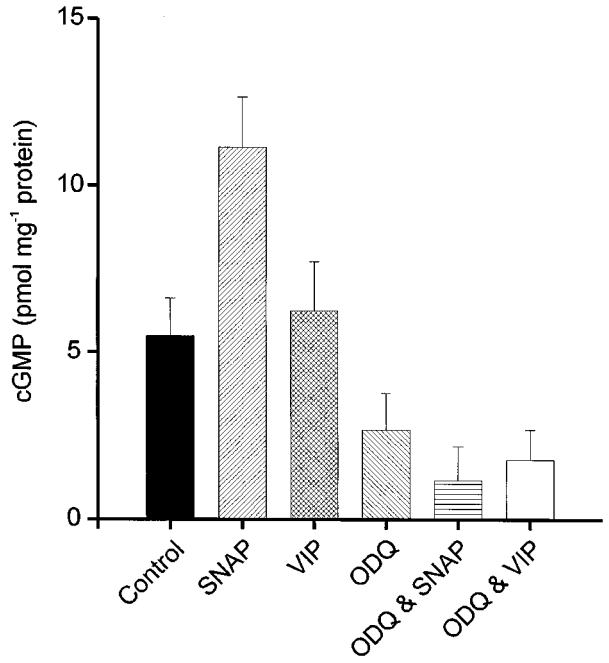

Figure 7 Effects of SNAP and VIP on tissue cyclic GMP levels. SNAP increased cyclic GMP levels in tissues ($P<0.01$). ODQ reduced basal levels of cyclic GMP ($P<0.01$) and blocked the effect of SNAP ($P<0.05$). VIP had no effect on cyclic GMP levels either before ($P>0.8$) or after ODQ ($P>0.15$). Data are means ± s.e.mean from six experiments.

There is substantial evidence that multiple transmitters mediate enteric inhibitory responses in the proximal stomach (see Lefebvre, 1993). NO and VIP are released in a response to field stimulation of fundus muscles, and the release of both transmitters is blocked by tetrodotoxin (Boeckxstaens *et al*., 1990; Grider & Makhlouf, 1987; D'Amato *et al*., 1992). ATP has also been suggested to contribute to enteric inhibitory responses (Mashimo *et al*., 1996), but the evidence for this agent is less convincing (see Discussion in Lefebvre, 1993). Physiological evidence also supports the idea of multiple transmitters since inhibition of NO synthesis incompletely inhibits relaxation responses to EFS or nicotinic agonists, and experimental manipulations to bind or break-down VIP inhibit responses in a manner addictive to inhibition of NO synthesis (Li & Rand, 1990; Barbier & Lefebvre, 1993; McLaren *et al*., 1993).

Effects of NO synthase inhibitors, oxyhaemoglobin on tone suggest constant background output of NO in fundus muscles. MDL12,330A had a similar effect suggesting that under 'unstimulated' conditions cyclic AMP-dependent mechanisms are also activated in these muscles. Previous studies of cat gastric fundus support the idea of background cyclic nucleotide production by showing significant relaxations of tone when cyclic GMP- and cyclic AMP-specific phosphodiesterase activities were inhibited (Barbier & Lefebvre, 1993).

Although NO released from inhibitory neurons elicits effects *via* cyclic GMP-dependent mechanisms, we noted somewhat different responses to ODQ and L-NNA or oxyhaemoglobin. L-NNA and oxyhaemoglobin caused persistent increases in tone, but after adding ODQ, tone increased transiently and then returned to approximately the basal level of tone. Previous studies in canine colonic muscle have noted non-specific inhibitory effects of ODQ on electrical slow waves and contractions and a similar discrepancy between the actions of ODQ and arginine analogues on basal activity (Franck *et al*., 1997). The inhibitory effect of ODQ may explain why tone was not increased in the present study by blocking basal production of cyclic GMP. Non-specific effects of ODQ, however, did not interfere with the inhibition of cyclic GMP formation and NO-dependent relaxations by ODQ, nor compromise our conclusion that the effects of VIP are independent of cyclic GMP-dependent mechanisms. Another difference between the effects of ODQ and L-NNA was the degree to which EFS-stimulated responses were reduced. ODQ nearly blocked responses, while L-NNA blocked responses by approximately 74% (at 8 Hz). It is unlikely that the greater effect of ODQ was due to block of responses initiated by VIP in post-junctional cells since relaxations to exogenous VIP were unaffected by ODQ. The difference in responses to ODQ and L-NNA might be explained by differences in the effectiveness of the compounds at the concentrations used. It is also possible that the effects of ODQ beyond those of L-NNA could be due to prejunctional inhibition of VIP release. Grider *et al*. (1992) and Jin *et al*. (1993) have reported that NO stimulated VIP release from enteric neurons, and this effect might be mediated *via* cyclic GMP. If ODQ strongly blocked pre- and post-junctional formation of cyclic GMP, then its effects may be a combination of post-junctional blockade of NO-dependent relaxation and pre-junctional inhibition of VIP release. If NO synthesis was only partially blocked by the dose of L-NNA used and only extracellular levels of NO were reduced by oxyhaemoglobin, then VIP release might have been unaffected by these compounds.

The source of NO in enteric neurotransmission has been questioned by some investigators (Grider *et al*., 1992; Jin *et al*., 1996). These authors have concluded that a significant portion of NO release (82% in rabbit; 48% in rat) during NANC responses in the gastric fundus is due to production of NO in post-junctional cells that is induced by VIP (e.g. Jin *et al*., 1996). They have also reported that mechanical responses to VIP are greatly attenuated by blockade of NO synthesis (Grider *et al*., 1992). Consistent with these data, these authors have also reported that; (i) SNP stimulates production of guanosine 3′,5′-cyclic monophosphate (cyclic GMP) and cyclic GMP-dependent protein kinase (cyclic G-kinase), and (ii) VIP stimulates production of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) and cyclic GMP, and both cyclic G-kinase and cyclic AMP-dependent protein kinase (cyclic A-kinase) in dispersed rabbit muscle cells (Murthy & Makhlouf, 1995).

Studies from several other laboratories, performed on intact tissues of several species, have generally not supported 'serial cascade' models of enteric inhibitory neurotransmission. The first problem arose from the observation that field stimulation of muscle strips elicits a rise in cyclic GMP without concomitant changes in cyclic AMP (Torphy *et al*., 1986) or activation of protein kinase A (Barnette *et al*., 1990). In the same studies VIP induced relaxation by cyclic AMP-dependent mechanisms without enhancing cyclic GMP levels. We also observed no correlation between VIP relaxations and enhancement in cyclic GMP levels in the present studies of the canine fundus. Secondly, most functional studies have found that relaxation responses to VIP are not affected by arginine analogues. This is a critical test, because stimulation by VIP leads to NO production in the serial cascade concept. Thus, blockade of NO synthesis should have dramatic effects on the ability of exogenous VIP to relax GI muscles. In studies of fundus muscles from dog (this study), cat (Barbier & Lefebvre, 1993), rat (McLaren *et al*., 1993); guinea-pig (Desai *et al*., 1994; Lefebvre *et al*., 1992); and pig (Lefebvre *et al*., 1995) arginine analogues had no significant effect on responses to VIP. Our observation that ODQ, an inhibitor of guanylyl

cyclase (Garthwaite *et al*,. 1995), completely blocks NO-dependent relaxation but has no effect on VIP-induced relaxations strongly argues that VIP does not activate NO-dependent or cyclic GMP-dependent mechanisms in the fundus. Finally, a recent study performed on fundus muscles from cyclic GMP-dependent protein kinase I (PKG-I) deficient mice shows evidence that the effects of NO and enteric inhibitory neurotransmission are highly dependent upon cyclic GMP-dependent mechanisms. Relaxation responses to VIP and cyclic AMP analogues were not affected in PKG-I mutant animals, suggesting that NO and VIP work *via* independent mechanisms in post-junctional cells (Pfeifer *et al*., 1998). Taken together, biochemical and physiological data derived from studies of intact muscles of several species strongly argue that the effects of VIP are not dependent upon pre- or post-junctional synthesis of NO.

Proponents of the serial cascade model have criticized many of the studies testing the effects of arginine analogues on VIP responses in intact muscle strips because excitatory agonists were used to raise tone (see Murthy *et al*., 1994). These authors have reported that agonists such as cholecystokinin octapeptide (CCK-8), carbachol, and phorbol 12-myristate 13-acetate blocked VIP-induced [^{3}H]-citrulline production (an index of NO production) in isolated muscle cells and muscle strips because these agonists activate protein kinase C (PKC) which they suggest inhibits the isoform of NOS expressed by smooth muscle cells. Further, their data indicated that any agonist or ionic condition that increased cytoplasmic Ca^{2+} (e.g. CCK-8, acetylcholine, ionomycin, and KCl) increased PKC activity. Taken together, these observations suggest that VIP-introduced production of NO would function only in cells in which Ca^{2+} levels (and therefore Ca^{2+}-dependent PKC activity) are low. In the present study we found that tone (which is associated with elevated levels of cytoplasmic Ca^{2+}; see Bayguinov & Sanders, 1998) was inhibited by VIP. Responses to VIP were not affected by L-NNA. It is possible that the independence of VIP and NO effects that we and others have observed could be due to suppression of VIP-induced NO formation in pre-contracted cells, but it is difficult to understand the physiological significance of an inhibitory mechanism that is suppressed by enhanced cytoplasmic Ca^{2+} (i.e. the state necessary for tone). It is also unclear why NOS in isolated muscle cells (which appear to generate spontaneous tone because they relax in response to VIP; see Grider *et al*., 1992; Murthy & Makhlouf, 1995) can be activated by VIP, while this mechanism is suppressed in intact muscles that develop spontaneous tone.

In summary, consistent with immunohistochemical evidence showing expression of VIP and NOS in varicose nerve terminals in the fundus (Lefebvre *et al*., 1995; Desai *et al*., 1994), our study supports the idea that enteric inhibitory neurons utilize NO and VIP as parallel neurotransmitters. The dominant transmitter appears to be NO. Post-junctional responses are mediated by distinct second messenger systems: NO activates responses *via* activation of guanylyl cyclase and the production of cyclic GMP and VIP activates adenylyl cyclase yielding cyclic AMP.

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