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The role of nitric oxide in inhibitory non-adrenergic non-cholinergic neurotransmission in the canine lower oesophageal sphincter

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1 The role of nitric oxide (NO) in non-adrenergic non-cholinergic (NANC) neurotransmission was studied on circular muscle strips of the canine lower oesophageal sphincter (LOS). Electrical field stimulation evoked frequency-dependent relaxations, which were resistant to adrenergic and cholinergic blockade and abolished by tetrodotoxin.

2 Exogenous administration of NO induced concentration-dependent and tetrodotoxin-resistant relaxations which mimicked those in response to electrical stimulation.

3 N^G-nitro-L-arginine (L-NNA), a stereospecific inhibitor of NO-biosynthesis, inhibited the relaxations induced by electrical stimulation but not those by exogenous NO or vasoactive intestinal polypeptide (VIP).

4 The effect of L-NNA was prevented by L-arginine, the precursor of the NO biosynthesis but not by its enantiomer D-arginine.

5 Haemoglobin abolished the NO-induced responses and reduced those evoked by electrical stimulation.

6 Cumulative administration of VIP induced concentration-dependent relaxations, which were slow in onset and sustained. A complete relaxation to VIP was not achieved and the relaxations were not affected by L-NNA.

7 In conclusion, our results provide evidence that NANC relaxations are mediated by NO, suggesting NO or a NO releasing substance as the final inhibitory NANC neurotransmitter in the canine LOS.

Keywords: Electrical stimulation (ES); lower oesophageal sphincter (LOS); nitric oxide (NO); non-adrenergic non-cholinergic nerves (NANC); vasoactive intestinal polypeptide (VIP); N^G-nitro-L-arginine (L-NNA)

Introduction

The lower oesophageal sphincter (LOS) is a specialized segment of the most distal part of the oesophagus. It plays an important role in preventing gastroesophageal reflux by maintaining an intraluminal pressure, higher than the fundus pressure. To allow the passage of food and liquids into the stomach, the LOS relaxes upon swallowing. These relaxations are mediated by a non-adrenergic non-cholinergic (NANC) neurotransmitter, since the inhibitory innervation of the gastrointestinal tract, including the LOS, is mainly provided by NANC neurones (Burnstock & Costa, 1973; Abrahamsson, 1986). Although the precise nature of the NANC neurotransmitter in the LOS is still debated, vasoactive intestinal polypeptide (VIP) is believed to be the most likely candidate. VIP is present in nerve terminals in the LOS region (Alumets *et al.*, 1979; Zimmerman *et al.*, 1989), it relaxes the LOS by direct action on the smooth muscle cells and increases intracellular adenosine 3':5'-cyclic monophasphate (cyclic AMP) contents (Bitar & Makhlouf, 1982). Furthermore, VIP is released by neural stimulation (Goyal & Rattan, 1980) and VIP antiserum partially inhibits the neurally induced relaxation of the feline LOS (Biancani *et al.*, 1984; Behar *et al.*, 1989).

However, a role for nitric oxide (NO) as neurotransmitter has been suggested in rat and mouse anococcygeus muscle (Gillespie *et al.*, 1989; Ramagopal & Leighton, 1989; Gillespie & Sheng, 1990; Gibson *et al.*, 1990), in rat cerebellum (Garthwaite *et al.*, 1988) and forebrain (Knowles *et al.*, 1989).

In previous studies, we provided evidence for the proposal that NO is a NANC neurotransmitter in the canine ileocolonic junction (Boeckxstaens *et al.*, 1990; 1991b,c; Bult *et al.*, 1990) and rat gastric fundus (Boeckxstaens *et al.*, 1991a).

The present study was designed to investigate the role of NO in the canine LOS by means of haemoglobin, which is known to trap NO avidly (Martin *et al.*, 1985) and N^G-nitro-L-arginine (L-NNA), a stereospecific inhibitor of NO biosynthesis (Ishii *et al.*, 1990; Moore *et al.*, 1990; Mülsch & Busse, 1990).

Methods

Tissue preparation

Mongrel dogs of either sex (body weight 10–20 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and a laparotomy was performed. Five cm of tissue above and below the LOS-region was removed, cut open longitudinally and rapidly rinsed in Krebs-Ringer buffer. The squamocolumnar border was located (Huizinga & Walton, 1989) and marked with steel pins and the mucosa was removed from the region below the border. Two muscle strips, 3 to 10 mm distal to the squamocolumnar border, were cut and each strip was again cut into 4 or 5 segments resulting in 8 to 10 circular muscle strips of approximately 2 mm wide and 10 mm long. The strips were tied at each end with silk thread and mounted in organ baths (25 ml, except for the VIP experiments: 5 ml) filled with modified Krebs-Ringer solution (mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, CaEDTA 0.026 and glucose 11.1), maintained at 37°C and continuously aerated with 95% O_2 and 5% CO_2.

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Isometric tension recording

Each muscle strip was placed between two platinum plate electrodes for field stimulation of the intramural nerves. Next they were connected to a strain gauge transducer (STATHAM UC2) for continuous measurement of isometric tension and an initial passive tension of 4 g was applied on each strip. After an equilibration period of 30 min, the strips were stimulated electrically with a GRASS-stimulator (8 Hz, 0.5 ms, 9 V, 100 mA) and a direct current amplifier in stimuli-trains of 10 s. Only tissue that gained tone spontaneously and relaxed on electrical stimulation was considered to be LOS tissue (Christensen *et al.*, 1973; Barnette *et al.*, 1990). These strips were allowed to equilibrate for at least 60 min before experimentation.

Experimental protocols

All experiments were performed during a 5-hydroxytryptamine (5-HT, 3 μM)-induced contraction and in the presence of atropine (1 μM) and guanethidine (3 μM). After each 5-HT-induced contraction the muscle strips were washed four times every 5 min.

First, dose-response curves to NO (0.1–30 μM, non-cumulative) and nitroglycerin (0.1–30 μM cumulative) were constructed on circular muscle strips of the LOS. Next, the effect of haemoglobin (10–30 μM), tetrodotoxin (1 μM), L-NNA (3–100 μM), hexamethonium (100 μM), phentolamine (10 μM) and propranolol (1 μM) was studied on the relaxations induced by electrical stimulation (ES, 0.25–16 Hz, 0.5 ms), NO (3 μM), isoprenaline (100 μM) and nitroglycerin (10 μM). The effect of L-NNA was re-examined in the presence of L-arginine (5 mM) and D-arginine (5 mM). Finally, we examined the inhibitory effect of L-NNA (30 μM) on a cumulative dose-response curve to VIP. All VIP experiments were performed in the presence of 0.1% bovine serum albumin.

All antagonists were added at least 10 min prior to the 5-HT-induced contraction.

Drugs used

The following drugs were used: L-arginine, D-arginine, bovine haemoglobin, bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.); atropine sulphate (Federa, Brussels, Belgium); isoprenaline hydrochloride, 5-hydroxytryptamine creatinine sulphate monohydrate, N^G-nitro-L-arginine, tetrodotoxin (Janssen Chimica, Beerse, Belgium); phentolamine, guanethidine monosulphate (Ciba-Geigy, Switzerland); nitroglycerin (Merck, Darmstadt, Germany), propranolol hydrochloride (ICI-Pharma, Belgium); VIP (UCB bioproducts, Braine-l'Alleud, Belgium).

All drugs were dissolved or diluted in distilled water and solutions were made on the day of experimentation. Ascorbic acid (570 μM) was added to the solutions of 5-hydroxytryptamine and isoprenaline. The stock solution of tetrodotoxin (1 mM in sodium citrate, pH 4.8) was stored at −20°C. VIP aliquots were stored at −20°C and further diluted on the day of experimentation. NO solutions and haemoglobin were prepared as described by Kelm *et al.* (1988). All drugs were added in volumes less than 0.5% of the bath volume.

Statistical analysis

Relaxations are expressed as percentage of the maximal relaxation induced by electrical field stimulation (16 Hz, 0.5 ms) at the beginning of experimentation (Barnette *et al.*, 1990). Results are shown as mean ± s.e.mean for the number of dogs indicated. The negative logarithm of the concentration of agonist that produced a response of 50% of the maximal response obtained with that agonist (pD_2 value), was calculated by linear regression analysis (Tallarida & Murray, 1981). All data were analyzed by Student's *t* test for paired and unpaired observations. *P* values of less than 0.05 were considered to be significant.

Results

During a 5-HT (3 μM)-induced contraction and in the presence of atropine (1 μM) and guanethidine (3 μM), electrical stimulation (0.25–16 Hz, 0.5 ms) induced frequency-dependent relaxations in the canine LOS (Figures 1, 3 and 4). These relaxations were not affected by hexamethonium (100 μM) or propranolol (1 μM) and phentolamine (10 μM) (Figure 1). Iso-

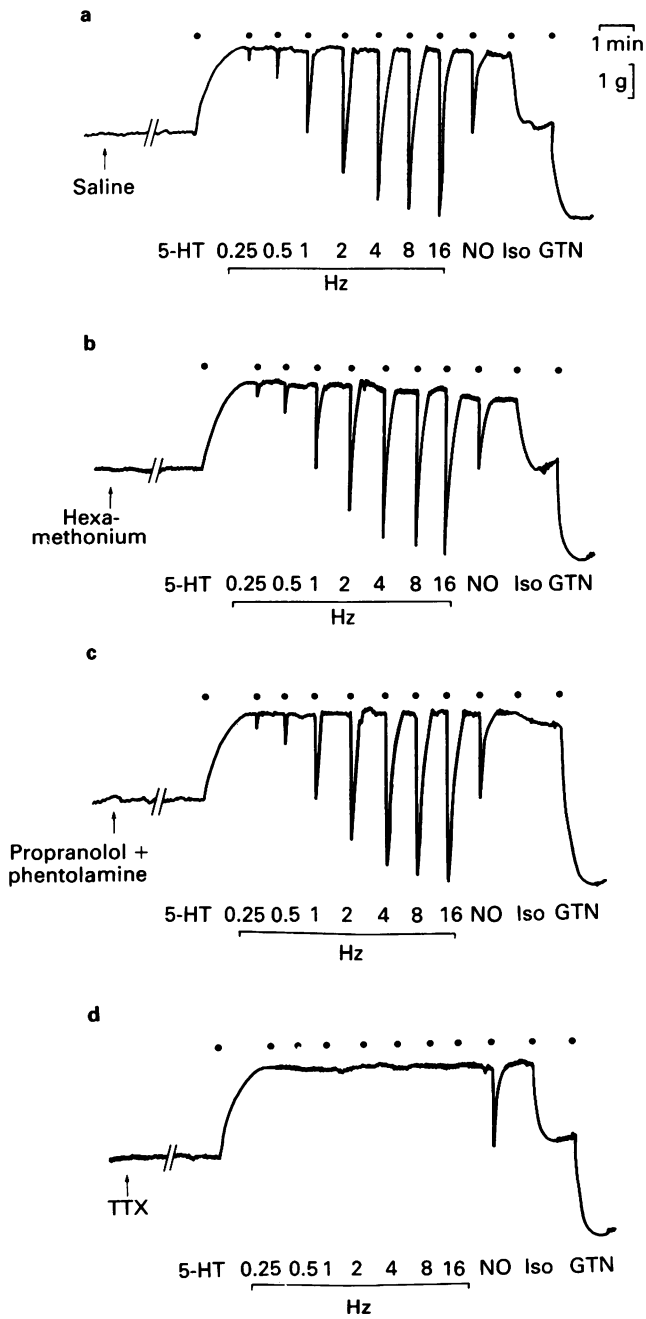

Figure 1 Isometric tension recordings showing (a) the control and the effects of (b) hexamethonium (100 μM), (c) propranolol (1 μM) plus phentolamine (10 μM), (d) tetrodotoxin (TTX, 1 μM) on the relaxations of the canine lower oesophageal sphincter induced by electrical stimulation (0.25–16 Hz, 0.5 ms), NO (3 μM), isoprenaline (Iso, 100 μM) and nitroglycerin (GTN, 10 μM). The experiments were performed during a 5-hydroxytryptamine (5-HT, 3 μM)-induced contraction and in the presence of atropine (1 μM) and guanethidine (3 μM). Similar results were obtained from five other experiments ($n = 6$). Tracing-breaks represent periods of tissue equilibration.

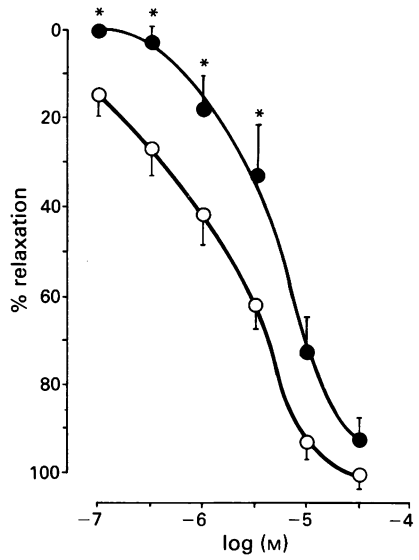

Figure 2 Effect of NO (●, 0.1–30 μM) and nitroglycerin (○, 0.1–30 μM) on canine lower oesophageal sphincter circular muscle strips. Experiments were performed during a 5-hydroxytryptamine-induced contraction and in the presence of atropine (1 μM) and guanethidine (10 μM). Results are shown as mean (s.e.mean shown by vertical bars) and are expressed as a percentage of the maximal relaxation induced by electrical field stimulation (16 Hz, 0.5 ms) at the beginning of experimentation ($n = 6$). * $P < 0.05$, significantly different, sensitivity to NO versus nitroglycerin, Student's t test for unpaired observations.

prenaline (100 μM) induced sustained relaxations (44 ± 9% of the relaxation to 16 Hz, 0.5 ms), which were not affected by hexamethonium (100 μM) but which were almost completely blocked in the presence of propranolol (1 μM) and phentolamine (10 μM) (Figure 1).

Administration of NO (0.1–30 μM, non-cumulative) and nitroglycerin (0.1–30 μM, cumulative) induced concentration-dependent relaxations (Figure 2) which were unaffected by hexamethonium (100 μM) or propranolol (1 μM) and phentolamine (10 μM) (Figures 1 and 2). The relaxations to NO mimicked those to electrical stimulation whereas the relaxations to nitroglycerin were sustained (Figure 1). The pD_2 for NO which was 5.40 ± 0.14 ($n = 5$) significantly differed from the pD_2 for nitroglycerin which was 5.87 ± 0.13 ($n = 5$).

The responses to electrical stimulation were abolished by TTX (1 μM) (Figure 1) and concentration-dependently inhibited by L-NNA (3–100 μM) and haemoglobin (10–30 μM) (Figure 3). In 6 experiments the maximal relaxation obtained by electrical stimulation (16 Hz, 0.5 ms) was inhibited from 100% to 0% by TTX (1 μM), to 20 ± 6% by L-NNA (100 μM) and to 72 ± 7% by haemoglobin (30 μM). The inhibitory effect of L-NNA was prevented by L-arginine (5 mM) but not by D-arginine (5 mM) (Figure 4). TTX and L-NNA did not affect the relaxations to NO, whereas they were abolished by haemoglobin (30 μM) (Figure 3).

Cumulative administration of VIP (1–300 nM) resulted in sustained, concentration-dependent relaxations which were slow in onset (Figure 3d). The highest concentration of VIP tested, relaxed the canine LOS muscle strips only by 52.5 ± 6.9% ($n = 4$), compared to the complete relaxation induced by electrical stimulation (16 Hz, 0.5 ms). The relaxations to VIP were not affected by L-NNA (30 μM) (Figure 4).

Discussion

The exact nature of the NANC neurotransmitter, released by inhibitory enteric neurones is still debated. Although VIP has been proposed as the most likely candidate in the canine LOS, the present results suggest NO or a NO releasing substance as the inhibitory NANC neurotransmitter.

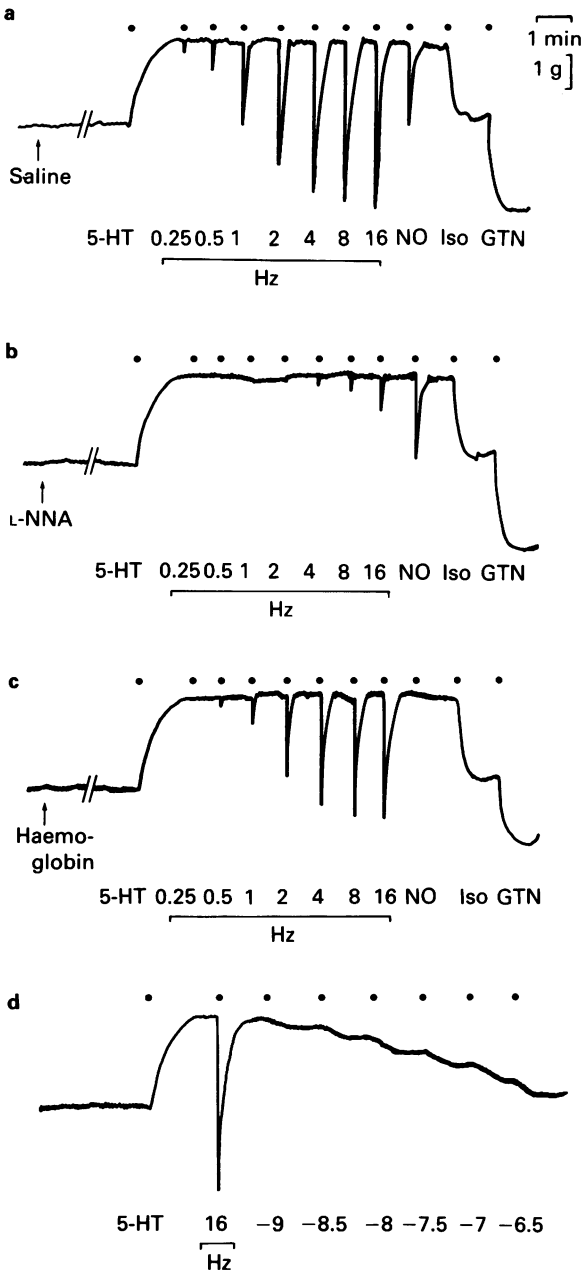

Figure 3 Isometric tension recordings of canine lower oesophageal sphincter circular muscle strips showing (a) the control and the effects of (b) N^G-nitro-L-arginine (L-NNA, 100 μM) and (c) haemoglobin (30 μM) on the relaxations induced by electrical stimulation (0.25–16 Hz, 0.5 ms), NO (3 μM), isoprenaline (Iso, 100 μM) and nitroglycerin (GTN, 10 μM). Tracing (d) shows the effect of cumulative administration of vasoactive intestinal polypeptide (VIP, 1–300 nM, log (M)). All experiments were performed during a 5-hydroxytryptamine-induced contraction and in the presence of atropine (1 μM) and guanethidine (3 μM). Similar results were obtained from additional experiments ($n = 6$, except for VIP $n = 4$). Tracing-breaks represent periods of tissue equilibration.

In the presence of adrenoceptor and cholinoceptor antagonists, electrical stimulation of circular muscle strips of the LOS caused relaxations which were abolished by tetrodotoxin, a nerve conductance blocker, suggesting that these responses resulted from NANC nerve stimulation. Since these relaxations were significantly reduced by L-NNA, an inhibitor of NO biosynthesis (Ishii *et al.*, 1990; Moore *et al.*, 1990; Mülsch & Busse, 1990), they were mediated by NO. This inhibition was stereospecific and competitive as it was prevented by L-arginine, a precursor of NO biosynthesis (Palmer *et al.*, 1988; Schmidt *et al.*, 1988) but not by its enantiomer, D-arginine. In

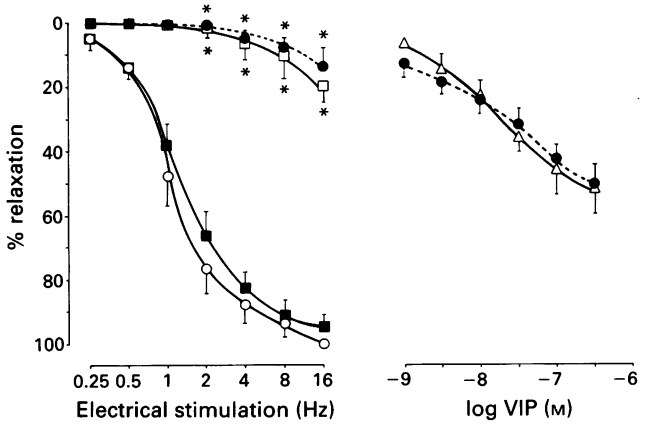

Figure 4 Effect of N^G-nitro-L-arginine (L-NNA, ●, 100 μM) on the NANC relaxations induced by electrical stimulation (○, 0.25–16 Hz, 0.5 ms) and vasoactive intestinal polypeptide (VIP, △, 1–300 nM) in the canine lower oesophageal sphincter. The effect of L-NNA on electrical stimulation was prevented by L-arginine (■, 5 mM) but not by D-arginine (□, 5 mM). L-NNA (30 μM) had no effect on the relaxations induced by VIP. Results are shown as mean (s.e.mean shown by vertical bars) ($n = 6$ for electrical stimulation and $n = 4$ for VIP). * $P < 0.05$, significantly different from control value, Student's t test for paired observations.

addition, exogenous administration of NO caused tetrodotoxin-resistant relaxations which were morphologically similar to those obtained by electrical stimulation. Since the relaxations to NO were not affected by L-NNA, the inhibitory effect of L-NNA on electrically-induced NANC relaxations was on the transmitter system rather than on the postjunctional effector cells. Compared to vascular smooth muscle, LOS tissue and other nonvascular smooth muscle (Buga *et al.*, 1989; Boeckxstaens *et al.*, 1990; 1991b,c) is relatively insensitive to NO. The pD_2 for NO in the canine LOS is 5.40 ± 0.14 ($n = 6$) whereas the pD_2 for NO in rabbit aortic rings is 6.82 ± 0.10 ($n = 10$, unpublished results). The NO solutions in these experiments were prepared identically for both tissues, indicating that the LOS is indeed less sensitive. Administration of nitroglycerin, by which NO is released intracellularly, also resulted in concentration-dependent relaxations.

Haemoglobin, which completely inactivates exogenous NO, significantly but not completely reduced the relaxations to electrical stimulation. This difference in efficacy of inhibition between the relaxation to NO and that to electrical stimulation may be explained by the large molecular size of haemoglobin so that only a small portion may have reached the neuromuscular junction.

Previously, a role in neurotransmission for NO, which accounts for the biological activity of vascular endothelium-derived relaxing factor (Palmer *et al.*, 1987), was also postulated in rat and mouse anococcygeus muscle (Gillespie *et al.*, 1989; Ramogopal & Leighton, 1989; Gillespie & Sheng, 1990; Gibson *et al.*, 1990), in rat cerebellum (Garthwaite *et al.*, 1988) and forebrain (Knowles *et al.*, 1989).

In previous studies we provided evidence for the proposal that NO or a NO releasing substance is the inhibitory NANC neurotransmitter in the canine ileocolonic junction (Boeckxstaens *et al.*, 1990; 1991b,c; Bult *et al.*, 1990) and rat gastric fundus (Boeckxstaens *et al.*, 1991a). In addition, the demonstration of the L-arginine: NO pathway in a variety of cells, suggests a more general role for this pathway in the regulation of cell function and communication (Moncada *et al.*, 1989).

Evidence has been obtained supporting the proposal that VIP is the inhibitory neurotransmitter in the LOS: VIP is released upon electrical stimulation (Biancani *et al.*, 1984) and VIP antiserum partly inhibits the relaxations to lower frequencies of electrical stimulation (Biancani *et al.*, 1984; Behar *et al.*, 1989). However, a total blockade was never achieved leading to the hypothesis that an additional neurotransmitter may be co-released by inhibitory NANC neurones (Goyal & Rattan, 1980; Behar *et al.*, 1989; Biancani *et al.*, 1989). Furthermore VIP-induced relaxations were slow in onset and sustained and were not affected by inhibitors of NO biosynthesis, in contrast to those induced by electrical stimulation. In addition, exogenous administration of even high concentrations of VIP (300 nM) failed to relax completely the canine LOS. This can be explained by the large molecular size of VIP, resulting in a limited ability to penetrate the synapse. Alternatively, it has been shown that the density of receptor binding sites for VIP in canine LOS is low (Zimmerman *et al.*, 1989), which might suggest a minor role for VIP in the inhibitory NANC mechanisms in the canine LOS. Finally, VIP-induced relaxations are mediated by a rise of intracellular cyclic AMP levels whereas electrically-induced relaxations result in a rise of intracellular cyclic GMP levels (Barnette *et al.*, 1990) providing evidence against VIP as mediator of these relaxations. Since nitrovasodilator compounds, including NO, activate guanylate cyclase and elevate cyclic GMP contents in vascular and nonvascular smooth muscle (Waldman & Murad, 1987), the combination of these results supports our hypothesis that NO is the final mediator in NANC neurotransmission in canine LOS.

In conclusion, we provide evidence that electrical stimulation of canine LOS circular muscle induced NANC relaxations which were significantly reduced by inhibitors of NO biosynthesis. Therefore, we propose NO or a NO releasing substance as the final neurotransmitter of inhibitory NANC nerves in the canine LOS.

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