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Evidence for nitric oxide as mediator of non-adrenergic, non-cholinergic relaxations induced by ATP and GABA in the canine gut

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1 The effects of haemoglobin, and the nitric oxide (NO) biosynthesis-inhibitors N^G-monomethyl-L-arginine (L-NMMA), its enantiomer D-NMMA, and N^G-nitro-L-arginine (L-NNA) were investigated on non-adrenergic non-cholinergic (NANC)-mediated relaxation of circular muscle strips of the canine terminal ileum and ileocolonic junction induced by electrical stimulation, adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA) and NO.

2 Tetrodotoxin, L-NMMA and L-NNA, but not D-NMMA, inhibited the relaxations induced by electrical stimulation, ATP and GABA, but not those in response to NO.

3 The inhibitory effect of L-NMMA and L-NNA was prevented by L-arginine, but not by D-arginine. L-Arginine did not potentiate any of the NANC relaxations.

4 Haemoglobin reduced the relaxation induced by electrical stimulation, ATP and GABA, and abolished those in response to NO.

5 Our results demonstrate that the ATP- and GABA-induced relaxations resulting from stimulaton of intramural NANC neurones, in addition to those induced by electrical impulses, are mediated by NO or a NO releasing substance and thus provide further evidence in support of the proposal that NO is the final inhibitory NANC neurotransmitter in the canine terminal ileum and ileocolonic junction.

Introduction

In the gastrointestinal tract, a major part of the inhibitory autonomic innervation appears to be provided by non-adrenergic non-cholinergic (NANC) nerves (Burnstock & Costa, 1973). However, the exact nature of the inhibitory neurotransmitter released by inhibitory NANC neurones still remains controversial. Depending on the tissue and/or species studied, mainly vasoactive intestinal polypeptide (VIP) (Goyal & Rattan, 1980; Grider *et al.*, 1985) or adenosine 5'-triphosphate (ATP) (Burnstock, 1972; 1981) have been suggested as the putative NANC neurotransmitter. Previously, we demonstrated the presence of an inhibitory NANC innervation in the canine terminal ileum and ileocolonic junction; stimulation of NANC neurones either by electrical impulses or by nicotinic receptor stimulation resulted in relaxations resistant to adrenoceptor and cholinoceptor blockade (Pelckmans *et al.*, 1989). The neurotransmitter released by these neurones is not VIP, ATP, γ-aminobutyric acid, 5-hydroxytryptamine, an opioid, somatostatin or substance P (Pelckmans *et al.*, 1989; Boeckxstaens *et al.*, 1990b,c,d,e). However, ATP, as well as GABA, stimulated purinoceptors and $GABA_A$ receptors respectively located on inhibitory NANC neurones resulting in the release of an unknown NANC neurotransmitter (Boeckxstaens *et al.*, 1990c,d).

Recently, we provided evidence in support of the proposal that nitric oxide (NO) or a related substance, which accounts for the biological activity of the vascular endothelium-derived relaxing factor (Palmer *et al.*, 1987), is the inhibitory NANC neurotransmitter in the canine ileocolonic junction (Boeckxstaens *et al.*, 1990a; Bult *et al.*, 1990). The NANC relaxations induced by electrical impulses or acetylcholine were blocked by inhibitors of NO biosynthesis, N^G-monomethyl-L-arginine (L-NMMA) (Palmer *et al.*, 1988) and N^G-nitro-L-arginine (L-NNA) (Ishii *et al.*, 1990; Moore *et al.*, 1990; Mülsch & Busse, 1990) and in addition, haemoglobin, which avidly binds NO (Ignarro *et al.*, 1987; Kelm *et al.*, 1988) reduced these NANC relaxations (Boeckxstaens *et al.*, 1990a). Similarly, in the rat and mouse anococcygeus muscle, NO or a NO releasing substance has been proposed as the transmitter of NANC nerves (Gillespie *et al.*, 1989; Ramagopal & Leighton, 1989; Gillespie & Sheng, 1990; Gibson *et al.*, 1990). Furthermore, we were able to demonstrate the release of NO in response to NANC nerve stimulation by electrical impulses and 1,1-dimethyl-4-phenylpiperazinium (DMPP) (Bult *et al.*, 1990).

In the present study, we investigated whether the NANC relaxations induced by ATP and GABA were also mediated by NO or a NO releasing substance by means of L-NMMA, its enantiomer D-NMMA, L-NNA and haemoglobin.

Methods

Tissue preparation

Mongrel dogs of either sex (body weight 10–30 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and a laparotomy was performed. The ileum and colon were resected 10 cm above and 3 cm below the ileocolonic junction respectively. After the resected specimen was cleaned and rinsed, the mucosa was removed from the ileum and ileocolonic junction by means of sharp dissection. Circular muscle strips were cut, approximately 1.5 cm long and 0.3 cm wide and mounted in organ baths (25 ml) (Pelckmans *et al.*, 1989) filled with a 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). The solution was maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2.

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Isometric tension recording

One end of each muscle strip was connected to a metal rod while the other end was attached to a strain gauge transducer (Statham UC2) for continuous recording of isometric tension. After the muscle strips were brought to the optimal point of their length-tension relationship (Pelckmans *et al.*, 1989), they were washed three times and then allowed to equilibrate for at least 45 min before experimentation.

Experimental protocols

All experiments were performed on muscle strips maximally contracted with noradrenaline (Pelckmans *et al.*, 1990) (30 μM; 2.7 $\pm$ 0.6 g in the ileum and 4.3 $\pm$ 0.5 g in the ileocolonic junction, mean $\pm$ s.e.mean; n = at least 6) and in the presence of 0.3 μM atropine. After each noradrenaline-induced contraction, the muscle strips were washed at least three times with an interval of 5 min, and a cycle time of at least 20 min.

In the first series of experiments, the effects of tetrodotoxin, haemoglobin, L-NMMA, its enantiomer D-NMMA, and L-NNA were studied on the relaxations (submaximal) induced by electrical stimulation (4 Hz, 1 ms), ATP (100 μM), GABA (100 μM) and NO (10 μM). In the second series of experiments, the inhibitory effect of L-NMMA and L-NNA was re-examined in the presence of L-arginine or D-arginine (2 mM). Electrical impulses (rectangular waves, 100 mA, 9 V) were delivered by a GRASS stimulator and a direct current (d.c.) amplifier in trains of stimuli of 10 s. Pharmacological agents were added at least 10 min prior to stimulation of the tissue.

Drugs used

The following drugs were used: adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA), D-arginine, L-arginine, bovine haemoglobin (Sigma Chemical Co., St. Louis, MO., U.S.A.), atropine sulphate (Federa, Brussels, Belgium), N^G-nitro-L-arginine (L-NNA), tetrodotoxin (Janssen Chimica, Beerse, Belgium), noradrenaline hydrogentartrate (Fluka AG, Buchs SG, Switzerland).

All drugs were administered as aqueous solutions except L-NNA which was dissolved in 65 mM HCl. Ascorbic acid (57 mM) was added to the stock solution of noradrenaline. These were prepared on the day of experimentation and administered in volumes not exceeding 0.5% of the bath volume. The stock solution of tetrodotoxin (1 mM, in sodium citrate, pH 4.8) was stored at -20°C. NO solutions and haemoglobin were prepared as described by Kelm *et al.* (1988). L-NMMA and D-NMMA were generously supplied by Dr S. Moncada (Wellcome Research Labs, Beckenham, Kent).

Presentation of results and statistical analysis

Results are expressed as percentage decrease of the noradrenaline-induced contraction and are shown as mean $\pm$ s.e.mean for the number of dogs indicated.

For statistical analysis, a two tailed Student's t test for paired observations was used. P values of less than 0.05 were considered to be significant.

Results

Effect of tetrodotoxin, L-NMMA, D-NMMA, L-NNA and haemoglobin on NANC relaxations induced by electrical stimulation, ATP, GABA or NO

During a noradrenaline (30 μM)-induced contraction and in the presence of 0.3 μM atropine, electrical stimulation (4 Hz, 1 ms), ATP (100 μM), GABA (100 μM) and NO (10 μM) induced submaximal relaxation of the canine terminal ileum and ileocolonic junction. These relaxations were qualitatively very similar as they were fast in onset and transient (Figures 1 and 2). Comparable to previous results, tetrodotoxin (0.6 μM), L-NMMA (100–300 μM), L-NNA (30–100 μM) and haemoglobin (10–30 μM) increased the basal tension in the muscle strips of the canine ileocolonic junction, but not in those of the terminal ileum (Figures 1 and 2). In both tissues, tetrodotoxin (0.6 μM) abolished and L-NMMA (100–300 μM) and L-NNA (30–100 μM) concentration-dependently inhibited the relaxations induced by electrical stimulation, ATP and GABA, whereas those induced by NO remained unaffected (Figures 1 and 2, Table 1). The inhibitory effect of L-NNA on these NANC relaxations was greater compared to L-NMMA in both the canine terminal ileum and ileocolonic junction (Figure 1b,c, Table 1). The enantiomer of L-NMMA, D-NMMA (100–300 μM), however, did not affect the relaxations (n = 3) (Figure 1d). Haemoglobin (10–30 μM) concentration-dependently reduced the relaxations in response to electrical stimulation, ATP and GABA, and abolished those induced by NO (Figure 2c, Table 2).

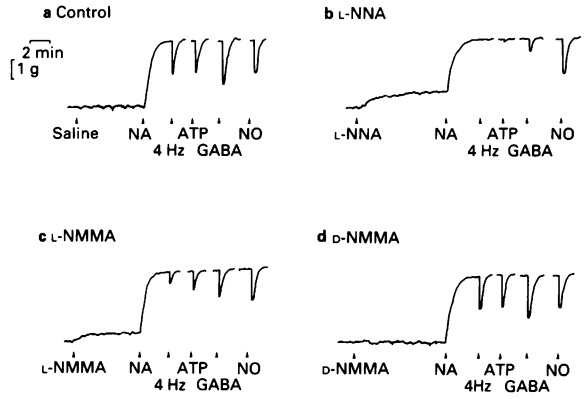

Figure 1 Isometric tension recordings showing (a) the control and the effect of (b) N^G-nitro-L-arginine (L-NNA, 100 μM), (c) N^G-monomethyl-L-arginine (L-NMMA, 300 μM) and (d) D-NMMA (300 μM) on NANC relaxations induced by electrical stimulation (4 Hz, 1 ms), adenosine 5'-triphosphate (ATP, 100 μM), γ-aminobutyric acid, (GABA, 100 μM) and nitric oxide (NO, 10 μM) in a muscle strip of the canine ileocolonic junction. L-NMMA and L-NNA, but not D-NMMA, inhibited the relaxations in response to electrical stimulation, ATP and GABA, but not those to NO. All effects were on a noradrenaline (NA, 30 μM)-induced contraction (4.3 $\pm$ 0.5 g, n = 6) in the presence of 0.3 μM atropine. Breaks in the tracings represent periods of tissue equilibration. Similar results were obtained in tissues from five other dogs and in the terminal ileum (n = 6).

After a wash-out period of 30–40 min, the effect of the inhibitors was removed as the amplitude of the NANC relaxations returned to their initial control values.

Effect of L-arginine or D-arginine on the inhibitory effect of L-NMMA and L-NNA

The presence of L-arginine (2 mM) prevented the inhibitory effect of both L-NMMA (100–300 μM) and L-NNA (30–100 μM) on the NANC relaxations induced by electrical stimulation, ATP and GABA in both the terminal ileum and ileocolonic junction (Table 1). In contrast, in the presence of D-arginine (2 mM), L-NMMA and L-NNA still inhibited these relaxations (Table 1). Preincubation with L-arginine did not potentiate any of the studied relaxations (n = 4, data not shown).

Discussion

In previous studies, we provided evidence for NO, a related substance or a NO releasing substance as the inhibitory NANC neurotransmitter in the canine terminal ileum and ileocolonic junction (Boeckxstaens *et al.*, 1990a; Bult *et al.*,

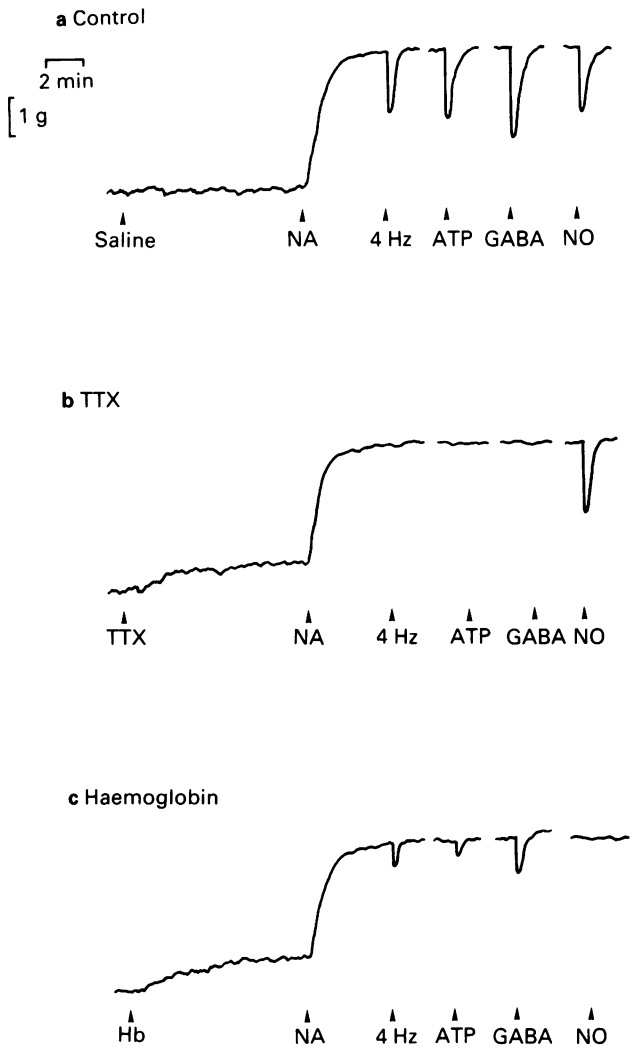

Figure 2 Isometric tension recordings showing (a) the control and the effect of (b) tetrodotoxin (TTX, 0.6 μM) and (c) haemoglobin (Hb, 30 μM) on NANC relaxations induced by electrical stimulation (4 Hz, 1 ms), adenosine 5'-triphosphate (ATP, 100 μM), γ-aminobutyric acid (GABA, 100 μM) and nitric oxide (NO, 10 μM) in a muscle strip of the canine ileocolonic junction. Tetrodotoxin abolished the relaxations in response to electrical stimulation, ATP and GABA, whereas those induced by NO remained unaffected. Haemoglobin reduced the relaxations in response to electrical impulses, ATP and GABA, whereas it abolished those to NO. All effects were on a noradrenaline (NA, 30 μM)-induced contraction (4.1 $\pm$ 0.4 g, $n = 6$) in the presence of 0.3 μM atropine. Breaks in the tracings represent periods of tissue equilibration. Similar results were obtained in tissues from five other dogs and in the terminal ileum ($n = 6$).

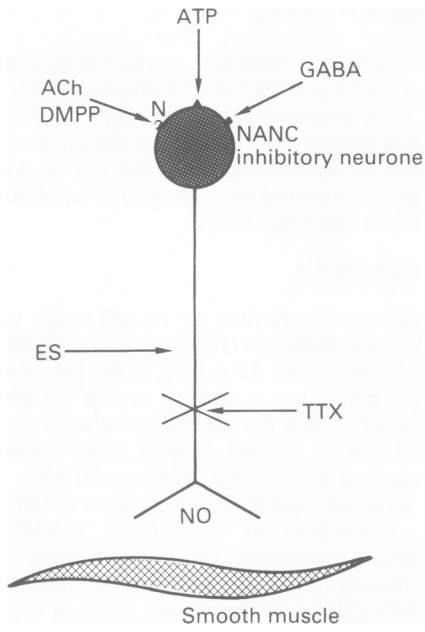

Figure 3 Schematic summary illustrating nitric oxide (NO) as the inhibitory neurotransmitter mediating the NANC relaxations in response to electrical stimulation (ES), adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA) and nicotinic receptor (N) stimulation by acetylcholine (ACh) and the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP) in the canine terminal ileum and ileocolonic junction.

1990). The NANC relaxations evoked by electrical impulses or nicotinic receptor stimulation were blocked by inhibitors of NO biosynthesis and by haemoglobin. In addition, we demonstrated the release of NO in response to NANC nerve stimulation. The present study illustrates that the neurally-mediated NANC relaxations induced by ATP or GABA are also mediated by NO or a NO releasing substance, suggesting that NO is the common final NANC neurotransmitter in the canine terminal ileum and ileocolonic junction (Figure 3).

As previously reported (Boeckxstaens *et al.*, 1990c,d), the relaxations induced by ATP or GABA, like those in response to electrical stimulation of the inhibitory NANC neurones, were abolished by the blocker of nerve conduction tetrodotoxin which indicates mediation by nerves. Both L-NMMA and L-NNA inhibited these relaxations in a stereospecific and competitive manner, since their effect was only prevented by the substrate of NO biosynthesis, L-arginine (Palmer *et al.*, 1988; Schmidt *et al.*, 1988) and not by D-arginine. Also the enantiomer of L-NMMA, D-NMMA, was without effect. Furthermore, as illustrated by the lack of effect of L-NMMA and L-NNA on the relaxation produced by NO, the blockade of the NANC-mediated relaxation was on the transmitter system rather than on the postjunctional effector cells. In addition, haemoglobin abolished the NO-induced relaxations and reduced the neurally-mediated responses. Considering the molecular size of haemoglobin, only a small proportion may have reached the neuromuscular junction which may explain the discrepancy in efficacy. Thus, those findings further favour NO or a NO releasing substance as mediator of the NANC relaxations induced by ATP and GABA in the canine terminal ileum and ileocolonic junction.

Finally, the rise in basal tension in the canine ileocolonic junction induced by tetrodotoxin and by inhibition of NO biosynthesis might suggest a constant background release of NO (Boeckxstaens *et al.*, 1990a). In contrast to our results, GABA released from myenteric neurones stimulates $GABA_A$ receptors on VIP and acetylcholine containing neurones to enhance the descending relaxation of the colonic peristaltic reflex via a predominant release of VIP (Grider & Makhlouf, 1990). In the rat duodenum on the other hand, $GABA_A$ receptor stimulation of intramural NANC neurones induced relaxation, possibly via the release of ATP (Maggi *et al.*, 1984; Manzini *et al.*, 1985). In the canine terminal ileum and ileocolonic junction however, ATP desensitization (Boeckxstaens *et al.*, 1990c) or trypsin (unpublished results) did not affect the GABA-induced relaxations and VIP failed to induce relaxation (Pelckmans *et al.*, 1989), indicating that it is unlikely that ATP or VIP mediates the responses to GABA.

NO or a related substance, which accounts for the biological activity of the vascular endothelium-derived relaxing factor (Palmer *et al.*, 1987), is synthesized from L-arginine (Palmer *et al.*, 1988; Schmidt *et al.*, 1988). This so-called L-arginine: NO pathway has been demonstrated in a variety of cells and thus might represent a more general pathway for the regulation of cell function and communication (Moncada *et al.*, 1989). In the canine ileocolonic junction, relaxation resulting from stimulation of NANC neurones by either elec-

Table 1 Effect of N^G-monomethyl-L-arginine (L-NMMA, 300 μM) and N^G-nitro-L-arginine (L-NNA, 100 μM) on relaxations induced by electrical stimulation, adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA) or nitric oxide (NO) in the absence or presence of L- or D-arginine (2 mM)

	Electrical stimulation (4 Hz, 1 ms)	*ATP* (100 μM)	*GABA* (100 μM)	*NO* (10 μM)
(a) *Ileum*				
Control	40 ± 6	49 ± 7	70 ± 6	53 ± 7
L-NMMA	11 ± 4*	27 ± 9*	47 ± 11*	48 ± 9
L-NMMA/L-arginine	35 ± 9	61 ± 5	78 ± 6	58 ± 8
L-NMMA/D-arginine	14 ± 5*	20 ± 9*	41 ± 7*	60 ± 8
L-NNA	0*	8 ± 8*	29 ± 14*	60 ± 8
L-NNA/L-arginine	38 ± 6	42 ± 6	78 ± 8	52 ± 12
L-NNA/D-arginine	1 ± 1*	0*	0*	46 ± 9
(b) *Ileocolonic junction*				
Control	39 ± 7	37 ± 7	74 ± 8	65 ± 7
L-NMMA	16 ± 2*	18 ± 2*	41 ± 9*	65 ± 7
L-NMMA/L-arginine	40 ± 10	33 ± 5	65 ± 8	55 ± 6
L-NMMA/D-arginine	27 ± 6*	25 ± 7*	52 ± 8*	63 ± 10
L-NNA	1 ± 1*	3 ± 2*	9 ± 5*	61 ± 8
L-NNA/L-arginine	33 ± 8	31 ± 5	51 ± 10*	61 ± 8
L-NNA/D-arginine	0*	0*	4 ± 2*	66 ± 6

The experiments were performed during a noradrenaline (30 μM)-induced contraction in the presence of 0.3 μM atropine. Results are shown as mean ± s.e.mean for 5–7 experiments and expressed as percentage decrease of the noradrenaline-induced contraction.
* $P < 0.05$, significantly different from controm, Student's t test for paired observations.

Table 2 Effect of haemoglobin on relaxations induced by electrical stimulation, adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA) or nitric oxide (NO)

	Electrical stimulation (4 Hz, 1 ms)	*ATP* (100 μM)	*GABA* (100 μM)	*NO* (10 μM)
(a) *Ileum*				
Control	50 ± 9	46 ± 6	79 ± 7	58 ± 6
Haemoglobin 10 μM	29 ± 5*	16 ± 7	30 ± 11*	0*
Haemoglobin 30 μM	14 ± 6*	5 ± 3*	21 ± 8*	0*
(b) *Ileocolonic junction*				
Control	49 ± 6	56 ± 1	71 ± 7	65 ± 9
Haemoglobin 10 μM	22 ± 5*	36 ± 10*	50 ± 12*	0*
Haemoglobin 30 μM	17 ± 1*	31 ± 10*	41 ± 12*	0*

The experiments were performed during a noradrenaline (30 μM)-induced contraction in the presence of 0.3 μM atropine. Results are shown as mean ± s.e.mean for 6–7 experiments and expressed as percentage decrease of the noradrenaline-induced contraction.
* $P < 0.05$, significantly different from control, Student's t test for paired observations.

trical impulses or chemical agents (acetylcholine, DMPP (unpublished results), ATP, GABA) are inhibited by blockade of the NO biosynthesis or by haemoglobin. Although we cannot exclude the possibility that another, as yet unidentified, NANC neurotransmitter induces the release of the NO-like factor from some non-neuronal cell, e.g. the smooth muscle, we favour the view that NO is the transmitter itself and represents the common pathway mediating the NANC relaxations in the canine ileocolonic junction (Figure 3). This mechanism is not confined to this region of the gastrointestinal tract and/or species as we obtained similar results in the rat gastric fundus (Boeckxstaens *et al.*, 1990f) and canine lower oesophageal sphincter (De Man *et al.*, unpublished observations). Furthermore, evidence for NO or a substance releasing NO as the transmitter of NANC nerves was also provided in the rat and mouse anococcygeus muscle (Gillespie *et al.*, 1989; Ramagopal & Leighton, 1989; Gillespie & Sheng, 1990; Gibson *et al.*, 1990). In addition, in the central nervous system, a role for NO in neurotransmission has already been postulated (Garthwaite *et al.*, 1988; Knowles *et al.*, 1989), further accentuating the importance of this L-arginine:NO pathway.

In conclusion, we provide evidence that the neurally-mediated relaxations induced by GABA or ATP are mediated by NO or a NO-releasing substance in a similar way as those in response to electrical impulses. Therefore, we propose NO as the common final neurotransmitter that mediates the NANC relaxations in the canine terminal ileum and ileocolonic junction (Figure 3).

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