Bioassay of nitric oxide released upon stimulation of non-adrenergic non-cholinergic nerves in the canine ileocolonic junction

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1 The release and the nature of the inhibitory non-adrenergic non-cholinergic (NANC) neurotransmitter was studied in the canine ileocolonic junction. A circular muscle strip of the canine ileocolonic junction served as donor tissue in a superfusion bioassay in which rings of rabbit aorta with the endothelium removed served as detector tissue.

2 The ileocolonic junction released a labile factor with vasodilator activity upon stimulation of non-adrenergic non-cholinergic (NANC) nerves in response to electrical impulses and the nicotinic receptor agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP). This release was respectively frequency- and concentration-dependent.

3 The release was reduced by the blocker of neuronal conductance, tetrodotoxin, and by the inhibitor of the nitric oxide (NO) biosynthesis N^G-nitro-L-arginine. The biological activity was enhanced by superoxide dismutase and eliminated by haemoglobin. Hexamethonium abolished only the release in response to DMPP.

4 Injection of adenosine 5'-triphosphate (ATP) or vasoactive intestinal polypeptide (VIP) onto the cascade induced relaxations of the rabbit aorta but they were different from those induced by NO or the transferable factor.

5 Based on organ bath experiments in which the reactivity of different parts of the circular smooth muscle layer of the ileocolonic junction was investigated, a muscle strip of superficial circular muscle with submucosa was chosen as the detector strip in the bioassay cascade.

6 The ileocolonic junction dose-dependently relaxed in response to nitroglycerin and NO. NO was much more potent in the rabbit aorta than in the canine ileocolonic junction. Equivalent amounts of the transferable factor, endothelium-derived relaxing factor and NO, as assessed by the relaxation of the rabbit aorta, failed to affect the ileocolonic junction.

7 In conclusion, our results demonstrate the release of a transferable vasorelaxant factor in response to NANC nerve stimulation which behaves pharmacologically like NO but not like ATP or VIP. Therefore, we suggest that NO or a NO releasing substance is the inhibitory NANC neurotransmitter in the canine ileocolonic junction.

Keywords: NO bioassay; endothelium-derived relaxing factor (EDRF); nitric oxide (NO); neurotransmission; non-adrenergic non-cholinergic nerves (NANC); ileocolonic junction

Introduction

Non-adrenergic non-cholinergic (NANC) nerves are believed to provide the main inhibitory autonomic nerve supply to gut smooth muscle (Burnstock & Costa, 1973; Burnstock, 1981). The exact nature of the transmitter(s) released by these nerves however is still debated. Adenosine 5'-triphosphate (ATP) (Burnstock, 1972) and vasoactive intestinal polypeptide (VIP) (Goyal & Rattan, 1980) are considered as the two main candidates depending on the tissue and/or species studied. Recently evidence has been presented for nitric oxide (NO) as an NANC inhibitory transmitter in some regions of the gut including the anococcygeus (Gillespie *et al.*, 1989; Ramagopal & Leighton, 1989), the ileocolonic junction and the stomach (Boeckxstaens *et al.*, 1990a,b; 1991a,b).

Previously, we demonstrated that electrical impulses, nicotinic receptor stimulation, ATP and γ -aminobutyric acid (GABA) stimulate NANC nerves resulting in relaxation of the canine ileocolonic junction (Pelckmans *et al.*, 1989; Boeckxstaens *et al.*, 1990c,d,e). These relaxations were mimicked and mediated by NO, suggesting NO as inhibitory NANC neurotransmitter (Boeckxstaens *et al.*, 1990a,b; 1991b). Using a

superfusion bioassay cascade, we also demonstrated the release of a NO-like factor in response to stimulation of the NANC nerves (Boeckxstaens *et al.*, 1990a; Bult *et al.*, 1990).

In the present study, the release of this transferable factor in response to electrical impulses and nicotinic receptor stimulation by 1,1-dimethyl-4-phenylpiperazinium (DMPP) was further characterized. In addition, we investigated the responsiveness of the canine ileocolonic junction placed in a bioassay cascade to the transferable factor, the endothelium-derived relaxing factor (EDRF), NO and nitroglycerin.

Methods

Tissue preparation

The ileocolonic junction as donor tissue in the bioassay cascade 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 the colon were resected 5 cm above and 3 cm below the ileocolonic junction respectively. After cleaning and rinsing of the resection specimen, the mucosa was removed (Pelckmans *et al.*, 1989)

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Figure 1 Schematic illustration showing the experimental set-up of the superfusion bioassay (ileocolonic junction: ICJ; noradrenaline: NA; rabbit aorta: RbA).

and the submucosa was peeled off from the ileocolonic junction. A circular muscle strip (about 4 mm wide and 2 cm long) was prepared and mounted in a perfusion chamber with a volume of about 0.5 ml and was perfused (3 ml min^{-1}) 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) maintained at 37°C . This solution contained superoxide dismutase (SOD, 20 units ml^{-1}), L-arginine ($50 \mu\text{M}$) and guanethidine ($3 \mu\text{M}$) and was aerated with a mixture of 80% N_2 , 15% O_2 and 5% CO_2 . The perfusion chamber contained two platinum ring electrodes, through which the muscle strip was pulled (Figure 1). Electrical impulses (rectangular waves, 25 mA, 9 V) were delivered by a GRASS stimulator and a direct current (d.c.) amplifier at an interval of at least 15 min resulting in reproducible responses.

Reactivity of the ileocolonic junction in organ baths After removal of the mucosa, strips (about 3 mm wide and 2 cm long) containing different tissue layers were cut by means of sharp dissection: muscle strips with (type A) or without submucosal layer (type C), muscle strips of superficial circular muscle with submucosa (type B) and muscle strips from which the superficial circular muscle and submucosa were removed (type D) (Figure 2). The muscle strips were mounted in organ baths containing a modified Krebs-Ringer solution aerated with 95% O_2 and 5% CO_2 at 37°C . They were connected to a strain gauge transducer (Statham UC2) for continuous measurement of isometric tension and used at the optimal point of the length-tension relationship (Pelckmans *et al.*, 1989).

The ileocolonic junction as detector tissue in the bioassay cascade Based on the organ bath experiments, a type B strip was chosen as detector in the bioassay cascade. This strip was mounted vertically immediately above a ring of rabbit abdominal aorta denuded of its endothelium and was connected to a strain gauge transducer for continuous measurement of isometric tension. After the muscle strip was placed at the optimal point of the length-tension relationship (Pelckmans *et al.*, 1989), the detector tissues were superfused

Figure 2 Schematic illustration showing the different layers (s = submucosa; c = circular muscle; m = myenteric plexus; l = longitudinal muscle) of the circular muscle strips (type A, B, C and D) of the canine ileocolonic junction.

by the effluent of the canine ileocolonic junction or by the perfusate of a segment of 5 cm of rabbit thoracic aorta and were contracted by an infusion of noradrenaline ($10 \mu\text{M}$). The sensitivity of the bioassay tissues was standardized by a bolus injection of nitroglycerin (1 nmol).

Rabbit aortic rings as detector tissue in the bioassay cascade New Zealand white rabbits (2000–2500 g) were killed by an overdose of sodium pentobarbitone. After a laparotomy, the abdominal aorta was removed and placed in modified Krebs-Ringer solution. Rings of abdominal aorta (3 mm wide) were cut and denuded of their endothelium by gentle rubbing. They were arranged either in a cascade with a delay of 2–3 s between the two rings or in parallel. The tissues were mounted under 8 g resting tension and their isometric tension was recorded. The aortic rings were superfused with the effluent of the canine ileocolonic junction and were contracted by an infusion of noradrenaline ($0.1 \mu\text{M}$) (Figure 1). A bolus of acetylcholine (3 nmol) was injected directly onto the aortic rings to verify the absence of endothelium (Furchgott & Zawadzki, 1980). Subsequently, atropine ($0.3 \mu\text{M}$) was introduced into the perfusate and the sensitivity of the bioassay tissues was standardized by a bolus injection of nitroglycerin (0.1 nmol).

The rabbit thoracic aorta as donor of endothelium-derived relaxing factor The rabbit thoracic aorta was removed and placed in modified Krebs-Ringer solution as described (Bult *et al.*, 1988). One cannula was inserted in the rostral opening, red blood cells were gently flushed away with modified Krebs-Ringer solution and the second cannula was placed in the caudal outlet. The aorta was positioned on a perspex support in an organ chamber filled with 400 ml modified Krebs-Ringer solution and perfused with modified Krebs-Ringer solution (3 ml min^{-1}).

Experimental protocols

Organ bath experiments After 45 min of equilibration, the muscle strips were stimulated with acetylcholine ($2 \mu\text{M}$) and after incubation with atropine ($0.3 \mu\text{M}$), the reactivity to noradrenaline ($30 \mu\text{M}$) was tested. Next, the effects of electrical stimulation (4 and 16 Hz, 1 ms, pulse train lasting 10 s), acetylcholine ($30 \mu\text{M}$), ATP ($100 \mu\text{M}$), GABA ($100 \mu\text{M}$) and nitroglycerin ($1 \mu\text{M}$) were studied on type B strips in the presence of atropine ($0.3 \mu\text{M}$) and compared with the responses obtained in type A strips. After each experiment, the preparations were placed in 4% formaldehyde, and sections were made for light microscopy.

Superfusion bioassay cascade In a first series of experiments, the effect of a transferable factor released by the canine ileocolonic junction in response to electrical stimulation (0.5–16 Hz, 0.5–2 ms, 25 mA, 9 V, pulse trains lasting 5–30 s) and infusion of the nicotinic agonist DMPP (3–100 μM , during 1 min) was investigated on rabbit aortic rings in the presence of atropine ($0.3 \mu\text{M}$), guanethidine ($3 \mu\text{M}$), L-arginine ($50 \mu\text{M}$) and SOD (20 units ml^{-1}). The effects of N^G -nitro-L-arginine (L-NNA, $100 \mu\text{M}$), tetrodotoxin ($1 \mu\text{M}$), L-arginine ($50 \mu\text{M}$), SOD (20 units ml^{-1}), haemoglobin ($0.1 \mu\text{M}$) and hexamethonium ($100 \mu\text{M}$) were studied on the release of the factor induced by electrical stimulation (16 Hz, 2 ms, pulse train lasting 20 s) or DMPP ($30 \mu\text{M}$). In addition, two aortic rings were arranged in a cascade to evaluate the stability of the transferable factor released in response to electrical stimulation or DMPP and of NO (0.1–0.5 nmol), ATP (1 μmol) and VIP (0.5 nmol) directly injected onto the cascade.

In a second series of experiments, the effect of the transferable factor released in response to electrical stimulation (16 Hz, 2 ms) was studied on the canine ileocolonic junction and the rabbit aorta. The latter was mounted in the bioassay cascade immediately under the ileocolonic junction. In addition, dose-responses curves to nitroglycerin (0.03–1 nmol) and

NO (0.1–100 nmol) directly injected onto the cascade were constructed and the effect of EDRF released by a perfused rabbit aorta in response to acetylcholine (30 nmol) was investigated.

Drug used

The following drugs were used: acetylcholine chloride, adenosine 5'-triphosphate (ATP), γ -aminobutyric acid (GABA), L-arginine, bovine haemoglobin, bovine serum albumin, 1,1-dimethyl-4-phenylpiperazinium (DMPP) (Sigma Chemical Co., St. Louis, MO, U.S.A.), atropine sulphate (Federa, Brussels, Belgium), guanethidine monosulphate (Ciba-Geigy, Switzerland), N^G -nitro-L-arginine (L-NNA), tetrodotoxin (Janssen Chimica, Beerse, Belgium), nitroglycerin (Merck, Darmstadt, Germany), noradrenaline hydrogentartrate (Fluka AG, Buchs SG, Switzerland), vasoactive intestinal polypeptide (VIP, UCB Bioproducts, Brain-l'Alleud, Belgium).

All drugs were administered as aqueous solutions except L-NNA which was dissolved in 0.065 M HCl. Ascorbic acid (0.57 mM) was added to the solution of noradrenaline. All solutions were prepared on the day of experimentation, except VIP (0.1 nM), haemoglobin (1 mM) and tetrodotoxin (1 mM), in sodium citrate, pH 4.8) which were stored at -20°C between experiments.

NO solutions and haemoglobin were prepared as described (Kelm *et al.*, 1988). Bovine recombinant superoxide dismutase (SOD) was a gift of Grünenthal GmbH (Aachen, Germany).

Presentation of results and statistical analysis

The results are expressed as g contraction or as percentage decrease of the noradrenaline-induced contraction of the rabbit aortic rings or of the muscle strip of the canine ileocolonic junction.

Values 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 or unpaired observations was used. *P* values of less than 0.05 were considered to be significant.

Results

Detection of the transferable factor by rabbit aortic rings

When the effluent of the unstimulated canine ileocolonic junction (mucosa and submucosa removed) was directed onto the cascade, the top aortic ring relaxed by $5 \pm 3\%$ ($n = 7$) of the noradrenaline ($0.1 \mu\text{M}$)-induced contraction. This relaxation was significantly increased to $18 \pm 6\%$ ($n = 7$) by SOD (20 units ml^{-1}). In the presence of atropine ($0.3 \mu\text{M}$), guanethidine ($3 \mu\text{M}$), L-arginine ($50 \mu\text{M}$) and SOD (20 units ml^{-1}), the tissue of the ileocolonic junction released more of this vasorelaxant factor upon electrical stimulation (0.5–32 Hz, 2 ms, pulse trains lasting 20 s) (Figures 3 and 4). The release was frequency-dependent (Figure 4) and varied with the pulse duration (0.5–2 ms) and the duration of the pulse train (5–30 s) (Table 1). In addition, infusion of the nicotinic agonist DMPP (0.3 – $100 \mu\text{M}$) concentration-dependently induced the release of a transferable factor by the ileocolonic junction (Figures 3 and 4). However, when the submucosal layer was still present, the release of vasorelaxant activity was not detected.

The relaxations of the rabbit aortic rings induced by the factor had the same morphology as those induced by nitroglycerin (0.1 nmol), NO (0.1 nmol) or ATP (1 μmol) directly injected onto the cascade (Figure 3). On the other hand, injection of VIP (0.5 nmol) over the bioassay tissues resulted in a slowly developing and long lasting relaxation (Figure 3). The biological activity of the factor declined to the same extent (electrical stimulation, $44 \pm 6\%$, $n = 7$; DMPP, $41 \pm 15\%$, $n = 4$) as the effect of NO ($42 \pm 5\%$, $n = 4$) during passage

Figure 3 Relaxation of rabbit aortic (RbA) rings arranged in a cascade by a transferable factor released by the canine ileocolonic junction (ICJ) in response to electrical impulses (16 Hz, 2 ms) and 1,1-dimethyl-4-phenylpiperazinium (DMPP, $30 \mu\text{M}$) and by injection of nitric oxide (NO, 0.1 nmol), ATP (1 mol) and vasoactive intestinal polypeptide (VIP, 0.5 nmol) onto the cascade (o.c.). The biological activity of the transferable factor and of NO, but not of ATP and VIP, decreased to the same extent during the delay of 3 s between upper and lower aortic ring. Breaks in the tracings represent periods of tissue equilibration. The experiments were performed in the presence of atropine ($0.3 \mu\text{M}$), guanethidine ($3 \mu\text{M}$), L-arginine ($50 \mu\text{M}$) and superoxide dismutase (20 units ml^{-1}). The rings of rabbit aorta were denuded of endothelium and contracted submaximally by a continuous infusion of noradrenaline ($0.1 \mu\text{M}$). The sensitivity of the bioassay was standardized by injection of nitroglycerin (GTN, 0.1 nmol). Similar results were obtained in 3 other experiments.

down the cascade, whereas that of ATP and VIP remained unchanged (Figure 3).

The relaxation of the aortic rings in response to electrical stimulation (16 Hz, 2 ms) or infusion of DMPP ($30 \mu\text{M}$) was significantly augmented from $5 \pm 5\%$ ($n = 7$) and $2 \pm 1\%$ ($n = 5$) to $28 \pm 8\%$ and $20 \pm 6\%$ respectively by SOD (20 units ml^{-1}) and from $9 \pm 4\%$ ($n = 4$) and $9 \pm 2\%$ ($n = 4$) to $23 \pm 3\%$ and $26 \pm 5\%$ respectively by L-arginine ($50 \mu\text{M}$). On the other hand, these relaxations were inhibited by haemoglobin ($0.1 \mu\text{M}$), L-NNA ($100 \mu\text{M}$) and tetrodotoxin ($1 \mu\text{M}$)

Figure 4 Relaxation of the top rabbit aortic ring induced by the frequency/concentration-dependent release of a vasorelaxant factor by the canine ileocolonic junction in response to electrical impulses ($\circ$, 0.5–32 Hz, 2 ms) and 1,1-dimethyl-4-phenylpiperazinium (DMPP, Δ , 3–100 μM) respectively. The experiments were performed as shown in Figure 3 in the presence of atropine ($0.3 \mu\text{M}$), guanethidine ($3 \mu\text{M}$), L-arginine ($50 \mu\text{M}$) and superoxide dismutase (20 units ml^{-1}). Results are shown as mean for 4 experiments (with s.e.mean shown by vertical bars) and are expressed as percentage decrease of the noradrenaline ($0.1 \mu\text{M}$)-induced contractions.

Table 1 Effect of pulse duration (0.5–2 ms, 20 s) and duration of pulse train (2 ms, 5–30 s) on the relaxation of the rabbit aorta to the transferable factor released by electrical stimulation (16 Hz)

	% relaxation	n
(a) Pulse duration (ms)		
0.5	19 ± 6	5
1	25 ± 4	5
2	30 ± 5	5
(b) Duration of pulse train (s)		
5	14 ± 5	4
10	27 ± 7	4
20	29 ± 6	4
30	36 ± 9	4

The experiments were performed as shown in Figure 3. Atropine (0.3 μ M), guanethidine (3 μ M), L-arginine (50 μ M) and superoxide dismutase (20 units ml⁻¹) were present. Results are shown as mean $\pm$ s.e.mean for the number of experiments (n) indicated and are expressed as percentage decrease of the noradrenaline (0.1 μ M)-induced contraction of the top aortic ring.

(Table 2). Hexamethonium (100 μ M) abolished the release of the factor induced by DMPP but had no effect on the release in response to electrical stimulation (Table 2). The relaxations induced by nitroglycerin (0.1 nmol), ATP (1 μ mol) or VIP (0.5 nmol) were not affected by SOD or haemoglobin (results not shown).

The canine ileocolonic junction as bioassay tissue

Organ bath experiments Histology confirmed the composition of the type A muscle strips, as schematically illustrated in Figure 2. Type B strips indeed only contained the submucosa and superficial circular muscle. The submucosa or the submucosa and the superficial circular muscle layer had been removed in type C or type D strips respectively, as indicated in Figure 2.

After equilibration, all different types of strips contracted to the same extent to acetylcholine (2 μ M, type A: 3.8 ± 0.6 g; type B: 4.0 ± 0.5 g; type C: 3.7 ± 0.8 g; type D: 3.7 ± 0.3 g, $n = 7$). However, only type A and type B strips contracted in a stable manner to noradrenaline (30 μ M) (3.2 ± 0.6 g and 2.9 ± 0.4 g respectively, $n = 7$). Removal of the submucosa

Table 2 Effect of haemoglobin, N^G-nitro-L-arginine (L-NNA), tetrodotoxin and hexamethonium on the relaxation of the rabbit aorta to the transferable factor released in response to electrical stimulation (16 Hz, 2 ms) and 1,1-dimethyl-4-phenylpiperazinium (DMPP, 30 μ M)

	Electrical stimulation (16 Hz, 2 ms) (% relaxation)	DMPP (30 μ M) (% relaxation)
Control	30 ± 8	21 ± 4
Haemoglobin 0.1 μ M	1 ± 1*	0*
L-NNA 0.1 mM	9 ± 4*	0*
Tetrodotoxin 1 μ M	2 ± 1*	3 ± 3*
Hexamethonium 0.1 mM	30 ± 7	0*

The experiments were performed as shown in Figure 3. Atropine (0.3 μ M), guanethidine (3 μ M), L-arginine (50 μ M) and superoxide dismutase (20 units ml⁻¹) were present. Results are shown as mean $\pm$ s.e.mean for 4–7 experiments and are expressed as percentage decrease of the noradrenaline (0.1 μ M)-induced contraction of the top aortic ring.

* $P < 0.05$, significantly different from control, Student's *t* test for paired observations.

Figure 5 Isometric tension recordings showing the reactivity of (a) type A, (b) type B, (c) type C and (d) type D circular muscle strips of the canine ileocolonic junction to noradrenaline (NA, 30 μ M). The different tissue layers of these muscle strips are schematically illustrated in Figure 2. In (a) and (b), the effect of electrical stimulation (4 and 16 Hz, 1 ms), acetylcholine (ACh, 30 μ M), ATP (100 μ M), γ -aminobutyric acid (GABA, 100 μ M) and nitroglycerin (GTN, 1 μ M) are shown during a noradrenaline-induced contraction. The experiments were performed in the presence of 0.3 μ M atropine. Similar results were obtained in 6 additional experiments.

(type C) resulted in a transient contraction to noradrenaline whereas the spontaneous activity was abolished by noradrenaline in type D muscle strips (Figure 5).

During a noradrenaline (30 μ M)-induced contraction in the presence of atropine (0.3 μ M), the NANC responses to electrical stimulation (4 and 16 Hz, 1 ms), acetylcholine (30 μ M), ATP (100 μ M), GABA (100 μ M) and nitroglycerin (1 μ M) were identical in the type A and type B muscle strips (Figure 5).

Superfusion bioassay cascade Type B muscle strips were superfused more homogeneously than type A strips, resulting in more reproducible, stable and sustained contractions to acetylcholine (2 μ M) or noradrenaline (10 μ M). Since the organ bath experiments demonstrated that these strips reacted in the same way as type A strips, all further experiments were performed on type B strips.

Infusion of 0.1 μ M noradrenaline on the detector tissues had no effect on the motor activity of the ileocolonic junction but contracted the rabbit aorta submaximally (3.7 ± 0.5 g, $n = 7$). During this contraction in the presence of atropine (0.3 μ M), guanethidine (3 μ M), L-arginine (50 μ M) and SOD (20 units ml⁻¹), EDRF released by the perfused rabbit aorta in response to acetylcholine (30 nmol), and the transferable factor released in response to electrical stimulation (16 Hz, 2 ms) of the ileocolonic junction induced a relaxation of $73 \pm 9\%$ ($n = 4$) and $38 \pm 5\%$ ($n = 5$) of the rabbit aortic ring respectively. The spontaneous activity of the ileocolonic junction was not affected.

Infusion of 10 μ M noradrenaline contracted both detector tissues (ileocolonic junction: 1.8 ± 0.2 g, $n = 9$; rabbit aorta: 4.7 ± 0.4 g, $n = 7$). Nitroglycerin (0.03–1 nmol) and NO (0.1–100 nmol), directly applied onto the cascade, dose-dependently relaxed both the canine ileocolonic junction and the rabbit aorta (Figures 6 and 7). However, NO relaxed the rabbit aorta more potently than the ileocolonic junction (Figure 7). In both tissues, nitroglycerin was more potent than NO (Figure 7). EDRF and the transferable factor now relaxed the rabbit aorta by $38 \pm 5\%$ ($n = 4$) and $12 \pm 3\%$ ($n = 5$) respectively, but failed to relax the ileocolonic junction (Figure 6). In the presence of 1 mM L-arginine, the relaxation of the aortic ring to the transferable factor was significantly increased to $37 \pm 8\%$ ($n = 3$); however, the factor did not relax the ileocolonic junction (Figure 6). Injection of 0.1–0.3 nmol nitroglycerin or 0.1–0.3 nmol NO produced relaxations equal to those of EDRF and the ileocolonic factor on the aortic ring, but only nitroglycerin induced relaxation of the ileocolonic junction (Figure 6).

Figure 6 Isometric tension recordings of the canine ileocolonic junction (ICJ) and the rabbit aorta (RbA) showing the effect of the transferable factor (electrical stimulation; 16 Hz, 2 ms), or nitroglycerin (GTN, 0.3 nmol) and NO (0.3 nmol) injected onto the cascade (o.c.). Endothelium-derived relaxing factor (EDRF) released in response to acetylcholine (ACh, 30 nmol) from a perfused rabbit aorta relaxed the aortic ring to the same degree. Breaks in the tracing represent periods of tissue equilibration. The numbers in parentheses are the doses expressed in nmol. The experiments were performed in the presence of atropine (0.3 μ M), guanethidine (3 μ M), L-arginine (1 mM) and superoxide dismutase (20 units ml^{-1}). If the effect of EDRF was tested, atropine was infused only onto the detector tissues. The ring of rabbit aorta was denuded of endothelium and both tissues were contracted by a continuous infusion of noradrenaline (10 μ M). The sensitivity of the bioassay was standardized by injection of nitroglycerin (1 nmol). Similar results were obtained in 2 other experiments.

Figure 7 Relaxation of the canine ileocolonic junction (filled symbols) and the rabbit aorta (open symbols) in response to nitroglycerin (O, 0.03–1 nmol) and NO (Δ , 0.1–100 nmol) injected onto the cascade. The experiments were performed as shown in Figure 6 in the presence of atropine (0.3 μ M), guanethidine (3 μ M), L-arginine (50 μ M) and superoxide dismutase (20 units ml^{-1}). Results are shown as mean for at least 5 experiments with s.e.mean shown by vertical bars and are expressed as percentage decrease of the noradrenaline (10 μ M)-induced contraction.

* $P < 0.05$, significantly different, sensitivity of the canine ileocolonic junction versus rabbit aorta to NO, Student's t test for unpaired observations.

† $P < 0.05$, significantly different, sensitivity to NO versus nitroglycerin in the canine ileocolonic junction, Student's t test for unpaired observations.

** $P < 0.05$, significantly different, sensitivity to NO versus nitroglycerin in the rabbit aorta, Student's t test for unpaired observations.

Discussion

Endothelium-derived relaxing factor (EDRF) is synthesized and released by the vascular endothelium in response to diverse substances (Furchgott & Zawadzki, 1980; Furchgott, 1984). This vasorelaxant factor is now thought to be nitric oxide (NO) (Palmer *et al.*, 1987) and/or S-nitrosocysteine which spontaneously decomposes with the liberation of NO (Myers *et al.*, 1990; Ignarro, 1990). NO is enzymatically synthesized from its precursor L-arginine (Palmer *et al.*, 1988a; Schmidt *et al.*, 1988), a pathway which has been demonstrated in several cell types underlying a variety of biological actions (Moncada *et al.*, 1989). In particular, formation of NO from L-arginine has been reported in the rat cerebellum (Garthwaite *et al.*, 1988; Dickie *et al.*, 1990) and forebrain (Knowles *et al.*, 1989). In addition, in the peripheral nervous system, evidence is available favouring a role of NO in NANC neurotransmission (Gillespie *et al.*, 1989; Ramagopal & Leighton, 1989; Boeckxstaens *et al.*, 1990a,b; 1991a,b; Buht *et al.*, 1990; Gibson *et al.*, 1990; Tucker *et al.*, 1990). Exogenous NO directly relaxes the smooth muscle and mimics the relaxations induced by stimulation of the intramural NANC nerves. Furthermore, inhibition of NO biosynthesis by L-arginine analogues reduces the NANC responses without affecting the postjunctional effector cells. Finally, we here demonstrate the release of NO in response to NANC nerve stimulation which is a fundamental criterion for NO to be considered as putative neurotransmitter (Burnstock *et al.*, 1986).

Stimulation of the NANC nerves in the canine ileocolonic junction by electrical impulses or the nicotinic agonist DMPP triggered the release of a labile transferable factor in a frequency/concentration-dependent manner. This stimulated release was tetrodotoxin-sensitive, indicating that neuronal structures were activated, rather than other cell populations such as endothelial or mononuclear cells, or micro-organisms. Recently, evidence has indeed been obtained that the enzyme which catalyzes NO formation, NO synthase, is present in the myenteric plexus and associated neuronal processes in the intestine and is present in particularly large amounts in neuronal axons (Bredt *et al.*, 1990). In addition, it has also been demonstrated that intact neuronal tissue can release NO upon stimulation (Dickie *et al.*, 1990). Although we cannot exclude the possibility that an as yet unidentified neurotransmitter induced the release of the transferable factor from some non-neuronal cell, e.g. the smooth muscle, we favour the view that NO or a NO releasing substance is the transmitter itself and is released by the NANC nerves.

The nature of the factor released is indeed non-adrenergic non-cholinergic since both atropine and guanethidine were present. In addition, the aortic rings failed to relax to acetylcholine or noradrenaline. As illustrated by the decrease of the biological activity down the cascade, the factor released by the ileocolonic junction is unstable, a feature which is not compatible with peptides like VIP, amines, vasodilator prostanooids or ATP, but rather with a substance like NO. Relaxations induced by NO indeed declined to the same extent as those in response to the transferable factor. In addition, the biological activity of both NO and the factor, but not of VIP and ATP, was increased by SOD, a substance that prolongs the half-life of NO through suppression of its inactivation by superoxide anion (Gryglewski *et al.*, 1986). Furthermore, L-arginine, the precursor of NO (Palmer *et al.*, 1988a; Schmidt *et al.*, 1988), increased the release of the factor whereas the latter was decreased by inhibition of NO biosynthesis. The enhanced release after L-arginine supplementation, which is generally not observed when vascular endothelial cells are used as the source of NO (Palmer *et al.*, 1989a,b), could be in agreement with the previous reported hypothesis of a continuous release of NO in the canine ileocolonic junction (Boeckxstaens *et al.*, 1990b). This was confirmed by the finding that the effluent of the unstimulated ileocolonic junction relaxed the aortic rings, an effect that was enhanced by

SOD. Previously, comparable evidence has been obtained illustrating basal release of EDRF from unstimulated endothelial cells (Griffith *et al.*, 1984; Gryglewski *et al.*, 1986). Finally, haemoglobin, known to trap NO avidly (Martin *et al.*, 1985), abolished the biological activity of the factor, but not that of ATP and VIP. These results indicate that NO, but not ATP or VIP, accounts for the vasorelaxant effect of the transferable factor, suggesting NO or a NO releasing substance as the inhibitory NANC neurotransmitter. Similar results were obtained with the NANC innervation of the canine lower oesophageal sphincter (unpublished observations) and the rat gastric fundus (Boeckxstaens *et al.*, 1991a) indicating that this mechanism is not confined to the tissue and/or species studied.

We examined the effect of the transferable factor on the ileocolonic junction itself and compared it with that of NO, nitroglycerin and EDRF. Preliminary organ bath experiments were performed to determine which part of the ileocolonic junction was most suitable as detector tissue in the bioassay cascade. These experiments revealed spatial differences in reactivity to noradrenaline in the circular muscle layer: the superficial part contracted to noradrenaline whereas the spontaneous activity of the deeper part of the circular muscle layers was abolished by noradrenaline. Spatial organisation of the spontaneous electrical activity and of the innervation has been reported previously in the circular smooth muscle of the canine colon (Smith *et al.*, 1987a,b; 1989). It was suggested that different transmitters and/or different receptors may be present through the thickness of the circular layer (Smith *et al.*, 1989), which might also explain our results. However, it should also be considered that differences in response to noradrenaline might result from injury to the smooth muscle cells due to tissue manipulation. However, all strips contracted to acetylcholine, indicating that the contractile apparatus of the smooth muscle cells was still functional. In addition, the type B strips behaved similarly to the type A strips. Placed in the bioassay cascade, only the type B strip contracted in a sustained manner to noradrenaline, justifying its choice as detector strip.

Submaximal contraction of the ileocolonic junction was achieved by an infusion of 10 μ M noradrenaline (Pelckmans *et al.*, 1990), a concentration which maximally contracted the rabbit aorta. Although this reduced the sensitivity of the rabbit aorta to nitroglycerin (Herman *et al.*, 1971) and the transferable factor, this experimental condition was optimal for investigation of the effect of different substances on the ileocolonic junction. However, even maximal stimulation of the release of the factor, as judged from the response of the aortic ring, was not sufficient to cause relaxation of the ileocolonic junction. In addition, amounts of NO or EDRF that matched the vasodilator activity of the factor also failed to affect the ileocolonic junction. This could be explained by a low sensitivity of this nonvascular smooth muscle to NO compared to the rabbit aorta. Indeed, only high amounts of NO relaxed the ileocolonic junction, suggesting that greater

amounts of the transferable factor and EDRF are also required to be effective. In contrast, nitroglycerin, a stable substance from which NO is liberated in the smooth muscle cell, was almost equipotent in the ileocolonic junction and in the rabbit aorta under these experimental conditions. This great difference in responsiveness to exogenous NO and to NO liberated from nitroglycerin in the canine ileocolonic junction may therefore rather indicate that a significant proportion of the exogenous NO is inactivated before it reaches the smooth muscle cells. The submucosa for example may trap or act as a biological barrier to NO. This is illustrated by the finding that release of the transferable factor was only detected from tissue from which the submucosa has been removed. High tissue concentrations of superoxide anions (Gryglewski *et al.*, 1986), quick oxidation or binding to the submucosa may all account for a higher degree of inactivation of NO compared to nitroglycerin. This might also explain the previously reported low sensitivity to NO and EDRF in other nonvascular smooth muscle (Shikano *et al.*, 1987; Dusing *et al.*, 1988; Gillespie & Sheng, 1988; Buga *et al.*, 1989; Cocks & Angus, 1990), since these bioassay tissues generally still contained their mucosa and submucosa. Vascular rings on the other hand were without endothelium and most probably lack such possible barriers to NO. Alternatively, the transferable factor, EDRF and NO may represent more selective and potent relaxants of vascular than of nonvascular smooth muscle.

In conclusion, we were able to demonstrate the release of an unstable vasorelaxant factor in response to NANC nerve stimulation, which behaves pharmacologically like NO and is synthesized from L-arginine. Since we previously also demonstrated that NO mimics and mediates the NANC relaxations in the canine ileocolonic junction (Boeckxstaens *et al.*, 1990a,c, d), we propose NO or a NO releasing substance as the putative NANC neurotransmitter in this tissue. In contrast to classical neurotransmitters, the instability of NO makes the classical inactivation by uptake or enzyme systems unnecessary. Furthermore, since NO is a highly lipophilic substance, it will diffuse easily through the postjunctional cellular membrane without binding to an extracellular receptor. Inside the cell, the haem group of guanylate cyclase will bind NO which will induce the synthesis of guanosine 3':5'-cyclic monophosphate (cyclic GMP) resulting in smooth muscle relaxation (for review, see Waldman & Murad, 1987). Therefore, if future experiments confirm the role of NO in NANC neurotransmission, it will be an odd and primitive neurotransmitter which is also reflected by its chemical composition.

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