Ca²⁺ dependency of the release of nitric oxide from non-adrenergic non-cholinergic nerves

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- 1 The role of Ca²⁺ in nitrergic neurotransmission was studied in the canine ileocolonic junction.
- 2 The specific N-type voltage-sensitive Ca²⁺ channel blocker ω -conotoxin GVIA (CTX, 10–100 nM) significantly reduced the electrically-evoked (2–16 Hz, 1–2 ms pulse width) non-adrenergic non-cholinergic (NANC) relaxations, preferentially affecting those to low frequency stimulation, in circular muscle strips of the ileocolonic junction. In contrast, the nerve-mediated NANC-relaxations in response to acetylcholine (30 μ M), γ -aminobutyric acid (100 μ M) and adenosine 5'-triphosphate (100 μ M), as well as the relaxations to nitric oxide (NO) (3–10 μ M) and nitroglycerin (1 μ M), remained unaffected.
- 3 A NO-related substance (NO-R), released from the ileocolonic junction in response to NANC nerve stimulation (4 and 16 Hz, 2 ms pulse width), was assayed with a superfusion bioassay cascade. CTX (50 nM) reduced the release of NO-R induced by electrical impulses (4 Hz: from $18 \pm 4\%$ to $6 \pm 4\%$; 16 Hz: from $33 \pm 2\%$ to $14 \pm 4\%$, $n = 5$), but not that in response to the nicotinic receptor agonist, 1,1-dimethyl-4-phenylpiperazinium (DMPP, 0.3 mM). In Ca²⁺-free medium, the release of NO-R evoked by electrical impulses or DMPP was inhibited. The L-type Ca²⁺ channel blockers verapamil (1–3 μ M) and nifedipine (1 μ M) had no effect.
- 4 From these results we conclude that the release of NO-R in response to NANC nerve stimulation is Ca²⁺-dependent. The electrically-evoked release of NO-R results from Ca²⁺ entry through CTX-sensitive N-type voltage-sensitive Ca²⁺ channels, whereas that induced by nicotinic receptor activation involves CTX-insensitive Ca²⁺ channels, different from the L- or N-type.

Keywords: Bioassay; ω -conotoxin; electrical stimulation; ileocolonic junction; inhibitory innervation; nitric oxide (NO); non-adrenergic non-cholinergic nerves

Introduction

Abundant evidence has accumulated in support of our hypothesis (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1990a) that nitric oxide (NO) or a NO related substance (NO-R) functions as a neurotransmitter of the inhibitory non-adrenergic non-cholinergic (NANC) innervation of the gut (for review see Sanders & Ward, 1992). Pharmacological, electrophysiological and immunocytochemical studies have all added important evidence in favour of this hypothesis, so that, at present, NO fulfils most, if not all, criteria to be considered as a neurotransmitter.

In the canine ileocolonic junction, we have previously demonstrated that NO mimicked and mediated NANC (nerve-induced) relaxations in response to electrical impulses, adenosine 5'-triphosphate (ATP), γ -aminobutyric acid (GABA), acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) (Boeckxstaens *et al.*, 1990a; 1991b; Bogers *et al.*, 1991). Furthermore, using a superfusion bioassay, we provided evidence indicating the release of a transferable factor in response to electrical and nicotinic receptor stimulation of the NANC nerves in this preparation (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1991c). This factor behaved pharmacologically like NO: its biological activity was abolished by haemoglobin, known to bind NO avidly (Martin *et al.*, 1985) and was increased by superoxide dismutase, which prolongs the half-life of NO (Gryglewski *et al.*, 1986). In addition, the release of the factor was blocked by inhibition of NO biosynthesis and tetrodotoxin, but augmented by L-arginine (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1991c), the substrate of NO

biosynthesis (Palmer *et al.*, 1988). These results indicated that NO fulfilled the criteria of a putative neurotransmitter released during nerve activation (Christensen & Wingate, 1983).

In general, transmitter release from nerve terminals depends on the increase of intracellular Ca²⁺ concentration resulting from Ca²⁺ influx through Ca²⁺ channels (Augustine *et al.*, 1987; Miller, 1987). These channels can be divided into two major classes: voltage-activated channels, which respond to changes in membrane potential and which are subdivided into low threshold (T-type) and high-threshold (L, N, and P-type) channels, and receptor-operated channels, which are insensitive to depolarizing stimuli, but open when neurotransmitters, hormones or drugs combine with receptors on the cell membrane (Bertolino & Llinás, 1992; Spedding & Paoletti, 1992). ω -Conotoxin GVIA (CTX) is a potent inhibitor of the N-type voltage-sensitive Ca²⁺ channels in neurones, but not in muscle (McCleskey *et al.*, 1987; Miller, 1987). This toxin has been shown to block nerve stimulation-induced NANC relaxations in the guinea-pig taenia caeci, rat gastric fundus and rat anococcygeus muscle (De Luca *et al.*, 1990), suggesting that Ca²⁺ channels are indeed linked to neurotransmitter release in the NANC nerve terminals. However, the role of Ca²⁺ in NO release from NANC nerves has not been fully elucidated. Therefore, we have studied the effect of CTX on nitrergic relaxations in the canine ileocolonic junction and its effect, as well as that of Ca²⁺-free medium and the L-type voltage-sensitive Ca²⁺ channel blockers, verapamil and nifedipine, on the release of the transferable factor evoked by electrical and chemical stimulation of the NANC nerves. A preliminary account of some of these results has been presented (De Man *et al.*, 1992).

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Methods

Organ bath experiments

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, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25, CaEDTA 0.026 and glucose 11.1). The solution was maintained at 37°C and aerated with a mixture of 95% O₂ and 5% CO₂.

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 30 µM noradrenaline 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.

The effects of CTX (1–100 nM) were studied on the relaxations induced by electrical stimulation (2–16 Hz, 1–2 ms pulse width), ATP (100 µM), GABA (100 µM), NO (3–10 µM) and nitroglycerin (1 µM). Electrical impulses (rectangular waves, 100 mA, 9 V) were delivered by a Grass stimulator and a direct current amplifier in trains of stimuli of 10 s. CTX was added at least 20 min prior to stimulation of the tissue. The agents added remained in the organ bath until washout after the nitroglycerin-induced relaxation (Figure 1).

Superfusion bioassay cascade

Preparation of the donor tissue After resection, cleaning and rinsing of the ileocolonic junction, the mucosa and submucosa was peeled off and a circular muscle strip was prepared and mounted in a perfusion chamber, as described previously (Boeckxstaens *et al.*, 1991c). The tissue was perfused (3 ml min⁻¹) with a modified Krebs-Ringer solution maintained at 37°C. This solution contained superoxide dismutase (20 units ml⁻¹), L-arginine (50 µM) and guanethidine (3 µM) and was aerated with a mixture of 80% N₂, 15% O₂ and 5% CO₂. The perfusion chamber contained two platinum ring electrodes, through which the muscle strip was pulled. Electrical impulses (rectangular waves, 25 mA, 9 V) were delivered by a Grass stimulator and a direct current amplifier at an interval of at least 15 min, resulting in reproducible responses.

Preparation of the detector tissue 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 µM). A bolus of acetylcholine (3 nmol) was injected directly into the aortic rings to verify the absence of endothelium (Furchgott & Zawadzki, 1980). Subsequently, atropine (0.3 µM) was introduced into the perfusate and the sensitivity of the bioassay tissues was standardized by a bolus injection of nitroglycerin (0.1 nmol).

Experimental protocols In a first series of experiments, the release of the transferable factor induced by electrical stimulation (4 and 16 Hz, 2 ms pulse width, pulse trains lasting 20 s) or the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP, 0.3 mM during 1 min) was investigated in the presence of modified Krebs-Ringer solution (control) and during infusion of CTX (50 nM).

In another series, the donor tissue was first incubated in Ca²⁺-free medium (composition in mm: NaCl 123, KCl 4.7, MgSO₄ 1.2, NaHCO₃ 25, NaEDTA 0.026 and glucose 11.1) for 20 min before it was placed in the perfusion chamber. Subsequently, it was superfused with Ca²⁺-free Krebs-Ringer solution, and after equilibration, it was stimulated with electrical impulses (8 Hz, 2 ms, pulse trains, lasting 20 s) and DMPP (0.3 mM for 1 min). The concentration of Ca²⁺ in the fluid superfusing the detector tissue was kept constant by mixing the Ca²⁺-free effluent of the donor tissue with a 2.5 mM CaCl₂ solution. Next, the release in response to these stimuli was repeated after perfusion with normal Ca²⁺ (2.5 mM) containing Krebs-Ringer solution.

In a final series of experiments, the effect of verapamil (1–3 µM) and nifedipine (1 µM) was investigated on the NO-R release. Muscle strips of the canine ileocolonic junction were incubated for 20 min in Krebs-Ringer solution containing either solvent, verapamil or nifedipine. Thereafter, the donor tissue was placed into the perfusion chamber and superfused with control Krebs-Ringer solution, or Krebs-Ringer solution containing verapamil or nifedipine respectively. After equilibration (5 min), the donor tissue was stimulated with electrical impulses (4 and 16 Hz, 2 ms, pulse trains lasting 20 s) and DMPP (0.3 mM for 1 min). In order to study the effect of verapamil or nifedipine on the NO-R release within the same muscle strip, experiments were also performed in which the release of NO-R was first investigated under control conditions. Thereafter, the donor tissue was perfused with Krebs-Ringer solution containing either verapamil or nifedipine and the release of NO-R evoked by electrical impulses and DMPP was reinvestigated. Again, the effluent of the donor tissue containing verapamil or nifedipine was mixed with 2.5 mM CaCl₂ to maintain the tone of the detector tissue.

Drugs used

The following drugs were used: acetylcholine chloride (ACh), adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA), L-arginine, ω-conotoxin GVIA (CTX), 1,1-dimethyl-4-phenylpiperazinium (DMPP), nifedipine (Sigma Chemical Co., St. Louis, MO, U.S.A.), atropine sulphate (Federa, Brussels, Belgium), guanethidine monosulphate (Ciba-Geigy, Switzerland), nitroglycerin (Merck, Darmstadt, Germany), noradrenaline hydrogentartrate (Fluka AG, Buchs SG, Switzerland), verapamil (Searle Lab., Brussels, Belgium).

All drugs were administered as aqueous solutions except nifedipine, which was dissolved in dimethylsulphoxide. Ascorbic acid (0.57 mM) was added to the solution of noradrenaline. All solutions were prepared on the day of experiments, except CTX which was stored at –20°C between experiments.

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

Presentation of results and statistical analysis

The results are expressed 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

Organ bath experiments

In the presence of atropine (0.3 μ M) and guanethidine (3 μ M), electrical impulses (2–16 Hz, 1–2 ms pulse width), ATP (100 μ M), GABA (100 μ M) and ACh (30 μ M) evoked NANC nerve-induced relaxations. These relaxations were mimicked by NO (3–10 μ M), whereas nitroglycerin (1 μ M) induced sustained relaxations (Figure 1). CTX (10–100 nM) concentration-dependently raised the basal tension of the ileocolonic junction (10 nM: 0.2 ± 0.1 g; 100 nM: 0.5 ± 0.1 g; $n = 7$) and concentration-dependently and irreversibly reduced the NANC relaxations induced by electrical impulses (Figures 1 and 2). The inhibitory effect was frequency-dependent; the lower frequencies (2–4 Hz) were preferentially reduced, whereas no effect was detected on the relaxations to 16 Hz (1 and 2 ms pulse width). The relaxations induced by ATP (control: $31 \pm 3\%$; CTX 100 nM: $30 \pm 8\%$, $n = 6$), GABA (control: $51 \pm 6\%$; CTX 100 nM: $48 \pm 6\%$, $n = 6$), ACh (control: $25 \pm 4\%$; CTX 100 nM: $26 \pm 5\%$, $n = 6$), NO (3 μ M, control: $37 \pm 7\%$; CTX 100 nM: $36 \pm 4\%$, $n = 6$) and nitroglycerin (control: $80 \pm 8\%$; CTX 100 nM: $90 \pm 5\%$, $n = 6$) were not affected by CTX (Figure 1).

Superfusion bioassay

Effect of CTX on the release of NO-R In the presence of atropine (0.3 μ M), guanethidine (3 μ M), superoxide dismutase

Figure 1 Typical tracing of a circular muscle strip of the canine ileocolonic junction showing the relaxations to electrical stimulation (2–16 Hz, 1–2 ms pulse width) (ACh, 30 μ M), adenosine 5'-triphosphate (ATP, 100 μ M), γ -aminobutyric acid (GABA, 100 μ M), nitric oxide (NO, 3 μ M) and nitroglycerin (GTN, 1 μ M) in (a) the absence and (b) the presence of ω -conotoxin (CTX, 0.1 μ M) during a noradrenaline (NA, 30 μ M)-induced contraction. Similar results were obtained in at least 5 other experiments. W = wash out.

Figure 2 Effect of ω -conotoxin (CTX, Δ , 1 nM; $\square$, 10 nM; $\circ$, 100 nM) on the NANC relaxations induced by electrical impulses ($\bullet$, 2–16 Hz, 1–2 ms pulse width) in the canine ileocolonic junction. Results are expressed as percentage relaxation of the noradrenaline (30 μ M)-induced contraction and shown as mean $\pm$ s.e.mean for at least 6 experiments. **P* < 0.05, significantly different from control, Student's *t* test for paired observations.

Figure 3 (a) Typical tracing of a rabbit aortic (RbAo) ring showing the effect of ω -conotoxin (CTX, 50 nM) on the relaxations to nitroglycerin (GTN, 0.1 nmol bolus injection) injected onto the cascade and on the release of a transferable factor characterized as NO or NO-R released by the canine ileocolonic junction (cICJ) in response to electrical impulses (4 and 16 Hz, 2 ms pulse width, pulse trains lasting 20 s) and 1,1-dimethyl-4-phenylpiperazinium (DMPP, 0.3 mM 1 min). The aortic ring was precontracted by noradrenaline (NA, 0.1 μ M). Breaks in the tracing represent periods of equilibration. (b) Effect of CTX (50 nM; hatched columns) on the relaxations induced by GTN (0.1 nmol bolus injection) and on the release of NO-R in response to electrical (4 and 16 Hz, 2 ms pulse width, pulse trains lasting 20 s) and nicotinic receptor stimulation (DMPP, 0.3 mM during 1 min) (open columns). Results are expressed as percentage of the noradrenaline (0.1 μ M)-induced contraction and shown as mean $\pm$ s.e.mean from at least 4 experiments. **P* < 0.05, significantly different from control, Student's *t* test for paired observations.

(20 units ml^{-1}) and L-arginine (50 μM), electrical impulses (4 and 16 Hz, 2 ms pulse width) and infusion of the nicotonic agonist, DMPP (0.3 mM for 1 min) induced the release of a transferable factor from the ileocolonic junction which relaxed the bioassay tissue (rabbit aortic rings) by $18 \pm 5\%$ ($n = 5$), $33 \pm 2\%$ ($n = 6$) and $25 \pm 3\%$ ($n = 4$), respectively, of the noradrenaline-induced contraction. Infusion with CTX (50 nM) did not affect the reactivity of the rabbit aortic rings to nitroglycerin (0.1 nmol bolus injection), but reduced the release of NO-R from the ileocolonic junction induced by electrical stimulation. CTX had no effect on the release evoked by DMPP (Figure 3). As with the results obtained in the organ bath experiments, the inhibitory effect of CTX was irreversible, even after a washout period of more than 60 min.

Effect of Ca^{2+} -free medium on the release of NO-R After incubation in, and during perfusion with, Ca^{2+} -free Krebs-Ringer solution, electrical (8 Hz, 2 ms pulse width) stimulation of the ileocolonic junction failed to induce the release of NO-R, whereas DMPP (0.3 mM during 1 min) still induced a relaxation varying from 0 to 20%, resulting in a mean of $8 \pm 5\%$ ($n = 4$). Normalisation of the Ca^{2+} concentration (2.5 mM) of the perfusate resulted in a significant increase of the release of NO-R in all experiments; electrical stimulation of the ileocolonic junction now induced a relaxation of the detector tissue of $20 \pm 3\%$ ($n = 9$), whereas DMPP evoked a relaxation of $22 \pm 5\%$ ($n = 4$) of the noradrenaline-induced

contraction (Figure 4). The sensitivity of the bioassay tissue to nitroglycerin (0.1 nmol bolus injected into the cascade) was not altered, since the concentration of Ca^{2+} in the Krebs-Ringer solution superfusing the detector tissue was kept constant (2.5 mM) via a CaCl_2 infusion.

Effect of verapamil and nifedipine on the release of NO-R After incubation in Krebs-Ringer solution containing solvent, verapamil (1–3 μM) or nifedipine (1 μM), the muscle strip of the ileocolonic junction was placed in the superfusion chamber and superfused with control solution or Krebs-Ringer containing verapamil or nifedipine respectively. Although verapamil and nifedipine were used in concentrations high enough to block acetylcholine-induced contractions in the canine ileocolonic junction (unpublished results), they did not reduce the release of NO-R evoked by electrical impulses (4 and 16 Hz, 2 ms pulse width, pulse trains lasting 20 s) and DMPP (0.3 mM during 1 min) nor did they affect the relaxation of the rabbit aortic rings to nitroglycerin (0.1 nmol bolus injection) (results not shown). Similar results were obtained when the release of NO-R was first determined under control conditions, and re-evaluated in the same muscle strip after infusion with verapamil or nifedipine.

Discussion

The role of Ca^{2+} in neurotransmitter release has been well established over many years and depends on Ca^{2+} entry into the presynaptic terminal (Augustine *et al.*, 1987; Miller, 1987). CTX, a potent inhibitor of the N-type voltage-sensitive Ca^{2+} channels of neurones (McCleskey *et al.*, 1987) has been shown to be a very useful tool in evaluating the role of Ca^{2+} entry during neurotransmission (De Luca *et al.*, 1990), especially because it has no effect on the Ca^{2+} channels in the smooth muscle (McCleskey *et al.*, 1987). In several tissues, this toxin inhibits neurotransmitter release from cholinergic and adrenergic, as well as NANC nerves (Maggi *et al.*, 1988; De Luca *et al.*, 1990). Also, in the present study, the release of NO or a NO related substance, recently proposed as the inhibitory NANC neurotransmitter in the gut (Boeckxstaens *et al.*, 1990a; Bult *et al.*, 1990), was inhibited by CTX, and in Ca^{2+} -free medium, indicating a crucial role for Ca^{2+} in nitrergic neurotransmission. Indirect evidence in favour of Ca^{2+} -dependency of NANC neurotransmission was also recently reported by Stark *et al.* (1991); solutions containing low Ca^{2+} and high Mg^{2+} blocked NANC nerve-induced inhibitory junction potentials.

Indeed, in a first series of experiments performed in organ baths, CTX raised the basal tension, and inhibited the nitrergic relaxations evoked by electrical stimulation of the NANC nerves. As in previous studies (Maggi *et al.*, 1988; De Luca *et al.*, 1990), this inhibition was irreversible and frequency-dependent; mainly the relaxations to lower frequency stimulation were reduced by CTX. As CTX did not inhibit the NO-induced relaxations, its effect was presynaptic and thus most likely resulted from reduction of the NO release. This was confirmed by actually measuring the NO or NO-R release in response to nerve stimulation via a bioassay method using rabbit aortic rings without endothelium as detector tissue. These results thus confirmed the involvement of presynaptic CTX-sensitive Ca^{2+} channels in the electrically-stimulated nitrergic neurotransmission. In contrast to the organ bath experiments, however, the release of NO-R in response to 16 Hz was significantly reduced. This can be explained by differences in experimental conditions: (1) the electrical current generated in the superfusion chamber equals 25 mA whereas that in the organ baths measures 100 mA; (2) the gas mixture aerating the Krebs-Ringer solution of the bioassay contains only 15% oxygen whereas oxygen represents 95% of the gas mixture used in the organ bath experiments; (3) the release of NO-R induced by 16 Hz (2 ms) was submaximal in the bioassay experiments, whereas

Figure 4 (a) Typical tracing of a rabbit aortic (RbAo) ring showing the relaxation to injection of nitroglycerin (GTN, 0.1 nmol bolus injection) and those in response to the release of NO-R evoked by electrical (8 Hz, 2 ms pulse width, pulse train lasting 20 s) and nicotinic receptor (1,1-dimethyl-4-phenylpiperazinium, DMPP, 0.3 mM during 1 min) stimulation of the canine ileocolonic junction (cICJ) in the absence and in the presence of Ca^{2+} (2.5 mM). The aortic ring was precontracted by noradrenaline (NA, 0.1 μM). Breaks in the tracing represent periods of equilibration. (b) Effect of Ca^{2+} -free medium (hatched columns) on the relaxations to GTN (0.1 nmol bolus injection) and on the release of NO-R by the canine ileocolonic junction (cICJ) in response to electrical impulses (8 Hz, 2 ms pulse width, pulse trains lasting 20 s) and DMPP (0.3 mM during 1 min) (open columns). Results are expressed as percentage of the noradrenaline (0.1 μM)-induced contraction and shown as mean $\pm$ s.e. mean from at least 4 experiments. * $P < 0.05$, significantly different from control, Student's *t* test for paired observations.

the relaxation to 16 Hz (2 ms) was maximal to supramaximal in the organ bath experiments. These points may all contribute to a more pronounced effect of CTX in the bioassay experiments. Furthermore, it should also be emphasized that the bioassay cascade in fact detects the amount of NO-R that has escaped from the neuromuscular space. Once the maximal functional response has been reached, increasing the intensity of nerve stimulation, although this might result in a higher release of NO-R (detected by the bioassay), will still induce the same maximal relaxation. Conversely, at maximal to supramaximal stimulation (16 Hz, 2 ms in the organ bath experiments), CTX might reduce the output of NO-R, as shown by the bioassay; however, this amount may still be high enough to induce a maximal functional effect, resulting in a discrepancy between bioassay and tension recording data.

As the L-type Ca²⁺ channel blockers verapamil and nifedipine had no effect, these Ca²⁺ channels can be identified as N-type channels. Further, Ca²⁺ dependency was demonstrated by the finding that electrically-evoked release of NO-R was abolished in Ca²⁺-free medium. The finding that CTX raised the basal tension of the ileocolonic junction might indicate a constant background release of NO, confirming our previous results (Boeckxstaens *et al.*, 1990a).

In contrast, drug-induced NANC neurotransmission was not affected by CTX; NANC relaxations in response to ATP, GABA or ACh, even of small amplitude, were not inhibited by CTX. Similarly, CTX did not reduce the DMPP-induced release of NO-R measured by bioassay. Experiments in Ca²⁺-free medium however showed that the NO-R release in response to DMPP was significantly reduced. These results indicate Ca²⁺ dependency of the NO-R release and suggest that nicotinic receptor activation triggers the influx of Ca²⁺ into the NANC nerve terminals through CTX-insensitive Ca²⁺ channels. As verapamil and nifedipine were also without effect, these channels differ from the N- or L-type channels; their exact nature still remains to be determined. It thus appears that chemical stimulation of NANC nerves is coupled to activation of Ca²⁺ channels different from those activated by electrical impulses. This would imply that different stimuli can open different Ca²⁺ channels. Alternatively, different populations of NANC neurones might be activated by electrical stimuli or receptor stimulation.

Interestingly, in two of four experiments, DMPP, in contrast to electrical impulses, still induced the release of relatively large amounts of NO-R in Ca²⁺-free medium. This might suggest mobilisation of intracellular Ca²⁺ to activate NO synthase. Alternatively, Ca²⁺-independent NO-R synthesis might be induced by DMPP. Recently, Kostka *et al.* (1993) reported Ca²⁺-independent NO synthase activity in myenteric synaptosomes of the canine ileum compatible with

the latter possibility. Under what circumstances and to what extent these mechanisms are involved in the NO-R release from NANC nerves by DMPP cannot be determined from our experiments.

At present, two distinct types of NO synthase have been identified: a constitutive Ca²⁺-calmodulin-dependent enzyme and an inducible Ca²⁺-independent enzyme (for review, see Moncada *et al.*, 1991). As the enzyme present in the neurones and nerve fibres of the myenteric plexus is the constitutive isoform (Bredt *et al.*, 1990), Ca²⁺ influx into varicosities during activation thus could increase NO synthesis. Whether the Ca²⁺-dependency of the NO release from NANC nerves results from its synthesis or its release cannot be determined from our experiments. However, based on the chemistry of NO, which implies that it is rather unlikely that NO is stored in vesicles, and the features of the neuronal NO synthase, it appears that Ca²⁺ mainly plays a role in the synthesis of the neurotransmitter.

A final and important point of discussion concerns the origin of the NO-R release. In previous studies (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1991a,c), we showed that blockade of the neuronal conductance inhibited the NO-R release. In the present study, CTX, known to inhibit neurotransmitter release via a presynaptic action (McCleskey *et al.*, 1987), also reduced the NO-R release. Based on these findings, together with the knowledge that NO synthase has been demonstrated in the nerve endings innervating gut smooth muscle, but not in the smooth muscle itself (Bredt *et al.*, 1990), we believe that the NO-R released is mainly of neuronal origin. Recently, it has been suggested that NO is released from the muscle cells by vasoactive intestinal polypeptide (VIP) in the guinea-pig gastric fundus (Grider *et al.*, 1992). However, VIP does not induce relaxations in the canine ileocolonic junction (Boeckxstaens *et al.*, 1990b), excluding this possibility. Furthermore, using a polyclonal antibody against NO synthase, morphological evidence is available demonstrating the absence of NO synthase immunoreactivity in the smooth muscle cells of the canine ileocolonic junction (Bogers *et al.*, 1993).

In conclusion, the present study demonstrates the Ca²⁺-dependency of the release of NO-R in response to NANC nerve stimulation. Electrically-evoked NO-R release results from Ca²⁺ entry through N-type CTX-sensitive Ca²⁺ channels, whereas that induced by chemical stimulation triggers the influx of Ca²⁺ via CTX-insensitive Ca²⁺ channels, different from the N- or L-type.

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