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Comparison of the pharmacological profile of S-nitrosothiols, nitric oxide and the nitrergic neurotransmitter in the canine ileocolonic junction

Joris G. De Man, Guy E. Boeckxstaens, Benedicte Y. De Winter, Tom G. Moreels, Menno E. Misset, Arnold G. Herman & [1]Paul A. Pelckmans

Divisions of Gastroenterology and Pharmacology, Faculty of Medicine, University of Antwerp (UIA), B-2610 Antwerpen-Wilrijk, Belgium

1 In organ bath experiments, hydroquinone (30-100 μM) and hydroxocobalamin (30-100 μM) concentration-dependently inhibited the relaxations induced by NO (0.3-30 μM) but not those by nitroglycerin (GTN, 1 μM) in the canine ileocolonic junction (ICJ). Hydroxocobalamin reduced the relaxation to low frequency (2 Hz) stimulation of the non-adrenergic, non-cholinergic (NANC) nerves, whereas hydroquinone only reduced the NANC nerve-mediated relaxations to electrical stimulation at 16 Hz, 0.5 ms.

2 Relaxations to S-nitroso-L-cysteine (CysNO, 1-30 μM), or S-nitroso-N-acetyl-D,L-penicillamine (SNAP, 1-30 μM) were not inhibited by hydroquinone (30-100 μM), hydroxocobalamin (30-100 μM), pyrogallol (30-100 μM) or L-cysteine (1-3 μM). Hydroquinone (100 μM) only reduced the relaxation to 10 μM CysNO. Hydroxocobalamin, but not hydroquinone, pyrogallol or L-cysteine, potentiated the relaxations to the lowest concentration (1 μM) of S-nitrosoglutathione (GSNO, 1-30 μM).

3 In the superfusion bioassay, hydroquinone (100 μM) and hydroxocobalamin (1 μM) concentration-dependently inhibited the biological activity of authentic NO (1-4 pmol) to the same extent as that of the transferable nitrergic factor, released from the canine ICJ in response to NANC nerve stimulation (8-16 Hz, 2 ms). Responses to GTN (10 pmol) or adenosine 5'-triphosphate (10 nmol) were not affected.

4 In conclusion, the nitrosothiols CysNO, SNAP and GSNO relax the canine ileocolonic junction, but these relaxations, pharmacologically, behave differently from the NANC nerve-mediated relaxations. From the bioassay experiments, we conclude that the nitrergic factor, released in response to NANC nerve stimulation of the canine ICJ, behaves pharmacologically like NO but not like a nitrosothiol. Therefore, we suggest NO, and not CysNO, SNAP or GSNO as the inhibitory NANC neurotransmitter in the canine ICJ.

Keywords: Bioassay; canine ileocolonic junction; nitrergic neurotransmission; nitric oxide; S-nitrosothiols; non adrenergic non cholinergic transmission

Introduction

Since nitric oxide (NO) was proposed as a non-adrenergic non-cholinergic (NANC) neurotransmitter in the alimentary tract (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1990), the so-called nitrergic transmission has been demonstrated throughout the whole gastrointestinal tract (for reviews see: Sanders & Ward, 1992; Stark & Szurszewski, 1992). At present the role of NO in NANC neurotransmission has been well-established, but the exact chemical identity of the nitrergic neurotransmitter remains controversial. It was reported that drugs like hydroquinone, pyrogallol and LY 83583, which act as free radical scavengers or superoxide anion generators, had a differential effect on relaxations induced by authentic NO compared to those induced by NANC nerve stimulation in the bovine retractor penis muscle (Gillespie & Sheng, 1990), the rat gastric fundus and mouse anococcygeus (Barbier & Lefebvre, 1994; Hobbs *et al.*, 1991). Furthermore, in the rat anococcygeus muscle, hydroxocobalamin, which is thought to act as a NO scavenger, clearly inhibited relaxations to NO but only those to low frequency stimulation of the NANC nerves (Rajanayagam *et al.*, 1993). It was therefore hypothesized that the inhibitory nitrergic NANC neurotransmitter is not free NO but rather a superoxide-resistant, NO-releasing molecule, such as a S-nitrosothiol. S-nitrosothiols have indeed been shown to relax vascular (Ignarro *et al.*, 1981; Myers *et al.*, 1990) and non-vascular, including gastrointestinal, smooth muscle (Gibson *et al.*, 1992; Kerr *et al.*, 1992; Knudsen *et al.*, 1992; Rand & Li, 1993; Liu *et al.*, 1994; Barbier & Lefebvre, 1994) and to mimic NANC hyperpolarizations in canine colon (Thornbury *et al.*, 1991) and rat gastric fundus (Kitamura *et al.*, 1993).

In order to investigate whether the inhibitory NANC neurotransmitter is NO or an S-nitrosothiol, we recently studied the effect of L-cysteine, shown to discriminate between NO and S-nitrosothiols (Feelisch *et al.*, 1994), and the effect of pyrogallol on the nitrergic neurotransmitter in the canine ileocolonic junction (ICJ), both in organ baths and in a bioassay set-up (Boeckxstaens *et al.*, 1994). In organ baths, pyrogallol and L-cysteine inhibited the NO-induced but not the NANC nerve-induced relaxations. It was hypothesized that this differential effect was due to their pharmacological inactivity at the neuromuscular junction, since in bioassay superfusion experiments, both agents inhibited the biological activity of the nitrergic neurotransmitter released in response to NANC nerve stimulation to the same extent as the biological activity of authentic NO (Boeckxstaens *et al.*, 1994). In contrast, responses to S-nitrosothiols were not affected, indicating that the inhibitory nitrergic NANC neurotransmitter is free NO rather than a S-nitrosothiol.

To investigate further whether the nitrergic neurotransmit-

[1] Author for correspondence.

ter is NO or a S-nitrosothiol, we studied the effect of the nitrosothiols S-nitroso-L-cysteine (CysNO), S-nitroso-glutathione (GSNO) and S-nitroso-N-acetyl-D,L-penicillamine (SNAP) in the canine ICJ. In addition, we compared the effect of hydroquinone, hydroxocobalamin, pyrogallol and L-cysteine on the relaxations to S-nitrosothiols, authentic NO and the endogenous nitrergic NANC neurotransmitter. Finally, using a superfusion bioassay, we investigated the effect of hydroquinone and hydroxocobalamin on the biological activity of authentic NO and the transferable nitrergic factor.

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 ICJ. After the resected specimen was cleaned and rinsed, the mucosa was removed from the ileum and ICJ by means of sharp dissection. Circular muscle strips of the ICJ 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.

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 0.1 μM substance P-contracted muscle strips and in the presence of 0.3 μM atropine. After each contraction, the muscle strips were washed four times with an interval of at least 5 min.

The effects of hydroquinone (30–100 μM) and hydroxocobalamin (30–100 μM) were investigated on the frequency-response curve to electrical stimulation (2–16 Hz, 0.5–2 ms), on the relaxations to nitroglycerin (GTN, 1 μM) and on the concentration-response curves to NO (0.3–30 μM), CysNO (1–30 μM), GSNO (1–30 μM) and SNAP (1–30 μM). Possible changes with time were evaluated in parallel muscle strips serving as time control. Electrical pulses (rectangular waves, 100 mA, 9 V) were delivered by a Grass stimulator and a direct current amplifier in trains of stimuli of 10 s.

Superfusion bioassay cascade

Preparation of the donor tissue After resection, cleaning and rinsing of the ICJ, 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.*, 1991). The tissue was perfused (3 ml min^{-1}) with a modified Krebs-Ringer solution maintained at 37°C. This solution contained L-arginine (50 μM), guanethidine (3 μM) and superoxide dismutase (20 u ml^{-1}) and was aerated with a mixture of 80% N_2, 15% O_2 and 5% CO_2. As hydroquinone acts as a superoxide- and/or radical scavenger, the experiments with hydroquinone were performed in the absence of exogenous superoxide dismutase. 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 in trains of stimuli of 20 s and with 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, denuded of their endothelium by gentle rubbing and arranged in parallel under 8 g resting tension for isometric tension recording. The aortic rings were contracted by an infusion of noradrenaline (0.1 μM) and then superfused with the effluent of the superfusion tube that contained the muscle strip of the canine ileocolonic junction. A bolus of acetylcholine (3 nmol) was injected directly onto the aortic rings to verify the absence of endothelium. 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 (GTN, 10 pmol).

Experimental protocols The effects of hydroquinone (100 μM) and hydroxocobalamin (1 μM) were investigated on the relaxations of the rabbit aortic ring in response to the nitrergic factor released in response to electrical stimulation (8–16 Hz, 2 ms) of the NANC nerves of the canine ICJ and on relaxations induced by bolus injections of authentic NO (1–4 pmol), GTN (10 pmol) and ATP (10 nmol).

Drugs

The following drugs were used: adenosine 5'-triphosphate, glutathione, hydroquinone, hydroxocobalamin, L-arginine, L-cysteine, N-acetyl-D,L-penicillamine, pyrogallol, substance P (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). Solutions of NO were prepared as described previously (Kelm *et al.*, 1988). Solutions of CysNO, GSNO and SNAP were prepared as described by Barbier & Lefebvre (1994).

Presentation of results and statistical analysis

Results are expressed as percentage decrease of the substance P- or noradrenaline-induced contraction and shown as mean ± s.e.mean for the number of dogs indicated. Differences were considered statistically significant for $P<0.05$, using Student's t test for paired observations.

Results

Organ bath experiments

Responses to NANC nerve stimulation Electrical stimulation of the canine ICJ (2–16 Hz, 0.5–2 ms) induced frequency-dependent NANC relaxations, previously shown to be mediated by NO or a NO-releasing substance (Boeckxstaens *et al.*, 1990). Hydroquinone (30–100 μM) only reduced the NANC nerve-induced relaxations to electrical stimulation at 16 Hz, 0.5 ms (Figure 1). Hydroxocobalamin (30–100 μM) did not affect the nitrergic NANC relaxations, except those to low frequency stimulation (2 Hz, 0.5 ms) which were inhibited by 100 μM hydroxocobalamin (Figure 1). None of these drugs affected the basal tension or the substance P-induced contraction of the canine ICJ.

Concentration-response curves to NO NO (0.3–30 μM) concentration-dependently relaxed the muscle strips of the canine ICJ. Hydroquinone (30–100 μM) and hydroxocobal-

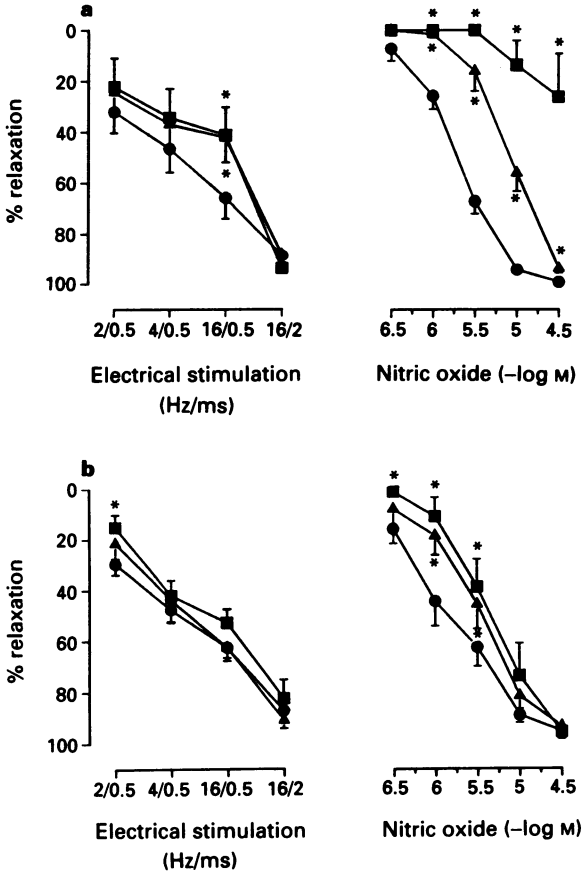

Figure 1 Effect of (a) hydroquinone (▲, 30 μM; ■, 100 μM) and (b) hydroxocobalamin (▲, 30 μM; ■, 100 μM) on the NANC relaxations induced by electrical stimulation (●, 2–16 Hz, 0.5–2 ms) and on the concentration-response curve to NO (●, 0.3–30 μM) in the canine ileocolonic junction. Results are expressed as percentage decrease of the substance P (0.1 μM)-induced contraction and shown as mean ± s.e.mean for $n = 7-8$ experiments. $^*P < 0.05$ is considered as significantly different from control; Student's t test for paired observations.

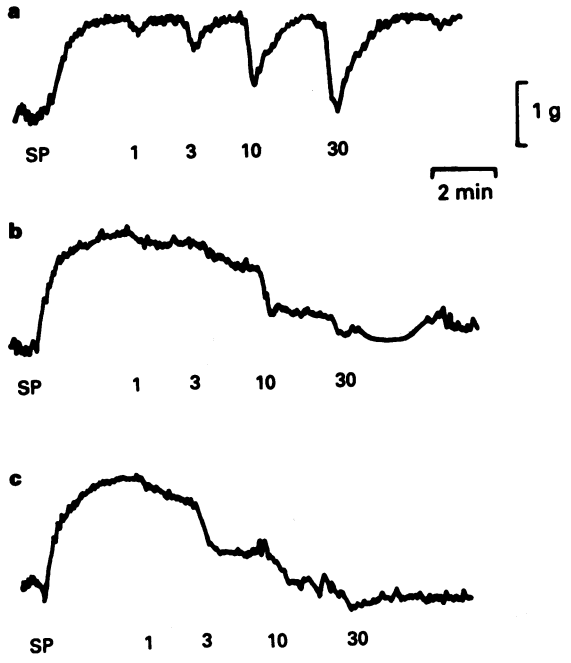

Figure 2 Typical tracings showing the concentration-dependent effect of (a) S-nitroso-L-cysteine (1–30 μM), (b) S-nitrosoglutathione (1–30 μM) and (c) S-nitroso-N-acetyl-D,L-penicillamine (1–30 μM) on the canine ICJ during a substance P (SP, 0.1 μM)-induced contraction.

amin (30–100 μM) significantly shifted the concentration-response curve to NO to the right (Figure 1). In addition, hydroquinone (100 μM) significantly reduced the maximal relaxation to NO from 100% to 26 ± 17% ($n = 6$) of the substance P-induced contraction. In contrast, hydroxocobalamin had no effect on the maximal response to NO.

Concentration-response curves to S-nitrosothiols CysNO, GSNO and SNAP (all 1–30 μM) relaxed the muscle strips of the canine ICJ. Relaxations to CysNO were transient and resembled those to NO and NANC nerve stimulation (Figure 2), but they were not affected by hydroquinone (30–100 μM), hydroxocobalamin (30–100 μM), pyrogallol (30–100 μM) or L-cysteine (1–3 μM) (Figure 3). However, the highest concentration of hydroquinone (100 μM) inhibited the relaxation to 10 μM CysNO. In contrast to NO and CysNO, relaxations to GSNO were sustained (Figure 2). They were not inhibited by pyrogallol (30–100 μM), hydroquinone (30–100 μM) or L-cysteine (1–3 μM). Hydroxocobalamin (30–100 μM) on the other hand potentiated the relaxations to the lower concentrations of GSNO (Figure 4). Relaxations to SNAP were also sustained (Figure 2) and were not significantly affected by hydroquinone (30–100 μM), hydroxocobalamin (30–100 μM), pyrogallol (30–100 μM) or L-cysteine (1–3 μM) (Figure 5).

Bioassay experiments

Responses to NANC nerve stimulation Electrical stimulation (8–16 Hz, 2 ms) of the canine ICJ induced the release of a

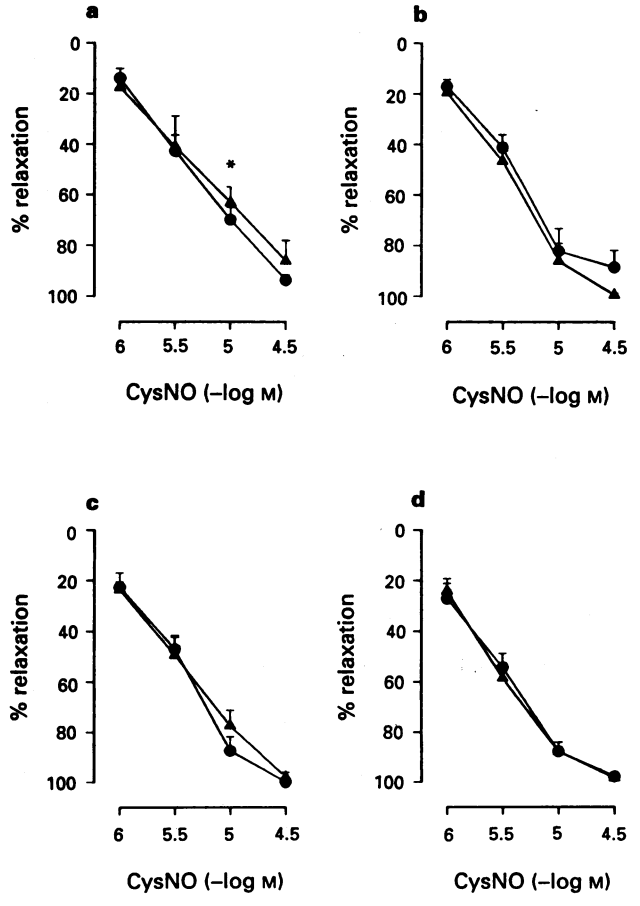

Figure 3 Effect of (a) hydroquinone (▲, 100 μM), (b) hydroxocobalamin (▲, 100 μM), (c) pyrogallol (▲, 100 μM) and (d) L-cysteine (▲, 3 μM) on the concentration-response curve to S-nitroso-L-cysteine (●, CysNO, 1–30 μM) in the canine ileocolonic junction. Results are expressed as percentage decrease of the substance P (0.1 μM)-induced contraction and shown as mean ± s.e.mean for $n = 5$ experiments. $^*P < 0.05$ is considered as significantly different from control; Student's t test for paired observations.

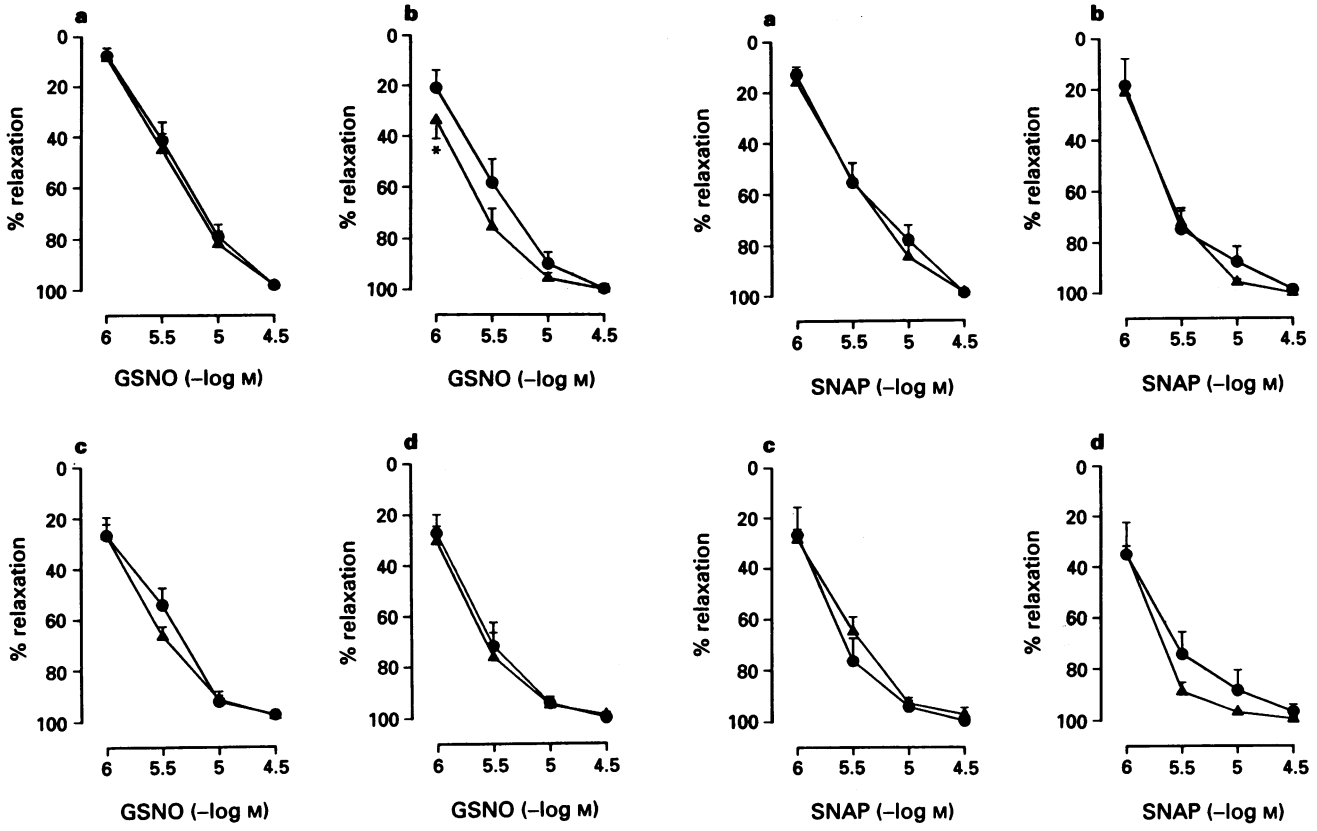

Figure 4 Effect of (a) hydroquinone (▲, 100 μM), (b) hydroxocobalamin (▲, 100 μM), (c) pyrogallol (▲, 100 μM) and (d) L-cysteine (▲, 3 μM) on the concentration-response curve to S-nitrosogluthathione (●, GSNO, 1–30 μM) in the canine ileocolonic junction. Results are expressed as percentage decrease of the substance P (0.1 μM)-induced contraction and shown as mean ± s.e.mean for $n = 5$ experiments. $*P < 0.05$ is considered as significantly different from control; Student's t test for paired observations.

Figure 5 Effect of (a) hydroquinone (▲, 100 μM), (b) hydroxocobalamin (▲, 100 μM), (c) pyrogallol (▲, 100 μM) and (d) L-cysteine (▲, 3 μM) on the concentration-response curve to S-nitroso-D,L-penicillamine (●, SNAP, 1–30 μM) in the canine ileocolonic junction. Results are expressed as percentage decrease of the substance P (0.1 μM)-induced contraction and shown as mean ± s.e.mean for $n = 5$ experiments. $*P < 0.05$ is considered as significantly different from control; Student's t test for paired observations.

vasorelaxant factor, previously characterized as NO or a NO-related substance (Boeckxstaens *et al.*, 1991). The biological activity of the transferable nitrergic vasorelaxant factor was significantly and concentration-dependently inhibited by hydroquinone (10–100 μM) or hydroxocobalamin (0.1–1 μM) (Figure 6). Hydroquinone or hydroxocobalamin did not affect the noradrenaline-induced contraction of the rabbit aorta or the relaxation to nitroglycerin (10 pmol) (Figure 6) and ATP (10 nmol) (results not shown).

Concentration-response curves to NO NO (1–4 pmol), injected into the effluent of the superfusion tube, induced concentration-dependent relaxations of the rabbit aorta which were significantly inhibited by hydroquinone (100 μM) and hydroxocobalamin (1 μM) (Figure 7). In Figure 7, the data obtained with the transferable factor under control conditions were plotted on the corresponding concentration-response curve to NO according to their value on the Y-axis. This figure shows that hydroquinone and hydroxocobalamin inhibit the relaxations to the nitrergic factor to the same extent as those to NO.

Discussion

Since substances like pyrogallol, hydroquinone, LY 83583 and hydroxocobalamin had different effects on NO as compared to NANC nerve-induced responses, it has been suggested that a NO releasing substance, such as a nitrosothiol, acts as NANC neurotransmitter rather than authentic NO.

To date, a number of S-nitrosothiols have been investigated (Thornbury *et al.*, 1991; Gibson *et al.*, 1992; Kerr *et al.*, 1992; Knudsen *et al.*, 1992; Kitamura *et al.*, 1993; Rand & Li, 1993; Barbier & Lefebvre, 1994; Liu *et al.*, 1994) as possible nitrergic NANC neurotransmitters. In the present study CysNO, GSNO and SNAP all concentration-dependently relaxed the canine ICJ. However, only the relaxations to CysNO were transient and resembled those to NANC nerve stimulation while the relaxations to GSNO and SNAP were sustained. None of the nitrosothiols had a pharmacological profile similar to that of nerve stimulation: hydroxocobalamin reduced the electrically-induced relaxations with lower amplitude, whereas it failed to reduce the relaxations induced by the nitrosothiols under study. Hydroxocobalamin even enhanced the relaxations to the lowest concentration of GSNO. Furthermore, hydroquinone failed to reduce the relaxations to SNAP and GSNO, whereas it reduced those to nerve stimulation at 16 Hz, 0.5 ms. Hydroquinone also inhibited the relaxation to 10 μM CysNO, however not to the same extent as the nerve-mediated relaxation at 16 Hz, 0.5 ms. Like the nerve-mediated relaxations (Boeckxstaens *et al.*, 1994), those induced by CysNO, SNAP or GSNO were not affected by L-cysteine and pyrogallol. However, based on the finding that hydroxocobalamin and hydroquinone affect the nerve-mediated responses differently from the responses to CysNO, SNAP and GSNO, these nitrosothiols can be excluded as possible neurotransmitters in the canine ileocolonic junction. Furthermore, S-nitrosothiols, which are

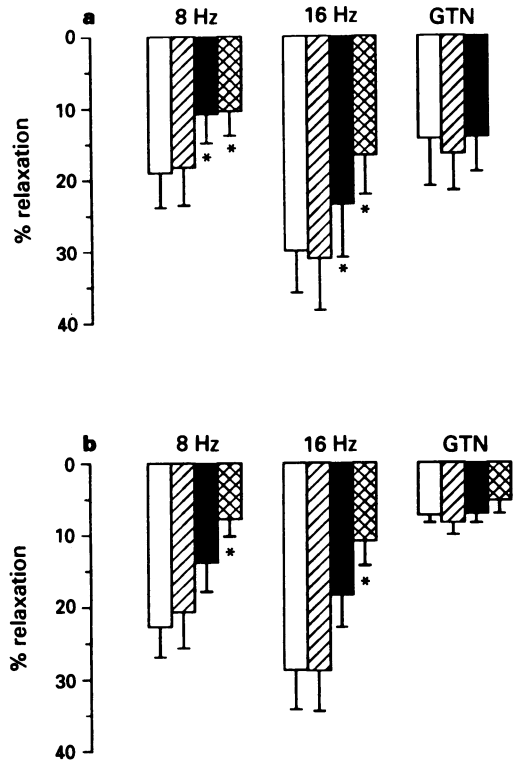

Figure 6 Concentration-dependent effect of (a) hydroquinone (hatched columns, 10 μM; solid columns, 30 μM; cross hatched columns, 100 μM) and (b) hydroxocobalamin (hatched columns 0.3 μM; solid columns, 1 μM; cross hatched columns, 3 μM) on the biological activity of the vasorelaxant nitrergic factor, released in response to NANC nerve stimulation (8–16 Hz, 2 ms) of the canine ICJ, and on the activity of nitroglycerin (GTN, 10 pmol). Results are expressed as percentage decrease of the noradrenaline (0.1 μM)-induced contraction and shown as mean ± s.e.mean for $n = 4-7$ experiments. $^*P < 0.05$ is considered as significantly different from control; Student's t test for paired observations.

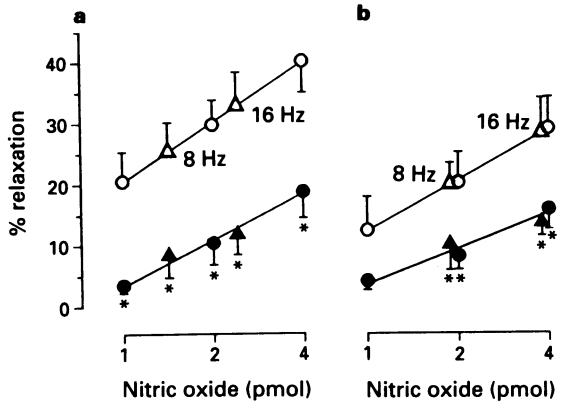

Figure 7 Effect of (a) hydroquinone (100 μM, filled symbols) and (b) hydroxocobalamin (1 μM, filled symbols) on the biological activity of NO (○, 1–4 pmol), injected in the effluent of the superfusion tube and on the activity of the vasorelaxant nitrergic factor, released in response to NANC nerve stimulation (△, 8–16 Hz, 2 ms) of the canine ICJ. Results are expressed as percentage decrease of the noradrenaline (0.1 μM)-induced contraction and shown as mean ± s.e.mean for $n = 6$ experiments. $^*P < 0.05$ is considered as significantly different from control; Student's t test for paired observations.

generally polarized substances (Kowaluk & Fung, 1990), are unlikely to permeate cell membranes whereas free NO is a highly permeable gas which can diffuse rapidly across cell membranes.

Although in the organ baths the pharmacological profile of exogenous NO is not identical to that of the endogenous nitrergic neurotransmitter, we do believe that the neurotransmitter released by the inhibitory NANC nerves is free NO. As reported earlier (Boeckxstaens *et al.*, 1994), the differential effect of pyrogallol and L-cysteine on NO- and NANC nerve-induced relaxations studied in organ baths, can be explained by inactivity of these substances at the site of the neuromuscular junction. Although these superoxide anion generators had no effect on NANC nerve-mediated relaxations in organ baths, in the bioassay set-up they inhibited the biological activity of NO and the nitrergic transferable factor, released in response to NANC nerve stimulation, with equal potency. Also in the present study, the data obtained from the organ bath experiments revealed a differential effect of hydroquinone and hydroxocobalamin on the responses to NO and to NANC nerve stimulation. However, in the bioassay experiments, the biological activity of the transferable factor and NO were affected to the same extent, suggesting that NO is released in response to NANC nerve stimulation. However, it has to be considered that the NANC nerves might actually release a nitrosothiol, from which NO is liberated either spontaneously or after contact with the effector cell (Kowaluk & Fung, 1990; Mathews & Kerr, 1993) and subsequently detected by the bioassay tissue. However, the biological activity of CysNO, SNAP or GSNO, even after they superfused the ileocolonic junction, was not affected by pyrogallol or L-cysteine (Boeckxstaens *et al.*, 1994) providing evidence against this possibility. Furthermore, it has to be emphasized that in the organ bath, exogenous NO is directly injected into the bathing fluid that contains the inhibitor under study, whereas this inhibitor has to move into the neuromuscular junction to block the biological activity of the endogenous neurotransmitter. This difference is not present in the bioassay, resulting in an identical pharmacological profile for NO and the nitrergic neurotransmitter, illustrating that the differential effect of pyrogallol, L-cysteine, hydroquinone and hydroxocobalamin in the organ baths results from their reduced activity at the neuromuscular junction. Such a reduced activity may be due to high tissue activity of superoxide dismutase, which was recently shown to protect effectively the nitrergic neurotransmitter in the rat anococcygeus against the action of pyrogallol (Liu & Szurszewski, 1994).

In conclusion, we have illustrated that the pharmacological profile of CysNO, SNAP and GSNO differs from that of the nitrergic neurotransmitter in the canine ileocolonic junction. Although NO and the nitrergic neurotransmitter were differently affected by hydroquinone and hydroxocobalamin in the organ bath experiments, they had an identical pharmacological profile in the superfusion bioassay. From these results, we suggest that the differential effect in the organ baths results from reduced activity of these inhibitors at the neuromuscular junction and conclude that the nitrergic NANC neurotransmitter of the canine ICJ is NO, and not CysNO, SNAP or GSNO.

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