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Evidence that NO acts as a redundant NANC inhibitory neurotransmitter in the guinea-pig isolated taenia coli

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1 The relative contribution of the putative transmitters, nitric oxide (NO) and an apamin-sensitive factor, possibly ATP, to inhibitory responses evoked by electrical field stimulation (EFS; 0.2–5 Hz, 0.2 ms duration, supra-maximal voltage for 10 s) of non-adrenergic, non-cholinergic (NANC) nerves was investigated in the guinea-pig isolated taenia coli contracted with histamine (1 μM).

2 Peak relaxations to EFS (0.2–5 Hz) were tetrodotoxin (1 μM)-sensitive, maximal at 0.2 Hz and completely resistant to the nitric oxide synthase inhibitor, N^G-nitro-L-arginine (L-NOARG; 100 μM) in either the presence or absence of atropine (1 μM). Furthermore, the specific inhibitor of soluble guanylyl cyclase, 1H-[1,2,4] oxadiazolo [4,3-a] quinoxaline-1-one (ODQ; 10 μM), the cytochrome P_{450} inhibitor and free radical generator, 7-ethoxyresorufin (7-ER; 10 μM) and the NO scavenger, oxyhaemoglobin (HbO; 30 μM) had no effect on EFS-induced relaxations alone and in combination with L-NOARG (100 μM).

3 Maximum relaxation to the NO donor, sodium nitroprusside (SNP; 1 μM) was significantly reduced by HbO (30 μM), abolished by 7-ER (10 μM) and ODQ (10 μM) but was unaffected by apamin (0.1 μM), an inhibitor of small conductance Ca^{2+}-activated K^+ channels.

4 The relaxation to EFS at 0.2 Hz was resistant to apamin but those to 0.5 and 5 Hz were significantly reduced. EFS (0.2–5 Hz)-evoked relaxations that persisted in the presence of apamin were further significantly inhibited by L-NOARG (100 μM) or ODQ (10 μM), but not by HbO (30 μM) or 7-ER (10 μM).

5 ATP (1–30 μM) produced concentration-dependent relaxations that were abolished by apamin (0.1 μM), unaffected by ODQ (10 μM) but only significantly reduced by L-NOARG (100 μM) at the lowest concentration of ATP (1 μM) used.

6 Nifedipine (0.3 μM), abolished contractions to 67 mM KCl, histamine (10 μM), endothelin-1 (0.03 μM), 5-hydroxytryptamine (5-HT; 10 μM) and the thromboxane-mimetic, 9-11-dideoxy-9α, 11α-methano-epoxy-prostaglandin $F_{2\alpha}$ (U46619; 0.1 μM).

7 The findings of the present study suggest that NO is released during NANC nerve stimulation, but plays no role in NANC relaxations in the guinea-pig taenia coli unless the effects of another apamin-sensitive, nerve-derived hyperpolarizing factor (NDHF) are blocked. Thus, we propose that in this tissue, NO acts as a 'backup' or redundant NANC nerve inhibitory transmitter and like NDHF mediates relaxation via hyperpolarization.

Keywords: NO; ATP; NANC; K^+ channels; cyclic GMP; backup; guinea-pig taenia coli

Introduction

Evidence from functional and morphological studies support the involvement of multiple transmitters in non-adrenergic non-cholinergic (NANC) inhibitory neurotransmission of gastrointestinal tissue. Although the exact mechanism of NANC-mediated relaxation of gut tissue is controversial, three putative mechanisms have been described; purinergic (Burnstock, 1972; 1990), nitrergic (Sanders & Ward, 1992; Rand, 1992; Rand & Li, 1995) and peptidergic (Hills *et al*., 1983; Grider *et al*., 1985; Furness *et al*., 1992). Purinergic nerves are believed to release adenosine 5′-triphosphate (ATP) or a related purine which activates apamin-sensitive K^+ channels to evoke inhibitory junctional potentials (Bennett *et al*., 1966; Burnstock *et al*., 1970; Costa *et al*., 1986; Burnstock, 1990; Bridgewater *et al*., 1995) and smooth muscle relaxation. Nitrergic nerves differ from conventional nerves in that they contain an enzyme, neuronal nitric oxide (NO) synthase (Bredt & Snyder, 1990; Bredt *et al*., 1991) which, when activated by an increase in Ca^{2+}, causes release of NO which then mediates relaxation of the underlying smooth muscle primarily by increasing guanosine 3′ : 5′-cyclic monophosphic (GMP). Finally, neuropeptides such as vasoactive intestinal peptide (VIP) (Furness *et al*., 1992), pituitary adenylyl cyclase-activating peptide (PACAP) (Sundler *et al*., 1992; McConalogue *et al*., 1995) and helospectin (Absood *et al*., 1992; Desai *et al*., 1992; Ny *et al*., 1994) have been localized in enteric nerves throughout the gut, and PACAP in particular, is released upon NANC nerve stimulation and mediates relaxation via apamin-sensitive hyperpolarizing mechanisms (Schworer *et al*., 1992; Jin *et al*., 1994; McConalogue *et al*., 1995). Whilst it is not clear whether these putative transmitters exist in separate or similar populations of nerves (Furness *et al*., 1992; Bridgewater *et al*., 1995; McConalogue *et al*., 1995) their differential contributions to NANC nerve-mediated smooth muscle relaxation are unknown.

We have recently questioned a direct role for NO in NANC neurotransmission in the rat anococcygeus (Selemidis & Cocks, 1997), a gut-associated smooth muscle generally regarded to be innervated only by nitrergic NANC nerves (Gillespie *et al*., 1989; Li & Rand, 1989; Hobbs & Gibson, 1990; Liu *et al*., 1991; Rand, 1992; Rand & Li, 1995). In that study, we provided indirect evidence that a nerve-derived hyperpolarizing factor, which we termed NDHF, accounted for all the response when the tissue was sub-maximally contracted by a depolarizing stimulus. NO, concomitantly released with NDHF, contributed to the response only if the effects of NDHF were blocked with nifedipine (Selemidis & Cocks, 1997). Thus, NO appeared to function as a 'backup' factor for NDHF. A similar but reverse-order backup mechanism has been demonstrated in blood vessels such as rabbit carotid artery (Cowan *et al*., 1993) and pig (Kilpatrick & Cocks, 1994)

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and cow coronary arteries (Drummond & Cocks, 1996) where the release of both NO and a non-NO hyperpolarizing factor (EDHF) (Felatou & Vanhoutte, 1988; Garland *et al*., 1995) from the endothelium mediate relaxation of the underlying smooth muscle. However, in these blood vessels, NO accounted for most of the relaxation to endothelium-dependent relaxing agents with EDHF able to compensate almost fully for NO when its synthesis was inhibited.

Although the guinea-pig taenia coli is innervated by NOS-containing enteric nerves (Furness *et al*., 1992), NANC nerve-mediated relaxations have been shown to be resistant to NOS inhibitors (Rand & Li, 1990; Knudsen & Tottrup, 1992; Piotrowski *et al*., 1993; Grider *et al*., 1994). In this tissue, the putative non-NO transmitters, ATP and PACAP, both evoke apamin-sensitive hyperpolarization and relaxation. However, apamin does not abolish similar responses to NANC nerve-stimulation. Therefore, the aim of the present study was to determine if, as in the rat anococcygeus (Selemidis & Cocks, 1997), NO is essentially redundant and acts mainly as a 'backup' relaxation mechanism for an apamin-sensitive, hyperpolarizing factor (NDHF) in the guinea-pig taenia coli.

Methods

Guinea-pigs (250–450 g; tri-coloured/albino; either sex) were killed by CO_2 asphyxiation. Strips of taenia coli, 10 mm long, including the same width of underlying circular smooth muscle but free of any mucosa were suspended via two silk ties vertically between a force-displacement transducer (Grass, FTO3, Quincy, MA, U.S.A.) and a micrometer-driven glass support in water-jacketed (37°C), 25 ml organ baths containing carbogenated (95% O_2; 5% CO_2) Krebs solution of the following composition (in mM: Na^+ 143.1, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 127.8, HCO_3^- 25.0, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.0, guanethidine 30 μM, atropine 1 μM (unless otherwise specified) and propranolol 1 μM). Tissues were left at zero passive tension for 60 min before being stretched to a baseline level of 1 g passive force. Changes in isometric force were amplified (Model 108; BMRI; Australia) and registered on single channel flat bed chart recorders (Rikadenki, model R-01, Japan). After a further equilibration period of 30 min, preparations were contracted maximally (F_{max}) with histamine (10 μM). After repeated washes to restore force to baseline levels, all tissues were subsequently contracted with histamine (1 μM) to $\sim$50% (F_{max}). Once a stable level of active force was achieved relaxations were then obtained to either electrical field stimulation (EFS; 0.2, 0.5 or 5 Hz, 0.2 ms duration, supramaximal voltage for 10 s) of NANC inhibitory nerves delivered in square wave pulses (S44 stimulator, Grass Instruments, Quincy, MA, U.S.A.) via platinum ring electrodes, ATP (1, 10 or 30 μM) or sodium nitroprusside (SNP, 1 μM). Only one relaxation response followed by a 30 min washout period to either EFS, a single concentration of ATP or SNP was obtained for each histamine (1 μM) contraction. Preparations received only one experimental protocol, either EFS, ATP or SNP.

Effect of NO inhibitors and apamin on EFS-induced relaxation

After control relaxations to EFS (0.2, 0.5 or 5 Hz) were obtained in the absence of drug treatments, the same tissues were either not treated or treated with N^G-nitro-L-arginine (L-NOARG, 100 μM), apamin (0.1 μM), 1H-[1,2,4] oxadiazolo [4,3-a] quinoxaline-1-one (ODQ, 10 μM), oxyhaemoglobin (HbO, 30 μM) or 7-ethoxyresorufin (7-ER, 10 μM). Some tissues treated with L-NOARG were also exposed to either apamin (0.1 μM), HbO (30 μM), ODQ (10 μM) or 7-ER (10 μM). Other combination treatments included apamin (0.1 μM) with either ODQ (10 μM), HbO (30 μM), 7-ER (10 μM), $NaHCO_3$ (1 mM) or DMSO (0.1%). All drugs were incubated for 30 min after which responses to EFS were repeated.

Responses to ATP and SNP

The effects of L-NOARG (100 μM), apamin (0.1 μM) and ODQ (10 μM) on responses to single concentrations of ATP (1, 10 or 30 μM) were obtained after the initial control responses and exposure to all drugs for 30 min. Parallel experiments without antagonists were conducted in tissues not treated with any drugs to serve as time controls. Similar but unpaired responses to SNP (1 μM) were obtained in separate preparations that were not treated or treated for 30 min with either (a) ODQ (10 μM), (b) HbO (30 μM), (c) 7-ER (10 μM) or apamin (0.1 μM).

Contractions to other agonists

Maximum contractions to a single concentration of either 67 mM KCl, histamine (10 μM), endothelin-1 (0.01 μM), 5-hydroxytryptamine (5-HT, 10 μM) or U46619 (0.1 μM) were obtained in the absence of nifedipine in separate preparations. After control responses, all preparations were either untreated (time control) or treated with nifedipine (0.3 μM) for 30 min before being re-exposed to the same concentration of agonist.

Statistics

Peak relaxation responses to EFS, ATP as well as the slower relaxations to SNP (measured at 10 s intervals for 30 s) were expressed as percentage reversal of the level of active force to histamine (1 μM). Differences in mean peak relaxations between controls and treatments were tested for significance by Student's paired *t* tests. Control relaxations to EFS and ATP of each treatment group were compared by a one-way analysis of variance (ANOVA) with multiple comparisons by the Tukey-Kramer method. To compare the effects of one treatment to another for all frequencies (ie. to compare whether the block by apamin was greater than that by apamin and HbO), the mean differences of each groups' control and treatment were compared with one another by ANOVA (Tukey-Kramer). In all cases, significance was accepted at the ($P<0.05$) level.

Drugs and their sources

Haemoglobin (HbO, bovine plasma), N^G-nitro-L-arginine (L-NOARG), histamine sulphate, guanethidine sulphate, tetrodotoxin-citrate, (−)-propranolol, 5-hydroxytryptamine creatinine sulphate (5-HT) (Sigma, MO, U.S.A.); 1H-[1,2,4] oxadiazolo [4,3-a] quinoxaline-1-one (ODQ), 7-ethoxyresorufin (7-ER), apamin, nifedipine, 9-11-dideoxy-9α, 11α-methano-epoxy-prostaglandin $F_{2\alpha}$ (U46619) (Sapphire Bioscience, Sydney, Australia); adenosine triphosphate (ATP), atropine sulphate, (Research Biochemicals International, U.S.A.); sodium nitroprusside (SNP, David Bull Laboratories, Melbourne, Australia); endothelin-1 (ET-1, Peninsula Laboratories, U.S.A.). A stock solution of haemoglobin (1 mM) was prepared by dissolution in 0.9% NaCl and then reduced with sodium dithionite ($Na_2S_2O_4$). The reduced HbO solution was run through a Sephadex (PD 10) size exclusion column previously exposed to 0.9% NaCl to remove excess $Na_2S_2O_4$. Stock solutions of L-NOARG (100 mM) were prepared in 1 M $NaHCO_3$, whereas ODQ (100 mM) and 7-ER (10 mM) were both dissolved in 100% dimethyl-sulphoxide (DMSO). A stock solution of nifedipine (10 mM) and U46619 (1 mM) were prepared in 100% ethanol. Stock solutions of all other drugs were prepared in distilled water. Subsequent dilutions of all stock solutions were made with distilled water.

All preparations exposed to HbO alone or in combination with other drugs were initially exposed to 10 μM HbO for 25 min. Before the contraction to histamine was obtained, preparations were subsequently exposed to a further 20 μM HbO to account for any protein denaturation. The final concentration of HbO was taken as 30 μM.

Results

EFS (0.2–5 Hz, 0.2 ms duration, supra-maximal voltage for 10 s) of histamine (1 μM)-contracted preparations in the presence of guanethidine (30 μM), atropine (1 μM) and propranolol (1 μM) caused tetrodotoxin (1 μM)-sensitive relaxations. The peak response was maximal at 0.2 Hz since it was not significantly different to those obtained with 0.5 and 5 Hz stimulation. Control relaxations to EFS (0.2–5 Hz) for all treatment groups were not significantly different from one another. Also repeated time control relaxations to all frequencies tested were not significantly different (data not shown).

Effect of L-NOARG on EFS-evoked relaxation

L-NOARG (100 μM), had no effect on either the time course (Figure 1) or peak relaxations to EFS (control: 0.2 Hz, $85.3\pm5.3\%$; 0.5 Hz, $91.6\pm1.5\%$; 5 Hz, $87.0\pm2.6\%$, L-NOARG: 0.2 Hz, $82.9\pm5.1\%$; 0.5 Hz, $84.78\pm5.6\%$; 5 Hz, $82.1\pm5.4\%$, $n=5$, Figure 3). Control relaxations to EFS in the absence of atropine tended to be smaller but were not significantly different from responses obtained in the presence of atropine. However, L-NOARG (100 μM) still had no effect on responses to EFS in the absence of atropine (Figure 2).

Effect of HbO, ODQ and 7-ER on EFS and SNP-evoked relaxations

The addition of L-NOARG (100 μM) alone, or in combination with either HbO (30 μM), ODQ (10 μM) or 7-ER (10 μM) had no significant effect on the contraction to histamine (1 μM). Neither, HbO, ODQ nor 7-ER, caused a significant inhibition of EFS (0.2–5 Hz)-induced relaxations alone (data not shown, $n=5$) or in combination with L-NOARG (100 μM) (Figure 3). The maximum relaxation elicited by the NO donor, SNP (1 μM), was abolished by ODQ and 7-ER, markedly reduced by HbO but unaffected by apamin (0.1 μM) (Figure 4).

Effects of apamin on EFS-evoked relaxation

Apamin (0.1 μM), had no effect on relaxations induced by EFS (0.2 Hz) (control: $76.5\pm4.7\%$; apamin treated: $64.3\pm6.4\%$, $n=5$). However, it caused a significant reduction in relaxations to 0.5 Hz (control: $90.0\pm3.0\%$; apamin: $69.4\pm4.7\%$, $n=6$) and 5 Hz (control; $94.2\pm1.5\%$, apamin $60.9\pm3.7\%$, $n=6$) (Figure 5). Increasing the concentration of apamin to 1 μM produced no additional block of EFS-induced relaxations

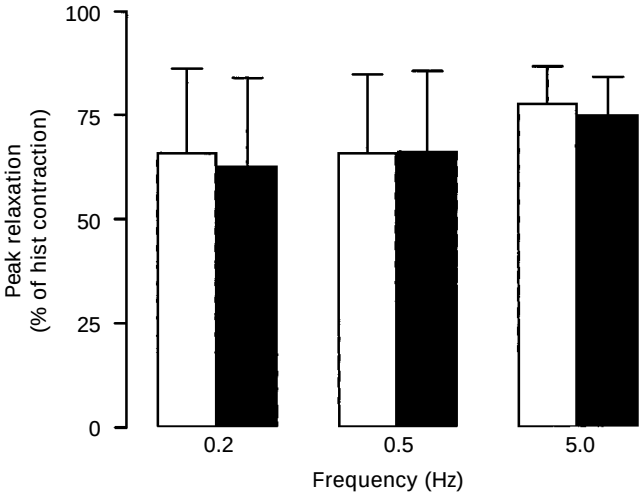


Figure 2 Pooled data showing peak relaxations (expressed as a % of the histamine (hist) contraction) elicited by electrical field stimulation (EFS; 0.2–5 Hz, 0.2 ms duration, supramaximal voltage for 10 s) of non-adrenergic nerves (absence of atropine) of guinea-pig taenia coli preparations pre-contracted with histamine (1 μM). The open columns represent the control relaxations and the solid columns the sequential relaxations obtained in the presence of L-NOARG (100 μM). The vertical error bars represent 1 s.e.mean from 4 experiments.

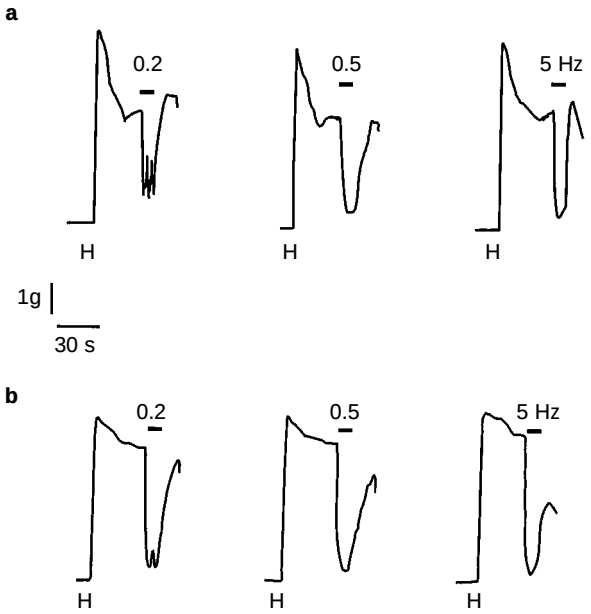
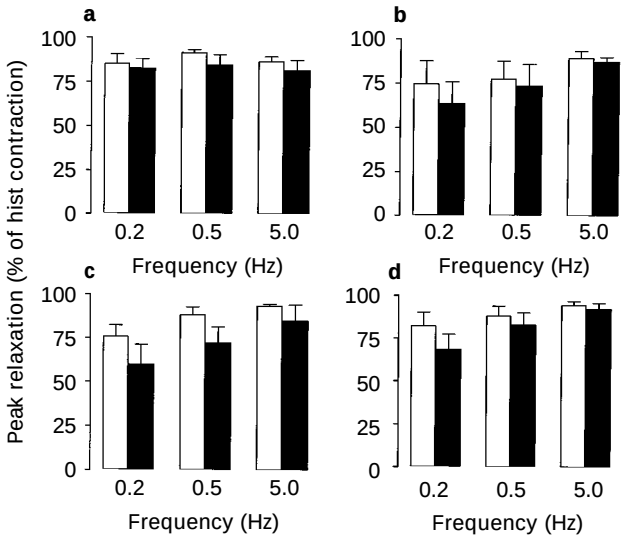


Figure 1 Digitized traces of original chart recordings showing only the second responses elicited by electrical field stimulation (EFS; 0.2–5 Hz, 0.2 ms duration, supramaximal voltage for 10 s) of NANC inhibitory nerves of guinea-pig isolated taenia coli. Preparations were pre-contracted with histamine (H; 1 μM) in (a) the absence and (b) the presence of L-NOARG (100 μM). Scale bars apply for both (a) and (b). The breaks in the traces represent 30 min.



> Figure 3 Pooled data showing peak relaxations (expressed as a % of the histamine (hist) contraction) elicited by electrical field stimulation (EFS; 0.2–5 Hz, 0.2 ms duration, supramaximal voltage for 10 s) of NANC inhibitory nerves of guinea-pig isolated taenia coli preparations pre-contracted with histamine (1 μM). The open columns represent the control relaxations and the solid columns the sequential responses obtained in the presence of either (a) L-NOARG (100 μM) or a combination of L-NOARG (100 μM) and (b) HbO (10 μM), (c) ODQ (10 μM) or (d) 7-ER (10 μM). The vertical error bars represent 1 s.e.mean from 6 experiments.

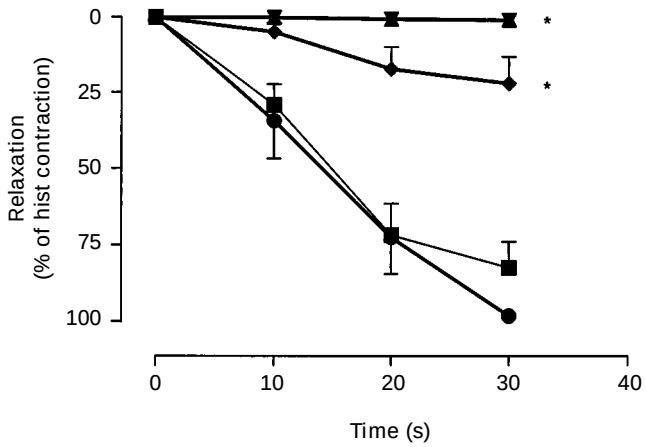


Figure 4 Relaxations elicited by sodium nitroprusside (SNP; 1 μM) in preparations of guinea-pig taenia coli pre-contracted with histamine (hist; 1 μM) in the absence (■, $n=10$) or presence of either HbO (30 μM; ◆, $n=5$), ODQ (10 μM; ▼, $n=5$), 7-ER (10 μM; ▲, $n=5$) or apamin (0.1 μM, ●, $n=6$). The relaxation measured every 10 s is expressed as a % of the histamine contraction. The vertical error bars represent 1 s.e.mean. *Denotes significant difference ($P<0.01$) from control.

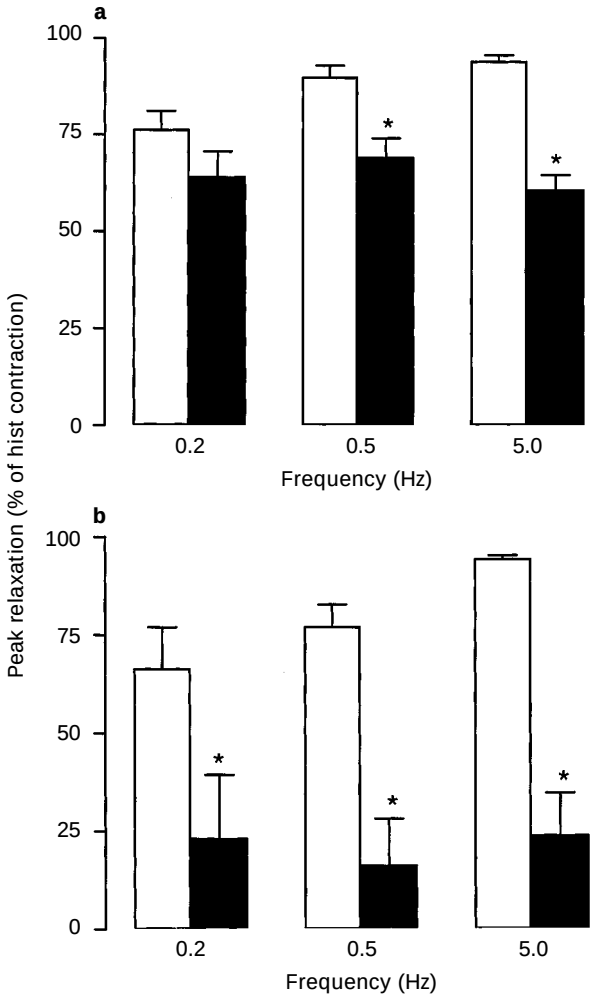


Figure 5 Pooled data showing peak relaxations (expressed as a % of the histamine (hist) contraction) to electrical field stimulation (0.2–5 Hz, 0.2 ms duration, supramaximal voltage for 20 s) of NANC inhibitory nerves in isolated preparations of guinea-pig taenia coli pre-contracted with histamine (1 μM). The open columns represent the control relaxations and the solid columns the sequential responses obtained in the presence of either (a) apamin (0.1 μM; $n=5$) or (b) a combination of apamin (0.1 μM; $n=6$) and L-NOARG (100 μM). The vertical error bars represent 1 s.e.mean.

(data not shown, $n=3$). However, addition of L-NOARG (100 μM) to apamin-treated tissues abolished relaxations at all frequencies of stimulation in 3 out of 6 preparations (Figure 5) and significantly reduced those in the remaining 3 (combination of all 6 experiments gave the following mean values for peak relaxations in the presence of both apamin and L-

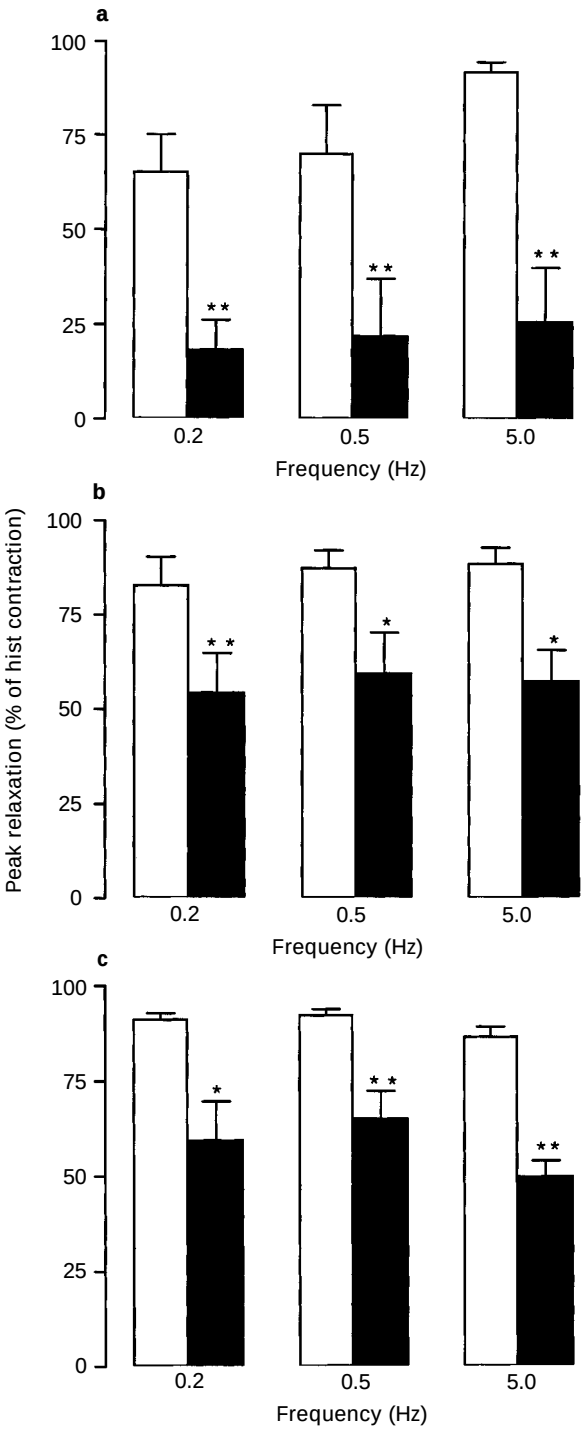


Figure 6 Pooled data showing peak relaxations (expressed as a % of the histamine (hist) contraction) elicited by electrical field stimulation (EFS; 0.2–5 Hz, 0.2 ms duration, supramaximal voltage for 10 s) of NANC inhibitory nerves in guinea-pig taenia coli preparations pre-contracted with histamine (1 μM). The open columns represent the control relaxations and the solid columns the sequential responses obtained in the presence of a combination of apamin (0.1 μM) and either (a) ODQ (10 μM), (b) 7-ER (10 μM) or (c) HbO (30 μM). The vertical error bars represent 1 s.e.mean from 5 experiments. Significant difference from controls denoted by $^*P<0.05$ and $^{**}P<0.01$.

NOARG: control; 0.2 Hz, 67.6±10.5%; 0.5 Hz, 77.3±5.5%; 5 Hz, 94.6±0.7%: apamin plus L-NOARG; 0.2 Hz, 23.3±16.1%; 0.5 Hz, 16.4±11.7%; 5 Hz, 24.1±10.6%, $n=6$) (Figure 5). A similar degree of inhibition of EFS-induced relaxations was obtained when apamin (0.1 μM) was combined with ODQ (10 μM). (Figure 6). The combination of apamin (0.1 μM) with either $NaHCO_3$ (1 mM) or DMSO (0.1%), the solvents for L-NOARG (100 μM) and ODQ (10 μM), respectively, produced no additional block of EFS-evoked relaxations than that observed by apamin alone (data not shown, $n=4$).

In the presence of apamin and either HbO or 7-ER, relaxations to all frequencies of EFS were significantly reduced when compared to the first control response in each case (Figure 6). However, these differences between the first control and second treatment responses were not significantly different from those obtained in the absence and presence of apamin alone (Figure 5). Although the pooled data show that there was no additional effect of HbO over the block caused by apamin alone (the combination of all 8 experiments gave the following mean values, control: 0.2 Hz, 91.4±1.4%; 0.5 Hz, 92.6±1.3%; 5 Hz, 86.8±2.4%: apamin plus HbO: 0.2 Hz, 59.8±10.0%; 0.5 Hz, 65.6±7.0%; 5 Hz, 50.3±4.0%) in 4 experiments the response to 0.2 Hz EFS remaining after apamin and HbO was 34.4±6.3%. In the remaining 4 experiments the block of EFS (0.2 Hz)-evoked responses by the combination of apamin and HbO (83.2±2.6%) was not different from that produced by apamin alone. The peak responses to 0.5 Hz and 5 Hz field stimulation in the presence of apamin and HbO were not as widely scattered as those to 0.2 Hz (Figure 7).

Responses to ATP

ATP (1–30 μM) caused concentration-dependent relaxations that were reproducible after 30 min (data not shown). The time course of these responses was similar to that evoked by EFS (Figure 8) and they were abolished by apamin (0.1 μM), unaffected by ODQ (10 μM) and only significantly reduced by L-NOARG (100 μM) at the lowest concentration of ATP (1 μM) used (Figure 9).

Effect of nifedipine

Maximum contractions to histamine (10 μM), 67 mM K^+, endothelin-1 (0.03 μM), 5-HT (10 μM) or U46619 (0.1 μM) which were all reproducible after 30 min were abolished by nifedipine (0.3 μM) (data not shown, $n=3$).

Discussion

The results of the present study suggest that whilst NO is co-released with a non-NO, apamin-sensitive transmitter from NANC inhibitory nerves in the guinea-pig taenia coli, it remains functionally 'silent'. This conclusion is supported by two findings. First, inhibition of either the synthesis of NO with the potent NO-synthase inhibitor, L-NOARG or its effect on smooth muscle via cyclic GMP accumulation with the selective guanylyl cyclase inhibitor, ODQ (Garthwaite *et al.*, 1995), had no effect on the relaxations to EFS. Furthermore, combined treatment with ODQ and L-NOARG similarly failed to have any inhibitory effects on these responses. The second finding that supports a backup role for NO in NANC inhibitory neurotransmission in the guinea-pig taenia coli was that in the presence of apamin, both L-NOARG and ODQ caused marked inhibition of relaxations to EFS. Together these results imply that although NO did not contribute to the inhibitory response to NANC nerve stimulation, it was concomitantly released with the dominant, apamin-sensitive transmitter. Another possible explanation for the lack of contribution of NO to NANC relaxations is that the apamin-sensitive transmitter causes relaxation of the taenia coli as well as inhibition of the release of NO also via an apamin-sensitive mechanism.

The reason for the use of combination treatments involving L-NOARG and NO inhibitors was to determine whether L-NOARG alone blocked the production of NO. The similar degree of block of apamin-resistant responses by both L-

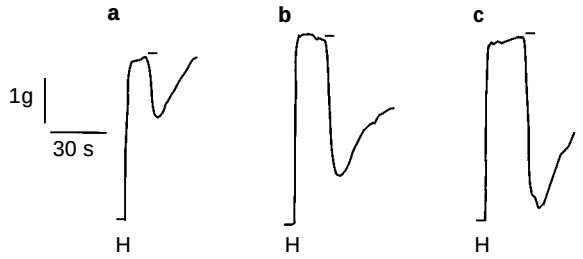


Figure 8 Digitized trace of original chart recording showing second response time control to (a) 1 μM, (b) 10 μM, (c) 30 μM ATP for 10 s in the guinea-pig taenia coli pre-contracted with histamine (H; 1 μM). Note the time course of the relaxations are similar to those evoked by electrical field stimulation (see Figure 1). The breaks in the traces represent 30 min.

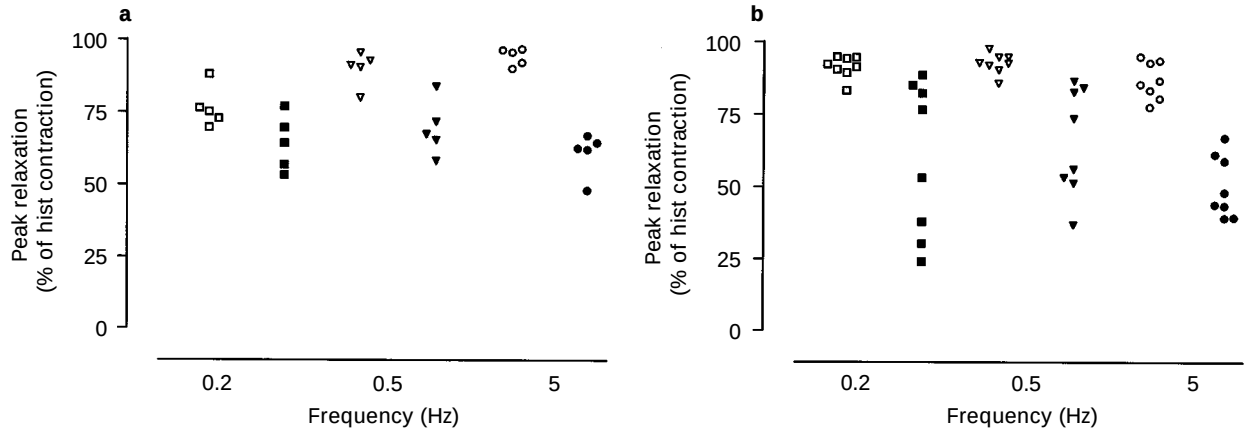


Figure 7 Scattergraphs showing the individual data of peak relaxations (expressed as a % of the histamine (hist) contraction) elicited by electrical field stimulation (EFS; 0.2–5 Hz, 0.2 ms duration, supramaximal voltage for 10 s) of NANC inhibitory nerves of guinea-pig isolated taenia coli contracted with histamine (1 μM). Relaxations to EFS in preparations treated with (a) apamin (0.1 μM, $n=5$, same data as in Figure 5) have been shown for comparison with those treated with (b) a combination of apamin (0.1 μM) and HbO (30 μM, $n=8$, same data as in Figure 6c). In both (a) and (b), open (control) and solid (treated) symbols denote individual responses to (□, ■) 0.2 Hz, (△, ▲) 0.5 Hz and (○, ●) 5 Hz.

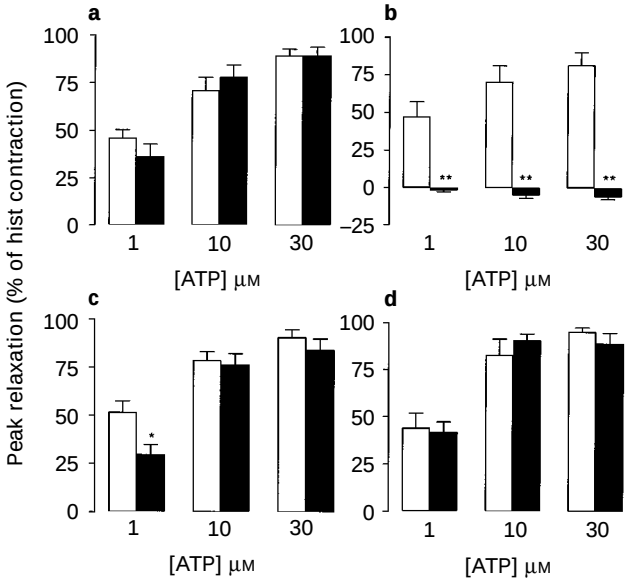


Figure 9 Pooled data showing peak relaxations (expressed as a % of the histamine (hist) contraction) to ATP in preparations of guinea-pig taenia coli pre-contracted with histamine (1 μM). The open columns represent the control relaxations and the solid columns the sequential responses obtained in the presence of (a) no drug treatment (time control, $n=6$), (b) apamin (0.1 μM, $n=4$), (c) L-NOARG (100 μM, $n=10$) and (d) ODQ (10 μM, $n=6$). Vertical error bars represent 1 s.e.mean. Significant difference from controls denoted by $*P<0.05$ and $**P<0.01$.

NOARG and ODQ combined with the finding that ODQ abolished the relaxation to SNP, strongly supports the conclusion that L-NOARG abolished NOS activation during NANC nerve stimulation in the guinea-pig taenia coli. However, the results obtained with the other NO inhibitors examined here, i.e. 7-ER and HbO, did not support this conclusion even though like ODQ both 7-ER and HbO blocked the relaxation to SNP. 7-ER, which is thought to inhibit NO through its ability to generate O_2^- radicals, has been shown to inhibit nitrergically mediated responses in the rat anococcygeus muscle (Li & Rand, 1996). However, Martin *et al.* (1994) found in the bovine retractor penis that an exogenous O_2^- radical generating system abolished responses to exogenous NO but only blocked responses to NANC nerve-derived NO if endogenous superoxide dismutase (SOD) was first inhibited. From these findings, Martin *et al.* (1994) suggested that endogenous SOD acted to protect neurally released NO from degradation by O_2^- radicals, at least in the bovine retractor penis muscle. A similar mechanism for protection by SOD of neurally released NO from O_2^- radicals in the guinea-pig taenia coli may explain why 7-ER was ineffective in removing endogenous NO in this tissue.

Although the group data for HbO plus apamin indicated that HbO also failed to improve the inhibition of NANC relaxations by apamin significantly, in half of the preparations examined it caused further block of the response at low frequency stimulation to a similar degree as L-NOARG. In the remaining half of the preparations, HbO had no effect on the block by apamin. Although the precise reason for this variability in block of apamin-resistant responses by HbO is unknown, it may relate to either the accessibility of HbO to or the rate of diffusion of NO within the neuroeffector junction, as has been evoked to explain a similar lack of effect of other NO-inactivating agents against nitrergic responses in the gut (Wood & Garthwaite, 1994). Such an explanation implies that when both the amount and consequently the rate of diffusion of neurally released NO are low at the neuroeffector junction, as may occur with low frequencies of stimulation, the probability of HbO binding to NO would be greater than that at higher frequencies of stimulation.

A similar functional backup system for NO released from NANC inhibitory nerves described here for the guinea-pig taenia coli has been demonstrated in the rat anococcygeus (Selemidis & Cocks, 1997), a smooth muscle preparation previously regarded to be innervated solely by nitrergic nerves (Gillespie *et al.*, 1989; Li & Rand, 1989; Hobbs & Gibson, 1990; Liu *et al.*, 1991; Rand, 1992; Rand & Li, 1995). Thus, like the guinea-pig taeni coli, NO was found to play little role in NANC-mediated relaxation of the rat anococcygeus (Selemidis & Cocks, 1997), since both L-NOARG and HbO had no effect on these responses. However, block of hyperpolarization-mediated relaxations due to NDHF with nifedipine in the anococcygeus unmasked relaxations mediated by NO which compensated for approximately half of the maximum response.

The mechanism via which gut smooth muscle preparations are contracted may be important when considering the potency of nitrergic-mediated relaxations (Gibson *et al.*, 1994) and redundancy of smooth muscle relaxing factors (Selemidis & Cocks, 1997). In the present study, the guinea-pig taenia coli was contracted apparently only by depolarization-dependent mechanisms, since contractions to a range of agents as well as K^+ -depolarization were blocked by nifedipine. However, in the rat anococcygeus higher concentrations of phenylephrine still caused near-maximum contractions after nifedipine, which again abolished contractions to K^+ -depolarization (Selemidis & Cocks, 1997). Thus, in the rat anococcygeus, any relaxation to EFS in the presence of nifedipine was probably due to a NO-dependent, hyperpolarization-independent mechanism. By contrast, since all the contractile response to histamine in the guinea-pig taenia coli was due to depolarization-induced Ca^{2+} influx, any NO-dependent relaxations to EFS after block of small conductance K^+ channels with apamin probably involved hyperpolarization.

NO has been shown to mediate relaxation of gastrointestinal smooth muscle via both cyclic GMP-dependent and independent mechanisms which may be both ionic (hyperpolarization) (Du *et al.*, 1991; Robertson *et al.*, 1993; Archer *et al.*, 1994; Hampl *et al.*, 1995; Koh *et al.*, 1995; Watson *et al.*, 1996; Yamakage *et al.*, 1996) or non-ionic (metabotropic) (Selemidis & Cocks, 1997). The apamin-insensitive K^+ channel which most likely mediates hyperpolarization and underlies the relaxation to NO in the guinea-pig taenia coli is probably regulated by cyclic GMP, since ODQ caused the same degree of block of apamin-insensitive responses as L-NOARG. However, cyclic GMP is unlikely to regulate the apamin-sensitive channel since apamin-sensitive relaxation to ATP was unaffected by ODQ. A similar ability of NO to hyperpolarize gut smooth muscle via an apamin-insensitive, cyclic GMP-dependent K^+ channel has been shown in the guinea-pig proximal colon (Watson *et al.*, 1996). The type of K^+ channel which mediated any NO-mediated hyperpolarization and relaxation in the rat anococcygeus was not examined in our previous study (Selemidis & Cocks, 1997). However, if K^+ channels were involved, then they may have also been cyclic GMP-dependent since relaxations to SNP were partially blocked by ODQ (Selemidis & Cocks, unpublished observations).

Thus, as in our earlier study (Selemidis & Cocks, 1997), we propose that a non-NO, hyperpolarizing factor, NDHF, which mediates relaxation via non-selective activation of K^+ channels, is the dominant NANC transmitter in the guinea-pig taenia coli. However, NO is co-released with NDHF and can mediate relaxation via hyperpolarization-and non-hyperpolarization-dependent mechanisms, both of which are dependent on cyclic GMP. Whether NO is able to contribute to smooth muscle relaxation depends on how the tissue is either contracted, or the sensitivity and activity of the K^+ channels it activates compared to those activated by NDHF. For example, the NDHF-activated, apamin-sensitive, K^+ channel in the guinea-pig taenia coli mediates approximately 80% of the relaxation to NANC nerve stimulation. If NO is co-released with NDHF, then the cyclic GMP-dependent K^+ channel it acti-

vates either has a longer latency or higher threshold for opening than that for the apamin-sensitive channel, or it is in some way inactivated by NDHF.

Since we were unable to contract the guinea-pig taenia coli with any agent in the presence of nifedipine we could not assess any metabotropic role for NO in mediating relaxation, as we could in the rat anococcygeus. Also, we did not address the identity of NDHF either here or in our previous study in the rat anococcygeus. However, in the guinea-pig taenia coli, the similar time course of relaxations to ATP and those to NANC nerve stimulation as well as the abolition of ATP-mediated relaxation with apamin, is consistent with previous suggestions that ATP is a NANC transmitter, at least in this tissue (Burnstock, 1972; Ferraro *et al*., 1980; Costa *et al*., 1986; Den Hertog *et al*., 1989; Hoyle *et al*., 1990; Burnstock, 1990). Since PACAP-mediated relaxations in the guinea-pig taenia coli are also similar to those to NANC nerve stimulation and are blocked by apamin (Schworer *et al*., 1992), it too needs to be considered as a candidate for NDHF.

Finally, contrary to previous studies (Knudsen & Tottrup, 1992; Ward *et al*., 1996), we failed to demonstrate any block of relaxations to EFS in the guinea-pig taenia coli with L-NOARG in the absence of atropine. By definition, EFS in the absence of atropine no longer represents NANC conditions. As such, the claim by Ward *et al*. (1996) that L-NOARG inhibited to a small, but significant degree, responses to NANC nerve stimulation only in the absence of atropine, needs to be considered with caution. Furthermore, their data also showed a small but significant inhibition by L-NOARG of responses to similar NANC nerve stimulation in the presence of atropine (see Figure 3, Ward *et al*., 1996). The higher concentration of histamine (3 μM) used by Ward *et al*. (1996) most likely contracted the taenia coli to higher levels of active force as iandicated by the smaller responses to EFS compared to those observed in the present study. Therefore, functional antagonism may have become important such that removal of NO with L-NOARG affected to a small degree, the response to EFS regardless of the presence of atropine. However, under these conditions atropine removal could accentuate this small role for NO by allowing excitatory cholinergic activity to antagonize further functionally any inhibitory nerve-mediated relaxations.

In conclusion, NANC nerve innervation in the guinea-pig taenia coli, a tissue where changes in activity of voltage-operated Ca^{2+} channels appears to be the major regulator of 'tone', provides a useful model for evaluating differential, hyperpolarizing mechanisms of nerve-mediated relaxation in the gut. An analogous, endothelium-dependent system for regulation of vascular smooth muscle tone by multiple and often redundant 'transmitter' factors highlights the physiological importance of endothelial cells and NANC nerves in the control of blood flow and gut motility.

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References

ABSOOD, A., EKBLAD, E., EKELUND, M., HAKANSON, R. & SUNDLER, F. (1992). Helospectin-like peptides in the gastrointestinal tract: immunocytochemical localization and immunochemical characterisation. *Neuroscience*, **46,** 431–438.

ARCHER, S.L., HUANG, J.M.C., HAMPL, V., NELSON, D.P., SHULTZ, P.J. & WEIR, E.K. (1994). Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.*, **91,** 7583–7587.

BENNETT, M.R., BURNSTOCK, G. & HOLMAN, M.E. (1966). Transmission from intramural inhibitory nerves to the smooth muscle of the guinea-pig taenia coli. *J. Physiol.*, **182,** 541–558.

BREDT, D.S., HWANG, P.M. & SNYDER, S.H. (1991). Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature*, **347,** 768–770.

BREDT, D.S. & SNYDER, S.H. (1990). Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 682–685.

BRIDGEWATER, M., CUNNANE, T.C. & BRADING, A.F. (1995). Characteristic features of inhibitory junction potentials evoked by single stimuli in the guinea-pig isolated taenia coli. *J. Physiol.*, **485,** 145–155.

BURNSTOCK, G. (1990). Overview: purinergic mechanisms. *Ann. New York Acad. Sci.*, **603,** 1–18.

BURNSTOCK, G. (1972). Purinergic nerves. *Pharmacol. Rev.*, **24,** 509–581.

BURNSTOCK, G., CAMPBELL, G., SATCHELL, D.G. & SMYTHE, A. (1970). Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br. J. Pharmacol.*, **40,** 668–688.

COSTA, M., FURNESS, J.B. & HUMPHREYS, C.M.S. (1986). Apamin distinguishes two types of relaxation mediated by enteric nerves in the guinea-pig gastrointestinal tract. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **332,** 79–88.

COWAN, C.L., PALACINO, J.J., NAJIBI, S. & COHEN, R.A. (1993). Potassium channel-mediated relaxation to acetylcholine in rabbit arteries. *J. Pharmacol. Exp. Ther.*, **266,** 1482–1489.

DEN HERTOG, A., NELEMANS, A. & DEN AKKER, J.V. (1989). The inhibitory action of suramin on the P_2-purinoceptor response in smooth muscle cells of guinea-pig taenia caeci. *Eur. J. Pharmacol.*, **166,** 531–534.

DESAI, H., UDDMAN, R., MALINA, J. & SUNDLER, F. (1992). Helospectin-like immunoreactivity in the esophagus. *Regul. Pept.*, **40,** 363–371.

DRUMMOND, G.R. & COCKS, T.M. (1996). Evidence for mediation by endothelium-derived hyperpolarising factor of relaxation to bradykinin in the bovine isolated coronary artery independently of voltage-operated Ca^{2+} channels. *Br. J. Pharmacol.*, **117,** 1035–1040.

DU, C., MURRAY, J., BATES, J.N. & CONKLIN, J.L. (1991). Nitric oxide: mediator of NANC hyperpolarisation of opposum esophageal smooth muscle. *Am. J. Physiol.*, **261,** G1012–G1016.

FELATOU, M. & VANHOUTTE, P.M. (1988). Endothelium-dependent hyperpolarisation of canine coronary smooth muscle. *Br. J. Pharmacol.*, **93,** 515–524.

FERRERO, J.D., COCKS, T.M. & BURNSTOCK, G. (1980). A comparison between ATP and bradykinin as possible mediators of the responses of smooth muscle to non-adrenergic, non-cholinergic nerves. *Eur. J. Pharmacol.*, **63,** 295–302.

FURNESS, J.B., POMPOLO, S., SHUTTLEWORTH, C.W.R. & BURLEIGH, D.E. (1992). Light-and electron-microscopic immunochemical analysis of nerve fibre types innervating the taenia of the guinea-pig caecum. *Cell. Tiss. Res.*, **270,** 125–137.

GARLAND, C.J., PLANE, F., KEMP, B. & COCKS, T.M. (1995). Endothelium-dependent hyperpolarisation: a role in the control of vascular tone. *Trends Pharmacol. Sci.*, **16,** 23–30.

GARTHWAITE, J., SOUTHAM, E., BOULTON, C.L., NIELSEN, E.B., SCHMIDT, K. & MAYER, B. (1995). Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4] oxadiazolo[4,3-a] quinoxalin-1-one. *Mol. Pharmacol.*, **48,** 184–188.

GIBSON, A., MCFADZEAN, I., TUCKER, J.F. & WAYMAN, C. (1994). Variable potency of nitrergic-nitrovasodilator relaxations of the mouse anococcygeus against different forms of induced tone. *Br. J. Pharmacol.*, **113,** 1494–1500.

GILLESPIE, J.S., LIU, X. & MARTIN, W. (1989). The effects of L-arginine and N^G-monomethyl L-arginine on the response of the rat anococcygeus muscle to NANC nerve stimulation. *Br. J. Pharmacol.*, **98,** 1080–1082.

GRIDER, J.R., CABLE, M.B., BITAR, K.N., SAID, S.I. & MAKLOUF, G.M. (1985). Vasoactive intestinal peptide: Relaxant neurotransmitter in tenia coli of the guinea-pig. *Gastroenterology*, **89,** 36–42.

GRIDER, J.R., KATSOULIS, S., SCHMIDT, W.E. & JIN, J.G. (1994). Regulation of the descending relaxation phase of intestinal peristalsis by PACAP. *J. Auton. Nerv. Syst.*, **50,** 151–159.

HAMPL, V., HUANG, J.M., WEIR, E.K. & ARCHER, S.L. (1995). Activation of the cGMP-dependent protein kinase mimics the stimulatory effect of nitric oxide and cGMP on calcium-gated potassium channels. *Physiol. Res.*, **44,** 39–44.

HILLS, J.M., COLLINS, C.S. & BURNSTOCK, G. (1983). The effects of vasoactive intestinal polypeptide on the electrical activity of guinea-pig intestinal smooth muscle. *Eur. J. Pharmacol.*, **88,** 371 – 376.

HOBBS, A.J. & GIBSON, A. (1990). L-N^G-nitroarginine and its methyl ester are potent inhibitors of non-adrenergic, non-cholinergic transmission in the rat anococcygeus. *Br. J. Pharmacol.*, **100,** 749 – 752.

HOYLE, C.H.V., KNIGHT, G.E. & BURNSTOCK, G. (1990). Suramin antagonizes responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. *Br. J. Pharmacol.*, **99,** 617 – 621.

JIN, J.G., KATSOULIS, S., SCHMIDT, W.E. & GRIDER, J.R. (1994). Inhibitory transmission in tenia coli mediated by distinct vasoactive intestinal peptide and apamin-sensitive pituitary adenylate cyclase activating peptide receptors. *J. Pharmacol. Exp. Ther.*, **270,** 433 – 439.

KILPATRICK, E.V. & COCKS, T.M. (1994). Evidence for differential roles of nitric oxide (NO) and hyperpolarisation in endothelium-dependent relaxation of pig isolated coronary artery. *Br. J. Pharmacol.*, **112,** 557 – 565.

KNUDSEN, M.A. & TOTTRUP, A. (1992). A possible role of the L-arginine-nitric oxide pathway in the modulation of cholinergic transmission in the guinea-pig taenia coli. *Br. J. Pharmacol.*, **107,** 837 – 841.

KOH, S.D., CAMPBELL, J.D., CARL, A. & SANDERS, K.M. (1995). Nitric oxide activates multiple potassium channels in canine colonic smooth muscle. *J. Physiol.*, **489,** 735 – 743.

LI, C.G. & RAND, M.J. (1989). Evidence for a role of nitric oxide in the neurotransmitter system mediating relaxation of the rat anococcygeus muscle. *Clin. Exp. Pharmacol. Physiol.*, **16,** 933 – 938.

LI, C.G. & RAND, M.J. (1996). Inhibition of NO-mediated responses by 7-ethoxyresorufin, a substrate and competitive inhibitor of cytochrome P_{450}. *Br. J. Pharmacol.*, **118,** 57 – 62.

LIU, X., GILLESPIE, J.S., GIBSON, I.F. & MARTIN, W. (1991). Effects of N^G-substituted analogues of L-arginine on NANC relaxation of the rat anococcygeus and bovine retractor penis muscles and bovine penile artery. *Br. J. Pharmacol.*, **104,** 53 – 58.

MARTIN, W., MCALLISTER, K.H.M. & PAISLEY, K. (1994). Nanc neurotransmission in the bovine retractor penis muscle is blocked by superoxide anion following inhibition of superoxide dismutase with diethyldithiocarbamate. *Neuropharmacology*, **33,** 1293 – 1301.

MCCONALOGUE, K., LYSTER, D.J.K. & FURNESS, J.B. (1995). Electrophysiological analysis of the actions of pituitary adenylyl cyclase activating peptide in the taenia of the guinea-pig caecum. *Naunyn-Schmeideberg's Arch. Pharmacol.*, **352,** 538 – 544.

NY, L., ALM, P., EKSTROM, P., HANNIBAL, J., LARSSON, B. & ANDERSSON, K.E. (1994). Nitric oxide synthase containing, peptide-containing and acetylcholinesterase-positive nerves in the cat lower oesophagus. *Histochem. J.*, **26,** 721 – 733.

PIOTROWSKI, W., SIMON, M.C. & BRENNAN, L. (1993). Effects of N^G-nitro-L-arginine and methylene blue on non-adrenergic, non-cholinergic responses of isolated guinea-pig taenia caeci. *Br. J. Pharmacol.*, **157p**.

RAND, M.J. (1992). Nitrergic transmission: Nitric oxide as a mediator of non-adrenergic, non-cholinergic neuro-effector transmission. *Clin. Exp. Pharmacol. Physiol.*, **19,** 147 – 169.

RAND, M.J. & LI, C.G. (1995). Nitric oxide as a neurotransmitter in peripheral nerves: Nature of transmitter and mechanism of transmission. *Annu. Rev. Physiol.*, **57,** 659 – 682.

RAND, M.J. & LI, C.G. (1990). Nitric oxide mediates non-adrenergic, non-cholinergic relaxation in some neuro-effector systems: Examples of nitrergic transmission. Xth International Congress of Pharmacology, Amsterdam. *Eur. J. Pharmacol.*, **183,** 1144.

ROBERTSON, B.E., SCHUBERT, R., HESCHELER, J. & NELSON, M.T. (1993). cGMP-dependent protein kinase activates Ca-activated K-channels in cerebral artery smooth muscle cells. *Am. J. Physiol.*, **265,** C299 – C303.

SANDERS, K.M. & WARD, S.M. (1992). Nitric oxide as a mediator of nonadrenergic, non cholinergic neurotransmission. *Am. J. Physiol.*, **262,** G379 – G392.

SCHWORER, H., KATSOULIS, S., CREUTZFELDT, W. & SCHMIDT, W.E. (1992). Pituitary adenylate cyclase activating peptide, a novel VIP-like gut brain peptide, relaxes the guinea-pig taenia caeci via apamin-sensitive potassium channels. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **346,** 511 – 514.

SELEMIDIS, S. & COCKS, T.M. (1997). Evidence that both nitric oxide (NO) and a non-NO hyperpolarising factor elicit NANC nerve-mediated relaxation in the rat isolated anococcygeus. *Br. J. Pharmacol.*, (in press).

SUNDLER, F., EKBLAD, E., ABSOOD, A., HAKANSON, R., KOVES, K. & ARIMURA, A. (1992). Pituitary adenylate cyclase activating peptide: a novel vasoactive intestinal peptide-like neuropeptide in the gut. *Neuroscience*, **46,** 439 – 454.

WARD, S.M., DALZIEL, H.H., KHOYI, M.A., WESTFALL, A.S., SANDERS, K.M. & WESTFALL, D.P. (1996). Hyperpolarisation and inhibition of contraction mediated by nitric oxide released from enteric inhibitory neurones in guinea-pig taenia coli. *Br. J. Pharmacol.*, **118,** 49 – 56.

WATSON, M.J., BYWATER, R.A.R., TAYLOR, G.S. & LANG, R.J. (1996). Effects of nitric oxide (NO) and NO donors on the membrane conductance of circular smooth muscle cells of the guinea-pig proximal colon. *Br. J. Pharmacol.*, **118,** 1605 – 1614.

WOOD, J. & GARTHWAITE, J. (1994). Models of the diffusional spread of nitric oxide: implications for neural nitric oxide signalling and its pharmacological properties. *Neuropharmacology*, **33,** 1235 – 1244.

YAMAKAGE, M., HIRSHMAN, C.A. & CROXTON, T.L. (1996). Sodium nitroprusside stimulates Ca^{2+}-activated K^+ channels in porcine tracheal smooth muscle cells. *Am. J. Physiol.*, **14,** L338 – L345.

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