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Evidence that nitric oxide acts as an inhibitory neurotransmitter supplying taenia from the guinea-pig caecum

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1 Nitric oxide synthase-containing nerve fibres are abundant within taenia of the guinea-pig caecum, but there is little previous evidence supporting a direct role for nitric oxide (NO) in responses to enteric inhibitory nerve stimulation. In this study we have attempted to identify an NO-dependent component of inhibitory transmission in isolated taenia coli.

2 Isometric tension was recorded in the presence of atropine and guanethidine (both 1 μM). Tone was raised with histamine (1 μM), and intrinsic inhibitory neurons stimulated using either a nicotinic agonist (1,1-dimethyl-4-phenylpiperazinium iodide; DMPP) or electrical field stimulation (EFS).

3 DMPP (1–100 μM) produced concentration-dependent biphasic relaxations, comprising an initial peak relaxation followed by a sustained relaxation. Responses to DMPP were antagonized by tetrodotoxin (1 μM) or apamin (0.3 μM) and abolished by hexamethonium (300 μM). L-nitro-arginine (L-NOARG; 100 μM) and oxyhaemoglobin (2%) both significantly reduced sustained relaxations produced by DMPP.

4 EFS (5 Hz, 30 s) also produced biphasic relaxations. Both L-NOARG and an inhibitor of soluble guanylate cyclase (ODQ, 1–10 μM) reduced the sustained component of EFS responses.

5 Two NO donors, sodium nitroprusside (SNP) and diethylenetriamine-nitric oxide adduct (DENO), produced concentration-dependent relaxations. Responses to SNP and DENO were antagonized by ODQ (1 μM) and by apamin (0.3 μM).

6 These results suggest that NO contributes directly to a component of inhibitory transmission in guinea-pig taenia coli. The actions of NO appear to be mediated *via* cyclic GMP synthesis, and may involve activation of small conductance calcium activated K^+ channels. A role for NO is most evident during sustained relaxations evoked by longer stimulus trains or chemical stimulation of intrinsic neurons.

Keywords: Nitric oxide; taenia coli; apamin; ATP; ODQ; co-transmission, nicotinic receptor; enteric nervous system; vasoactive intestinal peptide

Abbreviations: DENO, diethylenetriamine-nitric oxide adduct; DMPP 1,1-dimethyl-4-phenylpiperazinium iodide; EFS, electrical field stimulation; i.j.p., inhibitory junction potential; KRB, modified Krebs Ringer buffer; L-NOARG, L-nitroarginine; NO, nitric oxide; NOS, nitric oxide synthase; NOS-LI, nitric oxide synthase-like immunoreactivity; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one; PACAP, pituitary adenylate cyclase activating peptide; SNP, sodium nitroprusside; TTX, tetrodotoxin; VIP, vasoactive intestinal peptide

Introduction

The taenia of the guinea-pig caecum is one of the first tissues where neurotransmission to gastrointestinal (GI) smooth muscle was shown to involve transmitters other than acetylcholine or noradrenaline (Burnstock *et al*., 1964). Previous studies have suggested that ATP and VIP are inhibitory transmitters supplying this tissue (e.g. Burnstock *et al*., 1970; MacKenzie & Burnstock, 1980; Grider *et al*., 1985; Grider & Rivier, 1990). More recently, nitric oxide synthase-like immunoreactivity (NOS-LI) was found in intrinsic nerve fibres supplying the guinea-pig taenia coli and in neurons cultured from explants of myenteric plexus taken from guinea-pig caecum (Furness *et al*., 1992; 1994 Saffrey *et al*., 1992). NOS-LI was co-localized in neurons with vasoactive intestinal peptide (VIP) immunoreactivity and, based on counts of total fibres, it appears that VIP/NOS fibres are the inhibitory motor neurons that innervate this tissue (Furness *et al*., 1992). Thus, VIP and NO may act as co-transmitters in this tissue. Counts of neurons also suggest that other inhibitory transmitters (e.g. ATP) are likely to be released from a single population of enteric inhibitory neurons (Furness *et al*., 1992).

At least six previous studies have investigated whether NO synthesis is important in nerve-stimulated relaxations of guinea-pig taenia coli. Five of these studies have reported that inhibitors of NO synthesis have no effect on relaxations evoked by short trains (5–10 s) of nerve stimulation in the presence of atropine (Knudsen & Tottrup, 1992; Randall & Williams, 1992; Williams & Parsons, 1995; Ward *et al*., 1996; Selemidis *et al*., 1997). In a brief report, Piotrowski *et al*. (1993) found that L-NOARG caused a small decrease in the amplitude nerve-stimulated relaxations, but this effect was not mimicked by an inhibitor of guanylate cyclase activity (methylene blue), leading to the suggestion that this action of NO was not mediated by cyclic GMP formation (Piotrowski *et al*., 1993). Some reports suggest that the contribution of NO is masked, or redundant when other transmitter systems are present. For example, in one study, L-NOARG had no significant effect on nerve-stimulated relaxations unless it was added together with α-chymotrypsin, a non-specific inhibitor of neuropeptide-mediated transmission (Randall & Williams, 1992). Knudsen & Tottrup (1992) reported that a contribution of NO could be revealed in taenia preparations examined in the absence of

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atropine, and suggested that NO might act on the terminals of excitatory neurons and inhibit the release of acetylcholine. Ward *et al*. (1996) also found that the actions of L-NOARG in the taenia coli were dependent on the absence of atropine, but found no effects of NO on ^{3}H-acetylcholine release. Selemidis *et al*. (1997) found no influence of atropine on the effects of L-NOARG in the same tissue, but instead found that a role for NO could be revealed if preparations were pre-treated with apamin, a polypeptide toxin which blocks small conductance calcium-activated K^+ channels. It was proposed that a transmitter other than NO (for example ATP) is the primary inhibitory transmitter in the taenia coli, and it is only when the actions of this transmitter are blocked (e.g. with apamin), that other, 'redundant', transmitters mechanisms can be revealed (Selemidis *et al*., 1997).

In the present study we have re-examined the role of NO as an inhibitory neurotransmitter in taeni coli. We have tested the hypothesis that during relatively long periods of nerve stimulation, NO contributes significantly to sustained relaxations. In particular we were interested to determine whether a role for NO in enteric inhibitory neural responses could be observed in the presence of atropine and in the absence of apamin. Finally, we determined whether actions of NO in this tissue were mediated by stimulation of soluble guanylate cyclase, using a selective inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ).

Methods

Tissue preparation

Animals were killed by CO_2 asphixiation and cervical dislocation, the abdomen opened and the caecum removed and placed in modified Krebs-Ringer solution (KRB). These procedures were approved by the University of Nevada Laboratory Animal Use and Care Committee. Taenia, with underlying myenteric plexus, were cut from the surface of the caecum. This dissection was performed under a dissecting microscope, taking care to also remove some of the underlying circular muscle layer along the length of the segment. This procedure ensures that myenteric plexus is included in the segment. Segments (1 cm) were attached to strain gauges (FT03, Gould, or 1030 UFI) and placed in 5 or 10 ml organ baths containing KRB maintained at 37°C. A resting force of 1 g was applied to the strips before equilibrating for 90–120 min. Bathing solution was changed at 20 min intervals, and atropine and guanethidine (both 1 μM) were present throughout all experiments.

Nerve stimulation

To examine the effects of nerve stimulation or addition of NO donors, tone was raised with histamine (1 μM). In some experiments, stimulation of intrinsic neurons was achieved by electrical field stimulation (EFS, 30 s trains of 0.5 ms pulses, delivered at 5 Hz) applied *via* platinum ring electrodes. In these experiments, EFS was begun 2 min after addition of pre-contracting histamine. In other experiments, intrinsic neurons were stimulated using the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP). In some experiments, cumulative additions of DMPP (1–100 μM) were made, beginning 60 s after addition of pre-contracting histamine. Cumulative additions of DMPP were then made at 30 s intervals. In other experiments, responses to single concentrations of DMPP (30 μM) were assessed. In these experiments, DMPP was added 2 min after histamine addition. Responses to DMPP were assessed at 25 min intervals. No desensitization to DMPP was observed over this time course.

Statistical analysis All data are presented as means ± s.e.-mean and differences between groups were analysed using Student's *t*-tests, with a level of $P<0.05$ being considered significant.

Drugs and solutions

The KRB contained (in mM) NaCl 118.5, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 23.8, KH_2PO_4 1.2, dextrose 11. This solution had a pH of 7.4 at 37°C when bubbled to equilibrium with 95% O_2/5% CO_2. In some preliminary experiments, the KRB contained (in mM) NaCl 120.35, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.5, NaH_2PO_4 1.2, dextrose 11.5. This solution had a pH of 7.4 when bubbled to equilibrium with 97% O_2/3% CO_2. Oxyhaemoglobin was prepared as a lysate of canine erythrocytes according to the method of Bowman & Gillespie (1982), with the exception that red cells were lysed by 1 : 1 addition of distilled water. Atropine sulfate, guanethidine sulphate, histamine hydrochloride, apamin, tetrodotoxin, sodium nitroprusside and DMPP were all from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ) was from Tocris Cookson Inc (MO, U.S.A.), stored frozen in DMSO (10 mM aliquots) and diluted to working concentrations in KRB. Diethylenetriamine-nitric oxide adduct (DENO) was from RBI chemicals (Natick, MA, U.S.A.), stored frozen in aliquots (30 mM in H_2O) and diluted to working concentrations in KRB.

Results

Responses to DMPP

We first tested the role of NO-dependent inhibitory transmission in strips of taenia during stimulation by nicotinic agonist DMPP. After tone of the muscles was raised with histamine (1 μM), DMPP (1–100 μM) produced concentration-dependent relaxations (Figure 1). Responses were transient, with relaxation beginning soon after addition of DMPP and waning with time to approximately the level of tone before DMPP addition. Concentration-response curves were generated by cumulative addition of DMPP. Successive concentrations were added as soon as the response to the previous concentration reached a maximum (usually at intervals of 30 s). Relaxations to DMPP were abolished by hexamethonium (100 μM, $n=6$, data not shown), indicating that these responses were due to activation of nicotinic receptors. Tetrodotoxin (TTX) (1 μM) reduced responses to DMPP (1–30 μM), but did not abolish these responses. For example, 30 μM DMPP relaxed muscles by 92.1 ± 5.4 and 17.9 ± 5.3% under control conditions and in the presence of TTX, respectively ($P<0.002$). Higher concentrations of DMPP continued to elicit maximal relaxation in the presence of TTX (e.g. 96.8 ± 1.2%, with 100 μM DMPP, $P>0.05$, $n=4$).

L-NOARG (100 μM) did not significantly reduce the peak amplitude of DMPP-stimulated relaxations (e.g. 94.8% inhibition in control vs 86.8% inhibition after exposure to L-NOARG for 20 min, $P>0.2$, $n=4$). However, L-NOARG reduced the sustained component of DMPP-stimulated relaxations, and tone was restored to the control level more rapidly after an initial relaxation. Integration of the relaxation responses showed that L-NOARG significantly reduced

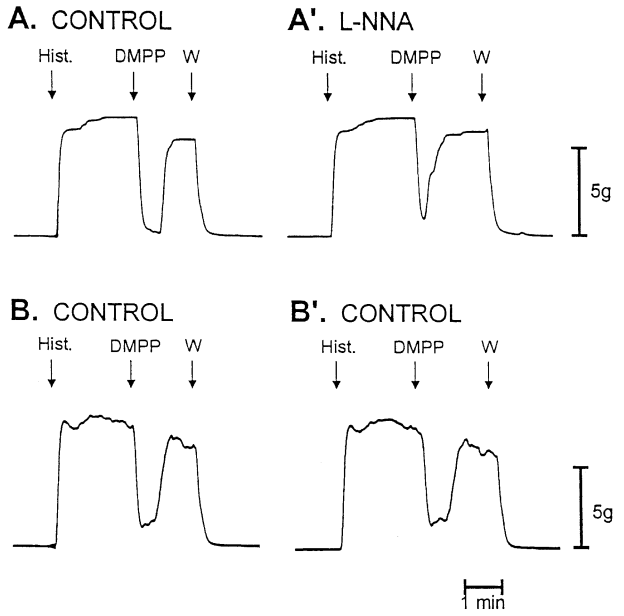

Figure 1 L-nitro-arginine (L-NOARG), an inhibitor of nitric oxide synthesis, removes a component of the inhibitory response to DMPP. (A) and (B) show control responses to DMPP addition (30 μM) in two tissues. (A′) shows that the sustained component of the DMPP response was significantly reduced after 30 min L-NOARG (100 μM) exposure. (B′) shows that, in the absence of L-NOARG, responses to DMPP were reproducible over the same time course.

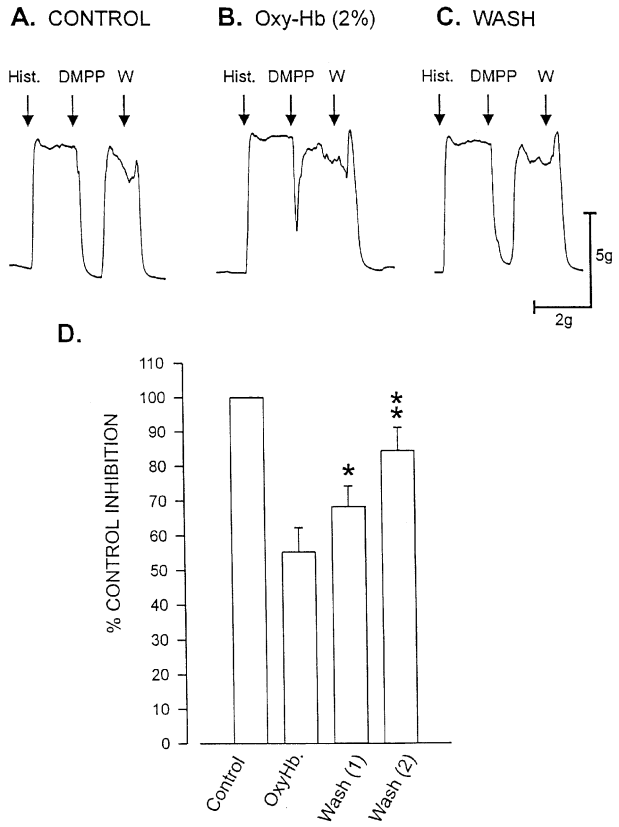

Figure 2 Oxyhaemoglobin, a scavenger of extracellular nitric oxide, removes a component of the inhibitory response to DMPP. (A) shows control relaxant response to a single exposure to DMPP (30 μM). Following 10 min exposure to oxyhaemoglobin (2%) and 30 min following the previous DMPP challenge, the response to DMPP was significantly attenuated (B). (C) shows that responses to DMPP were restored 60 min after washout of haemolgobin. Mean data showing integrated area of relaxation responses from six experiments is illustrated in (D). Wash (1) is 30 min after haemoglobin; Wash (2) is 60 min after haemoglobin. Bars represent means$\pm$s.e.mean of six experiments. Asterisks indicates means significantly different from responses in the presence of oxyhaemoglobin ($*P<0.05$; $**P<0.01$).

inhibitory responses (by $28.1\pm4.2\%$, $P<0.001$ as compared to time-matched controls; Figure 2).

The NO scavenger, oxyhaemoglobin (2%), also produced significant inhibition of relaxation responses (by $44.7\pm7.0\%$, $n=6$; Figure 3); and inhibitory responses to DMPP were restored after washout of haemoglobin (to 68.3 ± 5.9 and $84.47\pm6.7\%$ of control responses after 30 and 60 min, respectively).

Apamin (0.3 μM), a blocker of small conductance calcium activated K^+ channels, also antagonized responses to DMPP, but a significant portion of the relaxation elicited by 30–100 μM DMPP was resistant to apamin. L-NOARG inhibited the apamin-resistant relaxations produced by DMPP (Figure 3). L-NOARG also reduced TTX-resistant responses, suggesting that NO synthesis contributes to the TTX-sensitive component of DMPP action. The residual responses to high concentrations of DMPP in the presence of TTX may be due to stimulation of pre-junctional receptors on nerve-terminals to release inhibitory transmitter by a mechanism independent of action potential generation (see Rand & Li 1992; Shuttleworth *et al*., 1995).

We also tested the ability of DMPP to stimulate excitatory neurons in the preparation. These experiments were performed under conditions of low tone (i.e. muscles not pre-contracted with histamine) and without atropine in the bathing medium. Under these conditions, DMPP (1–100 μM) produced no resolvable contractile response. To avoid masking of excitatory effects by simultaneous effects of inhibitory neurotransmitters DMPP was also tested after most of the inhibitory responses were blocked using a combination of apamin (0.5 μM) and L-NOARG (100 μM). Under these conditions, DMPP (1–100 μM) produced no resolvable contraction (data not shown). Thus under the conditions of our experiments, DMPP appears to selectively activate enteric inhibitory neurons in the guinea-pig taenia coli.

Responses to EFS

Previous studies showed that relaxations produced by relatively brief periods of EFS (5–10 s) are unaffected by inhibitors of nitric oxide synthesis (see Introduction). The results with DMPP suggested that NO-dependent relaxation can be elicited in taenia coli when inhibitory neurons are activated by sustained stimulation. Parameters of EFS were chosen that produced sustained relaxation of similar time course to the responses elicited by DMPP. Stimulation at 5 Hz for 30 s produced relaxation consisting of two components: (1) an initial peak relaxation that reached a $85.5\pm5.6\%$ of maximum relaxation 17.8 ± 2.2 s after onset of stimulation ($n=8$) and (2) a sustained relaxation that persisted for the duration of the stimulus. At the termination of the stimulus, the sustained relaxation averaged $89.9\pm4.5\%$ of the peak response. All components of the relaxation response to EFS were abolished by TTX (1 μM; $n=5$).

L-NOARG (100 μM) did not significantly reduce the peak relaxation measured at either 5 or 10 s after the onset of the stimulus. Peak relaxation measured 5 s after the onset of stimulation was $63.2\pm5.4\%$ in control and $63.6\pm4.6\%$ in L-

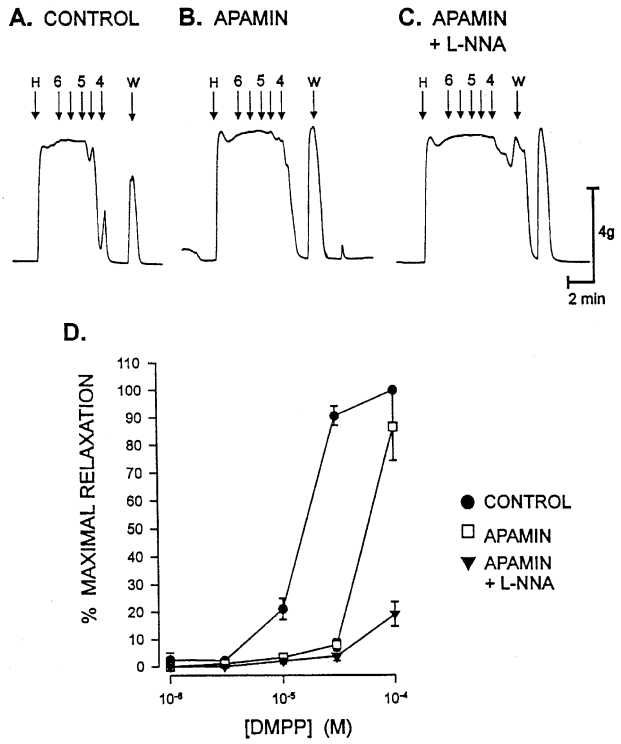

Figure 3 Apamin is an effective antagonist of DMPP-stimulated relaxations and L-NOARG reduces apamin-resistant relaxations. Numbers in (A, B and C) refer to $-\log_{10}$ DMPP molar concentrations. Control responses to cumulative DMPP additions (1–100 μM) are shown in (A). Apamin (0.3 μM) abolishes responses to low DMPP concentrations (1–0.3 μM), but 100 μM DMPP still produced maximal relaxation in the presence of apamin (B). Subsequent exposure to L-NOARG (100 μM) significantly reduced apamin-resistant DMPP responses (C). Results of six experiments are summarized in (D). Points represent means±s.e.mean of six experiments (control, apamin) or three experiments (apamin + L-NOARG).

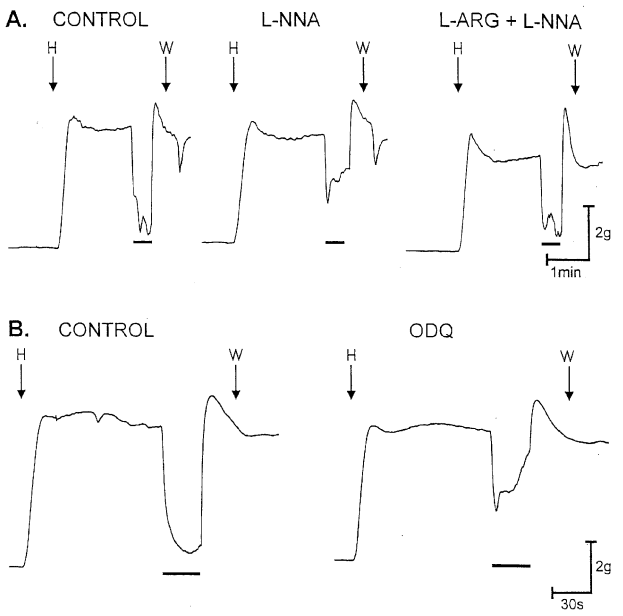

Figure 4 L-NOARG and ODQ antagonized sustained relaxations evoked by electrical field stimulation (EFS). (A) and (B) show responses from different muscle strips. Under control conditions, EFS (5 Hz, 30 s, indicated by horizontal bars) produced relaxations that were sustained throughout the period of stimulation (left panels). (A) shows that L-NOARG (100 μM) significantly reduced the sustained component of relaxation and this effect was reversed by addition of L-arginine (1 mM, in the continued presence of L-NOARG). (B) shows that ODQ (10 μM) also significantly reduced relaxations evoked by this relatively long period of EFS. Histamine additions and washouts are indicated by H and W respectively.

NOARG ($P=0.93$, $n=6$). Peak relaxation measured 10 s after onset of stimulation was 73.1±7.6% in control and 63.6±4.6% ($P=0.19$, $n=6$). However, L-NOARG treatment resulted in a significant decrease in the time taken to reach maximal relaxation during the stimulus train (i.e. 6.26±2.1 s, $P<0.001$ compared with control, $n=6$), and a significant decrease in the peak amplitude of relaxations (to 65.1±4.9% maximum amplitude, $P<0.05$, $n=6$). Most apparent was a reduction in the sustained component of relaxation (Figure 4A). At the end of the 30 s stimulation period, the amplitude of the sustained component was 45.7±6.0% of the control response ($P<0.002$, $n=6$), and the integrated area of inhibitory responses was reduced by 55.4±3.8% compared with control responses ($n=6$; $P<0.02$). L-arginine partially reversed the actions of L-NOARG, restoring the peak amplitude of relaxations to 79.3±3.8% of control responses; ($P<0.05$). In the presence of L-arginine, the integrated area of inhibitory responses was reduced by 29.1±6.2% compared with control responses.

Actions of nitric oxide donors

Sodium nitroprusside (SNP; 10 nM–10 μM) produced concentration-dependent relaxations (EC_{50} 0.29 μM, $n=4$) of histamine-contracted muscle strips. Apamin (0.3 μM) antagonized the actions of SNP; relaxations to 1 μM SNP were 84.0±14.9 and 19.3±3.1% in control and apamin, respectively ($P<0.05$, $n=4$) (Figure 5B). Responses to SNP were abolished by ODQ (1 μM) (Figure 5A).

A second NO donor, diethylenetriamine-nitric oxide adduct (DENO) was also tested because it was recently proposed that DENO and SNP activate different mechanisms in gastrointestinal smooth muscle (Goyal & He, 1997). DENO was less potent than SNP, and responses to single concentrations (0.3–1 mM) were tested. The actions of DENO were abolished by ODQ (10 μM, $n=4$) and apamin (0.3 μM, $n=6$) (Figure 6). DENO relaxations were 71.4±10.7 and 0.65±1.01% in control and apamin, respectively ($P<0.005$, $n=6$).

Discussion

The results of this study provide evidence that NO contributes to inhibitory neuromuscular transmission in the guinea-pig taenia coli. The most prominent role of NO appears to be in maintenance of inhibitory responses during sustained periods of inhibitory nerve stimulation. A role for NO could be demonstrated in the presence of atropine and absence of apamin, suggesting that NO acts directly as an inhibitory transmitter, rather than modulating cholinergic excitatory transmission or acting as a redundant transmitter that is active only when other transmitter mechanisms are blocked (see Introduction).

Inhibitory neuromuscular transmission to guinea-pig taenia caeci is often studied in the presence of atropine and guanethidine and responses are often referred to as non-adrenergic, non-cholinergic (NANC) relaxations. Under these conditions, Piotrowski and co-workers (1993) demonstrated that L-NOARG caused a small, but significant decrease in responses, however other studies have found little effect of NOS inhibitors (Knudsen & Tottrup, 1992; Randall & Williams,

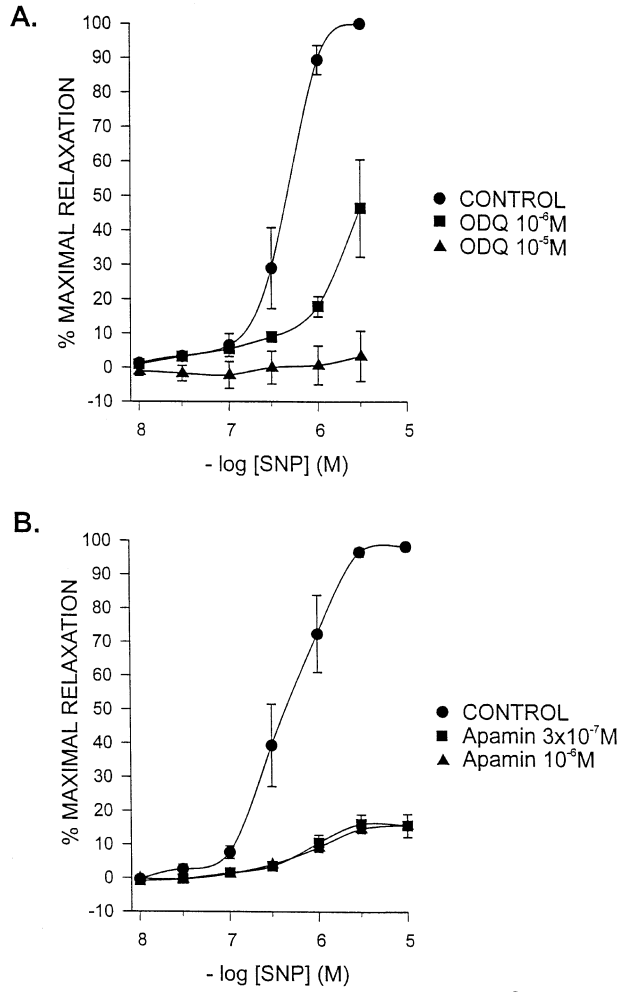

Figure 5 Apamin and ODQ antagonized responses to a nitric oxide donor. Sodium nitroprusside (SNP) produced concentration-dependent relaxations (A), that were largely reduced by ODQ (1 μM) and abolished by ODQ (10 μM). (B) shows control SNP relaxations and responses in the presence of apamin (0.3–1 μM).

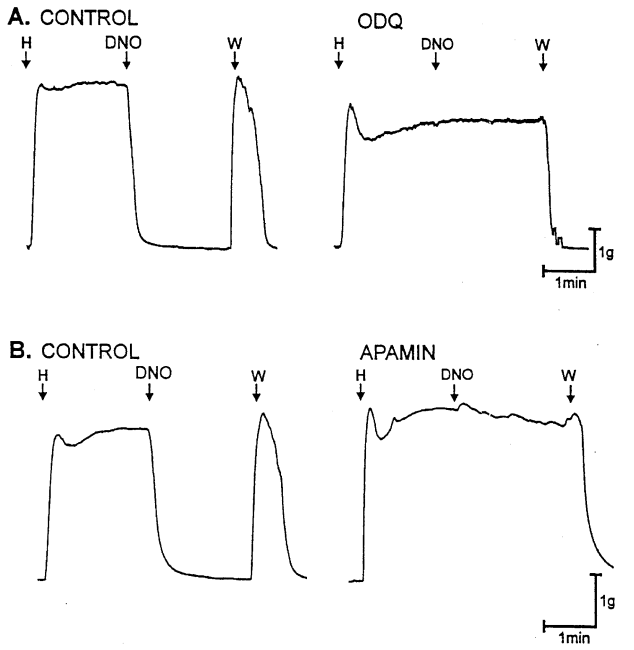

Figure 6 ODQ and apamin block responses to DENO. (A) and (B) show responses recorded from different muscle strips. DENO (1 mM) produced complete relaxation of histamine pre-contracted strips (left panels). These relaxations were abolished by prior exposure to ODQ (10 μM) or apamin (0.3 μM).

1992, Williams & Parsons, 1995; Ward *et al*., 1996; Selemidis *et al*., 1997). The different conclusions reached in previous studies may be due in part to differences in stimulus duration, and analysis of responses. Previous studies have usually used relatively short stimulus trains (5–10 s) which produce relaxations mediated predominantly by release of transmitter(s) other than NO. We tested the role of NO in responses to longer periods of nerve stimulation, because recent intracellular microelectrode recording studies have demonstrated a clear difference in the time course of NO-dependent and NO-independent components of enteric inhibitory transmission. In many tissues, stimulation of intrinsic neurons produces compound inhibitory junction potentials (i.j.p.s) recorded in gastrointestinal smooth muscle cells. These compound events typically involve an initial fast hyperpolarization that is resistant to NOS inhibitors, and a slower component of hyperpolarization that is NO-dependent. When single pulses of EFS are used, the fast component of the i.j.p. predominates, but the two components of compound i.j.p.s become easier to dissociate when relatively long stimulus trains are used. During sustained stimulation, the initial fast i.j.p. decays to a sustained level which is maintained throughout the stimulus and addition of L-NOARG reduces this sustained hyperpolarization (see He & Goyal, 1993; Stark *et al*., 1993; Keef *et al*., 1993, Shuttleworth *et al*., 1997). The guinea-pig taenia coli has a very prominent fast i.j.p., that is resistant to NOS inhibitors (Bridgewater *et al*., 1995; Ward *et al*., 1996). Previous studies have utilized relatively short stimulus trains (5–10 s) and found that L-NOARG produces no significant decrease in peak amplitude of relaxation, consistent with the notion that the fast i.j.p. underlies the rapid initial relaxation response (Ward *et al*., 1996). Our results are consistent with these reports, in that the peak relaxation measured 5 or 10 s after the onset of stimulation were not significantly reduced by L-NOARG. During relatively long trains of electrical stimuli (30 s) or sustained chemical stimulation with DMPP, the peak relaxation declined to a sustained, plateau level, and this component of the response was clearly sensitive to NOS inhibitors and oxyhaemoglobin. Taken together, these results suggest that nerve-stimulated relaxations of guinea-pig taenia coli are due to the summation of (1) a rapid onset, peak relaxation mediated mainly by a transmitter other than NO and (2) a sustained relaxation mediated by NO.

This conclusion predicts that nerve-stimulated relaxations of guinea-pig taenia coli will be most sensitive to NOS inhibitors when the fast (NO-independent) component of relaxation is selectively reduced and the relative contribution of NO synthesis to the peak relaxation is increased. This suggestion may explain why previous studies have found effects of NOS inhibitors in guinea-pig taenia coli only when (1) atropine is absent (Knudsen & Tottrup 1992; Ward *et al*., 1996) or (2) apamin is present (Selemidis *et al*., 1997). Intracellular microelectrode recording from guinea-pig taenia coli has shown that responses to single pulses of EFS are compound responses composed of a cholinergic excitatory junction potential (e.j.p.) blocked by atropine (Bennett, 1966), and fast a i.j.p., abolished by apamin (Vladimirova & Shuba, 1978; Maas, 1981) but unaffected by L-NOARG (Bridgewater *et al*., 1995; Ward *et al*., 1996). These two opposing effects both peak soon after the onset of stimulation (latency around

100 ms) and as such are functionally antagonistic. This antagonism was first demonstrated by Bennett (1966) who showed that atropine approximately doubled the amplitude of the fast i.j.p. (as compared with non-atropinized preparations), and later confirmed when block of the i.j.p. lead to a concomitant increase in e.j.p. amplitude (e.g. Vladimorva & Shuba, 1978; Maas, 1981). This suggests that the relaxation response observed immediately after nerve stimulation involves a balance between cholinergic excitatory, and non-NO-dependent inhibitory mechanisms. It therefore seems reasonable to suggest that in the absence of atropine, the relative importance of NO in the net relaxation response increases and the relaxation appears more sensitive to NOS inhibitors.

It is also possible that NO release modulates the acetylcholine release, explaining the dependence of NOS inhibitor effect on atropine (Knudsen & Tottrup 1992). However, it is noteworthy that experiments to directly test this hypothesis found no effect of NO on ^{3}H-ACh release (Ward *et al*., 1996).

The nicotinic agonist DMPP stimulates nicotinic receptors on myenteric neuron soma, leading to TTX-sensitive relaxations, and at high concentrations (e.g. 100 μM) also appears to stimulate receptors located a terminals of enteric inhibitory neurons producing TTX-insensitive relaxations (see also Rand & Li, 1992; Shuttleworth *et al*., 1995). In the present study, DMPP produced relaxations that included a sustained component sensitive to L-NOARG, demonstrating the involvement of NO, in the presence of atropine and absence of apamin. A previous report noted that DMPP-stimulated relaxations were unaffected by L-NOARG (Randall & Williams, 1992), however it is not clear from that brief report how these authors analysed DMPP-stimulated relaxations. It is noteworthy that in the present study, L-NOARG had no significant effect on the peak amplitude of DMPP-stimulated relaxations (the parameter most commonly measured in this type of study), but did significantly reduce the sustained component of relaxation.

In many smooth muscles, NO causes smooth muscle relaxation by stimulation of soluble guanylate cyclase, and intracellular accumulation of cyclic GMP. The involvement of cyclic GMP in NO-dependent relaxations of guinea-pig taenia was tested previously using methylene blue (Piotrowski *et al*., 1993). Methylene blue did not mimic the effects of L-NOARG, leading to the suggestion that NO causes relaxation in a cyclic GMP-dependent manner (Piotrowski *et al*., 1993). However methylene blue appears to be a relatively ineffective inhibitor of soluble guanylate cyclase in some gastrointestinal preparations, and we recently demonstrated that a novel soluble guanylate cyclase inhibitor (ODQ, Garthwaite *et al*., 1995) effectively antagonizes NO-dependent responses in these tissues (Franck *et al*., 1997). Selemidis & coworkers (1997) showed that ODQ antagonized NO-dependent responses in guinea-pig taenia caeci, and in the present study, ODQ reduced the sustained component of nerve-stimulated relaxations and effectively antagonized relaxations produced by two NO donors. These results strongly suggest that NO causes relaxation of guinea-pig taenia caeci by elevation of intracellular cyclic GMP.

Two NO donors were tested, SNP and DENO, because it was recently proposed that these agents preferentially generate different redox species of NO (NO^+ and $NO^\bullet$ respectively) and activate different hyperpolarization mechanisms in GI smooth muscle (Goyal & He, 1997). We found that both donors produced relaxations sensitive to ODQ. Furthermore, apamin, a blocker of small conductance calcium activated K^+ channels (SK), antagonized responses to SNP and DNO. This suggests that increases in cyclic GMP, or downstream events, can activate SK and contribute to hyperpolarization. It may be that stimulation by multiple transmitters in the guinea-pig taenia caeci (e.g. NO and ATP) converge upon the activation of SK channels.

In contrast to our findings, Selemidis *et al*. (1997) found that apamin did not antagonize responses to SNP. The reasons for these differences from our results are not known. It is interesting to note that Selemidis *et al*. (1997) found that NOS inhibitors were only effective in reducing the peak amplitude of nerve-stimulated relaxations when apamin was present. These authors concluded that NO is a redundant transmitter in the taenia coli, the action of which is not relevant unless other transmitter mechanisms are blocked. The present study suggests that NO participates in inhibitory transmission, even when apamin is absent, but that its contribution to the initial peak relaxation is masked by the co-release of another transmitter(s). From the discussion above, selective antagonism of the fast component of inhibitory transmission (e.g. with apamin in the hands of Selemidis *et al*., 1997) would be expected to increase the relative contribution of NO to the remaining response, and the response would, therefore, be more susceptible to NOS inhibitors. When the present data are considered together with previous reports, it seems inappropriate to term NO a redundant transmitter in this tissue. The frequency and burst durations of intrinsic motor neurons in the guinea-pig caecum *in vivo* are unknown, it is difficult to know whether the stimulus parameters used in this or other studies are physiologically relevant, however, it appears that sustained relaxations of guinea-pig taenia coli depend upon the co-release of NO.

It has recently been reported that VIP stimulates NO synthesis in GI smooth muscle cells, and raised the controversial hypothesis that NO may act as a second messenger for VIP action, rather than as a primary neurotransmitter (Murthy *et al*., 1995, but see also Keef *et al*., 1994; Desai *et al*., 1994). Previous studies suggest that VIP contributes nerve-stimulated relaxations of the taenia, since this transmitter is released from taenia following nicotinic receptor stimulation (Iselin *et al*., 1988), and VIP antagonists inhibit EFS-stimulated inhibitory responses (Grider & Rivier 1990). Interestingly, it has recently been reported that VIP does not stimulate NO synthesis in smooth muscle cells isolated from guinea-pig taenia caeci (Jin *et al*., 1993) suggesting that NO synthesis cannot couple VIP receptors to relaxation in this tissue. Therefore if NO is involved in inhibitory transmission, it is not as a consequence of VIP action, but rather as a co-transmitter with VIP. Since VIP-LI and NOS-LI are co-localized in the same neurons in the taenia (Furness *et al*., 1992), this suggests that these transmitters are co-released from the same neurons.

The transmitter(s) responsible for the initial relaxation in the taenia coli were not investigated in this study. Previous evidence has suggested that ATP mediates these responses (Burnstock *et al*., 1970, MacKenzie & Burnstock, 1980; Mass, 1981, Costa *et al*., 1986), and recently the peptide pituitary adenylate cyclase activating peptide (PACAP) has also been suggested as a transmitter in this tissue (Schwörer *et al*., 1992; McConalogue *et al*., 1995).

In conclusion, these data suggest a transmitter other than NO (e.g. ATP) is primarily responsible for initial, rapid onset relaxations in guinea-pig taenia coli, and NO release significantly contributes to sustained neurogenic relaxations. These transmitters appear to be released from a single neuronal population, resulting in the potential of inhibitory nerve stimulation to produce both rapid and sustained inhibition of muscle tone.

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