Involvement of cyclic GMP in non-adrenergic, non-cholinergic inhibitory neurotransmission in dog proximal colon

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1 Nitric oxide (NO) may serve as a non-adrenergic, non-cholinergic (NANC) neurotransmitter released from enteric inhibitory nerves in the gastrointestinal tract. We tested whether guanosine 3':5'-cyclic monophosphate (cyclic GMP) may serve as a second messenger in transducing the NO signal into inhibitory junction potentials (i.j.ps) and relaxation in the canine proximal colon.

2 The membrane permeable analogue of cyclic GMP, 8-bromo cyclic GMP (8-Br-cyclic GMP) mimicked the effects of NO by hyperpolarizing cells near the myenteric border of the circular muscle layer and shortening slow waves in cells near the submucosal surface of the circular muscle layer. 8-Br-cGMP also inhibited spontaneous phasic contractions.

3 The specific cyclic GMP phosphodiesterase inhibitor, M&B 22948, hyperpolarized cells near the myenteric border and prolonged the duration of i.j.ps. M&B 22948 also inhibited phasic contractile activity.

4 Methylene blue failed to reduce significantly the amplitude and duration of i.j.ps and had variable effects on contractions.

5 Cyclic GMP levels were assayed in unstimulated muscles and in muscles exposed to exogenous NO and electrical field stimulation. Both stimuli hyperpolarized membrane potential, inhibited contractions, and elevated cyclic GMP levels.

6 Treatment of muscles with L-N^G-nitroarginine methyl ester (L-NAME) increased spontaneous contractile activity and lowered cyclic GMP levels. The inhibitory effect of M&B 22948 on contractions was greatly reduced after muscles were treated with L-NAME.

7 These data support the concept that the effects of NANC nerve stimulation and NO (which may be one of the enteric inhibitory transmitters) may be mediated by cyclic GMP.

Keywords: Nitric oxide; non-adrenergic, non-cholinergic nerves; colonic motility; cyclic GMP; enteric nervous system, methylene blue, gastrointestinal motility

Introduction

Nitric oxide (NO) may serve as a neurotransmitter in non-adrenergic, non-cholinergic (NANC) responses in the oesophagus (Murray *et al.*, 1991; Tottrup *et al.*, 1991), stomach (Desai *et al.*, 1991; Boeckxstaens *et al.*, 1991), small bowel (Toda *et al.*, 1990; Stark *et al.*, 1991), ileocolonic sphincter (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1990; Ward *et al.*, 1992b), colon (Dalziel *et al.*, 1991; Thornbury *et al.*, 1991; Ward *et al.*, 1992a; Huizinga *et al.*, 1992) and internal anal sphincter (Rattan & Chakder, 1991). In many of these muscles relaxation of tonic contraction or reduction in the amplitude of phasic contractions are mediated via hyperpolarization responses known as inhibitory junction potentials (i.j.ps; Burnstock *et al.*, 1963; 1966). I.j.ps are thought to be due to a transient increase in potassium conductance (Tomita, 1972), and repetitive electrical field stimulation can lead to summation of i.j.ps, prolonged hyperpolarization, and sustained inhibition of contractile activity (e.g. Thornbury *et al.*, 1991). At least one K channel has been shown to be activated by NO stimulation (Thornbury *et al.*, 1991), but the transduction mechanism that causes this effect is unknown. In vascular smooth muscles, NO binds to a haeme group and activates soluble guanylate cyclase (Craven & DeRubertis, 1978). The production of guanosine 3':5'-cyclic monophosphate (cyclic GMP), and perhaps phosphorylation of cellular proteins by cyclic GMP-dependent protein kinase, is thought to transduce the NO signal and produce relaxation of smooth muscle cells (Rapoport & Murad, 1983).

Previous studies have also shown that electrical field stimulation evokes cyclic GMP formation in the lower oesophageal sphincter (Torphy *et al.*, 1986), and it is well known that elevation of cyclic GMP causes relaxation of a variety of smooth muscles (e.g. Barnette *et al.*, 1989; Katsuki *et al.*, 1977; Rattan & Moumami, 1988). Recent evidence has shown that cystamine and methylene blue can block hyperpolarization responses to sodium nitroprusside and electrical field stimulation in the opossum oesophagus (Du & Conklin, 1992). These observations suggest that cyclic GMP formation may mediate responses to NO (and therefore enteric inhibitory nerve responses) in gastrointestinal muscles. In the present study this hypothesis was tested by measuring the electrical and mechanical effects of elevation of cyclic GMP with the membrane permeable form of cyclic GMP, 8-bromo cyclic GMP and by delaying the metabolism of cyclic GMP with a specific cyclic GMP phosphodiesterase inhibitor, M&B 22948 (Weishaar *et al.*, 1986). The effects of enteric inhibitory nerve stimulation, inhibitors of NO synthesis, and exogenous NO on cyclic GMP levels in muscles of the canine proximal colon were also examined.

Methods

Mongrel dogs of either sex were anaesthetized with sodium pentobarbitone (45 mg kg⁻¹). The abdomen of each animal was opened and a segment of proximal colon, 4–12 cm from the ileocolonic sphincter, was removed. The colonic segment was opened along the mesenteric border and faecal material was removed by washing with Krebs-bicarbonate solution.

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The resulting sheet was pinned out in a dissecting dish. Muscle strips (1 mm by 10 mm) were cut parallel to the longitudinal muscle fibres for electrophysiological studies (see Smith *et al.*, 1987a,b) and parallel to the circular fibres for mechanical/biochemical studies, and mucosal tissues were removed. The muscles were maintained in Krebs-bicarbonate solution at $37.5 \pm 0.5^\circ\text{C}$. The Krebs-bicarbonate solution used in this study contained (in mM): NaCl 110, KCl 4.6, CaCl_2 2.5, NaHCO_3 24.8, KH_2PO_4 1.2, MgSO_4 1.2 and glucose 5.6. When equilibrated with 95% O_2 /5% CO_2 , this solution had a pH of 7.3–7.4. Atropine, phentolamine and propranolol (all at 10^{-6} M) were routinely added to block muscarinic receptors and adrenoceptors ('NANC solution'). In the presence of these agents, responses elicited by electrical field stimulation (EFS) were blocked by tetrodotoxin (10^{-6} M) and were therefore regarded as enteric inhibitory nerve responses.

Electrophysiology experiments

The electrophysiological chamber was continuously perfused with Krebs-bicarbonate solution, and muscles were allowed to equilibrate for at least 2 h before intracellular recordings were initiated. Smooth muscle cells near either the submucosal or myenteric borders of the circular muscle layer were impaled with glass microelectrodes to measure enteric inhibitory nerve responses (Smith *et al.*, 1987a,b). Transmembrane potential was measured with a standard electrometer (WPI M-7000), and outputs were displayed on an oscilloscope. Signals were recorded on magnetic tape and chart paper. Electrical field stimulation (EFS) was delivered as square wave pulses (0.5 ms duration, supramaximal voltage at a variety of frequencies) from a Grass S44 stimulator coupled via a stimulus isolation unit (Grass SIU5) to platinum wire electrodes placed on either side of the muscle strips.

Mechanical and biochemical experiments

Muscle strips, cut parallel to the circular fibres, were mounted in jacketed tissue baths under a resting tension of 1 g and allowed to equilibrate for 60–90 min. This degree of resting tension has been shown to produce optimal tension in canine colonic circular muscles (Keef *et al.*, 1991). During the equilibration period, some muscles developed spontaneous phasic contractions and others were mechanically quiescent. EFS (0.5 ms, supramaximal voltage at selected frequencies) was performed in a similar manner to that used in electrophysiological experiments. The Krebs-bicarbonate solution bathing the preparations was changed every 15 min. Mechanical responses were recorded with force transducers (Grass FT03) and a polygraph chart recorder. Mechanical responses were tabulated by measuring the area under the traces for a 3 min period before and one minute after addition of drugs. Effects are expressed as percentage change from control activity per minute.

To assay cyclic GMP levels, muscles were snap frozen by quickly removing the tissue holder from the tissue bath and freeze clamping the tissue with flat tongs that had been cooled in liquid N_2 . Frozen muscles were stored at -85°C and later assayed for cyclic GMP content (see below).

In some experiments the muscle strips were bisected, creating strips consisting of the submucosal and myenteric halves of the circular muscle layer. The myenteric strips contained the myenteric plexus and the longitudinal muscle layer, and submucosal strips contained submucosal elements including enteric ganglia as confirmed histologically. These 'isolated region' muscle strips were used to measure cyclic GMP levels in response to nitric oxide (NO) or EFS. These muscles were also used to compare the inhibitory effects of NO on contractions of the 2 regions. These muscles were pre-stimulated with ACh (3×10^{-7} M; in the presence of tetrodotoxin, 10^{-6} M), and the effects of various concentrations of NO on phasic contractions were studied.

Cyclic GMP determination

Cyclic GMP was assayed by an enzyme immunoassay method (Caymen Chemical Company, Ann Arbor, MI, U.S.A.). Samples were prepared for assay by homogenization in 6% TCA with glass Duall tissue grinders followed by extraction with water-saturated diethyl ether. Aqueous phases were then lyophilized to dryness and resuspended in 1.0 M potassium phosphate buffer (pH 7.4) before addition to duplicate microtiter plate wells. Cyclic GMP levels in samples and standards were detected following competition between cyclic GMP and the acetylcholinesterase-linked cyclic GMP tracer for specific antiserum binding sites. The antiserum complex, linked to acetylcholinesterase, was used to cleave Ellmans reagent, and absorbance was measured at 412 nm. Cyclic GMP content of samples was determined from a standard curve constructed from determination of known amounts of cyclic GMP added to the plate. Levels of cyclic GMP are expressed as pmol cyclic GMP mg^{-1} protein (determined by method of Bradford, 1976). Duplicate variation in the cyclic GMP assay was less than 3%.

Drugs and active agents

Nitric oxide: Stock solutions of NO were prepared by bubbling ice-cold, deoxygenated (sonication under vacuum followed by purging with 100% N_2 gas) distilled water with NO gas (99% pure) to give a saturated solution (1–1.5 mM; Ignarro *et al.*, 1987). In tension experiments NO was delivered to the tissues by addition of the appropriate volume of stock solution directly to the tissue chamber. The stated concentrations of NO have not been corrected for breakdown and therefore may be somewhat overestimated. Addition of water alone instead of NO solution had no effect on electrical or mechanical activity.

Other drugs: L- N^G -nitroarginine methyl ester (L-NAME), L- N^G -monomethyl arginine (L-NMMA) and acetylcholine (ACh) (Sigma) were made in stock solutions at 10^{-1} M. Propranolol (Sigma) was obtained as the hydrochloride salt. Atropine (Sigma) was used as the sulphate salt and phentolamine (Ciba Geigy) as the mesylate salt. Substance P (Sigma) was dissolved in phosphate buffer (10^{-4} M). M&B 22948 (zaprinast; a gift from Rhone-Poulenc Rorer, Dagenham, England) was dissolved in 0.1 N NaOH at a concentration of 10^{-2} M. Methylene blue and tetrodotoxin (TTX) were also obtained from Sigma. LY-83583 (6-anilo-5,8-quinolinedione; CalBiochem) was dissolved in a stock solution (10^{-2} M). Stock solutions were diluted to desired concentrations with Krebs-bicarbonate solution.

Data analysis

Statistical significance of differences between the means of data groups was determined by Student's *t* test for paired or unpaired data, as appropriate.

Results

Control electrical and mechanical responses

Cells near the myenteric border had average resting membrane potentials (RMP) of -45 ± 2 mV ($n = 24$ preparations) and exhibited small spontaneous electrical oscillations as previously described (Smith *et al.*, 1987b). Electrical field stimulation (EFS) induced hyperpolarization or inhibitory junction potentials (i.j.ps).

Cells at the submucosal border had more negative RMPs, averaging -82 ± 2 mV, ($n = 12$) and exhibited spontaneous slow wave activity as previously described (cf. Smith *et al.*, 1987a). EFS reduced the amplitude of slow waves during stimulation, and a 'rebound excitation' followed the period of stimulation (Ward *et al.*, 1992a).

The majority of circular muscle strips were spontaneously

mechanically active and exhibited small phasic contractions at the same frequency as the electrical slow waves (i.e. 5–6 cycles per min). These contractions were often irregular in amplitude and rarely exceeded 10% of the maximum contractile amplitude produced with ACh (10^{-4} M; Keef *et al.*, 1991). EFS (5–20 Hz) in the absence of antagonists consistently gave rise to an excitatory contractile response ($n=9$). In the presence of muscarinic receptor and adrenoceptor antagonists ('NANC solution'), EFS inhibited spontaneous contractions.

Effects of 8-bromo cyclic-GMP

Muscles were exposed to the membrane permeable analogue of cyclic GMP, 8-bromo cyclic GMP (8-Br-cyclic GMP) to determine whether increasing cyclic GMP would mimic the effects of NO. 8-Br-cyclic GMP (10^{-3} M) caused hyperpolarization of cells near the myenteric border averaging 23 ± 3 mV (from -50 ± 3.8 mV to -73 ± 2.8 mV; $n=5$, $P<0.005$). Oscillatory electrical activity was superimposed upon the hyperpolarization, this activity increased in amplitude from an average of 9.2 ± 1.5 mV prior to the addition of 8-Br-cyclic GMP to 32 ± 4.0 mV in the presence of 8-Br-cyclic GMP ($P<0.005$; $n=5$). Although the absolute amplitude of oscillatory activity increased in the presence of 8-Br-cyclic GMP, the maximum depolarization level reached during oscillations was reduced (see Figure 1a), suggesting that 8-Br-cyclic GMP would inhibit phasic contractions (Barajas-Lopez & Huizinga, 1989). This type of electrical activity has also been observed in response to NO, the NO-thiol conjugate, S-nitrosocysteine, and during sustained EFS (Thornbury *et al.*, 1991).

8-Br-cyclic GMP did not significantly hyperpolarize cells near the submucosal border (Figure 1b), which may have been due to the negative RMPs of these cells (-83 ± 2 mV; $n=6$). 8-Br-cyclic GMP decreased the frequency (from 4.6 ± 0.2 cycles min^{-1} to 3.0 ± 0.3 cycles min^{-1} ; $n=6$; $P<0.005$) and duration of slow waves (from 4.3 ± 0.2 s to 2.9 ± 0.4 s; $n=6$; $P<0.05$; Figure 1b). The amplitude of slow waves was unchanged by this compound. The effects of 8-Br-cyclic GMP persisted for at least 20–30 min after removal of the drug from the bath.

Figure 1 Effect of 8-Br-cyclic GMP (8-Br-cGMP) on electrical activities of a cell near the myenteric border (a) and a cell near the submucosal surface (b). In the myenteric cell, membrane potential was about -50 mV and small electrical oscillations were superimposed. Addition of 8-Br-cyclic GMP (at arrow, 10^{-3} M) caused a large hyperpolarization and larger amplitude oscillations were superimposed. Despite the large amplitude of these events, at their peaks they did not reach even the former resting potential level. (b) Shows slow waves recorded from a submucosal cell. Addition of 8-Br-cyclic GMP (10^{-3} M) had little or no effect on resting potential but shortened the duration of slow waves.

A separate series of experiments tested the effects of 8-Br-cyclic GMP (10^{-4} M) on the contractile activity of circular muscle strips. 8-Br-cyclic GMP (10^{-4} and 10^{-3} M) inhibited contractile activity in these muscles by an average of $51 \pm 12\%$ ($n=5$) and $86 \pm 3\%$ ($n=6$), respectively. The effects of 8-Br-cyclic GMP were persistent, and at least 30 min were required after removal of the drug (10^{-3} M) for full restoration of control activity. Figure 2 shows the inhibitory effects of 8-Br-cyclic GMP on contractile activity.

Effects of M&B 22948

In cells near the myenteric border M&B 22948 (10^{-4} M) caused hyperpolarization averaging 11 ± 4 mV (i.e. from -41 ± 1.9 mV under control conditions to -52 ± 4 mV after the addition of M&B 22948; $n=8$, $P<0.05$). In the 2 preparations, where M&B 22948 produced the largest hyperpolarizations, electrical oscillations were superimposed upon the hyperpolarizations (Figure 3a). These events were similar to the responses caused by nitric oxide, S-nitrosocysteine, and EFS at 1 Hz (see Thornbury *et al.*, 1991). At the submucosal border M&B 22948 (10^{-4} M) did not significantly alter resting membrane potential, slow wave upstroke and plateau amplitude, slow wave duration or frequency ($n=4$; Figure 3b).

Figure 2 Effects of 8-Br-cyclic GMP on mechanical activity of colonic muscles. Spontaneously active muscles were exposed to 10^{-4} to 10^{-3} M 8-Br-cyclic GMP for 10 min. Representative responses to 3 concentrations are shown. 8-Br-cyclic GMP reduced the amplitude of contractions in a concentration-dependent manner. Panels to right of each trace show restoration of control activity after wash periods noted in figure.

Figure 3 Effect of M&B 22948 (10^{-4} M) on electrical activities of cells near myenteric (a) and submucosal (b) boundaries of the circular muscle layer. Addition of M&B 22948 in (a) caused a large hyperpolarization similar to the effects of 8-Br-cyclic GMP. Large amplitude oscillations were superimposed upon the hyperpolarization response. M&B 22948 had little or no effect on slow waves at the submucosal border (b). All effects were reversible upon washout of the drug.

I.j.ps were elicited by trains of stimuli (1–10 pulses at 20 Hz) before and after incubation with M&B 22948 (10^{-4} M; 10 min). M&B 22948 significantly increased the duration of i.j.ps (e.g. from 3.5 ± 0.24 s to 10.8 ± 1.9 s with 6 pulse trains (0.5 ms at 20 Hz) $n = 6$; $P < 0.05$; see Figure 4). There was not a significant change in the absolute amplitude of i.j.ps in the presence of M&B 22948, but this result is somewhat difficult to interpret since this compound also hyperpolarized membrane potential. M&B 22948 also caused a change in the shape of i.j.ps (see Figure 4a). After M&B 22948, i.j.ps, reached a maximum level of hyperpolarization, partially repolarized, and then settled into a sustained 'plateau' hyperpolarization. Repolarization occurred in 2 phases, an initial rapid component, followed by a slow component with a time-constant of several seconds.

M&B 22948 (10^{-4} M) essentially abolished mechanical activity in intact strips of muscle ($n = 6$). This effect persisted while the drug was present, but was reversible upon washout.

Effects of methylene blue

Others have shown that methylene blue inhibits the soluble guanylate cyclase in smooth muscles (Ignarro *et al.*, 1986). Methylene blue (10^{-5} to 10^{-4} M) added for up to 1.5 h did not significantly affect membrane potential in cells near the myenteric border (-46 ± 3 mV to -41 ± 2 mV; $100 \mu\text{M}$) or i.j.p. amplitude and duration (i.e. 29 ± 4 mV to 27 ± 3 mV and 4.0 ± 0.4 s to 4.0 ± 0.4 s, respectively; P values > 0.5 ; $n = 7$), (Figure 5). We also tested the effects of LY-83583 (10^{-5} M; an inhibitor of soluble guanylate cyclase; Mulsch *et al.*, 1988) on i.j.ps. In 5 preparations, this compound failed to reduce i.j.p. amplitude or duration.

In addition to a lack of effects on i.j.ps, methylene blue (10^{-4} M) also had no significant effect on hyperpolarization

Figure 4 Effects of M&B 22948 on inhibitory junction potentials (i.j.ps). In (a) i.j.ps were elicited in a cell near the myenteric border by electrical field stimulation. Trace shows responses to short trains consisting of the number of pulses indicated. Addition of M&B 22948 (10^{-4} M) caused hyperpolarization (about 7 mV in this muscle) and an increase in the duration of the i.j.ps. (b) Shows a summary plot of i.j.p. duration as a function of the number of pulses in the stimulus train ($n = 6$): (○) control; (●) M&B 22948 (10^{-4} M).

Figure 5 Effects of methylene blue on i.j.ps; (a) shows continuous recording after exposure to methylene blue (10^{-4} M). In this cell methylene blue caused depolarization. Methylene blue did not significantly affect the amplitude or duration of i.j.ps. (see text for averaged values). (b) Methylene blue did not affect hyperpolarization induced by nitric oxide.

responses to NO ($5 \mu\text{l}$ bolus application to recording chamber; $n = 4$). In these experiments resting potential averaged -45 ± 2.1 mV and -43 ± 2.5 mV before and in the presence of methylene blue, respectively. NO caused an average 27 ± 2.5 mV hyperpolarization before methylene blue and 28.3 ± 1.4 hyperpolarization in the presence of the drug.

The effects of methylene blue were not tested at the sub-mucosal border, because we have previously shown that this compound depolarizes these cells due to non-specific effects, making interpretation of results very difficult (Sanders *et al.*, 1989). Methylene blue had variable effects on mechanical activity, which may have been due to the non-specific actions of this compound.

Effect of NO on cyclic GMP levels and mechanical activity

NO (10^{-5} M; muscles taken for assay 15 s after addition of NO) significantly increased cyclic GMP content (Figure 6) and caused concentration-dependent relaxation of colonic muscles (Figure 7).

Figure 6 Effects of NO on cyclic GMP levels in colonic circular muscles. Cyclic GMP was measured in isolated strips dissected from the myenteric and submucosal halves of the circular layer. NO (10^{-5} M) caused a significant increase in cyclic GMP in both regions ($P < 0.05$).

Figure 7 Effects of NO on contractile activities of intact circular muscle strip (a), submucosal muscle strip (b), and isolated myenteric muscle strip (c). Muscles were prestimulated with acetylcholine (3×10^{-7} M), and tetrodotoxin (10^{-6} M) was included in the bath solution. In each panel addition of NO (3×10^{-7} M) caused an interruption in the normal pattern of contractile activity. In (b), as the inhibitory effects of NO diminished there is a rebound contraction; (d), shows concentration-response relationships for myenteric (▼), submucosal (■) and intact (●) circular muscles. Data are averages and s.e.mean (vertical bars) from 6 experiments for each curve.

In some experiments muscles were bisected into the myenteric and submucosal regions (see Methods) and changes in cyclic GMP levels in each region in response to NO were assayed. Figure 6 shows that NO produced significant and similar increases in cyclic GMP levels in both myenteric and submucosal regions. NO also inhibited contractile activity in both regions (Figure 7). The concentration-response curve for the effects of NO on intact muscles was shifted to the right with respect to the responses of isolated myenteric and submucosal muscles, because NO may not have penetrated the intact muscles as completely before it was inactivated.

Effect of EFS on cyclic GMP levels

EFS (5 Hz, 0.5 ms, supramaximal voltage) for 15 s caused cessation of mechanical activity in intact muscle strips. Assay of cyclic GMP in intact muscles failed to detect significant changes in cyclic GMP levels in response to EFS. We also tested the effects of EFS on regional muscle strips consisting of: (i) the circular muscle along the submucosal surface of the circular layer, and (ii) circular muscle near the myenteric border. After equilibration and in the presence of the 'NANC solution', phasic contractions were stimulated with substance P (10^{-7} M). After 5 min in substance P, EFS (5 Hz,

0.5 ms, supramaximal voltage) was applied for 15 s. Cyclic GMP levels were elevated by EFS (Table 1). The increase in cyclic GMP produced by EFS was enhanced by M&B 22948.

Effect of L-NAME on spontaneous activity

Exposure of intact muscles to the NO-synthase inhibitor, L-NAME (10^{-4} M) significantly enhanced the amplitude of spontaneous contractions by an average of $256 \pm 46\%$ ($n = 8$; $P < 0.05$; Figure 8). L-NAME reduced cyclic GMP levels in 4 of 6 muscles studied (Figure 8).

After inhibition of spontaneous contractile activity with M&B 22948, L-NAME (10^{-4} M; $n = 3$) also reversed the effects of M&B 22948 by more than 95% (Figure 9). When

Table 1 Effects of NANC nerve stimulation on cyclic GMP levels in colonic muscles

Tissues	Control	NANC stimulation
Myenteric		
Basal	177 ± 75	392 ± 50
+ M&B22948	75 ± 43	257 ± 92
Submucosal		
Basal	117 ± 33	242 ± 52
+ M&B22948	81 ± 9.6	391 ± 115

Cyclic GMP was measured in substance P-stimulated segments of colonic smooth muscle with or without NANC nerve stimulation (5 Hz) in the presence and absence of the cyclic GMP-phosphodiesterase inhibitor M&B 22948 (10^{-4} M) as described in Methods. Contraction was monitored and tissues frozen in Liq. N₂ at 15 s following EFS or at matched times for control tissues. Values are expressed as the mean $\pm$ s.e. of three experiments.

* Significant difference from control: $P \leq 0.05$. **Significant difference from control: $P \leq 0.03$.

Figure 8 Effects of L-N^G-nitroarginine methyl ester (L-NAME) on spontaneous contractions and cyclic GMP levels: (a) shows effects of L-NAME (10^{-4} M) on the average spontaneous contractile amplitude; see example trace below graphs. L-NAME increased the force of phasic contractions from an average amplitude of 312 ± 111 mg to 752 ± 253 mg ($n = 8$). In another series of experiments, muscles were frozen for cyclic GMP assay before and 25 min after exposure to L-NAME (10^{-4} M; b). L-NAME decreased cyclic GMP in 4 of 6 of the muscles studied. Data suggest that some muscles may have spontaneous release of NO and therefore elevated levels of cyclic GMP. Block of NO synthesis can decrease production of cyclic GMP and decrease tonic inhibition.

Figure 9 L-N^G-nitroarginine methyl ester (L-NAME) prevents or reverses the effects of M&B 22948: (a) shows spontaneous contractile activity enhanced by L-NAME. After L-NAME, M&B 22948 caused much less reduction in contractile amplitude than in muscles untreated by L-NAME (see Figure 5 and b). (b) Shows inhibition of effects by M&B 22948. L-NAME reversed the inhibitory effect of M&B 22948. These data suggest that effects of M&B 22948 depend upon NO synthesis, and suggest that NO effects are mediated via cyclic GMP.

M&B 22948 (10^{-4} M) was added after treatment with L-NAME ($n=4$) contractile amplitude was reduced by $33 \pm 4\%$ (Figure 9a), whereas in muscles untreated with L-NAME, this concentration of M&B 22948 abolished mechanical activity (Figure 9b and see section above on M&B 22948).

Discussion

The results of this study provide additional support for the hypothesis that NO is an essential component of enteric inhibitory neurotransmission in the canine gastrointestinal tract. Previous studies showed that EFS increased cyclic GMP levels in the bovine retractor penis muscle (Bowman & Drummond, 1984), corpus cavernosum smooth muscle (Ignarro *et al.*, 1990), and oesophageal muscles (Torphy *et al.*, 1986). The present results add to this concept, suggesting that cyclic GMP may be involved in transducing enteric inhibitory neurotransmission in the proximal colon. This idea is supported by the following: (i) NO inhibited mechanical activity (we showed previously that NO hyperpolarized membrane potential; see Thornbury *et al.*, 1991) and elevated cyclic GMP, (ii) cyclic GMP (delivered as its membrane permeable analogue) mimicked the hyperpolarization and mechanical effects of NO and EFS, (iii) M&B 22948, a selective cyclic GMP-phosphodiesterase inhibitor, inhibited mechanical activity, hyperpolarized RMP, and prolonged i.j.ps. These observations satisfy several of the major criteria necessary for establishing cyclic GMP as a second messenger in enteric inhibitory neurotransmission in the canine colon (see discussion in Conklin & Du, 1992).

Few studies have previously shown an increase in cyclic GMP during EFS in GI muscles. We found that it was difficult to demonstrate changes in cyclic GMP in strips of colonic muscle consisting of the full thickness of the muscularis. This may have been due to a localization of the elevation of cyclic GMP within cells or tissues. We have previously reported that the enteric inhibitory innervation is functionally directed toward specific 'pacemaker regions' within the muscularis of the canine proximal colon (Smith *et al.*, 1989), and recent morphological studies have demonstrated nitric oxide synthase-like immunoreactivity in nerves near the submucosal and myenteric pacemaker regions (Ward *et al.*, 1992c). Changes in cyclic GMP were demonstrable in full thickness muscles when exogenous NO was applied (e.g. we observed a 2.5 fold change in cyclic GMP levels), but it was more difficult to demonstrate significant changes in cyclic GMP levels when nerves were activated. This suggests the possibility that NO released from nerves may reach only a fraction of the effector cells. As we have discussed previously, inhibitory innervation of the pacemaker regions is

sufficient to reduce the amplitude and durations of slow waves and therefore to reduce the amplitude of phasic contractions throughout the circular muscle layer (Smith *et al.*, 1989). Because responses to NO released from nerves could be specifically directed at pacemaker regions, we also analyzed cyclic GMP changes in muscles dissected from the pacemaker regions. In these strips EFS produced significant elevation in cyclic GMP levels and these effects were augmented by M&B 22948.

Different basal levels of cyclic GMP were observed in experiments that tested effects of NO versus EFS. There are 2 possible explanations for these differences in basal cyclic GMP: (i) the EFS experiments were performed in the presence of substance P which may lower cyclic GMP levels; (ii) cyclic GMP was normalized against protein content of samples and the relative protein content of the thin (regional) muscle strips may have been different from that in full-thickness or bisected muscle strips (as used in experiments depicted in Figures 6 and 8).

Others have reported that methylene blue, which is known to inhibit soluble guanylate cyclase in smooth muscles (Ignarro *et al.*, 1986), reduces enteric inhibitory nerve-mediated responses in gastrointestinal muscles (Conklin & Du, 1992; Huizinga *et al.*, 1992). In contrast we found that methylene blue had no effect on i.j.ps. These results seem contradictory to the involvement of guanylate cyclase in enteric inhibitory nerve responses, but there are also other explanations for the lack of effects of methylene blue as discussed below.

Cyclic GMP is produced by guanylate cyclases (for review see Tremblay *et al.*, 1988), and several investigators have shown that the enhancement in cyclic GMP levels resulting from stimulation with NO is due to activation of guanylate cyclase (e.g. Mittal & Murad, 1977). Methylene blue has been used frequently as a tool to determine whether cyclic GMP is involved in the transduction of agonist responses because it can inhibit the soluble isoforms of guanylate cyclase (Ignarro *et al.*, 1986). The pharmacology of methylene blue is complex and in some tissues other effects may accompany the inhibition of cyclic GMP formation. For example, we have shown that methylene blue (10^{-5} M) depolarizes colonic muscle cells near the submucosal surface of the circular muscle layer (Sanders *et al.*, 1989). This effect does not appear to be due to an inhibition of spontaneous cyclic GMP production because, in the present study, we found that M&B 22948 (which would tend to preserve spontaneously produced cyclic GMP) had little effect on cells near the submucosal border. Huizinga and co-workers (1992) have reported that methylene blue reduces nerve-mediated relaxations of colonic muscle strips but their data are less than convincing because: (i) methylene blue (used at 5×10^{-5} M) caused a significant potentiation of contractions (which is consistent with our finding that methylene blue depolarizes these muscles), and (ii) it is difficult to tell whether the failure of nerve stimulation to block contractions was due to a block of cyclic GMP formation or because there was insufficient inhibitory transmitter released by nerve stimulation to reverse the depolarization caused by methylene blue. In the present study we examined the effects of methylene blue on cells near the myenteric border of the circular layer because the membrane potentials of these cells are not greatly affected by this compound (Sanders *et al.*, 1989). Methylene blue did not significantly affect i.j.ps during exposures of up to 1.5 h.

It is also possible that in some preparations NO effects could be mediated by particulate forms of guanylate cyclase, which are not inhibited by methylene blue. For example, Horio & Murad (1991) have recently purified a particulate form of guanylate cyclase from retinal rod outer segments that is activated by sodium nitroprusside and NO. Others have also reported activation of particulate forms of G cyclase by NO and SNP (Lad & White, 1979; Waldman *et al.*, 1982), although one has to be somewhat cautious about some of the earlier studies because it is possible that a small

amount of soluble guanylate cyclase could have been trapped in the preparation of the particulate form of the enzyme (see Tremblay *et al.*, 1988).

At present the mechanisms by which cyclic GMP causes cellular responses in colonic smooth muscles is not fully understood. The electrical responses are likely to be due to changes in membrane conductance and cyclic GMP is known to regulate ion channels in other cells either directly (Fesenko *et al.*, 1985) or via activation of cyclic GMP-dependent protein kinase (Paupardin-Tritsch *et al.*, 1986). The relaxation response could be mediated by mechanisms in addition to the electrical effects. For example, cyclic GMP-dependent mechanisms affect the Ca^{2+} sensitivity of contractile proteins in gastrointestinal muscles (e.g. Ozaki *et al.*, 1992), and the rate of Ca^{2+} uptake or extrusion may also be regulated by cyclic GMP (Lincoln, 1989).

In summary, our findings are consistent with the

hypothesis that cyclic GMP may be a second messenger that transduces the enteric inhibitory transmitter signal into i.j.ps and relaxation in the proximal colon. There is growing evidence that at least a portion of the enteric inhibitory signal arises from release of NO, or a related compound. NO increased cyclic GMP levels in colonic muscle tissues and produced hyperpolarization and relaxation. Some pharmacological tools known to affect levels of cyclic GMP altered electrical and mechanical responses in ways consistent with mechanisms involving cyclic GMP.

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