British Journal of Pharmacology (1997) 122, 1223–1229

Effects of a novel guanylate cyclase inhibitor on nitric oxide-dependent inhibitory neurotransmission in canine proximal colon

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1 Previous studies suggested that nitric oxide (NO) may cause hyperpolarization and relaxation of canine colonic smooth muscle by both cGMP-dependent and cGMP-independent mechanisms. This hypothesis was tested using 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), a novel inhibitor of NO-stimulated guanylate cyclase.

2 In the presence of histamine (30 μM), atropine and indomethacin (both at 1 μM), electrical field stimulation of intrinsic neurons (EFS; 5 Hz) produced inhibition of phasic contractile activity that is due to NO synthesis. ODQ caused a concentration-dependent block of this response (10 nM to 10 μM).

3 Inhibitory junction potentials (IJPs) due to NO synthesis were recorded from muscle cells located near the myenteric border of the circular muscle layer, using intracellular microelectrodes. IJPs were abolished by ODQ (1–10 μM).

4 EFS (10–20 Hz) produced frequency-dependent inhibition of electrical slow waves recorded from cells located near the submucosal surface of the circular muscle layer. This inhibition is due to NO synthesis, and it was abolished by ODQ (1–10 μM).

5 Hyperpolarization and relaxation produced by an NO donor, sodium nitroprusside, were abolished by ODQ pretreatment (1–10 μM). In contrast, inhibitory responses to 8-Br-cGMP (1 mM) were unaffected by ODQ.

6 ODQ alone (1–10 μM) had no significant effect on spontaneous electrical or phasic contractile activity. In tissues pre-treated with L-NAME (300 μM), ODQ decreased the amplitude of spontaneous or histamine-stimulated phasic contractile activity.

7 These results suggest that electrical and mechanical effects of endogenously released and exogenously applied NO in canine colon are largely due to cGMP synthesis by ODQ-sensitive soluble guanylate cyclase. No evidence to support a direct (cGMP-independent) mechanism of NO action was found. ODQ also appears to cause a non-specific inhibition of muscle contractile activity; however, this effect does not contribute to block of NO-dependent effects.

Keywords: Nitric oxide; cyclic GMP; ODQ; guanylate cyclase; colon; gastrointestinal motility; enteric nervous system; sodium nitroprusside

Introduction

As a result of studies over the last 30 years, it has become clear that inhibitory neuromuscular transmission to gastrointestinal smooth (GI) muscle is mediated by a combination of inhibitory transmitters. In most tissues, responses to inhibitory nerve stimulation represent the summation of responses to multiple transmitters, and the relative contribution of each can be estimated by pharmacological dissection. Evidence has suggested that ATP, certain peptides (e.g. vasoactive intestinal peptide, pituitary adenylate cyclase activating peptide) and nitric oxide (NO) serve as inhibitory neurotransmitters. The circular muscle of the canine proximal colon is somewhat unique in that under most experimental conditions inhibitory transmission is completely abolished by nitric oxide synthase (NOS) inhibitors. This feature makes the canine colon a useful preparation to study mechanisms of NO-mediated enteric neurotransmission (see Sanders & Ward, 1992; Shuttleworth & Sanders, 1996).

A commonly described mechanism of NO action is stimulation of soluble guanylate cyclase, which increases intracellular cGMP concentration and leads to phosphorylation of various cellular targets by G kinase (Moncada *et al*., 1991; Bredt & Snyder, 1992; Lincoln & Cornwell, 1993; Garthwaite & Boulton, 1995a). Several observations indicate that NO stimulates guanylate cyclase in gastrointestinal smooth muscles: (1) NO and electrical stimulation of intrinsic neurons increase tissue cGMP levels (Torphy *et al*., 1986; Ward *et al*., 1992a; Chakder & Rattan, 1993; Suthamnatpong *et al*., 1993), (2) immunohistochemical methods have shown that NO stimulation increases cGMP levels in smooth muscle cells within intact muscle preparations (Shuttleworth *et al*., 1993b), and (3) NO increases cGMP levels in isolated smooth muscle cells (e.g. Jin *et al*., 1993). Pharmacological evidence that cGMP mediates the actions of NO in gastrointestinal smooth muscle includes: (1) membrane-permeable cGMP analogues mimic the actions of NO, causing membrane hyperpolarization, inhibition of phasic electrical activity and inhibition of contractions (Barnette *et al*., 1989; Ward *et al*., 1992a), (2) membrane-permeable cGMP analogues activate at least three types of K^+ channels in isolated myocytes, and current via these channels could mediate the hyperpolarization and inhibition of electrical activity observed in intact muscles (Thornbury *et al*., 1991; Koh *et al*., 1995), and (3) an inhibitor of cGMP-selective phosphodiesterase (zaprinast) caused hyperpolarization and prolonged NO-dependent inhibitory junction potentials (IJPs) due to release of NO from enteric neurons (Ward *et al*., 1992a).

Although this evidence is consistent with a major role for cGMP as a second messenger mediating NO-dependent responses, it is possible that a portion of the inhibitory responses could be due to additional mechanisms. This hypothesis is particularly important in light of recent reports that NO can directly activate K^+ channels in smooth muscles (Bolotina *et al*., 1994; Koh *et al*., 1995).

Previous studies have reported that inhibitors of soluble guanylate cyclase antagonize effects of neuronally-released NO

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in gastrointestinal smooth muscle (e.g. Conklin & Du, 1992; Huizinga *et al*., 1992; Meulemans *et al*., 1993; Yano *et al*., 1995; Rae & Muir, 1996). However, until recently, available agents which block soluble guanylate cyclase are either ineffective in GI smooth muscle or have significant non-specific actions which complicate their use. For example, the putative GC inhibitor LY83583 generates superoxide, breaks down NO and inhibits NO release (Mulsch *et al*., 1988; Barbier & Lefevre, 1992). Methylene blue is not a potent guanylate cyclase inhibitor, and has also been shown to inhibit NOS activity (Mayer *et al*., 1993; Luo *et al*., 1995). Methylene blue also causes a non-specific depolarization of canine colonic smooth muscle cells located at the submucosal border of the circular muscle layer, possibly due to blockade of K^+ channels (Sanders *et al*., 1989; Liu *et al*., 1993). Despite this problem, methylene blue has been tested on NO-dependent responses in canine proximal colon. Methylene blue did not block IJPs generated by release of NO from enteric neurons (Ward *et al*., 1992a), but it caused a slowly developing, partial inhibition of the inhibitory effect of NO on phasic contractions (Huizinga *et al*., 1992).

1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxaline-1-one (ODQ) was recently identified as a potent and selective inhibitor of NO-stimulated soluble guanylate cyclase (Garthwaite *et al*., 1995b). Unlike methylene blue and LY83583, this agent does not inhibit NOS activity, or generate superoxide radical. ODQ (1 nM – 1 μM) has been shown to block nerve-mediated relaxations of rabbit anococcygeus (Cellek *et al*., 1996) and at 1 – 10 μM, ODQ was reported to inhibit EDRF-stimulated relaxation of bovine pulmonary artery (Brunner *et al*., 1996). Young *et al*. (1996) showed that ODQ blocked actions of SNP and partially antagonized NO-dependent responses to nerve stimulation in mouse caecum longitudinal muscle, suggesting that neuronally-released NO could act in part by cGMP-independent mechanisms. In the present study, we have investigated the effects of ODQ on electrical and mechanical responses of canine colonic muscles to enteric nerve stimulation and exogenous NO donors to attempt to dissect the role of cGMP-dependent and -independent mechanisms in this tissue.

Methods

Mongrel dogs of either sex were obtained from vendors licensed by the U.S. Department of Agriculture. The use of dogs for these experiments was approved by the Institutional Animal Care and Use Committee. The animals were sacrificed with pentobarbitone sodium (100 mg kg^{-1}). The abdomen was opened and a segment of proximal colon 6 – 14 cm from the ileocaecal sphincter was removed. The colon was opened along the mesenteric border, washed of remaining fecal material, and pinned out in a dissecting dish containing oxygenated Krebs-Ringer bicarbonate (KRB) solution.

Mechanical experiments

Strips of muscle (approximately $1 \times 10 \times 1.3$ mm) were cut parallel to the circular muscle fibers and the mucosa was removed. These strips were attached to strain gauges (UFI 1030) and maintained in Krebs-bicarbonate buffer (KRB) at 37°C. Voltage outputs from the strain gauges were digitized and recorded using a computer-based acquisition system (Biopac MP100A, Biopac Systems, CA, U.S.A.). Records were displayed and analysed using Acknowledge software (v 3.2, Biopac Systems, CA, U.S.A.). A resting force of 1 g was applied to the strips before equilibrating for 90 – 120 min. Histamine (30 μM) was applied at 20 min intervals until stable responses, consisting of large amplitude phasic contractions (see Figures 1, 4), were observed. In order to emphasize nerve-mediated relaxations, the KRB in all experiments contained atropine (1 μM), and enteric inhibitory responses and responses to exogenous NO were elicited during stimulation with histamine (30 μM). Electrical field stimulation (EFS; 1 min trains of 0.5 ms duration pulses were delivered at 5 Hz, 15 V from a Grass S44 stimulator via platinum ring electrodes. Under these conditions, EFS produced tetrodotoxin-sensitive inhibition of phasic contractions during the period of stimulation. Contractile activity was assessed by calculating the area between the trace and a baseline corresponding to complete relaxation of the muscle strip. The baseline corresponding to complete relaxation was determined as the minimum level of tone of the preparation prior to the addition of histamine. This was verified as representing complete relaxation in preliminary experiments using a supramaximal concentration of sodium nitroprusside (SNP; 10 μM) in the absence of ODQ. The effectiveness of nerve stimulation was calculated by comparing the response during the 1 min recording period with contractile activity recorded 1 min prior to stimulation.

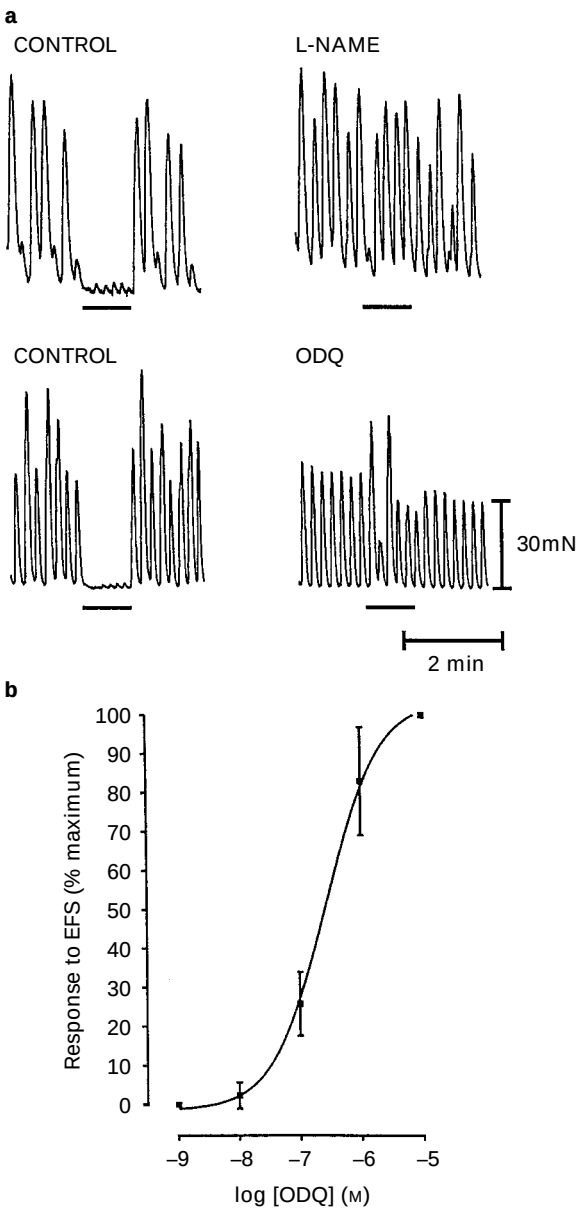

Figure 1 ODQ blocked NO-dependent inhibitory neurotransmission. (a) Effects of electrical field stimulation (EFS) on phasic contractile activity enhanced by histamine. The left traces are control responses to EFS (5 Hz; indicated by black bars). Phasic contractile activity was abolished during the stimulus. The response depended on NO synthesis, as it was blocked by L-NAME (300 μM; trace at top right). ODQ (1 μM, trace at bottom right) also blocked the inhibitory effects of EFS. L-NAME and ODQ were tested in muscles of several animals. (b) Summary of normalized data from eight experiments testing the effects of ODQ on EFS-stimulated inhibition of phasic contractile activity over a range of concentrations.

Intracellular electrical recording

Strips of muscle were pinned to the floor of an electrophysiological chamber and constantly perfused with warmed, oxygenated KRB maintained at 37°C. Muscle strips were allowed to equilibrate for 90–120 min before experiments were begun. Cells within the circular muscle layer were impaled with glass microelectrodes filled with 3 M KCl and having resistances of 30–50 M Ohm. Impalements were accepted based on previously discussed criteria (Sanders & Smith, 1986). EFS was applied from a Grass S88 stimulator via platinum wire electrodes placed on each side of the preparation. Membrane potential was measured with a high input impedance electrometer (WPI S-7100) and outputs were displayed on an oscilloscope (Tektronix 5111A). Analogue electrical signals were recorded on magnetic tape (Hewlett Packard 3964A) and reproduced on chart paper (Gould 2200). Analogue signals were also digitized and recorded using a computer-based system (Biopac Systems) as described above for mechanical data.

Circular smooth muscle cells located nearest to the longitudinal muscle layer typically have resting potentials of about −45 mV (Smith *et al*., 1987). Cells in this region of the circular muscle layer responded to field stimulation of enteric inhibitory neurons with large transient hyperpolarizations, referred to as inhibitory junction potentials (IJPs). The effects of drugs on IJPs was analysed by comparing IJP area, calculated as the area between the trace and a baseline corresponding to resting membrane potential.

Circular smooth muscle cells located near the submucosal surface of the circular muscle layer have more negative resting potentials (approximately −80 mV) and display regular spontaneous electrical slow waves in the absence of external stimulation (Smith *et al*., 1987). In this region, stimulation of inhibitory neurons causes a small degree of hyperpolarization and reduces the amplitude and duration of the slow waves (Ward *et al*., 1992b). Atropine (1 μM) was present throughout all electrophysiological experiments.

In all experiments, responses to nerve stimulation were calculated from digitized records using Acknowledge data analysis software (Biopac Systems, CA, U.S.A.). All data are expressed as mean±s.e.mean and significance of difference between groups was determined using Student's *t* tests.

Solutions and drugs

The Krebs-bicarbonate buffer (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 at 37°C when bubbled to equilibrium with 97% O_2/3% CO_2. 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxaline-1-one (ODQ) was obtained from Tocris Cookson Co. (MO, U.S.A.). L-arginine, L-N^G-nitroarginine methyl ester (L-NAME), L-Nitroarginine (L-NNA), atropine sulphate, histamine dihydrochloride, nifedipine were all obtained from Sigma Chemical Co. (MO, U.S.A.).

Results

Responses to neuronal release of NO

Strips of canine proximal colon circular muscle often show spontaneous phasic contractile activity, and the amplitude of this activity can be enhanced by excitatory agonists to serve as a background upon which to investigate inhibitory agonists and neurotransmission. With contractions enhanced by histamine (30 μM), electrical field stimulation (5 Hz, 0.5 ms, 1 min) inhibited phasic contractile activity. This response appeared to be due to neuronal release of NO as it was blocked by TTX (1 μM) and inhibitors of NO synthesis (i.e. L-NAME, 300 μM), as shown previously (Huizinga *et al*., 1992, Shuttleworth *et al*., 1993a). Pre-treatment of muscles with ODQ (1 nM to 10 μM) had no significant effect on histamine-stimulated contractions (see below) and caused a concentration-dependent antagonism of the inhibitory responses to EFS (Figure 1). ODQ abolished inhibitory effects of EFS and revealed a mean increase in phasic contractile activity during nerve stimulation (20.3±19.7% and 65.1±34.5% increase in phasic contractions by EFS compared to pre-stimulus contractions with 1 and 10 μM ODQ, respectively, $P<0.01$, $n=8$ for both). Figure 1b shows a summary of data from these eight experiments normalized against the maximal response to EFS observed in each strip. This data was used to calculate an apparent EC_{50} of 0.26±9.3 μM ($n=8$) for the effects of ODQ on responses to EFS under these conditions.

We also studied the effects of ODQ on inhibitory junction potentials (IJPs) recorded from circular muscle layer near the border with the myenteric plexus. Previous work has shown IJPs recorded from this region are NO-dependent and reduced or abolished by L-NAME (Dalziel *et al*., 1991). In the present study, EFS (single pulses 0.2 ms, 10 V, delivered at 0.03 Hz) produced IJPs with mean amplitude 20±2 mV from a mean resting potential of −48±1 mV ($n=7$). ODQ virtually abolished IJPs (to 3.0±0.6% and 1.9±0.8% of control values with 1 and 10 μM ODQ, respectively, $P<0.01$, $n=7$ (Figure 2b) without having any significant effect on resting membrane potential (RMP; −48±1 mV and −50±2 mV in control and ODQ, respectively, $P=0.23$, Ns, $n=7$).

Responses to neuronal release of NO were also assessed in intracellular recordings of slow waves from cells located near the submucosal border of the circular muscle layer. In the presence of atropine, EFS (10 pulses delivered at 1–20 Hz) produced a frequency-dependent inhibition of slow wave duration that, in two of eight experiments, was followed by a 'rebound' excitation manifest as a slow wave of greatly increased duration (Ward *et al*., 1992b). NOS inhibitors blocked nerve-stimulation inhibition and reveal a small, frequency-de-

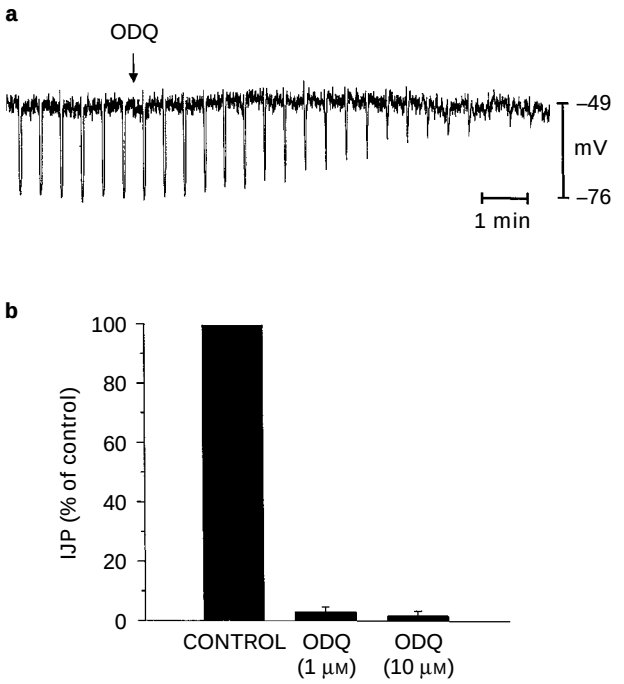

Figure 2 ODQ blocked inhibitory junction potentials (IJPs). (a) Continuous microelectrode recording from a smooth muscle cell near the myenteric border of the circular muscle layer. Single pulses of EFS were applied repetitively throughout the trace (at 0.03 Hz). Each pulse produced a large, transient hyperpolarization (IJP). Addition of ODQ (1 μM added at arrow), while maintaining the impalement, virtually abolished the IJPs. (b) Summary data from eight experiments in which the effects of 1 μM and 10 μM ODQ were tested sequentially on IJPs. IJPs were almost blocked by 1 μM ODQ and 10 μM ODQ had no significant additional effect.

pendent excitation during the stimulus which is related to the release of non-cholinergic excitatory transmitter(s) (Shuttleworth *et al*., 1993a). ODQ had no effect on unstimulated slow waves (1 μM, Table 1) but blocked EFS-evoked inhibition of electrical slow waves and unmasked a small degree of excitation during EFS at higher frequencies ($18.8 \pm 7.5\%$ excitation with ODQ 1 μM vs $57.4 \pm 3.9\%$ inhibition in control, 10 pulses at 10 Hz, $P<0.01$, $n=8$, Figure 3). Rebound excitation after EFS-induced inhibition of slow wave activity occurred in two of eight experiments, and this rebound was abolished by ODQ 1 μM.

Responses to sodium nitroprusside

As previously reported (e.g. Shuttleworth *et al*., 1995), SNP produced concentration-dependent inhibition of phasic contractile activity of circular muscle strips. Under control conditions, SNP (300 nM) reduced contractions by $46.5 \pm 7.4\%$. ODQ (1–10 μM) abolished the effects of SNP on contractile activity ($-2.9 \pm 5.9\%$ and $0.5 \pm 8.5\%$ inhibition of contractile activity, $P<0.01$ and $P<0.05$ for 1 and 10 μM ODQ, respectively, $n=7$; Figure 4). ODQ also antagonized effects of the exogenous SNP on electrical activity recorded from cells near the myenteric border of the circular muscle layer. The hyperpolarization response to SNP (1 μM) was reduced from 18 ± 2 mV to 3 ± 1 mV ($P<0.01$, $n=5$; Figure 5).

Responses to 8-Br-cGMP

Electrical and mechanical responses to NO can be mimicked by membrane permeable analogues of cGMP (Ward *et al*., 1992a). In the present study 8-Br-cGMP produced sustained inhibition of histamine-stimulated contractile activity, and these effects were not reduced by ODQ (i.e. $86.6 \pm 6.6\%$ reduction of phasic contractions under control conditions and $88.6 \pm 2.4\%$ reduction in the presence of 10 μM ODQ). In a series of intracellular recordings from cells near the myenteric border of the circular muscle, 8-Br-cGMP (1 mM) induced hyperpolarization averaging 18 ± 3 mV. This response was not affected by ODQ (i.e. 18 ± 2 mV of hyperpolarization in the presence of ODQ, 1 μM, $P>0.05$, $n=3$; Figure 5).

Effects of ODQ on spontaneous myogenic activity

Previous studies have provided evidence to suggest that basal release of NO exerts a tonic inhibitory influence on the circular muscle of the canine proximal colon. Part of the evidence comes from the observation that L-NAME exposure increases the amplitude of spontaneous and histamine-induced contractions (Ward *et al*., 1992a; Keef *et al*., 1994, 1997). In contrast to the actions of L-NAME, ODQ did not increase the amplitude of spontaneous and histamine-stimulated phasic contractions. In fact, ODQ (10 μM) alone produced a small (but not statistically significant) reduction in spontaneous contractions ($20.9 \pm 8.0\%$ inhibition of control activity, $P=0.13$, Ns, $n=8$) and histamine-stimulated contractions ($4.6 \pm 13.7\%$ inhibiton of control activity, $P=0.71$, Ns, $n=9$). When these experiments were performed in tissues pre-treated with L-NAME (300 μM), ODQ produced clear decreases in the amplitude of spontaneous and histamine-stimulated contractions. In the presence of L-NAME, ODQ (10 μM) caused a $70.6 \pm 7.5\%$ inhibition of spontaneous contractile activity ($P<0.05$, $n=8$) and a $41.5 \pm 5.6\%$ inhibition of contractions produced by histamine (30 μM, $P<0.01$, $n=8$).

ODQ did not affect any measured parameter of spontaneous electrical slow waves recorded from the submucosal border of the circular muscle layer. Furthermore, ODQ had no effect on the duration of slow waves stimulated with the calcium channel agonist Bay K 8644. The mean duration of slow waves in the presence of Bay K 8644 was 15.1 ± 2.7 s and 16.9 ± 4.4 s in the presence of ODQ (1 μM) and Bay K 8644 ($P=0.51$, Ns, $n=3$).

Table 1 Summary of experiments demonstrating that ODQ (10 μM) had no effect on electrical slow wave activity recorded at the submucosal border of the circular muscle layer ($n=5$)

	Control	*ODQ*
RMP (mV)	-82 ± 1	-82 ± 1
Peak amplitude (mV)	54 ± 1	50 ± 2
Plateau (mV)	46 ± 1	46 ± 2
Frequency (events/min)	4.0 ± 0.3	3.8 ± 0.3
Duration (s)	3.8 ± 0.3	3.7 ± 0.4

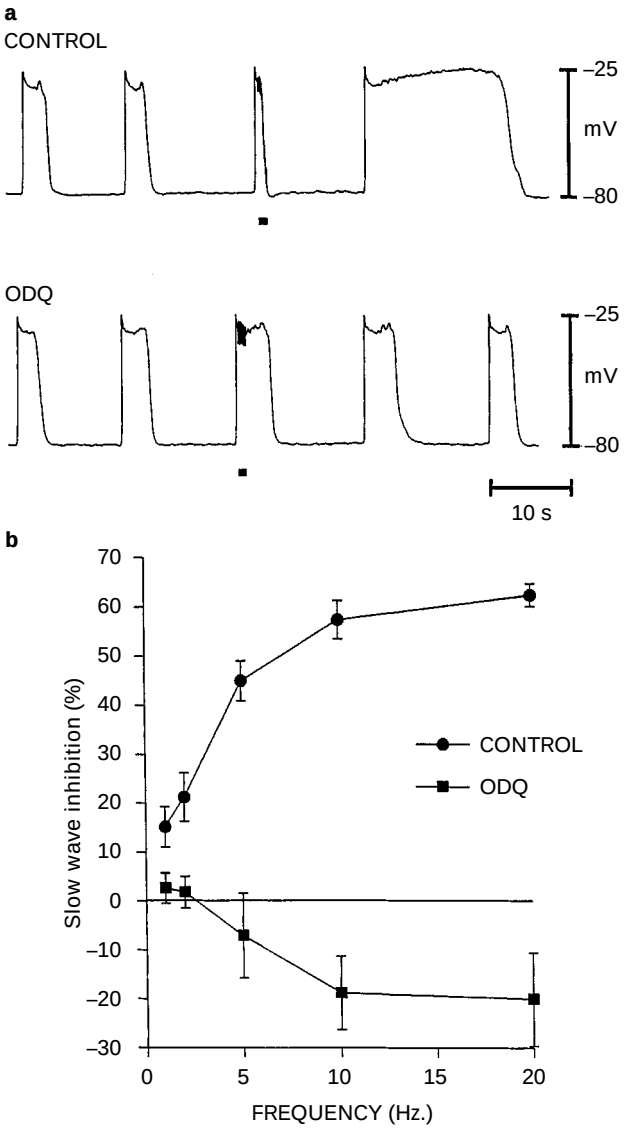

Figure 3 ODQ blocked EFS-stimulated shortening of slow waves. (a) Electrical slow waves recorded from a cell near the submucosal surface of the circular muscle layer. Under control conditions (top trace), EFS (10 Hz, 1 s, indicated by black bars) applied during the plateau phase of slow waves resulted in immediate repolarization and reduction in slow wave duration. In this example, the inhibitory response was followed by a large 'rebound' excitation, characterized by a slow wave of long duration. ODQ (1 μM), added while maintaining the same impalement, abolished both the inhibitory response to EFS, and the rebound excitation. (b) Summary data from nine similar experiments in which ODQ was tested on responses to a range of stimuli (i.e. 10 pulses delivered at 1–20 Hz). In control conditions (●), EFS produced a frequency-dependent inhibition of slow wave duration. In the presence of ODQ (■; 1 μM) inhibitory responses to 1 and 2 Hz were almost abolished, and at higher stimulus frequencies (5–20 Hz) a small increase in slow wave duration was observed when cGMP-dependent mechanisms were blocked.

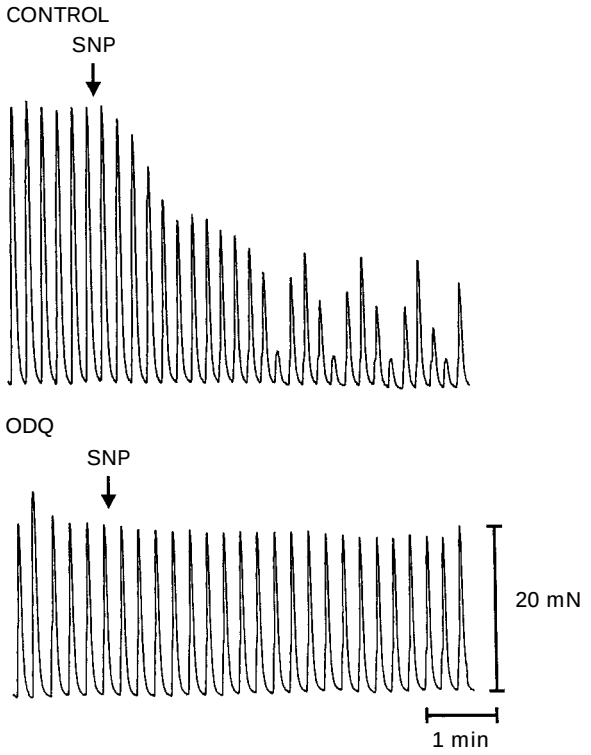

Figure 4 ODQ blocked the mechanical effects of an NO donor. The traces show a series of phasic contractions in the same muscle strip that were pre-stimulated by histamine (30 μM). L-NAME (300 μM) was present throughout. Under control conditions (top panel), SNP (1 μM) produced significant reduction in the force of the phasic contractions. Addition of ODQ (1 μM) blocked the inhibitory response to SNP.

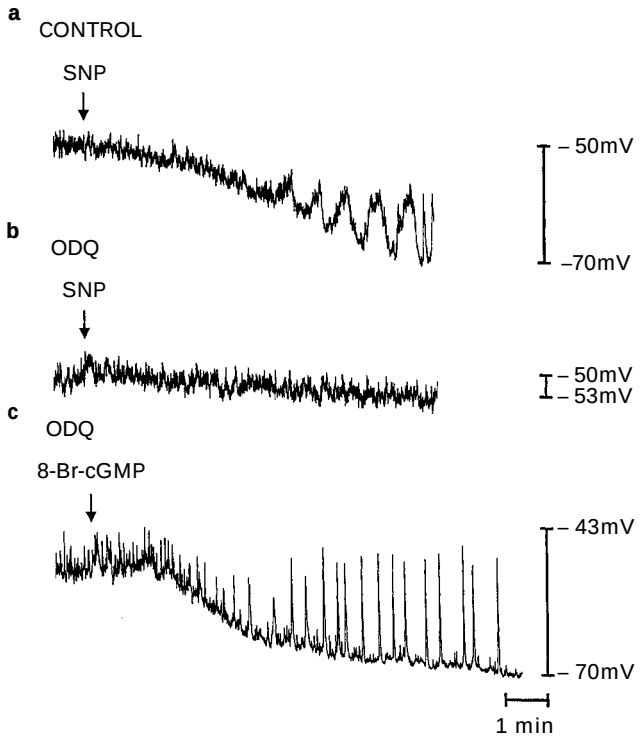

Figure 5 ODQ blocked the electrical effects of SNP but not of 8-Br-cGMP. All traces show microelectrode recordings from cells near the myenteric border of the circular muscle layer. (a) Under control conditions, SNP (1 μM) produced a hyperpolarization of resting membrane potential and initiated a series of rhythmic depolarizations (see Keef *et al.* (1997) for explanation of the rhythmic phenomenon induced by SNP). (b) In the presence of ODQ (1 μM), SNP had no significant effect on resting membrane potential. (c) In the presence of ODQ the membrane permeable cGMP analogue (8-Br-cGMP) still evoked a large hyperpolarization.

Discussion

Nitric oxide (NO) is a key mediator of inhibitory neurotransmission in the canine proximal colon; however, the mechanism(s) by which NO causes hyperpolarization and relaxation of colonic smooth muscle has been controversial. There is considerable evidence supporting the hypothesis that NO inhibits GI muscles by stimulation of soluble guanylate cyclase and elevation of intracellular cGMP. However, a major deficiency in testing this hypothesis has been the lack of agents which effectively inhibit guanylate cyclase (see Introduction). In the present study, we have shown that the guanylate cyclase inhibitor (ODQ) blocks the electrical and mechanical actions of NO. Inhibitory responses to neuronally-released and exogenously-applied NO were both blocked, while responses to 8-Br-cGMP were unaffected. In some preparations, NO-dependent inhibition of electrical slow waves was followed by a rebound excitatory event. ODQ blocked both inhibitory and excitatory phases of this response, consistent with previous suggestions that this rebound excitation is dependent on NO synthesis and action (see Ward *et al.*, 1992b).

In previous studies to test the involvement of cGMP in NO-dependent transmission, investigators have had to rely on the use of relatively weak guanylate cyclase inhibitors with a number of confounding non-specific actions. Huizinga *et al.* (1992) showed that methylene blue partially antagonized NO-dependent inhibition of contractile activity. This effect was accompanied by an increase in basal contractile activity which could be due to removal of basal cGMP synthesis. Unfortunately, in addition to non-specific actions on NOS and radical generation (see Introduction), methylene blue has direct non-specific actions on smooth muscle cells which could complicate these studies. When electrical slow waves are recorded at the submucosal border of the circular muscle layer, methylene blue causes a large, non-specific depolarization of smooth muscle cells which is unrelated to guanylate cyclase inhibition (Sanders *et al.*, 1989; Ward *et al.*, 1992a, but see also Liu *et al.*, 1993). Intracellular microelectrode recordings from smooth muscle cells located near the myenteric border of the circular muscle suggest that methylene blue does not selectively block the actions of NO in this tissue. Cells located in this region are already relatively depolarized (RMP approx -40 mV) and methylene blue has no effect on resting membrane potential, NO-dependent IJPs or hyperpolarization responses to exogenous NO (Ward *et al.*, 1992a). When taken together with the results of the present study, this suggests that methylene blue is not an effective inhibitor of guanylate cyclase in the canine proximal colon.

ODQ was effective at concentrations similar to those which abolish NO-stimulated cGMP formation in a variety of other tissues (Brunner *et al.*, 1995, 1996; Boulton *et al.*, 1995; Garthwaite *et al.*, 1995; Cellek *et al.*, 1996) and does not share with methylene blue its non-specific depolarization and contraction of muscle strips. In contrast, some inhibition of spontaneous contractile activity was noted in mechanical experiments. This is a noteworthy difference between the actions of ODQ and NOS inhibitors. Previous studies have shown that L-NAME exposure increases the amplitude of spontaneous and agonist-stimulated contractile activity and, together with other evidence, concluded that basal release of NO exerts a tonic inhibitory influence on colonic circular muscle (Ward *et al.*, 1992a; Keef *et al.*, 1994, 1997). In contrast, ODQ (10 μM) alone produced a small (but not statistically significant) reduction in spontaneous and histamine-stimulated contractions. When these experiments were performed in tissues pre-treated with L-NAME (100 μM), ODQ produced clear decreases in the amplitude of spontaneous and histamine-stimulated contractions.

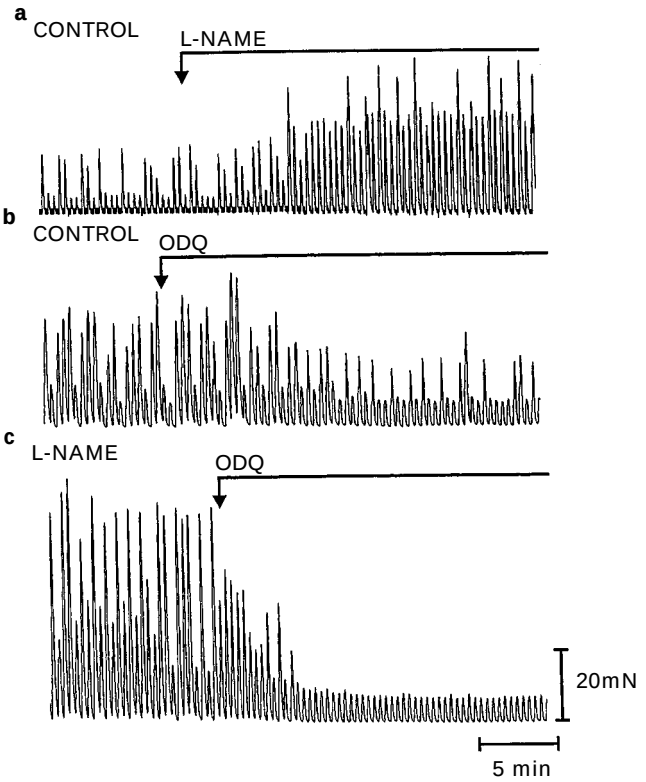

Figure 6 Spontaneous contractions were increased by L-NAME and decreased by ODQ. (a) L-NAME (300 μM) produced a sustained increase in the amplitude of spontaneous contractions in a circular muscle strip. This response is consistent with the hypothesis that muscle contractility is normally inhibited by basal release of NO (see Keef *et al*., 1997). (b) In contrast, ODQ (10 μM) caused a decrease in the amplitude of spontaneous contractions. (c) The inhibitory effect of ODQ was more clearly seen when tissues were pre-treated with L-NAME (300 μM).

This observation, combined with the results described above, suggests that ODQ may have two effects on spontaneous mechanical activity: (1) an excitatory effect resulting from block of actions of basally-released NO, and (2) a direct inhibitory effect of ODQ on smooth muscle excitability. The excitatory action of ODQ can be removed by pre-treating the tissue with L-NAME to reduce basal NO-stimulated cGMP levels in the muscle, thereby unmasking an inhibitory effect of ODQ on contractile force. The mechanism of the inhibitory action is currently unknown, but the fact that ODQ did not affect parameters of electrical slow waves raises the possibility that ODQ may have non-specific effects on excitation-contraction coupling.

It was recently reported that ODQ blocks NO-dependent inhibitory transmission in rabbit anococcygeus muscle (Cellek *et al*., 1996). Interestingly, these authors noted that ODQ alone produced an increase of approximately 10% in the basal tone of anococcygeus strips. This suggests that the nature of direct effects of ODQ on smooth muscle contractility may be dependent on the tissue or species tested. In longitudinal muscle of the mouse caecum, ODQ abolished responses to NO donors, and reduced responses to neuronally-released NO, but it was noted that ODQ was significantly less effective than L-NMMA (Young *et al*., 1996). This contrasts with our results in canine colon, where ODQ was more effective than L-NAME and suggests that there may also be tissue-specific differences in the role of cGMP-dependent mechanisms in NO-dependent neurotransmission.

The IJPs in canine proximal colon could be caused by direct activation of K^+ channels by neuronally-released NO, via an action independent on cGMP synthesis. NO was found to activate three classes of K^+ channel in isolated colonic myocytes, having conductances of 250 pS (BK channels, 82 pS (K_{NO1}) and <4 pS (K_{NO2}) (Koh *et al*., 1995). When tested in the cell-attached configuration, NO and cGMP analogues reversibly activated all three conductances. Interestingly, K_{NO1} and K_{NO2} were activated by NO, but not cGMP analogues in excised inside-out patches (Koh *et al*., 1995). It was suggested that SNP and NO may directly activate K^+ channels in excised patches by a redox modification similar to that proposed for NO activation of BK channels in coronary smooth muscle (Bolotina *et al*., 1994). The hypothesis that IJPs are due to direct ion channel activation was considered because of: (1) the rapid time course of the IJP (up to 45 mV s^{-1}), and (2) the lack of effect of methylene blue on IJPs (see Discussion in Koh *et al*., 1995). The fact that IJPs are abolished by ODQ suggests that IJPs are primarily due to cGMP-dependent mechanisms, and the increase in cGMP caused by NO is sufficiently rapid to produce events with a rise time of 45 mV s^{-1}. It is possible that redox modification of channel proteins is a non-physiological phenomenon, caused only by high concentrations of NO used in patch clamp studies. One caveat to this conclusion is the possibility that ODQ could directly inhibit the K^+ conductance(s) activated by NO. This possibility has not been directly tested, but it seems very unlikely because: (1) these channels are also activated by 8-Br-cGMP (Koh *et al*., 1995); and (2) ODQ did not influence the hyperpolarization response to 8-Br-cGMP. Thus, it is unlikely that ODQ blocks the ion channels responsible for NO- and cGMP-dependent hyperpolarization.

Specific blockers of BK channels do not block IJPs, suggesting that these events are mediated by cGMP-dependent stimulated openings of K^+ channels with conductances of 82 pS and <4 pS (Koh *et al*., 1995), or other as yet undiscovered cGMP-dependent conductances. Activation of K^+ channels would lead to hyperpolarization, and the subsequent inhibition of Ca^{2+}-influx would lead to relaxation. Relaxation could also be mediated by a G-kinase mediated decrease in Ca^{2+} current, or factors independent on membrane potential. For instance, cGMP-dependent mechanisms increase Ca^{2+} uptake in vascular smooth muscle cells (Lincoln & Cornwell, 1993) and decrease the Ca^{2+} sensitivity of the contractile apparatus in vascular smooth muscles (Nishimura & van Breemen, 1989).

In conclusion, the use of a blocker of the guanylate cyclase activated by NO suggests that most, if not all, NO-dependent inhibitory transmission in the circular muscle of the canine colon is mediated by cGMP. No evidence for direct (cGMP-independent) inhibition was found.

Supported by NIH grant PO1 DK 41315 and DFG Fr 1170/2-1.

References

BARBIER, A.J.M. & LEFEBVRE, R.A. (1992). Effect of LY83583 on relaxation induced by non-adrenergic non-cholinergic nerve stimulation and exogenous nitric oxide in the rat gastric fundus. *Eur. J. Pharmacol*., **21,** 331–334.

BARNETTE, M., TORPHY, T.J., GROUS, M., FINE, C. & ORMSBEE, H.S. (1989). Cyclic GMP: a potential mediator of neurally- and drug-induced relaxation of opossum lower esophageal sphincter. *J. Pharmacol. Exp. Ther*., **249,** 524–528.

BOLOTINA, V.M., NAJIBI, S., PALACINO, J.J., PAGANO, P.J. & COHEN, R.A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature*, **368,** 850–853.

BOULTON, C.L., SOUTHAM, E. & GARTHWAITE, J. (1995). Nitric oxide-dependent long-term potentiation is blocked by a specific inhibitor of soluble guanylyl cyclase. *Neuroscience*, **69,** 699–703.

BREDT, D.S. & SNYDER, S.H. (1992). Nitric oxide, a novel neuronal messenger. *Neuron*, **8,** 3 – 11.

BRUNNER, F., SCHMIDT, K., NIELSEN, E.B. & MAYER, B. (1996). Novel guanylyl cyclase inhibitor potently inhibits cyclic GMP accumulation in endothelial cells and relaxation of bovine pulmonary artery. *J. Pharmacol. Exp. Ther.*, **277,** 48 – 53.

BRUNNER, F., STESSEL, H. & KUKOVETZ, W.R. (1995). Novel guanylyl cyclase inhibitor, ODQ reveals role of nitric oxide, but not of cyclic GMP in endothelin-1 secretion. *FEBS Lett.*, **376,** 262 – 266.

CELLEK, S., KASAKOV, L. & MONCADA, S. (1996). Inhibition of nitrergic relaxations by a selective inhibitor of the soluble guanylate cyclase. *Br. J. Pharmacol.*, **118,** 137 – 140.

CHAKDER, S. & RATTAN, S. (1993). Involvement of cAMP and cGMP in relaxation of internal anal sphincter by neural stimulation, VIP, and NO. *Am. J. Physiol.*, **261,** G702 – G707.

CONKLIN, J.L. & DU, C. (1992). Guanylate cyclase inhibitors: effect on inhibitory junction potentials in esophageal smooth muscle. *Am. J. Physiol.*, **263,** G87 – G90.

DALZIEL, H.H., THORNBURY, K.D., WARD, S.M. & SANDERS, K.M. (1991). Involvement of nitric oxide synthetic pathway in inhibitory junction potentials in canine proximal colon. *Am. J. Physiol.*, **269,** G789 – G792.

GARTHWAITE, J. & BOULTON, C.L. (1995). Nitric oxide signalling in the central nervous system. *Ann. Rev. Physiol.*, **57,** 683 – 706.

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.

HUIZINGA, J.D., TOMLINSON, J. & PINTIN-QUEZADA, J. (1992). Involvement of nitric oxide in nerve-mediated inhibition and action of vasoactive intestinal peptide in colonic smooth muscle. *J. Pharmacol. Exp. Ther.*, **260,** 803 – 808.

JIN, J.G., MURTHY, K.S., GRIDER, J.R. & MAKHLOUF, G.M. (1993). Activation of distinct cAMP- and cGMP-dependent pathways by relaxant agents in isolated gastric muscle cells. *Am. J. Physiol.*, **264,** G470 – G477.

KEEF, K.D., MURRAY, D.C., SANDERS, K.M. & SMITH, T.K. (1997). Basal release of nitric oxide induces oscillatory motor pattern in canine colon. *J. Physiol. (Lond.)*, **499,** 773 – 786.

KEEF, K.D., SHUTTLEWORTH, C.W., XUE, C., BAYGUINOV, O., PUBLICOVER, N.G. & SANDERS, K.M. (1994). Relationship between nitric oxide and vasoactive intestinal polypeptide in enteric inhibitory neurotransmission. *Neuropharmacology*, **33,** 1303 – 1314.

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. (Lond.)*, **489,** 735 – 743.

LINCOLN, T.M. & CORNWELL, T.L. (1993). Intracellular cyclic GMP receptor proteins. *FASEB J.*, **7,** 328 – 338.

LIU, L.W., THUNEBERG, L., DANIEL, E.E. & HUIZINGA, J.D. (1993). Selective accumulation of methylene blue by interstitial cells of Cajal in canine colon. *Am. J. Physiol.*, **264,** G64 – G73.

LUO, D., DAS, S. & VINCENT, S.R. (1995). Effects of methylene blue and LY83583 on neuronal nitric oxide synthase and NADPH-diaphorase. *Eur. J. Pharmacol.*, **290,** 247 – 251.

MAYER, B., BRUNNER, F. & SCHMIDT, K. (1993). Inhibition of nitric oxide synthesis by methylene blue. *Biochem. Pharmacol.*, **45,** 367 – 374.

MEULEMANS, A.L., HELSEN, L.F. & SCHUURKES, J.A. (1993). Role of NO in vagally-mediated relaxations of guinea-pig stomach. *Naunyn Schmiedeberg's Arch. Pharmacol.*, **347,** 225 – 230.

MONCADA, S., PALMER, R.M. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharm. Rev.*, **43,** 109 – 142.

MULSCH, A., BUSSE, R., LIEBAU, S. & FORSTERMANN, U. (1988). LY83583 interferes with the release of endothelium-derived relaxing factor and inhibits soluble guanylate cyclase. *J. Pharm. Exp. Ther.*, **247,** 283 – 288.

NISHIMURA, J. & VAN BREEMEN, C. (1989). Direct regulation of smooth muscle contractile elements by second messengers. *Biochem. Biophys. Res. Commun.*, **163,** 929 – 935.

RAE, M.G. & MUIR, T.C. (1996). Neuronal mediators of inhibitory junction potentials and relaxation in the guinea-pig internal anal sphincter. *J. Physiol. (Lond.)*, **493,** 512 – 527.

SANDERS, K.M., BURKE, E.P. & STEVENS, R.J. (1989). Effects of methylene blue on rhythmic activity and membrane potential in the canine proximal colon. *Am. J. Physiol.*, **256,** G779 – G784.

SANDERS, K.M. & SMITH, T.K. (1986). Enteric neural regulation of slow waves in circular muscle of the canine proximal colon. *J. Physiol. (Lond.)*, **377,** 297 – 313.

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

SHUTTLEWORTH, C.W.R., BURNS, A.J., WARD, S.M., O'BRIEN, W.E. & SANDERS, K.M. (1995). Recycling of L-citrulline to sustain nitric oxide-dependent enteric neurotransmission. *Neuroscience*, **68,** 1295 – 1304.

SHUTTLEWORTH, C.W. & SANDERS, K.M. (1996). Involvement of nitric oxide in neuromuscular transmission in canine proximal colon. *Proc. Soc. Exp. Biol. Med.*, **211,** 16 – 23.

SHUTTLEWORTH, C.W., SANDERS, K.M. & KEEF, K.D. (1993a). Inhibition of nitric oxide synthesis reveals non-cholinergic excitatory neurotransmission in the canine proximal colon. *Br. J. Pharmacol.*, **109,** 739 – 747.

SHUTTLEWORTH, C.W., XUE, C., WARD, S.M., DE VENTE, J. & SANDERS, K.M. (1993b). Immunohistochemical localization of 3′,5′-cyclic guanosine monophosphate in the canine proximal colon: responses to nitric oxide and electrical stimulation of enteric inhibitory neurons. *Neuroscience*, **56,** 513 – 522.

SMITH, T.K., REED, J.B. & SANDERS, K.M. (1987). Origin and propagation of electrical slow waves in circular muscle of canine proximal colon. *Am. J. Physiol.*, **252,** C215 – C224.

SUTHAMNATPONG, N., MAEHARA, T., KANADA, A., TAKEUCHI, T. & HATA, F. (1993). Dissociation of cyclic GMP level from relaxation of the distal, but not the proximal colon of rats. *Japn. J. Pharmacol.*, **62,** 387 – 393.

THORNBURY, K.D., WARD, S.M., DALZIEL, H.H., CARL, A., WESTFALL, D.P. & SANDERS, K.M. (1991). Nitric oxide and nitrosocysteine mimic nonadrenergic, noncholinergic hyperpolarization in canine proximal colon. *Am. J. Physiol.*, **261,** G553 – G557.

TORPHY, T.J., FINE, C.F., BURMAN, M., BARNETTE, M.S. & ORMSBEE, H.S. (1986). Lower esophageal sphincter relaxation is associated with increased cyclic nucleotide content. *Am. J. Physiol.*, **251,** G786 – G793.

WARD, S.M., DALZIEL, H.H., BRADLEY, M.E., BUXTON, I.L., KEEF, K., WESTFALL, D.P. & SANDERS, K.M. (1992a). Involvement of cyclic GMP in non-adrenergic, non-cholinergic inhibitory neurotransmission in dog proximal colon. *Br. J. Pharmacol.*, **107,** 1075 – 1082.

WARD, S.M., DALZIEL, H.H., THORNBURY, K.D., WESTFALL, D.P. & SANDERS, K.M. (1992b). Nonadrenergic, noncholinergic inhibition and rebound excitation in canine colon depend on nitric oxide. *Am. J. Physiol.*, **262,** G237 – G243.

YANO, S., KIYOTA, Y., YAMAMOTO, M. & WATANABE, K. (1995). Pharmacological features of non-adrenergic non-cholinergic (NANC) relaxation induced by electrical vagal stimulation in isolated mouse stomach. *Japn. J. Pharmacol.*, **69,** 9 – 15.

YOUNG, H.M., CIAMPOLI, D., JOHNSON, P.J. & STEBBING, M.J. (1996). Inhibitory transmission to the longitudinal muscle of the mouse caecum is mediated largely by nitric oxide acting via guanylyl cyclase. *J. Auton. Ner. Syst.*, **61,** 103 – 108.

(Received June 13, 1997
Revised August 12, 1997
Accepted August 13, 1997)