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Role of nitric oxide in non-adrenergic, non-cholinergic inhibitory junction potentials in canine ileocolonic sphincter

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1 Electrical field stimulation causes neurally-mediated relaxation of the ileocolonic sphincter that is due to activation of non-adrenergic and non-cholinergic (NANC) nerves. Recent studies have suggested that nitric oxide (NO) is the neurotransmitter that mediates relaxation.

2 Using intracellular recording techniques, we have tested whether NANC inhibitory junction potentials (i.j.ps) in the canine ileocolonic sphincter are also mediated by NO.

3 Electrical field stimulation elicited excitatory and inhibitory junction potentials: e.j.ps were blocked by atropine (10^{-6} M) and tetrodotoxin (TTX; 10^{-6} M); i.j.ps were also blocked by TTX and partially blocked by apamin (10^{-6} M). I.j.ps were unaffected by atropine, phentolamine and propranolol (all at 10^{-6} M).

4 The arginine analogues, L-N^G-nitroarginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA), decreased the amplitude of i.j.ps and L-arginine, but not D-arginine, partially restored the i.j.ps.

5 I.j.ps were also inhibited by oxyhaemoglobin (1%), but not by methaemoglobin.

6 Exogenous NO (10^{-7} M to 3×10^{-5} M) caused concentration-dependent hyperpolarizations that were similar in amplitude to the NANC nerve-evoked i.j.ps. Hyperpolarizations to NO were unaffected by L-NAME, but were blocked by oxyhaemoglobin.

7 Tetrodotoxin, L-NAME and oxyhaemoglobin all caused depolarization of resting membrane potential.

8 The specific guanosine 3′:5′-cyclic monophosphate phosphodiesterase inhibitor, M&B 22948, caused hyperpolarization, increased the maximum level of hyperpolarization reached during i.j.ps, and increased the duration of i.j.ps.

9 These data further support the hypothesis that NANC neurotransmission in the ileocolonic sphincter is mediated by NO or an NO-releasing compound. The data also suggest that tonic release of NO, possibly from spontaneous firing of NANC nerves, may regulate resting membrane potential and tone in this sphincter.

Keywords: Non-adrenergic, non-cholinergic nerves; gastrointestinal motility; inhibitory junction potential

Introduction

Recent evidence has suggested that nitric oxide (NO) or a NO-releasing substance, may be the transmitter that mediates NANC inhibitory neurotransmission in the gut (Toda *et al.*, 1990; Bult *et al.*, 1990; Dalziel *et al.*, 1991). Early support for this hypothesis came from a series of mechanical studies in which strips of muscle from the canine ileocolonic sphincter relaxed in response to electrical field stimulation of NANC nerves (Boeckxstaens *et al.*, 1990a,c). These responses were blocked by arginine analogues that are known to inhibit specifically nitric oxide synthase (Palmer *et al.*, 1987; see also Figure 5 in Moncada *et al.*, 1991), the enzyme responsible for producing NO from L-arginine. The inhibition by arginine analogues was reversed by L-arginine, but not by D-arginine. Inhibitory responses were mimicked by exogenous NO, and responses to NANC nerve stimulation and exogenous NO were blocked by oxyhaemoglobin, which is known to be a scavenger of extracellular NO (Martin *et al.*, 1985). Investigations into the chemical identity of the substance that conveys the NANC inhibitory signal showed that field stimulation released a substance that behaved in a manner very similar to authentic NO in bioassay cascades (Boeckxstaens *et al.*, 1991). Taken together, these data suggest that NO may be a NANC inhibitory transmitter in the canine ileocolonic sphincter, and this work has stimulated many other investigations of this hypothesis. Recent studies of gastrointestinal (GI) muscles from nearly all levels of the GI tract and from several species have provided strong support for the notion that NO is the primary inhibitory neurotransmitter in the gut (Toda *et al.*, 1990; Bult *et al.*, 1990; Dalziel *et al.*, 1991; Thornbury *et al.*, 1991; Tottrup *et al.*, 1991).

Although NANC inhibitory nerves ultimately mediate relaxation in GI muscles, an important step in neurotransmission is the hyperpolarization response in postjunctional, smooth muscle membranes. Hyperpolarization, which inhibits electrical rhythmicity and decreases the open probability of voltage-dependent Ca^{2+} channels (Smith *et al.*, 1989; Langton *et al.*, 1989) is thought to be mediated by a transient increase in K^+ conductance (Tomita, 1972). We tested the hypothesis that inhibitory junction potentials in the ileocolonic sphincter are mediated by NO, or an NO-releasing compound, by examining the following: (i) the effects of NO synthase inhibitors on NANC inhibitory junction potentials, (ii) the effect of authentic NO on membrane potential, (iii) the effects of NO scavengers on NANC i.j.ps and exogenous NO, (iv) and in an attempt to try and explain the mechanism involved in NANC i.j.ps, we have examined the effect of the guanosine 3′:5′-cyclic monophosphate (cyclic GMP) phosphodiesterase inhibitor M&B 22948 on the NANC i.j.p.

Methods

Mongrel dogs of either sex were anaesthetized with sodium pentobarbitone (45 mg kg^{-1}). The abdomen was opened and a segment of bowel (8 cm) from the distal ileum to the proximal colon was removed. This segment contained the ileocolonic sphincter. The fascia attaching the caecum to the ileum was cut and the caecum was removed. The ileocolonic sphincter region was bisected by a longitudinal cut from small bowel to colon and the luminal contents were removed by washing

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with Krebs-bicarbonate solution. The resulting sheet was pinned-out with the mucosal side up in a dish of oxygenated Krebs-bicarbonate solution. The ring of circular muscle identified as the ileocolonic sphincter was very prominent (Figure 1). Preparations for intracellular recording were made by cutting muscle strips 1–2 mm wide and 2–3 cm long in planes transverse and parallel to the sphincter. The mucosal layers were dissected away from the underlying smooth muscle. Muscle strips were placed between two parallel platinum electrodes in a 2 ml electrophysiological recording chamber and pinned to the Sylgard floor (Dalziel *et al.*, 1991). The muscles were allowed to equilibrate for approximately 1 h before intracellular experiments were performed. Throughout experiments, preparations were maintained at 37.5 ± 0.5°C by constant perfusion with pre-warmed, pre-oxygenated Krebs-bicarbonate solution having the following composition (mM): Na^+ 137.4, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 134, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, dextrose 11.5. Equilibration of the solution with 97% O_2:3% CO_2 achieved a final pH of 7.3 to 7.4.

Cells near the submucosal surface of the sphincteric ring were impaled with glass microelectrodes filled with 3 M KCl and having resistances ranging from 30 to 50 MΩ. Transmembrane potentials was measured with a standard electrometer (WPI M-7000), and outputs were displayed on an oscilloscope (Tektronix 5111). Electrical signals were recorded on magnetic tape (Hewlett-Packard 3964A) and by a chart recorder (Gould 2200). The preparations were electrically field stimulated with an electronic stimulator (Grass S44) and a stimulus isolation unit hooked to the platinum electrodes. I.j.ps were elicited by trains of 3 square wave pulses (0.5 ms duration, supramaximal voltage, 20 Hz). These short trains were delivered every 10–20 s throughout the experimental period.

Solution and drugs

L-N^G-nitroarginine, methylester (L-NAME), N^G-monomethyl-L-arginine (L-NMMA), L-arginine, D-arginine, and propranolol (all Sigma) were used as hydrochloride salts. The sulphate salt of atropine (Sigma), and the mesylate salt of phentolamine (Ciba Geigy) were used. Drugs were dissolved in distilled water as stock solutions of 10^{-1} or 10^{-2} M and further serial dilutions were made in Krebs-bicarbonate solution as required. M&B 22948 (Zaprinast), a gift from Rhone-Poulenc Rorer, was dissolved in 0.1 M NaOH (10^{-1} M) and diluted to the final desired concentration in Krebs-bicarbonate solution as required. Drugs were introduced to a 20 ml side reservoir from which the bath was perfused at a rate of 5 ml min^{-1}. The lag time for perfusion of the recording chamber from the reservoir was 1–2 s.

NO stock solution was prepared by bubbling ice-cold, deoxygenated (sonication under vacuum followed by purging with pure nitrogen gas) distilled water with NO gas (99% pure) to give a saturated solution (1–1.5 mM; Ignarro *et al.*, 1987). NO was diluted in Krebs-bicarbonate solution to the desired concentration immediately before exposing the muscles to this solution. Oxyhaemoglobin was prepared as a haemolysate of canine blood according to the method of Bowman & Gillespie (1982) with the exception that red cells were lysed by 1:1 addition of distilled water. Methaemoglobin was prepared in a similar manner, but potassium ferricyanide was used to convert oxyhaemoglobin to methaemoglobin prior to dialysis (Thornbury *et al.*, 1991).

Data are expressed as mean ± s.e. and paired or unpaired Student's *t* tests were used for determination of statistical significance where appropriate; *n* values refer to number of muscles used in each experiment.

Results

Circular muscle cells in the ileocolonic sphincter had average resting membrane potentials of -55 ± 2 mV and either displayed small spontaneous oscillations in membrane potential (55%) or were electrically quiescent ($n = 30$). Electrical field stimulation produced a transient depolarization followed by a more sustained hyperpolarization (Figure 2). The depolarization was identified as a cholinergically-mediated excitatory junction potential (e.j.p.) because it was blocked by atropine (10^{-6} M). The hyperpolarization response persisted in the presence of atropine, phentolamine and propranolol (all at 10^{-6} M). These non-adrenergic and non-cholinergic (NANC) responses were identified as inhibitory junction potentials (i.j.ps) elicited by intramural nerves because they were reduced or abolished by tetrodotoxin (10^{-6} M; Figure 3). In 50% of the preparations i.j.ps consisted of two components: an initial fast hyperpolarization phase which partially recovered within 8 s followed by a sustained hyperpolarization phase that persisted for about 20 s. In the remainder of the preparations the initial fast phase could not be distinguished from the sustained component. I.j.ps averaged 15 ± 1 mV in amplitude and 15 ± 0.9 s in duration (4.6 ± 0.4 s in duration at half maximal amplitude; $n = 30$ muscles from 12 dogs).

I.j.ps could be recorded from muscles for up to 8 h under control conditions. Throughout the period of a single cell impalement the amplitude of the i.j.p. remained stable under control conditions. The duration of impalements varied considerably but recordings were maintained for up to 3 h.

Others have shown that an extract from bee venom, apamin, inhibits i.j.ps and neurally-mediated mechanical relaxations (Banks *et al.*, 1979; Shuba & Vladimirova, 1980). Apamin has been shown to block small conductance, Ca^{2+}-activated K channels in a variety of preparations (cf. Capoid & Ogden, 1989). There appears to be apamin-sensitive and insensitive components to the NANC inhibitory response in GI muscles (Costa *et al.*, 1986). We tested the effects of apamin

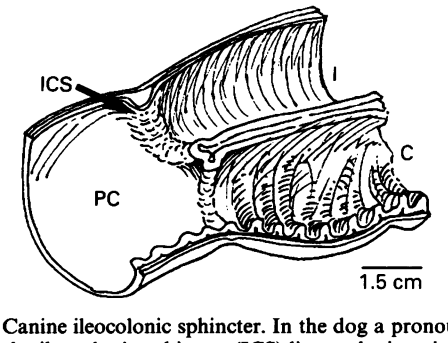

Figure 1 Canine ileocolonic sphincter. In the dog a pronounced ring of muscle, the ileocolonic sphincter (ICS) lies at the junction between the terminal ileum (I) and proximal colon (PC). The caecum (C) lies below this region. Muscle cells within the sphincteric ring of circular muscle were impaled for electrophysiological studies.

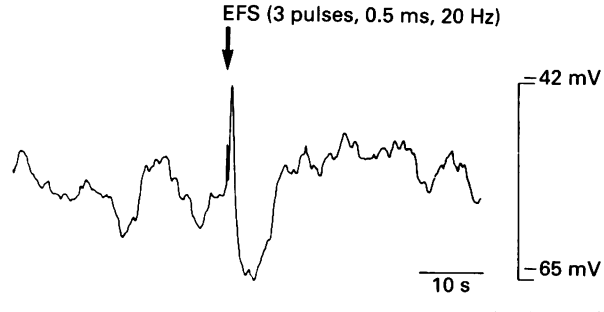

Figure 2 Responses to electrical field stimulation (stimulus applied at arrow) were characterized by an initial depolarization (e.j.p.), followed by a more sustained hyperpolarization response (i.j.p.). I.j.ps were non-adrenergic and non-cholinergic (NANC) responses. In many muscles membrane potential spontaneously oscillated (see irregular baseline before and after response to electrical field stimulation) during the course of experiments.

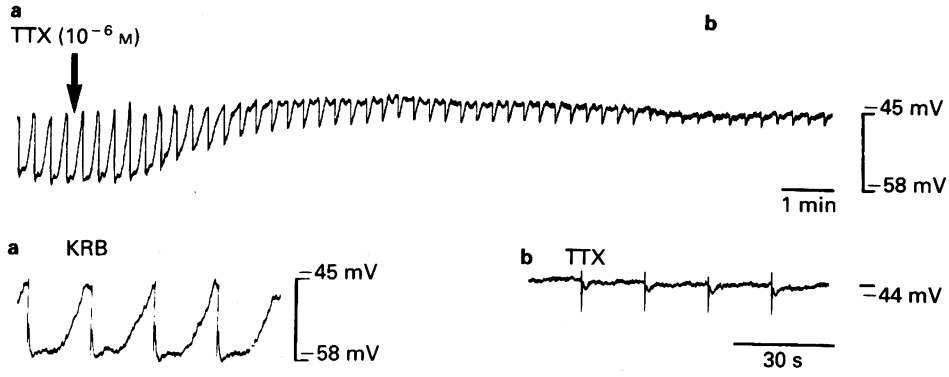

Figure 3 Effects of tetrodotoxin (TTX; 10^{-6} M) on i.j.ps. Top trace shows time-course of response to TTX. I.j.ps were elicted every 20 s. TTX reduced amplitude of i.j.ps. I.j.ps during control (a) and at maximal effect of TTX (b) are shown at expanded time scale in bottom panels.

on i.j.ps in the ileocolonic sphincter. Apamin (10^{-6} M) reduced the amplitude of the fast hyperpolarization phase from an average of 21 ± 3 mV to 14 ± 1.5 mV ($n = 4$, $P < 0.05$), but did not affect the duration or the slow component of the i.j.p. (Figure 4). These data suggest that different mechanisms, perhaps different K channels, mediate the slow and fast components of the i.j.p.

Nitric oxide (NO) is produced from L-arginine by the enzyme nitric oxide synthase (cf. Moncada *et al.*, 1991). NO synthesis is stereospecific and can be competitively inhibited by the L-arginine analogues, L-NAME and L-NMMA. Exposure of ileocolonic muscle strips to L-NAME (10^{-4} M) caused a depolarization in resting membrane potential (from −54 ± 3 mV to −47 ± 1 mV) and decreased the amplitudes

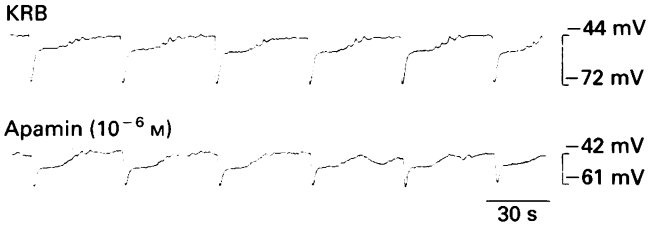

Figure 4 Effects of apamin (10^{-6} M) on i.j.ps. Top trace shows repetitive i.j.ps recorded in Krebs-bicarbonate solution. Bottom trace shows i.j.ps after exposure to apamin for 15 min. Apamin reduced amplitude of fast phase of i.j.p., but did not affect slow phase and duration of i.j.ps.

of i.j.ps (from 14 ± 1 mV to 5 ± 1 mV; $n = 10$, $P < 0.001$ respectively). In muscles with 2 phase i.j.ps, both phases were inhibited by L-NAME. Figure 5 shows an example of the effects of L-NAME (10^{-4} M) on resting potential and i.j.ps. The average time for L-NAME to produce a maximal reduction in the amplitude of the i.j.p. was 19.5 ± 1 min. The effects of L-NAME were fully reversible upon washout. L-NMMA (10^{-4} M) also reduced the i.j.p. amplitude from 16 ± 1 mV to 6 ± 1.5 mV ($n = 6$) and was also fully reversible upon washout.

The inhibitory action of L-arginine analogues on NANC nerve-induced relaxations of the canine ileocolonic sphincter have been shown to be reversed by L-arginine, but not by the stereoisomer D-arginine (Boeckxstaens *et al.*, 1990a). We tested whether i.j.ps inhibited by L-NAME could be restored by L-arginine, but not by D-arginine. Muscles were exposed to L-NAME (10^{-4} M) for 15 min. Then L-arginine (1 mM) was added to the perfusion solution. L-Arginine partially reversed the inhibition of i.j.ps caused by L-NAME (Figure 6). In these experiments the average i.j.p. amplitude before the addition of L-NAME was 14 mV ± 1.8 mV ($n = 5$), L-NAME reduced the average i.j.p. to 5 mV ± 1.2 mV, and L-arginine reversed the L-NAME effect from 5 mV ± 1.2 mV to 8.4 mV ± 1.0 mV ($P < 0.01$). Restoration of i.j.ps in the presence of L-NAME was stereospecific, addition of D-arginine did not restore i.j.ps inhibited by L-NAME ($n = 6$; Figure 7).

Oxyhaemoglobin (1%), which has been previously shown to scavenge NO (Martin *et al.*, 1985), significantly reduced the amplitude of the NANC nerve-induced i.j.ps from an average of 16 mV ± 4 mV to 4 mV ± 2 mV ($n = 5$, $P < 0.05$; see Figure

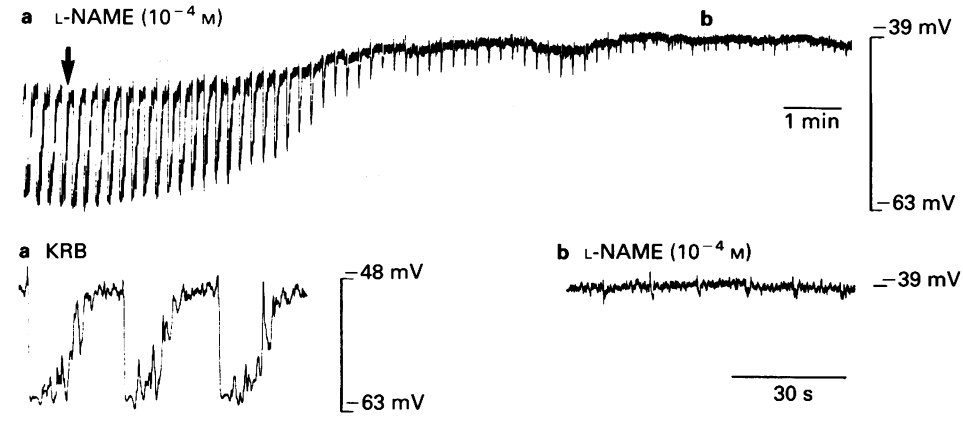

Figure 5 Effect of L-N^G-nitroarginine methyl ester (L-NAME) on i.j.ps. L-NAME (10^{-4} M) was added at arrow. This caused depolarization of membrane potential and a reduction in the amplitude of i.j.ps. Top panel shows time-course of effects. Bottom panels show i.j.ps before L-NAME (a) and at maximal effect of L-NAME. Similar effects occurred in response to N^G-monomethyl L-arginine (data not shown).

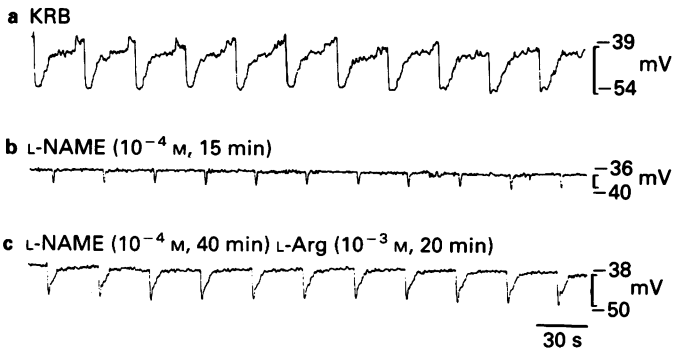

Figure 6 Inhibitory effects of L-N^G-nitroarginine methyl ester (L-NAME) on i.j.ps were reversed by L-arginine: (a) repetitive i.j.ps elicited in control conditions; (b) reduction in i.j.ps in the presence of L-NAME (10^{-4} M); (c) partial restoration of i.j.ps by addition of L-arginine (L-Arg, 1 mM)

8). This effect took an average of 13 ± 2 min to fully develop. Methaemoglobin (1%) did not affect i.j.ps.

Arginine analogues and oxyhaemoglobin reduced the amplitude of i.j.ps, but these agents did not abolish these events. This may have been due to: (i) the concentration of oxyhaemoglobin and arginine analogues used may have been insufficient to inhibit totally NO synthesis or sequester all NO released, or (ii) another substance, perhaps co-released with NO, could be responsible for the remaining portion of the i.j.p. In two experiments we tested the effects of L-NAME (10^{-4} M) and oxyhaemoglobin (1%) together. Combination of these two agents completely abolished i.j.ps.

Experiments were also performed to determine whether exogenous NO could mimic the hyperpolarization produced by NANC nerve stimulation. NO was added to the bath at estimated concentrations ranging from 10^{-7} M to 3×10^{-5} M as previously described (Thornbury *et al.*, 1991). NO caused concentration-dependent hyperpolarizations that were similar in amplitude to the NANC nerve-evoked i.j.ps. Figure 9 shows responses to several concentrations of NO obtained while maintaining a single impalement. In a series of experiments 3×10^{-6} M NO caused hyperpolarization averaging 8.1 mV ± 1.6 mV (i.e. from −51.4 ± 2.8 mV to −59.5 ± 2.7 mV; $n = 10$; $P < 0.001$) and 10^{-5} M NO caused hyperpolarization averaging 11.3 mV ± 1.0 mV (from −56 ± 1.8 mV to −67.3 ± 1.9 mV; $n = 16$ from 8 dogs;

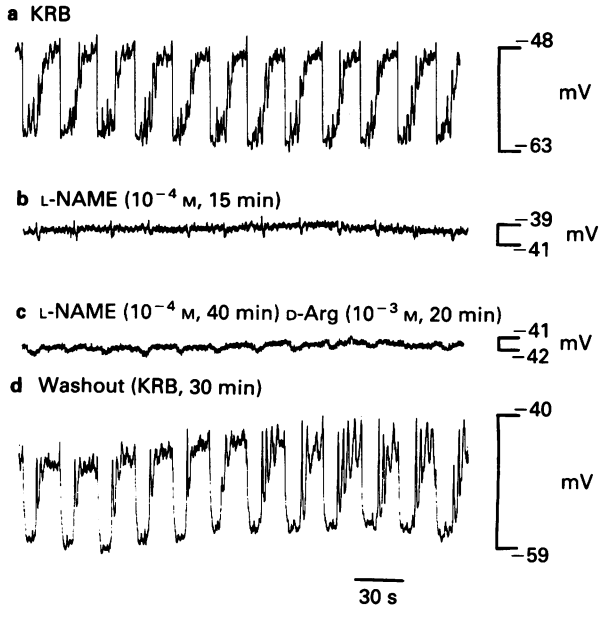

Figure 7 I.j.ps inhibited by L-N^G-nitroarginine methyl ester (L-NAME) were not restored by D-arginine; (a) repetitive i.j.ps elicited in control conditions; (b) reduction in i.j.ps in the presence of L-NAME (10^{-4} M); (c) failure to restore i.j.ps by addition of D-arginine (D-Arg, 1 mM); (d) washout of L-NAME and restoration of i.j.ps.

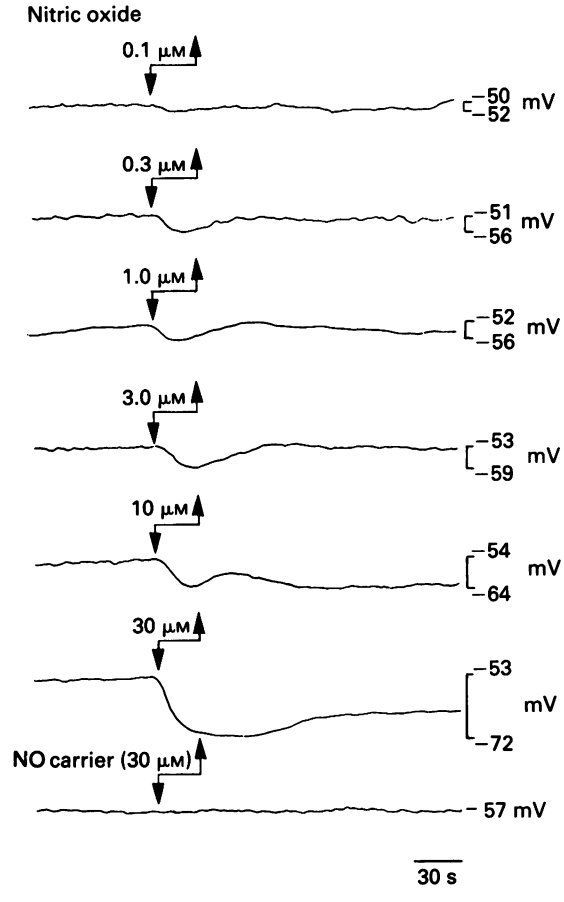

Figure 9 Effects of exogenous nitric oxide on membrane potential. While maintaining a single impalement, the muscle was exposed to several concentrations of NO. This caused a concentration-dependent hyperpolarization response (estimated concentrations shown above each trace). Bottom panel shows that the solution that NO was dissolved in (i.e. amount of NO carrier needed to make 3×10^{-5} M dilution of NO) had no effect alone.

Figure 8 Effects of oxyhaemoglobin: repetitive i.j.ps were elicited, and at the arrow, oxyhaemoglobin (oxy-Hb; 1%). This caused depolarization of membrane potential and a reduction in the amplitude of i.j.ps.

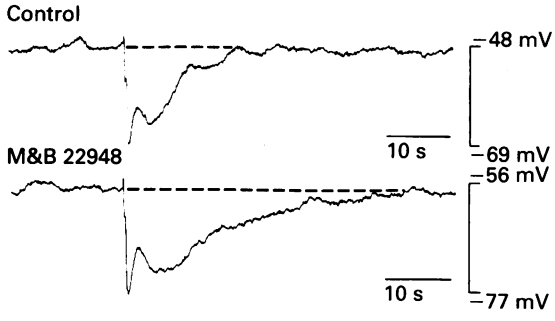

Figure 10 Effect of M&B 22948 on i.j.ps: traces show superimposed i.j.ps recorded before (control) and in the presence of M&B 22984 (10^{-4} M). The specific cyclic GMP phosphodiesterase inhibitor, M&B 22948 caused an enhancement in the duration of i.j.ps (see text for details).

$P < 0.0001$). L-NAME (10^{-4} M) did not significantly affect the hyperpolarization caused by NO. NO (3×10^{-6} M) caused an average 9.2 mV ± 2.7 mV hyperpolarization in the presence of L-NAME (from −54.4 mV ± 6.3 to −63.6 ± 2.5 mV; $n = 5$), and 10^{-5} M NO caused an average 12.2 mV ± 1.0 mV hyperpolarization (from −59 mV ± 4.6 mV to −71.2 mV ± 4.3 mV; $n = 5$). Oxyhaemoglobin (1%), an NO scavenger, did however significantly reduce the NO-induced hyperpolarization. Hyperpolarizations caused by exogenous NO were reduced by oxyhaemoglobin (i.e. from 6.0 ± 1 mV with 3×10^{-6} M NO to 1 ± 1 mV; $n = 4$, $P < 0.05$; and from 13 ± 1 mV with 10^{-5} M NO to 1 ± 0.4 mV; $n = 4$, $P < 0.005$).

In vascular muscles the receptor for NO appears to be the haemoprotein of soluble guanylate cyclase (Rapoport & Murad, 1983). Binding of NO increases the production of cyclic GMP (Craven & DeRubertis, 1978), and elevation of cyclic GMP is associated with relaxation in smooth muscles (Ignarro & Kadowitz, 1985). NANC effects may be mediated by cyclic GMP in GI smooth muscles. In order to determine whether cyclic GMP participates in the generation of NANC i.j.ps, we tested the effects of the specific cyclic GMP phosphodiesterase inhibitor M&B 22948 (Kukovetz *et al.*, 1982) on i.j.ps. Addition of M&B 22948 (10^{-4} M) caused an 8 mV ± 3 mV hyperpolarization in resting membrane potential from −59 mV to −67 mV ($n = 5$; $P < 0.05$). Although the absolute amplitude of the fast component of the i.j.p. was not significantly affected by M&B 22948, the duration of the second component was increased from 13.4 ± 3.2 s to 20.6 ± 3.2 s ($P < 0.01$) in the presence of M&B 22948. Figure 10 shows superposition of i.j.ps before and in the presence of M&B 22948. I.j.ps are likely to be due to an increase in K conductance (Tomita, 1972). Therefore, the fact that the amplitude of the fast phase was maintained despite the 8 mV hyperpolarization in resting potential caused by M&B 22948, suggests that the increase in K conductance during i.j.ps was enhanced by M&B 22948.

Discussion

The ileocolonic sphincter (ICS) is formed by a thickening of the circular muscle layer, and it divides the terminal ileum from the proximal colon. It serves as a true sphincter; tonic contraction maintains a region of high pressure (e.g. 66 cmH_2O in dogs; see Kelley *et al.*, 1966). The ICS is thought to cause retention of ileal contents to increase absorption and to prevent reflux of colonic contents into the ileum to help prevent bacterial overgrowth of the small bowel (see Papasova, 1989). Distension of the ileum causes the pressure in the ICS to decrease (Kelley *et al.*, 1966; Kelley & De Weese, 1969), and this is a neurally-mediated reflex (Pahlin & Kewenter, 1975). Extrinsic innervation of the ICS comes from vagal nerves and from sympathetic nerves originating in the superior and inferior mesenteric ganglia, and a dense intrinsic innervation arises from the myenteric plexus (Papasova, 1989). Stimulation of either group of extrinsic nerves enhances sphincteric pressure and stops trans-sphincteric flow (Pahlin, 1975; Pahlin & Kewenter, 1976). Low frequency stimulation of vagal nerves however, inhibits sphincteric pressure (Pahlin & Kewenter, 1975), suggesting a class of low threshold inhibitory neurones running with the vagus. Electrical field stimulation of ICS muscles *in vitro* yields a pronounced relaxation in the presence of drugs to block adrenoceptors and cholinoceptors (Conklin & Christensen, 1975; Papasova & Mizhorkova, 1981; Boeckxstaens *et al.*, 1990d). These non-adrenergic, non-cholinergic inhibitory nerves relax the ICS and allow ileal contents to pass into the colon (see Papasova, 1989). The intrinsic nerves that convey NANC inhibitory input to the smooth muscle of the canine ICS can be activated by acetylcholine (ACh) via nicotinic receptors and by γ-aminobutyric acid (GABA) via $GABA_A$ receptors (Pelckmans *et al.*, 1989; Boeckxstaens *et al.*, 1990b).

Several putative mediators of NANC relaxation have been investigated; adenosine 5′-triphosphate (ATP) and vasoactive intestinal polypeptide (VIP) have generally been considered the strongest candidates (see Hoyle & Burnstock, 1989). However, studies have suggested that neither of these transmitter candidates mediate NANC relaxations in the canine ICS. For example, desensitization of receptors for ATP inhibited further relaxations in response to exogenous ATP, but did not affect relaxations induced by electrical field stimulation or ACh (i.e. acting through nicotinic receptors as described above; Boeckxstaens *et al.*, 1990d). VIP appears to be ineffective in causing relaxation or hyperpolarization in the ICS (MacKenzie & Szurszewski, 1984; Boeckxstaens *et al.*, 1990d). Recent work has shown that field stimulation of NANC nerves in ICS muscles releases a substance that was identified as NO on the basis of its effects on bioassay tissues, chemical stability, neutralization by haemoglobin, and inhibition by arginine analogues (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1991).

The membrane potentials of ICS cells is relatively positive (i.e. −55 mV in the present study; −43 mV in guinea-pig ICS (Kubota, 1983); and −55 mV in another study of the canine ICS (MacKenzie & Szurszewski, 1983), and small spontaneous oscillations in membrane potential are observed in many cells. The resting potentials and oscillations are in the range where voltage-dependent Ca^{2+} channels are activated in many smooth muscle cells, including neighbouring circular muscle cells of the canine proximal colon (Langton *et al.*, 1989; Ward *et al.*, 1990). It is possible that the spontaneous mechanical tone in ICS muscles might be related to a small, continuous leak of Ca^{2+} into cells. Addition of excitatory agonists, such as noradrenaline, causes depolarization (Ward & Sanders, unpublished observations), and this would be expected to increase the open probability of voltage-dependent Ca^{2+} channels and increase the influx of Ca^{2+}. ICS muscles are also capable of generating action potentials, but these were rarely observed in the present study or in a previous study of the canine ICS (MacKenzie & Szurszewski, 1983). Therefore, this muscle appears to depend upon the resting membrane potential as an important means of regulating the influx of Ca^{2+} and the force of contraction.

The transmitter released by NANC nerves produces i.j.ps and hyperpolarizes membrane potential. Previous studies have shown that the membrane hyperpolarization elicited by NANC nerve stimulation causes relaxation (MacKenzie & Szurszewski, 1983), and it is likely that this mechanism explains the NANC-induced relaxations described by Boeckxstaens and colleagues (cf. Boeckxstaens *et al.*, 1990a). In the present study we have obtained data suggesting that NANC i.j.ps in the canine ICS are due to release and the actions of NO, and it is likely that the NO-mediated i.j.ps are the mechanism behind NANC relaxations in the ICS (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1990a; 1991). The fact that exogenous NO

causes hyperpolarization and mimics the membrane response to field stimulation in ICS muscles further supports the hypothesis that NO is the NANC inhibitory transmitter in these muscles.

At present the mechanism of the NANC inhibitory junction potentials is unknown. Most investigators have concluded that i.j.ps are due to a transient increase in K conductance (cf. Tomita, 1972). We found that apamin blocks a portion of the i.j.p. in ICS muscles, suggesting the involvement of small conductance Ca^{2+}-activated K channels (Capoid & Ogden, 1989). However, the inability of apamin to abolish i.j.ps suggests either that the potency of this agent for the K channels mediating i.j.ps is weak or that more than a single class of channels is involved. The link between NO and K channels is poorly understood. We have found in colonic circular muscle cells that NO increases the open probability of large conductance Ca^{2+}-activated K channels (Thornbury *et al.*, 1991). Whether these channels have a role in NANC responses in ICS muscles will require further investigation.

The receptor for NO in smooth muscle cells appears to be soluble guanylate cyclase (Rapoport & Murad, 1983). Our data suggest that effects of NO on membrane potential are also mediated via cyclic GMP. M&B 22948, a specific inhibitor of cyclic GMP phosphodiesterase (Kukovetz *et al.*, 1982), caused hyperpolarization and prolonged the duration of i.j.ps.

Boeckxstaens and colleagues found that addition of arginine analogues (e.g. L-NNA) or oxyhaemoglobin raised basal tone (Boeckxstaens *et al.*, 1990a). These data suggest that there is tonic release of NO in ICS that may regulate basal tone. Other muscles, such as the anococcygeus (Gillespie *et al.*, 1989) and gastric antrum (Ozaki *et al.*, 1992), also appear to be influenced by the tonic release of NO. In the present study we observed an electrophysiological correlate of the increase in basal tone in response to arginine analogues and oxyhaemoglobin. L-NAME and oxyhaemoglobin caused depolarization of resting potential, and this effect may explain the rise in tone in response to these agents. These findings suggest that constant release of NO maintains membrane potential at a more negative level than would occur in the absence of NO. The source of the tonic release of NO is unknown at present but it is possible that NO could leak from nerves, endothelial cells lining blood vessels, or other cell types that express NO synthase (cf. Moncada *et al.*, 1991). The fact that TTX raises basal mechanical tone in ileocolonic muscles (Boeckxstaens, 1991) suggests that a portion of the NO may come from spontaneous neural activity. We also found that M&B 22948 caused a significant hyperpolarization of the membrane potential. If tonic release of NO stimulates production of cyclic GMP and cyclic GMP serves as the second messenger to mediate the hyperpolarization response, then inhibiting metabolism of cyclic GMP should increase the influence of NO on membrane potential and cause hyperpolarization.

In summary, experiments on the canine ICS have suggested that NO is the inhibitory transmitter that mediates the NANC relaxation. In this, and most other GI smooth muscles, relaxation is preceded by a hyperpolarization response known as an i.j.p. We have shown that i.j.ps in the canine ICS are likely to be mediated by NO. NO is presumably coupled to hyperpolarization via the enhanced production of cyclic GMP.

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