Basal release of nitric oxide induces an oscillatory motor pattern in canine colon

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1. The consequences of intrinsic, basal nitric oxide release on electrical and contractile activity of canine proximal colon were examined. Membrane potential and contraction were simultaneously recorded from the circular muscle in the presence of drugs to block adrenergic and cholinergic responses.

2. Electrical slow waves were recorded from muscle cells near the submucosal surface of the circular layer. Spontaneous contractions were initiated by each slow wave. Contractile amplitude increased 1·9-fold when nerves were blocked with tetrodotoxin (TTX, 1 μM).

3. Muscle cells near the myenteric surface displayed myenteric potential oscillations (MPOs) averaging 16 cycles per minute (c.p.m.) in frequency and 10 mV in amplitude. Twenty-five per cent of muscles displayed an additional slow, neurogenic oscillation (mean frequency, 1 c.p.m.; amplitude, 14 mV) superimposed upon the MPO rhythm.

4. The nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine (L-NA, 100 μM; $n = 16$) abolished neurogenic oscillations, depolarized cells, and increased MPO upstroke velocity, amplitude and frequency. The actions of L-NA were mimicked by N^{ω}-nitro-L-arginine methylester (L-NAME, 100 μM) and oxyhaemoglobin (3%).

5. Spontaneous contractions were increased 2·3-fold by L-NA, and TTX had no effect on contractions after addition of L-NA.

6. The NO-donor sodium nitroprusside (SNP, 1 μM) reversed the electrical and mechanical effects of L-NA and initiated slow oscillations similar to the neurogenic oscillations. Slow oscillations were also evoked with *S*-nitroso-*N*-acetylpenicillamine (SNAP, 1 μM). The effects of NO donors were blocked by oxyhaemoglobin.

7. Slow electrical oscillations could not be elicited by SNP after removal of a thin strip of circular muscle along the myenteric edge.

8. These data suggest that the spontaneous electrical and contractile activity of the proximal colon is tonically suppressed by basal release of NO. Basal NO causes an oscillatory pattern of electrical and mechanical activity. This activity does not require patterned firing of nerves; rather a continuous, low level release of NO would be capable of producing the neurogenic oscillatory behaviour. The slow oscillatory activity depends upon the presence of the myenteric region of the circular muscle layer, which contains cell bodies of enteric neurons and interstitial cells of Cajal.

Many regions of the gastrointestinal (GI) tract are maintained in a state of inhibition which has been proposed to be due to release of inhibitory transmitter from spontaneously active nerves (Wood, 1972). Nitric oxide (NO) is now widely recognized as an important inhibitory neurotransmitter in the GI tract (Bult, Boexkxstaens, Pelckmans, Jordaens, Van Maercke & Herman, 1990; Thornbury, Ward, Dalziel, Carl, Westfall & Sanders, 1991; Stark, Bauer & Szurszewski, 1991; Sanders & Ward, 1992). A number of studies suggest that the contractile activity of various GI muscles can be suppressed by basal NO release since spontaneous contractions or tone are enhanced by either arginine analogues or oxyhaemoglobin (e.g. Boeckxstaens, Pelckmans, Bult, De Man, Herman & Van Maercke, 1990; Ward *et al.* 1992*a*; Ozaki, Blondfield, Hori, Publicover, Kato & Sanders, 1992; Keef, Du, Ward, McGreggor & Sanders, 1993). The source of this basal NO release is unclear since several cell types in addition to neurons (Bredt, Hwang & Snyder, 1991; Ward, Xue, Shuttleworth, Bredt, Snyder & Sanders, 1992*b*; Young, Furness, Shuttleworth, Bredt & Snyder, 1992; Belai *et al.* 1992) express NO synthase in GI tissues. These include (1) interstitial cells (Xue, Pollock,

Schmidt, Ward & Sanders, 1994), (2) endothelial cells (Pollock, Nakane, Buttery, Martinez, Springall & Polak, 1993; Xue *et al.* 1994), (3) epithelial cells (Torihashi *et al.* 1995), and (4) macrophages (Xie *et al.* 1992). Smooth muscle has also been proposed as a source of NO in GI muscles (Grider, Murthy, Jin & Makhlouf, 1992).

Although contractile activities of the small bowel and colon are inhibited by basal NO release, these muscles are not quiescent, i.e. muscle strips of these organs typically exhibit on-going rhythmic electrical and mechanical activity. Some investigators have suggested that oscillatory activity may be generated by patterned discharge from enteric nerves. In a recent study of the spontaneous activity of the mouse colon, for example, it was hypothesized that periods of quiescence were due to firing of enteric inhibitory nitrergic neurons, and myoelectrical–contractile complexes occurred when inhibitory neural activity was suppressed (Lyster, Bywater & Taylor, 1995). Although it is clear that many of the stereotypical motor patterns of GI motility are regulated by the enteric nervous system (see Wood, 1994), electrical rhythmicity is a property of GI muscles, and the oscillatory nature of spontaneous activity in the colon may not require sophisticated patterns of neural discharge.

Canine colonic circular muscle is driven by two discrete populations of pacemakers (Smith Reed & Sanders, 1987*a*, *b*). Cells along the submucosal surface of the circular muscle layer generate a 5 cycles per minute (c.p.m.) slow wave rhythm, whereas cells at the border between the circular and longitudinal muscle layers generate a 17 c.p.m. rhythm known as myenteric potential oscillations (MPOs; Smith *et al.* 1987*b*). Slow waves and MPOs summate within the circular muscle, resulting in a complicated pattern of electrical activity. The summed electrical activity initiates colonic contractions (Smith *et al.* 1987*a*). In addition to myogenic electrical activity, a slow electrical oscillation caused by the release of neurotransmitters is sometimes superimposed upon colonic electrical activity (Smith, Reed & Sanders, 1989). The purpose of the present study was to: (1) determine the role of NO in tonic inhibitory neural regulation of the canine colon, (2) characterize the actions of basal NO release on the electrical and contractile activity of the circular muscle layer, (3) determine whether neurogenic oscillations are due to patterned neural firing (periodic release of inhibitory transmitter; see Lyster *et al.* 1995) or continuous basal release of inhibitory transmitter, and (4) determine whether specialized cells in pacemaker regions mediate the actions of NO. Preliminary accounts of this work have appeared in abstract form (Keef, Murray & Smith, 1995).

METHODS

Experimental protocol

Mongrel dogs of either sex were killed with an overdose of sodium pentobarbitone (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, cleared of remaining faecal material and pinned-out in a dissecting dish containing oxygenated Krebs–Ringer bicarbonate solution (KRB) of the following composition (mM): NaCl, 118·5; KCl, 4·7; $CaCl_2$, 2·5; $MgCl_2$, 1·2; $NaHCO_3$, 23·8; KH_2PO_4, 1·2; and dextrose, 11·0. This solution had a pH of 7·4 at 37 °C when bubbled to equilibrium with 95% O_2–5% CO_2. Unless otherwise specified, experiments were performed under non-cholinergic, non-adrenergic (NANC) conditions (i.e. in the presence of 1 μM each of atropine, phentolamine and propranolol).

Strips (15 mm long) of the tunica muscularis were cut parallel to the circular muscle fibres with a knife consisting of a pair of parallel scalpel blades set 1·5 mm apart. The mucosa was removed from strips by sharp dissection. After dissection, the muscle strips were pinned in cross-section to the floor of an electrophysiological chamber. Two-thirds (i.e. 10 mm) of the length of the muscle strips were carefully pinned to facilitate intracellular recording, and the remaining third was unpinned. The end of the unpinned region was attached to a Statham tension transducer (Fig. 1). In this configuration (referred to as Type I muscle strips) the membrane potential at any point through the circular muscle layer (i.e. from the submucosal edge to the myenteric edge) could be recorded while simultaneously recording contractile activity of the entire muscle strip. The Type I configuration was used throughout this study unless otherwise stated in the text.

In experiments to determine the role of the pacemaker regions in the actions of sodium nitroprusside (SNP), strips of muscle were prepared by removing specific regions of the circular and longitudinal muscle layers with a fine diamond-edged knife. The resulting strips were of three types. Type II muscle strips were prepared by cutting away the submucosal edge and approximately one-third of the thickness of the circular muscle layer. Type III muscle strips were prepared by removing approximately half of the longitudinal muscle layer and the submucosal two-thirds of the circular muscle layer. The resulting Type III strips were composed of the myenteric pacemaker region and a third of the circular muscle layer. Type IV muscle strips were prepared by removing the submucosal edge, the longitudinal muscle layer, and a third of the circular muscle from the myenteric edge. The resulting Type IV strips were composed of approximately one-third of the circular muscle layer without either the submucosal or myenteric pacemaker regions. Type III and IV muscle strips were prepared from single intact cross-section strips of muscularis. Cells at the same relative positions through the thickness of the circular muscle layer were impaled in Types III and IV muscle strips. Schematic representations of these preparations are shown in Fig. 10.

Muscles were stretched to a resting tension of 1 g because this has previously been shown to produce optimal length (L_o) for contraction (see Keef, Ward, Stevens, Frey & Sanders, 1992). Muscle strips were superfused with warmed, oxygenated KRB solution. Temperature was monitored by a thermistor probe submerged in the perfusion solution near the muscle and was maintained at 37 ± 0·5 °C. Muscles were allowed to equilibrate for approximately 2 h before experiments were undertaken.

Muscle cells were impaled with glass microelectrodes filled with 3 M KCl with resistances ranging from 40 to 80 MΩ. Impalements were accepted based on previously discussed criteria (Sanders & Smith, 1986). Membrane potential was measured with a high input impedance electrometer (WPI Duo 773), and outputs were displayed on an oscilloscope (Nicolet 3091). Analog electrical and mechanical signals were digitized, recorded on videotape (Vetter 875), and reproduced on chart paper (Gould 2200). In experiments with oxyhaemoglobin the earth electrode was connected to the

recording chamber via an agar salt bridge to avoid problems of offset encountered with the haemolysate. In Type I muscle strips intracellular recordings were made at several positions through the circular muscle layer. Since the thickness of muscles varied, the point of impalement within the circular muscle layer was expressed as a percentage of the distance through the muscle layer (i.e. the submucosal edge of the circular muscle was considered 0% and the myenteric edge was considered 100%). The majority of recordings were made from cells at the 5, 50 and 95% points through the circular layer. Impalements were made within a 200 μm width of the pinned region directly adjacent to the unpinned region from which contractions were recorded.

Analysis of data

Several different parameters of electrical activity were tabulated. Resting membrane potential of cells near the submucosal edge of the circular muscle (5%) was determined as the most negative potential between slow waves. Maximum depolarization during slow waves was determined as the peak level of depolarization attained during the plateau phase. Resting membrane potential of cells in the myenteric region (95%) was determined as the mean of the most negative values occurring between MPOs. Peak depolarization was determined as the mean maximal level of depolarization reached during MPOs. 'Resting' membrane potential of muscles exhibiting neurogenic or SNP-induced slow oscillations was defined as the mean of the most negative values of membrane potential. The amplitude of slow oscillations was determined by subtracting 'resting' membrane potential from the mean of the most negative membrane potentials attained during the depolarization phase of the slow oscillation. Slow wave duration was determined as the time between 10% depolarization and 90% repolarization.

Significant differences between means were calculated by Student's two-tailed paired or unpaired t tests and values were considered significantly different when $P < 0{\cdot}05$. Only one muscle strip per animal was used and thus n values represent both the number of animals and the number of muscle strips used. Each cell impaled at a particular position (e.g. 5%) within the 200 μm recording width revealed a very similar electrical pattern due to the extensive electrical coupling between cells (see Smith *et al.* 1987*a*). In contrast, there were substantial differences in the electrical pattern recorded from a particular position in muscle strips taken from different animals. For this reason we have quoted electrical patterns in terms of the number of muscle strips which manifested the pattern. Data are expressed as means ± standard error of the mean (S.E.M.).

Drugs

Drugs used in this study included tetrodotoxin, atropine sulphate, phentolamine hydrochloride, propranolol hydrochloride, N^{ω}-nitro-L-arginine (L-NA), N^{ω}-nitro-L-arginine methylester (L-NAME) and sodium nitroprusside (all purchased from Sigma). *S*-nitroso-acetyl-penicillamine (SNAP) was obtained from Research Biochemicals (Natick, MA, USA). Oxyhaemoglobin was prepared from 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. The final dilution of oxyhaemoglobin used was 3%.

RESULTS

Relationship between electrical activity and contraction

Electrical slow waves were recorded from all cells impaled near the submucosal surface of the circular muscle layer. In 67% of muscles (i.e. 16 of 24 strips) spontaneous slow waves were of equal duration from event to event (see Fig. 2*A* and Table 1). In these muscles, slow waves depolarized to a mean level of $-38 \pm 1{\cdot}2$ mV ($n = 16$). In 33% of muscles an alternating pattern of long- and short-duration slow waves was observed (Fig. 2*B* and Table 1), as previously described (Sanders & Smith, 1986). Although we observed different slow wave patterns in different muscle strips, cells impaled

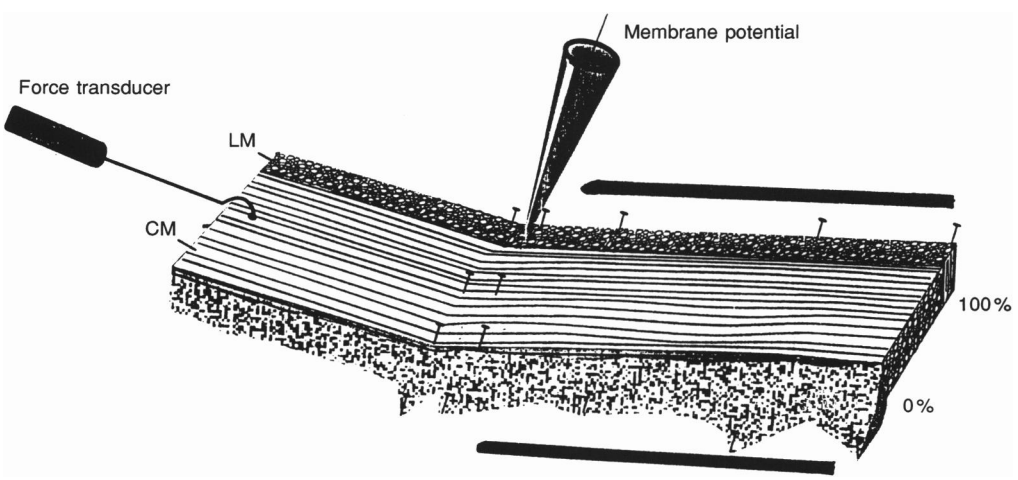

Figure 1. Diagram of the preparation used for simultaneous measurements of membrane potential and contractions

One end of the muscle strip was pinned to the floor of the recording chamber exposing the entire tunica muscularis, consisting of the circular (CM) and longitudinal (LM) muscle layers. The other end of the muscle was attached with a suture to a force transducer. Cells at 5, 50 and 95% of the distance through the CM were impaled with a glass microelectrode to measure membrane potential at representative points, and contractions were recorded from the full thickness of the CM (note that 0 and 100% were considered the submucosal and myenteric surfaces of the CM, respectively).

Table 1. Effect of L-NA and SNP on slow wave parameters

			Slow wave duration		Slow wave amplitude		
	Treatment	n	A Long (s)	B Short (s)	C Long (mV)	D Short (mV)	Percentage of muscles
1	Control	8	22·0 ± 1·8	5·2 ± 0·3	41·8 ± 2·2	38·2 ± 0·4	33
2	Control	16	—	4·9 ± 0·3	—	35·5 ± 1·3	67
3	L-NA	7	25·0 ± 1·7	5·2 ± 0·3	43·2 ± 1·8	37·0 ± 1·5	46
4	L-NA	8	—	4·8 ± 0·6	—	34·7 ± 1·2	54
5	L-NA + SNP	2	31·2	4·3	46·8	40	22
6	L-NA + SNP	7	—	3·8 ± 0·3*	—	34·4 ± 1·4	78

Shown are mean values ± S.E.M. for the length (s) and amplitude (mV) of long- and short-duration slow waves under control conditions (rows 1 and 2) *vs.* L-NA (rows 3 and 4) *vs.* L-NA plus SNP (rows 5 and 6). n values represent the number of muscle strips tested. Rows 1, 3 and 5 indicate mean values for muscles exhibiting the intermittent long-/short-duration pattern and rows 2, 4, 6 indicate mean values for muscles exhibiting equal-duration slow waves. The percentage of muscles with the intermittent pattern increased from 33% of control muscles (row 1) to 46% of muscles in the presence of L-NA (row 3) and decreased to 22% of muscles with combined L-NA and SNP (row 5). L-NA, N^{ω}-nitro-L-arginine (100 μM). Sodium nitroprusside (SNP, 1 μM). Of the following statistical comparisons (Student's two-tailed, unpaired t test): 1A *vs.* 3A; 1B *vs.* 2B; 1B *vs.* 3B; 2B *vs.* 4B; 2B *vs.* 6B; 3B *vs.* 4B; 1C *vs.* 3C; 1D *vs.* 2D; 1D *vs.* 3D; 2D *vs.* 4D; 2D *vs.* 6D; and 3D *vs.* 4D, only 2B *vs.* 6B was statistically different ($P < 0·05$) and is indicated with an asterisk.

within the submucosal region of a given strip each revealed the same slow wave pattern (e.g. 5 additional impalements in the tissue represented in Fig. 2*B* produced recordings characterized by an alternating pattern of long- and short-duration slow waves). The maximal level of depolarization reached during long- and short-duration slow waves did not differ significantly (i.e. −35 ± 2·2 and −38 ± 1·1 mV, respectively; $P > 0·05$).

Slow waves were associated with small spontaneous phasic contractions recorded from the entire circular muscle strip (Fig. 2). Spontaneous contractions were noted in 85% of preparations (28 of 33 strips). The frequency of equal-duration slow waves (i.e. 4·7 ± 0·2 c.p.m.) was identical to the frequency of contractions (i.e. 4·7 ± 0·2 c.p.m.; $n = 16$). Long-duration contractions were associated with long-duration slow waves ($n = 8$; Fig. 2*B*). The relationship

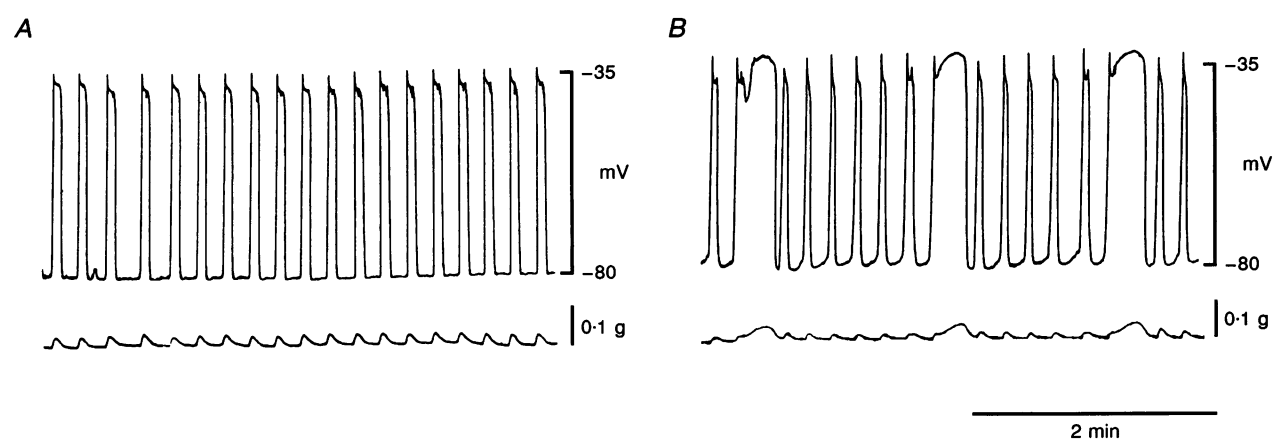

Figure 2. Examples of the two types of electrical activity (top traces) recorded near the submucosal surface of the circular muscle layer in different preparations

Contractions were recorded simultaneously from the entire circular muscle layer (bottom traces). Panel *A* shows a typical example of the pattern of slow waves recorded in 67% of muscle strips. Each slow wave was similar in amplitude and duration and coupled to a small phasic contraction. Slow wave frequency in this example is 4·5 c.p.m. and mean duration is 5·5 s. Panel *B* shows an example of the alternating pattern of long- and short-duration slow waves observed in 33% of muscles. The frequency of short-duration slow waves in this example is 5 c.p.m. and mean slow wave durations are 19·7 s (long slow waves) and 4·8 s (short slow waves). Longer-duration slow waves gave rise to contractions of greater amplitude and duration than short-duration slow waves. Time scale in *B* applies to *A*.

between the pattern of slow waves and the pattern of phasic contractions is consistent with previous descriptions of the coupling between electrical and mechanical activities in this preparation (e.g. Smith *et al.* 1987*a*). Since phasic contractions correlated with the pattern of slow waves, the contractile record, which represent a sum of the activity of the entire circular muscle layer, could be used as an indirect indicator of slow wave activity when impalements were made in circular muscle cells near the myenteric edge (see below).

The amplitude of spontaneous contractions associated with short-duration slow waves varied substantially among preparations (range, 7–200 mg; mean, 25 ± 5 mg; $n = 16$), with the largest spontaneous contractions representing approximately 5% of the maximum ACh-induced (100 μM) contraction (see Keef *et al.* 1992). There was no significant difference in the amplitude of contractions occurring during short-duration slow waves in muscles with equal-duration slow waves *versus* muscles with the intermittent long-/short-duration pattern (25 ± 5 mg ($n = 16$) *versus* 26 ± 5 mg ($n = 8$); $P > 0{\cdot}05$). In contrast, long-duration slow waves were associated with a 2·4 ± 0·7-fold significant increase in contractile amplitude ($n = 8$; $P < 0{\cdot}05$).

Cells were also impaled within the interior (i.e. at 50% of the distance through the muscle layer) and near the myenteric edge of the circular muscle (at 95%; see Fig. 1). Resting membrane potential (most negative potential) and slow wave amplitude decreased as a function of distance from the submucosal surface (Fig. 3), as previously reported (see Smith *et al.* 1987*a*,*b*). The typical electrical activity recorded from cells near the myenteric surface consisted of MPOs; in 69% of muscle strips) with a mean peak amplitude of 9·6 mV (Table 2 and Fig. 4*A*). In 25% of muscle strips (8 of 32) slower electrical oscillations were apparent (Fig. 4*B*), and these events have been attributed to neurogenic influences in previous studies (Smith *et al.* 1989; Lyster *et al.* 1995). In muscle strips exhibiting neurogenic oscillations this pattern was noted in each cell impaled in the myenteric region of the circular muscle layer. Neurogenic oscillations reached significantly more hyperpolarized levels (i.e. −64·2 ± 1·2 mV; $n = 8$; $P < 0{\cdot}05$) than those observed in preparations without this type of activity (Fig. 3*A*). The neurogenic oscillations averaged 14 ± 1·9 mV in amplitude and occurred at a regular frequency which averaged 1·0 ± 0·1 c.p.m. (see Table 2).

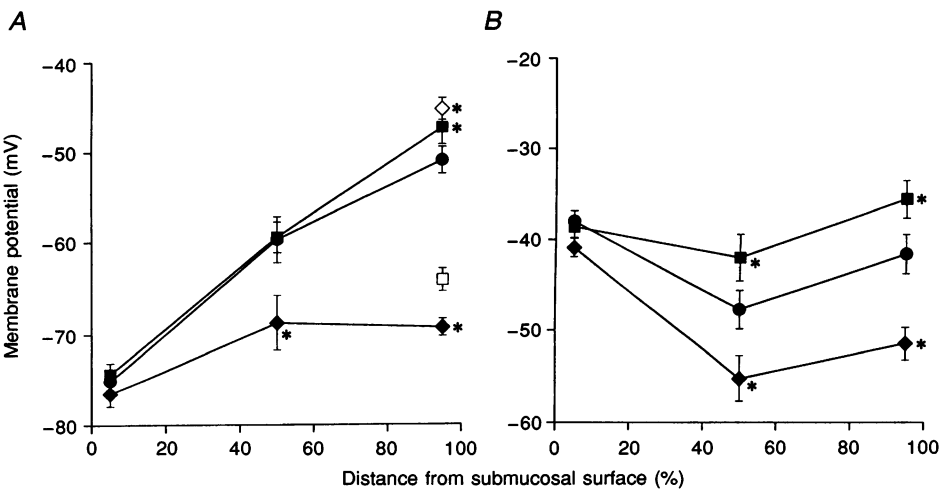

Figure 3. Comparison of resting membrane potential (*A*) and peak depolarization of spontaneous electrical events (*B*) at 5, 50 and 95% of the distance from the submucosal surface of the circular muscle in the presence or absence of L-NA (100 μM) or L-NA (100 μM) plus SNP (1 μM)

A, under control conditions in 75% of muscles (●) a significant gradient in the resting membrane potential was observed from the submucosal edge (most negative) to the myenteric edge (least polarized). In the remaining 25% of muscles neurogenic oscillations were observed in which membrane potential periodically reached significantly more negative levels (□). L-NA caused a significant depolarization of the resting membrane potential near the myenteric surface in preparations without neurogenic oscillations (● *vs.* ■) and in preparations with neurogenic oscillations (□ *versus* ◇). Subsequent addition of SNP (◆) in the continued presence of L-NA caused significant hyperpolarization of the resting membrane potential below the control level in the middle and near the myenteric edge (● *vs.* ◆). *B*, in contrast to the resting membrane potential (*A*) no significant gradient in peak depolarization was observed between the submucosal surface and the myenteric surface. Addition of L-NA significantly increased peak depolarization of cells in the middle of the muscle and near the myenteric surface (● *vs.* ■). SNP reversed this effect, leading to a significant decrease in peak depolarization in the middle of the muscle and near the myenteric surface (● *vs.* ◆). Data for panels *A* and *B* consist of means ± S.E.M. in cells impaled at 5, 50 and 95% of the distance through the muscle layer from 5–14 muscle strips. Asterisks indicate significant differences obtained from the comparisons indicated above.

Table 2. Time course and amplitude of electrical events recorded at the myenteric border in the presence and absence of L-NA and SNP

	MPO				Slow oscillation			
Treatment	n	Frequency (c.p.m.)	Amplitude (mV)	dV/dt ($V\ s^{-1}$)	n	Frequency (c.p.m.)	Amplitude (mV)	Occurrence (%)
Control	18	16·1 ± 0·8	9·6 ± 0·2	0·012 ± 0·002	32	1·0 ± 0·1	14·1 ± 1·9	25
L-NA	14	20·5 ± 1·2*	13·2 ± 1·5*	0·020 ± 0·004*	21	0	0	0
L-NA + SNP	12	16·0 ± 2·0	4·4 ± 0·8*	0·007 ± 0·001*	19	1·1 ± 0·1	13·4 ± 1·4	74

Shown are mean values ± S.E.M. n values indicate the number of muscle strips tested. The mean values shown on the left (MPO) were determined from all muscle strips tested (e.g. 18 of 18 control muscles). The mean values listed on the right were determined in those tissues which exhibited slow electrical oscillations (e.g. 8 of 32 control muscle strips or 25% exhibited spontaneous electrical oscillations). L-NA, N^{ω}-nitro-L-arginine (100 μM); Sodium nitroprusside (SNP, 1 μM). Asterisks indicate values significantly different ($P < 0·05$) from control.

In most muscles (i.e. 29 of 35, or 83%) we did not observe an obvious relationship between the electrical activity recorded near the myenteric edge of the circular muscle and contractions recorded from the entire circular layer (e.g. Fig. 4*A*). In a few cases there was a relationship between these activities (e.g. see Fig. 9*A*). Occasionally, spikes were superimposed on MPOs, and these events were associated with contractions at the slow wave frequency (Fig. 4*B*).

Inhibition of nitric oxide synthesis enhances electrical and mechanical activity

Addition of the arginine analogue L-NA (100 μM) significantly increased the amplitude of spontaneous phasic contractions by a mean of 2·3 ± 0·3-fold ($n = 16$; $P < 0·05$), suggesting that there was ongoing release of nitric oxide (NO) in these muscles. In addition, the percentage of muscles in which intermittent long-duration slow waves were recorded increased from 33 to 46% of muscles (see Fig. 5*A* and Table 1). Fast oscillations of membrane potential were superimposed upon the plateau phase of slow waves in 40% of muscles after addition of L-NA. The amplitude and time course of short- and long-duration slow waves were not significantly enhanced by L-NA (Table 1).

The neural blocker tetrodotoxin (TTX) was tested to determine whether spontaneous release of NO is neural in origin. TTX (1 μM) significantly increased the mean spontaneous contractile amplitude by 1·9 ± 0·4-fold ($n = 19$; $P < 0·05$). Subsequent addition of L-NA in the presence of TTX gave rise to a further 0·6-fold increase in contractile amplitude (total increase with L-NA plus TTX was 2·5 ± 0·4-fold greater than control; $n = 6$; $P < 0·05$). In contrast, TTX had no additional effect when added in the presence of L-NA ($n = 10$). Thus, it appears that a

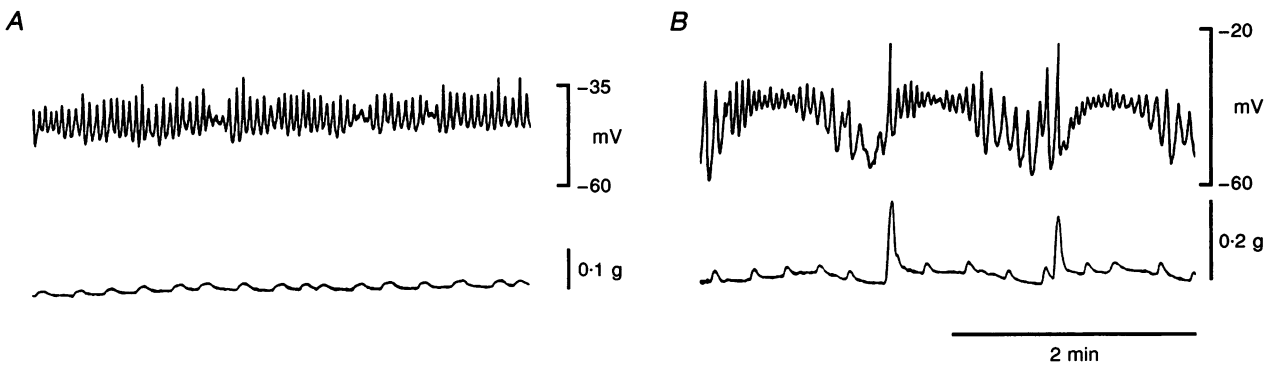

Figure 4. Examples of the two types of electrical activity (top traces) recorded near the myenteric surface of the circular muscle in different preparations

Contractions were recorded simultaneously from the entire circular muscle layer (bottom traces). *A*, example of the typical MPO activity recorded from 69% of preparations. In this example there was no obvious relationship between contractions and MPOs. Rather, contractions occurred in conjunction with slow waves, as shown in Fig. 2. *B*, in another preparation neurogenic oscillations are shown superimposed upon MPOs. Neurogenic oscillations were observed in 25% of muscles. Contractile tone was less during the hyperpolarizing phase of the neurogenic oscillation and spike potentials were associated with larger amplitude phasic contractions. Time scale in *B* applies to *A*.

portion of the spontaneous release of NO is due to activation and propagation of action potentials in enteric neurons, and part is due to action potential-independent release of NO.

In muscles without neurogenic oscillations L-NA caused a significant depolarization of resting membrane potential in the myenteric region from $-51{\cdot}0 \pm 1{\cdot}5$ to $-47{\cdot}3 \pm 1{\cdot}8$ mV ($n = 10$; $P < 0{\cdot}05$; Fig. 3*A*). In muscles which exhibited neurogenic oscillations L-NA abolished the oscillations (see Fig. 7*B*) and depolarized cells from $-64{\cdot}2 \pm 1{\cdot}2$ to $-45{\cdot}3 \pm 1{\cdot}2$ mV ($n = 4$; $P < 0{\cdot}05$; Fig. 3*A*). L-NA also significantly increased the peak depolarization reached during MPOs ($P < 0{\cdot}05$; Fig. 3*B*), as well as the frequency, amplitude and upstroke velocity of MPOs (Fig. 6*B* and Table 2; $P < 0{\cdot}05$). The number of tissues in which there was a correlation between electrical activity recorded in the myenteric region and contraction of the whole tissue increased after addition of L-NA (i.e. 17% in control *vs.* 34% in the presence of L-NA; see Figs 6*B* and 7*B* for examples of correlated activity). Likewise, following addition of L-NA, the number of muscles with occasional spike potentials increased (i.e. from 19 to 24% of muscles). Contractile amplitude increased 3- to 6-fold when a spike occurred (Fig. 7*B*).

To determine whether the blockade of neurogenic oscillations by L-NA was specific for the NO pathway we tested two additional blockers, i.e. L-NAME and oxyhaemoglobin. Neurogenic oscillations were entirely blocked with

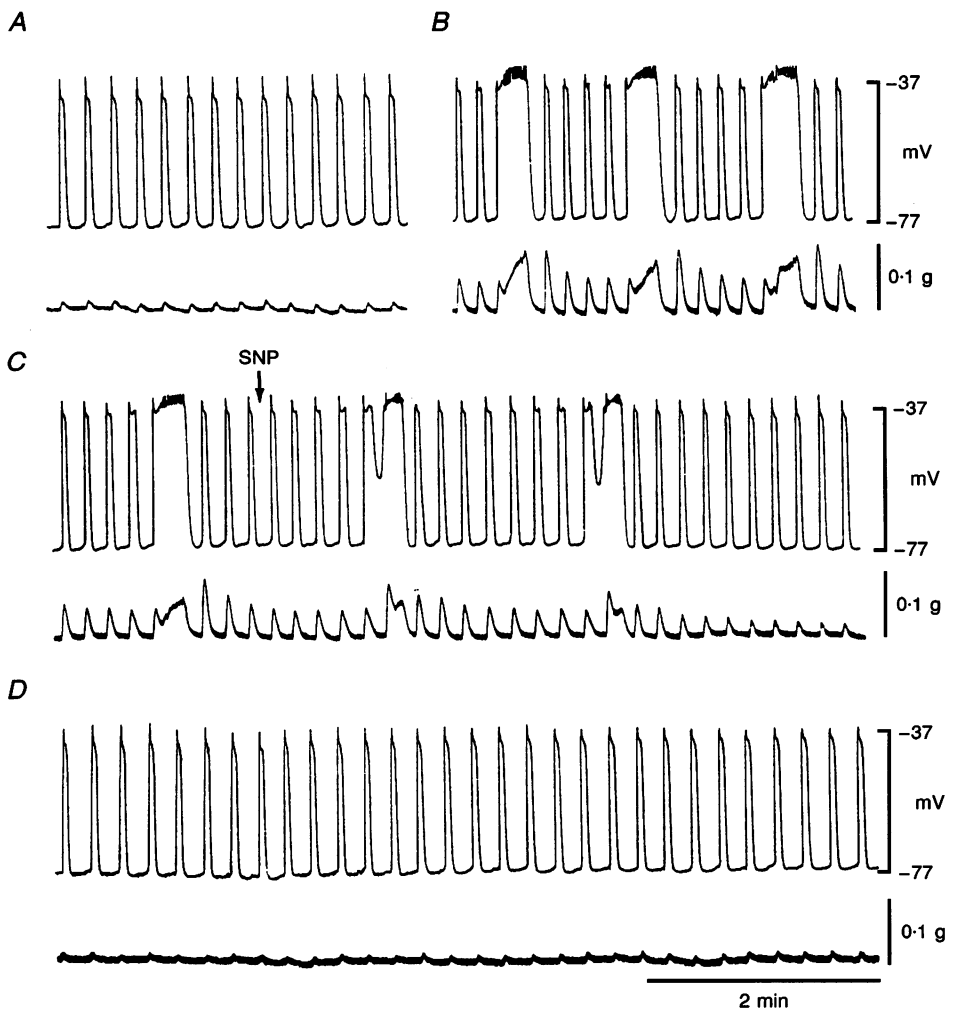

Figure 5. Effects of L-NA and SNP on slow waves in a single muscle strip

A, control recording of slow waves from a cell near the submucosal surface (upper trace) along with contractions recorded from the entire circular muscle layer (lower trace). *B*, recording from another cell in the same region in the presence of L-NA (100 μM; added 6 min before the start of this record). Exposure to L-NA resulted in a large increase in the amplitude of contractions and the appearance of intermittent long-duration slow waves with superimposed rapid oscillations. There was no significant change in the time course of short-duration slow waves (see Table 1 for summarized data). *C*, recording from a third cell in the same region after 19 min in the continued presence of L-NA. SNP (1 μM) was added to the superfusion medium at the arrow. This abolished the long-duration slow waves and reduced the force of phasic contractions. *D*, continued recording from the same cell as *C*, demonstrating that long-duration slow waves were abolished throughout the exposure to SNP. Time scale applies to all panels.

L-NAME (100 μM; see Fig. 6*B*), and this compound also decreased resting membrane potential from $-62{\cdot}3 \pm 1{\cdot}8$ to $-46{\cdot}8 \pm 1{\cdot}6$ mV ($n = 3$; $P < 0{\cdot}05$). Oxyhaemoglobin (3%) decreased resting membrane potential from $-60{\cdot}2$ to $-45{\cdot}3$ mV in the one preparation tested and also blocked neurogenic oscillations.

SNP reversed the actions of L-NA

Addition of SNP (1 μM), in the continued presence of L-NA, reduced the amplitude of contractions by 90% (i.e. from $63{\cdot}1 \pm 17{\cdot}5$ mg in L-NA to $6{\cdot}3 \pm 1{\cdot}5$ mg in L-NA and SNP; $n = 7$). The decrease in contractile force was associated with a significant hyperpolarization of membrane potential near the myenteric surface and within the interior of the muscle ($P < 0{\cdot}05$; Fig. 3*A*). SNP also decreased peak depolarization ($P < 0{\cdot}05$; Fig. 3*B*) as well as the amplitude, frequency and upstroke velocity of MPOs ($P < 0{\cdot}05$; Table 2). The spikes observed in some muscles after L-NA were abolished by SNP (Fig. 7*C*).

In 26% of muscles (i.e. 5 of 19 muscles) SNP (1 μM) caused sustained hyperpolarization of cells near the myenteric edge from $-56{\cdot}3 \pm 1{\cdot}1$ to $-68{\cdot}8 \pm 1{\cdot}8$ mV ($P < 0{\cdot}05$). In the remaining preparations (i.e. 14 of 19 muscles or 74%), constant exposure to SNP induced slow oscillations in membrane potential (Fig. 7*C*). This activity developed within 4–5 min of adding SNP and persisted for the duration of electrical recording (in one case 3 h). The SNP-induced slow oscillations were statistically identical in time course and magnitude to the neurogenic slow oscillations observed in 25% of preparations under control conditions (see Table 2). SNP restored the neurogenic pattern of slow oscillations to muscles which had displayed this behaviour before addition of L-NA ($n = 4$; Fig. 7), and induced slow oscillations in muscles which did not exhibit this behaviour spontaneously ($n = 11$). SNP-induced oscillations persisted in the presence of TTX (1 μM; $n = 4$) but were abolished by 3% oxyhaemoglobin ($n = 3$). The SNP-induced oscillations were greatest in amplitude near the myenteric surface ($14{\cdot}1 \pm 1{\cdot}9$ mV; $n = 8$), significantly smaller in the central region ($8{\cdot}5 \pm 1{\cdot}6$ mV; $n = 6$; $P < 0{\cdot}05$), and were not observed in cells near the submucosal surface ($n = 9$; Fig. 8), consistent with the characteristics of the neurogenic oscillations (Smith *et al.* 1989). Application of the NO donor SNAP (1 μM) also caused slow electrical oscillations (i.e. frequency, $1{\cdot}1 \pm 0{\cdot}1$ c.p.m.; amplitude, $12{\cdot}2 \pm 2{\cdot}5$ mV; $n = 3$, Fig. 9*A*), which did not differ in time course and magnitude from those elicited with SNP ($1{\cdot}0 \pm 0{\cdot}1$ c.p.m.; $14{\cdot}1 \pm 1{\cdot}9$ mV; $n = 8$). The SNAP-induced slow electrical oscillations were also abolished by 3% oxyhaemoglobin ($n = 3$; Fig. 9*B*).

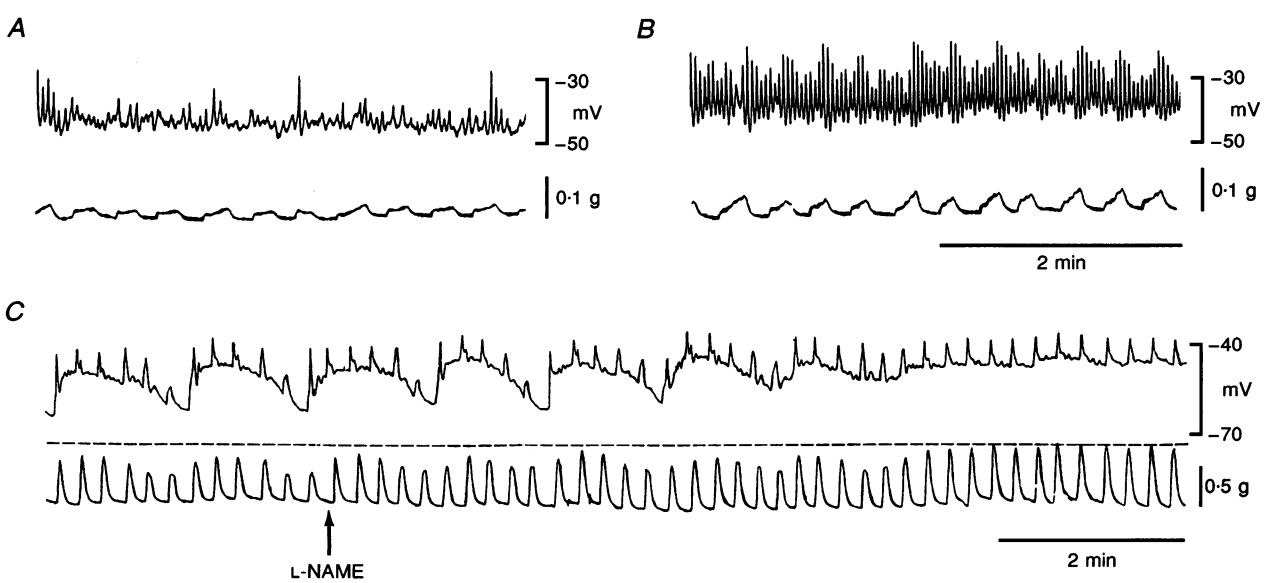

Figure. 6. Effect of L-NA on electrical activity (top traces each panel) recorded from cells near the myenteric surface and contractions (bottom traces) of the entire circular muscle layer in two different preparations

A, typical electrical activity recorded from a cell located 95% distance from the submucosal surface. This tissue exhibited MPOs but no neurogenic oscillations. There was little or no relationship between MPOs and contractions (time scale for *A* indicated in *B*). *B*, recording from the same cell as in *A* 6 min after addition of 100 μM L-NA to the superfusion medium. L-NA caused depolarization, and increased the frequency, amplitude and dV/dt of MPOs (see Table 2 for summarized data). *C*, recording from another tissue which exhibited neurogenic oscillations. This cell was located at 75% of the distance from the submucosal surface and small amplitude slow waves are apparent superimposed upon the neurogenic rhythm. The slow waves are associated with phasic contractions. Note the fluctuation in contractile amplitude which accompanies the rise and fall of neurogenic oscillations. L-NAME (100 μM) was added at the arrow, and this caused depolarization and abolition of the neurogenic oscillations.

SNP did not significantly hyperpolarize cells near the submucosal surface of the circular muscle layer ($n = 9$; Fig. 3*A*; $P < 0{\cdot}05$), but it decreased the duration of slow waves (Table 1). Four of the nine muscles tested exhibited an alternating pattern of long- and short-duration slow waves. Of these four muscles, SNP abolished the long-duration slow waves in two (e.g. Fig. 5*C*) and did not abolish them in two (e.g. Fig. 8*C*). Overall, therefore, 22% of muscles exhibited long-duration slow waves in L-NA plus SNP (i.e. 2 of 9 muscles) *versus* 46% of muscles bathed in L-NA alone (i.e. 7 of 15 muscles) *versus* 33% of control muscles (i.e. 8 of 24; see Table 1). SNP completely abolished the small fast electrical oscillations that were occasionally superimposed upon slow waves. These data show that SNP (1 μM) reversed the effects of L-NA in the submucosal region.

The myenteric pacemaker region is required for generation of NO-dependent slow oscillations

To determine whether the pacemaker regions contribute to the generation of SNP-induced slow electrical oscillations, additional experiments were performed in which the myenteric and/or submucosal pacemaker regions (Smith *et al.* 1987*a*,*b*) were removed (see descriptions of Type II–IV muscle strips in the Methods). In the presence of L-NA (100 μM) all muscle strips containing the myenteric pacemaker region (i.e. Types I–III) exhibited MPOs and had similar resting potentials (i.e. $-49 \pm 1{\cdot}7$ mV ($n = 10$) in Type I; $-50{\cdot}3 \pm 1{\cdot}8$ mV ($n = 3$) in Type II; -51 ± 2 mV ($n = 4$) in Type III). In contrast, Type IV muscles, which had the myenteric and submucosal pacemaker areas removed, were electrically quiescent. Resting membrane potentials were significantly more negative in Type IV strips than in Type III strips at similar points of impalement (i.e. $-62{\cdot}4 \pm 1{\cdot}5$ mV *vs.* -51 ± 2 mV, respectively; $n = 4$; $P < 0{\cdot}05$). The difference in resting membrane potentials in Types III and IV muscle strips may be due to the presence of pacemaker cells in the Type III strips, as previously suggested (Liu & Huizinga, 1993).

SNP (1 μM) induced slow electrical oscillations in muscles with the submucosal pacemaker region removed (i.e. Types II and III muscles; see Fig. 10*B* and *C*), and the time course and magnitude of these oscillations was not significantly different from the oscillations recorded from intact muscle

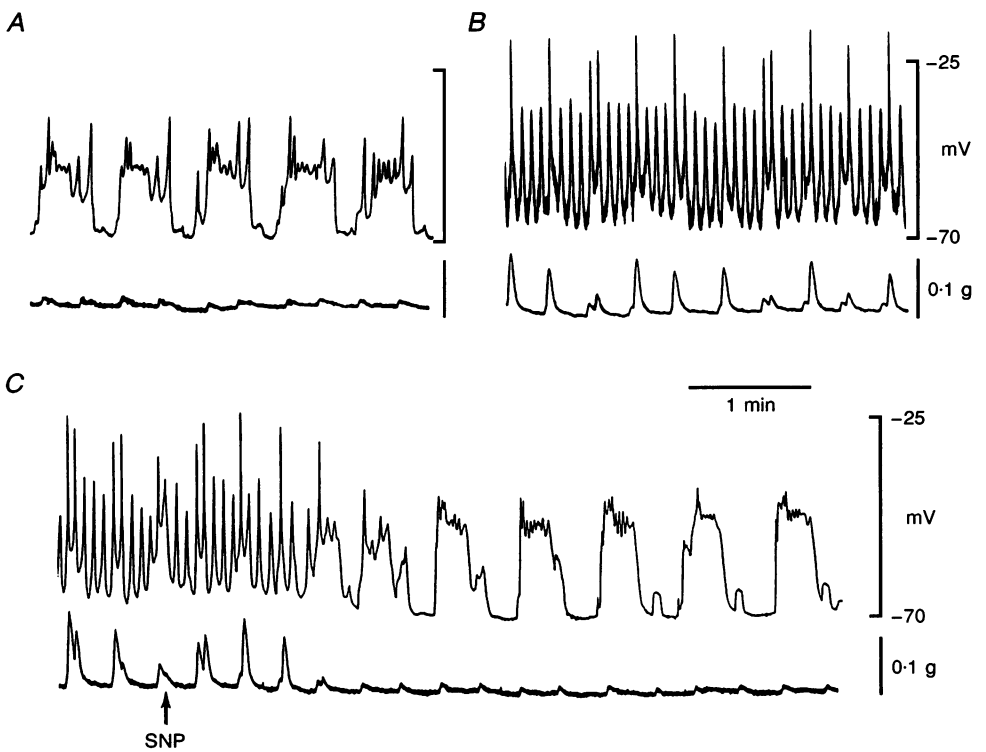

Figure 7. Effect of L-NA and SNP on electrical activity recorded from cells near the myenteric surface in a single muscle strip

A, MPOs with superimposed neurogenic oscillations were recorded from cells in this preparation near the myenteric surface in the absence of L-NA as shown in this representative recording. *B*, L-NA (10 μM) enhanced the amplitude of MPOs, caused occasional spikes, and abolished the neurogenic oscillations as shown in this recording obtained from a different cell 20 min after application of L-NA. Note the increase in amplitude of phasic contractions which is associated with these changes in electrical activity. Time scale applies to all panels. *C*, in a third cell impaled near the myenteric surface the effects of SNP (1 μM) are shown. SNP was added to the superfusate at the arrow (L-NA present throughout) and resulted in the development of a slow electrical rhythm which was very similar to the control neurogenic oscillations.

strips (i.e. amplitudes were 14·1 ± 1·9 mV ($n = 8$), 13·7 ± 2·1 mV ($n = 3$) and 17·1 ± 1·7 mV ($n = 4$) and frequencies were 1·1 ± 0·1 c.p.m. ($n = 8$), 1·2 ± 0·2 c.p.m. ($n = 3$) and 1·1 ± 0·1 c.p.m. ($n = 4$) for Types I, II and III, respectively). SNP failed to induce slow electrical oscillations in preparations with both pacemaker regions removed (Type IV preparations). In these muscles SNP caused a small hyperpolarization (i.e. from −62·4 ± 1·5 to −66·5 ± 2·1 mV; $n = 4$; Fig. 10*D*), but it did not induce oscillations. The lack of slow oscillations in Type IV muscles could have been due to the more negative resting membrane potentials recorded in these muscles. However, cells in intact muscle strips (Type I) at 50% of the distance through the circular muscle layer had resting potentials similar (−60·6 ± 1·1 mV; $n = 10$) to Type IV muscles (−62·4 ± 1·5; $n = 4$), and SNP induced slow oscillations (8·5 ± 1·6 mV amplitude; $n = 6$) in the intact muscles. These data suggest that cells within the myenteric pacemaker region are required for induction of NO-induced slow electrical oscillations.

DISCUSSION

This study demonstrates that the 'spontaneous' electrical and mechanical behaviour of canine colonic muscle is modulated by basal release of NO. NO appears to originate, at least in part, from an action potential-dependent mechanism in enteric neurons because more than half of the tonic inhibitory drive was blocked by TTX. Similar tonic neural inhibition was first proposed in the feline small bowel by Wood (1972). The remaining NO-dependent tonic inhibition appears to involve NO, which is released in a TTX-insensitive manner. Possible sources for TTX-insensitive NO release include nerve terminals (Ward *et al.* 1992*b*), interstitial cells of Cajal (Publicover, Hammond & Sanders, 1993; Xue *et al.* 1994), endothelial cells of small blood vessels (Pollock *et al.* 1993), macrophages (Xie *et al.* 1992), and smooth muscle cells (Grider *et al.* 1992). Continuous suppression of visceral smooth muscle activity by NO is a common occurrence, and evidence for this phenomenon was noted in the first published studies

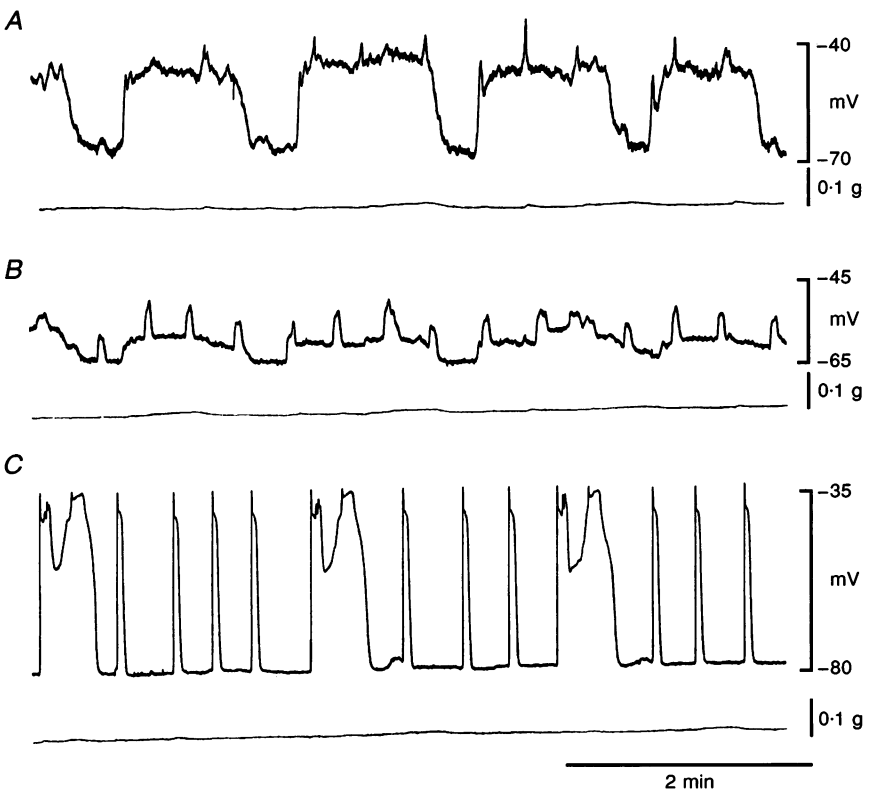

Figure 8. SNP-induced electrical oscillations decrease with distance from the myenteric edge (100 μM L-NA and 1 μM SNP present throughout)

A, SNP-induced slow oscillations recorded near the myenteric surface (at 95% of the distance through the muscle layer) were largest in amplitude as shown in this typical example. *B*, in another cell recorded from the middle of the circular muscle (at 50%) slow oscillations were still resolvable but were of smaller amplitude than at the myenteric edge (see text for mean values). *C*, in a third recording made from a cell located near the submucosal surface (at 5%) slow electrical oscillations are not resolvable. The long-duration slow waves which persisted in this preparation in the presence of SNP (see Table 1) were not synchronized with the SNP-induced slow oscillations. In the absence of L-NA and SNP there were no slow electrical oscillations in this preparation. Time scale applies to all panels.

investigating the role of NO in neural regulation of smooth muscles (e.g. Gillespie, Liu & Martin, 1989; Li & Rand, 1989; Boeckxstaens *et al.* 1990). These early studies were performed on tonic muscles, and it was found that inhibition of NO synthesis increased contractile tone. The results of the present study suggest that in addition to tonically reducing the amplitude of phasic contractions, basal release of NO is responsible for producing a new rhythmic pattern of electrical and mechanical activity. This activity is superimposed upon myogenic pacemaker potentials, and may have important consequences in the overall output of colonic motility.

In a previous study of the canine colon we noted large spontaneous membrane hyperpolarizations that originated in the myenteric region and were manifest in both the circular and longitudinal muscle layers (Smith *et al.* 1989). In that study the cyclical nature of these events was not discussed. Spontaneous hyperpolarizations were insensitive to hexamethonium but were blocked by morphine or TTX, leading to the conclusion that the oscillations were neurogenic in origin. We speculated that the spontaneous hyperpolarizations could be due to periodic release of inhibitory transmitters giving rise to 'spontaneous' inhibitory junction potentials (Smith *et al.* 1989). At the time, the transmitter mediating enteric inhibitory responses in the canine colon was unknown, so it was not possible to test whether restoration of the transmitter after inhibition of nerves would restore the oscillatory pattern. Since the duration of each neurogenic hyperpolarization was much longer than the duration of a single inhibitory junction potential we speculated that the periods of hyperpolarizations were due to trains of action potentials followed by periods of quiescence. Indeed, such a mechanism was also suggested more recently for TTX-sensitive oscillatory myogenic activity in mouse colon (Lyster *et al.* 1995). In this muscle periods of myogenic inhibition were proposed to be due to activation of inhibitory motoneurons, while periods of excitation were attributed to suppression of inhibitory inputs together with activation of excitatory inputs. Such a mechanism requires phasically active enteric neurons and

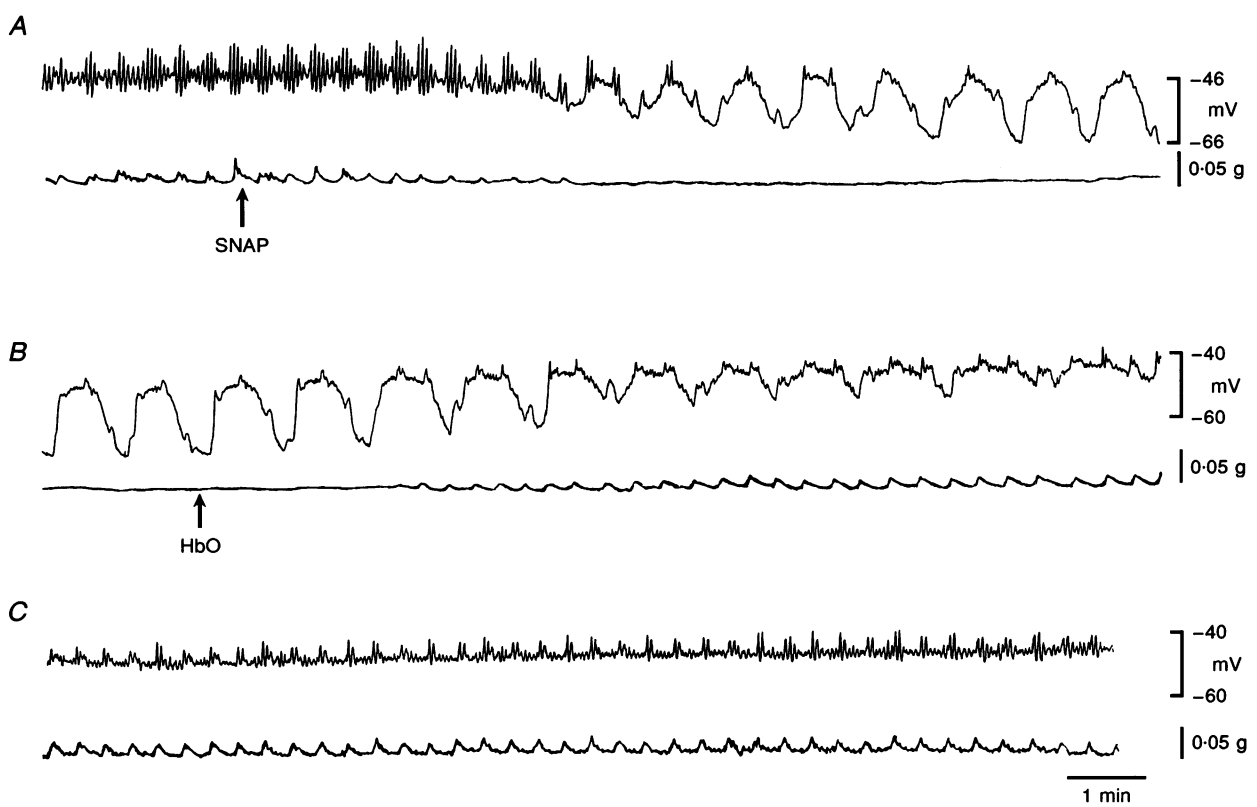

Figure 9. SNAP also induced NO-dependent slow oscillations, and this pattern of activity was reversed by oxyhaemoglobin (HbO)

A, MPOs (top trace), recorded from a cell near the myenteric edge of the circular muscle, waxed and waned in amplitude. Contractions (bottom trace) occurred with each burst of MPOs. SNAP (1 μM; added to superfusate at the arrow) induced slow electrical oscillations which were similar to the events obtained with SNP (e.g. compare with Figs 7*C*, 8*A* and 10*A–C*). *B*, in the continued presence of SNAP addition of HbO (arrow) gradually reduced the amplitude of slow oscillations (trace *B* begins 3 min after the end of trace *A*). *C*, in the continued presence of SNAP and HbO the slow electrical oscillations were completely abolished (trace *C* begins 5 min after the end of trace *B*). Electrical traces in all panels are excerpts from a continuous impalement of a single cell.

significant co-ordination between inhibitory and excitatory motor inputs. Previous studies have reported the existence of bursting neurons in the enteric nervous system (see Wood, 1994), but the findings of the current study suggest that the neurogenic oscillatory behaviour of the proximal colon (Smith *et al.* 1989; Lyster *et al.* 1995) can be accomplished by tonic levels of NO since: (1) neurogenic oscillations were blocked by arginine analogues or oxyhaemo-globin, and (2) an electrical pattern virtually indistinguishable from neurogenic oscillations could be evoked by constant levels of SNP or SNAP. Oscillations induced by NO donors were unlikely to be related to neural activity since they could be obtained in the combined presence of atropine, phentolamine, propranolol, L-NA and TTX.

SNP gave rise to slow electrical oscillations in 74% of preparations. In contrast, only 25% of control muscles exhibited spontaneous neurogenic oscillations. The greater effect of NO donated by SNP or SNAP *versus* neurally released NO is likely to be concentration dependent (e.g. basal release of NO may not raise tissue levels of NO to the same extent as exogenous application of 1 μM NO donors). In a previous study it was reported that continuous activation of enteric inhibitory nerves with electrical field stimulation at 1 Hz gave rise to slow fluctuations in membrane potential which were similar to the spontaneous neurogenic oscillations reported in this study (Thornbury *et al.* 1991). Thus, in preparations that do not have sufficient basal release of NO to elicit neurogenic oscillations, supplementation of NO levels by stimulation of inhibitory neurons can generate this pattern of activity. In intact colonic segments, the prevalence of the neurogenic pattern of oscillations might also be enhanced by reflex activation of inhibitory motoneurons (Furness & Costa, 1977).

In intact muscle strips the electrical changes evoked by inhibition of basal NO release, sequestration of basal NO, and application of NO donors were much greater in the myenteric pacemaker region than in the submucosal pacemaker region. Large amplitude, slow electrical oscillations were elicited by SNP in preparations lacking the submucosal pacemaker region, whereas SNP did not elicit slow oscillations in preparations lacking the myenteric pacemaker region. We have also noted that neurogenic oscillations and SNP-induced slow oscillations are greatest in amplitude at the myenteric surface and decrease as a function of distance from the myenteric edge (Smith *et al.* 1989, and present

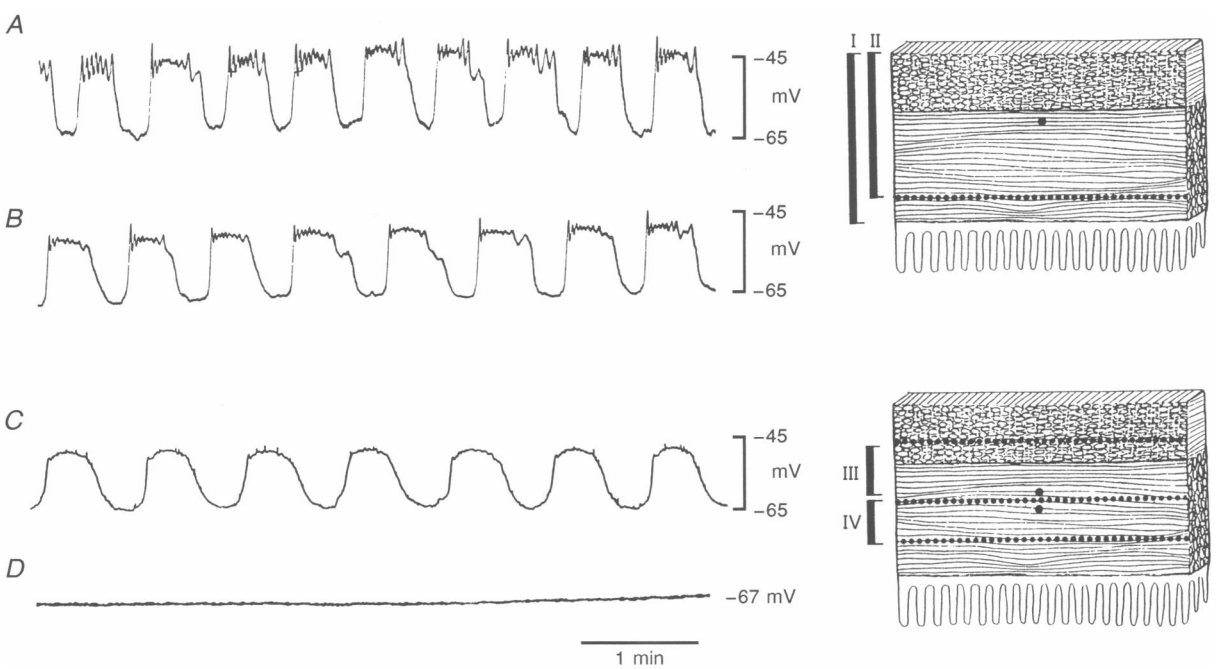

Figure 10. Slow electrical oscillations induced by NO donors depend upon the integrity of the myenteric pacemaker region

Intracellular recordings were made in Types I–IV muscle strips shown schematically on the right (see Methods for additional details). Dotted lines indicate where the muscle was cut and larger black dots indicate where the microelectrode was placed. Two intact muscle strips were used to produce all four sections. All muscles were pretreated with SNP (1 μM) to elicit slow electrical oscillations (100 μM L-NA present throughout). *A*, recording from an intact (Type I) muscle strip. Typical slow electrical oscillations were observed. *B*, recording from another cell in the same muscle 45 min after removal of the submucosal pacemaker region (Type II). As shown in this example, SNP-induced oscillations were still present in these muscles. *C*, slow oscillations were also observed in Type III muscle strip, as shown in this recording. *D*, in the absence of both pacemaker regions (Type IV muscles) membrane potential did not oscillate in the presence of SNP, as shown in this recording from a Type IV muscle strip.

study). These observations suggest that cells within the myenteric pacemaker region may be responsible for these particular effects of NO. At present we do not know whether NO elicits slow oscillations via its effects on enteric neurons, interstitial cells of Cajal (ICC), or the smooth muscle cells that populate the immediate vicinity of the pacemaker region. It is interesting to note that a recent study of neurogenic responses in the mouse stomach has suggested that NO-dependent effects are mediated by ICC (Burns, Lomax, Torihashi, Sanders & Ward, 1996). It is possible that removal of the interstitial cell population within the myenteric pacemaker region in the canine colon is responsible for the reduction in responses to SNP in this tissue as well.

Blockade of NO synthesis with L-NA enhanced the frequency, amplitude and upstroke velocity of MPOs while SNP had the opposite effect. Greater depolarization of cells tends to enhance excitation–contraction coupling, and therefore regulation of electrical activity is likely to contribute to the modulation of contractile amplitude by NO. The ionic mechanisms responsible for the electrical effects of NO are not entirely clear. NO increases K^+ conductance in canine colonic muscles (Thornbury *et al.* 1991; Koh Campbell, Carl & Sanders, 1995) and the effects of NO on K^+ conductances of ICC or smooth muscle cells could account for the hyperpolarizing action of NO. NO and/or cGMP have also been shown to suppress voltage-dependent Ca^{2+} channel currents in smooth muscle cells (Clapp & Gurney, 1991; Ishikawa, Hume & Keef, 1993; Koh & Sanders, 1996), and effects of NO on Ca^{2+} channels in either smooth muscle cells or ICC could affect electrical rhythmicity. The actions of NO on pacemaker activity may therefore involve effects on both K^+ and Ca^{2+} channels, as well as other undescribed ionic pathways.

The slow oscillatory electrical behaviour which can be produced by constant exposure to NO in this study can be elicited by other agonists that increase K^+ conductance. For example, forskolin, which activates a 4-aminopyridine-sensitive K^+ conductance in the canine colon, produced a similar oscillatory behaviour (Smith *et al.* 1993; Du, Carl, Smith, Khoyi, Sanders & Keef, 1994). The neurogenic pattern characterized in the present study appears to be attributable to release of NO from nerves. However, the general pattern of slow electrical oscillations is likely to be a more fundamental characteristic of the overall current–voltage relationship of the pacemaker/smooth muscle apparatus which can develop as K^+ conductance increases.

Although the effects of NO near the submucosal surface were less than near the myenteric surface, blockade of NO synthesis with L-NA did lead to a small increase in the percentage of muscles exhibiting a pattern of long- and short-duration slow waves (i.e. from 33 to 46% of muscles). Many agonists evoke this slow wave pattern in the canine colon (e.g. Huizinga, Chang, Diamant & El-Sharkawy, 1984; Sanders & Smith, 1986; Keef *et al.* 1992), but the ionic mechanisms responsible are still unclear. The NO donor SNP reversed the effects of NO blockade and led to a further reduction in the percentage of muscles with this pattern to 22% of muscles. These results suggest that NO may also affect the mechanisms which regulate slow waves. Whether this involves modulation of K^+ and/or Ca^{2+} conductance or some other ionic conductance is not known. It should be noted that stimuli that enhance tissue levels of cAMP also block the pattern of long- and short-duration slow waves (Smith *et al.* 1993).

In conclusion, our results suggest that spontaneous electrical and contractile activity of colonic muscles is tonically suppressed by basal release of NO from nerves. The highly patterned activity resulting from basal NO release, does not require organized, intermittent neural activity; rather a continuous, low level of tonic NO release could produce this oscillatory activity. The oscillatory behaviour induced by NO is superimposed upon myogenic rhythmicity and appears to result from stimulation of the pacemaker/smooth muscle apparatus within the myenteric region of the tunica muscularis.

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