British Journal of Pharmacology (1996) 118, 1605-1614

Effects of nitric oxide (NO) and NO donors on the membrane conductance of circular smooth muscle cells of the guinea-pig proximal colon

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1 The membrane conductance changes underlying the membrane hyperpolarizations induced by nitric oxide (NO), S-nitroso-L-cysteine (NC) and sodium nitroprusside (SNP) were investigated in the circular smooth muscle cells of the guinea-pig proximal colon, by use of standard intracellular microelectrode recording techniques.

2 NO (1%), NC (2.5-25 μM) and SNP (1-1000 μM) induced membrane hyperpolarization in a concentration-dependent manner, the hyperpolarizations to NO and NC developing more rapidly than those to SNP. The slower-developing responses to SNP were mimicked by the membrane permeable analogue of guanosine 3':5' cyclic-monophosphate (cyclic GMP), 8-bromo-cyclic GMP (500 μM), and by isoprenaline (10 μM).

3 The hyperpolarizations to NC and SNP were reduced in a low Ca^{2+} (0.25 mM) saline and upon the addition of haemoglobin (20 μM), but were not effected by N^G-nitro-L-arginine (L-NOARG) (100 μM) or ω-conotoxin GVIA (100 nM). The hyperpolarizations to SNP were also significantly reduced by methylene blue (50 μM).

4 Apamin (250 nM) depolarized the membrane potential approximately 10 mV and reduced the initial transient component of the hyperpolarization to NO (1%) and NC (25 μM), but had no effects on the hyperpolarizations to SNP and cyclic GMP. Tetraethylammonium (TEA) (5-15 mM), had little effect on the membrane responses to NO(1%), NC(2.5-25 μM), SNP(100-1000 μM) or cyclic GMP(500 μM). However, TEA (5-15 mM) reduced the membrane hyperpolarizations to SNP (10 μM) and isoprenaline (10 μM) in a concentration-dependent manner. The hyperpolarization to isoprenaline (10 μM) remaining in the presence of 15 mM TEA was blocked by ouabain (10 μM).

5 The amplitude of electrotonic potentials (1 s duration) elicited during NO donor hyperpolarizations were little changed or only slightly reduced (5-25%). However, the amplitude of the electrotonic potentials elicited during maintained electrically-induced hyperpolarizations of similar amplitude were significantly increased (30-150%), suggesting that the non-linear membrane properties of the proximal colon partially mask an increase in membrane conductance elicited during the NO donor hyperpolarizations.

6 Membrane hyperpolarization in the presence of an NO donor, 8-bromo-cyclic GMP, isoprenaline, or upon application of a maintained hyperpolarizing electrical current, often evoked oscillations of the membrane potential. These oscillations were prevented by Cs^+ (1 mM).

7 These results indicate that NO and NC hyperpolarize the circular muscle of the proximal colon by activating at least two TEA-resistant membrane K^+ conductances, one of which is sensitive to apamin blockade. The K^+ conductance increases activated by SNP or 8-bromo-cyclic GMP were little effected by apamin, perhaps suggesting a common mechanism. In contrast, the hyperpolarization to isoprenaline appears to involve the activation of TEA-sensitive Ca^{2+}-activated K^+ ('BK') channels, as well as a Na:K ATPase. Finally, the 'background' membrane conductance of the circular muscle cells of the proximal colon decreased upon membrane hyperpolarization to reveal oscillations of the membrane potential which may well represent 'pacemaker' or 'slow wave' activity.

Keywords: Nitric oxide; NO donors; NANC inhibition; apamin; K^+ channel blockers; membrane conductance; circular muscle of guinea-pig proximal colon

Introduction

In many gastrointestinal preparations, transmural electrical stimulation produces inhibition of the mechanical and electrical activity of the smooth muscle layers which remains in the presence of a muscarinic antagonist and after sympathetic denervation (Furness, 1969; Maggi & Giuliani, 1993). Muscle relaxation in most preparations is preceded by a transient hyperpolarization of the membrane potential, the inhibitory junction potential (i.j.p.), which prevents action potential discharge and therefore the Ca^{2+} entry necessary for the maintenance of muscle tone. In the guinea-pig colon, the initial component of this non-adrenergic, non-cholinergic (NANC) relaxation and i.j.p., in response to electrical stimulation or distension, can be blocked by apamin, a blocker of the small-conductance ('SK') Ca^{2+}-activated K^+ channels (Blatz & Magelby, 1986) and by suramin, the putative P_2-purinoceptor antagonist. On the other hand, the 'apamin-resistant' component of the NANC relaxation or i.j.p. can be antagonized by inhibitors of nitric oxide synthase (NOS) (Maggi & Giuliani, 1993; Zagorodnyuk & Maggi, 1994; Watson *et al.*, 1992; 1996), suggesting that NANC inhibition in the colon arises from the neuronal release of both ATP and nitric oxide (NO), or a related NO-donor compound. Such a pharmacological division of the NANC i.j.p. into a

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transient apamin-sensitive i.j.p. and a slower NO-dependent i.j.p., however, does not necessarily apply to other gastrointestinal preparations or species studied. In the rat colon, the i.j.p. is fully blocked by apamin and little affected by NOS inhibition (Suthamnatpong *et al.*, 1994), while the opposite applies in the dog colon and opossum oesophagus (Daniel *et al.*, 1983; Jury *et al.*, 1985; 1992; Smith *et al.*, 1989; Dalziel *et al.*, 1991; Du *et al.*, 1991). In the human colon and the dog pylorus or duodenum, the component of the i.j.p. that is blocked by NOS inhibition is also partially blocked by apamin (Bayguinov *et al.*, 1992; Bayguinov & Sanders 1993; Keef *et al.*, 1993). In contrast, NOS inhibition reduces both the apamin-sensitive and apamin-resistant components of the i.j.p. recorded in the dog ileocolonic sphincter (Ward *et al.*, 1992c). In addition, many gastrointestinal preparations often display a period of 'rebound' contraction and post-stimulus depolarization (PSD) following the NANC i.j.p. and relaxation which have been attributed to the activation of a depolarizing cation-selective inward rectifier current at negative potentials (Benham *et al.*, 1987), or to the release of excitatory tachykinins (Bywater *et al.*, 1983; Giuliani *et al.*, 1993; Zagorodnyuk & Maggi, 1994), NO and/or prostaglandins (Ward *et al.*, 1992b; Lyster *et al.*, 1993; Saha *et al.*, 1993; Venkova & Krier 1994).

Attempts to mimic the effects of endogenously-released NO with the direct application of NO, or substances which donate NO, have also revealed a confusing variety of responses, depending on both the gastrointestinal preparation and the nature of the NO donor. In dog colon, pyloric sphincter and duodenum, NO and S-nitroso-L-cysteine (NC) both induce apamin-sensitive membrane hyperpolarization and rebound PSD which are associated with an increase in the internal levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Bayguinov *et al.*, 1992; Ward *et al.*, 1992a; Bayguinov & Sanders, 1993). Similar hyperpolarizing responses to NO in human colon, however, are not reduced by apamin (Keef *et al.*, 1993). In the rat gastric fundus, the hyperpolarizing responses to NC, but not sodium nitroprusside (SNP), are partially inhibited by apamin (Kitamura *et al.*, 1993). In single cells of the dog colon, NC also increases the activity of apamin-resistant/TEA sensitive large conductance Ca^{2+}-activated K^+ ('BK') channels recorded in cell-attached membrane patches (Thornbury *et al.*, 1991). However, the hyperpolarizing responses of the rat gastric fundus to NC are not blocked by concentrations of tetraethylammonium (TEA) (1 mM) which would be expected to block 'BK' channels, nor by other K^+ channels blockers such as 4-aminopyridine (4-AP, 1–10 mM) and glibenclamide (1 μM) (Kitamura *et al.*, 1993). Responses to both NC and SNP, however, were reduced significantly by 10 mM TEA. The application of 8-bromo-cyclic GMP also induced membrane hyperpolarization in the rat gastric fundus which are unaffected by apamin (1 μM), TEA (1 mM) or glibenclamide (1 μM) (Kitamura *et al.*, 1993).

The membrane conductance changes underlying the apamin-sensitive i.j.p. has long been thought to involve an increase in the membrane K^+ conductance (Tomita, 1972; den Hertog & Jager, 1975). On the other hand, the apamin-resistant i.j.p. has been reported to be associated with little change in membrane conductance (Lyster *et al.*, 1992), an increase in the membrane K^+ conductance (Jury *et al.*, 1985; 1992; Christinck *et al.*, 1990; Kitamura *et al.*, 1993), or with a decreased Cl^- conductance (Crist *et al.*, 1991). In the present experiments, we examine the nature of the membrane conductance changes underlying the hyperpolarizations and rebound PSDs of the circular muscle of the guinea-pig proximal colon induced by the NO donors, NO, NC and SNP. We also compare the nature of these NO donor conductance changes with the conductance changes which occur during the hyperpolarizations induced upon the application of 8-bromo-cyclic GMP or upon the injection of a constant hyperpolarizing electrical current. These results have been presented previously in brief (Watson *et al.*, 1993; 1994a).

Methods

Segments of proximal colon (5 cm in length, cut 1 cm distal to the caecum) were dissected from guinea-pigs (weighing 300–600 g), killed previously by a blow to the head and exsanguination, and placed in a dissection dish containing physiological saline (see below), constantly bubbled with a 95% O_2: 5%CO_2 gas mixture. 'T'-shaped preparations of the muscle wall were dissected from these colon segments such that the horizontal bar of the 'T' was cut parallel to the longitudinal muscle and the vertical portion of the 'T' was cut in the circumferential direction. The circular muscle axis of these 'T' preparations was placed between two large AgCl:Ag plate electrodes, which were used to inject hyperpolarizing and depolarizing currents. Two small silver wires (2 mm apart), attached to a differential amplifier, were located in the partition compartment to monitor the generated field strength (V cm^{-1}), as an estimate of current amplitude. Changes in the membrane conductance were estimated by plotting the steady-state amplitude (V) of the resulting electrotonic potentials against the amplitude of applied depolarizing and hyperpolarizing currents (I) (1–10 V cm^{-1} of 1 s duration). Intrinsic NANC nerves were activated by transmural electrical stimulation (single or 3–8 pulses at 20–50 Hz and 2–30 V) delivered by a pair of Ag/AgCl wires, placed just oral to the vertical portion of the 'T'. Membrane potentials recordings were made from circular smooth muscle cells approximately 2 mm outside the plate electrodes and 2 mm anal to the transmural stimulating wires.

Electrophysiological recordings

Membrane potential changes in cells of the circular muscle layer were recorded with glass microelectrodes (1.5 mm o.d.: Clarke Electromedical Instruments) (resistances of 30–100 MΩ when filled with 2 M KCl) attached to a unity-gain pre-amplifier (WPI M4A). A second 'reference' microelectrode was placed approximately 0.5 mm from the recording microelectrode. The position of this reference electrode was carefully positioned to monitor and annul any extracellular potential changes before, during and after application of extracellular current injection. The voltage difference between these two electrodes was recorded differentially. This 'corrected' membrane potential and current field strength were recorded on a 4 channel FM tape recorder (Tandberg Instrumentation Recorder.Series-115). Records were later digitized and analyzed using a Labmaster TL-1 (Axon Instruments) analog-to-digital board and a personal computer running Axotape 2.02 (Axon Instruments) and Sigmaplot 5.0 (Jandel Scientific) software.

Physiological solutions

The physiological saline had the following composition (mM): Na^+ 146, K^+ 5, Ca^{2+} 2.5; Mg^{2+} 1, Cl^- 126, $H_2PO_4^-$ 1, SO_4^{2-} 1, HCO_3^- 25, glucose 11. This solution was constantly bubbled with a 95% O_2:5%CO_2 gas mixture that maintained the pH between 7.3 and 7.4. In some experiments a low Ca^{2+} (0.25 mM), high Mg^{2+} (3.25 mM) solution was used.

Drugs

In all experiments, the 'control' saline contained nifedipine (1 μM, Bayer) and hyoscine (1 μM, Sigma) to suppress tissue movement. The following drugs were prepared as stock solutions (10 μM–10 mM): ω-conotoxin GVIA (Peninsular), N^G-nitro-L-arginine (L-NOARG), L-arginine (Nova Biochem), caesium chloride (Cs^+), sodium nitroprusside (SNP) (May and Baker), isoprenaline, apamin, tetrodotoxin, 4-aminopyridine (4-AP), glibenclamide, tetraethylammonium (TEA), methylene blue, 8-bromo-cyclic GMP and ouabain (all Sigma, U.S.A.). Nifedipine was dissolved in ethanol. Stock solutions of S-nitroso-L-cysteine (NC) (10 mM) were prepared on a weekly basis by the method described by Field

et al. (1978), while haemoglobin was prepared from human type IV haemoglobin (Sigma) (Bowman *et al.*, 1982). Infusion of the vehicle for NC (for 20 s) had no effect on the membrane potential or conductance. Stock solutions of sodium nitroprusside (May and Baker) were dissolved in distilled water and kept frozen for no more than 7 days. NO solutions were prepared daily by a method similar to that described by Stark *et al.* (1991) and added directly to the bathing solution. All stock solutions were kept at 4°C and diluted to their final concentration in physiological saline as indicated.

Statistical analysis of data

The membrane potential and the amplitude and time-to-peak of the NO donor hyperpolarizations and evoked electrotonic potentials were expressed as mean ± s.e.mean, with n indicating the number of preparations. Where appropriate, Students t tests or 1 to 3-way analyses of variance (ANOVA) were used to determine statistical significance between either averaged data or the slopes and shape of the V-I plots. Results were deemed statistically significant when $P<0.05$.

Results

In the presence of nifedipine (1 μM) and hyoscine (1 μM), the circular smooth muscle cells of the proximal colon had a membrane potential of about −60 mV (see below) and sometimes displayed slow oscillations of potential at a frequency of 17 ± 2.9 min^{-1} ($n=6$). Spontaneous hyperpolarizations (5–15 mV in amplitude) similar in time course to nerve-evoked NANC i.j.ps were also sometimes observed.

Effects of nitric oxide (NO) and NO donors

Application of NO (0.1–1% for 5s) hyperpolarized the circular muscle of the proximal colon in a concentration-dependent manner (see Figure 1a). The hyperpolarization consisted of an initial transient hyperpolarization which reached a maximum amplitude of 27.5 ± 1.8 mV ($n=5$) within 4 s, and then decayed to a less hyperpolarized level for several seconds. The NO donors, S-nitroso-L-cysteine (NC) (2.5–25 μM) (Figure 1c) and sodium nitroprusside (SNP) (1 μM–1 mM) (Figure 1b,d) (applied for 20 s–1 min) also hyperpolarized the proximal colon in a concentration-dependent manner, with 25 μM NC and 1 mM SNP respectively inducing maximal membrane hyperpolarizations of 25.5 ± 1.7 ($n=3$) and 16.5 ± 0.5 ($n=3$) mV. Half-maximal membrane hyperpolarizations were obtained with approximately 5 μM NC and 50 μM SNP, respectively. Rhythmic oscillations in the membrane potential (with a frequency of 21 ± 2.8 min^{-1}) were often but not always recorded during these hyperpolarizations to NC (2.5–25 μM) and SNP (100 μM and 1 mM) (Figure 1). Following these hyperpolarizations, a small rebound PSD (3–6 mV) lasting for tens of seconds was also often recorded. In some experiments, the enteric inhibitory nerves were also stimulated (3 pulses at 2–30 V and 20–50 Hz) before and during the application of NO, NC or SNP. The amplitude of these electrically-evoked i.j.ps was always reduced during the hyperpolarizations evoked upon the application of an NO donor (Figure 1b) (see also Kitamura *et al.*, 1993).

Effects of 8-bromo-cyclic GMP or isoprenaline

The addition of 8-bromo-cyclic GMP (500 μM), the membrane permeable cyclic GMP analogue (Figure 1e), or isoprenaline (10 μM) (Figure 1f) also induced membrane

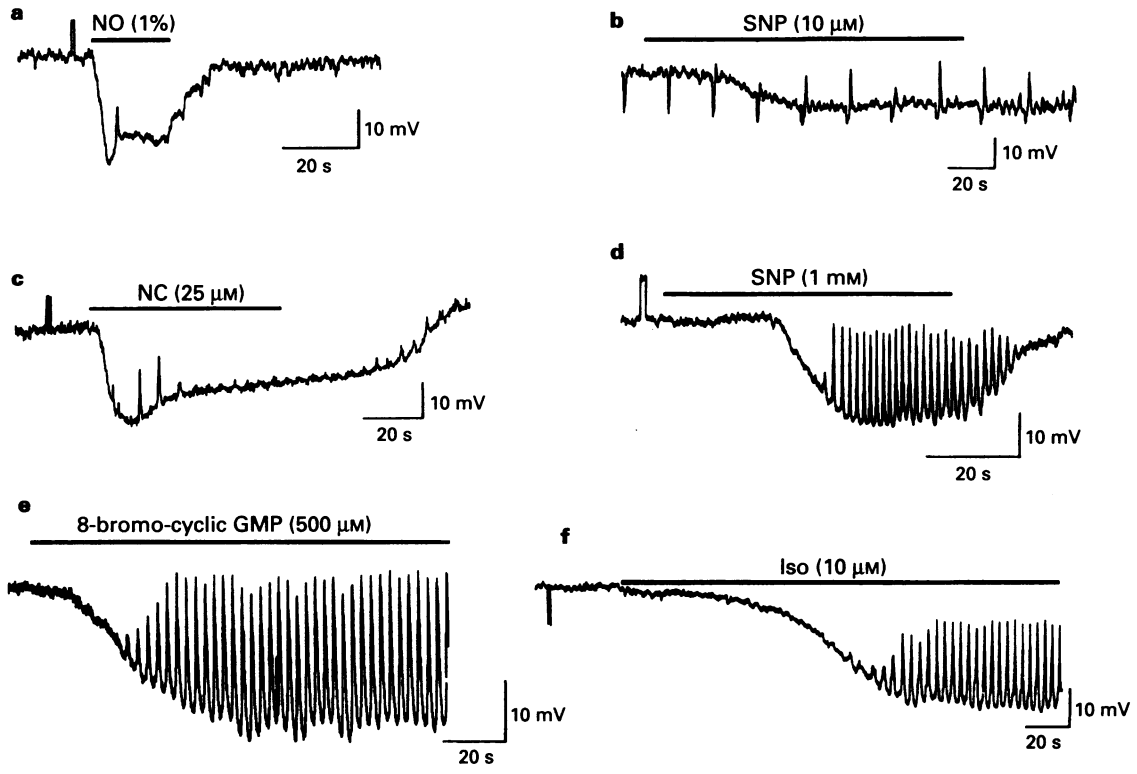

Figure 1 The effects of nitric oxide (NO, 1%)(a), sodium nitroprusside (SNP, 10–1000 μM) (b,d), S-nitroso-L-cysteine (NC, 25 μM)(c), 8-bromo-cyclic GMP (500 μM)(e) and isoprenaline (Iso, 10 μM)(f) on the membrane potential of the circular smooth muscle of the guinea-pig proximal colon, previously arrested with nifedipine (1 μM) and hyoscine (1 μM). In (b), NANC i.j.ps were also recorded upon stimulation of the enteric inhibitory motor nerves (3 pulses at 2–30 V and 20–50 Hz every 20 s) before and during the application of SNP (10 μM). Note that i.j.p. amplitudes were always reduced during the hyperpolarizations evoked upon the application of an NO donor.

hyperpolarization of 16±0.7 mV ($n=7$) and 21.1±2.0 mV ($n=5$) respectively in the proximal colon. However, these hyperpolarizations were slower in time course than those induced by NO, NC or SNP; e.g. a 1–2 min exposure to 8-bromo-cyclic GMP induced a hyperpolarization with a time to maximal amplitude of 206±15.4 s ($n=7$) and which persisted for 10–20 min. In approximately 50% of preparations, membrane oscillations (frequency 7–15 min^{-1}) were induced during the membrane hyperpolarization to cyclic GMP or isoprenaline (Figure 1e,f).

Effects of L-NOARG and haemoglobin

L-NOARG, an inhibitor of nitric oxide synthase (NOS) was applied to the proximal colon to investigate whether the effects of NO, or the NO donor compounds, were mediated via the

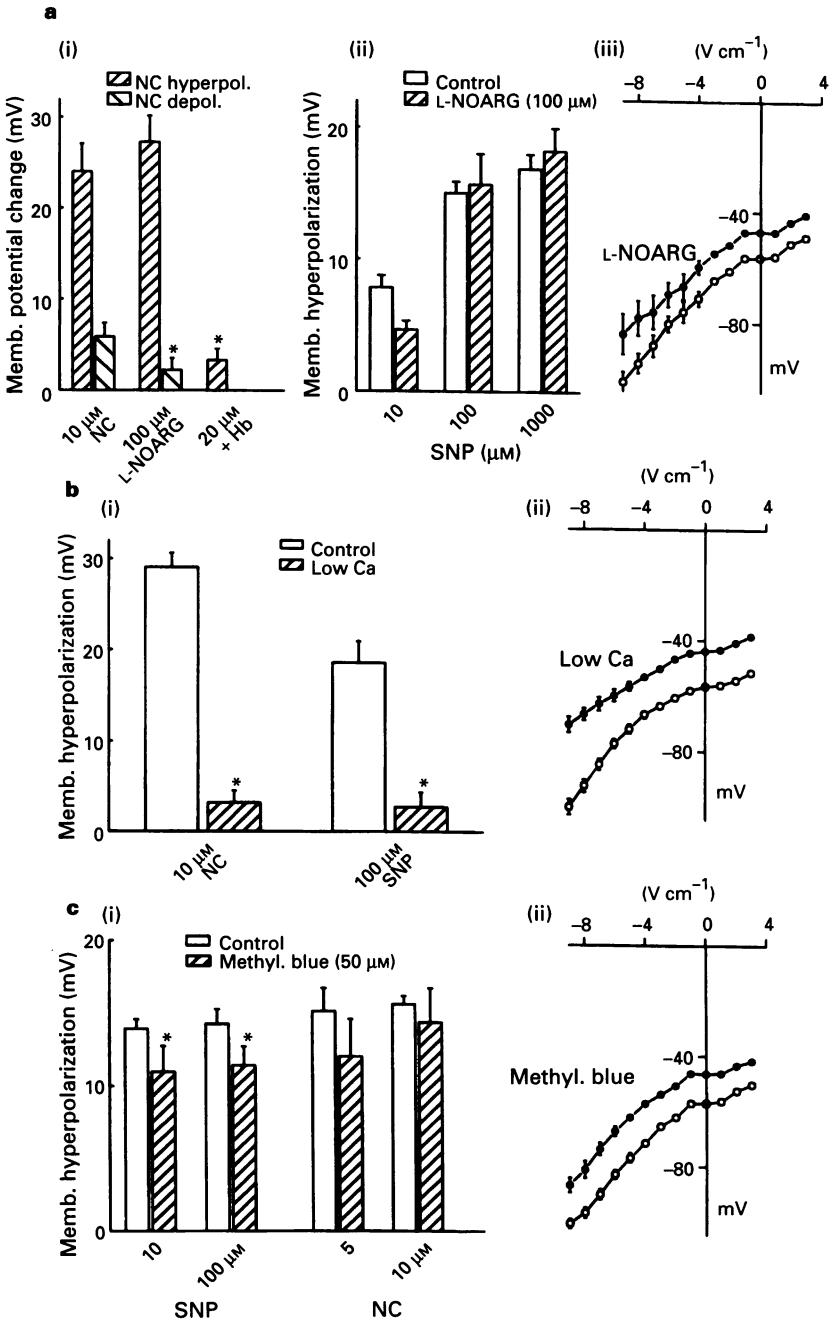

Figure 2 Effects of N^G-nitro-L-arginine (L-NOARG, 100 µM)(a), a low Ca^{2+} (0.25 mM) saline (b) and methylene blue (50 µM)(c) on the hyperpolarizing and rebound depolarizing (PSD) membrane responses to S-nitroso-L-cysteine (NC, 10–100 µM) and sodium nitroprusside (SNP, 10–1000 µM). (a), L-NOARG (100 µM) had no effect on the hyperpolarizations to NC (10–100 µM) (a(i), NC hyperpol.) or SNP (10–1000 µM) (a(ii)), but significantly reduced the amplitude of the NC-induced rebound PSD (a(i), NC depol.). This reduction of the PSD is perhaps associated with the 10 mV depolarization of the voltage-current (*V-I*) relationship of the proximal colon (a(iii)). Haemoglobin (Hb, 20 µM), added in the presence of L-NOARG, significantly reduced the responses to NC (a(i)). (b) Exposure to low Ca (0.25 mM) saline significantly reduced the membrane responses to NC (10 µM) and SNP (100 µM)(b(i) Control) and shifted the *V-I* plots 10 mV in the depolarized direction (b(ii)). (c) Methylene blue significantly reduced the membrane responses to SNP (10–100 µM), but not to NC (5–10 µM) (c(i)), and depolarized the *V-I* plot 10 mV (c(ii)). In all panels * denotes a significant difference at $P<0.05$.

production and release of NO from NANC nerves, smooth muscle or interstitial cells (Publicover *et al.*, 1993; Grider *et al.*, 1994). The addition of L-NOARG (100 μM) caused a significant depolarization of the membrane potential to -47.0 ± 0.6 mV (control -56.3 ± 0.3 mV; $n=4-6$, unpaired t test), but did not significantly change the slope or shape of the *V-I* curve recorded in response to various depolarizing (1–3 V cm^{-1}) and hyperpolarizing (1–9 V cm^{-1}) currents (Figure 2a(iii)). The hyperpolarizations induced by NC (10 μM) ($n=7$) (Figure 2a(i)) or SNP (10–1000 μM) ($n=3$) (Figure 2a(ii)) were little affected in the presence of L-NOARG; the amplitude of the NC-induced PSD, however, was significantly reduced ($n=4$, paired t test) (Figure 2a(i)). In the continued presence of L-NOARG, the addition of the NO scavenger, haemoglobin (20 μM), significantly reduced the hyperpolarization produced by 10 μM NC ($n=4$, paired t test) (Figure 2a(i)). Haemoglobin (10–20 μM for 20 min), however, had no effect on the concentration-response curve to SNP, even though the membrane potential depolarized to -56.2 ± 1.3 mV (control membrane potential -59.6 ± 0.9 mV) ($n=4$) (data not shown).

Effects of low Ca^{2+}: high Mg^{2+} solution

Replacing the normal physiological saline with a low Ca^{2+} (0.25 mM): high Mg^{2+} (3.25 mM) solution (for 10–30 min) depolarized the membrane potential to -43.8 ± 0.7 mV (control -56.6 ± 0.4 mV) ($n=10-16$), which was associated with a significant decrease in the slope of the *V-I* plot at potentials negative to -70 mV (3 way ANOVA) (Figure 2b(ii)). In the presence of this low Ca^{2+} saline, the hyperpolarizations to both NC (10 μM) and SNP (100 μM) were significantly reduced ($n=4-8$, unpaired t test) (Figure 2b(i)). Under these conditions, the NANC i.j.ps were also reduced and finally abolished in a time-dependent manner (Watson *et al.*, 1994b; 1996). In contrast, the addition of the neuronal 'N-type' Ca^{2+} channel blocker, ω-conotoxin GVIA (0.1 μM), depolarized the membrane potential to -46.6 ± 1.2 mV (control -56.8 ± 1.2 mV),

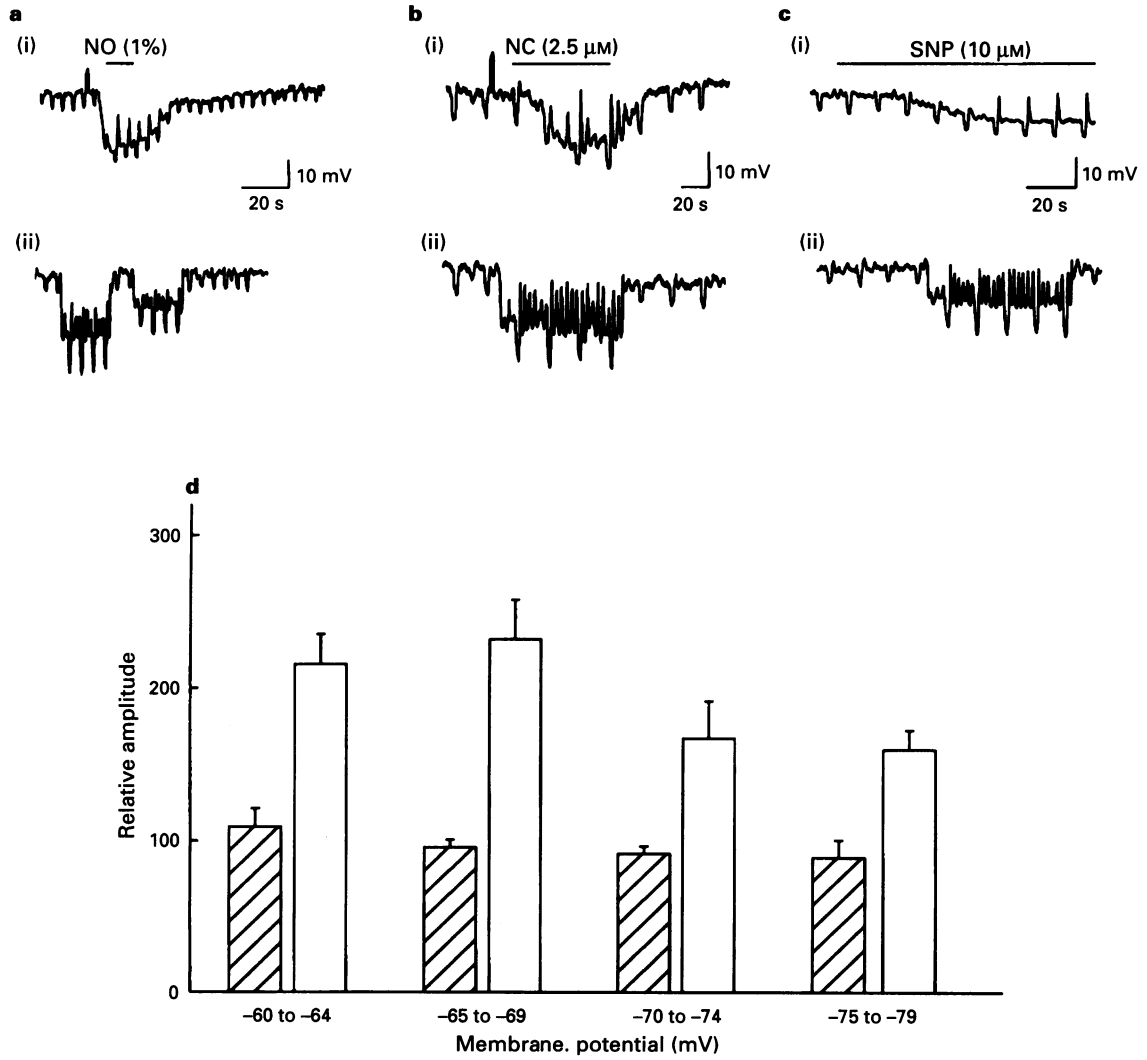

Figure 3 Effects of membrane hyperpolarization on the amplitude of electrotonic potentials (stimulus strength 5–8 V cm^{-1}, 1 s in duration) evoked during exposure to NO (1%) (a(i)), NC (2.5 μM) (b(i)) and SNP (10 μM) (c(i)), or during electrically-induced conditioning membrane hyperpolarization (a(ii), b(ii), c(ii)) in the same cell. Electrical hyperpolarizations were matched in amplitude with their corresponding drug-induced responses. (d) The data obtained upon application of the 3 NO donors and cyclic GMP have been pooled and compared with the electrotonic potentials evoked during the electrically-induced membrane hyperpolarization. Electrotonic potential amplitudes were expressed as a percentage of the electrotonic potential at the control membrane potential, binned into four groups (-60 to -64, -65 to -69, -70 to -74 and -75 to -79 mV) and plotted against the membrane potential. The amplitude of the electrotonic potentials produced during the electrically-induced hyperpolarizations (open columns) was significantly larger than the electrotonic potentials recorded during the agonist-induced hyperpolarizations (hatched columns) at all membrane potentials.

but had no effect on the amplitude or time to peak of the membrane hyperpolarizations to NC (5–10 μM) ($n=4$) or NO (1%) ($n=3$) (data not shown).

Effects of inhibiting the cytosolic guanylyl cyclase

Methylene blue has been shown to inhibit guanylyl cyclase in a variety of smooth muscle preparations (Gruetter *et al.*, 1981; Torphy *et al.*, 1986). In the present study, methylene blue (50 μM) depolarized the proximal colon to -46.5 ± 0.7 mV (control -57.0 ± 0.7 mV) ($n=14-17$), associated with a parallel shift of the *V-I* plot in the depolarized direction (Figure 2c(ii)). In the presence of methylene blue, the hyperpolarizations to SNP (10–100 μM) ($n=6$) were significantly reduced (paired *t* test). The hyperpolarizations to NC (5–10 μM) ($n=5$), however, were not significantly reduced in methylene blue (Figure 2c(i)).

Membrane conductance changes during electrically- or drug-induced hyperpolarization

The addition of NC, SNP, NO and 8-bromo-cyclic GMP usually hyperpolarized the circular muscle of the proximal colon between 5–30 mV (see Figure 1). We attempted to estimate the changes in the membrane conductance during these hyperpolarizations by recording the changes in electrotonic potential amplitudes elicited upon application of a short hyperpolarizing current (5–8 V cm^{-1}, 1 s in duration every 5–20 s). In Figure 3, it can be seen that the electrotonic potentials recorded during the time course of the hyperpolarizations to NO (1%) ($n=4$) (Figure 3a(i)), NC (2.5 μM) ($n=5$) (Figure 3b(i)), SNP (10–100 μM) ($n=5$) (Figure 3c(i)) and 8-bromo-cyclic GMP (500 μM) ($n=4$) (data not shown) were similar, or only slightly reduced, compared to those recorded at the control membrane potential. However, electrotonic potentials elicited upon an electrically-induced conditioning hyperpolarization of similar amplitude were larger than the control electrotonic potentials (Figure 3a(ii),b(ii),c(ii)). In Figure 3d, the data obtained upon the application of the three NO donors and cyclic GMP have been pooled. Electrotonic potential amplitudes, recorded during the application of an NO donor (NO, NC and SNP), cyclic GMP or electrically-evoked hyperpolarization, were expressed as a percentage of the electrotonic potential at the control membrane potential, binned into groups (−60 to −64, −65 to −69, −70 to −74 and −75 to −79 mV) and plotted against membrane potential. It can be seen that the amplitude of the electrotonic potentials produced during the electrically-induced conditioning hyperpolarizations (open columns) were significantly larger than the electrotonic potentials elicited upon exposure to an agonist (hatched columns) at all membrane potentials (1 way ANOVA) (Figure 3d).

We have interpreted these results as follows: (*i*) the smooth muscle cells of the proximal colon have non-linear membrane properties such that their membrane conductance decreases at negative potentials, as evident by the increased amplitude of the electrotonic potentials; (*ii*), the application of the NO donors or 8-bromo-cyclic GMP elicited membrane hyperpolarization by increasing the membrane conductance, and (*iii*), the little or no change in amplitude of the electrotonic potentials recorded during the application of these agents arises from the summation and near cancellation of these opposite voltage- and drug-activated changes in the membrane conductance.

In an attempt to demonstrate directly an NO-donor increase in the membrane conductance, a variable depolarizing current was applied to the proximal colon to maintain the membrane at the control potential during the application of NC (25 μM) (1 min). Under these conditions, the effects of the non-linear membrane properties of the proximal colon should be avoided (Cayabyab & Daniel, 1995). Depolarizing currents were either applied throughout the membrane responses to NC, or at the peak amplitude of the NC-induced hyperpolarization (Figure 4a). Hyperpolarizing electrotonic potentials

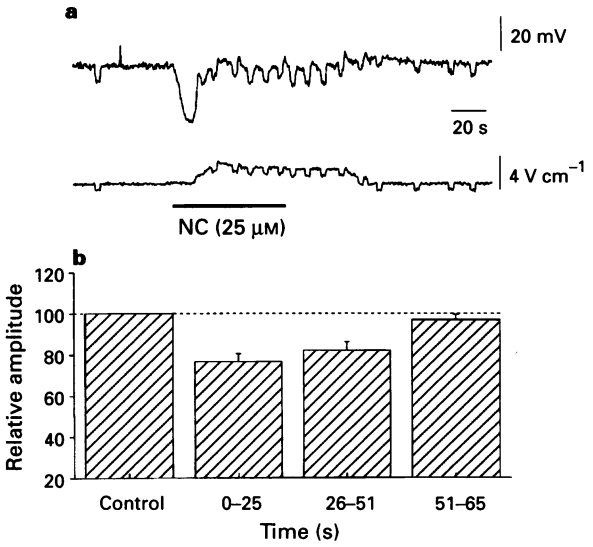

Figure 4 Estimating the increase in the membrane conductance to NC (25 μM) while maintaining the membrane at its control potential by applying a variable depolarizing current(a). (b) The amplitude of all electrotonic potentials (2 V cm^{-1} every 10 s) were expressed as a percentage of their controls, grouped into three time periods (0–25 s, 26–51 s and 51–65 s), averaged ($n=4$) and plotted against time.

(5–10 mV in amplitude) were then elicited during these conditioning depolarizations and compared with control. When depolarizing currents were applied throughout, the electrotonic potentials were reduced $22\pm3\%$ and $31\pm2\%$ ($n=4$) respectively over the initial 0–30 s and following 30–60 s of NC application. In four experiments in which the membrane potential was returned to control levels at the peak of the NC hyperpolarization (Figure 4a), test electrotonic potentials were reduced $24\pm4\%$ during the initial 0–25 s period ($P<0.05$ ANOVA) and $18\pm4\%$ ($n=4$) ($P>0.05$ ANOVA) during the following 26–51 s period, returning to control amplitudes within 60 s (Figure 4b). These reductions of the electrotonic potential amplitude occurred in the periods corresponding to the hyperpolarizing and PSD phases of the NC response, suggesting that they both arise from an increase in the membrane conductance.

Effects of K^+ channel blockers

Recently, NO donors have been demonstrated to increase the activity of 'BK' channels in both cell-attached (Thornbury *et al.*, 1991) and excised (Bolotina *et al.*, 1994; McPhee & Lang, 1994; Watson *et al.*, 1994b) smooth muscle membrane patches. The effects of the K^+ channel blockers, TEA, charybdotoxin, 4-aminopyridine and glibenclamide were therefore investigated in an attempt to establish the nature of the membrane conductance increases induced by NO and the NO donors. In common with the rat gastric fundus (Kitamura *et al.*, 1993), apamin blocked the initial transient component of the NC- and NO-induced hyperpolarizations (Watson *et al.*, 1994a,b) (Figure 5a(i),(ii)). The peak amplitude of the hyperpolarization to NC (25 μM) was reduced to 16.9 ± 1.7 mV in apamin (250 nM) (control amplitude of 26.8 ± 0.9 mV; $n=3$). This decrease was associated with an increase in the time to peak response to NC to 21.6 ± 1.6 s (control 7.6 ± 1.34 s, $n=3$) and with a membrane depolarization to -43.5 ± 1.2 mV (control, -52.8 ± 0.9 mV, $n=9$). In contrast, apamin had no effect on the hyperpolarizations produced by SNP (100 μM) ($n=4$) (Figure 5b(i),(ii)) or 8-bromo-cyclic GMP (500 μM) ($n=3$) (data not shown), although the oscillations increased in amplitude (Watson *et al.*, 1994a). In addition, charybdotoxin (100 nM), a blocker of 'BK' channels, was without effect on the NC (25 μM) hyperpolarizations induced in the presence of

apamin ($n=2$) (Figure 5a(iii)). Finally, 4-aminopyridine (5 mM) and glibenclamide (1 μM) both depolarized the proximal colon to near −50 mV (−51.2±0.6 ($n=3$) and −53.5±1.5 ($n=3$) mV respectively), but had little effect on the hyperpolarizations to NC (25 μM); being 24±0.5 ($n=3$) and 25.5±2.5 ($n=3$) mV in 4-AP and glibenclamide respectively (control amplitude 27.5±0.8 mV, $n=28$).

Activation of β-adrenoceptors in many smooth muscles also causes membrane hyperpolarization (Bülbring & Tomita 1987), thought to be due, in part, to the cyclic-AMP-mediated activation of charybdotoxin/TEA-sensitive 'BK' channels (Kume *et al.*, 1993). The effects of TEA (5–15 mM) on the amplitude to the membrane hyperpolarizations to NC (25 μM) and isoprenaline (10 μM) are therefore compared in Table 1. First, it can be seen that TEA (5–15 mM) produced a concentration-dependent depolarization of the circular muscle of the proximal colon which was associated with parallel shift of the *V-I* plot (data not shown). Second, it is clear that TEA was effective at blocking, in a concentration-dependent manner, the membrane hyperpolarizations to isoprenaline (25 μM), but not to NC (10 μM). Finally, the addition of ouabain (10 μM), blocker of the plasmalemmal Na:K ATPase, reduced the hyperpolarizations to NC (25 μM), recorded in the presence of 15 mM TEA, approximately 50% (14.3±2.3 mV) ($n=3$) and completely abolished the residual hyperpolarization recorded in response to isoprenaline (10 μM). In the absence of TEA, ouabain (10 μM) depolarized the proximal colon to −55.2±0.7 mV ($n=4$). This depolarization was associated with a large decrease in the slope of the *V-I* plot, suggesting a significant increase in the membrane conductance. Under these conditions, ouabain again reduced the NC (25 μM) hyperpolarization to 14.0±2.2 mV ($n=4$) (Watson *et al.*, 1993).

In the presence of TEA (5 mM), the membrane hyperpolarization to 10 μM SNP (7.8±0.9 mV, $n=12$) was significantly smaller than the control SNP (10 μM) hyperpolarization in the absence of TEA (10.2±1.1 mV) (ANOVA). Membrane responses to higher concentrations of SNP (100 μM (18.0±1.9 mV, $n=12$) and 1 mM (19.3±3.0 mV, $n=9$)), however, were not significantly affected by 5 mM TEA (respectively 15±0.9 and 16.9±1.1 mV in TEA) (ANOVA). Finally, the hyperpolarizations to NO (1%) (31.5±1.8 mV, $n=5$) and cyclic GMP (500 μM) (13.2±3.0 mV, $n=5$) in 5 mM TEA were also not significantly different from their control amplitudes (27.5±1.85 and 9.2±1.9 mV respectively, unpaired *t* test).

Effects of Cs^+

Rhythmic oscillations of the membrane potential were often recorded superimposed on the hyperpolarizations induced by NO donors, isoprenaline and 8-bromo-cyclic GMP. Such membrane oscillations were also recorded during prolonged (>4 s) electrically-induced hyperpolarizations (Figure 6b top panel), suggesting that additional voltage- and time-dependent conductance changes are activated by membrane hyperpolarization itself. We have applied Cs^+ (1 mM), a blocker of cation-selective inward rectifier (I_f) channels in smooth muscle (Benham *et al.*, 1987), to investigate whether the activation of these channels contribute to the initiation of the membrane oscillations. The addition of Cs^+ (1 mM) had little effect on the membrane potential or on the amplitude of the electrotonic potentials generated by short depolarizing and hyperpolarizing electrotonic potentials, 1 s in duration ($n=6-8$) (Figure 6c(ii)). The electrotonic potentials generated with longer electrotonic potentials (4 s duration), however, were reduced in amplitude at potentials negative to −50 mV ($n=8-10$) (Fig-

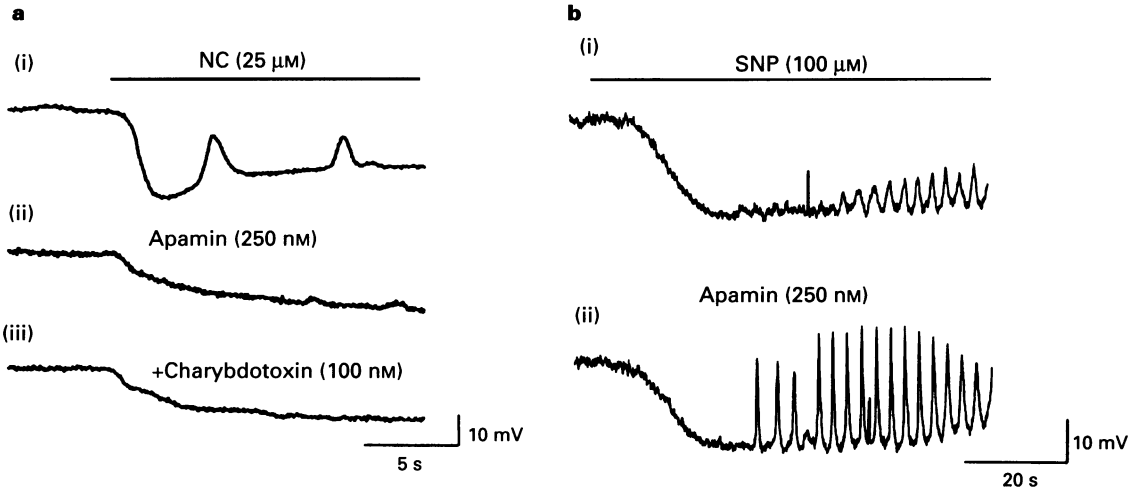

Figure 5 Effects of apamin and charybdotoxin on NO donor hyperpolarizations. Apamin (250 nM) blocked the initial transient component of the membrane response to NC (25 μM)(a(i), (ii)) but had no effect on the hyperpolarizations to SNP (100 μM)(b(i)(ii)). Charybdotoxin (100 nM) also had no effect on the apamin-resistant hyperpolarization to NC (25 μM)(a(iii)).

Table 1 Effects of tetraethylammonium and ouabain on the hyperpolarizations of the proximal colon induced by S-nitroso-L-cysteine (25 μM) and isoprenaline (10 μM)

Blocker	*Membrane potential* (mV)	*S-nitroso-L-cysteine* (mV)	*Isoprenaline* (mV)
Control	−61.6±0.4	27.5±0.8 ($n=28$)	21.1±2.0 ($n=5$)
TEA (5 mM)	−51.9±0.7	29.8±2.1 ($n=4$)	18.3±1.6 ($n=4$)
TEA (10 mM)	−50.5±0.9	25.2±6.6 ($n=4$)	10.3±2.8 ($n=4$)
TEA (15 mM)	−46.8±1.2	27.5±1.5 ($n=4$)	5.6±2.8 ($n=3$)
TEA (15 mM) + ouabain (10 μM)	−50.0±0.8	14.3±2.3 ($n=3$)	0 ($n=3$)

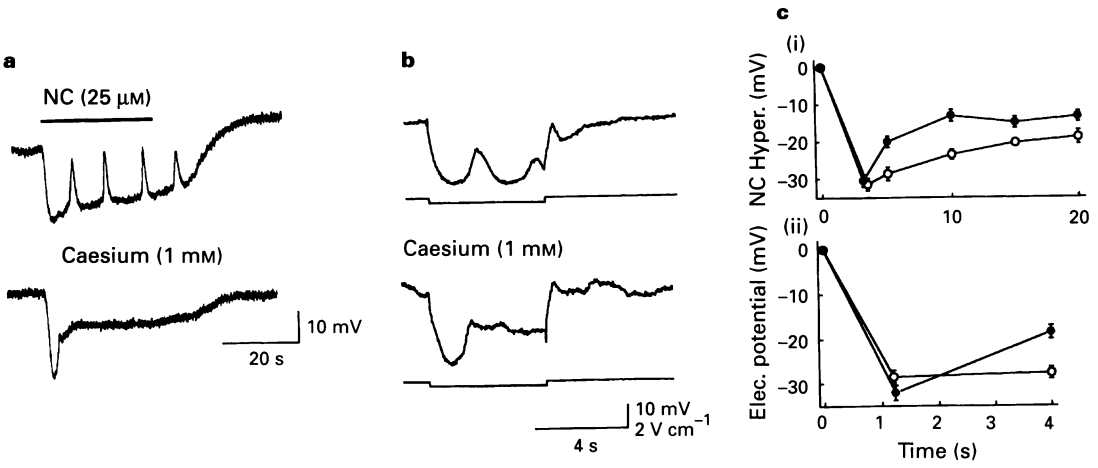

Figure 6 Effects of caesium (1 mM) on the membrane oscillations observed during the hyperpolarizations to either NC (25 μM) (a) or prolonged (4 s) electrical current injection (2 V cm^{-1})(b). (c) Plot of the time course of the NC hyperpolarizations (c(i)) and the peak amplitude and steady state level of the 4 s electrotonic potentials (c(ii)) in the absence (○) and presence (●) of caesium (1 mM).

ure 6b lower panel, c(ii)). In the presence of Cs^+ (1 mM), the peak amplitude (30.6±1.6 mV) and time to peak (3.1±0.3 s) of the NC (25 μM)-induced hyperpolarization was not changed from control (31.5±1.7 mV and 3.4±0.1 s respectively, $n=11$) (Cayabyab & Daniel, 1995). However, the rapid oscillations in membrane potential during the NC hyperpolarization were abolished (Figure 6a lower panel) such that the membrane potential settled at a significantly less negative level than that reached during a control NC hyperpolarization (ANOVA) (Figure 6c(i)). The oscillations of membrane potential recorded during the application of long (>4 s) hyperpolarizing electrotonic potentials were also abolished by Cs^+ (1 mM) (Figure 6b lower panel). These results suggest that a slow activation of inward rectifier channels at potentials negative to −50 mV contributes to the generation of the membrane oscillations at negative potentials.

Discussion

We have confirmed recent reports (Maggi & Giuliani, 1993; Zagorodnyuk & Maggi, 1994) that the electrically- or stretch-evoked NANC i.j.p. recorded in the circular smooth muscle cells of the guinea-pig proximal and distal colon can be divided into two apamin-sensitive and apamin-resistant components (Watson *et al.*, 1993; 1996). The apamin-resistant i.j.p. and rebound PSD were both reduced or abolished upon the addition of L-NOARG, or haemoglobin. These effects of L-NOARG were partially reversed in the presence of excess L-arginine, suggesting that NO, or a related NO-donor compound, is likely to be the apamin-resistant inhibitory transmitter (Maggi & Giuliani, 1993; Zagorodnyuk & Maggi, 1994; Watson *et al.*, 1993; 1996). In the present experiments, we have demonstrated that NO, NC, SNP and cyclic GMP all mimic the L-NOARG-sensitive i.j.p., hyperpolarizing the membrane potential of the guinea-pig proximal colon. In common with results obtained in the rat gastric fundus and dog colon (Thornbury *et al.*, 1991; Kitamura *et al.*, 1993), the hyperpolarizations to NO and NC were more rapidly-developing and more sensitive to blockade by haemoglobin than those produced by SNP. We have demonstrated that the NO donor and cyclic GMP hyperpolarizations are also associated with an increase in the membrane K^+ conductance (see below), although this conductance increase was difficult to quantify due to the contamination of a voltage-dependent decrease in the membrane conductance at potentials negative to −50 mV.

In the proximal colon of the guinea-pig, blockade of 'SK' Ca^{2+}-activated K^+ channels with apamin reduced the initial phases of the NANC i.j.p. (Maggi & Giuliani, 1993; Zagorodnyuk & Maggi, 1994, Watson *et al.*, 1996) and the hyperpolarizations to NC or NO, but had no effect on the hyperpolarizations to SNP (Figure 5) or 8-bromo-cyclic GMP (Kitamura *et al.*, 1993). NC and NO also appeared to be directly activating 'SK' channels as the time to peak and maximal amplitude of the hyperpolarizations to NO and NC were unaffected by ω-contoxin GVIA or L-NOARG and methylene blue, blockers of neurally-released NO and cytosolic guanylyl cyclase respectively. In contrast, the hyperpolarizations to SNP were longer-lasting, sensitive to methylene blue and mimicked by 8-bromo-cyclic GMP. These results suggest that exogenous NO, and NO released from S-nitroso-L-cysteine can induce membrane hyperpolarization by directly activating apamin-sensitive 'SK' channels (Kitamura *et al.*, 1993), perhaps the same channels activated by the L-NOARG-insensitive NANC inhibitory transmitter (Watson *et al.*, 1996). In contrast, NO release from SNP or upon NANC nerve stimulation induced an apamin-resistant hyperpolarization upon the activation of intracellular guanylyl cyclase and synthesis of cyclic GMP (Ward *et al.*, 1992a; Kitamura *et al.*, 1993; Watson *et al.*, 1994a; Cayabyab & Daniel, 1995).

Hyperpolarizing responses to the three NO donors, in the absence or presence of apamin, were reduced or abolished when the extracellular Ca^{2+} was nominally removed, but mostly not affected by TEA (Table 1), charybdotoxin (Figure 5a), glibenclamide, 4-AP, nifedipine or ω-conotoxin GVIA (Kitamura *et al.*, 1993; Cabyabyab & Daniel, 1995). These results suggest that NO-donor induced hyperpolarizations are dependent on external Ca^{2+}, but not on the flow of Ca^{2+} through either neuronal or post-junctional 'L'- or 'N-type' Ca^{2+} channels. As in the rat gastric fundus (Kitamura *et al.*, 1993), these NO donor hyperpolarizations do not arise from the activation of the following K^+ channels: (*i*) 'BK' Ca^{2+}-activated K^+ channels; (*ii*) cromakalim-activated and/or ATP-dependent K^+ channels; (*iii*) 4-AP-sensitive, transiently-opening 'A-type' K^+ channels, and (*iv*) 'delayed rectifier' K^+ channels, most of which have been previously identified in single cells of the proximal colon at both the single cell and single channel level (Vogalis *et al.*, 1993; Vogalis & Lang, 1994). These result remain puzzling, however, as the activity of TEA-sensitive 'BK' channels recorded in single smooth muscle cells isolated from the guinea-pig (Watson *et al.*, 1994b) and dog colon (Ward *et al.*, 1994b) has been shown to be increased by the NO donors, NC and SNP. In contrast, the blockade of the hyperpolarizations to isoprenaline by TEA (Table 1) suggests the involvement of 'BK' and perhaps 'delayed rectifier' K^+ channels (Vogalis *et al.*, 1993), consistent with the previously demonstrated increase of 'BK' channel activity in sin-

gle tracheal muscle cells mediated by cyclic-AMP and protein kinase A (Kume *et al.*, 1989). Clearly, these inconsistencies require further study.

Membrane hyperpolarization induced by NO donors, isoprenaline and 8-bromo-cyclic GMP, or by prolonged (>4 s) current injection, shifted the membrane potential through a voltage range which was non-linear and often unstable as evident by the rhythmic oscillations at negative potentials. Such voltage- and time-dependent changes in the membrane conductance, were overcome in the present experiments by electrically returning the membrane potential to control levels before estimating the conductance changes associated with the NO donor, NC. In these experiments, the test electrotonic potentials were generally reduced 20–25% over the first 25 s of NC (25 μM) exposure and 20–30% over the following 30 s. Using the method of Bolton *et al.* (1981), we have estimated that the decreases in the electrotonic potentials correspond to a 1.6–1.7 fold (0–25 s) and 1.5–2.1 fold (30–60 s) increase in the membrane conductance; where Δ conductance $= (P_1/P_2)^2$, P_1 and P_2 are the amplitudes of the electrotonic potentials in the absence and presence of NC respectively. These increases in membrane conductance are occurring in the time periods corresponding to the initial apamin-sensitive and the following apamin-resistant hyperpolarization, as well as the rebound PSD, perhaps suggesting that they all arise from an increase in the membrane conductance. If this is the case, the hyperpolarizations must be due to an increase in the membrane K^+ conductance, while the PSD must represent an increased membrane cationic and/or Cl^- conductance (Cayabyab & Daniel, 1995).

The nature/origin of the potential oscillations at negative potentials remain to be elucidated. The linear current-voltage relationship of single circular muscle cells of the guinea-pig proximal colon at potentials negative to −60 mV, recorded in the presence of nifedipine at room temperature (Vogalis *et al.*, 1993) or 35°C, suggest that these oscillations do not arise from the activation of previously-characterized voltage-operated channels. However, given the well known voltage-dependent action of nifedipine, we cannot exclude the possibility that there is a voltage-dependent unblocking of 'L-type' Ca^{2+} channels at negative potentials. These unblocked Ca^{2+} channels could well contribute to the upstroke of these potential oscillations. The blockade of the oscillation with Cs^{2+}, however, suggests that the activation and deactivation of I_f channels could also contribute to both the upstroke and falling phase of these potential oscillations. However, these channels have yet to be characterized in the proximal colon, either at the whole cell or single channel level.

Alternatively, these membrane oscillations at negative potentials could well represent propagated 'pacemaker' potentials or 'slow waves' becoming more clearly recorded when the membrane conductance of the 'driven' areas of the proximal colon is decreased with membrane hyperpolarization. Such a decrease in membrane conductance would improve the cable properties of the circular smooth muscle which would allow better propagation of any signals from pacemaker areas such as interstitial cells. Evidence in support of this suggestion is perhaps illustrated in Figure 5b, where the amplitude and time course of these potential oscillations became larger and more regular when the membrane conductance was decreased with apamin, similar increases in oscillation amplitudes were observed in indomethacin (10 μM) (data not shown). Finally, the frequency of these potential oscillations was little affected by the membrane potential, although their amplitudes were increased with membrane hyperpolarization. These results are consistent with the often described properties of 'slow waves' in many gastrointestinal smooth muscles (Prosser, 1978). The ionic mechanisms underlying these hyperpolarization-induced membrane oscillations remain to be elucidated.

In summary, the hyperpolarizations induced by the NO donors used in the present experiments are associated with an increase in at least two yet-to-be characterized membrane conductances, one of which was sensitive to blockade by apamin and activated by only NO and NC. The slower-developing apamin-resistant hyperpolarizations to NO, SNP and NC were mimicked by 8-bromo-cyclic GMP, perhaps suggesting that NO donors can also activate a cyclic GMP-activated K^+ conductance. In addition, membrane hyperpolarization itself induces voltage-dependent changes in the membrane conductance which masks the conductance changes during the NANC i.j.p. (Watson *et al.*, 1996) and NO donor hyperpolarizations.

Supported by the National Health and Medical Research Council of Australia.

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(Received September 1, 1995
Revised March 18, 1996
Accepted April 3, 1996)