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Effects of nitric oxide donors, S-nitroso-L-cysteine and sodium nitroprusside, on the whole-cell and single channel currents in single myocytes of the guinea-pig proximal colon

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1 The nature of the membrane channels underlying the membrane conductance changes induced by the nitric oxide (NO) donors, S-nitroso-L-cysteine (NOCys) and sodium nitroprusside (SNP) were investigated in single myocytes isolated from the circular muscle layer of the guinea-pig proximal colon, by use of standard whole-cell and single channel recording techniques.

2 Under voltage clamp, depolarizing steps from −60 mV elicited a rapidly-developing, little-inactivating outward K^+ current (I_K) at potentials positive to −40 mV (at 20–25°C). The steady-state level (I_{SS}) of this K^+ current increased in amplitude as the step potential was made to more positive potentials. If the depolarizing steps were made from a holding potential of −80 mV an additional rapidly activating and inactivating outward K^+ current was also elicited, superimposed on I_K.

3 At 20–25°C, NOCys (2.5 μM), SNP (100 μM) and 8-bromo-cyclic GMP (500 μM) increased the amplitude of I_{SS} of I_K elicited from a holding potential of −60 mV. In contrast, NOCys (2–5 μM) had little effect on I_{SS} at 35°C. Higher concentrations (⩾5 μM at 20–25°C and ⩾10 μM at 35°C) of NOCys decreased the peak amplitude (I_{Peak}) and I_{SS} of I_K in a concentration-dependent manner. This blockade of I_K with NOCys was always associated with an increase of the holding current (I_{Hold}), due to the activation of a membrane conductance with a reversal potential between 0 and +30 mV and which was reduced approximately 50% upon the addition of Cd^{2+} (1 mM).

4 NOCys (2.5 to 10 μM) or SNP (100 μM) increased the activity of large conductance Ca^{2+}-activated (BK) K^+ channels in both cell-attached and excised inside-out patches, bathed in either a symmetrical high K^+ (130 mM) or an asymmetrically K^+ (6 mM_{out}: 130 mM_{in}) physiological saline. Increases in BK channel activity in NOCys (10 μM) or SNP (100 μM) were associated with an increase in the probability of BK channel opening ($N.P_o$), and with a negative shift of the plots of $\ln(N.P_o)$ against the patch potential, with little change in the slopes of these plots. In cell-attached patches, the increase in $N.P_o$ with NOCys was often associated with a decrease in the BK single channel conductance.

5 In both cell-attached and excised patches, NOCys (2.5 to 10 μM) also activated an additional population of channels which allowed inward current flow at potentials positive to E_K. In excised inside-out patches bathed in asymmetrical K^+ physiological saline, these single channel currents were 2–3 pA in amplitude at −30 mV and reversed in direction near +10 mV, even if the NaCl (126 mM) concentration in the pipette solution had been replaced with an equimolar concentration of Na gluconate.

6 Under current clamp, NOCys (2.5 μM) and SNP (100 μM) had variable effects on the membrane potential of colonic myocytes, inducing either a small membrane hyperpolarization of <5 mV, or a slowly-developing membrane depolarization of about 5 mV. In contrast, NOCys (5 μM) produced a transient membrane hyperpolarization which was followed by a large depolarization of the membrane potential to positive potentials. The electrotonic potentials elicited in response to an injection of constant hyperpolarizing current (10 pA for 400 ms) were little changed during the NOCys (5 μM)-induced membrane hyperpolarization, but significantly reduced (to 61% of control) during the periods of membrane depolarization.

7 It was concluded that NOCys and SNP, directly increased the number of active BK channels in the membrane of colonic myocytes which leads to a small rapidly oscillating membrane hyperpolarization. The following rebound depolarization in NOCys arises from both the direct opening of a population of cationic channels and the blockade of voltage- and Ca-activated K^+ conductances. Finally, the apamin-sensitive K^+ channels underlying the initial transient hyperpolarization recorded in the intact proximal colon, in response to nerve-released or directly-applied NO, have yet to be identified at the single channel or whole-cell current level.

Keywords: Nitric oxide; NO donors; whole cell and single channel K^+ currents; BK channels; patch clamp; guinea-pig proximal colon

Introduction

Electrical or reflex stimulation of enteric inhibitory motor nerves produces an inhibition of the electrical and mechanical activity of the circular muscle layer in many gastrointestinal preparations. After this period of muscle inhibition, there is often a period of membrane depolarization and 'rebound' contraction; both of these periods of non-adrenergic, non-cholinergic (NANC) inhibition and excitation remaining in the presence of muscarinic antagonists and after sympathetic denervation (Furness, 1969; Maggi & Giuliani, 1993). In the

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circular muscle layer of the guinea-pig colon, the initial transient component of the NANC relaxation and membrane hyperpolarization (the inhibitory junction potential, i.j.p.) recorded in response to electrical stimulation or distension, can be blocked by apamin, a blocker of small-conductance Ca^{2+}-activated K^+ (SK) channels (Blatz & Magelby, 1986). In contrast, the 'apamin-resistant' components of the NANC relaxation and i.j.p., as well as a substantial portion of the rebound membrane depolarization, can be blocked by inhibitors of nitric oxide synthase (NOS), suggesting that the neural release of nitric oxide (NO) or a related compound plays a part in the generation of both the apamin-resistant NANC i.j.p. and rebound depolarization (Maggi & Giuliani, 1993; Zagorodnyuk & Maggi, 1994; Watson *et al*., 1996b). Similar demonstrations of endogenously-released NO having both inhibitory and excitatory actions have been demonstrated in the guinea-pig ileum (Lyster *et al*., 1992), opossum oesophagus (Saha *et al*., 1993) and in the dog and cat colon (Thornbury *et al*., 1991; Ward *et al*., 1992b).

In the proximal colon of the guinea-pig, apamin also blocked the initial phase of the membrane hyperpolarization recorded in response to directly-applied NO, or the NO donor, S-nitroso-L-cysteine (NOCys), to reveal a slower-developing membrane hyperpolarization which was mimicked by sodium nitroprusside (SNP), isoprenaline or 8-bromo-cyclic GMP (Kitamura *et al*., 1993; Watson *et al*., 1996a). The hyperpolarizations to SNP, but not NOCys or NO, were reduced upon blockade of cytosolic guanylyl cyclase activity with methylene blue (Watson *et al*., 1996a). Thus, the transmitter responsible for the apamin-sensitive i.j.p. and NO both appear to activate directly SK channels (Kitamura *et al*., 1993; Watson *et al*., 1996b). In addition, NO, released from exogenously-applied NO donors or upon NANC nerve stimulation, induces an apamin-resistant membrane hyperpolarization dependent upon the activation of guanylyl cyclase (Kitamura *et al*., 1993; Cayabyab & Daniel, 1995). However, in contrast, in the canine pyloric sphincter, duodenum and colon, NO and NOCys induce an apamin-sensitive membrane hyperpolarization and rebound depolarization which were associated with an increase in guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels (Bayguinov *et al*., 1992; Ward *et al*., 1992a; Bayguinov & Sanders, 1993).

We have recently shown in the guinea-pig proximal colon that the NO-induced membrane hyperpolarization and rebound depolarization were both associated with an increase in the membrane conductance (Watson *et al*., 1996a,b). We also demonstrated that the membrane hyperpolarization to an NO donor or cyclic GMP was associated with an increase in the membrane K^+ conductance, while the rebound depolarization was likely to be associated with an increase in the membrane conductance to cations or CI^- ions (Jury *et al*., 1985; Christinck *et al*., 1990; Crist *et al*., 1991; Kitamura *et al*., 1993; Watson *et al*., 1996a). These increases in membrane conductance were superimposed on a complex 'background' membrane conductance which was voltage-dependent, decreasing at potentials negative of -50 mV. This non-linear background membrane conductance tended to mask the conductance changes during the NANC i.j.p. and NO donor responses (Watson *et al*., 1996a,b). In the present study, we have examined the nature of some of the ionic channels underlying these changes in membrane conductance induced by the NO donors, NOCys and SNP, in freshly-dispersed single myocytes of the circular muscle layer of the guinea-pig proximal colon, using standard whole-cell and single channel recording techniques. Some of these results have been presented previously in a preliminary form (Waston *et al*., 1994).

Methods

Single cell preparation

Segments of proximal colon were cut open longitudinally and pinned out, mucosal surface uppermost, in a dissecting dish. The mucosa was removed by careful dissection and the circular muscle layer peeled away from the underlying longitudinal layer. Strips of circular muscle were cut into small pieces (2–5 mm^2) and rinsed in low-Ca^{2+} (30 μM) physiological saline (PS; see below) for 2–10 min. (at 37°C). The muscle pieces were then transferred to low-Ca^{2+} PS containing: collagenase Type 1(0.6 mg ml^{-1}; Worthington); bovine serum albumin (2 mg ml^{-1}; Sigma) and trypsin inhibitor (0.2 mg ml^{-1}; Sigma). The muscle pieces were incubated in the dispersal medium for 60 min after which they were re-suspended in low-Ca^{2+} PS and gently agitated for 10 to 20 min. Single smooth muscle myocytes were obtained by gentle trituration with a wide-bore glass pipette. Cells were allowed to settle to the glass-bottom of the recording chamber mounted on an inverted microscope over a period of 5 to 10 min; the solution was then exchanged for normal Ca^{2+} (1.5 mM) PS (Vogalis *et al*., 1993; Vogalis & Lang, 1994).

Whole-cell and single channel current recording

Patch pipettes were drawn from glass capillary tubing (1.5–1.8 mm o.d.; Kimax-51, Kimble, U.S.A.) on a programmable micropipette puller (Sachs-Flaming PC-84, Sutter Instruments) and their tips fire polished (MF-84 Narishige). Pipette resistances ranged from 2–7 MΩ when filled with pipette solution. Whole-cell membrane potential and currents were recorded by use of an Axopatch 200 (Axon Instruments) and conventional patch-clamp techniques. Current and voltage signals were stored on video tape via a Digital Data Recorder VR10B (Instrutech Corp) and later digitized (at 2 kHz) onto a computer hard disc for analysis with a Labmaster TM125 Interface (Axon Instruments) and pClamp 5.5.1 software (Axon Instruments).

Single channel analysis

Open channel amplitudes and durations were calculated at each patch potential (V_m) (mV) by use of all points amplitude histograms. Closed and opened levels were fitted with single gaussian distributions (by means of a simplex least squares algorithm), the difference between the mean of each gaussian giving an estimate of the amplitude (I)(pA) of the BK channel currents at each potential. The area under each fitted gaussian was used to calculate the relative probability of the patch current being at each current level (P_L) by dividing the summed time spent at each level (L) by the total observation time. The averaged probability of opening for N channels ($N.P_o$) was therefore

$$N.P_0 = \sum_{L=0}^{N} L.P_L$$

Typically, $\ln(N.P_o)$ was plotted against V_m and fitted to the straight line

$$\ln\,(N.P_0) = (V - V_{0.5})/K + \ln\,(N)$$

where $V_{0.5}$ and K represent the voltage of half -maximal activation and the slope of the line, respectively (Singer & Walsh, 1987).

Quantitative data are expressed as means ± s.e. (n = number of preparations). Differences between means were tested for

significance ($P<0.05$) by use of Student's paired t test, unless otherwise stated.

Solutions

Single smooth muscle myocytes were dispersed in a low-Ca^{2+} PS containing (mM): NaCl 126, KCl 6, sodium *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (Na-HEPES) 10, $MgCl_2$ 1, D-glucose 10, ethylene glycol-*bis* (*β*-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) 0.3 and $CaCl_2$ 50 μM, which gave a calculated free Ca^{2+} concentration of 15 nM; pH was adjusted to 7.4 with 5 M NaOH. Cells were first bathed in a 'normal' PS which was the same as above, except that the $CaCl_2$ concentration was 1.5 mM. During the whole-cell voltage clamp experiments, the pipette solution contained (mM): KCl 126, NaHEPES 10, Na_2 ATP 3, EGTA 0.3, $MgCl_2$ 3 and D-glucose 10; pH was set at 7.4 with 5 M KOH.

Single channel recordings were made from cell-attached or excised inside-out patches with a pipette solution consisting of either the normal PS (as above) or a 'high K^+' saline of the following composition (mM): KCl 130, NaHEPES 10, $MgCl_2$ 1, D-glucose 10, EGTA 0.3 and $CaCl_2$ 50 μM. The pH of the solution was adjusted to 7.4 with 5 M KOH. Both pipette solutions contained 1 μM nifedipine. During recordings from cell-attached patches, cells were bathed in the high K^+ saline to zero the membrane potential; all single channel recordings were made at room temperature (20–25°C). In one series of recordings from four excised inside-out patches, the NaCl concentration in the pipette solution was replaced with an equimolar concentration of Na gluconate (126 mM); changes in the junction potential caused upon gluconate substitution were minimized by the use of a 3 M KCl-agar electrode.

Drugs

Stock solutions of S-nitroso-L-cysteine (NOCys) (10 and 100 μM) were prepared every week, by use of the method described by Field *et al*. (1978). Stock solutions of sodium nitroprusside (SNP) (May & Baker) were dissolved in distilled water and kept frozen until needed. All stock solutions were stored at 4°C and diluted to their final concentrations in PS and added directly to the organ bath. The following drugs were also used; apamin, tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP) and 8-bromo-cyclic GMP (all Sigma, U.S.A.) and nifedipine (Bayer).

Results

At room temperature (20–25°C), single myocytes from the circular muscle of the proximal colon, bathed in PS containing 1.5 mM Ca^{2+} and 1–3 μM nifedipine, had membrane potentials between −40 and −50 mV (e.g. -48.9 ± 1.2 mV, $n=15$) when recorded under current clamp. However, under voltage clamp, cells were usually kept at a holding potential of −60 mV as this approximated the recorded membrane potential of the circular muscle cells in the guinea-pig intact proximal colon (at 35°C), superfused with nifedipine and hyoscine (Watson *et al*., 1996a,b). At 20–25°C, step depolarizations (320 ms duration) to potentials positive to −40 mV evoked a net outward current (I_K) which developed more rapidly and increased in amplitude as the step was made to more positive potentials. This I_K showed little inactivation over 320 ms and often displayed small spontaneous oscillations of current at positive potentials (Figure 1a(i),b(i),c(i)). Plots of the averaged current-voltage (*I*-V) relationship ($n=7$ cells) of the steady state current (I_{SS}) (measured 270–320 ms after the onset of a depolarizing step) of I_K revealed that the *I*-V relationship was linear at potentials between −90 and −40 mV and displayed a marked upward curvature (outward rectification) at positive potentials (data not shown).

The voltage-dependence of activation and inactivation, as well as the pharmacological sensitivity, of the K^+ channel populations underlying I_K have been investigated previously (Vogalis *et al*., 1993; Vogalis & Lang, 1994). A substantial proportion of I_{SS} at positive potentials is blocked by concentrations of tetraethylammonium (TEA) (1–5 mM), which readily block current flow through large conductance Ca^{2+}-activated (BK) K^+ channels in this preparation (Vogalis & Lang, 1994), to reveal a slowly-developing delayed rectifier-like K^+ current ($I_{K(DR)}$), activated at potentials positive to −20 mV. This $I_{K(DR)}$ is, in turn, blocked by 10–15 mM TEA (Vogalis *et al*., 1993). Myocytes, bathed in 2 μM nifedipine and 10–15 mM TEA and held at more negative holding potentials (e.g. −80 mV), display an additional transient outward current ($I_{K(TO)}$) upon depolarization to potentials positive to −40 mV. This $I_{K(TO)}$ is half-maximally available for opening at −72 mV (at 20–25°C in 2 μM nifedipine) and sensitive to blockade by 4-aminopyridine (4-AP)(2–5 mM) (Vogalis *et al*., 1993; Vogalis & Lang, 1994). However, when myocytes are held at −60 mV (at 20–25°C) and bathed in nifedipine-containing PS, only about 5% of these $I_{K(TO)}$ channels would be available for opening upon membrane depolarization. Thus the majority of I_K evoked from −60 mV (at 20–25°C) would be due to the flow of current through BK and $I_{K(DR)}$ channels.

Effects of NOCys

The application of 2.5 μM NOCys to cells, held at −60 mV (at 20–25°C), increased I_{SS} at all potentials positive to 0 mV (Figure 1a (i-ii)). At +40 mV, the absolute value of I_{SS} in the presence of NOCys (2.5 μM) was measured as 277 ± 45 pA, significantly larger than the control I_{SS} (172 ± 27 pA; $n=5$, $P<0.05$). This increase in I_{SS} was often associated with an increase in the oscillations recorded during the depolarizing step. Ramped depolarizations (between −100 and +100 mV, over 0.5s) were also applied (every 20s) to these colonic myocytes so that the time-dependent effects of NOCys could be investigated. 'Instantaneous' current-voltage (*I*-V) plots were created by plotting the membrane currents generated by the ramped depolarizations against V_m. In Figure 1a (iii), it can be seen that NOCys (2.5 μM) increased the outward component of the ramp currents at potentials positive to −20 mV. These effects of NOCys (2.5 μM) were mimicked, in part, by both sodium nitroprusside (SNP) (Figure 1b) and 8-bromo-cyclic GMP (500 μM) (Figure 1c). SNP (100 μM) increased I_{SS}, evoked by stepped depolarizations to +40 mV to 337 ± 94 pA (control I_{SS} was 234 ± 90 pA; $n=3$, $P<0.05$), while in 8-bromo-cyclic GMP (500 μM) I_{SS} (at + 10 mV) was 147 ± 77 pA (control I_{SS} was 73.7 ± 19 pA; $P>0.05$, $n=3$). These effects of SNP and 8-bromo-cyclic GMP were both associated with an increase in the ramp current at positive potentials (Figure 1b(iii),c(iii)).

However, the application of higher concentrations of NOCys ($\geqslant5$ μM) (at 20–25°C), caused a rapid reduction of I_K at positive potentials (Figure 2a(i)). At +40 mV, 5 μM NOCys decreased the averaged value of I_{SS} from 277 ± 45 pA to -12.5 ± 3 pA ($n=5$, $P<0.05$). This decrease in I_{SS} was always associated with a significant increase in the current required to hold the cell at −60 mV (the holding current, I_{Hold}) from -38.9 ± 2.7 to -154 ± 30 pA ($n=5$, $P<0.05$) (Figure 2a(ii)).

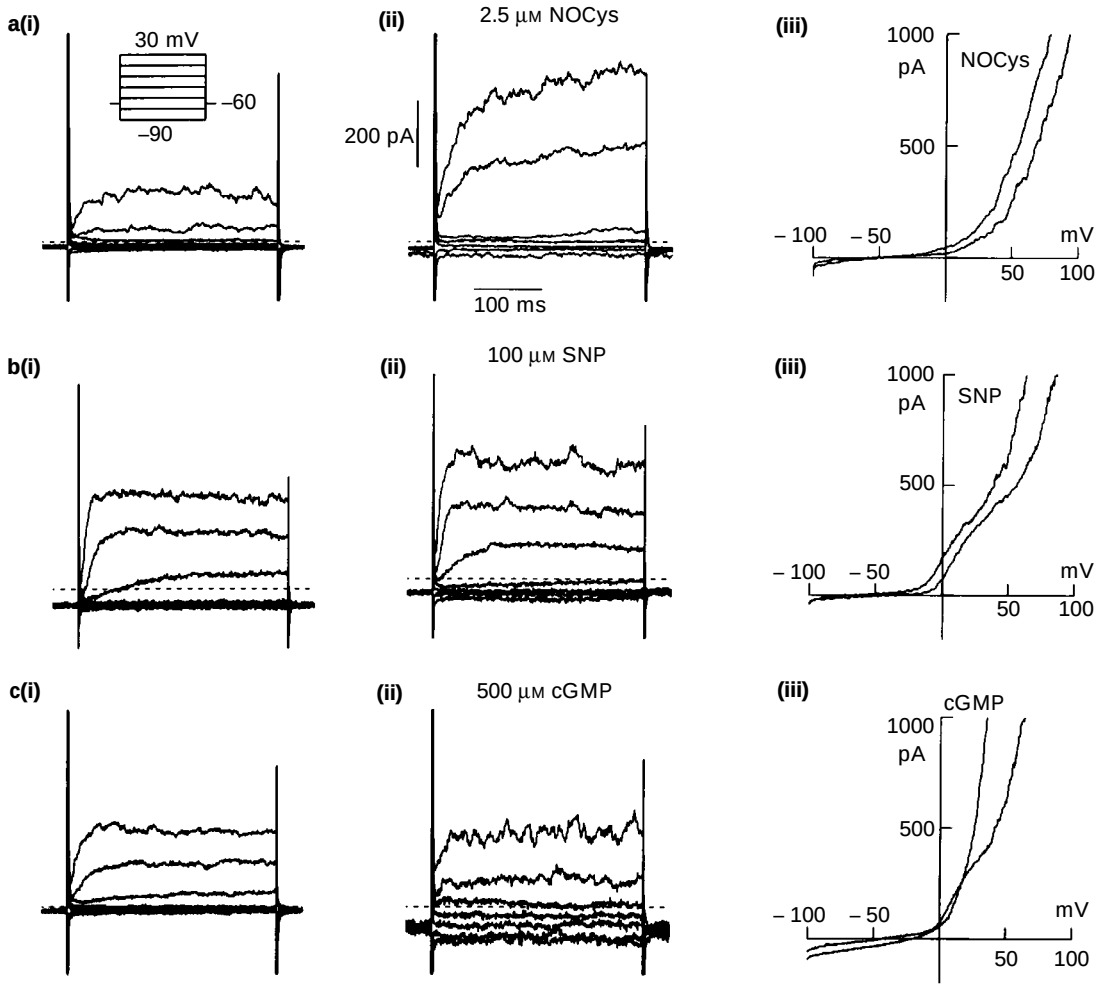

Figure 1 Effects of S-nitroso-L-cysteine (NOCys; (2.5 μM)(a), sodium nitroprusside (SNP; 100 μM)(b) and 8-bromo-cyclic GMP (cGMP; 500 μM)(c) on the whole-cell currents (I_K) recorded in single myocytes of the circular muscle layer of the guinea-pig proximal colon, bathed in nifedipine (1 μM)-containing physiological saline (PS). Whole-cell currents recorded (at 25°C) in response to step depolarizations to potentials every 20 mV between −90 and + 30 mV (from a holding potential of −60 mV) were obtained in control PS (a(i),b(i),c(i)), or after 1–2 min, exposure to 2.5 μM NOCys (a(ii)), SNP (100 μM) (b(ii)) or cGMP (500 μM) (c(ii)). All three agents increased the steady state current (I_{SS}) (measured 270–320 ms after the onset of each step depolarization) of I_K at potentials positive to O mV. NOCys (2.5 μM) (a(iii)), SNP (100 μM) (b(iii)) and cGMP (500 μM) (c(iii)) also increased in the outward direction the instantaneous current-voltage (*I*-V) plots obtained by plotting against membrane potential, the whole-cell currents recorded in response to ramped depolarizations (between −100 and +100 mV over 0.5 s) applied from a holding potential of −60 mV.

At 35°C, I_K, evoked upon membrane depolarization from a holding potential of −60 mV developed an initial transient peak current (I_{Peak}) which decayed slowly (Figure 2b(i)). This I_{Peak} was significantly greater than I_{SS} recorded at 35°C or room temperature. For example, the averaged amplitude of I_{Peak} at +40 mV (at 34–37°C) was 462±52 pA; significantly greater than I_{SS} recorded at either 35°C (255±31 pA; $P<0.05$, $n=16$, paired t test) or 20–25°C (303±26 pA; $P<0.05$, $n=22$, unpaired t test). The development of this I_{Peak} presumably arises from the effects of temperature on the kinetics of activation and inactivation of both $I_{K(DR)}$ and $I_{K(TO)}$ channels, as has been described previously in circular muscle cells from the dog proximal colon (Thornbury *et al.*, 1992). The addition of 2.5 or 5 μM NOCys had little effect on either I_{Hold} or the time course of I_K ($n=7$) recorded at 35°C (Figure 2b(i)). However, the addition of 10–15 μM NOCys caused a concentration-dependent increase in I_{Hold} which was accompanied by a decrease in I_{SS} ($n=7$). In general, 2 to 4 fold higher concentrations of NOCys were required to increase I_{Hold} and reduce I_{SS} at 35°C (Figure 2b(ii)), than at 20–25°C (Figure 2a(ii)).

Effects of Cd^{2+}

The time-dependent effects of higher concentrations of NOCys (5 μM) on single colonic myocytes under voltage clamp (at 20–25°C) are illustrated in Figure 3. Cells were held at −60 mV, stepped (to +20 mV) or ramped (between −100 and +100 mV, over 0.5s) depolarizations were applied every 20s (Figure 3a(i)). In the presence of NOCys (5 μM), I_{Hold} reached a peak amplitude of −320±49 pA ($n=8$) after about 20–60s (Figure 3a(ii)). This increase in I_{Hold} was associated with a time-dependent decrease in I_{SS} recorded during the depolarizing steps to +20 mV (Figure 3B(i)a,b), and with an increase in the slope and a rightward shift of the instantaneous *I*-V curves when the ramp currents were plotted against V_m (Figure 3B(ii)d,e). The *I*-V characteristics of this NOCys-activated conductance change were obtained by subtracting the *I*-V plot in the presence of

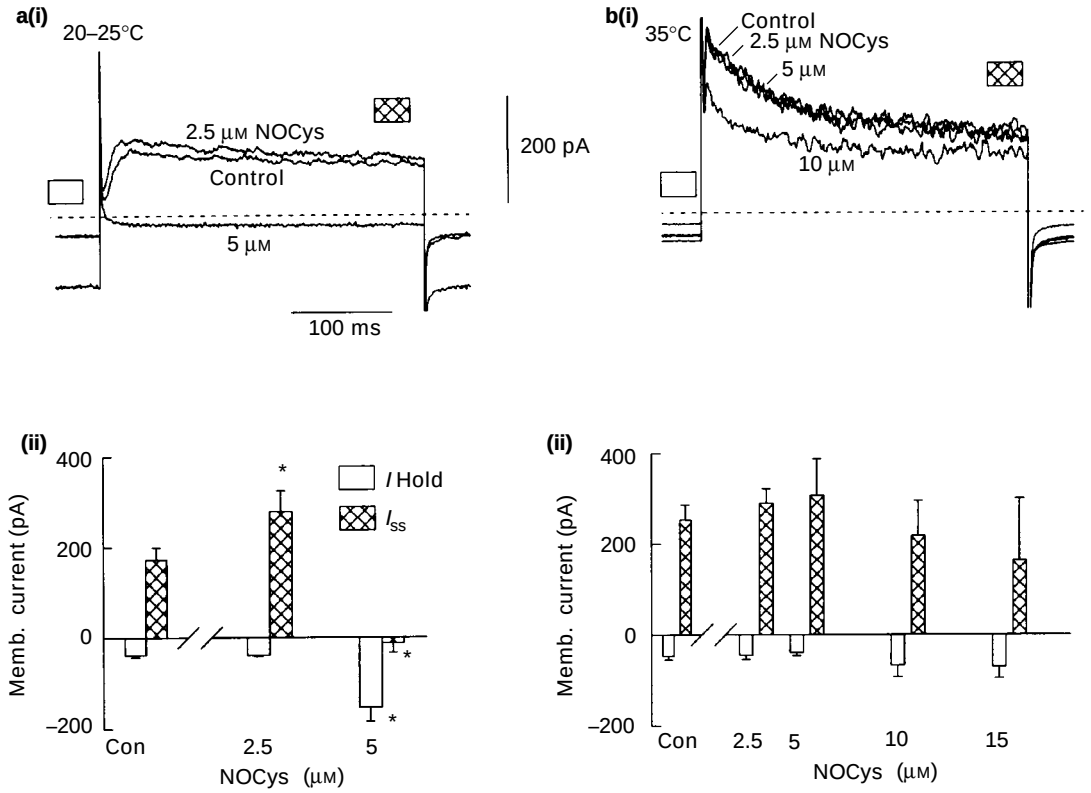

Figure 2 Summary of the effects of NOCys (2.5 to 15 μM) on the whole cell I_K recorded in single colonic myocytes at 20–25°C (a) ($n = 5$) or 35°C (b) ($n = 7$). Examples of I_K, recorded upon stepping to +40 mV from a holding potential of −60 mV, in the presence of NOCys (2.5 to 10 μM) were superimposed with I_K in control PS. The averaged amplitudes of I_{SS} of I_K and the averaged currents required to hold cells at −60 mV (I_{Hold}), in the absence (Con) and presence of NOCys (2.5 to 15 μM) have been compared in the lower panels (a(ii) and b(ii)).

NOCys (Figure 3B(ii)e) from the control *I*-V plot (Figure 3B(ii)d) and plotting the remaining current against V_m (Figure 3B(iii)). It can be seen that this NOCys-activated *I*-V plot was essentially linear at potentials negative to +20 mV and showed inward rectification at potentials more positive. In all cells, this NOCys-activated inward current (at −60 mV) slowly decayed over 5–10 min, either in the continued presence of NOCys or after NOCys washout. However, values of I_{Hold} and I_{SS} never completely returned to control values. This partial recovery after NOCys exposure was also reflected by the decrease in the slope of the *I*-V plots generated by the ramp depolarizations, particularly at negative potentials.

In smooth muscle, heavy metal ions such as cadmium (Cd^{2+}) have previously been shown to be effective non-selective blockers of both voltage-operated Ca^{2+} channels (Vogalis *et al*., 1993) and non-selective cationic channels (Inoue, 1991). In Figure 3A(ii), the addition of Cd^{2+} (1 mM) caused an immediate reduction in the amplitude the NOCys (5 μM)-activated inward current. In three cells, the amplitude of I_{Hold} (at −60mV) after a 2 min exposure to NOCys (5 μM) was -233 ± 33 pA; upon the addition of Cd^{2+}, I_{Hold} was reduced to -79 ± 18 pA ($P < 0.05$). The blockade of I_{SS} and of I_K by NOCys was also partially reversed in the presence of Cd^{2+} (Figure 3B(i)b,c). The slope of the NOCys-activated current (Figure 3B(iii), obtained upon subtraction of the control ramp current (Figure 3B(ii)d), at potentials negative to +20 mV was reduced approximately 50% in the presence of Cd^{2+} (1 mM). However, the inward rectification at positive potentials was reduced in Cd^{2+}, to reveal a substantial outward rectification consistant with the increase in I_{SS} at +20 mV (Figure 3B(i)c). In contrast, gadolinium (Gd^{3+}) (1 mM), another blocker of non-selective cationic channels in smooth muscle, had no effect on the increase of I_{Hold} induced upon the addition of 5 μM NOCys ($n = 2$).

The random oscillations of the whole-cell current, routinely recorded during depolarizing steps to potentials between −20 and +40 mV (from a holding potential of −60 mV) and characteristic of the opening and closing of BK channels, were mostly blocked in the presence of Cd^{2+} (1 mM) (Figure 4A(i)a,b). This blockade of BK channel activity was associated with a reduction of the instantaneous *I*-V curves generated from the ramped depolarizations, particularly at potentials positive to +20 mV (Figure 4A(ii)a,b). The remaining, slowly-decaying whole current evoked from a holding potential of −60 mV can, therefore, be considered to reflect the current flow through $I_{K(DR)}$ channels. Under these conditions, NOCys (2.5 μM) appeared to have little effect on the whole-cell currents recorded during either depolarizing steps to +20 mV (Figure 4A(i)b,c) or ramped depolarizations (between −100 and +100 mV, over 0.5s) (Figure 4A(iii)b,c). A higher concentration of NOCys (5 μM) applied to this colonic myocyte, bathed in Cd^{2+}-containing PS, still induced an inward current which was greatly reduced in duration (Figure 4B(i)), decaying rapidly to a sustained level (between −80 and −100 pA) similar to that obtained when Cd^{2+} was added after the application of NOCys (Figure 3A(ii)). The whole-cell currents, recorded during a stepped depolarization to +20 mV, were still blocked by 5 μm NOCys (Figure 4C(i)a,b,c,d), while the *I*-V plots generated by the ramped depolarization revealed that the inward current induced by NOCys was smaller at negative potentials (Figure 4C(ii)g), compared to the inward currents induced in the absence of Cd^{2+} (Figure 3B(ii)e). These effects of both NOCys and Cd^{2+}

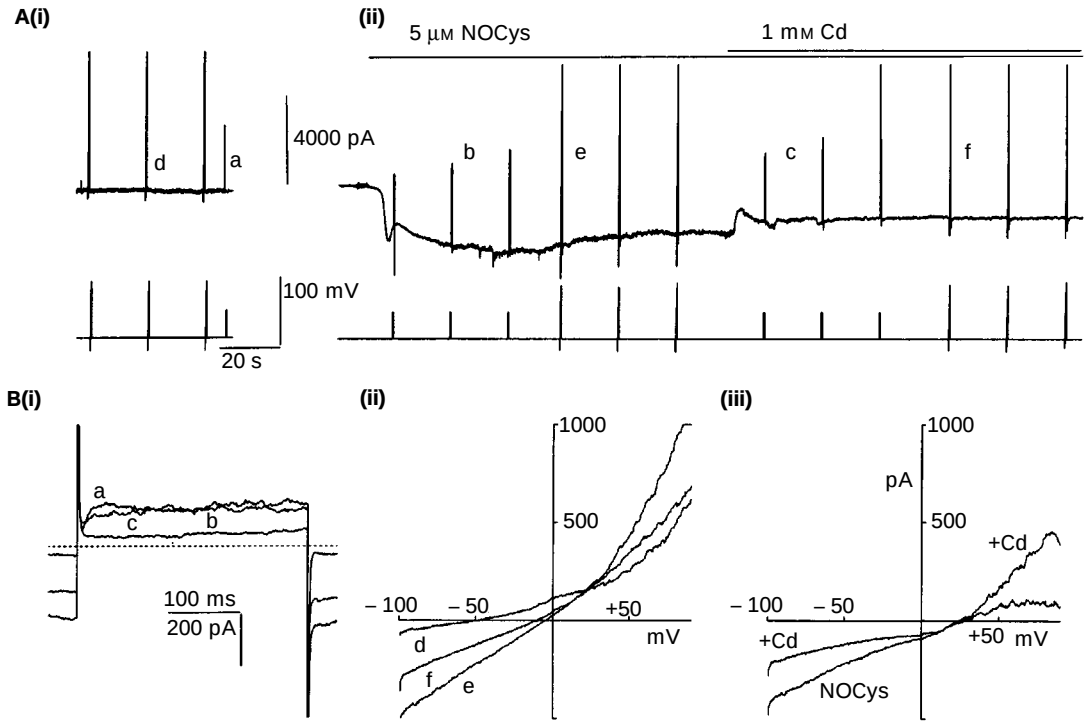

Figure 3 Cd^{2+} (1 mM) partially blocked the effects of NOCys (5 μM) on the whole cell membrane currents recorded in single colonic myocytes, held at −60 mV (at 25°C). Ramped depolarizations (between −100 and +100 mV over 0.5 s) or stepped depolarizations (to +20 mV) were applied continuously, every 20 s. (A) Recordings of membrane current (upper trace) and potential (lower trace) in the absence (A(i)) and presence (A(ii)) of NOCys (5 μM). (A(ii)) NOCys (5 μM) induced a time-dependent increase in I_{Hold} which was rapidly reduced (by approximately 60%) upon the addition of 1 mM Cd^{2+}. (B(i)) the whole-cell membrane currents recorded during the stepped depolarizations (indicated in A) before (B(i)a) and during the application of 5 μM NOCys (B(i)b), and NOCys +1 mM Cd^{2+} (B(i)c) have been superimposed, illustrating the effects of NOCys and Cd^{2+} on I_{SS} of I_K. The membrane current responses to the ramped depolarizations indicated in (A(d-f)) have been plotted against the patch potential in (B(ii)). It can be seen that NOCys (5 μM) induced an increase in the slope of the *I*-V curves, this increase was partially reversed upon the addition of Cd^{2+}. (B(iii)) the *I*-V plot of the NOCys-activated current was obtained by subtracting the ramp current recorded in control PS from the ramp currents recorded in 5 μM NOCys, and NOCys +1 mM Cd^{2+}, and plotting the remaining currents against the membrane potential.

were again partially reversible as both the *I*-V plot (Figure 4C(iii)i,h) and the whole-cell currents recorded at +20 mV (Figure 4C(i)e) returned to near control levels after 10 min washout in control PS containing 1 mM Cd^{2+}.

Effects of NOCys on single channel activity in cell-attached and excised membrane patches

The whole-cell voltage clamp experiments described above suggest that NOCys (<5 μM), SNP (100 μM) and 8-bromo-cyclic GMP (500 μM) (at 20–25°C) all induced a small increase in the amplitude of I_K activated at positive potentials. However, higher concentrations of NOCys (>5 μM) induced a blockade of I_K. This blockade was always associated with the activation of an inward current (at −60 mV) which reversed near 0 to +30 mV, suggesting the activation of a membrane conductance which was poorly selective for cations and/or Cl^-. Experiments were therefore performed in the cell-attached and isolated inside-out patch configuration in an attempt to identify the ionic channels associated with the modulations of these outward and inward currents.

In symmetrical high K^+(130 mM_{out}: 130 mM_{in}) saline, single channel openings were recorded in both cell-attached and excised patches maintained at potentials positive to +10 mV; the amplitude and frequency of these single channel openings increased with membrane depolarization. Typically, these single channels were 5–8 pA at +40 mV. These channels were K^+ selective as they had a reversal potential of 0 mV in the symmetrical high K^+ saline, and a reversal potential between −70 and −80 mV when the patch pipette contained a low K^+ (6 mM) PS. TEA (0.5–5 mM) added to the pipette solution reduced the amplitude of the current flowing through these channels in a concentration-dependent manner, suggesting that these channel currents represent the flow of current through BK channels found in many smooth muscles (Vogalis & Lang, 1994).

The addition of 2.5 μM NOCys produced a delayed increase in the activity of BK channels recorded in cell-attached patches, bathed in either a symmetrical high K^+ saline or an asymmetrical K^+ (6 mM_{out}: 130 mM_{in}) PS (Figure 5). Usually, there were only a few BK channel openings at +40 mV before the addition of NOCys (Figure 5a), such that $N.P_o$ was 0.04 ± 0.03 ($n=6$). Ramped depolarizations from −80 to +80 mV (over 500 ms) also activated relatively few channel openings, even at positive potentials (Figure 5a(i)). Following the addition of 2.5 μM NOCys, 3–5 channels became continuously active after a delay of 5–20s (Figure 5a); after 2 min, $N.P_o$ increased to 1.12 ± 0.03 ($n=6$; $P<0.05$). The ramped depolarizations also activated larger outward currents at positive potentials (Figure 5a(i–ii)). This activity peaked after 2–3 min and gradually decreased over a period of 1–10 min.

This increase in $N.P_o$ in NOCys (2.5–10 μM) was observed only at potentials at which BK channels were previously active, there was no increase in $N.P_o$ at negative potentials. Plots of

$N.P_o$ (Figure 5c(ii)) or $\ln(N.P_o)$ (Figure 5c(iii)) of the active BK channels against V_m revealed that the data obtained in control saline (open circles) and 5 μm NOCys (solid circles) were nearly superimposable. In 10 μm NOCys (hollow triangles), this activation curve was shifted to the left. The straight lines fitted to the lower values of $\ln(N.P_o)$ vs V_m plots revealed that the slopes (K) of these lines were little affected, suggesting that the voltage sensitivity of these BK channels was such that $N.P_o$ increased e fold upon a membrane depolarization of 15.8 mV in control lPS, and 20.2 and 17.8 mV, respectively, in 5 and 10 μm NOCys. Similar parallel shifts of the activation curves, at low P_o values and a constant internal Ca^{2+} concentration were observed in two other cell-attached patches, and in three excised patches (see below). Such parallel shifts have previously been suggested to reflect a sudden change in N (Singer & Walsh, 1987). It seems likely, therefore, that the predominant effect of NOCys in the present experiments is to increase the number of active BK channels in these cell-attached patches.

A consistent observation during the experiments with cell-attached patches was that the increased BK channel activity recorded in higher concentrations of NOCys ($\geqslant 5$ μM) was associated with a decrease in the recorded BK channel amplitudes (Figure 5b,c). BK channel amplitudes, as estimated

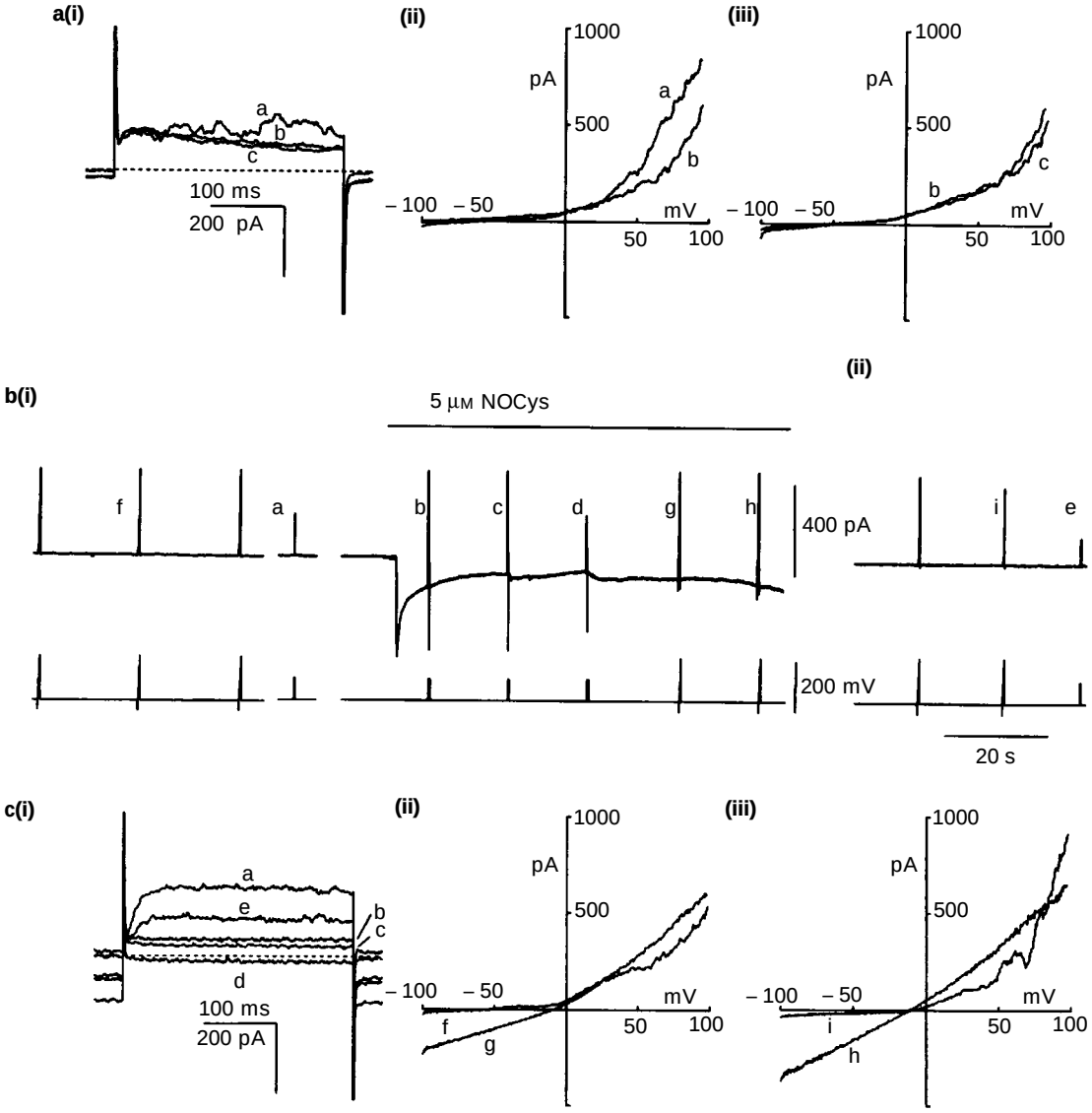

Figure 4 Effects of NOCys (2.5 to 5 μM) on the whole cell membrane currents recorded in colonic myocytes bathed in 1 mM Cd^{2+}(1 mM). Stepped depolarizations (to +20 mV) (A(i)) or ramped depolarizations (between −100 and +100 mV over 0.5 s) (A(ii-iii)) were applied (every 20 s) to myocytes held at −60 mV (at 25°C). (A(i)) Superimposed whole cell currents (at 20 mV) in control PS (A(i)a) and in the presence of 1 mM Cd^{2+} (A(i)b) or after 2 min exposure to Cd^{2+} (1 mM) +2.5 μM NOCys (A(i)c); the whole cell current in Cd^{2+} was little affected by NOCys (2.5 μM). (A(ii-iii) *I-V* plots generated from the ramped depolarizations in control PS (A(ii)a) and in the presence of 1 mM Cd^{2+} (A(ii)b, (iii)b) or after 2 min exposure to Cd^{2+} (1 mM) +2.5 μM NOCys (A(iii)c). (B(i-ii)) Recordings of membrane current (upper trace) and potential (lower trace) in a colonic myocyte continuously bathed in Cd^{2+} (1 mM), in the absence and presence (B(i)), and 10 min after the washout (B(ii)), of NOCys (5 μM). (B(i)) NOCys (5 μM) application, in the presence of Cd^{2+}, induced a transient increase in I_{Hold} (to −430 pA) which decayed to a steady state level between −80 and −100 pA. (C(ii)) The whole-cell membrane currents recorded during the stepped depolarizations (indicated in B) in the presence of Cd^{2+} (C(i)a) and upon the addition of 5 μM NOCys (C(i)b,c,d) were superimposed. The effects of NOCys were partially reversible, as illustrated by the partial recovery of I_{Hold} and I_K (C(i)e) 10 min after the reintroduction of control PS containing 1 mM Cd^{2+}. (C(ii-iii)) Superimposed *I*-V plots generated by the ramp depolarizations in Cd^{2+} (1 mM)(C(ii)f), upon the addition of NOCys (5 μM) (C(ii)g, (iii)h) and 10 min after removal of the NOCys (C(iii)i):

from the all points histograms (see Methods), were calculated at various membrane potentials (+10 to +80 mV) (Figure 5b(i–iii)) and plotted against V_m (Figure 5c(i)) ($n=3$). The addition of 10 μm NOCys caused a substantial increase in BK channel activity at potentials > + 20 mV (Figure 5b(i–iii)). In this experiment, the slope of the *I*-V plot of these BK channels

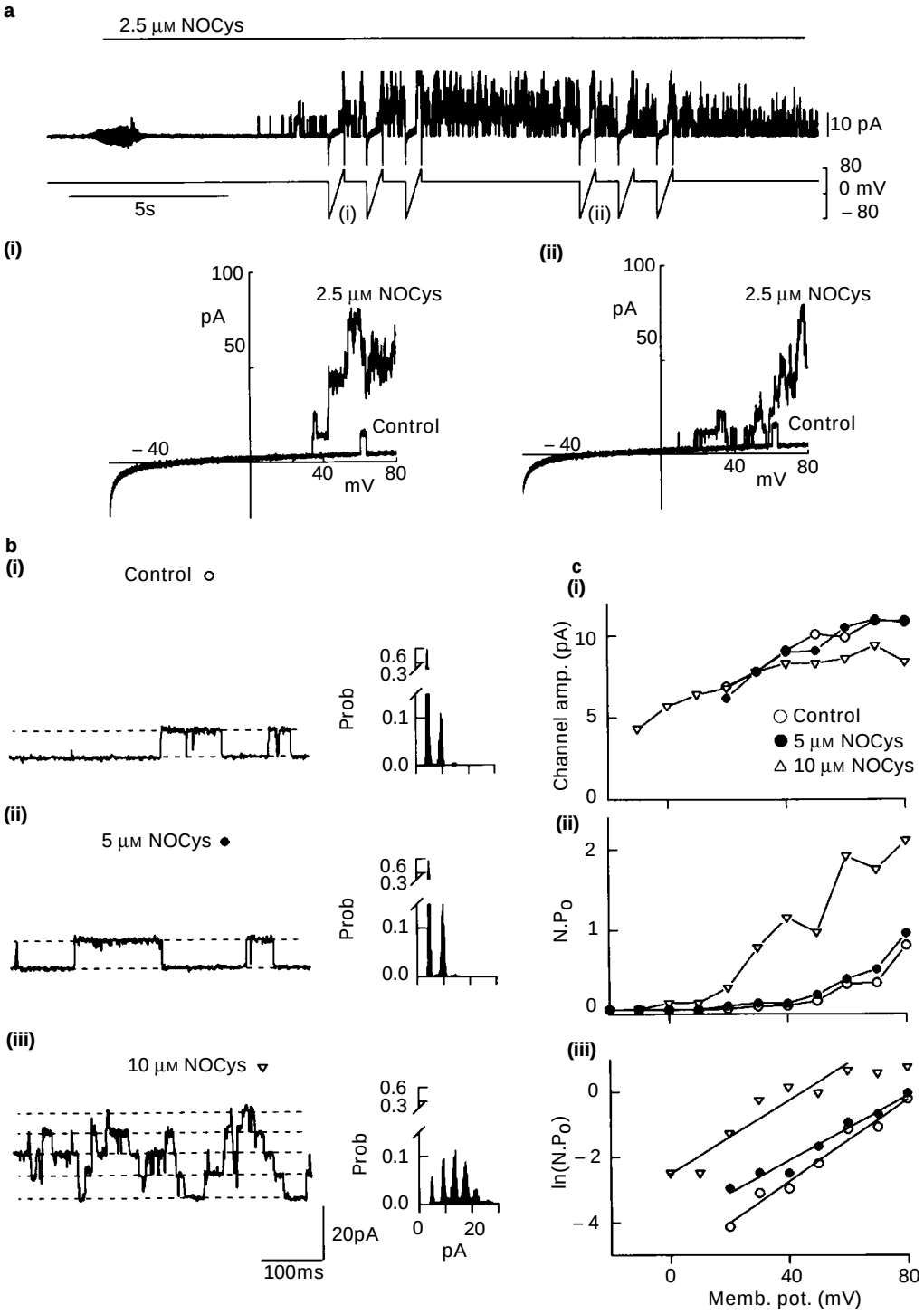

Figure 5 Effects of NOCys (2.5 to 10 μM) on BK channel activity in a cell-attached patch of a guinea-pig colonic myocyte. Patches were held at +40 mV and bathed in an asymmetrically K^+ (6 mM_{out}: 130 mM_{in}) PS and ramped depolarizations (between −80 and +80 mV over 0.5 s) applied as indicated. Typical membrane currents recorded during these ramped depolarizations in NOCys (2.5 μM) (highlighted by (i–ii) in (a), upper panel) were plotted against patch potential in the lower panels of (a), superimposed on the ramped currents recorded in control saline. NOCys (2.5 μM) increased BK channel activity at +40 mV after a delay of some 5 s, this was associated with a large increase in BK channel activity recorded at positive potentials during the ramped depolarizations (a, lower panels). (b) The frequency of BK channels activity increased in the presence of 5 μM (b(ii)) and 10 μM (b(iii)) NOCys in a concentration-dependent manner. The single channel amplitude and probability of BK channel opening ($N.P_o$) was calculated from the centres and integrals of the gaussian distributions fitted to the all points histograms (see Methods) of the active BK channels. The calculated amplitudes (c(i)), $N.P_o$ (c(ii)) and $\ln(N.P_o)$ (c(iii)) of the active BK channels in control saline and in the presence of 5 μM and 10 μM NOCys were plotted against membrane potential. The slopes (K) of the straight lines fitted to the lower values ($N.P_o < 0.1$) of the $\ln(N.P_o)$ vs V_m plots were little affected, such that $N.P_o$ increased e fold with 15.8 mV depolarization in control saline, and with 20.2 and 17.8 mV, respectively, in 5 μM and 10 μM NOCys.

in 10 μm NOCys (Figure 5c(i), open triangles) (calculated between +20 and +60 mV) gave a single channel conductance of 41 pS; compared with 99 and 83 pS in 5 μM NOCys (Figure 5c(i), solid circles) and control saline (Figure 5c(i), open circles), respectively.

Such reductions of the single channel conductance of the BK channels active in the presence of NOCys were not observed when excised inside-out patches were used. It has recently been shown that exogenously applied NO and the NO donors, NOCys and SNP, can directly increase the activity of BK channels, in excised smooth muscle membranes via a mechanism independent of cyclic GMP (Bolotina *et al*., 1994; Watson *et al*., 1994; McPhee & Lang, 1994). In Figure 6a, it can be seen that the BK channel activity in an excised patch (bathed in a symmetrical high K^+ saline) recorded either at a constant potential of +40 mV (Figure 6a(i–ii)), or at positive potentials during ramped depolarizations (between −80 and +80 mV over 500 ms) (Figure 6b), was readily increased by 2.5 μm NOCys. In 5 experiments, 2.5 μM NOCys significantly increased $N.P_o$ at +40 mV to 1.31±0.25 (control $N.P_o$ 0.76±0.1; $P<0.05$). In two excised inside-out patches, SNP (100 μM) also increased $N.P_o$ to 2.47 and 2.01, respectively (from control $N.P_o$ of 2.22 and 1.65, respectively).

In both cell-attached and excised patches, the NOCys-induced increase in BK channel activity at positive potentials was often associated with the activation of an additional population of channel currents of smaller amplitude, active at both negative and positive potentials. Typical examples of these smaller conductance NOCys (2.5 μM)-activated channel currents are illustrated in Figure 6c(ii). In these experiments ($n=4$), NOCys-activated channel currents were recorded in excised inside-out patches which were bathed in the asymmetrically K^+ PS, except that the NaCl in the pipette PS had been replaced by Na gluconate (126 mM). Under these conditions (at 20°C), both E_{Cl} and E_{Na} would be set

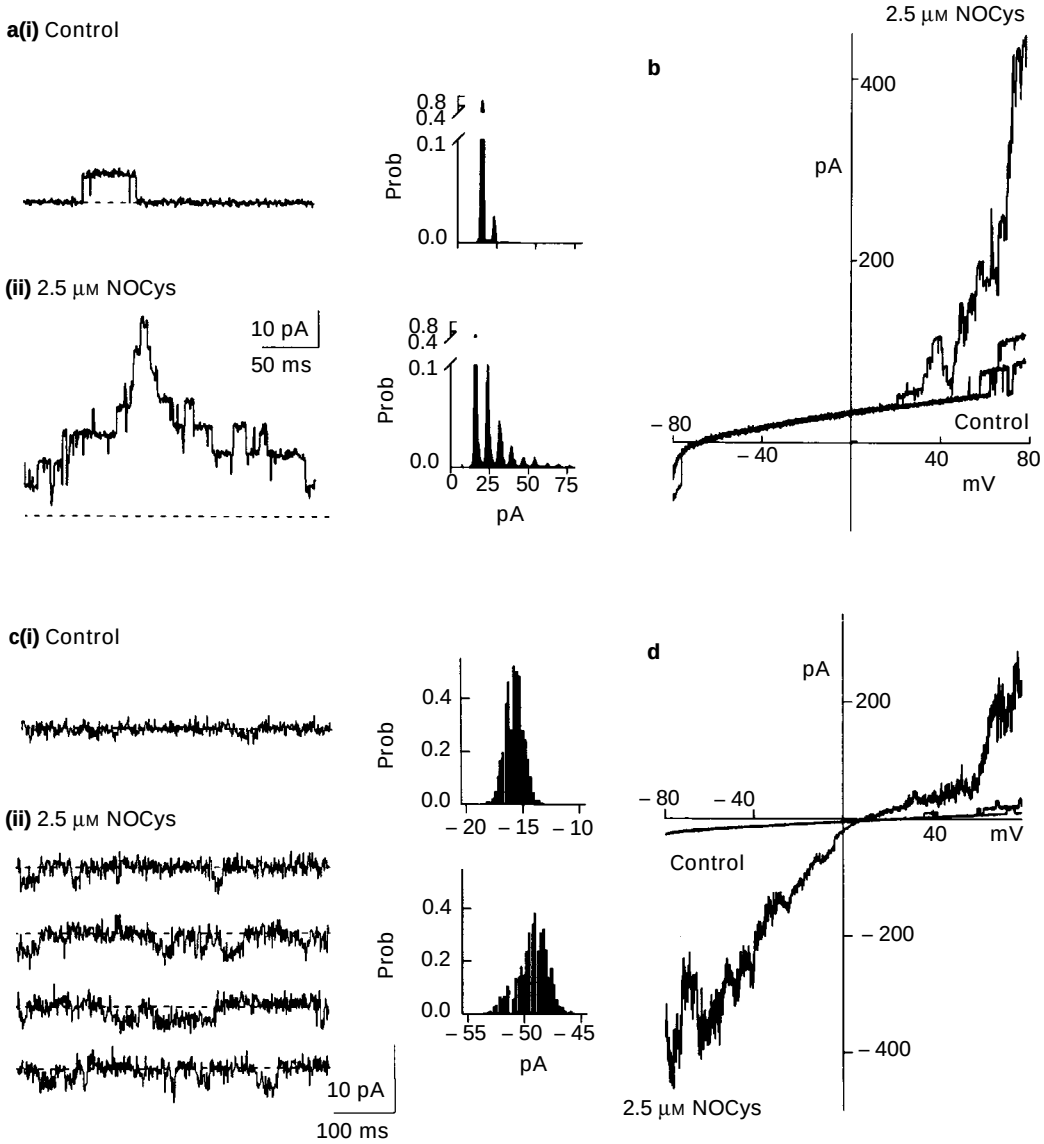

Figure 6 Effects of NOCys (2.5 μM) on channel activity in excised inside-out patches of guinea-pig colonic myocytes. BK channel activity recorded at + 40 mV (a), or during ramped depolarizations (between −80 and +80 mV, over 0.5 s) (b), in the absence (a(i)) and presence (a(ii)) of NOCys (2.5 μM); patches were exposed to a symmetrical high K^+ (130 mM) saline on either side of the membrane. (c), Typical examples of cationic channel currents recorded at a constant potential of −30 mV (c(ii)) or during ramped depolarizations (d) in excised inside-out patches bathed in asymmetrical K^+ (6 mM_{out}: 130 mM_{in}) saline. Note also that 126 mM NaCl in the pipette saline had been replaced with Na gluconate.

near +65 mV, E_K would be set near −76 mV. NOCys 2.5 μM again increased BK channel activity only at positive potentials (data not shown). Additional channel currents, smaller in amplitude and which were inward at potentials positive to E_K, and negative of 0 mV, were also readily recorded. At −30 mV, these inward channel currents were estimated by eye to be about 2–3 pA, the presence of these channel openings could also be detected by the development of a marked shoulder on the left hand side of the all points amplitude histogram (at −30 mV) (Figure 6(i–ii)). Ramp depolarizations between −80 and 80 mV revealed that these smaller conductance channel reversed near +10 mV and showed a marked inward rectification at negative potentials (Figure 6d). These data suggest that these smaller conductance channels are poorly selective for cations and perhaps represent the channels responsible for the NOCys-activated inward current recorded under whole-cell voltage clamp. These cationic channels have not been examined further in this study.

Effects of NOCys and SNP on the membrane potential

NOCys (2–25 μM) induces membrane responses in the intact guinea-pig proximal colon (at 37°C), bathed in nifedipine (1 μM) and hyoscine (1 μM), that consist of an initial transient apamin-sensitive hyperpolarization (about 20–30 mV in amplitude) which decays to a less hyperpolarized plateau, upon which oscillations of membrane potentials are often superimposed (Watson *et al.*, 1996a,b). In the present experiments, the exposure of single colonic myocytes to NOCys (2.5 μM) or SNP (100 μM) produced variable effects on the MP. In some cells, NOCys (2.5 μM)($n=6$) (Figure 7a(i)) and SNP (100 μM)($n=5$) (Figure 7a(ii)) induced an increase in the 'noise' of the membrane potential, due to the activation of small transient hyperpolarizations which fused into a net membrane hyperpolarization to -47.3 ± 0.9 ($P<0.05$) and -46.4 ± 1.1 mV ($P<0.05$), respectively, after 1–2 min. (respective control Vm of -42.4 ± 0.7 and -44.1 ± 0.7 mV). The amplitude of the electrotonic potentials, induced by

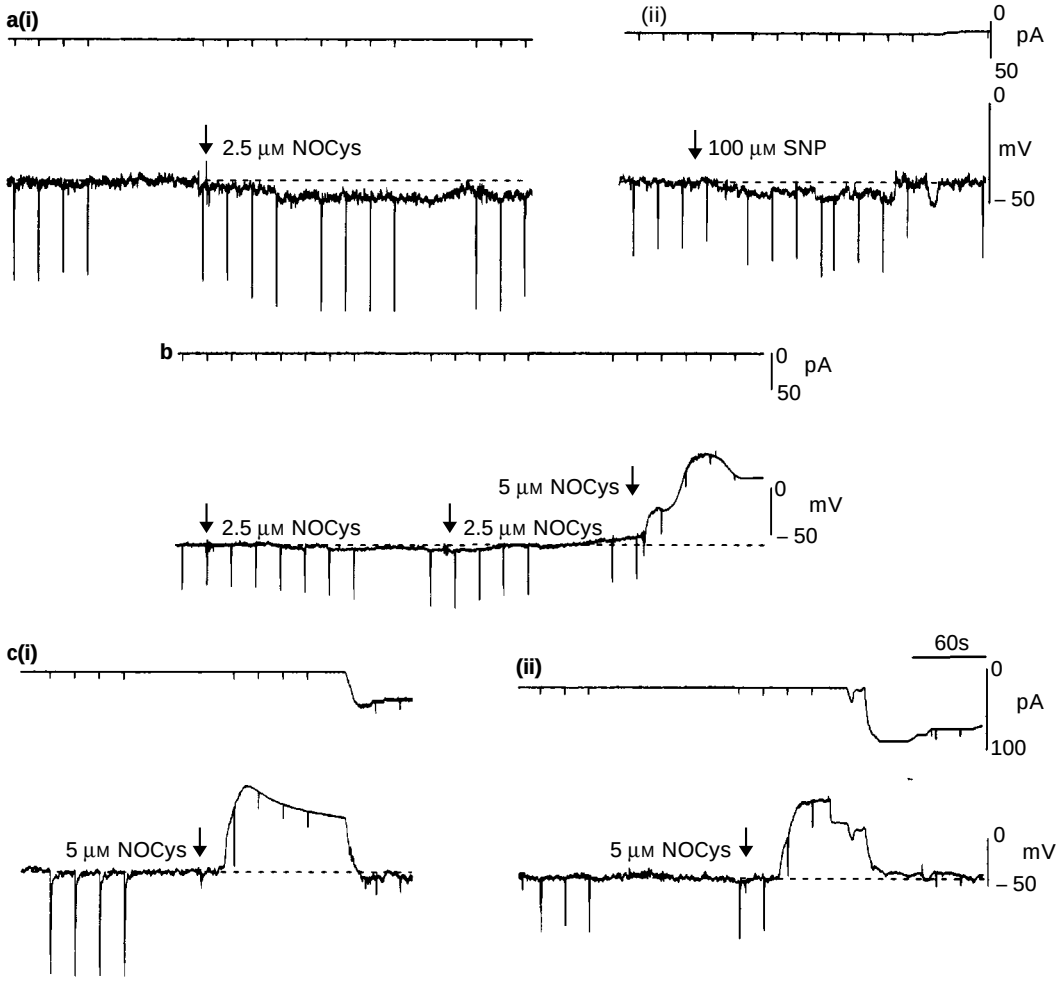

Figure 7 Effects of NOCys (2.5 to 5 μM) and SNP (100 μM) on the membrane potential of single colonic myocytes. NOCys (2.5 μM) (a(i)) and SNP (100 μM) (a(ii)) induced a membrane hyperpolarization of 2–4 mV associated with an increase in the potential 'noise' and with a 10% increase in the amplitude of the electrotonic potentials evoked by the stepped injection of hyperpolarizing current, 10 pA in amplitude and 0.5 s in duration. Returning the membrane potential back to control values by the application of a constant depolarizing current restored the amplitude of the electrotonic potentials to control values (a(ii)). (b) In some cells, the application of NOCys (2.5 μM) led to a membrane depolarization of 3–5 mV; 5 μM NOCys always depolarized the membrane potential to potentials positive to O mV. In the presence of 5 μM NOCys, electrotonic potentials recorded near the peak of the depolarizing response were reduced to 61% ($n=5$) of the control electrotonic potentials (c(i–ii)). When the membrane potential was returned to control values, by passing a constant hyperpolarizing current, the electrotonic potentials remained smaller than control (c(i-ii)).

injections of hyperpolarizing currents (10 pA for 400 ms), in NOCys (2.5 μM) and SNP (100 μM) were 111% ($P<0.05$) (Figure 7a(i)) and 107% ($P>0.05$) (Figure 7a(ii)) of those recorded in control PS. Repolarizing the MP, by passing a constant current, restored the amplitude of the electrotonic potentials to their control values (Figure 7a(ii)). In other cells, NOCys (2.5 μM) ($n=4$) (Figure 7b) and SNP (100 μM) ($n=9$) (data not shown) induced membrane depolarization to -40.5 ± 1.5 ($P<0.05$) and -45 ± 1.1 mV (P 0.05), respectively; from their respective control V_ms of -43.3 ± 0.8 and -51.6 ± 1.1 mV. NOCys (5 μM) produced a large depolarization to $+27\pm3.6$ mV ($n=5$) (Figure 7b). In some cells, this depolarization to NOCys (5 μM) was preceded by a transient membrane hyperpolarization to -50.4 ± 1.9 mV (control Vm of -45.3 ± 0.6 mV) ($n=6$; ($P<0.05$). The electrotonic potentials elicited in response to an injection of constant hyperpolarizing current were little affected during the NOCys-induced membrane hyperpolarization ($n=6$, ($P>0.05$), but reduced to 61% of the control electrotonic potentials ($n=5$; ($P<0.05$) during the period of membrane depolarization (Figure 6c(i–ii)). In three cells, the amplitude of the electrotonic potentials recorded when the V_m was returned to near control values in the continued presence of NOCys (5 μM) (by passing a constant inward current of 50 to 100 pA) was 17% ($n=3$) of control, compared with 36% near the peak of the NOCys-induced depolarization (Figure 7c(i–ii)).

Discussion

In the guinea-pig intact proximal colon, we have recently demonstrated that repetitive NANC nerve stimulation and the application of NO, or the NO donor compound NOCys, produced complex membrane potential responses in the circular muscle layer consisting of a transient hyperpolarization which decays to a sustained, often-oscillating hyperpolarized level, followed by a long-lasting rebound depolarization (Watson *et al*., 1996a,b). All three components were demonstrated to arise from increases in the membrane conductance. The initial transient hyperpolarization was suggested to arise from the opening of apamin-sensitive SK channels, while the following other two responses have been associated, in part, with an increased generation of cyclic GMP (Bayguinov *et al*., 1992; Ward *et al*., 1992a; Bayguinov & Sanders, 1993; Watson *et al*., 1996a,b).

In the present experiments, we have demonstrated that NOCys (2.5 μM), SNP (100 μM) and 8-bromo-cyclic GMP (500 μM) increased I_{SS} of I_K recorded in single colonic myocytes under whole-cell voltage clamp. We have also demonstrated that NOCys (2.5 to 20 μM) increased the probability of BK channel opening $(N.P)_o$ in cell-attached patches, by increasing the number of 'active' channels within a patch, and not by changing the voltage sensitivity of these BK channels. Although not detailed in the present experiments, isoprenaline (20 μM) also induced a similar leftward shift of the plots of $\ln(N.P)_o$ against V_m, significantly increasing $N.P_o$ (at $+40$ mV) to 0.76 ± 0.06 (control $N.P_0$ of 0.32 ± 0.07; $n=3$, $P<0.05$) after a delay of 2–3 min (Watson & Lang, unpublished data). This isoprenaline-induced increase in BK channel activity in cell-attached patches is likely to be via the activation of a G protein, adenylate cyclase and protein kinase A, as has been found in cell-attached patches of single myocytes of rabbit trachea and taenia caeci, and guinea-pig basilar arteries (Kume *et al*., 1989; Fan *et al*., 1993; Song & Simard, 1995). However, the effects of NOCys and SNP do not necessarily require the intermediary action of a cyclic GMP-dependent kinase (Tangiguchi *et al*., 1993; Robertson *et al*., 1995) as BK channel activity was also increased in excised inside-out patches, in the absence of cyclic GMP, ATP, GTP or any kinase activity. Such a direct modulation of BK channel activity by NO and sulphhydryl oxidizing/reducing agents is well established in vascular myocytes (Bolotina *et al*., 1994; Lee *et al*., 1994; Park *et al*., 1995). However, in the gastrointestinal tract, this direct action of NO donors has not been demonstrated previously. For example, in the dog (Thornbury *et al*., 1992) and rabbit (Lu *et al*., 1994) colon and opposum oesophagus (Murray *et al*., 1995), NO donors increased the amplitude of BK whole-cell currents, as well as the activity of single BK channels recorded in cell-attached patches. This increase in BK channel activity was mimicked by membrane-permeable analogues of cyclic GMP, but blocked by inhibitors of guanylate cyclase activity (Lu *et al*., 1994; Murray *et al*., 1995). In excised membrane patches of the dog colon, BK channel activity was not affected by the NO donor, S-nitro-N-acetyl penicillamine (SNAP), even though the activity of two populations of voltage-activated small conductance K^+ channels was increased, in a manner prevented by both reducing and alkylating agents (Koh *et al*., 1995). However, in the circular muscle of the guinea-pig proximal colon, it is likely that the number of active BK channels within a membrane can be modulated by both: (i) the phosphorylation of the BK channel protein/s by cyclic nucleotide-dependent protein kinases (Kume *et al*., 1989; Fan *et al*., 1993; Tangiguchi *et al*., 1993; Murray *et al*., 1995; Robertson *et al*., 1995; Song & Simard, 1995), and (ii), the oxidation of thiol groups on the BK channels or associated proteins. These speculations require further experimentation.

Higher concentrations of NOCys (>5 μM) reduced I_K triggered from -60 mV in a manner always associated with the activation of a cationic conductance. In excised inside-out patches, NOCys and SNP also directly opened a population of small conductance (approximately 100 pS) cationic channels, which were continually active at both depolarized and hyperpolarized holding potentials (Koivisto & Nedergaard, 1995). In general, the increase in I_{Hold} in the presence of NOCys was larger in cells bathed in low Ca^{2+} PS than cells perfused with normal PS. However, the inhibition of I_K by these agents was little altered. The reduction in I_K, therefore, is not likely to be caused by an increase in cytoplasmic concentrations of Ca^{2+} ($[Ca^{2+}]_{in}$) entering through these cationic channels (Gelband & Hume, 1995), particularly as rises in internal cyclic GMP are generally associated with the activation of mechanisms which lower $[Ca^{2+}]_{in}$. However, the persistant opening of these cationic channels, presumably permeable to Na^+, would tend to increase the internal concentration of Na^+ ($[Na^+]_{in}$), particularly within the submembrane cytosolic compartment. Such a rise in $[Na^+]_{in}$ may well block the current flow through BK, $I_{K(DR)}$ and $I_{K(TO)}$ channels which underlie the generation of I_K. Such a direct blockade of current flow through BK channels was demonstrated in Figure 5, where the NO donor-induced increase in BK activity in cell-attached patches was accompanied by a progressive decrease in BK channel amplitude. This Na^+-induced blockade was not observed when BK channel activity (at $+40$ mV) was increased by NOCys or Ca^{2+} in excised inside-out patches, although a reduction of BK channel amplitudes at very positive potentials ($>+80$ mV), previously ascribed to a blockade by internal Na^+, has been shown in a number of excised smooth muscle patches (Benham *et al*., 1986; Singer & Walsh, 1987). In summary, although it is possible that the decrease in I_K and the activation of the cationic channels occur upon cyclic nucleotide formation

(Nakayama & Fozzard, 1988; Drain *et al*., 1994; Goulding *et al*., 1994; Zufall *et al*., 1994; Scamps, 1996), it is more likely that the reduction of I_K arises from a time-dependent increase in $[Na^+]_{in}$ which competes for K^+ on the cytosolic side of the channels underlying I_K, reducing their outward current flow.

In the present experiments, the NO donors, NOCys and SNP, increased BK channel activity (see also Thornbury *et al*., 1991; Lu *et al*., 1994; Murray *et al*., 1995), which presumably underlies the small slowly-developing hyperpolarizations recorded in some cells, even at negative potentials. The activation of the 100 pS cationic channels presumably underlies the membrane depolarizations recorded in some cells to lower concentrations of NOCys, or SNP, and in all cells when higher concentrations of NOCys were used. However, the hyperpolarizing components of the membrane responses of the intact guinea-pig proximal colon (Watson *et al*., 1996a) or rat gastric fundus (Kitamura *et al*., 1993) to NOCys (2.25 μM), SNP (>100 μM) or 8-bromo-cyclic GMP (500 μM), are not readily blocked by concentrations of TEA (>1 mM) which would be expected to block BK channels. Nor are these hyperpolarizations blocked by other K^+ channel blockers such as 4-AP (1–10 mM) and glibenclamide (1 μM), suggesting that $I_{K(TO)}$ and ATP-dependent K^+ channels are not involved. The membrane hyperpolarizations to SNP, isoprenaline and 8-bromo-cyclic GMP in the intact tissue were further distinguished from the responses to NO or NOCys by their slowly-developing time course, by their resistance to apamin and their blockade by TEA concentrations which would be expected to block both BK and $I_{K(DR)}$ channels (Kitamura *et al*., 1993; Vogalis *et al*., 1993; Vogalis & Lang, 1994; Watson *et al*., 1996a). Clearly, many of the K^+ channel populations being activated by NO have yet to be identified. However, the blockade of I_K in the presence of high concentrations of NOCys may well be masking any modulations of the K^+ channels underlying I_K induced by these agents. It is likely that the precise determination of the nature of the K^+ channels opened by NO donors or isoprenaline needs to be carried out at the single channel level in excised inside-out patches, bathed in Na^+ free conditions. Under these conditions, the activity of other voltage- and NO-gated K^+ channels may well be unmasked (Koh *et al*., 1995).

In summary, it seems likely that the complex membrane hyperpolarizations and rebound depolarizations in intact, nifedipine-arrested preparations of the guinea-pig proximal colon, recorded upon NANC nerve stimulation or after the application of various NO donors, arises from changes in a number of membrane conductances. First, the initial transient hyperpolarization appears to involve apamin-sensitive SK channels opened by both NO and the transmitter/s released after blockade of NOS activity (ATP) (Maggi & Giuliani, 1993; Zagorodnyuk & Maggi, 1994). These channels have yet to be identified at the single channel or whole-cell current level. Second, the maintained, often-oscillating hyperpolarized level, obtained after the subsidence of the initial transient hyperpolarizing component, reflects a summation of (i), a diminishing component of the apamin-sensitive hyperpolarization, (ii) the slowly-developing hyperpolarization recorded in the presence of apamin; perhaps involving, in part, an increase in BK channel activity induced directly by NO and indirectly by cyclic GMP, and (iii) the cationic conductance activated directly by NO and indirectly by cyclic GMP formation. The development of the rebound depolarization presumably reflects a continued activation of the cationic conductance, which would be augmented by the blockade of both voltage- and Ca-activated K^+ conductances presumably as the $[Na^+]_{in}$ rises.

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