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Nitric oxide (NO)-induced activation of large conductance Ca^{2+}-dependent K^+ channels (BK_{Ca}) in smooth muscle cells isolated from the rat mesenteric artery

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1 To assess the action of nitric oxide (NO) and NO-donors on K^+ current evoked either by voltage ramps or steps, patch clamp recordings were made from smooth muscle cells freshly isolated from secondary and tertiary branches of the rat mesenteric artery.

2 Inside-out patches contained channels, the open probability of which increased with $[Ca^{2+}]_i$. The channels had a linear slope conductance of 212 ± 5 pS ($n=12$) in symmetrical (140 mM) K^+ solutions which reversed in direction at 4.4 mV. In addition, the channels showed K^+ selectivity, in that the reversal potential shifted in a manner similar to that predicted by the Nernst potential for K^+. Barium (1 mM) applied to the intracellular face of the channel produced a voltage-dependent block and external tetraethylammonium (TEA; at 1 mM) caused a large reduction in the unitary current amplitude. Taken together, these observations indicate that the channel most closely resembled BK_{Ca}.

3 In five out of six inside-out patches, NO (45 or 67 μM) produced an increase in BK_{Ca} activity. In inside-out patches, BK_{Ca} activity was also enhanced in some patches with 100 or 200 μM 3-morpholino-sydnonimine (SIN-1) (4/11) and 100 μM sodium nitroprusside (SNP) (3/8). The variability in channel opening with the NO donors may reflect variability in the release of NO from these compounds.

4 In inside-out patches, 100 μM SIN-1 failed to increase BK_{Ca} activity (in all 4 patches tested), while at a higher (500 μM) concentration SIN-1 had a direct blocking effect on the channels ($n=3$). NO applied directly to inside-out patches increased ($P<0.05$) BK_{Ca} activity in two patches.

5 In the majority of cells (6 out of 7), application of NO (45 or 67 μM) evoked an increase in the amplitude of whole-cell currents in perforated patches. This action was not affected by the soluble guanylyl cyclase inhibitor, 1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one (ODQ). An increase in whole-cell current was also evoked with either of the NO donors, SIN-1 or SNP (each at 100 μM). With SIN-1, the increase in current was blocked with the BK_{Ca} channel blocker, iberiotoxin (50 nM).

6 With conventional whole-cell voltage clamp, the increase in the outward K^+ current evoked with SIN-1 (50–300 μM) showed considerable variability. Either no effect was obtained (11 out of 18 cells), or in the remaining cells, an average increase in current amplitude of $38.7 \pm 10.2\%$ was recorded at 40 mV.

7 In cell-attached patches, large conductance voltage-dependent K^+ channels were stimulated by SIN-1 (100 μM) applied to the cell ($n=5$ patches).

8 These data indicate that NO and its donors can directly stimulate BK_{Ca} activity in cells isolated from the rat mesenteric artery. The ability of NO directly to open BK_{Ca} channels could play an important functional role in NO-induced relaxation of the vascular smooth muscle cells in this small resistance artery.

Keywords: Nitric oxide; potassium channels, calcium activated; smooth muscle, arterial; arteries, mesenteric resistance

Introduction

Nitric oxide (NO) stimulates potent vasodilatation by relaxing vascular smooth muscle cells. The relaxation is thought to be mediated mainly by the activation of soluble guanylyl cyclase, leading to increased intracellular levels of cyclic GMP and the subsequent activation of protein kinases (Archer *et al*., 1994). In addition to this action on guanylyl cyclase, other actions of NO have been reported, which include interactions with protein thiol groups to form S-nitrosyl compounds, which modulate enzyme activity and receptor function (Schulz & Triggle, 1994; Broillet & Firestein, 1996).

In the vasculature, the endothelium is the main source of NO which diffuses readily to the adjacent smooth muscle cells. In a recent study, the NO donor, 3-morpholino-sydnonimine hydrochloride (SIN-1) was found to stimulate complete relaxation of preconstricted, rat isolated mesenteric arteries. Surprisingly, in the presence of basal NO synthesis by the endothelium the relaxation appeared to be due to the activation of charybdotoxin-sensitive potassium channels. However, after basal NO synthesis was blocked, relaxation was due to a combination of both potassium channel and guanylyl cyclase activation (Plane *et al*., 1996). A charybdotoxin-sensitive K^+ channel has also recently been implicated in the relaxation of guinea-pig pulmonary artery rings with the NO donors s-nitroso-N-acetyl-penicillamine (SNAP) and SIN-1 (Bialecki & Stinson-Fisher, 1995).

The suggestion that NO can activate a large, Ca^{2+}-dependent potassium channel (BK_{Ca}) independently of guanosine 3′:5′-cyclic monophosphate (cyclic GMP), is controversial. NO has been shown to stimulate BK_{Ca} channels directly in vascular smooth muscle cells (Bolotina *et al*., 1994). However, it has also been shown to stimulate these channels through a cyclic GMP-dependent mechanism (George & Shibata, 1995). Moreover, SNAP did not modify BK_{Ca} channel activity in excised patches from canine colonic smooth muscle cells (Koh *et al*., 1995). However, in the latter study,

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NO did stimulate voltage-gated K^+ channels of small and intermediate conductance (4 and 80 pS, respectively) independent of any change in cyclic GMP.

We have presented evidence recently that indicates that NO might directly open charybdotoxin-sensitive potassium channels and that this action makes a major contribution to smooth muscle relaxation (Plane *et al*., 1996). In the present study, we have investigated the possibility that NO directly activates potassium channels in freshly isolated smooth muscle cells from the rat mesenteric artery using patch-clamp techniques. We provide direct evidence that NO can activate BK_{Ca} channels independently of cyclic GMP in these smooth muscle cells.

Methods

Male Wistar rats (250–300 g) were killed by cervical dislocation. A segment of lower ileum plus attached mesentery was removed. Secondary and tertiary mesenteric arteries were cleaned of connective tissue and cut open longitudinally into 1–3 mm strips. The strips of arteries were placed in a refrigerator (4°C) for 1 h. The tissue was then transferred to an enzyme mixture at 37°C containing 1 mg ml^{-1} collagenase, 2 mg ml^{-1} bovine serum albumin (BSA) and 0.70 mg ml^{-1} pronase (all from Sigma) for 15–20 min. This enzyme mixture had a volume of 3 ml and contained 15 μM [Ca^{2+}]. The strips were then transferred to a nominally Ca^{2+}-free solution which was enzyme free, and gently triturated with a large diameter (approx. 1 mm) Pasteur pipette and then with a smaller diameter (approx. 0.5 mm) Pasteur pipette until the suspension became cloudy. The resulting cell suspension was kept at 4°C and used within 9 h of isolation. A drop of the cell suspension was placed in the centre of a 35 mm tissue culture dish (Nunc) and mounted on an inverted microscope (Nikon 200 UK). On occasions, strips of mesenteric artery, were kept overnight in 2 mg ml^{-1} BSA and 15 μM $CaCl_2$ and then in the morning the collagenase/pronase was added. The tissue was incubated at 37°C for 10–15 min and triturated as described earlier. Results obtained from these cells were similar to those obtained from the acutely dissociated cells, therefore results were pooled.

The external (Tyrode) solution contained (in mM): NaCl 130, KCl 5, $MgCl_2$ 1.2, HEPES 10, glucose 10, $CaCl_2$ 1.2; pH 7.3 (buffered with NaOH). The pipette solution contained (in mM): KCl 130, Na_2ATP 2, $MgCl_2$ 3, HEPES 5, EGTA 1; pH 7.3 (buffered with KOH). The above two solutions were used for the whole-cell and outside-out patch recordings. For the inside-out, cell-attached and perforated patch experiments, the pipette (extracellular) solution contained (in mM): KCl 140, HEPES 10, $CaCl_2$ 1, $MgCl_2$ 1; pH 7.3 (buffered with KOH). The intracellular solutions containing 0.1, 1.0 and 10 μM free Ca^{2+} were made up from different combinations of EGTA and $CaCl_2$ as has been used previously to study large conductance Ca^{2+}-activated K^+ channels in rat pulmonary artery smooth muscle cells (Albarwani *et al*., 1994). To prevent K_{ATP} channel activation, 2 mM Na_2ATP was added to the intracellular solutions.

Voltage-clamp and current-clamp recordings were made using different configurations of the patch-clamp technique at 20–24°C (Hamill *et al*., 1981). Current clamp recordings were made with the perforated patch (see below) to assess the mean resting membrane potential which was -45.6 ± 3.6 mV ($n = 5$). The perforated patch method was employed using amphotericin. A 30 mg ml^{-1} stock solution of amphotericin B was made and vortexed for several seconds. Ten microlitres of this solution was mixed with 1 ml of the pipette solution. Tips were dipped in the pipette solution for a few seconds and then backfilled with the amphotericin containing pipette solution. Adequate access to the cell interior was achieved when the access resistance was $\leqslant 20$ MΩ which was usually obtained after 5–10 min after gigaohm seal formation. Patch pipettes were made from borosilicate glass (Clarke, Electromedical Instruments o.d. 1.5 mm, i.d. 0.86 mm) and had resistances of 3–8 MΩ after fire polishing. The patch clamp amplifier was an axopatch 1D (Axon Instruments Inc) and data were stored on a 486 PC after digitization by a digidata 1200 interface (Axon Instruments Inc). Voltage-clamp command paradigms and data analysis were performed using Pclamp 6.20 software (Axon Instruments Inc) and FigP (Biosoft Cambridge, U.K.). In voltage clamp, outward currents were evoked by using voltage ramps and on occasions voltage steps. The voltage was usually ramped from -100 to $+50$ mV for a duration of 600 ms from a holding potential of -70 mV. Leakage current was determined by measuring the mean inward current when the cell was hyperpolarized from the holding potential. Both whole-cell and single-channel currents were filtered at 5 kHz (-3 dB). Single-channel currents were digitized at 10 kHz. Open probability (P_o) was determined from the following relation

$$P_o = \sum_{i=1}^{N} t_i i/(TN)$$

where t_i is the time spent with i = 1,2,.....N channels open and where N is the number of channels, i = unitary current and T is the sample length (30 s). The maximum number of conductance levels (N) was observed under conditions of high P_o (ie at large positive potentials and/or with high [Ca^{2+}]). In some experiments, the effects of changes of membrane voltage on P_o of BK_{Ca} were studied and here the Boltzmann function was used.

$$P_o/P_{max} = (1 + \exp((V - V_{1/2})/k))^{-1}$$

where P_{max} is the maximal P_o, $V_{1/2}$ is the potential at which P_{max} is half-maximal. V is the membrane voltage and k the slope factor (voltage required to produce an e fold change in P_o) is a reflection of the steepness of the voltage dependence. Unitary current amplitude was determined from amplitude histograms fitted with Gaussian functions.

The pipette solutions were frozen in aliquots at -18°C and defrosted and filtered before use (pore size 0.2 μm, Gelman sciences). Liquid junction potentials between the pipette and bath solutions were measured using a 3 M KCl reference electrode and found to be $\leqslant 4$ mV, hence corrections for these potentials were not made. Drugs were applied using a local perfusion pipette which had a diameter of approximately 200 μm. The perfusion pipette was positioned approximately 200–400 μm from the cell/patch under study.

SIN-1 was obtained from Tocris Cookson Ltd (Langford, Bristol, U.K.) and made up as a 10 mM stock solution in degassed water. NO was made up as a saturated solution of 446 μM in degassed Tyrode solution. Either 200 or 300 μl of this stock was injected directly into the culture dish (approx. volume 2 ml) resulting in an approximate concentration of 45 or 67 μM. Owing to the spontaneous breakdown of NO, the exact concentration of NO was not known, but nevertheless, is likely to be somewhat less than these values. ODQ (1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one; an inhibitor of soluble guanylate cyclase) was also obtained from Tocris Cookson Ltd and made up as a 10 mM stock solution in dimethylsulphoxide (DMSO). Iberiotoxin (IBTX), tetraethylammonium chloride (TEA), barium chloride and sodium nitroprusside (SNP) were

all purchased from Sigma. Results are expressed as mean ± s.e.mean unless otherwise stated. Statistical comparisons were made using the Student's paired *t* test, a *P* value <0.05 was considered significant.

Results

Characteristics of BK_{Ca} in excised patches

BK_{Ca} channels in excised patches showed clear activation with intracellular Ca^{2+} (Figure 1a). In symmetrical (140 mM) K^+ solutions, and between ±60 mV, the slope conductance was linear. The average slope conductance was 212±5 pS ($n=12$) and the mean reversal potential was 4.4 mV for channels present in either 1.0 or 10 μM Ca^{2+}. The data points in symmetrical KCl solutions were fitted by linear regression, and a permeability value P_K of 4.3×10^{-13} cm^3 s^{-1} was calculated from the relation

$$I_K = P_K(VF^2[K^+])/(RT)$$

where V is the patch potential, F is the Faraday constant, R is the gas constant and T is the absolute temperature.

The channel permeability value calculated above was used in the constant field (GHK) equation (Goldmann, 1943). The *I*-V relationship in outside-out patches was well fitted using the value of 4.3×10^{-13} cm s^{-1}. In inside-out patches, when 140 mM K^+ in the bath medium was replaced by 5 mM K^+, the *I*-V relationship showed rectification and the reversal potential shifted in a manner that would be predicted from the Nernst relationship for K^+. Under the conditions employed, the exact reversal potential was not determined owing to the poor signal to noise ratio and the large number of channels activated close to this reversal potential. However, the channel amplitude was slightly

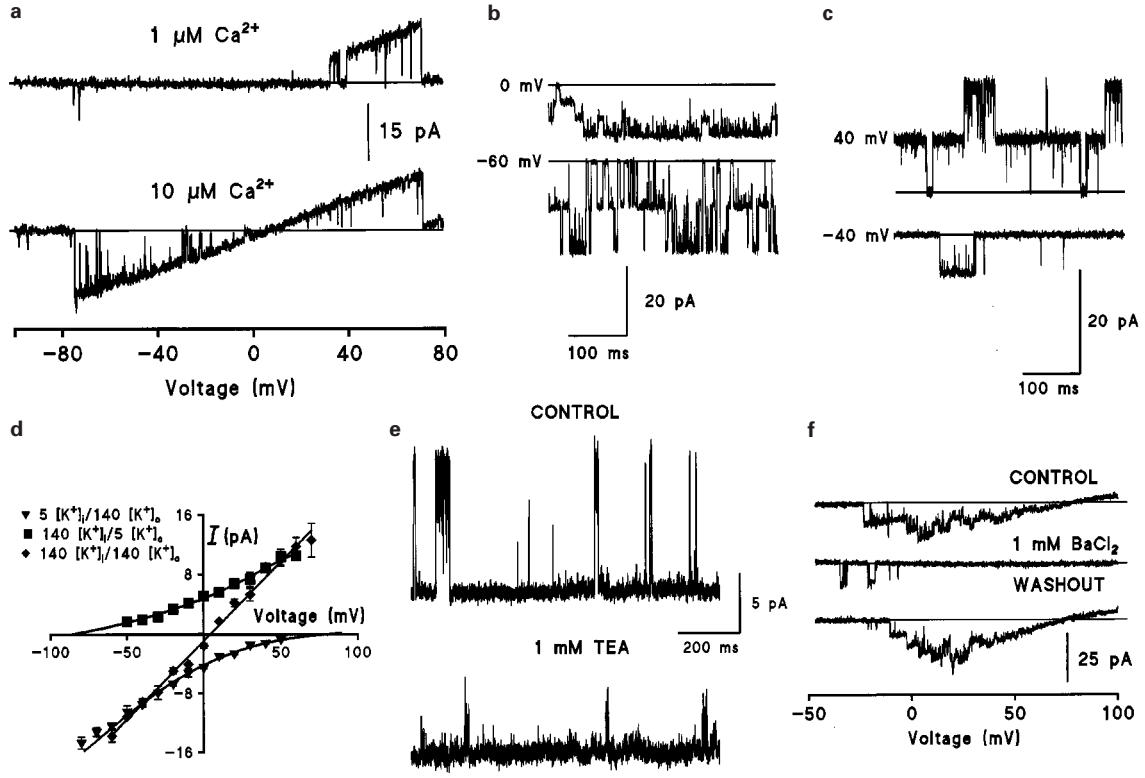

Figure 1 Classification of BK_{Ca} channels in excised patches from rat mesenteric arteries. (a) activation of BK_{Ca} channel by an increase in $[Ca^{2+}]_i$. With 1 μM Ca^{2+}, the BK_{Ca} channel was activated only at positive potentials (top trace). The slope conductance of the channel was 222 pS and the extrapolated reversal potential was 5 mV. Increasing the $[Ca^{2+}]$ to 10 μM stimulated a channel with similar conductance (225 pS; bottom trace). The channel now activated at negative potentials with a reversal potential of 4 mV. The voltage was ramped from −100 to 80 mV over a period of 600 ms and symmetrical (140 mM) K^+ solutions were used. Only a single BK_{Ca} channel was apparent in this inside-out patch. (b) Examples of BK_{Ca} channel activity in an inside-out patch with asymmetrical (140/5 mM) K^+ solutions at patch potentials of 0 and −60 mV. Unitary current amplitude decreased with voltage. However, the open probability increased. (c) BK_{Ca} channel activity in an inside-out patch with symmetrical (140 mM) K^+ solutions at patch potentials of 40 and −40 mV. Unitary currents were outward at 40 mV and inward at −40 mV. Two channels can be seen to be open at 40 mV whereas only one channel was active at −40 mV. The solid horizontal lines represent the closed level. (d) *I*-V relationships for the BK_{Ca} channel. The solid squares show *I*-V data from outside-out patches fitted with the GHK equation using a P_K value of 4.3×10^{-13} cm^3 s^{-1}. The solid diamonds show *I*-V relationships from 12 inside-out patches in symmetrical (140 mM) K^+ solution. Data points were fitted by linear regression and had an average slope conductance of 212±5 pS and a reversal potential of 4.4 mV. The solid triangles show *I*-V relationship from 21 inside-out patches in 140 mM $[K^+]_o$ and 5 mM $[K^+]_i$. These data were fitted with the GHK equation using a P_K value of 3.90×10^{-13} cm^3 s^{-1}. The vertical lines represent s.e.mean. (e) Reduction of unitary current amplitude with 1 mM TEA. The unitary current amplitude was significantly ($P<0.05$) decreased from 10.5 to 3.1 pA in the presence of 1 mM TEA which was applied to the extracellular aspect of an outside-out patch. The patch potential was 60 mV. The reduction in unitary current amplitude by TEA is voltage-dependent (decreasing with increasing potential, Nelson & Quayle, 1995). Hence the reduction in current amplitude at +60 mV may not be as large as that seen at −60 mV. The P_o was somewhat variable ranging from low to high, this was an example of the former. (f) Voltage-dependent block of BK_{Ca} with 1 mM barium applied to the intracellular face of an inside-out patch. The patch was ramped from 100 to −50 mV and several BK_{Ca} channels were activated. With barium, BK_{Ca} channels were inhibited and only brief channel openings occurred at negative potentials. The inhibition was relieved on washout. Asymmetrical (140/5 mM) K^+ solutions were used and the $[Ca^{2+}]$ was 1 μM.

smaller throughout the voltage range compared to similar ionic conditions found in the outside-out patches.

For inside-out patches, a permeability (P_K) value of 4.3×10^{-13} cm^3 s^{-1} did not describe the *I*-V curve well. However, a P_K value of 3.9×10^{-13} cm^3 s^{-1} did adequately fit these data. The reduction in unitary current amplitude observed in inside-out patches may reflect an effect of intracellular [Na^+] on BK_{Ca} as described previously (Singer & Walsh, 1984; Yellen, 1984). Another typical feature of BK_{Ca} channels is that the open probability is voltage-dependent. Examples of unitary current activity in asymmetrical and symmetrical K^+ solutions at different

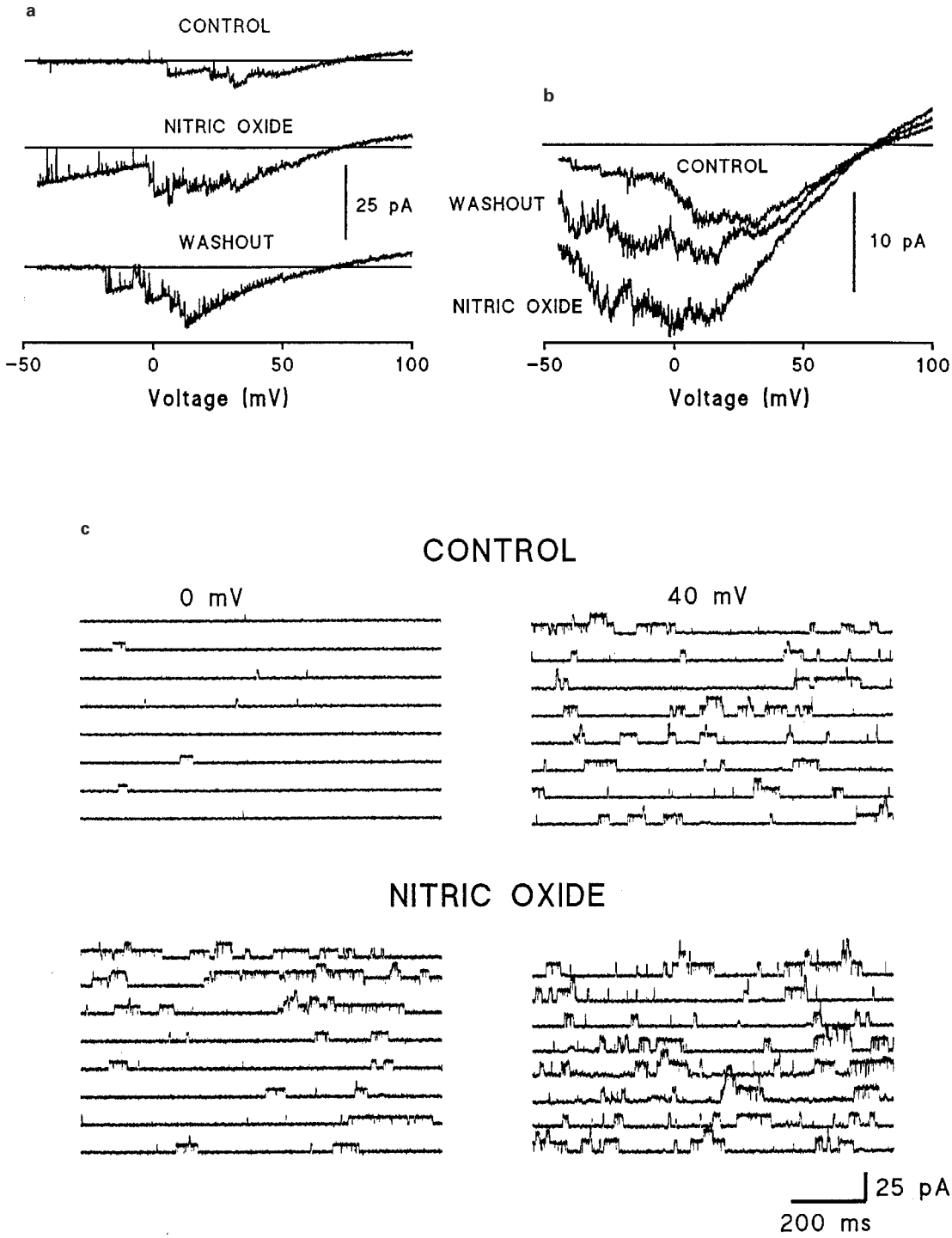

Figure 2 NO-evoked stimulation of BK_{Ca} channels in excised patches. Inside-out patch: (a) the inside-out patch was ramped between 100 and −50 mV. In control conditions, BK_{Ca} channels were primarily activated at positive potentials (voltage-dependent). With NO (approx 67 μM) present, BK_{Ca} channels were now activated at negative potentials and the current was enhanced. The effect of NO was partially reversed on washout. (b). Average current from 12 trials is illustrated. NO evoked a large increase in BK_{Ca} activity which was partly reversible. Asymmetrical (140/5 mM) K^+ solutions were used and the [Ca^{2+}] was 1 μM. Outside-out patch: (c) the top two panels show BK_{Ca} activity at 0 and 40 mV, respectively, P_o at 0 mV was 0.003 and at 40 mV, it increased to 0.158. The bottom two panels show increased activity of BK_{Ca} channels recorded in the presence of NO (approx. 45 μM). The P_o was now 0.22 at 0 mV and 0.27 at +40 mV. The open probability was significantly increased with NO at both 0 and 40 mV ($P < 0.05$).

potentials (displaying the voltage-dependence) and the *I*-V relationships under these different ionic conditions are shown in Figure 1b, c and d, respectively.

External TEA is an effective blocker of BK_{Ca} in vascular smooth muscle ($K_D = 0.2$ mM, Nelson & Quayle, 1995). Application of 1 mM TEA significantly ($P < 0.05$) reduced the

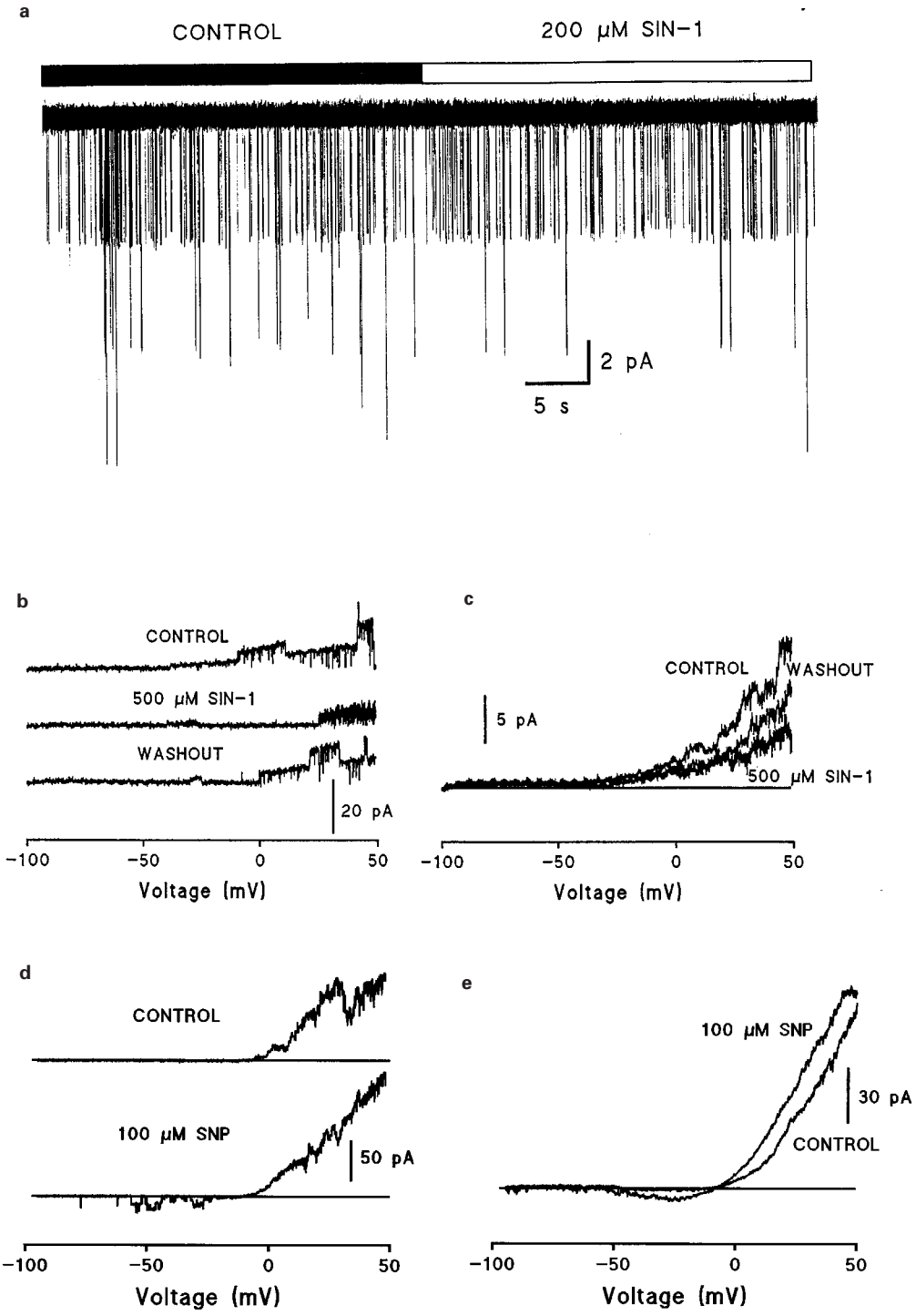

Figure 3 Actions of SIN-1 and SNP on BK_{Ca} channels in excised patches from rat mesenteric smooth muscle cells. (a) Lack of effect of SIN-1 on BK_{Ca} channels. A continuous 60 s record from an inside-out patch containing BK_{Ca} channels in control and in the presence of 200 μM SIN-1. The application of SIN-1 (for 30 s) to the intracellular aspect of the patch had no effect on BK_{Ca} channel activity. The patch potential was 0 mV. Asymmetrical (140/5 mM) K^+ solutions were used and the $[Ca^{2+}]$ was 1 μM. (b and c) Blocking effect of 500 μM SIN-1 on BK_{Ca} channels. (b) The effect of SIN-1 on BK_{Ca} channels in an outside-out patch. The patch was ramped between −100 and 50 mV. BK_{Ca} channels were primarily activated at positive potentials. With SIN-1, BK_{Ca} channels were inhibited and a flickery block occurred. This block with SIN-1 was partially reversed on washout. (c) Average current from 24 trials and the inhibitory action of SIN-1 on BK_{Ca} channels and its partial reversal with washout. (d and e) Effect of 100 μM SNP on BK_{Ca} channels in inside-out patches. In (d) the top trace shows control current from a patch ramped between −100 and 50 mV. BK_{Ca} channels were primarily activated at positive voltages. The bottom trace shows an increase in current at positive voltages and the opening of channels at negative potentials with 100 μM SNP. The panel in (e) shows the average current from 24 trials revealing the increase in BK_{Ca} activity in response to SNP. Solutions used contained symmetrical (140 mM) K^+ and the $[Ca^{2+}]$ was 1 μM. This patch contained multiple BK_{Ca} channels.

unitary current amplitude of BK_{Ca} ($n=4$) without reducing the number of channel openings ($P>0.05$). Barium applied to the intracellular face of inside-out patches has been shown to produce a voltage-dependent block of BK_{Ca} channels in vascular smooth muscle (Benham *et al*., 1985). Barium 1 mM produced a voltage-dependent block (P_o decreased with voltage) of BK_{Ca} in four inside-out patches but had no effect on unitary current amplitude. Figure 1e and f show the reduction in current amplitude with 1 mM TEA and the voltage-dependent block by barium, respectively. In addition, iberiotoxin (50 nM) inhibited the open probability of BK_{Ca} channels in outside-out patches, also without affecting unitary current amplitude ($n=2$ patches, data not shown).

Effect of nitric oxide (NO) and nitric oxide donors in excised patches

A direct effect of NO and its donors on BK_{Ca} channels was investigated using excised patches. In 5 out of 6 inside-out patches, NO (45 or 67 μM) increased the activity of BK_{Ca}. This action of NO on BK_{Ca} was partly reversible upon washout. Figure 2 shows the stimulant effect of NO on BK_{Ca} channels in inside-out patches. Figure 2a shows unitary current activity with a single voltage ramp ($100>-50$ mV), while Figure 2b shows the mean current from 12 trials. The unitary current amplitude of BK_{Ca} channels was unaffected by NO. Moreover, NO failed to activate any activity in patches that did not contain BK_{Ca} channels (ie silent patches, $n=3$). The increase in BK_{Ca} activity described with NO was variable ranging from 25–1018%, with a mean enhancement of 432 ± 225% at -40 mV ($n=5$). BK_{Ca} activity was also stimulated by NO in outside-out patches. Figure 2c shows this effect of NO on BK_{Ca} channels in an outside-out patch at 0 and 40 mV. In one other outside-out patch, NO, produced a significant ($P<0.05$) increase in P_o of BK_{Ca}.

In seven out of eleven inside-out patches, application of SIN-1 (100–200 μM) to the intracellular aspect of the patch did not enhance BK_{Ca} activity. In the other 4 patches, only a small increase (20 ± 3.6%, $P>0.05$) in BK_{Ca} activity was observed. The lack of effect of 200 μM SIN-1 on BK_{Ca} channels at 0 mV in one inside-out patch is shown in Figure 3a. Similarly, in four outside-out patches, BK_{Ca} channel activity was not affected by 100 μM SIN-1. However, at higher (500 μM) concentrations, SIN-1 had an inhibitory (blocking) action on BK_{Ca} channels ($n=3$ patches, see Figure 3b and c). The inhibitory effect of SIN-1 appeared to resemble a form of ion-channel block, but this was not investigated further. The application of SNP also activated BK_{Ca} channels in 3 out of 8 inside-out patches. Figure 3d and e illustrates the increase in BK_{Ca} activity when SNP was applied to the intracellular face of an inside-out patch. The average increase in BK_{Ca} activity with 100 μM SNP was 23 ± 4.2% ($n=3$, $P<0.05$) at 40 mV.

Perforated patch recording

Authentic NO (45 or 67 μM) applied to single cells evoked an increase in the outward current of 256.6 ± 56% ($n=6$). Generally, the effect of NO was rapid and partially reversible. The action of NO on outward current is illustrated in Figure 4a. In two separate cells, the application of 20 μM ODQ (a soluble guanylate cyclase inhibitor) together with NO failed to prevent an increase in the outward current (Figure 4b). ODQ (20 μM) alone had no effect on whole-cell outward K^+ currents (mean increase of 3.6 ± 1.1% $n=5$, $P>0.05$) or single BK_{Ca} channels in outside-out patches ($n=2$, data not shown).

In eight out of ten cells, an increase in outward K^+ current followed the application of SIN-1. The average increase with 100 μM SIN-1 was 112 ± 36.9% ($n=8$) at 40 mV. Figure 5a shows the stimulant effect of SIN-1 on outward currents evoked by a voltage ramp. SNP 100 μM also increased outward current by 52.9 ± 18.7% ($n=3$, data not shown). The increase in the outward current was attributed to the activation of large conductance Ca^{2+}-activated K^+ channels, as it was abolished in the presence of 50 nM iberiotoxin (IBTX). Outward currents were evoked by voltage steps from -60 mV in 10 mV increments to 70 mV for a duration of 220 ms. These outward currents were noisy, especially at large positive potentials. Figure 5b shows the *I*-V relationship from a typical cell. The inset depicts the noisy outward current at a test potential of 40 mV. In the presence of IBTX, the outward current was reduced and no enhancement of the current was observed with the subsequent application of SIN-1 (100 μM). Overall, SIN-1 (100 μM) increased the current by only 1.1 ± 13.4% ($P>0.05$) in the presence of IBTX in eight cells.

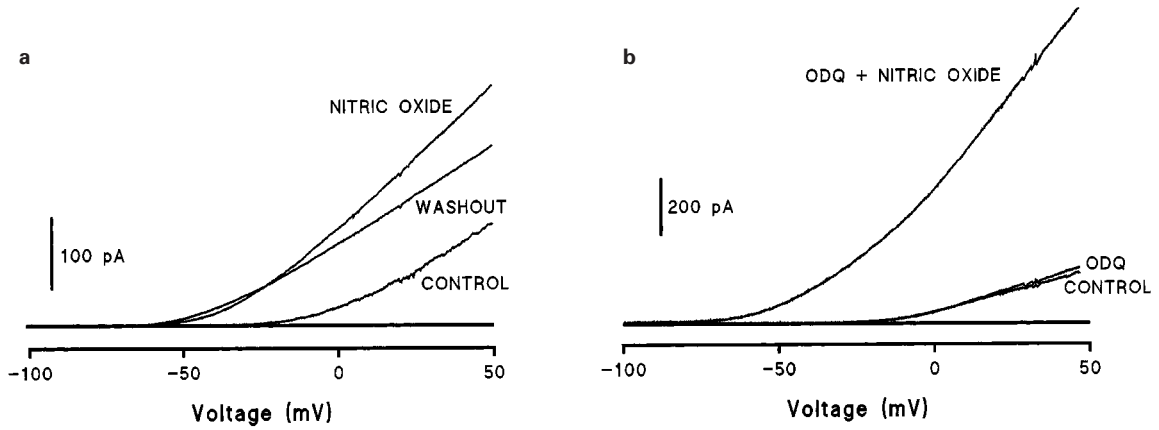

Figure 4 Effect of NO on whole-cell currents evoked by voltage ramps using the perforated patch method. (a) Three traces showing whole-cell control currents, currents in the presence of NO (approx. 65 μM) and after washout. Outward currents were evoked by ramping the cell between -100 and 50 mV. NO produced a large increase in the outward current which was partially reversed on washout. (b) Three traces showing whole-cell control currents, currents with 20 μM ODQ present and in the presence of both 20 μM ODQ and NO (approx. 65 μM). ODQ on its own had little effect on whole-cell currents and did not prevent the increase in outward current evoked by NO. Outward currents were evoked by ramping the cell between -100 and 50 mV.

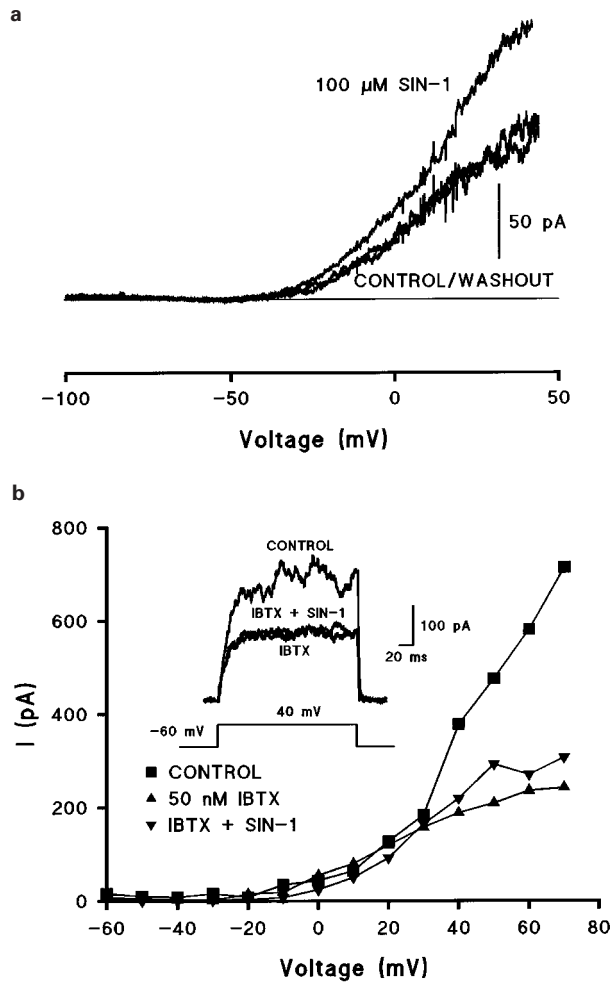

Figure 5 Effect of 100 μM SIN-1 on whole-cell currents in perforated patches. (a) Whole-cell currents evoked by the voltage ramp before, during and after 100 μM SIN-1. Outward current was stimulated in the presence of SIN-1, an effect which was fully reversible. (b) The *I*-V relationship in a cell held at −60 mV and stepped to 70 mV in increments of 10 mV for 200 ms. The peak currents in control solution, in the presence of 50 nM IBTX and in the presence of 50 nM IBTX and 100 μM SIN-1 are shown. The inset depicts control currents, and currents with either IBTX or IBTX with 100 μM SIN-1 present at a test potential of 40 mV. In the presence of IBTX, SIN-1 failed to enhance the outward current.

Conventional whole-cell recording and cell-attached patches

With the conventional whole-cell voltage clamp technique, responses to SIN-1 (50–300 μM) were found to be much more variable than in the perforated patch experiments. The majority of cells (11 out of 18) showed no increase in outward K^+ current upon the addition of SIN-1. In the remaining cells, the application of SIN-1, produced a modest increase in the outward current (measured at +40 mV) of $38.7 \pm 10.2\%$ ($P < 0.05$). In these cells, two showed full reversal upon washout, whereas in others, no reversal was obtained. In two other cells, the outward current increased in size following washout of SIN-1. SIN-1 increased the outward current and this effect was most evident at positive voltages. It seems unlikely that the increase in the outward current was mediated by ATP-dependent K^+ channels (K_{ATP}), since 2 mM ATP was included in the patch pipette solution which would be sufficient to inhibit the opening of K_{ATP}. The outward current was primarily carried by K^+ channels, since TEA (2 mM) reduced the current by $77.4 \pm 2.9\%$ at 40 mV ($n = 3$, data not shown).

The abolition of SIN-1-evoked whole cell outward current in the presence of IBTX, provided further evidence that the channel activated by NO was BK_{Ca}. This was further investigated using cell-attached patches, with the cells continuously bathed in Tyrode solution. In most patches (24 out of 30), unitary currents were absent, even with patch depolarization. Unitary currents were observed at the resting potential (pipette potential of 0 mV) and channel activity increased with depolarization, while the current amplitude decreased ($n = 6$ patches). From these six patches, the average slope conductance was 111.6 ± 13.8 pS and the mean reversal (pipette) potential was -43.4 ± 3.2 mV. The open probability (P_o) was determined at different pipette potentials, and the activation curve fitted with Boltzmann functions to derive the parameters $V_{1/2}$ and k. The values of $V_{1/2}$ and k were found to vary considerably between patches. For example, in one patch for a 141 pS channel, $V_{1/2}$ was 42 mV and k was 8.2 mV, whilst, from another patch, a 148 pS channel had a $V_{1/2}$ of −48 mV and k was 24.4 mV.

The application of SIN-1 to cells either had (a) no effect on a previously quiescent patch ($n = 6$ patches), (b) activated a voltage-dependent ion channel with a slope conductance of 139 pS (patch was previously silent) or (c) increased the open probability of channels already present in the patch which had a mean slope conductance of 120.7 ± 3.5 pS ($n = 4$) and an extrapolated reversal potential of -39.7 ± 3.5 mV ($n = 4$). The open probability in each of these four cell-attached patches was significantly enhanced by the application of SIN-1 ($P < 0.05$). For example, in one patch, open probability at 0 mV (resting potential) increased from 0.048 ± 0.004 to 0.278 ± 0.090 with SIN-1. The effect of SIN-1 in either activating this channel or increasing the activity of channels already present in the patch was delayed (up to a few minutes) and was not readily reversible upon washout.

The actual patch potential is given by the following relationship

$$V_p = V_m - V_{pip}$$

where V_p = patch potential, V_m = membrane potential and V_{pip} = pipette potential. The average reversal potential (pipette potential) from unitary currents observed in cell-attached patches with or without SIN-1 was -47.3 ± 4.2 mV ($n = 11$). The average resting membrane potential obtained from perforated patch recordings was -45.6 ± 3.6 mV ($n = 5$).

From the above relationship, the patch potential at which reversal occurred was close to 0 mV. The cell has a high $[K^+]$ and with the high $[K^+]$ present in the patch pipette E_K would be expected to be close to 0 mV. This suggests that these channels are K^+-selective. In one other cell-attached patch, the application of 10 mM caffeine to the cell activated a 140 pS channel which had a reversal (pipette) potential of −38 mV (data not shown) indicating that a large conductance K^+ channel was activated by a rise in $[Ca^{2+}]_i$.

Discussion

The results described in this study suggest that NO and NO donors can each directly activate large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) in rat mesenteric artery smooth muscle cells. The channels had a large conductance, they were Ca^{2+}-dependent and showed K^+ selectivity all characteristics of BK_{Ca} channels. In addition, the unitary current amplitude in excised patches was greatly reduced by 1 mM TEA, which has

a K_D value around 0.2 mM for BK_{Ca} channels in vascular smooth muscle. This is in contrast to the delayed rectifier (K_V) channels where the K_D for TEA is around 10 mM (Nelson & Quayle, 1995). A voltage-dependent block by internally applied barium (1 mM) was also observed, similar to that reported for BK_{Ca} channels in rat renal arteries (Gebremedhin *et al*., 1996).

In the whole-cell configuration, responses to NO were blocked with iberiotoxin, which is a selective inhibitor of BK_{Ca} ($K_D < 10$ nM; Nelson & Quayle, 1995) and at the concentration used (50 nM) should not affect K_v. Any stimulation of K_v by NO could have been observed if present, but no increase in outward current occurred under these conditions. TEA (at 2 mM selective for BK_{Ca}) also produced a large block (approx. 77%) of the outward current again supporting the suggestion that the majority of the outward current was carried by BK_{Ca} in these experiments (*cf* Yuan *et al*., 1996). The channel present in cell-attached patches had a large conductance, showed voltage-dependence and a reversal potential close to E_K. The application of SIN-1 or caffeine each activated a similar large conductance channel which also reversed close to E_K. These data all support the suggestion that a calcium-dependent potassium channel, probably BK_{Ca} was present in the mesenteric cells and could be activated by NO.

The conductance increase was unlikely to reflect the activation of K_{ATP} channels, since it is known that in rat mesenteric arteries these channels are only revealed when the cell is metabolically inhibited, and only display a small (20 pS) conductance which is not voltage-dependent (Zhang & Bolton, 1995). In that study, a larger (around 150 pS) voltage-dependent K^+ channel was also described and defined as BK_{Ca}. BK_{Ca} channels with relatively low conductance have also been observed in cell-attached patches in rabbit vascular myocytes. The low relative conductance may reflect a blocking action due to intracellular [Mg^{2+}] (Morales *et al*., 1996). However, the overall channel characteristics were very similar to the channel we describe here.

Smooth muscle BK_{Ca} channels have previously been shown to be stimulated by NO in a number of vascular and non-vascular preparations. Although this action of NO reflects a modulation of BK_{Ca} by cyclic GMP-dependent protein kinases (Robertson *et al*., 1993; Zhou *et al*., 1996; Sansom *et al*., 1997), evidence now indicates that NO can also act directly to modulate BK_{Ca} (Bolotina *et al*., 1994) or act through a combination of both of these mechanisms (Peng *et al*., 1996).

The fact that the selective inhibitor of guanylyl cyclase, ODQ, did not prevent the increase in the whole-cell outward current evoked by NO in perforated patch recordings, shows that NO can directly activate BK_{Ca} in the mesenteric artery. But on comparing data from the whole-cell experiments, it is clear that the percentage of cells responding to NO is lower with conventional whole-cell recording compared to the perforated patch. One possible explanation is that soluble guanylyl cyclase may have dialyzed out of the cell in the former. In that case, the actions of NO in the intact mesenteric artery might also have a cyclic GMP-dependent component.

It is not clear whether the direct (and possibly an indirect effect via cyclic GMP) action of NO occurs on all BK_{Ca} channels or whether separate isoforms of BK_{Ca} might be differentially modulated. BK_{Ca} channels are composed of two dissimilar subunits, α (pore forming) and β (modulatory). Injection of the α subunits alone into *Xenopus* oocytes can produce functional BK_{Ca} channels. However, injection of both α and β subunits, produced BK_{Ca} channels which were more sensitive to both voltage and calcium than BK_{Ca} channels formed by α subunits alone (McManus *et al*., 1995). Two different isoforms of BK_{Ca} with quite different calcium sensitivities have been described in bovine mesenteric arteries (Sansom & Stockand, 1994). This finding coupled with a recent study (Tanaka *et al*., 1997) has led to the suggestion that different subtypes (isoforms) of BK_{Ca} may exist within a particular cell type. (Sansom & Stockand, 1994; Tanaka *et al*., 1997). Alternatively or additionally the distribution of channels may vary between cells within the artery wall (Archer *et al*., 1996; Michelakis *et al*., 1997). Three different types of smooth muscle cells have been described in rat pulmonary arteries based on their respective sensitivity to blockers and the density of K_{Ca}/K_v channels (Archer *et al*., 1996). So the pulmonary artery consists of a mosaic of cells that have different K^+ channel subtypes. These confer upon cells a differential ability to respond to agents such as NO (Michelakis *et al*., 1997). Therefore, a diversity of cell function might underlie the overall responses to vasodilators/vasoconstrictors within or between vascular beds. The variability in the responses to NO may reside ultimately in the density/expression of BK_{Ca} channels in rat mesenteric smooth muscle cells, so that electrophysiological diversity might be a major determinant of differences in vascular reactivity.

The precise mechanism by which NO directly activates BK_{Ca} in the mesenteric artery will require further investigation. However, the mechanisms may be the same as that described by Bolotina *et al*. (1994). In this study, the direct effect of NO on BK_{Ca} was prevented by N-ethylmaleimide (NEM) which modifies protein sulphydryl groups so that they cannot be nitrosylated. NO-induced inhibition of the NMDA receptor has also been shown to involve reactions with membrane bound thiol groups on the NMDA receptor-channel complex independently of cyclic GMP (Lei *et al*., 1992). These authors suggested that the redox state/location of the channel/protein may govern the extent of NO modulation. As well as modulating the activity of BK_{Ca} in cultured vascular smooth muscle cells (Bolotina *et al*., 1994), NO has also been shown to modulate directly both delayed rectifier (K_V) and BK_{Ca} channels in rat pulmonary arteries (Yuan *et al*., 1996; Zhao *et al*., 1997) and rat brain BK_{Ca} channels reconstituted into lipid bilayers (Shin *et al*., 1997).

Although the mechanism(s) by which NO stimulates BK_{Ca} is not clear from our experiments, it probably does not include a change in channel conductance. The maximum current amplitude and the threshold of current activation were displaced towards more negative potentials by NO (Figures 2a,b and 5a,b). The enhancement was very variable (25–1018%), and did not appear to reflect a change in permeability as the reversal potential and conductance of spontaneous channels and those activated by SIN-1 were similar. Also, the effect of NO was consistent with a leftward shift of the activation curve. Such shifts are thought to reflect a sudden change in N (Singer & Walsh, 1987), hence the predominant effect of NO and its donors would be to increase the number of active BK_{Ca} channels. Out data suggest that NO may act like an increase in $[Ca^{2+}]_i$ or the BK channel opener NS 1619 (Edwards *et al*., 1994). Both these agents shift the activation curve to the left without altering the slope (k).

One potential problem associated with using NO donors such as SIN-1 and SNP is that they release other by-products which also have the ability to interact with and possibly influence channel activity. SIN-1 generates both NO and O_2^- (superoxide), free radicals which can combine with NO to produce peroxynitrite ($OONO^-$) (Feelish & Stamler, 1996). The peroxynitrite group may oxidize thiol groups or interact with residues within the channel pore and cause inhibition of

BK_{Ca} (see Figure 4b and c). Both inhibition (by NO) and stimulation (by $OONO^-$) of voltage-dependent Ca^{2+} channels from ferret heart muscle have been described with NO donors such as SIN-1 (Campbell *et al*., 1996). Free radical formation might explain the inhibitory action on BK_{Ca} which we observed with high concentrations of SIN-1 in outside-out patches.

Our data from freshly isolated cells from the rat mesenteric artery indicate that NO can directly stimulate BK_{Ca} channels independently of any effect through cyclic GMP. These observations support our functional studies in the same artery, which suggest that the major relaxation mechanism evoked by NO (SIN-1) is via a cyclic GMP-independent activation of potassium channels (Plane *et al*., 1996). In that study, NO-evoked relaxation was not modified by the guanylyl cyclase inhibitor ODQ, but was abolished in the presence of the potassium channel blocker charybdotoxin. In some way, the basal release of NO seems to down-regulate the cyclic GMP-dependent pathway for relaxation, as inhibition of basal NO release either with NO synthase inhibitors or by removal of the endothelium, alters this profile. In the absence of basal NO, relaxation was resistant to either charybdotoxin or ODQ alone, but totally abolished by these agents in combination (Plane *et al*., 1996). Interestingly, a similar influence of basal NO does not appear to operate to this extent in a larger artery, the carotid. In this vessel, ODQ alone did significantly reduce relaxation to NO, although a combination of ODQ and charybdotoxin was still required for complete inhibition of relaxation (Plane *et al*., 1998), This indicates that different mechanisms for smooth muscle relaxation to NO predominate in different parts of the circulation.

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