

Sodium (2-sulfonatoethyl) methanethiosulfonate prevents *S*-nitroso-L-cysteine activation of Ca^{2+} -activated K^+ (BK_{Ca}) channels in myocytes of the guinea-pig taenia caeca

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1 The modulation of large conductance Ca^{2+} -activated K^+ (BK_{Ca}) channels by the nitric oxide (NO^{\cdot}) donor, *S*-nitroso-L-cysteine (NOCys) and three sulfhydryl-specific methanethiosulfonate (MTS) reagents, positively charged 2-aminoethyl MTS hydrobromide (MTSEA $\text{C}_3\text{H}_9\text{NO}_2\text{S}_2\text{HBr}$) and [2-(trimethylammonium) ethyl MTS bromide (MTSET $\text{C}_6\text{H}_{16}\text{NO}_2\text{S}_2\text{Br}$), and negatively charged sodium (2-sulfonatoethyl) MTS (MTSES $\text{C}_3\text{H}_7\text{O}_5\text{S}_3\text{Na}$) were compared in excised inside-out membrane patches of the guinea-pig taenia caeca.

2 In membrane patches bathed in a low Ca^{2+} (15 nM) high K^+ physiological salt solution, 1–3 BK_{Ca} channels opened with a low probability ($N.P_o$) of 0.019 ± 0.011 at 0 mV. $N.P_o$ readily increased with membrane depolarization, raised Ca^{2+} concentration or upon the addition of NOCys (10 μM for 2–5 min) such that 5–15 open BK_{Ca} channels were evident.

3 MTSEA (2.5 mM) decreased, while MTSES (2.5 mM) increased $N.P_o$ (at 0 mV) and the number of open BK_{Ca} channels at positive potentials. These changes in channel activity remained after a prolonged washout of these two MTS reagents.

4 MTSET (2.5 mM) increased $N.P_o$ (at 0 mV) and voltage-dependently decreased BK_{Ca} current amplitudes in a manner readily reversed upon washout.

5 Pre-exposure of excised membrane patches to MTSES or *N*-ethylmaleimide (NEM 1 mM), a specific alkylating agent of cysteine sulfhydryls, but not MTSEA or MTSET, prevented the excitatory actions of NOCys (10 μM).

6 It was concluded that NOCys-evoked increases in BK_{Ca} channel activity occur *via* the *S*-nitrosylation of cysteine residues within basic regions of the channel α subunit that have an accessible water interface.

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Abbreviations: BK_{Ca} , large conductance Ca^{2+} -activated K^+ ; *I*–*V*, current-voltage; DTT, dithiothreitol; MTS, methanethiosulfonate; MTSEA, 2-aminoethyl MTS hydrobromide; MTSET, [2-(trimethylammonium) ethyl MTS bromide; MTSES, sodium (2-sulfonatoethyl) MTS; Na-HEPES, sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; NOCys, *S*-nitroso-L-cysteine; NEM, *N*-ethylmaleimide; $N.P_o$, probability of opening; NO^{\cdot} , nitric oxide; NO^+ , nitrosonium; ODQ, 1H-[1,2,4] oxadiazolo [4,3-*a*] quinoxalin-1-one; PSS, physiological salt solution; RSH, sulfhydryl; RSNO, *S*-nitrosothiol; R-S-S-R, disulfide

Introduction

Large conductance Ca^{2+} -activated K^+ (BK_{Ca}) channels are abundant in most smooth muscles and are generally thought to be involved in controlling the electrical excitability of the membrane potential that regulates voltage-activated Ca^{2+} entry and muscle contraction. Although the gating of smooth muscle and neuronal BK_{Ca} channels is under the control of the membrane potential, the internal concentration of free Ca^{2+} and the phosphorylation state of the channel protein, both native (Bolotina *et al.*, 1994; Park *et al.*, 1995; Thuringer & Findlay, 1997; Mistry & Garland, 1998; Ahern *et al.*, 1999; Gong *et al.*, 2000; Lang *et al.*, 2000) and cloned α subunits (DiChiara & Reinhart, 1997; Jeong *et al.*, 2001; Tang *et al.*, 2001; Chung *et al.*, 2002) of BK_{Ca} channels can be directly

modulated by the cell redox potential. Oxidation of sulfhydryl (thiol or RSH)-containing cysteine residues, particularly in the presence of trace amounts of metal ions, leads to the formation of disulfides (R-S-S-R) that are physiologically reduced back to thiols by endogenous redox agents such as glutathione. It is likely that there are multiple sites of redox modulation as oxidizing agents have been reported to both increase (Park *et al.*, 1995; Thuringer & Findlay, 1997) and decrease (Park *et al.*, 1995; DiChiara & Reinhart, 1997; Wang *et al.*, 1997; Tang *et al.*, 2001) the probability of the opening of BK_{Ca} channels. Moreover, these sites of redox modulation are on cysteine residues, as *N*-ethylmaleimide (NEM), a specific alkylating agent of free sulfhydryls on cysteine residues, prevents the action of the oxidizing agents, thimerosal and diamide (Wang *et al.*, 1997). These reactive cysteine groups are not on the extracellular surface or within the pore of the BK_{Ca}

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channel since membrane-impermeant oxidizing agents are only effective when applied to the cytosolic surface of excised membrane patches and neither the oxidation nor the reduction of the BK_{Ca} channel protein has any effect on the unit conductance of the BK_{Ca} channels (Wang *et al.*, 1997; Lang *et al.*, 2000).

Nitric oxide (NO[·]) or reactive nitrogen species formed by NO[·] released from intestinal inhibitory motor nerves and vascular endothelial cells have been demonstrated *in vitro* to increase the opening of large (BK_{Ca}), intermediate and small conductance Ca²⁺-activated K⁺ channels (Bolotina *et al.*, 1994; Koh *et al.*, 1995). Applied NO[·] donors increase the opening of native and cloned smooth muscle BK_{Ca} (Koh *et al.*, 1995; Fukao *et al.*, 1999) and TREK-1 (Koh *et al.*, 2001) channels indirectly *via* mechanisms involving the activation of cytosolic guanylyl cyclases and channel phosphorylation. However, a direct NO[·] donor activation of BK_{Ca} channels in excised membrane patches of vascular and intestinal smooth muscle in the presence of specific guanylyl cyclase blockers such as 1H-[1,2,4] oxidiazolo [4,3-*a*] quinoxalin-1-one (ODQ) has also been demonstrated (Bolotina *et al.*, 1994; Mistry & Garland, 1998; Lang *et al.*, 2000). This activation has been suggested to involve the binding of NO[·], or an oxidized derivative, NO⁺ or N₂O₃, to thiols to form *S*-nitrosothiols (RSNO) that may well lead to disulfide formation if another reduced thiol is in close proximity (Abderrahmane *et al.*, 1998; Lang *et al.*, 2000).

The purpose of this study was to examine pharmacologically which cysteine residues within the BK_{Ca} channel protein(s) in excised membrane patches of the guinea-pig taenia caeca are likely candidates for *S*-nitrosylation by the NO[·] donor, *S*-nitroso-L-cysteine (NOCys). We have used three methanethio-sulfonate (MTS) reagents, 2-aminoethyl MTS hydrobromide (MTSEA), [2-(triethylammonium) ethyl] MTS bromide (MTSET) and sodium (2-sulfonatoethyl) MTS (MTSES), which specifically convert sulfhydryls to disulfides in manner that is only reversible upon exposure to thiols such as dithiothreitol (DTT) (Akabas *et al.*, 1992; Kuner *et al.*, 1996; Karlin & Akabas, 1998). We have examined whether the irreversible alkanethiolation of cysteine thiols by MTS reagents can also interrupt the excitatory actions of NOCys. Any action of these MTS reagents on the gating of smooth muscle BK_{Ca} channels or the excitatory effects of NOCys has been interpreted in terms of accessibility to cysteine residues with respect to the volume and net charge of the surrounding water-accessible protein surface (Lang & Harvey, 2002). We have assumed that membrane-permeable MTSEA will have the greatest access to cysteine thiols within both hydrophobic and hydrophilic regions of the subunits of the BK_{Ca} channel protein. In contrast, MTSET and MTSES will have fairly equal access to water-facing cysteines of the BK_{Ca} channel, being restricted only by the charge of the neighboring protein region. Some of these results have been published previously in brief (Lang & Harvey, 2002).

Methods

Single-cell preparation

Guinea-pigs were killed by stunning and exsanguination, the cecum was exposed through an abdominal incision and one of

the taenia caeca dissected free. All experiments were carried using procedures approved by the Physiological Department Animal Ethics Committee at Monash University. Single smooth muscle myocytes were enzymatically dispersed from 5 mm strips of guinea-pig taenia caeca in a low-Ca²⁺ (50 μM) physiological salt solution (PSS) (see below) with added collagenase (0.5 mg ml⁻¹, Worthington, Sciman), trypsin inhibitor (0.5 mg ml⁻¹, Sigma) and bovine serum albumin (2 mg ml⁻¹, Sigma) at 37°C (see Lang *et al.*, 2000). Single cells were either stored at 4°C or plated onto the glass base of a recording chamber mounted (0.1–0.2 ml) on an inverted microscope (Olympus CK2). Cells were allowed 5–10 min to settle and then perfused (3 ml min⁻¹) for 2–5 min with a normal-Ca²⁺ (1.5 mM) containing PSS (see below) prior to commencing patch-clamp experiments.

Solutions

Cells were dispersed in a low-Ca²⁺ PSS containing (mM): NaCl 126, KCl 6, sodium *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid (Na-HEPES) 10, MgCl₂ 1, D-glucose 10, EGTA 0.3 and CaCl₂ 50 μM. The pH of the solution was adjusted to 7.4 with 5 M NaOH. Following dispersion, the Ca²⁺ concentration was increased to 1.5 mM before cell storage or experimentation. The patch pipette usually contained normal Ca²⁺ (1.5 mM) PSS, the presence or absence of this concentration Ca²⁺ in the pipette solution had no effect on the NO[·]-dependent activation of the BK_{Ca} channels (Lang & Watson, 1998; Lang *et al.*, 2000). After the formation of a seal (> 2 GΩ) between the pipette and the smooth muscle membrane, cells were bathed in a high K⁺, low Ca²⁺ PSS containing (mM): KCl 130, Na-HEPES 10, MgCl₂ 1, D-glucose 10, EGTA 0.3 and CaCl₂ 50 μM, which gives a computed free Ca²⁺ concentration of 15 nM (CABUFFER). The solution was adjusted to 7.4 with 5 M KOH. Inside-out patches were then excised into this high K⁺ PSS, establishing a near-physiological Ca²⁺ and K⁺ (6 mM [K]_o, 1.5 mM [Ca]_o; 130 mM [K]_i, 15 nM [Ca]_i) gradient across the membrane patch. All recordings were made at room temperature (22–25°C).

Single channel recording and analysis

Patch pipettes were drawn from glass capillary tubing (1.5–1.8 mm) (Clarke Electromedical Inst.) on a micropipette puller (Sachs-Flaming PC-84, Sutter Instruments) and pipette tips were heat polished (MF-84 Narishige). Pipette resistances ranged between 4 and 10 MΩ. Command potentials were delivered and channel currents recorded using an Axopatch 200 amplifier (3-dB cutoff frequency 1 kHz, 4-pole Bessel filter) (Axon Instruments). Patch potentials and channel currents were stored on videotape via a Digital Data Recorder VR10B (Instrutech Corp.) and later digitized (at 2 kHz) onto a computer hard disc for analysis using a DigiData 1200 Interface (Axon Instruments) and pClamp 6 software (Axon Instruments). Channel openings were recorded for 30–120 s at holding patch potentials between –80 and +80 mV. Patches were also subjected to a ramped depolarization (between –80 and +80 mV over 1 s) from a holding potential between 0 and +40 mV. These ramp-evoked channel currents (*I*) were then plotted against the patch potential (*V*) to give 'instantaneous' current–voltage (*I*–*V*) plots, which were used to follow any time-dependent changes in BK_{Ca} channel activity.

BK_{Ca} channel current levels at each holding potential were first plotted as an all-points amplitude histogram. Closed and opened levels were fitted with single Gaussian distributions (using a simplex least-squares algorithm), the difference between the mean of each Gaussian giving an estimate of the amplitude (I) (pA) of the BK_{Ca} channel currents at each patch potential (V) (mV). 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 by the total observation time. The averaged open probability for N channels ($N.P_o$) was therefore

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

To establish the Ca²⁺ sensitivity of the our BK_{Ca} channels after exposure to NEM (1 mM for 5 min), average currents (I) arising from the opening of multiple BK_{Ca} channels were recorded in excised macropatches using a repetitive ($\times 10$) multi-stepped depolarization protocol to potentials between -60 and $+90$ mV from a holding potential of -80 mV. Averaged current (I), conductance ($g = I/(V - E_R)$) (using a BK_{Ca} channel reversal potential (E_R) of -70 mV) (Lang *et al.*, 2000) and normalized conductance (g/g_{Max}) values were calculated and plotted against V . Activation curves were fitted with Boltzmann equations to obtain a measure of the voltage of half-maximal activation ($V_{0.5}$) and the slope, K . The maximum conductance (between $+60$ and $+90$ mV) of the individual macropatches bathed in 15, 150 and 300 nM Ca²⁺ was normalized as a fraction of the conductance obtained in 1.5 μM Ca²⁺ (y), plotted against the Ca²⁺ concentration (x) and fitted to the Hill equation

$$y = y_0 + x^n(x^n + C^n)^{-1}$$

where C is the half-maximal concentration of Ca²⁺ and n is the Hill coefficient.

Data were expressed as the mean \pm s.e.m N indicates the number of experiments. Statistical significance was determined using Student's t -test, $P < 0.05$ was considered to be significantly different.

Drugs

Stock solutions of *S*-nitroso-L-cysteine (NOCys) (100 mM) were prepared as previously described (Lang *et al.*, 2000). *N*-ethylmaleimide (NEM) and the methanethiosulfonate (MTS) reagents, 2-aminoethyl MTS hydrobromide (MTSEA), [2-(triethylammonium)ethyl] MTS bromide (MTSET) and sodium (2-sulfonatoethyl)MTS (MTSES) (Toronto Research Chemicals Inc.) were dissolved in distilled water just prior to experimentation and stored at 4°C. The infusion of the vehicle for NOCys had no effect on BK_{Ca} channel activity (Lang *et al.*, 2000). All stock solutions were diluted with PSS and applied as a bolus (1–100 μl) to the bath solution (volume of 0.2 ml); the concentrations indicated are the calculated final concentrations.

Exposures to the MTS reagents were mostly performed at 0 mV and for only relatively short periods (2–5 min) to maximize the reaction of the charged MTS reagents with ionized thiols ($-S^-$) on cysteines at a water-accessible surface. This protocol would also minimize any influences of the electrical field on the permeation of the MTS reagents as well

as any nonspecific interactions of these MTS reagents with other unionized thiols on imbedded or lipid-accessible cysteines (Karlin & Akabas, 1998).

Results

Effects of NOCys

Single BK_{Ca} channel openings were seldom recorded at potentials < 0 mV in excised membrane patches exposed to the physiological K⁺ gradient ($[K]_o$ 6 mM; $[K]_i$ 130 mM) and a cytosolic Ca²⁺ concentration of 15 nM. In one set of 11 experiments, BK_{Ca} channel currents at 0 mV were 7.4 ± 0.3 pA (range 6.6–8.3 pA) in amplitude and opened with a probability ($N.P_o$) of 0.019 ± 0.011 (range 0.001–0.053). Both current amplitude and $N.P_o$ of these BK_{Ca} channels increased with either maintained or ramped (Figure 1bi) depolarization of the membrane potential to positive potentials such that three to five simultaneously opening channels were evident (Lang & Watson, 1998; Lang *et al.*, 2000).

The application of the relatively unstable *S*-nitrosothiol compound, *S*-nitroso-L-cysteine (NOCys 10 μM , with a half-life of 80 s in PSS) to the cytosolic surface of the excised membrane patches increased BK_{Ca} channel activity after a delay of about 60 s (Figure 1a) (Lang *et al.*, 2000). In nine experiments, $N.P_o$ (at 0 mV) was 0.024 ± 0.006 (range 0.001–0.068) in control PSS and 0.585 ± 0.308 (range 0.043–0.912) ($P < 0.05$, paired t -test) after 2–5 min exposure to 10 μM NOCys. After the washout of NOCys, $N.P_o$ values slowly returned to near-control values over 15–20 min (Lang & Watson, 1998; Lang *et al.*, 2000).

The voltage-dependent effects of NOCys were examined using three to five ramped depolarizations applied 2 s apart before (Figure 1a, bi), during (Figure 1a, bii) and after (Figure 1biii) the peak response to NOCys (10 μM). During these ramp depolarizations, the number of active channels opening simultaneously at positive potentials increased 2–10 fold in the presence of NOCys (10 μM). Finally, more BK_{Ca} channel openings were evident at potentials negative of 0 mV as might be expected with a substantial shift to the left of these 'activation' curves (Figure 1bii) (Lang *et al.*, 2000).

Effect of NEM

N-ethylmaleimide (NEM) is a specific alkylating agent of free sulfhydryl groups on cysteine residues that should prevent any NOCys-evoked formation of *S*-nitrosothiols (RSNO) or any disulfide-bonding formation with vicinal RSNOs or cysteine thiols. NEM (1 mM for 5 min) applied to the cytosolic surface of excised patches decreased BK_{Ca} channel opening at $+20$ mV (Figure 2a) and at positive potentials during ramped depolarizations to $+100$ mV (Figure 2bi ii), significantly decreasing $N.P_o$ (at 20 mV) from 0.0283 ± 0.0067 in control PSS to 0.0096 ± 0.0025 ($P < 0.05$, $n = 11$) (Figure 2a) (Lang *et al.*, 2000). Figure 2 also illustrates that a pre-exposure to NEM (1 mM for 5 min) prevented the NOCys (10 μM)-evoked increase in BK_{Ca} channel opening at $+20$ mV (Figure 2a) or during ramped depolarizations (Figure 2biii); the NOCys-induced increase in $N.P_o$ after an exposure (5 min) to and washout (10 min) of NEM (1 mM) being $172 \pm 78\%$ ($P > 0.05$, $n = 6$) of the $N.P_o$ recorded after the washout of the NEM (Lang *et al.*, 2000). This increase in $N.P_o$ can be compared with

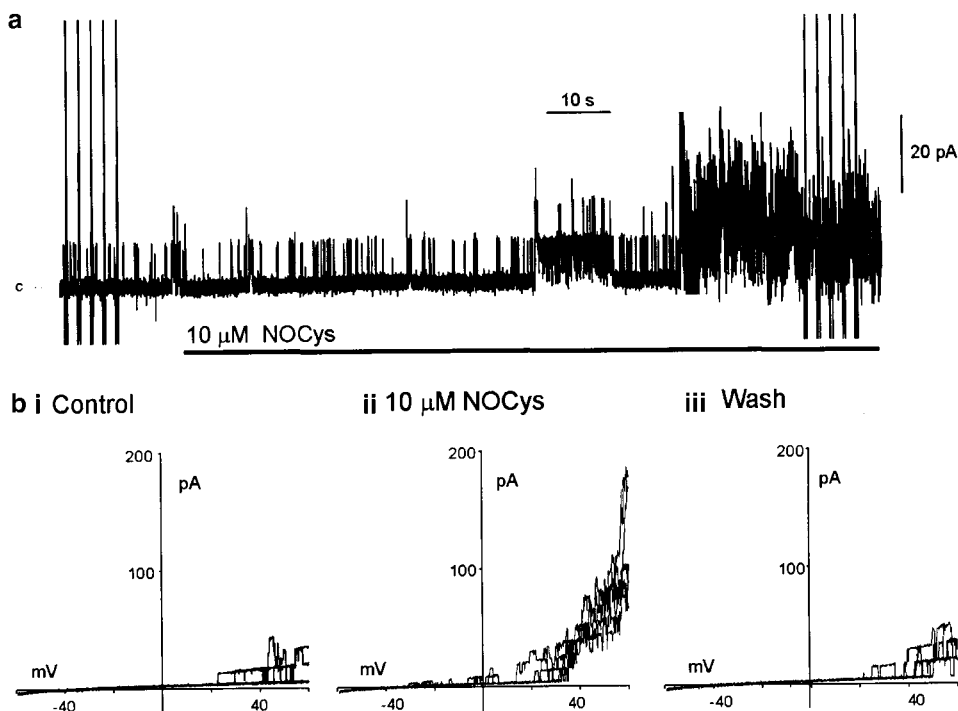


Figure 1 NOCys activation of BK_{Ca} channels in excised patches of the guinea-pig taenia caeca. (a) Addition of NOCys (10 μM) to the cytosolic surface increased BK_{Ca} channel activity (at 0 mV) in membrane patches bathed in a near-physiological K⁺ gradient, c denotes the closed level. (b) comparison of BK_{Ca} openings evoked by ramped depolarizations (three ramp depolarizations between -60 and +60 mV every 10 s) in the absence (bi) and 2 (bii) and 5 min (biii) after the addition of NOCys (10 μM) to the bathing solution, holding potential 0 mV.

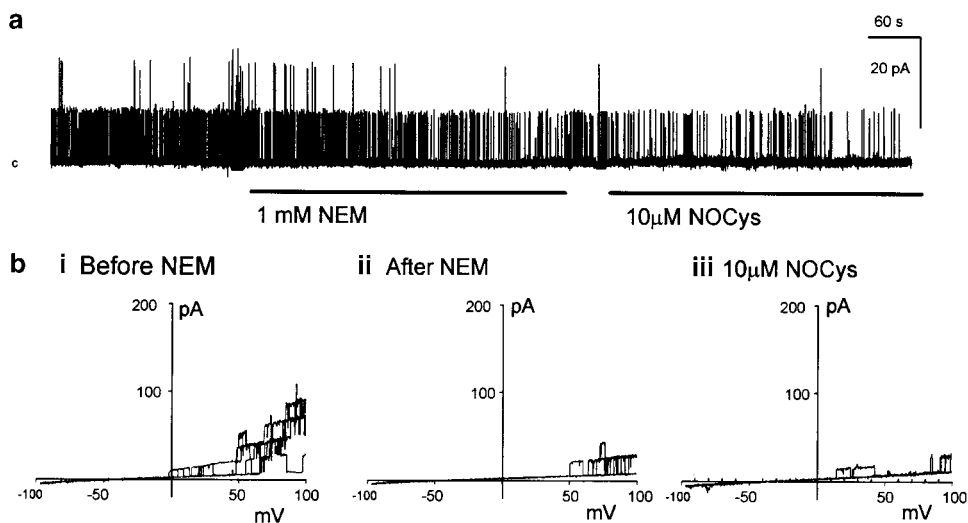


Figure 2 Alkylation of cysteine thiols with *N*-ethylmaleimide (NEM) prevented the excitatory action of NOCys. (a) Application of NEM (1 mM) to the cytosolic surface irreversibly decreased BK_{Ca} channel activity at 20 mV. The subsequent application of NOCys (10 μM) had little effect on BK_{Ca} channel activity. (b) comparison of BK_{Ca} openings evoked by ramped depolarizations (three ramp depolarizations between -100 and +100 mV every 10 s) in the absence (bi) and 5 min (bii) after the exposure to NEM (1 mM), holding potential 0 mV. (biii) Pre-exposure to NEM (1 mM for 5 min) prevented the excitatory actions of NOCys (10 μM) on ramped evoked BK_{Ca} channel openings.

the 1968-fold increase of *N.P_o* induced by NOCys (10 μM) applied to control membrane patches (see above).

The effects of NEM exposure on BK_{Ca} channel activation were examined in more detail in seven experiments. In 15 nM Ca²⁺ PSS, averaged macropatch currents at all potentials positive to 0 mV were reduced after a 5 min exposure to and

5 min washout of NEM (1 mM) (Figure 3A, Ba, Ca). When currents recorded before (Figure 3Ca, D, hollow squares) and after (Figure 3Ca, D filled squares) exposure to NEM (1 mM for 5 min) were expressed as normalized conductance, *V*_{0.5} was not significantly altered by NEM (62.8 ± 1.1 mV in control 15 nM Ca²⁺ PSS and 62.03 ± 3.7 mV after NEM exposure,

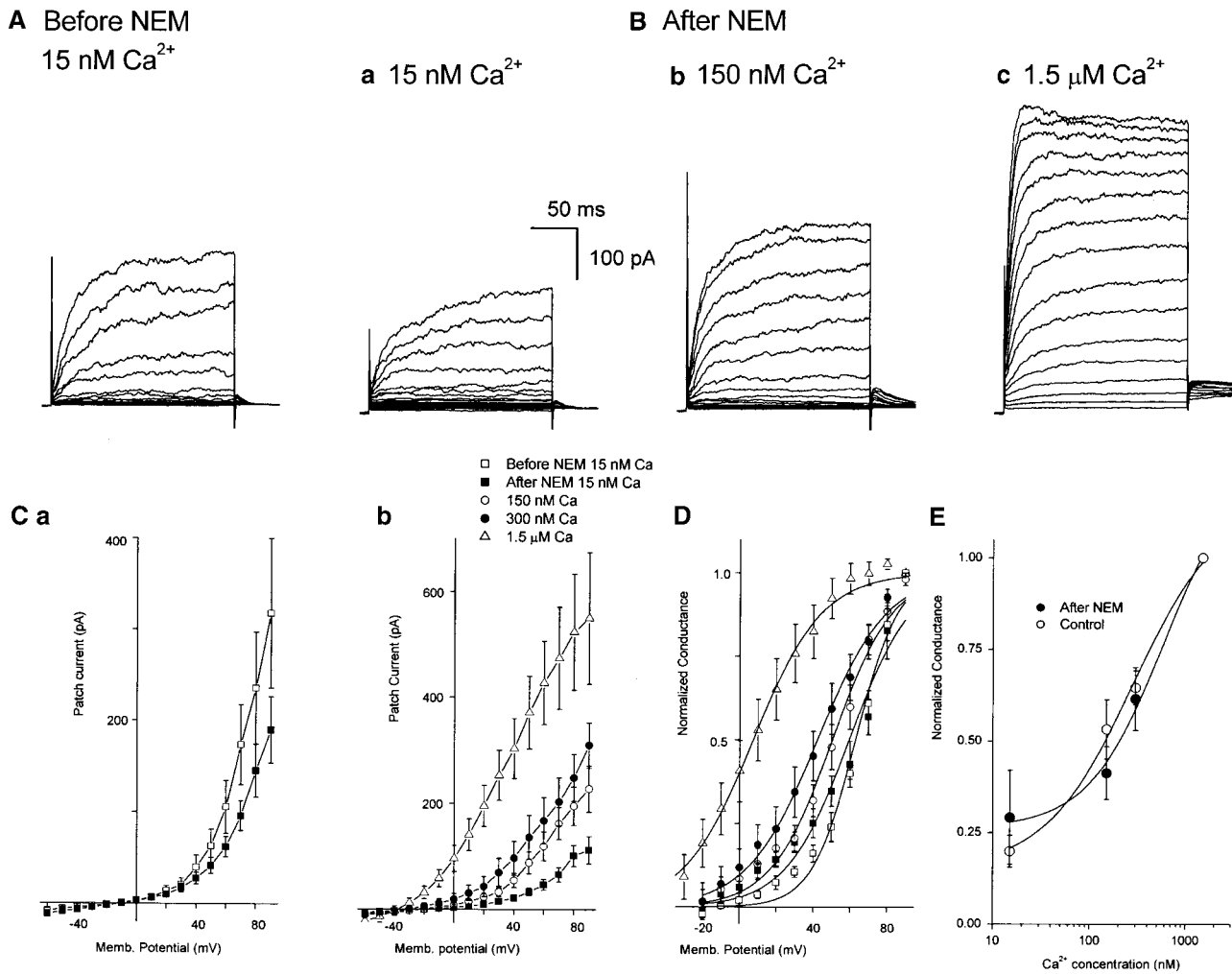


Figure 3 *N*-ethylmaleimide (NEM, 1 mM for 5 min) does not affect BK_{Ca} channel sensitivity to Ca²⁺. Typical macroscopic currents (A,B) and current–voltage relationships (C) for BK_{Ca} before (A, Ca hollow squares) and 5 min after (Ba, Ca,b filled squares) an exposure to NEM (1 mM for 5 min). Each current trace represents the average of 10 steps to test potentials every 10 mV between –60 and +90 mV from a holding potential of –80 mV. After an NEM exposure, the macroscopic BK_{Ca} currents (Ba–c) and resulting *I*–*V* relationships (Cb) were concentration-dependently increased by the concentration of Ca²⁺ bathing the cytosolic surface of the patch. Each data point represents the average of six to seven experiments. (D) Normalizing individual experiments to the maximum conductance reveals that NEM caused a significant increase in *K* (11.8 ± 1.5 mV in control 15 nM Ca²⁺ PSS and 16.9 ± 1.5 mV after NEM, respectively; *P* < 0.05, *n* = 6) without significantly altering *V*_{0.5} (62.8 ± 1.1 mV in control 15 nM Ca²⁺ PSS and 62.03 ± 3.7 mV after NEM exposure, respectively; *P* > 0.05, *n* = 6). Raising the cytosolic Ca²⁺ concentrations also shifted the Boltzmann (activation) curves fitted to the averaged data (*n* = 6–7) in a negative direction such that *V*_{0.5} and *K* were 59.5 and 17 mV, 50.4 and 16.4 mV, 42.3 and 18.8 mV, and 7.4 and 18.7 mV in 15, 150, 300 nM and 1.5 μM Ca²⁺ PSS, respectively. (E) Characteristics of Ca²⁺-dependent activation of BK_{Ca} channel currents either in control conditions or after an exposure to NEM. Maximal conductance recorded at test potentials between +60 and +90 mV from either control patches (hollow circles) (*n* = 3–6) or patches previously exposed to NEM (1 mM for 5 min) (filled circles) (*n* = 3–6) were normalized as a fraction of the conductance recorded in 1.5 μM Ca²⁺ PSS, averaged and plotted against Ca²⁺ concentration. Plots were fitted with the Hill equation to give a half-maximal activation (*C*) of 277 nM and a Hill coefficient (*n*) of 0.97 in control, and 576 nM and 1.12, respectively, after NEM exposure.

respectively; *P* > 0.05, *n* = 6). However, the slope *K* was significantly increased (11.8 ± 1.5 mV in control 15 nM Ca²⁺ PSS and 16.9 ± 1.5 mV after NEM, respectively; *P* < 0.05, *n* = 6) (Figure 3C,D).

In Figure 3Cb, a 5 min exposure to and 5 min washout of NEM (1 mM) did not prevent the increase in the macropatch current evoked by either membrane depolarization (Figure 3Cb) or when the Ca²⁺ concentration in the PSS bathing the cytosolic surface was raised from 15 (Figure 3Cb, filled squares) to 150 nM (Figure 3Cb, hollow circles), 300 nM (Figure 3Cb, filled circles) or 1.5 μM (Figure 3Cb, hollow triangles).

When macropatch currents were expressed as normalized conductance it was apparent that there was a concentration-dependent leftward shift of *V*_{0.5} with Ca²⁺ concentration, such that *V*_{0.5} obtained from the fitted Boltzmann curves to the averaged data in Figure 3D was 59.5, 50.4, 42.3 and 7.4 in 15, 150, 300 nM and 1.5 μM Ca²⁺ PSS, respectively. Raising the cytosolic Ca²⁺ concentration had little effect on *K*, being 17, 16.4, 18.8 and 18.7 mV in 15, 150, 300 nM and 1.5 μM Ca²⁺ PSS, respectively. Finally, a clear dependence of channel activation on Ca²⁺ concentration was obtained when the maximum conductance (between +60 and +90 mV) of the

individual macropatches bathed in 15, 150 and 300 nM Ca^{2+} was normalized as a fraction of the conductance obtained in 1.5 μM Ca^{2+} , averaged and plotted against the log of the Ca^{2+} concentration (Figure 3E, filled circles). This plot is not significantly different from that obtained when three control patches, not exposed to NEM, were bathed in the four concentrations of Ca^{2+} (Figure 3E, hollow circles). When fitted with the Hill equation, the half-maximal activation (C) of these BK_{Ca} channels before and after an exposure to NEM were 277 and 576 nM Ca^{2+} , respectively; the Hill coefficients were 0.97 and 1.12, respectively.

Effect of MTSEA

Although positively charged, MTSEA has been demonstrated to cross cell membranes suggesting that this MTS reagent readily changes from a protonated (membrane impermeant) to a deprotonated (membrane permeable) form (Karlin & Akabas, 1998). We have assumed that MTSEA will have access to cysteine residues that are present in water-accessible regions not surrounded by basic amino acids and as well as cysteine residues in the hydrophobic regions of the BK_{Ca} channel protein.

The application of MTSEA (2.5 mM for 5 min) to the cytosolic surface of excised patches rapidly reduced BK_{Ca} channel openings recorded at 0 mV (Figure 4a) or during

ramped depolarizations (Figure 4ci-ii). In 13 experiments, $N.P_o$ (at 0 mV) was reduced to $68 \pm 14\%$ ($P < 0.05$ paired t -test) of control after 2 min exposure to MTSEA (2.5 mM). This inhibitory effect of MTSEA was irreversible, even after 2–5 min washout of the MTSEA-containing PSS, involving multiple exchanges of the bath volume (Figure 4b).

The possibility that MTSEA might be permeating through the excised patch membrane and reacting with cysteine groups facing the extracellular surface of the channel protein was eliminated by observing that the MTSEA-induced decrease in $N.P_o$ still occurred when the pipette solution contained L-cysteine (10 mM) ($n = 4$). Under these conditions, it would be expected that any MTSEA reaching the extracellular surface of the BK_{Ca} channel protein(s) would be bound by the excess L-cysteine in the pipette saline.

In three experiments, the amplitude of the BK_{Ca} channel currents were recorded at various potentials between -60 and $+80$ mV in the presence (Figure 8a_{ii} filled circles) and absence (Figure 8a_{ii} hollow circles) of MTSEA (2.5 mM). BK_{Ca} channel current amplitudes at all potentials were not affected by MTSEA. At 0 mV, the averaged channel current amplitude was 7 ± 0.2 pA in the presence of MTSEA, and 6.8 ± 0.2 and 7.03 ± 0.01 pA, respectively, prior to and after the exposure to MTSEA ($n = 13$, $P > 0.05$ paired t -test) (Figure 8a_i).

Pre-exposure of membrane patches to MTSEA did not prevent the 418-fold increase in $N.P_o$ evoked in the presence of

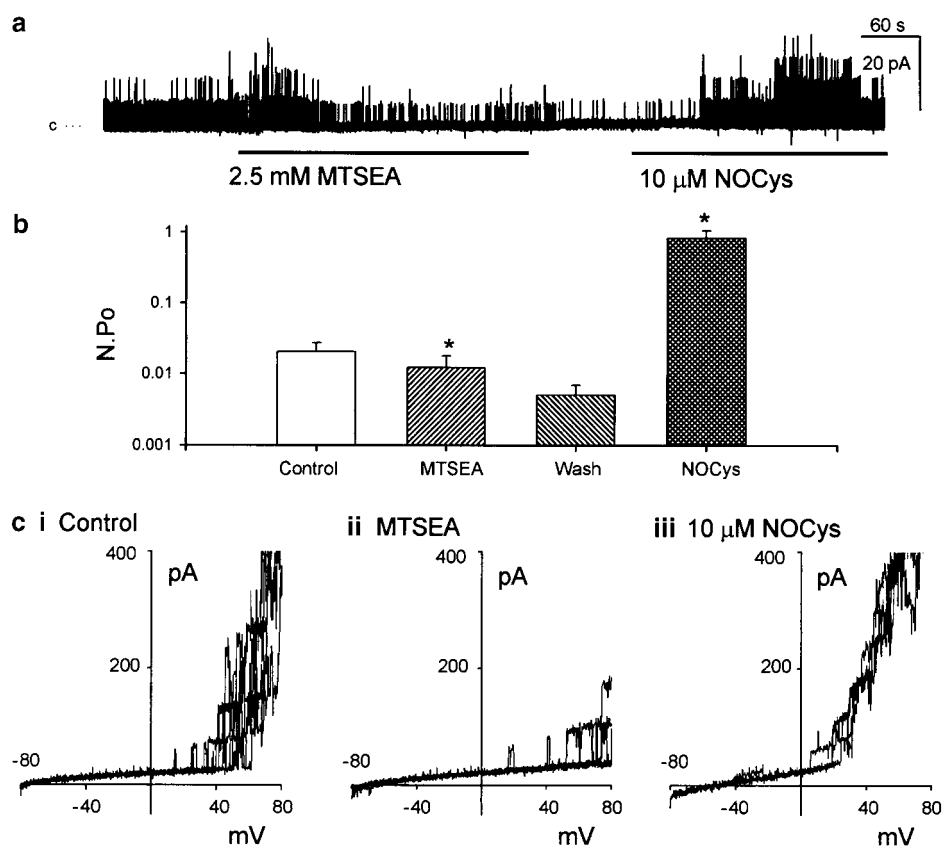


Figure 4 Positively charged but membrane permeable MTS reagent, MTSEA decreased BK_{Ca} channel activity but did not prevent the excitatory actions of NOCys. Application of MTSEA (2.5 mM for 5 min), to the cytosolic surface decreased BK_{Ca} channel activity ($N.P_o$) recorded at either 0 mV (a, b) or during ramped depolarizations between -80 and $+80$ mV (ci, ii). This reduction in $N.P_o$ remained upon washout of the MTSEA (b). The subsequent application of NOCys (10 μM) still increased $N.P_o$ at 0 mV (a, b) as well as the number of channel openings evoked by a ramp depolarization (ciii). The significant effects of all agents were established using Student's paired t -tests. *denotes $P < 0.05$.

NOCys ($10\ \mu\text{M}$) (Figure 4). $N.P_o$ in the presence of NOCys ($10\ \mu\text{M}$) applied after an exposure (2 min) and washout (2–5 min) of MTSEA, was 0.83 ± 0.22 compared to the value of $N.P_o$ (0.005 ± 0.002) ($n = 10$, $P < 0.05$) obtained following the washout of the MTS reagent (Figure 4b).

Effect of MTSET

Permanently positively charged and therefore membrane-impermeant MTSET would be expected to have access to cysteine residues within non-basic surface regions of the channel protein with a water interface. A 2 min exposure to MTSET (2.5 mM) significantly increased $N.P_o$ (at 0 mV) 22-fold ($n = 10$, $P < 0.05$) above control values (Figure 5a,b), as well as the number of BK_{Ca} channel openings evoked during a ramped depolarization (Figure 5c). However, MTSET also decreased BK_{Ca} channel amplitudes at 0 mV to 4.2 ± 0.7 pA ($n = 11$) (compared with 7.4 ± 0.3 pA in control $P < 0.05$ paired *t*-test) (Figure 8bi). In Figure 8bii, the averaged *I*–*V* plots ($n = 5$) obtained in the presence (Figure 8bii, hollow circles) or absence (Figure 8bii, filled circles) of MTSET have been compared. It can be seen that the slope of the *I*–*V* plot in the presence of MTSET is markedly reduced such that the slope conductance (at 0 mV) of these BK_{Ca} channels was 155 pS in control PSS and 95 pS in MTSET-containing PSS. In addition, the relative amplitude of these BK_{Ca} channel currents in

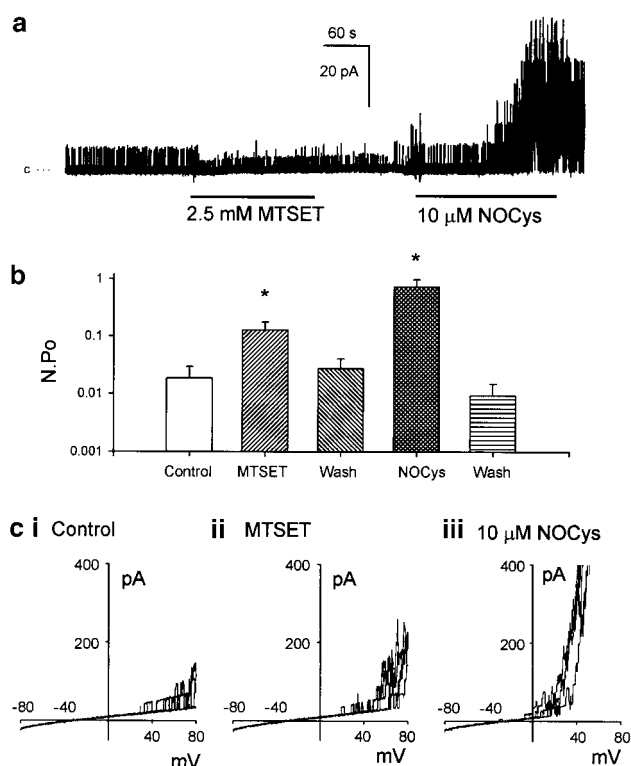


Figure 5 Positively charged MTS reagent MTSET increased BK_{Ca} channel activity (at 0 mV) in a manner reversed upon washout. MTSET did not prevent the excitatory actions of NOCys. Application of MTSET (2.5 mM for 5 min) significantly increased $N.P_o$ recorded at 0 mV (a, b) as well as the BK_{Ca} channel openings at positive potentials (ci, ii). This increased in $N.P_o$ was abolished upon washout of MTSET (b). After an exposure to MTSET, NOCys ($10\ \mu\text{M}$) still significantly increased BK_{Ca} channel openings at 0 mV (a, b) and positive potentials (ciii).

MTSET, expressed as a fraction of their respective control amplitudes (Figure 8bii, inset), decreased at more positive potentials suggesting a voltage-dependent blockade of current flow at positive potentials.

These MTSET-evoked changes in BK_{Ca} channel amplitude and $N.P_o$ were not maintained upon washout of the MTS reagent. Both $N.P_o$ (Figure 5b) and channel current amplitudes (Figure 8bi) returning to near-control values upon washout. This rapid reversibility of the effects of MTSET suggests that the observed effects of MTSET were nonspecific and not involving the conversion of cysteine sulfhydryls to a disulfide, which is presumably only reversible upon the addition of a thiol such as DTT. Moreover, the pre-exposure of membrane patches to MTSET did not prevent the 92-fold increase ($n = 10$, $P < 0.5$) in $N.P_o$ (0.742 ± 0.238) evoked by NOCys ($10\ \mu\text{M}$) (Figure 5a) applied after the washout of MTSET ($N.P_o$ of 0.0279 ± 0.013) (Figure 5a, b).

Effect of MTSES

Exposure of the cytosolic surface of excised membrane patches to negatively charged MTSES (2.5 mM for 5 min) evoked a rapid increase in BK_{Ca} channel activity at 0 mV (Figure 6a) and during ramped depolarizations to positive potentials (Figure 6ci, ii) such that $N.P_o$ (at 0 mV) in the presence of MTSET was significantly increased 11-fold above control ($n = 7$, $P < 0.05$ paired *t*-test) (Figure 6b). In contrast to MTSET, the amplitude of these BK_{Ca} channel currents either at 0 mV (Figure 8ci) ($n = 7$) or measured at various potentials between -40 and $+80$ mV (Figure 8cii) ($n = 3$) were not significantly affected by MTSES.

In contrast to MTSEA and MTSET, previously exposing excised membrane patches to MTSES (2.5 mM for 2 min) prevented the further activation of BK_{Ca} channels by NOCys ($10\ \mu\text{M}$) (Figure 6a). It can be seen in Figure 6b that the MTSES-evoked increase in $N.P_o$ was still evident after a 2-min washout of the MTS reagent. Moreover, the subsequent application of NOCys ($10\ \mu\text{M}$) did not evoke a further increase in $N.P_o$, being only $147 \pm 39\%$ ($n = 6$, $P > 0.05$ paired *t*-test) of the $N.P_o$ values recorded during the washout period after the exposure to MTSES (Figure 6b).

Finally, this exposure to MTSES did not prevent the increase in $N.P_o$ (at $+20$ mV) evoked by either membrane depolarization (Figure 6c) or when the cytosolic Ca^{2+} concentration was raised from 15 to 80 nM ($n = 3$) (Figure 7).

Discussion

BK_{Ca} channels exist as a tetrameric complex of two subunits: the pore-forming α subunit and the regulatory β subunit. The primary sequence of the α subunit obtained from different tissues is almost identical being encoded by a single gene, KCNMA1, previously termed 'slowpoke' or 'd_{slo}' in *Drosophila* (Butler *et al.*, 1993; Pallanck & Ganetzky, 1994; McCobb *et al.*, 1995; Vogalis *et al.*, 1996; Meera *et al.*, 1997; Tanaka *et al.*, 1997; Vergara *et al.*, 1998; Jiang *et al.*, 1999; Wallner *et al.*, 1999; Ha *et al.*, 2000). In contrast, β subunits are encoded by four genes (KCNMB1-4) (Tanaka *et al.*, 1997; Jiang *et al.*, 1999; Orio *et al.*, 2002). The minimum molecular structure required to create a functional BK_{Ca} channel is a tetramer recombination of the α subunit. In addition to

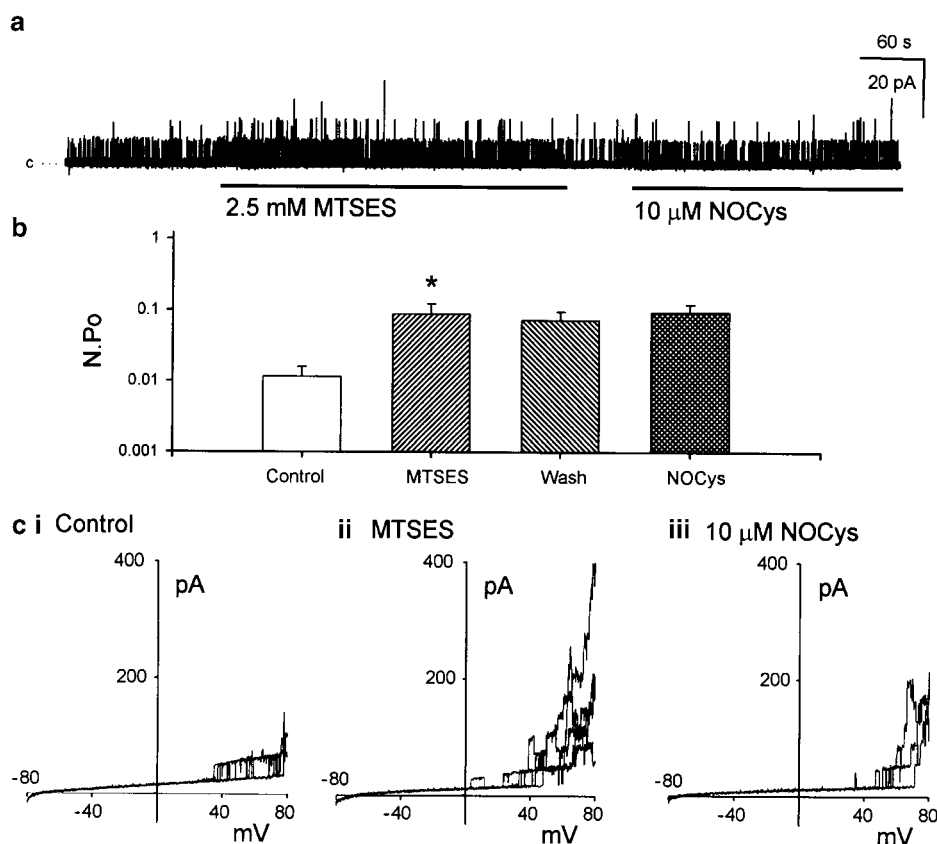


Figure 6 Negatively charged MTS reagent MTSES, irreversibly increased BK_{Ca} channel activity (at 0 mV) and prevented the excitatory actions of NOCys. Application of MTSES (2.5 mM for 5 min) significantly increased *N.P_o* recorded at 0 mV (a, b) as well as the BK_{Ca} channel openings evoked at positive potentials (ci, ii). This increase in *N.P_o* remained upon washout of MTSES (b). Pre-exposure to MTSES prevented any further NOCys (10 μ M)-evoked increases of *N.P_o* at 0 mV (a, b) or at positive potentials (ciii).

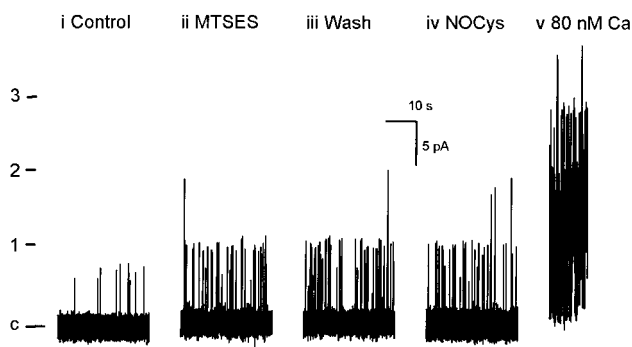


Figure 7 Comparing the effects of NOCys (10 μ M for 2 min) and raising the cytosolic Ca²⁺ concentration from 15 to 80 nM on BK_{Ca} channel activity (at 0 mV) after exposure and washout of MTSES (2.5 mM for 5 min). c denotes the closed level, 1–3 denote number of superimposed open channels.

containing the six membrane-spanning domains (S1–S6), the S4 voltage sensor and the pore domain between S5 and S6, which identifies this channel as a member of the S4 superfamily of voltage-gated K⁺ channels (*K_v*), the α subunit also contains a seventh transmembrane domain (S0) that locates the amino terminus in the extracellular domain. Additional hydrophobic cytoplasmic-located segments (S7–S10) are located within the long carboxy-terminal domain. A tetramerization domain (BK-T1) has been located in the hydrophilic region between S6 and S7, while Ca²⁺ binding has been

located to a region ('calcium bowl') between S9 and S10, which is highly conserved in all cloned BK_{Ca} channels (Orio *et al.*, 2002). However, replacing all of the negatively charged aspartate residues within the calcium bowl with uncharged asparagine only reduced Ca²⁺ binding and its associated Hill coefficient by approximately 50% (Bian *et al.*, 2001), suggesting that there are multiple sites in the carboxyl terminal involved in Ca²⁺ binding and channel activation. Alternate splicing at two to four sites between S8 and S10 of the α subunit changes the kinetics and Ca²⁺ sensitivity of expressed α subunit channels. However, larger changes in channel kinetics are generally created when α subunits are co-expressed with β subunits (Orio *et al.*, 2002).

β subunits (B1–B4) consist of two transmembrane segments connected by an extracellular 'loop' so that both termini are in the cytoplasmic domain (Knaus *et al.*, 1994; Meera *et al.*, 1996; Vogalis *et al.*, 1996; Orio *et al.*, 2002). B1 is highly expressed in smooth muscle, while B2 is expressed preferentially in brain and chromaffin cells, B3 is found in the testis, pancreas and spleen and B4 in the brain (Wallner *et al.*, 1996; Wallner *et al.*, 1999; Orio *et al.*, 2002). The β subunit increases the sensitivity of the α subunit to Ca²⁺, particularly at high [Ca]_i (McCobb *et al.*, 1995; McManus *et al.*, 1995; Vogalis *et al.*, 1996; Jiang *et al.*, 1999) and modifies charybdotoxin binding and the α subunit selectivity for K⁺ ions (McManus *et al.*, 1995). The presence of the β subunit is also necessary for the extracellular binding and activation of BK_{Ca} channels by 17- β estradiol (Valverde *et al.*, 1999). Targeted genetic deletion

of smooth muscle B1 in mice increases arterial tone and blood pressure, as well as modulates bladder action potential discharge and contraction (Brenner *et al.*, 2000; Petkov *et al.*, 2001).

The specific oxidation of sulfur-containing side chains within methionine residues in cloned human *slo* (*hslo*) channels expressed in HEK 293 cells with chloramine-T increases channel opening in a manner partially reversed by the enzyme peptide methionine sulfoxide reductase plus DTT (Hoshi & Heinemann, 2001; Tang *et al.*, 2001). In contrast, the specific oxidation of cysteine thiols with NEM decreases $N.P_o$ (Figure 2) in excised membrane patches of the guinea-pig taenia ceca (Lang *et al.*, 2000), which could be arising from the decrease in the BK_{Ca} channel sensitivity to voltage, evident by the small but significant increase in K (Figure 3C, D). However, exposure to NEM did not affect the sensitivity of the BK_{Ca} channel activation to raising the cytosolic concentration of Ca^{2+} (Figure 3). These results are consistent with the NEM-evoked decrease in BK_{Ca} channel activity in excised patches from the rabbit aorta (Bolotina *et al.*, 1994) and horse trachea (Wang *et al.*, 1997), and the NEM-evoked reduction of the whole-cell current of HEK 293 cells expressing rat *slo* (*rslo*) channels (Jeong *et al.*, 2001). In contrast, NEM has also been demonstrated to increase BK_{Ca} channel activity in excised patches pulled from nerve terminals of the rat posterior pituitary (Ahern *et al.*, 1999) or after isolation from bovine airways and incorporation into a lipid bilayer (Abderrahmane *et al.*, 1998).

The alkylation of cysteine thiols by NEM should prevent any NO^{\cdot} donor-evoked S-nitrosylation, or the conversion of neighboring thiols to a disulfide. Cytosolically applied NEM inhibited both the NOCys activation of BK_{Ca} channels in excised patches from the guinea-pig taenia ceca (Figure 2) (Lang *et al.*, 2000) and the NO^{\cdot} activation of BK_{Ca} channel gating in pituitary nerve terminals (Ahern *et al.*, 1999). However, NEM does not prevent the increase in *rslo* channel activity evoked by the NO^{\cdot} donor, S-nitroso-N-acetylpenicillamine (SNAP) (Jeong *et al.*, 2001). These variable effects of NEM on control and NO^{\cdot} -evoked BK_{Ca} and *slo* channel activity may well be arising from differences in the amino-acid sequence and therefore the microenvironment of the native BK_{Ca} and the *rslo* channels under study. In particular, there may well be a number of different spliced variants of the α subunit of the BK_{Ca} channel being expressed in the smooth muscle cells of the taenia ceca. These differing spliced variants could be combining in varying proportions with a number of differing spliced variants of the β subunit to form a variety of BK_{Ca} channels, which could explain the increases and decreases in channel gating observed in the presence of oxidizing agents and NEM, respectively. However, in the present experiments, it is clear that cysteine residues lying on the cytosolic surface of the BK_{Ca} channel protein(s) are the sites of NEM action.

Although positively charged, MTSEA has been reported to cross cell membranes and lipid bilayers (Karlin & Akabas, 1998). Thus, we have assumed that MTSEA will have the greatest access to the cysteine residues of the α subunit both within the hydrophobic regions and hydrophilic regions. In our experiments, MTSEA evoked a significant decrease in BK_{Ca} channel opening that remained upon washout, suggesting an irreversible binding of MTSEA to cysteine residues (Figure 4) (Tang *et al.*, 2001).

However, pre-exposure and washout of membrane patches to MTSEA did not prevent the excitatory effects of NOCys. This suggests that the cysteine residues bound by MTSEA are not the targets of S-nitrosylation in the presence of NOCys. Permanently positively charged MTSET would be expected to have access to cysteine residues within all acidic regions of the channel protein with a water interface. However, the reduction in BK_{Ca} channel amplitude (Figure 8b) and increase in $N.P_o$ (Figure 5) observed in the presence of MTSET were not maintained upon washout. These data suggest that the actions of MTSET do not involve the conversion of cysteine sulfhydryls to a disulfide. In fact, the MTSET-evoked reduction of channel current amplitude appeared similar to the rapid open-channel blockade observed in the presence of high concentrations of tetraethylammonium chloride or procaine applied to the cytosolic surface of smooth muscle BK_{Ca} channels (Benham *et al.*, 1985). These observations have not been studied further.

Only MTSES evoked an increase in $N.P_o$ that remained after washout. Moreover, only MTSES prevented any further increase in $N.P_o$ when NOCys was added (Figure 6). This specificity of MTSES is not based on the size of the MTS reagents used or on the volume of the water interface of the protein surface at which these agents are interacting. The $-SO_3$ group of MTSES is smaller than the $-N(CH_3)$ group of MTSET (0.58 nm) but larger than the $-NH_3$ group of MTSEA (0.36 nm). If the size of the head group was the essential factor restricting MTS reagent binding to the cysteines S-nitrosylated by NOCys, then MTSES and MTSET would be expected to have equal access (Akabas *et al.*, 1992). As MTSET did not prevent the excitatory actions of NOCys, we can conclude that the S-nitrosylatable cysteines lie in regions of the channel protein that would select against cations, within basic water-facing regions of the channel protein that would exclude MTSEA and MTSET. The gating of cloned α subunits of the BK_{Ca} channels isolated from rat and human brain and expressed in immortal cells lines have been demonstrated to be modulated by NO^{\cdot} donors (Jeong *et al.*, 2001), redox reagents (DiChiara & Reinhart, 1997; Tang *et al.*, 2001; Chung *et al.*, 2002) and NEM (Jeong *et al.*, 2001). These data suggest that it is unlikely that the β subunit plays any role in NOCys- or redox-evoked changes in BK_{Ca} channel activity.

There is considerable (>90%) homology in the sequences of the alpha subunit of BK_{Ca} channels (alpha-*slo*) isolated from the mouse and rat brain, dog intestine and human aorta and myometrium. In the published sequences of α subunit of the BK_{Ca} channels found in human myometrium (Accession No. AAK91504 or GI 15215553) and aorta (Accession No. AAA92290 or GI 758791) and the dog colon (Accession No. AAA84000, GI 1127824) there are 20–25 cysteine residues in the amino-acid sequence. Only five to six cysteine residues lie within hydrophobic regions leaving 15–20 cysteine residues as potential targets for S-nitrosylation by NOCys or alkylation by NEM. A detailed examination of the sequence of the alpha subunit of the human brain BK_{Ca} channel (alpha-*hbr1*) reveals that there are only two pairs of cysteine residues in the C terminal domain that are separated or surrounded by basic amino acids. The sequences surrounding these cysteines are also highly conserved in all species, except for some minor substitutions of basic amino acids in the rat and mouse. Alternate RNA splicing occurs in alpha-*hbr1* at three sites between S8 and S9 and at one site between S9 and S10 in the

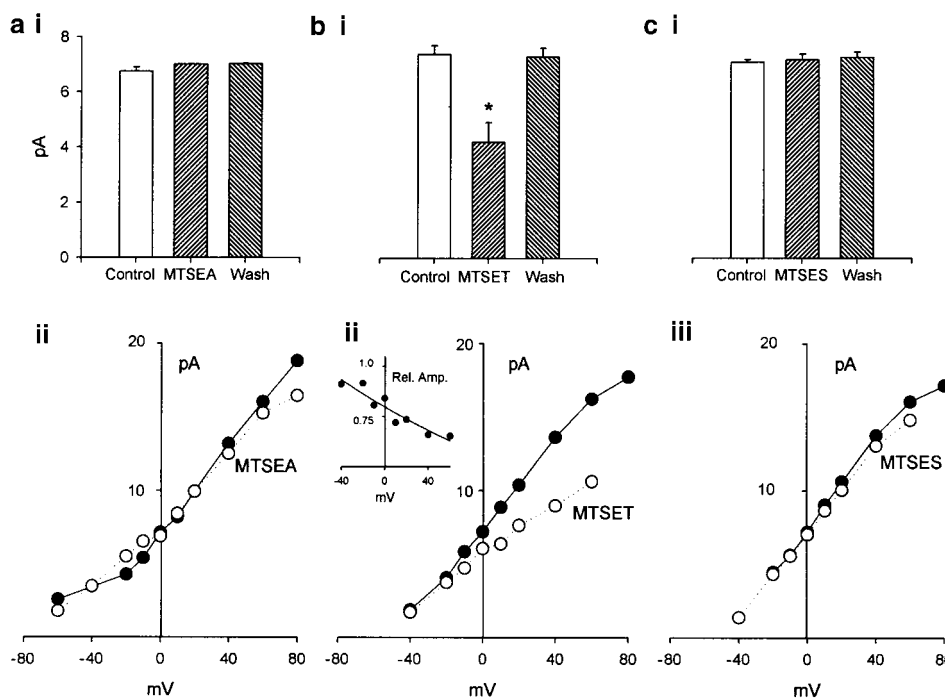


Figure 8 Summary of the effects of MTS reagents on the I - V properties of BK_{Ca} channels in excised patches of the guinea-pig taenia caeca. BK_{Ca} current amplitudes were measured either at 0 mV (ai, $n = 13$; bi, $n = 11$; ci, $n = 7$) or at every 10 mV between -60 and $+80$ mV in control saline (filled circles), in the presence of MTSEA (a(ii), $n = 3$), MTSET (b(ii), $n = 5$) or MTSES (c(ii), $n = 3$) and after washout of the MTS reagents (ai, bi, ci). MTSEA and MTSES did not significantly alter BK_{Ca} current amplitudes at 0 mV (ai, ci) or any other potential (a(ii), c(ii)). In contrast, MTSET significantly reduced channel amplitudes at 0 mV (bi) compared to control amplitudes, as well as the slope of the I - V plot between -40 and $+80$ mV (b(ii)). b(ii) Inset illustrates the current amplitudes in MSET expressed as a fraction of the control channel amplitudes (Rel. Amp.) plotted against V . The continuous line through the data points represents an exponential function, fitted by least squares: $f = 0.798 \exp(-0.004x)$.

C-terminal domain. In the human brain, the insertion of a number of alternate exons at these sites has led to the identification of nine constructs (*hbr1*–*hbr9*), which can insert one additional cysteine residue (*hbr4*) or three (*hbr2*, *hbr8*, *hbr9*) to six to nine (*hbr3*, *hbr7*) additional basic amino acids, all of which may well have functional consequences on the sensitivity of the channel to redox reagents, Ca²⁺ or nitrogen reactive species (Tseng-Crank *et al.*, 1994).

It is likely that the BK_{Ca} channels in the guinea-pig taenia caeca are equally diverse in their expression, distribution and sensitivity to NO[•], which limits the identification of the reactive cysteine residues in the present study. A systematic study involving the expression of α subunit constructs of known molecular structure with single mutations of individual cysteine residues to nonreactive alanine in an immortal cell line and the retesting of the effects of NEM, MTSES and NOCys

will be needed before we can identify unequivocally which cysteine thiols are involved in the S-nitrosylation mechanisms examined in the present experiments.

In summary, we have demonstrated that NEM prevents NOCys-evoked increases in BK_{Ca} channel (Ahern *et al.*, 1999; Lang *et al.*, 2000) activity, indicating that the S-nitrosylation of cysteine residues of the α subunit plays an important part of NOCys action. As negatively charged MTSES also increased $N.P_o$ and prevented the further activation by NOCys, it is likely that the cysteine residues reactive to NOCys lie within basic regions of the α subunit, which exclude positively charged MTSEA and MTSET.

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