Redox Regulation of Large Conductance Ca\(^{2+}\)-activated K\(^+\) Channels in Smooth Muscle Cells

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ABSTRACT The effects of sulphydryl reduction/oxidation on the gating of large-conductance, Ca\(^{2+}\)-activated K\(^+\) (maxi-K) channels were examined in excised patches from tracheal myocytes. Channel activity was modified by sulphydryl redox agents applied to the cytosolic surface, but not the extracellular surface, of membrane patches. Sulphydryl reducing agents dithiothreitol, \(\beta\)-mercaptoethanol, and GSH augmented, whereas sulphydryl oxidizing agents diamide, thimerosal, and 2,2′-dithiodipryridine inhibited, channel activity in a concentration-dependent manner. Channel stimulation by reduction and inhibition by oxidation persisted following washout of the compounds, but the effects of reduction were reversed by subsequent oxidation, and vice versa. The thiol-specific reagents N-ethylmaleimide and (2-aminoethyl)methanethiosulfonate inhibited channel activity and prevented the effect of subsequent sulphydryl oxidation. Measurements of macroscopic currents in inside-out patches indicate that reduction only shifted the voltage/\(nP_o\) relationship without an effect on the maximum conductance of the patch, suggesting that the increase in \(nP_o\), following reduction did not result from recruitment of more functional channels but rather from changes of channel gating. We conclude that redox modulation of cysteine thiol groups, which probably involves thiol/disulfide exchange, alters maxi-K channel gating, and that this modulation likely affects channel activity under physiological conditions.

KEY WORDS: \(K_{\text{Ca}}\) channels • sulphydryl • disulfide • thiol

INTRODUCTION Alterations in the redox state of cysteine residues constitute an important mechanism for the regulation of cellular functions. The thiol group of cysteine residues is the most reactive of any amino acid side chain, existing as free thiols, or, in the presence of appropriate electron acceptors, disulfides formed between vicinal thiols (Creighton, 1984, 1993). Thiol/disulfide redox state exchanges are generally reversible (Gilbert, 1995), their proportions varying in response to changes in cellular redox potential, which in turn affects the biological activities of enzymes, receptors, transporters, and transcription factors (Gilbert, 1990).

Redox modification of cysteine sulphydryl groups may also be an important mechanism of controlling ion channel function. Redox agents alter the function of several channels including sarcoplasmic reticulum Ca\(^{2+}\)-release channels in skeletal muscle (Zaidi et al., 1989), \(N\)-methyl-\(D\)-aspartate receptor channels in the brain (Sucher and Lipton, 1991), voltage-dependent (Ruppersberg et al., 1991), ATP-regulated \(K^+\) channels (Islam et al., 1993), and nonselective cation channels in guinea pig ventricular myocytes (Jabr and Cole, 1995) and in yeast *Saccharomyces cerevisiae* vacuolar membranes (Bertl and Slayman, 1990). These observations are characterized by the opposite actions of sulphydryl reducing and oxidizing agents on channel function and the reciprocal reversal of their effects. In the case of Kv1.4 potassium channels, fast inactivation of the channel is dependent on the reduced redox status of a cysteine residue in the ball-domain (Ruppersberg et al., 1991).

Large conductance, Ca\(^{2+}\)-activated K\(^+\) (maxi-K) channels are present in a wide variety of cell types. In smooth muscle cells, maxi-K channels are important determinants of vasomotor tone (Brayden and Nelson, 1992) and of the cellular responses to hormones and neurotransmitters (Cole et al., 1989; Kume et al., 1989; Toro et al., 1990; Kume and Kotlikoff, 1991; Anwer et al., 1992; Kume et al., 1994). In experiments examining the modulatory actions of protein kinases, protein phosphatases, and G protein subunits, we observed a...
marked stimulation of maxi-K channel activity by control buffer solutions prepared to mimic those used to suspend the proteins. By a process of elimination, the active components of these buffers were identified as β-mercaptoethanol (β-ME) and dithiothreitol (DTT), both of which are reducing compounds commonly used to prevent oxidation of protein sulfhydryl groups. We hypothesized that the activity of maxi-K channels is regulated by the redox state of critical sulfhydryl groups in the channel protein or an associated regulatory protein, involving exchanges between free thiols and disulfides. In the present study, we examined the effects of several types of sulfhydryl-modifying agents on maxi-K channel activity in isolated membrane patches from tracheal smooth muscle cells. We demonstrate that channel activity is markedly affected by alterations in cytosolic redox potential; channel activity is augmented in reducing, and inhibited in oxidizing, conditions, and the action of oxidizing agents is eliminated following alkylation of the sulfhydryl side chain. The mechanism of channel modulation appears to be an effect on channel gating since the number of functional channels does not change after reduction.

MATERIALS AND METHODS

Cell Dissociation

Smooth muscle cells were dissociated from tracheas obtained from horses killed by intravenous injection of pentobarbital sodium. The horses were killed for teaching purposes and euthanasia procedures were in accordance with the guidelines set by the Institutional Animal Care and Use Committee of the University of Pennsylvania. After dissecting away connective tissue on the adventitial side, a piece of trachealis (1.5 × 1.5 cm) was cut and cannulated with an 18-gauge needle between the mucosa and muscle layers. The tissue was tied on the needle, suspended in a warmed, jacketed chamber, and perfused with dissociation solution containing 1,750 U collagenase D (Boehringer Mannheim Corp., Indianapolis, IN), 25 U elastase (Worthington Biochemical Corp., Freehold, NJ), and 5 mg trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) in 5 ml of medium M199. After 10–20 min perfusion, the tissue was transferred to a petri dish, and the tissue was minced and centrifuged at 5°C and 1,000 rpm for 5 min.

Patch-clamp

Maxi-K channel activity was measured in inside-out and outside-out patches as previously described (Kume and Kotlikoff, 1991). Gigaohm seals were obtained using heat-polished borosilicate glass pipettes with a resistance of 3–7 MΩ. For inside-out patches, the bath solution was (in mM): KCl 135.0, MgCl₂ 2.0, CaCl₂ 1.8 or 2.2, EGTA 3.0, HEPES 10.0, pH adjusted to 7.2 with KOH solution. Free Ca²⁺ in this solution was either 290 nM (CaCl₂ = 1.8 mM) or 500 nM (CaCl₂ = 2.2 mM) (Fabiani, 1988). The pipette solution contained in (mM): NaCl 136.0, KCl 5.0, CaCl₂ 1.8, MgCl₂ 1.0, HEPES 10.0, with pH adjusted to 7.4 with NaOH solution. Solutions were reversed for outside-out patches. Patches were examined at a holding potential of 0–40 mV, depending on the level of control channel activity. After a patch was obtained, an equilibration period of ~5 min was allowed; patches that showed large fluctuations in channel activity over this period were discarded. Thereafter, channel activity was recorded continuously until the end of the experiment. Drug actions were observed for 5–10 min after addition to the bath solution either by pipette (at 1% of the bath volume) or by perfusion (in a volume of at least 10 times the bath volume). Unitary currents were amplified (EPC-7; List-Medical-Electronic, Darmstadt, Germany), displayed on a computer (pClamp software; Axon Instruments, Foster City, CA) and simultaneously stored on a modified Sony Digital Audio Tape Deck (DC-700). The stored data were later digitized using Axotape or pClamp software (Axon Instruments) at a sampling rate of 2.5 and 5 kHz after filtration (~3dB, 8-pole lowpass Bessel filter; Frequency Devices, Inc., Haverhill, MA) at 1 and 2 kHz, respectively.

Reagents

DTT, β-ME, thimerosal, diamide, 2, 2'-dithiodipyrindine (DTDP), and N-ethylmaleimide (NEM) were purchased from Sigma Chemical Co. Reduced glutathione (GSH) was purchased from Calbiochem-Novabiochem Corp. (La Jolla, California). (2-Aminothio)ethanethiol (MTSEA) was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Except for DTDP, all the agents were dissolved in bath solution. DTDP was first dissolved in ethanol to a concentration of 500 mM, and then diluted with bath solution to the final concentration of 50 μM. None of the agents had an effect on the pH of the bath solution at the applied concentration ranges. The effect of redox agents on free calcium in the solutions used was determined using fura-2. DTT, β-ME, DTDP, NEM, thimerosal, and MTSEA had no effect on free calcium at the highest concentrations used. The fluorescence of GSH and diamide prevented a similar determination.

Statistics

SigmaStat for Windows (version 1.0; Jandel Corp., San Rafael, CA) was used for statistical analyses. Significance was determined by either paired t tests or one way analysis of variance (ANOVA) for repeated measures, depending on the number of treatments that the patch received. When a significant effect was detected with ANOVA, Student-Newman-Keuls test was used for pair-wise comparisons. P < 0.05 was considered statistically significant. All results are expressed as mean ± SE.

RESULTS

Effects of Sulphydryl Redox Agents on Maxi-K Channel Activity

Channel activity in inside-out patches was concentration-dependently augmented by exposure of the cytosolic patch surface to the sulphydryl reducing agents DTT (10 μM to 1 mM, in logarithmic increments; Fig. 1), β-ME (10 μM to 10 mM, in logarithmic increments; Fig. 2), and GSH (50 or 170 μM; see Figs. 6 and 8). At the highest concentrations applied, open-state probability (nPET) increased 7.7-fold after addition of DTT (0.028 ± 0.009 to 0.216 ± 0.062, n = 9), 4.3-fold after β-ME (0.029 ± 0.014 to 0.125 ± 0.028, n = 6), and 8.4-fold after GSH (0.049 ± 0.016 to 0.406 ± 0.126, n = 6). An augmenting effect was generally observed within 1 min of drug application, followed by a continued increase in channel activity during the next 2 or 3 min; thereafter chan-
nel activity remained constant. The time course of a typical experiment in which DTT augmented channel activity is shown in Fig. 3; the stimulatory effect of DTT was not reversed by perfusing the bath for 5 min with 10–20 times the bath volume.

Conversely, application of sulfhydryl oxidizing agents to the patch membrane cytosolic surface reduced channel open-state probability. As shown in Fig. 4, concentrations as low as 5 μM diamide significantly inhibited channel activity, and nP_o was only 10% of the control level at 5 mM of diamide (0.391 ± 0.085 to 0.040 ± 0.011, n = 7). Channel activity was similarly inhibited by DTDP; at a concentration of 50 μM, DTDP significantly reduced nP_o from 0.383 ± 0.062 to 0.171 ± 0.033 (n = 7) (Fig. 5). To eliminate possible solvent effects, the same concentration (0.01%) of ethanol was included in the bath solution during the control period, and DTDP was applied by perfusing at least 10 times the bath volume.

To confirm that the change in channel activity following a redox agent resulted from redox state modification, we tested whether alteration of channel activity produced by sulfhydryl reduction could be reversed by subsequent oxidation, and vice versa. As shown in Fig. 6, GSH (50 μM) markedly augmented maxi-K channel activity, which was reversed by diamide (500 μM). In five experiments, nP_o increased 4.5-fold after GSH (0.022 ± 0.002 to 0.100 ± 0.020), and was then reduced to 8% of the stimulated value (0.100 ± 0.020 to 0.008 ± 0.002) following diamide. Likewise, inhibition of channel activity following sulfhydryl oxidation was reversed by sulfhydryl reduction. As shown on the left panel of Fig. 7, the oxidizing agent thimerosal (10 μM) applied to the cytosolic surface of inside-out patches reduced channel nP_o, which persisted after washout but was reversed by DTT (1 mM). Three identically designed experiments using diamide instead of thimer-
sal produced similar results; nPo dropped from 0.342 ± 0.086 to 0.093 ± 0.024 following diamide (5 mM), and remained at the low level after washing for 5 min (0.091 ± 0.037). Two of the three patches were exposed to DTT (1 mM) following diamide. In these patches nPo increased by 14.4-fold and 21.1-fold, respectively. The new levels of nPo were 4.6 and 2.7 times of the initial control values, respectively. The opposite actions of sulfhydryl reducing and oxidizing agents, and the reciprocal reversal of their effects, suggest that the activity of maxi-K channels is dependent on the redox status of one or more sulfhydryl groups on the channel protein, or an associated regulatory protein.

Redox Modulation Occurs at the Cytosolic Patch Surface

To determine whether the sulfhydryl group(s) involved in the response to redox agents is located on the intracellular or extracellular side of the membrane, we compared the effects of reducing and oxidizing agents applied to the bath solution in inside-out and outside-out patches. As shown in Fig. 7, thimerosal markedly inhibited channel activity when added to the bath solution of inside-out, but not outside-out, patches. Immediately after application of thimerosal to the cytosolic surface of inside-out patches, channel activity was rapidly inhibited but not abolished. Large deflections were due to bath addition. The slow decline in channel activity likely reflects some thimerosal permeation. Similarly, the relatively mem-

**Figure 3.** Time course of the effect of dithiothreitol. (A) Bar graph shows the mean open-state probability before, during, and after washout of 1 mM DTT from four inside-out patches. The effect of DTT was not reversed after over 5 min washout. * indicates statistical significance (P < 0.05). n = the number of patches. (B) The time course of a representative experiment (holding potential = 0 mV). After exposure to DTT (1 mM), channel activity increased within 1 min and reached a plateau within 3 min. The increase in channel activity was not reversed following washout of DTT. (C) Current traces from data points indicated in B.

**Figure 4.** The oxidizing agent diamide inhibits maxi-K channel activity in inside-out patches. (A) Continuous current trace showing a representative experiment in which the cytoplasmic surface of an inside-out patch was exposed to 5 mM diamide (holding potential = 0 mV). Channel activity was rapidly inhibited but not abolished. Large deflections were due to bath addition. (B) Mean data from 7 patches in which increasing concentrations of diamide were sequentially added to inside-out patches. Diamide inhibited maxi-K activity in a concentration-dependent fashion between 5 μM and 5 mM. * indicates statistical significance.

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brane-impermeant reducing agent GSH (DiPaola et al., 1989) also was shown to be effective only in inside-out patches. As shown in Fig. 8, GSH (170 μM) increased the nPo of maxi-K channels by more than eightfold (n = 6) when applied to inside-out patches (n = 6) but had no effect in outside-out patches (n = 4). The effect of GSH on inside-out patches could be observed within 30 s in continuous traces. The configuration dependence and time course of the action of these relatively impermeant oxidizing and reducing agents on channel activity suggest that the cysteine residue(s) responsible for redox regulation is located on the cytoplasmic aspect of the cell membrane.

Modification of Sulfhydryl Groups by NEM and MTSEA Inhibits Maxi-K Channel Activity and Prevents the Effect of Patch Oxidation

We reasoned that the action of redox agents on maxi-K channel activity was due to the modification of the sulfhydryl group of cysteine residues, and the formation or breakdown of one or more disulfide bonds. To confirm that the modification of reactive thiols underlies the observed effect of redox agents, we examined the response of maxi-K channels to oxidizing agents in NEM-treated patches. NEM alkylates free sulfhydryl groups (Creighton, 1993). If free thiols are involved in the responses to oxidizing agents, covalent modification of the free thiols by NEM should prevent disulfide bond formation, and attendant alterations in channel activity, following exposure to oxidizing agents. As shown in Fig. 9 A, addition of NEM (1 mM) to the bath solution rapidly inhibited maxi-K channel opening in inside-out patches and prevented the subsequent inhibition of channel activity upon exposure to diamide (0.5 mM). The concentration of diamide applied was 100-fold greater than that required to significantly inhibit channel activity in nonalkylated patches (compare Fig. 4). The results of six similar experiments are summarized in Fig. 9 B.

We also examined the effect of MTSEA on channel activity. MTSEA is a thiol-specific reagent that covalently modifies free thiol groups on cysteine residues.

Figure 5. DTDP inhibits maxi-K channel activity. (A) Addition of the oxidizing agent 2,2'-dithiodipyridine (DTDP) also inhibited channel activity. Continuous current trace of a representative experiment showing the effect of 50 μM DTDP on maxi-K channel activity (holding potential = 10 mV). Large deflections were produced by a temporary increase of the gain of the patch-clamp amplifier to mark the drug addition event. (B) Average results from 7 patches exposed to 50 μM DTDP. * indicates statistical significance.

Figure 6. Oxidation reverses the stimulatory effect of patch reduction. (A) A continuous recording showing the stimulation of maxi-K activity by the reducing agent GSH, and the reversal of this effect after addition of an excess of the oxidizing agent diamide (holding potential = 40 mV). (B) Mean open-state probability during control, GSH treatment, and GSH plus diamide from 5 inside-out patches. * indicates statistical significance compared with control and GSH + diamide. n = the number of patches.
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(Akabas et al., 1992; Stauffer and Karlin, 1994). As shown in Fig. 10, MTSEA (2.5 mM) itself markedly inhibited channel activity, and this effect was not reversed by washout. Moreover, MTSEA eliminated the inhibitory action of thimerosal (10 mM). In the experiment shown, channel activity was increased by stepping to positive voltages, so that inhibitory effects would not be obscured by the low nPo following MTSEA. In MTSEA-treated patches ($n = 3$), subsequent exposure to thimerosal had no effect on channel activity. Experiments were also performed to determine whether MTSEA and NEM, both covalent modifiers of cysteine thiol groups, were functionally competitive. After channel inhibition by MTSEA, exposure to NEM did not further inhibit channel activity. In four patches, nPo was 0.0105 ± 0.0041 before and 0.0113 ± 0.0078 after NEM (data not shown). These data indicate that the inhibitory action of oxidizing agents on maxi-K channels requires the presence of reactive sulfhydryl groups. Moreover, the inhibition of maxi-K channel activity by NEM and MTSEA provides further evidence of the relationship between the state of critical cysteine sulfhydryl group(s) and channel activity. Taken together, these results provide further evidence that the modulation of maxi-K channel activity by redox reagents results from a chemical modification of sulfhydryl groups.

**Mechanism of Channel Modulation by Sulfhydryl Redox Agents**

The increase in maxi-K channel nPo, after sulfhydryl reduction could occur either by the recruitment of maxi-K channels that are unavailable for K* conductance in the oxidized state (increased n), or by an increase in the open probability (P_o) of available channels. To determine if an increase in available maxi-K channel
number is involved in the augmentation of channel activity after the reduction of cytosolic sulfhydryl groups, the effect of β-ME on macroscopic currents was examined in inside-out patches. The contribution of delayed rectifier potassium channels to the macroscopic currents was minimized by holding patches at −20 mV and by including 4-aminopyridine (5 mM) in the pipette solution (Boyle et al., 1992). Macroscopic currents from inside-out patches were averaged from voltage-clamp steps to 130 mV to determine whether the maximum conductance of individual patches increased after sulfhydryl reduction. An example of such an experiment and the normalized conductance for six patches before and after reduction is shown in Fig. 11. β-ME shifted the conductance-voltage relationship of the evoked currents by −17.7 mV, without altering either the maximum conductance or the slope values (14.8 and 16.6 for control and β-ME, respectively) of the Boltzmann fits (n = 6). It is apparent that sulfhydryl reduction has no influence on available channel numbers, and the major response to it is a shift in the voltage-Po relationship resulting from changes in channel gating. Typical traces illustrating single channel kinetics before and after treatment with thiol reagents are shown in Fig. 12. The traces indicate that reduction increased open-state probability primarily by decreasing the mean shut intervals between openings, and that the open-state duration was not substantially altered. Conversely, patch oxidation decreased nPo by increasing mean shut intervals (Fig. 12, bottom). Kinetic analysis indicated that mean open times were not different after exposure to thiol modifying agents, whereas the duration of shut intervals was decreased after sulfhydryl reduction (GSH) and increased after sulfhydryl oxidation (thimerosal) and alkylation (NEM) (data not shown). While the

Figure 9. The thiol alkylating agent NEM inhibits maxi-K channels and blocks the effect of oxidation. (A) Continuous trace showing the effects of Nethylmaleimide (NEM (1 mM)) and subsequent diamide (0.5 mM) on maxi-K activity in an inside-out patch (holding potential = 10 mV). After recording control (control 1) activity, the patch was exposed to NEM for 5 min and then washed. Channel activity was recorded for about 3 min (control 2) before addition of diamide (0.5 mM). (B) Mean data from six similar experiments. NEM inhibited maxi-K channel activity significantly (indicated by *), and diamide had no additional effect.

Figure 10. The thiol modifying reagent MTSEA inhibits Maxi-K channels and blocks the effect of oxidation. (A) The time course of maxi-K channel activity following addition of 2.5 mM (2-aminoethyl)methanethiosulfonate (MTSEA) to an inside-out patch. MTSEA rapidly inhibited channel activity, and this effect was not reversed after washout. Channel activity was increased by stepping from 0 to +30 mV before examining the inhibitory action of thimerosal (10 μM). Thimerosal had no effect on channel activity in patches previously exposed to MTSEA. (B) Current traces from the periods indicated.
presence of multiple channels in patches of smooth muscle membranes complicates the kinetic analysis (particularly the interpretation of closed times), these results together with the findings illustrated in Fig. 11 suggest that reducing agents increase open-state probability by increasing the probability that a shut channel will open, rather than altering the open-state dwell time.

**Discussion**

Our results demonstrate that maxi-K channels in smooth muscle cells are regulated by agents that alter the redox state of sulphydryl groups. We have shown that sulphydryl reduction increases nPo of the channel whereas oxidation has the opposite effect. Maxi-K channels in patches pulled from smooth muscle cells appear to exist in a mixed redox state, since either reduction or oxidation markedly affected channel activity. This mixed redox state could occur either because some channels in the patch exist in the reduced state whereas others are in the oxidized state, or because each channel has more than one redox modulatory site existing in different redox states. Since membrane patches from airway myocytes always contain multiple channels, we could not differentiate between these two possibilities.

We compared the normalized conductance-voltage curves constructed from macroscopic currents in response to step-depolarization during control and after sulphydryl reduction to determine whether reduction would lead to an increase in the maximum conductance of the patch. The conductance-voltage curve was shifted leftward following β-ME, but there was no increase in the maximal conductance, suggesting that the increase in nPo resulted from an increase in the open probability of initially available channels (Fig. 11) and that reduction does not lead to the opening of previously silent channels.

The thiol specificity of the redox agents was confirmed by experiments with NEM and MTSEA. NEM is a thiol-alkylating agent, which is commonly used to trap protein thiols in their existing redox states (Creighton, 1984; Gilbert, 1995). Alkylation by NEM removes free thiols and should therefore prevent the formation of disulfides in response to sulphydryl oxidizing agents. In the present study, maxi-K channel activity was inhibited after exposure to NEM. In addition, the inhibitory effect of diamide was abolished by NEM pretreatment, as would be predicted for a thiol-specific action (Petroilli et al., 1994). We did not test the effect of reducing agents following NEM pretreatment because NEM alkylates free thiols but not disulfides, and, therefore, reducing agents may still work after NEM pretreatment in channels existing in “mixed” redox states. We also used MTSEA, which reacts specifically and rapidly with thiols to form mixed disulfides (Akabas et al., 1992; Stauffer and Karlin, 1994), to investigate the thiol-specific nature of redox modulation. As with NEM, exposure of inside-out patches to MTSEA inhibited maxi-K channel activity and blocked the modulatory action of oxidizing agents. After treatment of inside-out patches with MTSEA, thimerosal no longer inhibited channel activity (Fig. 10). Moreover, the inhibitory actions of MTSEA and NEM were mutually exclusive, in that following treatment with MTSEA, NEM no longer modulated channel activity. Taken together, these experiments strongly support the hypothesis that the inhibition of channel activity by oxidizing agents results from reactions involving one or more protein thiol groups.

The mechanisms of action of the redox agents used are well known. Reducing and oxidizing agents exert
their effects through two sequential thiol-disulfide exchanges with a mixed disulfide of the redox agent and a protein cysteine residue as the intermediate (Creighton, 1993; Brocklehurst, 1979; Kosower and Kosower, 1995). The opposite actions of sulfhydryl reducing and oxidizing agents and the reciprocal reversal of their actions on nPo are consistent with a mechanism of thiol/disulfide exchange.

It is not clear how changes in redox state regulate maxi-K channel activity. Since the activity of this channel is highly dependent on Ca\(^{2+}\), one possibility for the change in nPo is that thiol/disulfide exchanges are associated with changes in protein conformation that influence channel Ca\(^{2+}\)-binding affinity, which determines the rate constant for channel gating. Free protein thiols can exist either in the reduced state (-SH) or as thiolate anions (-S\(^2\)) depending on pH and the pKa of the thiol under consideration. One possibility is that one or more sulfhydryl groups close to the Ca\(^{2+}\)-binding region of the channel exists in the anion form, contributing to the Ca\(^{2+}\)-binding affinity. The formation of disulfides, or side chain modification by NEM or MTSEA, would eliminate the negative charge and lower the Ca\(^{2+}\)-binding affinity. Although cysteine thiols have an intrinsic pKa in the range of 9.0–9.5 (Creighton, 1993), thiols attached to protein molecules may deviate from this typical value by many orders of magnitude, due to electrostatic interactions within the protein (Gilbert, 1990). For example, the pKa of one of the two thiols in the active site of lipoamide dehydrogenase is < 5.5 (Gilbert, 1990).

Thiol/disulfide exchange involves covalent modifications, which occur only when appropriate electron acceptors (oxidizing agents) or donors (reducing agents) are present. We found that alterations of channel activity after redox modification were not reversed by washout of the redox agents, but were rapidly reversed by exposure to the counteracting reagents. The covalent nature of the modification likely underlies the fact that channel rundown is not commonly observed following patch excision, even though the reducing power of cytosolic solution is likely stronger than routine patch-clamp solutions.

We believe that the modulatory actions reported here are likely to be of physiological relevance. The intracellular concentration of GSH ranges from 0.1 to 10 mM (Meister, 1995), and in the present study GSH augmented channel activity significantly at a concentration as low as 50 \(\mu\)M, and increased channel activity over eightfold at 170 \(\mu\)M for GSH. Data were filtered at 2 kHz and sampled at 5 kHz.

![Figure 12. The action of thiol reagents on single channel kinetics. Representative traces of single channel kinetics before and after thiol modification. The major kinetic feature of oxidation by thimerosal (top) or alkylation by NEM (middle) was an increase in the duration of the long shut intervals, with no effect on open times. Conversely, sulfhydryl reduction by GSH markedly decreased long closed times without affecting open times (bottom).](image-url)
as kinases and phosphatases, and will therefore tend to confuse the interpretation of these experiments. This complication can be particularly pernicious, since we have observed that the augmenting effect of DTT on maxi-K channels is removed by boiling the buffer solution. Therefore experiments using protein preparations containing sulfhydryl reducing agents should be interpreted with caution, and boiling of the protein preparation alone is not adequate to rule out buffer actions.

In summary, we report the modulation of maxi-K channel gating by alterations in the redox state of the patch. The site of redox modulation is likely a cytosolic cysteine residue or residues(s), since the poorly membrane-permeant reducing agent GSH and oxidizing agent thimerosal altered maxi-K channel activity only when applied to the intracellular side of the patch membrane. Alkylation of sulfhydryl groups ablated the redox modulation. We have recently performed experiments on the recombinant α-subunit of maxi-K channels expressed in Xenopus oocytes and shown that the expressed channels are modulated by redox agents in a similar manner. Experiments are currently underway to locate the cysteine residue(s) responsible for the redox modulation.

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