Cysteine Modification Alters Voltage- and Ca\(^{2+}\)-dependent Gating of Large Conductance (BK) Potassium Channels

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ABSTRACT The Ca\(^{2+}\)-activated K\(^{+}\) (BK) channel α-subunit contains many cysteine residues within its large COOH-terminal tail domain. To probe the function of this domain, we examined effects of cysteine-modifying reagents on channel gating. Application of MTSET, MTSES, or NEM to mSlo1 or hSlo1 channels changed the voltage and Ca\(^{2+}\) dependence of steady-state activation. These reagents appear to modify the same cysteines but have different effects on function. MTSET increases I\(_{K}\) and shifts the G\(_{K}-V\) relation to more negative voltages, whereas MTSES and NEM shift the G\(_{K}-V\) in the opposite direction. Steady-state activation was altered in the presence or absence of Ca\(^{2+}\) and at negative potentials where voltage sensors are not activated. Combinations of [Ca\(^{2+}\)] and voltage were also identified where P\(_{o}\) is not changed by cysteine modification. Interpretation of our results in terms of an allosteric model indicate that cysteine modification alters Ca\(^{2+}\) binding and the relative stability of closed and open conformations as well as the coupling of voltage sensor activation and Ca\(^{2+}\) binding and to channel opening. To identify modification-sensitive residues, we examined effects of MTS reagents on mutant channels lacking one or more cysteines. Surprisingly, the effects of MTSES on both voltage- and Ca\(^{2+}\)-dependent gating were abolished by replacing a single cysteine (C430) with alanine. C430 lies in the RCK1 (regulator of K\(^{+}\) conductance) domain within a series of eight residues that is unique to BK channels. Deletion of these residues shifted the G\(_{K}-V\) relation by >−80 mV. Thus we have identified a region that appears to strongly influence RCK domain function, but is absent from RCK domains of known structure. C430A did not eliminate effects of MTSET on apparent Ca\(^{2+}\) affinity. However an additional mutation, C615S, in the Haem binding site reduced the effects of MTSET, consistent with a role for this region in Ca\(^{2+}\) binding.

KEY WORDS: calcium • potassium channel • BK channel • cysteine • RCK domain

INTRODUCTION

Large conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK) channels participate in many important physiological processes through their ability to respond to changes in membrane voltage and intracellular Ca\(^{2+}\). Therefore, mechanisms that underlie or regulate voltage- and Ca\(^{2+}\)-dependent gating are important to understand. The pore-forming α-subunit of BK channels contains a core domain resembling that of other voltage-dependent K\(^{+}\) channels with six transmembrane segments (S1–S6) including a charge S4 voltage sensor (Adelman et al., 1992; Butler et al., 1993). In addition, the channel contains a cytosolic COOH-terminal tail domain that represents almost 70% of the channel protein as well as a unique NH\(_{2}\)-terminal S0 transmembrane segment (Meera et al., 1997). Regions within the tail domain have been identified that influence channel activation by micromolar Ca\(^{2+}\) (Schreiber and Salkoff, 1997; Schreiber et al., 1999; Bao et al., 2002, 2004; Qian et al., 2002; Xia et al., 2002) as well as many other aspects of channel function under physiological or pathophysiological conditions, including regulation by millimolar Ca\(^{2+}\) or Mg\(^{2+}\) (Shi et al., 2002; Xia et al., 2002), protons (Avdonin et al., 2003), Heam-binding (Tang et al., 2003), phosphorylation (Schubert and Nelson, 2001), and oxidation (Tang et al., 2001, 2004).

Although considerable progress has been made in identifying functional elements within the BK channel tail, many features of the structure and function of this very large domain remain unknown. Even where important regions have been characterized, it is often unclear how they interact with each other or the transmembrane core to influence channel activation. An important aspect of the mechanisms by which tail domain elements influence function must be their eventual coupling to channel opening, a linkage that potentially involves multiple protein domains and interactions, including effects on voltage- or Ca\(^{2+}\)-dependent gating. Thus, it is likely that the tail domain contains not only regulatory elements that interact directly with signaling molecules, but also regions that are critical for coupling the action of these regulatory elements to the channel gate.

Two RCK (regulator of K\(^{+}\) conductance) homology domains (RCK1 and RCK2) have been identified within the NH\(_{2}\)-terminal half of the BK channel tail, encompassing several functionally important regions.

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Abbreviation used in this paper: WT, wild type.
The crystal structure of RCK domains are known for several prokaryotic proteins, including the Ca\(^{2+}\)-dependent MthK channel (Jiang et al., 2001, 2002), providing a potential model of BK channel structure and function. By analogy with MthK, conformational changes in the BK channel RCK domains are proposed to be coupled to the pore through the S6–RCK1 linker (Jiang et al., 2002). Effects of linker modification on BK channel gating appear consistent with this hypothesis (Niu et al., 2004). However, the RCK domains of BK and MthK channels share <20% sequence identity, suggesting that their structures are not identical and that important differences may exist regarding interactions that occur within the RCK domain and with the rest of the channel protein. For example, a low-affinity Mg\(^{2+}\)/Ca\(^{2+}\) binding site has been identified in the RCK1 domain that is not present in MthK (Shi et al., 2002; Xia et al., 2002). In addition, Mg\(^{2+}\)-dependent activation of BK channels is inhibited by mutations in the S4 and S4–S5 linker, suggesting that interactions exist between the RCK1 domain and voltage sensor, whereas MthK is a two transmembrane channel that has no voltage sensor (Hu et al., 2003).

In the present study, we probed the function of the tail domain by studying changes in BK channel gating produced by various cysteine-modifying reagents (MTSES, MTSET, and NEM) applied to the intracellular side of the channel. Of the 30 cysteine residues present in the mSlo1 BK channel α-subunit, only 3 are in the core domain, and these are located in extracellular regions of the NH\(_2\) terminus, S1–S2 linker, and pore loop. The majority of cysteines (24) are scattered throughout the tail domain and three more are located in the intracellular S0–S1 linker. Cysteine modification by thiol-specific MTS reagents or oxidation has been shown to inhibit BK channel activity (Dichiara and Reinhart, 1997; Tang et al., 2001, 2004; Erxleben et al., 2002). However, the functional changes and cysteines that underlie these changes have been only partially characterized.

Here, we determine the effects of cysteine modification on steady-state and kinetic properties of I\(_{Ks}\) over a wide range of conditions to determine the impact of modification on voltage- and Ca\(^{2+}\)-dependent gating as well as the relative stability of the closed and open conformation. BK channel gating is well described in terms of allosteric mechanisms where the closed–open conformational change does not require Ca\(^{2+}\) binding or voltage sensor activation but is promoted by either (Cox et al., 1997a; Horrigan and Aldrich, 1999, 2002; Horrigan et al., 1999; Rothberg and Magleby, 1999, 2000; Cui and Aldrich, 2000). By measuring steady-state activation under conditions that include 0 Ca\(^{2+}\) and extreme negative voltages, we can isolate effects on the closed–open transition from those on Ca\(^{2+}\)- or voltage-dependent gating mechanisms. Moreover, we can distinguish whether perturbations to Ca\(^{2+}\)- or voltage-dependent gating reflect changes in the activation of Ca\(^{2+}\) or voltage sensors or a change in the coupling of these sensors to channel opening (Horrigan and Aldrich, 2002).

By characterizing in detail the changes in BK channel gating produced by cysteine modification, we seek not only to identify important regions of the tail domain but also to understand the mechanisms by which these regions influence channel function. C911 has been identified previously in hSlo1 as a key cysteine whose modification is largely responsible for inhibitory effects of cysteine oxidation or MTSEA on BK channel activation (Tang et al., 2004). Modification of C911 is thought to inhibit Ca\(^{2+}\) binding to the nearby Ca\(^{2+}\)-bowl domain (Schreiber and Salkoff, 1997). However, the effects on channel gating of cysteine-modifying reagents in our experiments differ from those described by Tang et al. (2004), and involve additional sites of action. Thus, we find that modification of C615 by MTSET also reduces apparent Ca\(^{2+}\) affinity, suggesting that the Heam-binding site, which includes C615, may influence Ca\(^{2+}\) binding. In addition, we find that modification of C430 in the RCK1 domain alters the relative stability of closed and open conformations as well as the coupling of both voltage sensor activation and Ca\(^{2+}\) binding to channel opening. The impact of C430 is particularly interesting because it lies in a region of the RCK domain that is not present in MthK. The effects on gating of C430 modification, or deletion of a region including C430, are discussed in light of known features of RCK structure and the recent proposal that the BK channel RCK domain exhibits spring-like mechanical properties (Niu et al., 2004).

**Materials and Methods**

**Mutagenesis and Channel Expression**

Experiments were performed with the mbk5 clone of the mouse homologue of the Slo1 gene (mSlo1) (Butler et al., 1993) provided by L. Salkoff (Washington University, St. Louis, MO) in a BlueScript vector (Stratagene), or human homologue hSlo1 (hbr1) (Tseng-Crank et al., 1994) provided by T. Hoshi (University of Pennsylvania, Philadelphia, PA) in a pCI-neo vector (Promega). hSlo1 point mutants (C348A, C422A, C430A, C615S, C6285, C6305, and C911A) and C(1–13)A, C(18–29)A, and C(1–13)AC(18–29)A constructs were also provided by T. Hoshi (Tang et al., 2001; Avdonin et al., 2003). For constructs containing multiple C to A mutations, the numbers in parentheses refer to sequentially numbered cysteines where the actual position in hSlo1 or mSlo1 are 14, 53, 54, 56, 141, 277, 348, 422, 430, 485, 498, 554, 557, 612, 615, 628, 630, 695, 722, 797, 800, 820, 911, 975, 995, 1001, 1011, 1028, and 1051. The hSlo1-ΔD construct in which eight amino acids were deleted (428–435) was prepared by overlap extension PCR, subcloned into full-length hSlo1-pCI-neo, and verified by sequencing. cRNA for mSlo1 was synthesized.
from BamH1-linearized cDNA using T3 polymerase and for hSlo1 and hSlo1 mutants from NotI-linearized cDNA using T7 polymerase. *Xenopus* oocytes were injected with ~0.5–5 ng of cRNA 4–20 d before recording and maintained at room temperature.

**Electrophysiology**

Currents were recorded using the patch clamp technique in the inside out configuration (Hamill et al., 1981). Upon excision, patches were washed with at least 20× volumes of internal solution. K+ currents were recorded with internal solutions containing (in mM) 110 KMeSO4 and 20 HEPES and an external (pipette) solution containing 100 KMeSO4, 10 KCl, 2 MgCl2, 20 HEPES. Internal solutions contained 40 μM (+)-18-crown-6-tetracarboxylic acid to chelate contaminant Ba2+ (Diaz et al., 1996; Neya, 1996; Cox et al., 1997b). “0 Ca2+” solutions contained 2 mM EGTA reducing free Ca2+ to an estimated 0.8 mM based on the presence of ~10 μM contaminant Ca2+ (Cox et al., 1997b). Ca2+ solutions were buffered with 5 mM HEDTA, and free Ca2+ was measured with a Ca2+ electrode (Orion Research Inc.). Nominal [Ca2+] reported as 1, 5, 10, 20, and 70 μM correspond to measured concentrations of 1.3, 4.4, 9.9, 17, and 66 μM, respectively. Ca2+ was added as CaCl2 and [Cl–] was adjusted to 10 mM with HCl. The pH of all solutions was 7.2. Solutions were prepared and experiments performed at room temperature (22–24°C).

Electrodes were pulled from thick-walled 1010 glass (World Precision Instruments), coated with wax (KERR sticky wax) and fire polished before use. Pipette access resistance measured in the bath solution (0.8–1.5 MΩ) was used as an estimate of series resistance (Rg) to correct the voltage at which Ig was recorded. The corrected voltage was used in determining membrane conductance (Gm) from tail current measurements and in plotting the voltage dependence of Gm. Series resistance error was <15 mV for all data presented.

Data were acquired with an Axopatch 200B amplifier (Axon Instruments) set in patch mode with the amplifier’s internal 4-pole Bessel filter set at 100 kHz. Currents were subsequently filtered by an 8-pole Bessel filter (Frequency Devices Inc.) at 20 kHz and sampled at 100 kHz with an 18 bit A/D converter (Instrutech ITC-18). A P/4 protocol was used for leak subtraction (Armstrong and Bezanilla, 1974) with a holding potential of 0 mV for all data presented.

**RESULTS**

**Effects of Cysteine-modifying Reagents on mSlo1 Channel Function**

To study the effects on BK channel gating of modifying cysteine residues, membrane patches expressing mSlo1 channels were excised from *Xenopus* oocytes in the inside-out configuration and the thiol-reactive reagents, MTSES, MTSET, or NEM, were applied by exchanging the bath solution. MTSES (−) and MTSET (+) are charged and presumably modify only cysteines accessible from the intracellular solution, whereas NEM is membrane permeable and may modify additional sites. Spontaneous changes in BK channel gating, including a shift in the Gm–V relation to more positive voltages, are often observed following patch excision and may reflect oxidation of cysteine and/or methionine residues (DiChiara and Reinhart, 1997; Tang et al., 2001). Therefore, we typically waited ~30 min following patch excision to allow the Gm–V relation to stabilize, before acquiring control records and applying cysteine-modifying reagents. While this procedure may allow some cysteines to become oxidized, it assures that effects of cysteine-modifying reagents were studied in the virtual absence of spontaneous changes in gating.

During reagent application, the membrane was held at ~80 mV, and macroscopic potassium currents (Ik) were evoked by brief 25-ms test pulses every 10 s to a potential near the half-activation voltage (Vh).
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Figure 1. Modification and block of mSlo1 channels by MTSES, MTSET, and NEM. (A–C) $I_K$ evoked by 25-ms pulses to the indicated voltage before (control) and during treatment with the indicated cysteine-modifying reagent. MTSES in 0 Ca$^{2+}$ or NEM in 5 μM Ca$^{2+}$ cause a slow decrease in both steady-state and tail $I_K$, whereas MTSET in 0 Ca$^{2+}$ causes a rapid increase. Test pulses were delivered every 10 s from a holding potential of −80 mV, and reagent was applied by exchanging the bath solution. $I_K$ during the pulse and tail currents immediately following the pulse are plotted on different time scales (see scale bars). $I_K$ was plotted for each test pulse in the case of MTSET and MTSET but was plotted for every third pulse in the case of NEM. (D and E) Instantaneous $I_{K-V}$ relations in the presence or absence of MTS reagents demonstrate that channels are reversibly blocked at positive voltages. (D) The effect of 1 mM Na-MTSES (Δ) and 1 mM NaCl (O) are similar, suggesting that the Na$^+$ counter ion is responsible for the blocking effect of MTSES. (E) 1000 μM MTSET (O) blocks $I_K$ by 50% at +200 mV, but a much smaller effect is seen with 100 μM MTSET (Δ). $I-V$s were determined by maximally activating channels with a test pulse ($V = +200$ mV, [Ca$^{2+}$] = 5 μM) and measuring $I_K$ at different voltages immediately following the test pulse. Controls obtained before reagent application and after washout (filled symbols) were normalized at −80 mV. $I-V$s in the presence of reagent were measured after steady-state modification and were normalized to $I_K$ (−80) of the corresponding washout $I-V$. (F–H) $G_{K-V}$ relations determined from isochronal tail currents at −80 mV before (control) and after steady-state modification by the indicated reagent, corresponding to the conditions in A–C. Control and modified $G-V$s were measured in the same solutions (in the absence of reagent) and are fit by Boltzmann functions. MTSES in 0 Ca$^{2+}$ increases $V_h$ and decreases $G_{K_{\text{max}}}$ (control: $V_h = 210$ mV, $G_{K_{\text{max}}} = 71$ nS, $z = 1.397$); MTSES: $V_h = 249$ mV, $G_{K_{\text{max}}} = 43$ nS, $z = 1.059$). A similar effect is observed for NEM in 5 μM Ca$^{2+}$ (control: $V_h = 147$ mV, $G_{K_{\text{max}}} = 89$ nS, $z = 1.011$); NEM: $V_h = 168$ mV, $G_{K_{\text{max}}} = 68$ nS, $z = 0.907$). However, MTSET in 0 Ca$^{2+}$ produces a decrease in $V_h$ with $G_{K_{\text{max}}}$ held constant (control: $V_h = 237$ mV, $G_{K_{\text{max}}} = 105$ nS, $z = 1.13$); MTSET: $V_h = 207$ mV, $G_{K_{\text{max}}} = 105$ nS, $z = 1.048$).

(A–C) shows the effect on $I_K$ of 1 mM MTSES, 100 μM MTSET, and 20 mM NEM, respectively. MTSES, applied in the absence of Ca$^{2+}$, produced a gradual decrease in both $I_K$ evoked at +160 mV and tail current following the test pulse (Fig. 1 A). An approximate 60% steady-state decrease in current amplitude was observed after 250 s. By contrast, MTSET in 0 Ca$^{2+}$ produced a rapid twofold increase in $I_K$ within the 10-s interval between test pulses (Fig. 1 B). NEM, like MTSES, produced a gradual decrease in $I_K$, achieving an 80% steady-state reduction in 300 s in the presence of 5 μM Ca$^{2+}$ (Fig. 1 C).
Most effects of MTSES, MTSET, or NEM were not reversed by washout of the reagent and presumably reflect modification of cysteine residues. However, a small increase in outward current was often observed immediately upon washout of MTSES or MTSET. This increase reflects relief of channel block by the MTS reagent or its counter-ion. Instantaneous current–voltage relations obtained in the presence and absence of MTSES (Fig. 1 D) or MTSET (Fig. 1 E) demonstrate this blocking effect. At negative voltages, \( I_k \) is unaffected by washout. However, the I–V relation, which is relatively linear in the absence of reagent, rectifies in the presence of MTSES or MTSET such that \( I_k \) is reduced increasingly at more positive voltages. This rectification is consistent with rapid voltage-dependent block by a positively charged ion from the intracellular solution. In the case of MTSET(+), the reagent itself may block the channel. In the case of MTSES(−), the counter-ion Na\(^+\) is known to block BK channels (Kehl, 1996; Morales et al., 1996); and 1 mM NaCl reproduces the effect of 1 mM Na-MTSES on the I–V relation (Fig. 1 D).

To minimize the influence of channel block on analysis of cysteine modification, concentrations of MTSET and MTSES were limited to 100 \( \mu M \) and 1 mM, respectively. In addition, the time course of modification and conductance–voltage (\( G_k\)-V) relations were determined by measuring tail currents at a voltage (−80 mV) where block is negligible. Finally, reagent was washed out after steady-state modification when possible so that \( I_k \) could be compared before and after modification in the absence of reagent. \( G_k\)-V relations obtained in this way before and after modification by MTSES (Fig. 1 F), MTSET (Fig. 1 G), and NEM (Fig. 1 H) indicate that changes in \( I_k \) amplitude observed in Fig. 1 (A–C) reflect a shift of the \( G_k\)-V along the voltage axis and, for MTSES and NEM, a decrease in \( G_{k_{max}} \) estimated from fits to a Boltzmann function.

Effects of Cysteine Modification are \( Ca^{2+} \) Dependent

Because BK channel activation is \( Ca^{2+} \) dependent, we examined the effects of applying cysteine-modifying reagents in different \([Ca^{2+}]_i\), from 0 to 70 \( \mu M \). At each \([Ca^{2+}]_i\), test pulse voltage was less than or equal to \( V_h \) such that \( I_k \) should change if the \( G_k\)-V relation is altered. However, marked differences in the extent to which \( I_k \) changed were observed in different \([Ca^{2+}]_i\), (Fig. 2).

In contrast to the \( I_k \) decrease produced by MTSES in 0 \( Ca^{2+} \) (Fig. 1 A), MTSES in 5 \( \mu M \) \( Ca^{2+} \) had almost no effect on \( I_k \) and produced only a small immediate decrease in outward current, consistent with Na+ block (Fig. 2 A). Fig. 2 B plots \( G_k \) determined from tail current amplitude versus time for experiments in 0 \( Ca^{2+} \) and 5 \( \mu M \) \( Ca^{2+} \), similar to those in Fig. 1 A and Fig. 2 A. In 0 \( Ca^{2+} \), \( G_k \) was stable before MTSES was applied and then decreased by almost 80% to a steady state within 500 s, with a time course that can be approximated by an exponential function (line, \( \tau = 61 \) s). In 5 \( \mu M \) \( Ca^{2+} \), \( G_{k_{max}} \) decreased by <10% over the same time period. This small slow decrease cannot be distinguished from the spontaneous rundown of \( G_k \) that sometimes occurs in the absence of reagent presumably due to channel oxidation. A similar failure of MTSES to alter \( G_{k_{max}} \) was observed at all \([Ca^{2+}]_i\), >1 \( \mu M \) (unpublished data).

MTSET and NEM, unlike MTSES, significantly altered \( G_k \) in 5 \( \mu M \) \( Ca^{2+} \). 100 \( \mu M \) MTSET increased \( G_k \) by 50% with a time course, as in 0 \( Ca^{2+} \), that was too rapid to resolve (Fig. 2 D). 20 \( mM \) NEM decreased \( G_k \) by >80% with an exponential time course (\( \tau = 127 \) s; Fig. 2 F). However, conditions were also identified where changes in \( G_k \) produced by MTSET or NEM are small. Surprisingly, MTSET did not alter \( G_k \) in 1 \( \mu M \) \( Ca^{2+} \) (Fig. 2, C and D), although increases were observed in both higher (5 \( \mu M \)) and lower (0) \([Ca^{2+}]_i\), (Fig. 2 D). Conversely, the effect of NEM on \( G_k \) in 0 \( Ca^{2+} \) (Fig. 2, E and F) or 70 \( \mu M \) \( Ca^{2+} \) (Fig. 2 F) was much less than that observed in 5 \( \mu M \) \( Ca^{2+} \).

Cysteine Modification is Not Prevented by \( Ca^{2+} \) Binding

The results in Fig. 2 demonstrate that interaction among cysteine-modifying reagents, \( Ca^{2+} \), and BK channel gating is complex. Not only are the effects of MTSES, MTSET, and NEM on \( G_k \) \( Ca^{2+} \) dependent, but the pattern of \( Ca^{2+} \) dependence for each reagent is different. To understand these results, it’s important to distinguish between channel modification and the modification effect. Modification refers to the reaction of reagent with the BK channel protein, most likely forming a covalent bond with one or more cysteine residues. The modification effect is the change in channel function produced by cysteine modification. Although a modification effect indicates that cysteines have been modified, the converse is not necessarily true. That is, failure to observe a modification effect does not indicate a lack of cysteine modification if modification has no effect on function or produces complex changes in channel gating that are difficult to detect.

Two mechanisms could account for the failure to observe a modification effect at certain \([Ca^{2+}]_i\), in Fig. 2. First, the accessibility of cysteine residues to reagent might be state dependent such that the rate of modification is influenced by \( Ca^{2+} \) binding. That is, modification of key cysteines might be inhibited by the presence or absence of \( Ca^{2+} \). Second, cysteine modification might alter the \( Ca^{2+} \) sensitivity of channel gating such that the change in \( G_k \) produced by modification at each \([Ca^{2+}]_i\), is different. Experiments shown in Fig. 3 suggest that the latter mechanism accounts for the results in Fig. 2.

To test whether failure to observe a modification effect reflects a failure to modify cysteines, we first ap-
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Applied reagent at \([\text{Ca}^{2+}]_i\) where no modification effect is observed and then assayed for modification by either changing \([\text{Ca}^{2+}]_i\) (Fig. 3, A and B) or applying a second reagent (Fig. 3, C and D).

Fig. 3 A plots G–V relations in 20 and 70 μM Ca\(^{2+}\) before and after application of NEM. The corresponding sequence of solution changes and \(V_h\) measurements are indicated in Fig. 3 B. G–V\(^s\) were initially measured in 20 and 70 μM Ca\(^{2+}\), and then 20 mM NEM was applied for 300 s in the presence of 70 μM Ca\(^{2+}\), producing no change in \(V_h\) (Fig. 3 B) or \(G_K\) (Fig. 2 F). G–V\(^s\) obtained in 70 μM Ca\(^{2+}\) before application of NEM and after washout are indistinguishable (Fig. 3 A). However, the G–V in 20 μM Ca\(^{2+}\) measured after washout of NEM was shifted by \(-16\) mV relative to the control, similar to a \(-22 \pm 5\) mV shift (mean \(\pm\) SEM) observed when NEM was applied in the presence of 20 μM Ca\(^{2+}\). Thus NEM in 70 μM Ca\(^{2+}\) does modify cysteines and alter channel gating despite failure to alter steady-state activation in 70 μM Ca\(^{2+}\).

Fig. 3 (C and D) plots \(G_K\) in 5 μM Ca\(^{2+}\) as MTSES is applied for 200 s before applying NEM or MTSET, respectively. When applied individually in 5 μM Ca\(^{2+}\) (\(V_{\text{test}} = +160\) mV) or 0 μM Ca\(^{2+}\) (\(V_{\text{test}} = +200\) mV), the time course of \(G_K\) decay in 0 Ca\(^{2+}\) is fit by an exponential function (line, \(\tau = 61\) s). (C) MTSET has little effect on \(I_K\) evoked by \(+200\) mV pulses in 1 μM Ca\(^{2+}\). (D) \(G_K\) in 0 Ca\(^{2+}\) (\(V_{\text{test}} = +200\) mV) or 5 μM Ca\(^{2+}\) (\(V_{\text{test}} = +140\) mV) is increased rapidly by 100 μM MTSET, but no similar response is observed in 1 μM Ca\(^{2+}\) (\(V_{\text{test}} = +180\) mV). (E) NEM reduces both outward and tail \(I_K\) evoked by \(+200\) mV test pulses in 0 Ca\(^{2+}\). (F) \(G_K\) is slowly reduced by 20 mM NEM in 0 Ca\(^{2+}\) (\(V_{\text{test}} = +200\) mV), 5 μM Ca\(^{2+}\) (\(V_{\text{test}} = +140\) mV), or 70 μM Ca\(^{2+}\) (\(V_{\text{test}} = +40\) mV). However, the decrease is greatest in 5 μM Ca\(^{2+}\), where the time course of \(G_K\) decay is fit by an exponential function (line, \(\tau = 127\) s).

Figure 2. The effects of cysteine-modifying reagents on \(I_K\) are Ca\(^{2+}\)-dependent. (A) \(I_K\) evoked during test pulses to +200 mV in 5 μM Ca\(^{2+}\) is reduced immediately by 1 mM MTSES without altering tail current, consistent with reversible block (see Fig. 1 D) rather than a change in \(P_o\). (B) \(G_K\), determined for each test pulse from tail current amplitude at −80 mV and normalized to \(t = 0\), is plotted versus time as 1 mM MTSES is applied either in 5 μM Ca\(^{2+}\) (\(V_{\text{test}} = +160\) mV) or 0 μM Ca\(^{2+}\) (\(V_{\text{test}} = +200\) mV). The time course of \(G_K\) decay in 0 Ca\(^{2+}\) is fit by an exponential function (line, \(\tau = 61\) s). (C) MTSET has little effect on \(I_K\) evoked by \(+200\) mV pulses in 1 μM Ca\(^{2+}\). (D) \(G_K\) in 0 Ca\(^{2+}\) (\(V_{\text{test}} = +200\) mV) or 5 μM Ca\(^{2+}\) (\(V_{\text{test}} = +140\) mV) is increased rapidly by 100 μM MTSET, but no similar response is observed in 1 μM Ca\(^{2+}\) (\(V_{\text{test}} = +180\) mV). (E) NEM reduces both outward and tail \(I_K\) evoked by \(+200\) mV test pulses in 0 Ca\(^{2+}\). (F) \(G_K\) is slowly reduced by 20 mM NEM in 0 Ca\(^{2+}\) (\(V_{\text{test}} = +200\) mV), 5 μM Ca\(^{2+}\) (\(V_{\text{test}} = +140\) mV), or 70 μM Ca\(^{2+}\) (\(V_{\text{test}} = +40\) mV). However, the decrease is greatest in 5 μM Ca\(^{2+}\), where the time course of \(G_K\) decay is fit by an exponential function (line, \(\tau = 127\) s).
that underlie the modification effects of MTSET and NEM. This conclusion is supported by observations that MTSET pretreatment prevents the modification effects of NEM and MTSES (unpublished data). Thus, all three reagents appear to modify the same cysteine residues.

**Effects of Cysteine Modification on I\(_K\) Kinetics**

Changes in steady-state activation produced by cysteine modification are accompanied by changes in I\(_K\) kinetics (Fig. 4). Comparison of normalized currents evoked by voltage pulses before and after MTSES treatment in 0 Ca\(^{2+}\) (Fig. 4 A) shows that modification slows I\(_K\) activation at +240 mV 2.4-fold but has no effect on tail current decay at −80 mV. This is also evident in Fig. 4 B, which plots the time constants of I\(_K\) relaxation (\(\tau(I_K)\)) at different voltages in 0, 5, and 70 \(\mu\)M Ca\(^{2+}\) before and after steady-state modification. In 0 Ca\(^{2+}\), \(\tau(I_K)\) is increased at V \(\geq +200\) mV but is unchanged from +240 to +180 mV. That \(\tau(I_K)\) increases only at the most positive voltages suggests that cysteine modification slows the rate-limiting forward transitions from closed to open (activation) without altering the reverse transition (deactivation), consistent with a decrease in open probability and shift of the G–V to more positive voltages in 0 Ca\(^{2+}\) (Fig. 1 F). Likewise, \(\tau(I_K)\) is not altered in 70 \(\mu\)M Ca\(^{2+}\) (Fig. 4 B), consistent with the lack of a G–V shift in high [Ca\(^{2+}\)]. However, in 5 \(\mu\)M Ca\(^{2+}\), activation is slowed (Fig. 4, A and B), although I\(_K\) amplitude is unchanged (Fig. 2 B; Fig. 3, C and D). This result provides additional evidence that channels are modified even when steady-state activation is unchanged, and suggests that MTSES does not act merely to alter the rate-limiting transition.

MTSET slows I\(_K\) deactivation without affecting activation (Fig. 4, C and D), an effect opposite to that of MTSES. The time course of I\(_K\) evoked during a pulse to +240 mV in 0 Ca\(^{2+}\) is unchanged by MTSET, but the tail current decay at −80 mV is slowed (Fig. 4 C). \(\tau(I_K)\)–V relationships in 0, 5, or 70 \(\mu\)M Ca\(^{2+}\) (Fig. 4 D) exhibit similar responses to MTSET, increasing at voltages more negative than peak \(\tau(I_K)\) and unchanged at the most positive voltages. A slowing of deactivation is consistent with the increase in steady-state activation observed at these [Ca\(^{2+}\)]. Likewise, in 1 \(\mu\)M Ca\(^{2+}\), no change in either I\(_K\) kinetics (Fig. 4 C) or amplitude (Fig. 2 D) is observed.

Effects of NEM on the \(\tau(I_K)\)–V relation were not examined. However, the response to test pulses in 0, 10, 50, and 100 \(\mu\)M Ca\(^{2+}\) was examined before and after treatment with 20 mM NEM for 300 s in 70 \(\mu\)M Ca\(^{2+}\), producing no change in \(V_h\). However, subsequent measurement of \(V_h\) in 20 \(\mu\)M Ca\(^{2+}\) was shifted by −16 mV relative to the control, indicating that channels were modified by NEM in 70 \(\mu\)M Ca\(^{2+}\). (C and D) G\(_K\) measured from test pulse tail currents in 5 \(\mu\)M Ca\(^{2+}\) (\(V_{test} = +160\) mV) (*) are plotted versus time as patches are pretreated with 1 mM MTSES for the indicated period before applying 20 mM NEM (C) or 100 \(\mu\)M MTSET (D). MTSES pretreatment prevents the effects of NEM or MTSET, indicating that channels were modified by MTSES in 5 \(\mu\)M Ca\(^{2+}\) and that MTSES modifies the same cysteines as MTSET and NEM. The responses to NEM or MTSET without MTSES pretreatment are indicated by solid lines taken from Fig. 2, B and D, respectively.
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and 70 μM Ca\(^{2+}\) (Fig. 4 E) reveals a slowing of I\(_K\) activation with little effect on tail current decay, similar to the effect of MTSES and consistent with the decrease in steady-state activation produced by NEM (Fig. 2 F).

**Cysteine Modification Alters the Ca\(^{2+}\) Dependence of Steady-state Activation**

The above results indicate that cysteines are modified even at [Ca\(^{2+}\)]\(_i\) where no change in steady-state activation is evident. Therefore the apparent Ca\(^{2+}\) sensitivity of MTSES, MTSET, and NEM action (Figs. 1 and 2) must reflect effects of cysteine modification on the Ca\(^{2+}\) dependence of channel gating rather than effects of Ca\(^{2+}\) binding on cysteine accessibility. To further characterize changes in Ca\(^{2+}\)-dependent gating, we determined G\(_K\)–V relations at many [Ca\(^{2+}\)]\(_i\) before and after steady-state modification (Figs. 5 and 6). In these experiments, control data were recorded at various [Ca\(^{2+}\)]\(_i\), and then cysteine-modifying reagents were applied at [Ca\(^{2+}\)]\(_i\) where the extent of modification could be monitored, as in Fig. 1, until a steady-state current amplitude was attained. Subsequently, the reagent was washed out and G–Vs for the modified channels were determined in different [Ca\(^{2+}\)]\(_i\).

**Figure 4.** Effects of cysteine modification on I\(_K\) kinetics. (A, C, and E) Normalized I\(_K\) evoked by 25-ms pulses to the indicated voltages from a holding potential of ~80 mV are compared before and after steady-state modification by MTSES (A), MTSET (C), or NEM (E) at different [Ca\(^{2+}\)]\(_i\). Traces were normalized to steady-state I\(_K\) determined from exponential fits (lines). Tail currents are plotted on an expanded scale. (B and D) Time constants of I\(_K\) relaxation (τ(I\(_K\))) are plotted versus voltage for 0, 5, and 70 μM Ca\(^{2+}\) before (open symbols) and after steady-state modification (closed symbols) by MTSES (B) or MTSET (D). The three pairs of curves in each graph are from separate patches, each corresponding to a different [Ca\(^{2+}\)]\(_i\). τ(I\(_K\)) was determined at the most positive voltages by fitting I\(_K\) activation with an exponential function as in A, C, and E. At more negative voltages, channels were activated by a 25-ms test pulse, and τ(I\(_K\)) was determined from the tail current decay at different voltages.
than remains constant. These two differences may be related and probably reflect that membrane patches in the current study were excised for an extended period (~30 min) before G–Vs were recorded.

That G_{K_{max}} appears to decrease in low Ca^{2+} is relevant to our analysis because estimation of V_{h} is dependent on the value of G_{K_{max}} used to fit the G–V relation. If G_{K_{max}} is not constant, the V_{h}[Ca^{2+}] relation will be influenced by our ability to estimate G_{K_{max}} at each [Ca^{2+}]. In high [Ca^{2+}] (>5 μM), G_{K_{max}} was usually measured directly from the saturation of G_{K} at positive voltages. In 1–5 μM Ca^{2+}, examples exist where G_{K} was observed to saturate at voltages approaching +300 mV (e.g., Fig. 5 B). However, G_{K_{max}} at these [Ca^{2+}] was more typically estimated by fitting a nearly saturating G–V and allowing all parameters to vary freely as in Fig. 5 A. In this way, G_{K_{max}} in 1 μM was estimated to be 66 ± 12% (mean ± SEM) of that in 70 μM Ca^{2+}. For [Ca^{2+}] ≤ 1 μM, G_{K_{max}} could not be reliably estimated and was therefore approximated by the value determined in 1 μM Ca^{2+} (e.g., Fig. 5 A). G_{K–V} relations fit in this way from different experiments were normalized to G_{K_{max}} and averaged to illustrate the effect of Ca^{2+} on V_{h} (Fig. 6 A). As in previous studies, the normalized G–V shifts along the voltage axis in response to Ca^{2+} with little change in shape. The mean V_{h}[Ca^{2+}] relation obtained from these data (filled symbols, Fig. 5 C) is also similar in shape to that obtained from a previous study (open symbols) (Horrigan and Aldrich, 2002) but is shifted to more positive voltages.

The effects of cysteine-modifying reagents on the Ca^{2+} dependence of steady-state activation are shown in Fig. 6. Normalized G–V relations (mean ± SEM) obtained following modification by MTSES (Fig. 6 B), MTSET (Fig. 6 C), or NEM (Fig. 6 D) are similar in shape to the control (Fig. 6 A). The apparent charge (z) determined from Boltzmann fits to these data differ only at Ca^{2+} < 1 μM (Fig. 6 E). However, comparison of V_{h}[Ca^{2+}] relations for control and modified channels indicate that each reagent has a distinct effect on the Ca^{2+} dependence of steady-state activation (Fig. 6, E–G). MTSES increases V_{h} at low Ca^{2+} (≤ 1 μM) but not high Ca^{2+} (Fig. 6 E). MTSET fails to alter V_{h} over a range of [Ca^{2+}] from 0.5 to 2 μM but decreases V_{h} at both higher and lower [Ca^{2+}] (Fig. 6 F). NEM appears to shift the V_{h}[Ca^{2+}] relation along the Ca^{2+} axis such that V_{h} increases over a wide range of intermediate [Ca^{2+}] but is unchanged in 0 Ca^{2+} or 70 μM Ca^{2+}.

**Effects of Cysteine Modification on Steady-state Activation at Extreme Negative Voltages**

The complex effects of MTSES, MTSET, and NEM on the V_{h}[Ca^{2+}] relation suggest that cysteine modification alters multiple features of BK channel function. Shifts in the G–V relation may be caused by changes in either Ca^{2+}- or voltage-dependent gating or the energetics of channel opening (Cox et al., 1997a; Cui and Aldrich, 2000; Horrigan and Aldrich, 2002). However, changes in any one of these processes cannot account for the different effects on V_{h} produced by these reagents. To better determine how each aspect of gating is altered by cysteine modification, we examined steady-state activation at extreme negative voltages (Fig. 7).

BK channels can open in a Ca^{2+}-dependent manner at negative voltages where voltage sensors are not activated...
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(Horrigan et al., 1999). This phenomena is evident as a weakly voltage-dependent plateau in the $P_O$–$V$ relation at $V < -80$ mV when $P_o$ is plotted on a log scale versus voltage. Control $P_O$–$V$ relations (mean ± SEM) 0 Ca$^{2+}$ and 70 μM Ca$^{2+}$ are plotted in this way in Fig. 7 A. In 0 Ca$^{2+}$, $P_O$ at −120 mV is very small ($\sim 10^{-7}$), representing the closed–open equilibrium in the absence of Ca$^{2+}$ binding or voltage sensor activation. However, $P_O$ increases by almost four orders of magnitude in 70 μM Ca$^{2+}$ (Fig. 7 A), reflecting the strong interaction between Ca$^{2+}$ binding and channel opening. Thus, $P_O$ at extreme negative voltages provides information about Ca$^{2+}$-dependent gating and the energetics of channel opening in isolation from voltage-dependent gating.

The effects of MTSET on $P_O$ at extreme negative voltages are surprisingly different than those at more positive voltages. $P_O$–$V$ relations in 0, 5, and 70 μM Ca$^{2+}$ all increase at positive voltages in response to MTSET (Fig. 7 B), corresponding to approximate 40-mV decreases in $V_h$ (Fig. 6 G). However, at −120 mV, $P_O$ decreases in 5 μM Ca$^{2+}$, increases in 70 μM Ca$^{2+}$, and is relatively unchanged in 0 Ca$^{2+}$ (Fig. 7, B and C). That the effects of MTSET on $P_o$ at positive and negative voltages are different imply that cysteine modification alters voltage-dependent gating. That MTSET changes $P_O$ in a Ca$^{2+}$-dependent manner indicates that Ca$^{2+}$-dependent gating is also perturbed. However, failure to change $P_O$ in 0 Ca$^{2+}$ sug-
suggests that MTSET does not merely alter the energetics of channel opening.

To characterize the changes in gating that occur in the absence of voltage sensor activation, we determined the ratio $R_{\text{mod}} = \frac{P_{O}(\text{modified})}{P_{O}(\text{control})}$ at $-120$ mV in various $[\text{Ca}^{2+}]$ for MTSET, MTSES, and NEM. Steady-state currents were recorded at negative voltages before and after modification from macro-patches containing several hundred channels. Because $P_{O}$ is small at $-120$ mV, single channel currents are observed (Fig. 7 C) and $P_{O}$, was determined from amplitude histograms (Fig. 7 D, see materials and methods). In most cases, measurements were made at several voltages from $-160$ to $-80$ mV to confirm that $P_{O}$ is weakly voltage dependent at $-120$ mV. However, $I_{K}$ at more positive voltages was often too large to record. Therefore, the number of channels in the patch ($N$) was not routinely determined, and $R_{\text{mod}}$ was evaluated as $\frac{N \cdot P_{O}(\text{modified})}{N \cdot P_{O}(\text{control})}$. At $-120$ mV, $P_{O}$ at negative voltages was estimated as $\frac{N \cdot P_{O}}{N}$, where $P_{O}$ is determined from amplitude histograms and $N$ is estimated from $G_{\text{Kmax}}$ measured from macroscopic $I_{K}$ (see materials and methods). At more positive voltages, $P_{O}$ was estimated as $\frac{G_{K}}{G_{\text{Kmax}}}$. 

The effects of MTSET (E), MTSES (F), and NEM (G) on $P_{O}$ at different $[\text{Ca}^{2+}]$ are characterized by plotting the ratio $R_{\text{mod}} = \frac{N \cdot P_{O}(\text{modified})}{N \cdot P_{O}(\text{control})}$ at $-120$ mV (mean ± SEM).
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changed. However, for MTSES and NEM, \( R_{\text{mod}} = 0.2 \), indicating that these reagents, unlike MTSET, inhibit channel opening in the absence of voltage sensor activation or Ca\textsuperscript{2+} binding. All three reagents also cause a decrease in \( P_0 \) (\(-120 \text{ mV}\)) at intermediate [Ca\textsuperscript{2+}] from \( \sim 1-10 \mu\text{M} \) (i.e., \( R_{\text{mod}} < 1 \)). However, in higher [Ca\textsuperscript{2+}], \( P_0 \) is increased by MTSET and to a lesser extent by NEM but is relatively unchanged by MTSES.

Although each reagent has distinct effects on \( P_0 \) (\(-120 \text{ mV}\)), they all enhance the response to Ca\textsuperscript{2+}. That is, the Ca\textsuperscript{2+}-dependent change in \( P_0 \) from 0 Ca\textsuperscript{2+} to 70 \( \mu\text{M} \) Ca\textsuperscript{2+} at negative voltages is increased by cysteine modification. This effect is evident for MTSET from the \( P_o \)-V relations in Fig. 7 B. Moreover, that \( R_{\text{mod}} \) in 70 \( \mu\text{M} \) Ca\textsuperscript{2+} is always greater than \( R_{\text{mod}} \) in 0 Ca\textsuperscript{2+} indicates that this enhancement occurs for all three reagents (Fig. 7, E–G). Such an increase in the response to Ca\textsuperscript{2+} suggests that the coupling between Ca\textsuperscript{2+} binding and channel opening is strengthened by cysteine modification.

Identification of Modification-sensitive Cysteines

BK channel function is sensitive to modification of multiple cysteine residues (Tang et al., 2004). To identify these modification-sensitive residues, we examined the effects of MTSET, MTSES, and NEM on mutant constructs of hSlo1 that lack one or more cysteines. hSlo1 channels contain 29 cysteines, all of which are present
in mSlo1. Aside from an additional 56-amino acid COOH-terminal sequence including one cysteine present in mSlo1, hSlo1 shares 99% sequence identity with mSlo1 and exhibits similar functional properties (Brenner et al., 2000; Tang et al., 2001). The response of hSlo1 to cysteine-modifying reagents was not as extensively characterized as mSlo1. However, we tested the effects of MTSES and MTSET under many of the conditions described above for mSlo1 and observed no appreciable difference in terms of the direction and magnitude of changes in $V_h$ and $P_o(-120)$ (unpublished data).

Initially, we examined several constructs in which multiple cysteines were replaced by alanine. When all 29 cysteines are replaced, channels express poorly and are difficult to study (Tang et al., 2004). However, constructs lacking either the first 13 (C(1–13)A) or last 12 cysteines (C(18–29)A) express macroscopic currents. We tested the effects of MTSET, MTSES, and NEM on these two constructs in 5 μM Ca$^{2+}$. In both cases, $G_k$ was greatly altered by modifying reagents (Fig. 8, A and B), indicating that modification-sensitive cysteines remain. Moreover, a construct in which both sets of cysteines were replaced by alanine (C(1–13)A/C(18–29)A) expressed poorly but exhibited a clear decrease in open probability in response to NEM (Fig. 8 C).

The ability of C(18–29)A to respond to both MTSET and MTSES (Fig. 8 B) is somewhat surprising, given that the same construct is reported to be insensitive to another MTS reagent (MTSEA), owing to the removal of a key cysteine at position 911 (Tang et al., 2004). We also examined the effect of MTSET on a point mutant C911A (Fig. 8 B) and observed a response similar to that of C(18–29)A. Thus, it is clear that modification of C911 cannot be solely responsible for the effects of MTSES and MTSET in the present study.

Although mutants were affected by cysteine-modifying reagents, their response was not identical to the wild type (WT). C(1–13)A $G_k$ is decreased by NEM (Fig. 8 A), similar to the WT (Fig. 2 F). However MTSET decreases C(1–13)A $G_k$ as opposed to an increase for the WT (Fig. 2 D). In contrast, $G_k$ for C(18–29)A or C911A was immediately increased by MTSET (Fig. 8 B). However, this rapid increase was followed by a slow decay that is not observed with the WT. In addition, C(18–29)A $G_k$ is decreased by MTSES in 5 μM Ca$^{2+}$, whereas WT $G_k$ at the same [Ca$^2+$] is unaffected by MTSES (Fig. 2 B).

Differences in the response of WT, C(1–13)A, and C(18–29)A channels to cysteine-modifying reagents suggest that some of the removed cysteines may be modification sensitive. However, these differences are difficult to characterize and interpret owing to changes in channel function produced by the mutations. Both C(1–13)A and C(18–29)A required higher voltages to activate than the WT (Tang et al., 2004) and therefore could not be studied in the absence of Ca$^{2+}$. Given the complex interactions that occur between cysteine modification and channel function in the WT, it is possible that a change in gating caused by mutation could affect the response to cysteine modification. It is also possible that extensive mutation of cysteines could alter channel structure such that the accessibility of the remaining cysteines to modifying reagents is different than in the WT.

To minimize effects of mutation on channel structure or function, we examined the response to MTS reagents of several mutants where single cysteines were replaced. We focused on two regions in the COOH-terminal tail domain, the RCK1 domain and four central cysteines (C14-C17). The RCK1 domain is known to be functionally important and contains several cysteine residues, making it a likely candidate for modulation by cysteine modification. The response of C(1–13)A/C(18–29)A to NEM in Fig. 8 C suggests that at least one of the four remaining central cysteines may be modification sensitive in the WT. A cysteine residue was identified in each of these two regions that contribute significantly to the response to MTS reagents (Figs. 9 and 10).

$C_{430}$ in the RCK1 Domain Underlies Multiple Effects of MTSES and MTSET

In the RCK1 domain, we tested the effect of MTSET on point mutants in which the first three cysteines (C7–C9) were replaced individually by alanine (C348A, C422A, C430A). $G_k$–$V$ relations for all three mutants in 70 μM Ca$^{2+}$ (Fig. 8, D–F) were similar to the WT (Fig. 3 A). Likewise, $G_k$ evoked from C348A or C422A in 70 μM Ca$^{2+}$ was rapidly increased by MTSET in a manner similar to the WT, producing a shift in the G–V relation to more negative voltages (Fig. 8, D and E). However C430A eliminated the effect of MTSET on the G–V in 70 μM Ca$^{2+}$ (Fig. 8 F) and greatly reduced the effects of MTSET and MTSES at all [Ca$^{2+}$] (Figs. 9 and 10).

$G_k$–$V$ relations for C430A, in different [Ca$^{2+}$] (Fig. 9 A), are similar to those of WT channels and exhibit similar $V_h$ except at low [Ca$^{2+}$] (<1 μM), where C430A is more difficult to activate and $V_h$ is increased. The difference between $V_h$: [Ca$^{2+}$] relations for WT and C430A (Fig. 9 B) resemble those produced by modification of the WT with MTSES (Fig. 6 F), suggesting that removal of C430 or its modification by MTSES may have similar effects on channel function. Consistent with the idea that MTSES acts primarily by modifying C430, C430A is almost insensitive to MTSES. In 0 Ca$^{2+}$, MTSES decreases WT $G_k$ by 80% but has no detectable effect on C430A under the same conditions (Fig. 9 C).

Similarly, MTSES had no significant effect on the $V_h$: [Ca$^{2+}$] relation for C430A (Fig. 9 E). Likewise, MTSES produced little change in $P_o(-120)$ for C430A (i.e., $R_{mod}$ ~ 1; see Fig. 12 C).
Mutation of C430 reduces, but does not eliminate, effects of MTSET on channel gating. In 0 Ca\(^{2+}\), MTSET produces a rapid increase in WT \(I_k\), but C430A exhibits a slow decrease (Fig. 9 D). \(V_h\) for the mutant also increases slightly in 0 Ca\(^{2+}\) although no change in \(V_h\) is detected at higher [Ca\(^{2+}\)] (Fig. 9 F). Although C430A almost abolishes the effects of MTSET on \(V_h\), it does not eliminate changes in \(P_{O_{120}}\) (Fig. 10 A). Similar to the WT, \(P_{O_{120}}\) for C430A is unaffected by MTSET in 0 Ca\(^{2+}\) but is markedly decreased in 10 \(\mu\)M Ca\(^{2+}\) (Fig. 10 A). In contrast, MTSET has no effect on \(P_{O_{120}}\) in 70 \(\mu\)M Ca\(^{2+}\) (Fig. 10 A), whereas the WT shows an approximate fivefold increase (Fig. 7 C). \(G_k-V\) relations for C430A measured from individual patches immediately before and after treatment with MTSET confirm that there is little change in \(V_h\) in 10 or 70 \(\mu\)M Ca\(^{2+}\) and a small change in 0 Ca\(^{2+}\) (Fig. 10 B).

The differences in the response of WT and C430A channels to MTSET suggest that modification of C430 by MTSET may act to both increase \(P_{O_{120}}\) (−120 mV) in the presence of Ca\(^{2+}\) and shift the \(G_k-V\) relations to more negative voltages. At intermediate [Ca\(^{2+}\)], an increase in \(P_{O_{120}}\) may be masked by a decrease in \(P_{O_{120}}\) produced by modification of an additional site. This hypothesis is supported by the observation that the decrease in \(P_{O_{120}}\) produced by MTSET in 10 \(\mu\)M Ca\(^{2+}\) is at least twofold greater for C430A than for the WT (see Fig. 12 E).

**C615 in the Heme-binding Site Is Sensitive to MTSET**

The response of C(1–13)A/C(18–29)C to NEM (Fig. 7 C) suggests that at least one of four remaining cysteines in this construct (C612, C615, C628, C630) is also important for the effects of cysteine modification. We examined the effect of point mutations in three of these residues. Since the C430 site appears responsible in large part for changes in \(V_h\) and increases in

*Figure 9. C430A reduces the response to MTSES and MTSET. (A) \(G_k-V\) relations for C430A channels in different [Ca\(^{2+}\)] from a single patch (0 [\(\bullet\)], 0.1 [\(\square\), 0.3 [\(\blacksquare\), 0.6 [\(\Box\), 1.3 [\(\triangle\), 4.4 [\(\Delta\), 10 [\(\blacktriangledown\), 66 [\(\blacktriangledown\]). (B) \(V_h-[\text{Ca}^{2+}]\) relations (mean ± SEM) are compared for WT mSlo1 (\(\bullet\), hSlo1 (\(\blacktriangle\), and C430A (\(\blacktriangleleft\)) channels, indicating that C430A \(G-Vs\) are shifted to more positive voltages than the WT at low [Ca\(^{2+}\)]. (C) MTSES decreases WT \(G_k(\bullet)\) in 0 Ca\(^{2+}\) (from Fig. 2 B) but has no detectable effect on C430A \(G_k(\triangleleft)\) under the same conditions \((V_{test}=+200\text{mV})\). (D) MTSET rapidly increases WT \(G_k(\bullet)\) in 0 Ca\(^{2+}\) (from Fig. 2 D), but decreases C430A \(G_k(\triangleleft)\) under the same conditions \((V_{test}=+200\text{mV})\). (E and F) \(V_h-[\text{Ca}^{2+}]\) relations (mean ± SEM) for C430A before (\(\blacktriangle\)) and after treatment with 1 mM MTSES for 300 s (\(\blacktriangledown\)) or 100 \(\mu\)M MTSET for 30 s (\(\square\)) are almost indistinguishable. For clarity, only mean \(V_h\) is shown after modification, but variance was similar to the control.*
P$_{O}$(-120) in high Ca$^{2+}$, we screened for the ability of MTSET to decrease P$_{O}$(-120) in 10 μM Ca$^{2+}$. C628S and C630S both exhibited substantial decreases in P$_{O}$(-120) like the WT channel (unpublished data). However, C615S, in the Heam-binding site, eliminated this effect of MTSET (Fig. 10 C, middle). Despite a decreased response to MTSET in 10 μM Ca$^{2+}$, C615S still exhibits a marked increase in P$_{O}$(-120) in 70 μM Ca$^{2+}$, similar to the WT. However, C430A eliminates the increase in P$_{O}$(-120) observed for the WT in 70 μM Ca$^{2+}$.

**Figure 10.** C430A and C615S alter effects of cysteine modification on P$_{o}$ at negative voltages. (A) Steady-state I$_{K}$ at -120 mV (top) for C430A channels from three patches in different [Ca$^{2+}$] before and after treatment with 100 μM MTSET for 60 s. Corresponding amplitude histograms (bottom) determined from 20-s recordings indicate that P$_{o}$ is relatively unchanged in 0 Ca$^{2+}$ and decreased in 10 μM Ca$^{2+}$, similar to the WT. However, C430A eliminates the increase in P$_{o}$(-120) observed for the WT in 70 μM Ca$^{2+}$.

**Deletion of the RCK1 αD–βD Linker Enhances Channel Activation**

The sensitivity of C430 to modification and mutation suggests that this residue and/or nearby residues in the RCK1 domain play important roles in channel gating. The RCK1 domain is thought to have a Rossmann-fold topology containing a series of alternating α-helices and β-sheets (Jiang et al., 2001). Alignment of BK channel sequences with RCK domains of known structure indicate that C430 is located between α-helix D (αD) and β-sheet D (βD) (Jiang et al., 2001, 2002). Fig. 11A compares the sequences of mSlo1, hSlo1, and the fly homologue dSlo1 in this region with that of the MthK chan-
nel based on an alignment from Jiang et al. (2002) of BK channels with eight prokaryotic channels and transporters containing RCK domains. The αD–βD linker of MthK and other prokaryotic RCK domains contains only two amino acids. However BK channels contain an additional eight-amino acid domain including C430. The absence of this domain from the prokaryotic channels suggests it is not critical to RCK domain structure. Yet its sequence is highly conserved among BK channels, suggesting a possible functional role.

To examine the role of the αD–βD linker, we deleted the extra eight-amino acid domain from hSlo1 (hSlo1-ΔD, Fig. 11A) and compared its function to that of the WT. Like the WT, hSlo1-ΔD exhibited robust expression and responded rapidly to changes in voltage or Ca\(^{2+}\) (not depicted), consistent with the notion that the deletion does not radically disrupt channel structure. Likewise, G\(_V\)-V relations were similar in shape to the WT and exhibit a large shift in response to Ca\(^{2+}\) (Fig. 11B). However, the G\(_V\)-V relations in 0 Ca and 70 μM Ca\(^{2+}\) were shifted by −84 and −101 mV, respectively, relative to the WT. That is, hSlo1-ΔD was much easier to activate. This difference does not reflect a change in rundown (i.e., oxidation) caused by removal of C430 since the G–V for C430A (Fig. 9B) and hSlo1-ΔD were shifted in opposite directions relative to the WT. WT, hSlo1-ΔD, and C430A channels all exhibited time-dependent shifts of the G–V to more positive voltages and were studied a similar time after patch excision. Thus, the αD–βD linker has a large impact on channel gating, suggesting that effects of C430 modification could to some extent reflect perturbation of neighboring residues in the linker and/or the interaction of linker residues with the rest of the channel.

**Discussion**

We have studied the effects of cysteine-modifying reagents on native cysteines in the BK channel α-subunit to identify regions of the large COOH-terminal tail domain that influence channel function and to understand the gating mechanisms that are influenced by these regions. A similar strategy for structure–function analysis would involve mutating random sites within the tail domain. However, our approach has the advantage that modification of native cysteines is already known to impact channel gating and that we are able to compare changes in function before and after cysteine modification in the same patch. BK channels exhibit significant patch-to-patch variation in gating properties that can complicate the comparison of subtle differences between mutant and WT channels.

Our results demonstrate that the effects of cysteine-modifying reagents on BK channel function are complex in terms of the changes in channel gating, sensitivity to different modifying adducts, and sites of action.

![Figure 11](image_url)

**Figure 11.** Deletion of the αD–βD linker in the RCK1 domain enhances channel activation. (A) Amino acid sequence comparison of the MthK channel with BK channel homologues for human (hSlo1), mouse (mSlo1), and fly (dSlo1) as well as a deletion construct (hSlo-ΔD), covering the αD–βD region of the RCK1 domain. Alignments are taken from Jiang et al. (2002) and based on comparison of RCK domains for hSlo1, dSlo1, and eight prokaryotic channels and transporters including MthK. Shaded residues are semi-conserved among the aligned sequences. Locations of αD and βD are from the MthK crystal structure. Eight amino acids, present in BK channels but absent in MthK, were deleted from hSlo1 to make the hSlo-ΔD construct. (B) Mean G\(_V\)-V relations for hSlo1 ([•, ▲]) and hSlo-ΔD ([○, △]) are compared in 0 Ca\(^{2+}\) (circles) and 70 μM Ca\(^{2+}\) (triangles). Lines are fits to Boltzmann functions (0 Ca\(^{2+}\): hSlo1 \(V_m = 282\) mV, \(z = 0.79\) e; hSlo-ΔD \(V_m = +179\) mV, \(z = 0.95\) e; 70 μM Ca\(^{2+}\): hSlo1 \(V_m = +76\) mV, \(z = 1.03\) e; hSlo-ΔD \(V_m = -25\) mV, \(z = 0.70\) e).
function that can be related mainly to the coupling between Ca\(^{2+}\) binding and voltage sensor activation and channel opening. Deletion of a unique sequence including C430 in the RCK1 domain also has significant impact on function. These results are relevant to understanding the role of the RCK domain in BK channel gating.

MTSET, MTSES, and NEM appear to modify the same residues based on the ability of pretreatment with one to prevent the effect of another. However, each reagent has a distinct effect on channel function. MTSES and NEM shift the G–V to more positive voltages, whereas MTSET shifts the G–V in the opposite direction. NEM and MTSES reduce P\(_O\)(–120) dramatically in 0 Ca\(^{2+}\), whereas MTSET has no effect on P\(_O\)(–120) under the same conditions. In general, changes in P\(_O\) and NEM shift the G–V to more positive voltages, and voltage dependence. Moreover, conditions exist where each reagent has little effect on steady-state activation. NEM does not change V\(_h\) in 0 Ca\(^{2+}\) or 70 \(\mu\)M Ca\(^{2+}\). MTSET has little effect on V\(_h\) over a narrow range of intermediate Ca\(^{2+}\) near 1 \(\mu\)M. MTSES has no effect on V\(_h\) for [Ca\(^{2+}\)] > 1 \(\mu\)M. Failure to observe a change in steady-state activation under these conditions does not represent a failure to modify cysteines. The changes in channel function produced by each reagent were not dependent on the conditions under which the modifying reagents were applied. Rather, under certain conditions of [Ca\(^{2+}\)] and voltage, changes in gating do not manifest as a change in steady-state activation. In some cases, changes in I\(_k\) kinetics were observed when V\(_h\) was unchanged.

It is not surprising that conditions exist where cysteine modification leaves P\(_o\) unchanged. For example, if Ca\(^{2+}\) binding is altered by modification, then channel gating in the absence of Ca\(^{2+}\) should be unchanged (Tang et al., 2004). Likewise, if voltage sensor movement is altered, P\(_o\) should be unchanged at negative potentials where voltage sensors are not activated. In addition, it is likely that modification of multiple cysteines can have opposing effects on gating that cancel out and leave P\(_o\) unchanged over a narrow range of conditions. For example, MTSET in 5 \(\mu\)M Ca\(^{2+}\) leaves P\(_o\) unchanged near 0 mV while increasing P\(_o\)(V\(_h\)) and decreasing P\(_o\)(–120 mV) (Fig. 7 B). In this case, the lack of a change near 0 mV probably reflects the opposing effects of modifying C430 and C615, because mutation of these sites almost abolishes the effects on P\(_o\) at V\(_h\) and –120 mV, respectively. That MTSET leaves V\(_h\) unchanged over a relatively narrow range of [Ca\(^{2+}\)] (0.5–2 \(\mu\)M) is also likely to reflect action at multiple cysteines.

**Mechanisms of Action**

The activation of BK channels by Ca\(^{2+}\) and voltage can be described in terms of allosteric mechanisms (Horrigan and Aldrich, 2002). Channels undergo a closed to open (C-O) conformational change that occurs with low probability in the absence of Ca\(^{2+}\) and voltage sensor activation. Opening does not require Ca\(^{2+}\) binding or voltage sensor activation but is enhanced by either. That is, Ca\(^{2+}\) and voltage act almost independently to increase P\(_o\).

To understand how these gating mechanisms are perturbed by cysteine modification, we examined changes in steady-state activation produced by MTSET, MTSES, and NEM over a wide range of [Ca\(^{2+}\)] and voltage. These conditions include [Ca\(^{2+}\)] where high-affinity Ca\(^{2+}\)-binding sites should be vacant or saturated, and negative potentials that should force voltage sensors into a resting state. The changes in channel function produced under these limiting conditions in many cases provide a direct indication of the changes in gating mechanism.

That all three reagents alter channel function in the absence of Ca\(^{2+}\) indicates that none act merely to perturb Ca\(^{2+}\) binding. In 0 Ca\(^{2+}\), V\(_h\) is altered by MTSET or MTSES, and P\(_O\)(–120 mV) is decreased markedly by MTSES or NEM. The decreases in P\(_O\)(–120) indicate that the C-O equilibrium constant is reduced in the absence of Ca\(^{2+}\) binding or voltage sensor activation. The ability of MTSET to alter V\(_h\) without changing P\(_O\)(–120) implies that voltage-dependent gating must also be modified. That MTSET alters P\(_O\)(–120) only in the presence of Ca\(^{2+}\) indicates that Ca\(^{2+}\)-dependent gating must also be modified. Taken together, these results under limiting conditions indicate that many aspects of the gating mechanism are altered by cysteine modification. Thus, it is not surprising that modifying reagents produce complicated changes in the Ca\(^{2+}\) and voltage dependence of P\(_o\) under nonlimiting conditions where voltage, Ca\(^{2+}\) binding, and the energetics of the C-O transition all contribute to steady-state activation.

**Modeling Changes in Steady-state Activation**

To quantify changes in the gating mechanism and to account for results under nonlimiting conditions, we compared the changes in V\(_h\) and P\(_O\)(–120 mV) at various [Ca\(^{2+}\)] to the predictions of an allosteric model (Fig. 12). The model (Fig. 12 A, Scheme I), used previously to describe the ionic and gating currents of mSlo1 (Horrigan and Aldrich, 2002), includes a closed-open (C-O) conformational change characterized, in the absence of Ca\(^{2+}\) and voltage sensor activation, by an equilibrium constant L\(_o\), and charge z\(_q\). Voltage sensors in each of four identical subunits are represented by a resting to activated (R-A) conformational change with an equilibrium constant J\(_o\) and charge z\(_j\). Ca\(^{2+}\) binding is depicted as four identical binding sites that equilibrate between Ca\(^{2+}\)-free (X) and bound
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(X-Ca\(^{2+}\)) states with second order equilibrium constant \(K = [\text{Ca}^{2+}]/K_D\). \(K_D\) is the dissociation constant for Ca\(^{2+}\) when the channel is closed. The interaction between Ca\(^{2+}\) binding and channel opening is represented by an allosteric factor \(C\) where the C-O equilibrium increases C-fold for each Ca\(^{2+}\) bound. The interaction of voltage sensor activation with channel opening is represented by an allosteric factor \(D\) where the C-O equilibrium constant increases D-fold for each voltage sensor activated. A weak interaction between Ca\(^{2+}\) binding and voltage sensor activation (E) is also included (Horrigan and Aldrich, 2002). The equilibrium properties of this gating mechanism are fully described by eight parameters:

\[
P_o = \frac{L(1 + KC + JD + JKCDCE)^4}{L(1 + KC + JD + JKCDCE)^4 + (1 + J + K + JKKE)},
\]

where

\[
L = L_0 \exp\left(\frac{z_1V}{kT}\right);
\]

\[
J = J_0 \exp\left(\frac{z_1V}{kT}\right);
\]

\[
K = \frac{[\text{Ca}^{2+}]}{K_D}.
\]

Before modeling the effects of cysteine modification, parameters were determined for the control data. As noted above, G–Vs were shifted to more positive volt-
ages relative to previous studies and exhibit an apparent decrease in $G_{\text{max}}$ at low Ca$^{2+}$. Mean $G_{\text{v}}$--V relations for mSlo1 are plotted in Fig. 12 B together with predictions of the model using parameters from a previous study (dashed lines; Table I, A parameters) and with the parameters adjusted to fit the data (solid lines; Table I, B parameters). A decrease in D from 25 to 12, representing a 23% reduction in the energy of coupling between voltage sensor activation and channel opening, was sufficient to reproduce both the positive shift in $V_{h}$ and the shape of the G--V in 0 Ca$^{2+}$. A similar effect can also be produced by decreasing $L_{0}$ 15-fold (unpublished data). However, such a reduction in the G-O equilibrium constant is not consistent with $P_{\text{o}}(-120)$, as discussed below; and only minor adjustments were made to $L_{0}$ and C while $K_{0}$, $z_{L}$, $z_{J}$, and $J_{0}$ were left unchanged. The only other significant adjustment was a decrease in E that had no effect on the G--V in 0 Ca$^{2+}$ and little impact in high Ca$^{2+}$ but improved fits at intermediate [Ca$^{2+}$] where the model otherwise predicts a greater sensitivity to Ca$^{2+}$ than is actually observed. E is best determined by gating current measurements that were not performed in the present study (Horrigan and Aldrich, 2002). Therefore E was decreased from 2.4 to 1, to eliminate the weak interaction between Ca$^{2+}$ binding and voltage sensor activation and simplify the model.

The model does not predict that $G_{\text{max}}$ decreases in low Ca$^{2+}$ but rather that the slope of the G--V is reduced such that $G_{\text{max}}$ is not achieved until extreme positive voltages. In practice, we cannot distinguish between this prediction and the data given that the apparent saturation of $G_{h}$ in low Ca$^{2+}$ depends on measurements at very positive voltages, near +300 mV. Channel block by trace metal ions such as Ba$^{2+}$ might influence such measurements (Diaz et al., 1996; Neyton, 1996; Cox et al., 1997b), although a Ba$^{2+}$ chelator was included in the internal solution to minimize blocking effects (see MATERIALS AND METHODS).

Despite differences between the present and previous data, there are also similarities that indicate most parameters are unchanged. The Ca$^{2+}$ dependence of $V_{h}$ is similar to previous studies (Fig. 9 B), suggesting that parameters associated with Ca$^{2+}$-dependent gating ($K_{0}$, C) are similar. Moreover, $P_{\text{o}}$ at negative voltages was almost identical to previous measurements (Horrigan et al., 1999; Horrigan and Aldrich, 2002). $L_{0}$ was only adjusted slightly to fit the $P_{\text{o}}$--V relation in 0 Ca$^{2+}$ (line, Fig. 7 A). The model reproduces not only the magnitude of $P_{\text{o}}(-120)$ but also the voltage dependence of $P_{\text{o}}$, supporting the assumption that the charge associated with channel opening ($z_{L}$) as well as parameters associated with voltage sensor activation ($z_{L}, J_{0}$) are not altered. The allosteric factor C was adjusted slightly to fit the large increase in $P_{\text{o}}(-120)$ between 0 and 70 

\[
\begin{align*}
\text{TABLE I} & \\
\text{Scheme I: Parameters} & \\
L_{0} & 9.8 \times 10^{-7} & 11 \mu M & 8 & 25 & 0.032 & 2.4 \\
K_{0} & 1.3 \times 10^{-6} & 11 \mu M & 8.6 & 12 & 0.032 & 1 \\
C & 2.6 \times 10^{-7} & 11 \mu M & 12.5 & 12 & 0.032 & 1 \\
D & 2.6 \times 10^{-7} & 11 \mu M & 15.5 & 12 & 0.032 & 1 \\
J_{0} & 2.6 \times 10^{-7} & 11 \mu M & 12.5 & 15 & 0.024 & 1 \\
E & 1.3 \times 10^{-6} & 35 \mu M & 8.6 & 6 & 0.032 & 1 \\
F & 1.3 \times 10^{-6} & 35 \mu M & 10.5 & 12 & 0.032 & 1 \\
G & 1.3 \times 10^{-6} & 35 \mu M & 15.5 & 12 & 0.032 & 1 \\
H & 1.3 \times 10^{-6} & 35 \mu M & 15.5 & 14 & 0.045 & 1 \\
I & 1.3 \times 10^{-6} & 35 \mu M & 15.5 & 12 & 0.032 & 1 \\
J & 1.3 \times 10^{-6} & 25 \mu M & 15.5 & 12 & 0.032 & 1 \\
K & 1.3 \times 10^{-6} & 25 \mu M & 15.5 & 14 & 0.045 & 1 \\
L & 2.6 \times 10^{-7} & 11 \mu M & 17 & 12 & 0.032 & 1 \\
M & 2.6 \times 10^{-7} & 35 \mu M & 17 & 12 & 0.032 & 1 \\
N & 2.6 \times 10^{-7} & 35 \mu M & 17 & 17.5 & 0.020 & 1 \\
\end{align*}
\]

For all fits, $z_{L} = 0.3 \varepsilon$, $z_{J} = 0.58 \varepsilon$.

\[\mu M\text{Ca}^{2+}\text{ (lines, Fig. 7 A). The } K_{0} \text{ for Ca}^{2+} \text{ was left unchanged because the increase in mean } P_{\text{o}}(-120) \text{ between 0 and 4.4 } \mu M \text{Ca}^{2+} \text{ was } 145 \pm 55\text{-fold (mean } \pm \text{ SEM, } n = 3) \text{, not significantly different than the 100-fold change predicted by the model.}

\text{Modeling the Effects of Cysteine Modification}

Once parameters were adjusted to fit the control data, we determined which parameters could be changed to reproduce the effects of cysteine-modifying reagents. Fig. 12 (C, E, and G) plots the $R_{\text{mod}}$--[Ca$^{2+}$] relations for MTSES, MTSET, and NEM, respectively, for WT and mutant channels, where $R_{\text{mod}} = NP_{\text{o}}(\text{modified})/ NP_{\text{o}}(\text{control})$ at -120 mV. Individual data points are plotted, as opposed to averages, in Fig. 9, because different numbers of measurements were made at each [Ca$^{2+}$] and each point represents a different patch. Fig. 12 (D, F, and H) plots the $G_{\text{k}}$--V shift ($\Delta V_{h}$) produced by channel modification (mean $\pm$ SEM), equivalent to the difference between control and modified $V_{h}$--[Ca$^{2+}$] relations in Fig. 6. Based on the similar shape of $G_{\text{k}}$--V relations in high [Ca$^{2+}$] before and after modification (Fig. 3 A; Fig. 6 E; Fig. 7 B; Fig. 8 F), we conclude that the charge associated with voltage sensor activation ($z_{J}$) or channel opening ($z_{L}$) is not altered, leaving six parameters that can be adjusted ($L_{0}$, $K_{0}$, C, D, E, and $J_{0}$). Although $R_{\text{mod}}$ exhibits considerable scatter, we focused on fitting this data first because only three free parameters ($L_{0}$, $K_{0}$, and C) influence $P_{\text{o}}(-120)$.

\text{The Effects of MTSES}

MTSES decreases $P_{\text{o}}(-120)$ fivefold in 0 Ca$^{2+}$ (i.e., $R_{\text{mod}} = 0.2$), indicating a fivefold decrease in the C-O equilibrium constant $L_{0}$. A decrease in $L_{0}$ by itself
should reduce \(P_o\) (−120) at all \([Ca^{2+}]\) equally. However, \(R_{mod}\) is smallest in 0 \(Ca^{2+}\) and approaches unity in high \(Ca^{2+}\). To reproduce a \(Ca^{2+}\)-dependent increase in \(R_{mod}\) requires that the allosteric factor \(C\) be increased. Together, a decrease in \(I_o\) and increase in \(C\) reasonably approximate the \(R_{mod}–[Ca^{2+}]\) relationship (solid line, Fig. 12 D; Table I, C1) and predict the shape, although not the magnitude, of the \(\Delta V_h–[Ca^{2+}]\) relation (Fig. 12 D, C1), including that \(V_h\) is altered only at very low \([Ca^{2+}]\) < 1 \(\mu M\). The magnitude of the \(\Delta V_h–[Ca^{2+}]\) relation can be reproduced without altering the \(R_{mod}–[Ca^{2+}]\) relation by increasing the allosteric factor \(D\) (Fig. 12 D, C2; Table I, C2). The fit is further improved in high \(Ca^{2+}\) if the voltage sensor equilibrium constant \(J_0\) is decreased slightly (Fig. 12 D, C3; Table I, C3). The mutant C430A shows little response to MTSES (Fig. 11 E), implying that changes in \(I_o\), \(C\), \(D\), and \(J_0\) used to fit the WT data reflect modification of C430.

**The Effects of MTSET**

MTSET has effects on \(R_{mod}\) (Fig. 12 E) and \(\Delta V_h\) (Fig. 12 F) that differ from those of MTSES and involve action at more than one cysteine residue. However, as shown below, MTSET shares with MTSES an ability to increase \(C\) and \(D\) by modifying C430.

That MTSET modifies residues in addition to C430 is evident from the marked decrease in \(R_{mod}\) produced at intermediate \([Ca^{2+}]\) for C430A (Fig. 12 E). Such an effect is consistent with a decrease in \(Ca^{2+}\)-affinity such that \(Ca^{2+}\) bound at intermediate \([Ca^{2+}]\) is decreased whereas \(Ca^{2+}\) occupancy in 0 \(Ca^{2+}\) and saturating \(Ca^{2+}\) are unchanged. The model predicts a U-shaped \(R_{mod}–[Ca^{2+}]\) relation when \(K_p\) is increased from 11 to 35 \(\mu M\) without changing other parameters (Fig. 12 E, D1; Table I, D3). This approximates the 10-fold decrease in \(R_{mod}\) near 5 \(\mu M\) \(Ca^{2+}\) for C430A, but underestimates \(R_{mod}\) at high \(Ca^{2+}\). An improved fit can be obtained by increasing \(C\) slightly from 8.6 to 10.5 (Fig. 12 E, D2; Table I, D2). Similarly, the WT data can be fit by further increasing \(C\) to 15.5 with \(K_p = 35 \mu M\) (Fig. 12 E, D1; Table I, D1). The increase in \(C\) appears sufficient to account for the difference between the response of C430A and WT, implying that modification of C430 increases \(C\). The mutation C615S eliminates the change in \(R_{mod}\) at 5 \(\mu M\) \(Ca^{2+}\) but not in high \(Ca^{2+}\), suggesting that modification of C615 increases \(K_p\) with little effect on \(C\). However, C615 is not solely responsible for the change in \(K_p\). If we assume that MTSET acts only to increase \(C\), then the model predicts \(R_{mod}\) will increase over a 10-fold lower \(Ca^{2+}\) range than is actually observed for C615S (Fig. 12 E, F1; Table I, F1). The data can be fit by assuming \(K_p\) is increased to 25 \(\mu M\) (Fig. 12 E, F2; Table I, F2). That is, modification of a site other than C615 or C430 increases \(K_p\) from 11 to 25 \(\mu M\), and modification of C615 must further increase \(K_p\) to 35 \(\mu M\) to account for the response of the WT. A possible candidate for the additional site as discussed below is C911 described by Tang et al. (2004).

The parameters that fit the \(R_{mod}–[Ca^{2+}]\) relation for the WT channel also reproduce the basic shape of the \(\Delta V_h–[Ca^{2+}]\) relation (Fig. 12 E, F1; Table I, E1) but overestimate \(\Delta V_h\), especially in 0 \(Ca^{2+}\). That the G–V is shifted in 0 \(Ca^{2+}\) (\(\Delta V_h = -34 \pm 7 mV\)) although \(P_o\) (−120) is not changed (\(R_{mod} = 1.00 \pm 0.05\)) indicates that voltage-dependent gating must be altered. The data can be fit by increasing \(D\) and \(J_0\) (Fig. 12 F, E2; Table I, E2). As in the case of MTSES, the increase in \(D\) was necessary to fit \(\Delta V_h\) in 0 \(Ca^{2+}\), whereas the adjustment of \(J_0\) had a smaller effect. A similar \(\Delta V_h–[Ca^{2+}]\) relation is predicted for C615S (Fig. 12 F, F3; Table I, F3), with the difference that \(\Delta V_h\) is always negative for the mutant, consistent with the approximate −20-mV G–V shifts observed for 1 and 5 \(\mu M\) \(Ca^{2+}\) in Fig. 10 C.

Although the C430A mutant exhibits a marked decrease in \(P_o\) (−120) at intermediate \([Ca^{2+}]\), it exhibits little or no change in \(V_h\) in response to MTSET (Fig. 9 F). That \(V_h\) and \(P_o\) (−120) don’t change for C430A in 0 \(Ca^{2+}\) and saturating \(Ca^{2+}\) is consistent with the increases in \(C\) and \(D\) used to fit the WT response being caused by modification of C430. However, the failure of \(V_h\) to change at all \([Ca^{2+}]\) is not readily explained by the model since an increase in \(K_p\) used to fit the \(R_{mod}–[Ca^{2+}]\) relation generally predicts a bell-shaped \(\Delta V_h–[Ca^{2+}]\) relation. One factor that may influence our ability to fit the mutant data is that effects of cysteine modification on C430A or C615S were modeled using control parameters identical to those of the WT. This may be an oversimplification because \(V_h–[Ca^{2+}]\) relations for WT and C430A differ in low \([Ca^{2+}]\) (Fig. 9 B). However, mutants were not as well characterized as the WT and not all control parameters could be determined.

**The Effects of NEM**

The effect of NEM on \(P_o\) (−120) resembles that of MTSES with \(R_{mod} = 0.2\) in 0 \(Ca^{2+}\) and increasing at higher \([Ca^{2+}]\) (Fig. 12 G). These effects imply that NEM, like MTSES, decreases \(I_o\) and increases \(C\). When \(I_o\) is decreased fivefold and \(C\) is increased twofold, the model approximates \(R_{mod}\) in 0 \(Ca^{2+}\) and high \([Ca^{2+}]\) (Fig. 12 G, G1; Table I, G1). However, the model also predicts that \(R_{mod}\) will increase rapidly near \([Ca^{2+}] = 1 \mu M\), as for MTSES (Fig. 12 C), whereas the data increases over a 10-fold higher \(Ca^{2+}\) range, suggesting that \(K_p\) has increased. An increase in \(K_p\) from 11 to 35 \(\mu M\) reproduces this behavior (Fig. 12 G, G2; Table I, G2). Improved fits to \(R_{mod}\) were obtained with a slightly lower 30 \(\mu M\) \(K_p\) (unpublished data). However, a higher \(K_p\) provided better fits to \(\Delta V_h\) (below).

The \(\Delta V_h–Ca^{2+}\) relation for NEM is bell shaped (Fig. 12 H), consistent with an increase in \(K_p\). The para-
ters used to fit the $R_{\text{mod}}$–$\text{Ca}^{2+}$ relation reproduce $\Delta V_h$ for $\text{Ca}^{2+} \geq 5 \, \mu M$, but greatly overestimate $\Delta V_h$ in 0 $\text{Ca}^{2+}$, reflecting the fivefold decrease in $I_h$ (Fig. 12 H, G2; Table I, G2). This discrepancy can be accounted for, as with MTSES (Fig. 12 D), by increasing $D$ to fit the 0 $\text{Ca}^{2+}$ data and decreasing $J_0$ to improve the fit in high $\text{Ca}^{2+}$ (Fig. 12 H, G3; Table I, G3).

**Limitations of the Model and Data**

The model incorporates several simplifying assumptions, including that $\text{Ca}^{2+}$-binding sites are identical. At $[\text{Ca}^{2+}]$ used in our experiments, contributions of low-affinity $\text{Mg}^{2+}$/\text{Ca}^{2+}-binding sites to channel gating are negligible (Shi and Cui, 2001; Zhang et al., 2001). However, recent studies suggest that BK channels may contain more than one high-affinity $\text{Ca}^{2+}$-binding site per subunit (Bao et al., 2002; Xia et al., 2002). Possible heterogeneity in binding sites should not affect our conclusion that cysteine modification strengthens the coupling between $\text{Ca}^{2+}$ binding and channel opening and can also decrease binding affinity. However, the magnitude of changes in $C$ and $K_0$ could differ for each binding site. Selective effects of modifying reagents on different binding sites would certainly improve fits to the $R_{\text{mod}}$ and $\Delta V_h$–$[\text{Ca}^{2+}]$ relations owing to the increased number of free parameters. Multiple binding sites might help explain why the MTSET data sometimes changed over a narrower range of $[\text{Ca}^{2+}]$ than predicted by the model (Fig. 12 E). That being said, Scheme I, with a single type of binding site, was able to predict the contribution of C911 to our results is relatively small. First, mutants lacking C911 (C(18–29)A and C911A) still responded robustly to MTSET (Fig. 8 B), although the response exhibits an additional slow component that is not evident in the WT. Likewise C(18–29)A $I_h$ is strongly inhibited by MTSES (Fig. 8 B). Moreover, the mutation C430A virtually abolishes the effects of MTSES in our experiments, suggesting either that C911 was not modified by MTSES or that its modification had no effect on gating. Tang et al. (2004) did not report whether effects of MTSES were prevented by mutation of C911, so it is possible that MTSES modification of C911 has no effect on function. Another possibility is that channels in our experiments are partially oxidized owing to an ~30-min delay between patch excision and recording. Therefore, it is conceivable that modification of C911 was prevented by selective oxidation of this site. If C911 is oxidized to form a disulfide bond with another cysteine, then removal of C911 could expose the remaining disulfide partner to modification by MTSET, perhaps accounting for the additional slow component of MTSET action observed in mutants lacking C911.

Whether or not C911 is modified, our experiments identify additional modification-sensitive cysteines (C430, C615) and show that the functional consequences of their modification differ from those attributed to C911. In contrast to the effects of MTSEA reported by Tang et al. (2004), we find that cysteine modification alters steady-state activation in 0 $\text{Ca}^{2+}$ as well as in the presence of $\text{Ca}^{2+}$ and that effects may be inhibitory or excitatory depending on the modifying reagent, $[\text{Ca}^{2+}]$, and voltage. Differences in the effect of each reagent imply that modification of critical cysteines can be sensitive to the modifying adduct, not merely the absence of free thiol groups. For all reagents, we find that cysteine modification enhances the $\text{Ca}^{2+}$-dependent increase in $P_o(\sim-120)$, whereas Tang et al. (2004) saw a decrease in the response to $\text{Ca}^{2+}$ with MTSEA. We also observe effects on voltage-dependent gating even though there are no internally accessible cysteine residues in potential voltage-sensing domains (S2, S3, S4).

While we didn’t study effects of oxidation, an interesting question is whether oxidation of C430 or C615 could alter BK channel function under pathophysiological conditions. An important feature of our results is that effects of cysteine modification are complex and can leave steady-state activation virtually unchanged over a range of $[\text{Ca}^{2+}]$ and voltage while producing marked changes in $P_o$ under different conditions. Therefore, demonstration by Tang et al. (2004) that mutation of C911 virtually eliminates the effect of oxidation on $I_h$ in 4.7 $\mu M$ $\text{Ca}^{2+}$ at voltages near $-20$ mV...
does not rule out that oxidation of additional sites effect \( P_o \) under other conditions. Along these lines, it is worth noting that the effect of MTSET on mSlo1 was almost undetectable under conditions similar to those used by Tang et al. (2004) (e.g., -20 mV in 4.4 \( \mu \)M Ca\(^{2+}\), Fig. 7 B), whereas significant changes in \( P_o \), were observed at more positive or negative voltages or in different [Ca\(^{2+}\)].

**Conclusions**

Although the effects of MTSET, MTSES, and NEM on steady-state activation appear different, the above analysis suggests that their mechanisms of action are similar. MTSES and NEM both decrease the C-O equilibrium constant \( L_o \) fivefold. In all cases, modification of the WT channel is characterized by a 1.5–2-fold increase in the allosteric factor \( C \), representing a 0.9–1.6 kCal/M increase in the energy of coupling between Ca\(^{2+}\) binding and channel opening. In addition, changes in voltage-dependent gating, in particular an increase in \( D \) (0.4–0.8 kCal/M), were required for all three reagents. Changes in the voltage sensor equilibrium constant \( J_o \) equivalent to a small 12–20-mV shift in the Q–V relation were also helpful in fitting the data, but had a relatively minor impact compared with \( L_o \), \( C \), and \( D \). Most changes in \( L_o \), \( C \), and \( D \) produced by MTS reagents were abolished by the C430A mutation and can therefore be attributed to modification of this position. In addition, MTSET and NEM produce an approximate threefold increase in \( K_o \). The effect of MTSET on \( K_o \) appears unaffected by C430A but is reduced by C615S. Thus, it is likely that MTSET and NEM reduce Ca\(^{2+}\) affinity by modifying C615 as well as additional cysteines. C615 lies in a high-affinity Haem-binding site, and the mutation C615S essentially eliminates the sensitivity of hSlo1 channels to inhibition by Heamin (Tang et al., 2003). Thus a role of C615 in regulating Ca\(^{2+}\) affinity could be relevant to the mechanism of Heamin action.

Differences in the effects of MTSET, MTSES, and NEM can be attributed primarily to differential effects on \( L_o \) and \( K_o \). The ability of MTSET to shift the G–V in the opposite direction of MTSES or NEM is due primarily to the fivefold decrease in \( L_o \) produced by MTSES or NEM but not by MTSET. The marked decrease in \( P_o (−120) \) produced by MTSET in intermediate [Ca\(^{2+}\)] is caused by an increase in \( K_o \) that is not produced by MTSES. Thus, some effects of cysteine modification are dependent on the modifying adduct while others are not.

Although all reagents tested increased the coupling between Ca\(^{2+}\) binding and channel opening (C), it is significant that changes in C can occur independent of changes in \( L_o \) and \( K_o \). That is, MTSET increases C without altering \( L_o \), and modification of C430 by any reagent increased C without altering \( K_o \). In the allosteric model, C represents the ratio of \( K_o \) for the closed and open conformation (\( C = K_o / K_{o\text{open}} \)). Thus changes in a Ca\(^{2+}\)-binding site, which might be expected to perturb Ca\(^{2+}\) affinity for both closed and open conformations, would likely result in a change in \( K_o \) and possibly also in C. That C430 modification increases C without altering \( K_o \) is therefore consistent with a role of the C430 region in coupling that does not involve the Ca\(^{2+}\)-binding site but rather the linkage between binding site and gate. The relationship between C and \( L_o \) is also relevant to the mechanism of linkage. For example, if the linkage acts to inhibit channel opening and is relieved by Ca\(^{2+}\) binding, then an increase in C might be expected to decrease the C-O equilibrium constant in 0 Ca\(^{2+}\) (\( L_o \)) without altering channel opening in saturating Ca\(^{2+}\). This is exactly what we observe in response to MTSES modification (Fig. 12 C). However, a counter example is provided by MTSET, which increases channel opening in saturating Ca\(^{2+}\) without altering \( L_o \). Thus, we cannot conclude whether the linkage between Ca binding and channel opening is inhibitory or excitatory, but suggest that understanding the difference between MTSET and MTSES action might prove relevant to addressing this question in the future.

It is remarkable that modification of a single site (C430) alters several aspects of gating and that the effect on \( L_o \) is dependent on the modifying adduct, whereas changes in \( C \) and \( D \) occur for all reagents. That C430 is located in the RCK1 domain may help explain the diversity of these effects on channel function. The RCK1 domain has been implicated in Ca\(^{2+}\)-dependent gating, where it is thought to play a role either in Ca\(^{2+}\) binding or in coupling the binding of Ca\(^{2+}\) to channel opening (Bao et al., 2002; Xia et al., 2002). In either case, the RCK1 domain must be linked to channel opening. Thus is reasonable that perturbations of the RCK1 domain could effect C (coupling) and \( L_o \) (channel opening). In addition, the ability of S4 mutations to inhibit Mg\(^{2+}\)-dependent activation suggests that interactions exist between RCK1 and the voltage sensor (Hu et al., 2003). Therefore, it is also reasonable that perturbations of the RCK1 domain could effect voltage-dependent gating (\( D, J_o \)).

The effects of C430 modification may reflect perturbation of interactions between the \( \alpha D\beta D \) linker and other parts of the channel. C430 is located within a sequence of eight amino acids in the \( \alpha D\beta D \) linker that is conserved in BK channels but absent from prokaryotic RCK domains. Thus, the structure of this region and its interactions with the rest of the channel are unknown. But it is likely that such interactions occur because the BK-specific residues are not required for the basic Rossmann-fold RCK structure, yet their deletion greatly enhances channel activation. It is possible that
interactions involving the αD–βD linker are electrostatic in nature because 4 out of 10 linker residues are charged, and COOH-terminal to C430, 4 out of 7 residues are negatively charged. Changes in electrostatic interactions might account for the different impact of positive (MTSET), negative (MTSES), and neutral (NEM) modifying adducts on I_V. The high negative charge density near C430 could also explain why modification by MTSET(+) is much more rapid than MTSES(−), although these reagents were applied at concentrations that produce similar reaction rates with neutral thiol compounds (Stauffer and Karlin, 1994).

Modification of C430 might also affect channel function by perturbing the nearby αD helix. In the MthK channel, αD helices in different RCK domains participate in a hydrophobic dimer interface that is involved in forming the RCK gating ring and thought to be important for transmitting Ca²⁺ binding to channel opening (Jiang et al., 2002). In addition, αD participates in a salt bridge with βE within the same RCK domain, disruption of which in hSlo1 alters channel function (Jiang et al., 2001). Thus, modification of C430 near αD might alter BK channel function by perturbing the RCK1–RCK2 interaction and/or the αD–βE salt bridge.

Finally, another feature of the RCK1 domain that may be relevant to the broad effects of C430 modification on gating is its mechanical properties. Niu et al. (2004) found that reductions in the length of the S6–RCK1 linker increase open probability in the presence or absence of Ca²⁺, and concluded that the S6–RCK1 linker and the RCK gating ring have spring-like properties that exert tension on the S6 gate and govern open probability. They also proposed that most of the spring is in the gating ring. Consistent with this hypothesis, we find that deletion of the RCK1 αD–βD linker shifts the G–V relations in both 0 and 70 μM Ca²⁺ to more negative voltages similar to the effect of shortening the S6–RCK1 linker. If modification of C430 alters the mechanical properties of the gating ring, then it could potentially impact multiple features of gating by altering the transduction of local conformational changes in the COOH-terminal tail domain into changes in channel opening.

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