KCNE Peptides Differently Affect Voltage Sensor Equilibrium and Equilibration Rates in KCNQ1 K⁺ Channels

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KCNQ1 voltage-gated K⁺ channels assemble with the family of KCNE type I transmembrane peptides to afford membrane-embedded complexes with diverse channel gating properties. KCNQ1/KCNE1 complexes generate the very slowly activating cardiac I_{Ks} current, whereas assembly with KCNE3 produces a constitutively conducting complex involved in K⁺ recycling in epithelia. To determine whether these two KCNE peptides influence voltage sensing in KCNQ1 channels, we monitored the position of the S4 voltage sensor in KCNQ1/KCNE complexes using cysteine accessibility experiments. A panel of KCNQ1 S4 cysteine mutants was expressed in *Xenopus* oocytes, treated with the membrane-impermeant cysteine-specific reagent 2-(trimethylammonium) ethyl methanethiosulfonate (MTSET), and the voltage-dependent accessibility of each mutant was determined. Of these S4 cysteine mutants, three (R228C, G229C, I230C) were modified by MTSET only when KCNQ1 was depolarized. We then employed these state-dependent residues to determine how assembly with KCNE1 and KCNE3 affects KCNQ1 voltage sensor equilibrium and equilibration rates. In the presence of KCNE1, MTSET modification rates for the majority of the cysteine mutants were ~10-fold slower, as was recently reported to indicate that the kinetics of the KCNQ1 voltage sensor are slowed by KCNE1 (Nakajo, K., and Y. Kubo. 2007 *J. Gen. Physiol.* 130:269–281). Since MTS modification rates reflect an amalgam of reagent accessibility, chemical reactivity, and protein conformational changes, we varied the depolarization pulse duration to determine whether KCNE1 slows the equilibration rate of the voltage sensors. Using the state-dependent cysteine mutants, we determined that MTSET modification rates were essentially independent of depolarization pulse duration. These results demonstrate that upon depolarization the voltage sensors reach equilibrium quickly in the presence of KCNE1 and the slow gating of the channel complex is not due to slowly moving voltage sensors. In contrast, all cysteine substitutions in the S4 of KCNQ1/KCNE3 complexes were freely accessible to MTSET independent of voltage, which is consistent with KCNE3 shifting the voltage sensor equilibrium to favor the active state at hyperpolarizing potentials. In total, these results suggest that KCNE peptides differently modulate the voltage sensor in KCNQ1 K⁺ channels.

INTRODUCTION

Electrical excitability depends on the coordinated openings and closings of voltage-gated cation channels. The voltage sensitivity of these integral membrane proteins is mediated by a voltage sensor, a dynamic membrane-embedded domain composed of four transmembrane helices (S1–S4) that moves in response to changes in membrane potential (Long et al., 2005a,b). The S4 segment of the voltage sensor possesses a high concentration of positively charged amino acids, which accounts for most of the charges per channel that move across the membrane's electric field (Aggarwal and MacKinnon, 1996; Seoh et al., 1996). The trajectory and distance transversed by the S4 segment is an ongoing debate; however, all investigations agree that S4 moves between a resting and active state (Jiang et al., 2003; Chanda et al., 2005; Posson et al., 2005; Ruta et al., 2005; Darman et al., 2006). The shuttling of S4 charges between these two states has been followed in several voltage-gated channels using cysteine accessibility methodologies (Yang and Horn, 1995; Larsson et al., 1996; Yang et al., 1996;

Yusaf et al., 1996). These studies have shown that some residues in S4 are state-dependent: inaccessible to aqueous reagents at rest, but upon membrane depolarization they become exposed to the extracellular milieu and modifiable. For voltage-gated Na⁺, K⁺, and Ca²⁺ channels, depolarization shifts the equilibrium of the S4 segments to the active state, favoring an open activation gate that permits the rapid flow of ions across the membrane. Conversely, the codependent relationship between the S4 segment and activation gate is inversely coupled in hyperpolarized-activated cyclic-nucleotide-gated (HCN) channels; hyperpolarization shifts the sensor to the resting state and opens the activation gate (Mannikko et al., 2002; Vemana et al., 2004). In both classes of voltage-gated channels, the state of the S4 is tightly coupled to the equilibrium of the activation gate (Yellen, 1998).

KCNQ1 (Q1) channels are voltage-gated K⁺ channels that are found in both electrically excitable and

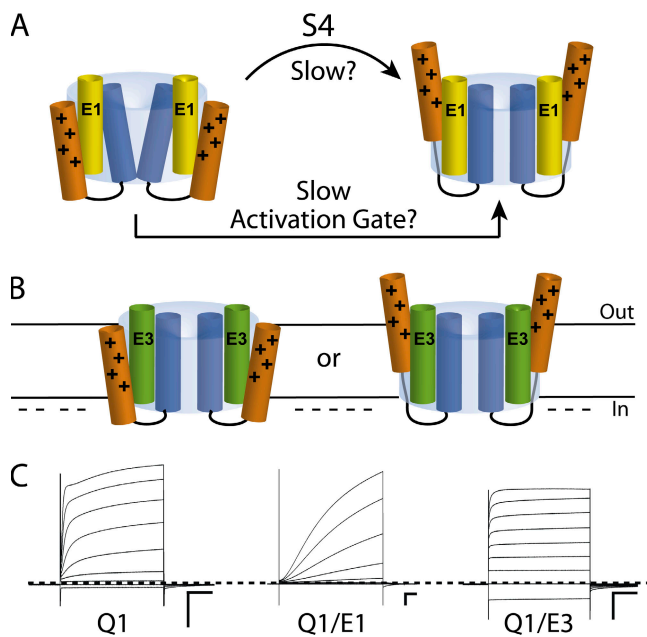Correspondence to William R. Kobertz: william.kobertz@umassmed.edu

Figure 1. Cartoon models depicting possible mechanisms for KCNE1 and KCNE3 modulation of KCNQ1 channels (A) In Q1/E1 channel complexes, slow gating arises from either the slow transition of S4 voltage-sensing domains (orange with positive charges) from resting to active positions, or from slow activation gate opening. (B) Q1/E3 complexes are open at hyperpolarizing potentials (denoted by negative charges along the cytoplasmic membrane) because S4 voltage sensors are uncoupled from the opening of the gate (left), or because the equilibrium of voltage sensors is shifted to favor the activated state (right). (C) TEVC recordings of Q1 channels alone and partnered with E1 and E3 peptides in ND96 solution (Materials and methods). Oocytes were held at −80 mV, and currents were elicited from 4-s command voltages from −100 mV to +40 mV in 20-mV increments. Scale bars represent 0.5 μA and 0.5 s. Dashed line indicates zero current.

nonexcitable cells. To meet the potassium flux requirements in this variety of tissues, Q1 channels coassemble with the family of KCNE type I transmembrane peptides, which substantially alter the voltage sensitivity of the channel (McCrossan and Abbott, 2004). Although currents from homomeric Q1 channels have not been observed in native tissues, it is a voltage-dependent delayed rectifier K$^+$ channel when expressed in standard cell lines and *Xenopus* oocytes (Barhanin et al., 1996; Sanguinetti et al., 1996). Q1 coassembly with KCNE1 (E1) peptides forms a complex that generates the cardiac I$_{Ks}$ current: an incredibly slowly activating current involved in maintaining the rhythmicity of the heartbeat (Barhanin et al., 1996; Sanguinetti et al., 1996). Deactivation kinetics of the Q1/E1 complex are also slowed when compared with homoterameric Q1 channels. In contrast, epithelial Q1/E3 complexes are open at both hyperpolarizing and depolarizing potentials and are weakly voltage dependent (Schroeder et al., 2000). If Q1/E3 complexes do open and close, the gating kinetics of these

transitions are nearly instantaneous. The three other KCNE peptides (E2, E4, E5) also profoundly affect Q1 voltage gating, converting it into a leak channel, a non-conducting channel, and a severely right-shifted channel, respectively (Tinel et al., 2000; Angelo et al., 2002; Grunnet et al., 2002). Most structure–function studies examining KCNE peptide interactions with Q1 have focused on the pore-forming domain (S5–S6) of the channel (Tai and Goldstein, 1998; Tapper and George, 2001; Melman et al., 2004; Panaghie et al., 2006). Unlike most Kv-type channels that possess five to seven net positive charges in S4, Q1 has only a net charge of +3. Recently, Abbott and colleagues have linked KCNE peptides' strong influence on Q1 voltage sensitivity to the channel's charge-poor S4 (Panaghie and Abbott, 2007). By adding charges to the S4 of Q1, they showed that the channel is unaffected by E3 whereas charge removal from KCNQ4 renders this channel susceptible to E3's modulatory effects, suggesting a connection between KCNE peptides and the voltage sensor. From this work, they proposed that E3 either uncouples the voltage sensor from the cytoplasmic gate or "locks" the voltage sensor in the active state; however, the position and equilibrium of the voltage sensor was not directly examined and thus they could not definitively differentiate between these two models.

Here, we experimentally address the following question: Do E1 and E3 peptides affect the voltage-dependent equilibrium and equilibration rate of the Q1 voltage sensor? For Q1/E1 complexes, the strikingly slow activation kinetics can arise from increasing the energy barrier for one of the two steps of activation: (1) S4 moving from the resting to active state or (2) activation gate opening (Fig. 1 A). For the constitutively conducting Q1/E3 complexes, E3 either uncouples the voltage sensor from the activation gate or it shifts the equilibrium of the voltage sensor such that it significantly resides in the active state at hyperpolarized potentials, as hypothesized by Abbott and coworkers (Panaghie and Abbott, 2007) (Fig. 1 B). To directly test these sets of possibilities, we identified S4 residues in unpartnered Q1 channels whose rates of modification increased upon depolarization in cysteine accessibility experiments, and then used these state-dependent residues to examine the position and equilibrium of S4 in Q1/E1 and Q1/E3 complexes. We find that the state-dependent S4 residues in Q1/E1 complexes are modified essentially independent of pulse duration, suggesting that E1 does not affect the time it takes for the voltage sensors to reach equilibrium. In contrast, all modifiable S4 residues in Q1/E3 complexes are rapidly modified irrespective of membrane voltage, indicating that the voltage sensor frequently resides in the active state when E3 is present. These diametrically opposed effects demonstrate the manifold nature of KCNE modulation of Q1 channels.
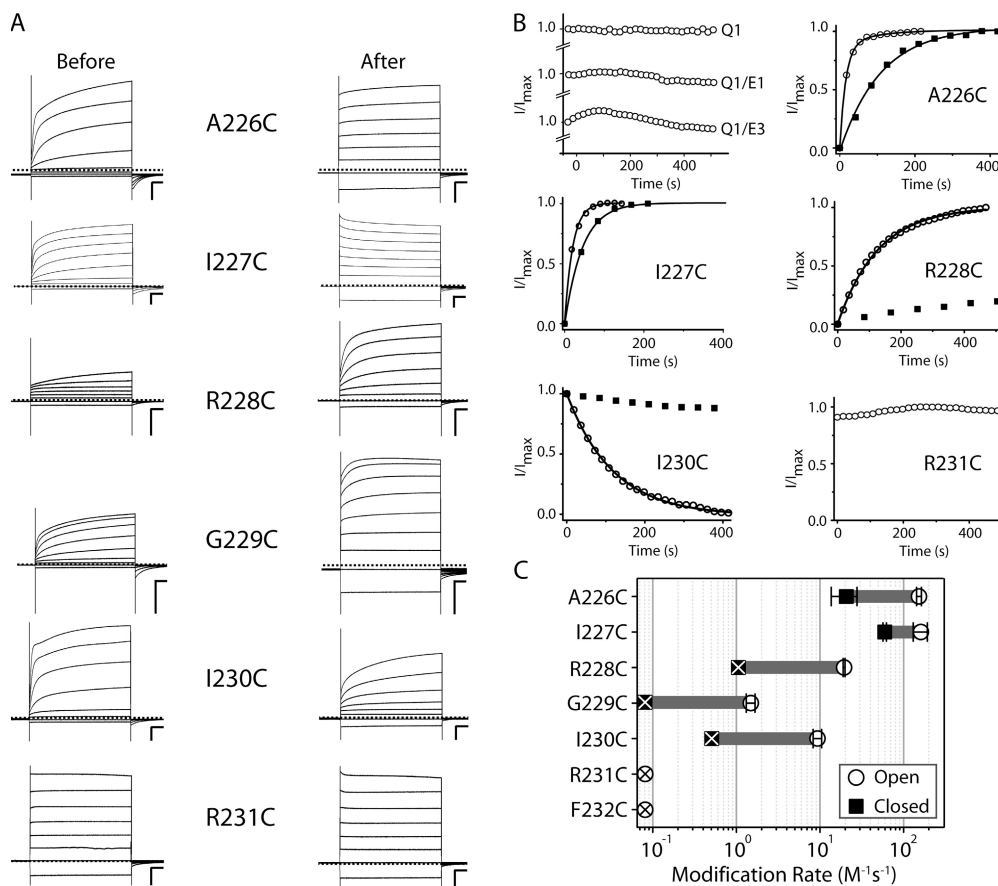
Figure 2. S4 cysteine substitutions in KCNQ1 show state-dependent MTSET modification. (A) TEVC recordings of representative Q1 channels with cysteine substitutions in S4 expressed in *Xenopus* oocytes before and after MTSET modification. Oocytes were held at −80 mV, and currents were elicited from 4-s command voltages from −100 mV to +40 mV in 20-mV increments. Scale bars represent 0.5 µA and 0.5 s. Dashed line indicates zero current. (B) Change in current monitored over time using 40-mV test pulses with continuous perfusion of MTSET. For negative controls, 800 µM MTSET was used for Q1 and Q1/E3; 1600 µM for Q1/E1 and the data were plotted on the same y-axis scale as the cysteine mutants and are separated by line hatches. Open circles represent the "open" protocol where channels were depolarized for 11% of total time; filled squares: "closed" protocol, 0.6% of total time. Currents from the open and closed protocols were measured ∼5 ms before the end of the shortest depolarizing pulse and were normalized to the maximal change in current for comparison. Curves were fit to single or double exponentials to calculate reaction rate constants (Table I). (C) Comparison of MTSET modification rates for Q1 S4 cysteine substitutions in the open (open circles) or closed (filled squares) protocols. The gray bar gives the fold-change in rate between the open and closed protocols. X-out open circles indicate no observed change of current using open protocol; X-out squares are an estimate of reaction rate in the closed protocol based on the extent of modification determined by switching to the open protocol. Data were averaged from three to six oocytes ± SEM.

MATERIALS AND METHODS

Mutagenesis and In Vitro Transcription
Human Q1, E1, and E3 were subcloned into vectors containing the 5′ and 3′ UTRs from the *Xenopus* β-globin gene for optimal protein expression. Single cysteine point mutations were introduced into Q1 using cassette mutagenesis and confirmed by DNA sequencing of the mutated insert. The cDNA plasmids were linearized by MluI digestion, and cRNA was synthesized by run-off transcription using SP6 or T7 RNA polymerase (Promega).

Electrophysiology
Oocytes were surgically removed from *Xenopus laevis* and defolliculated using 2 mg/ml collagenase (Worthington Biochemical Corp.) in OR2 containing (in mM) 82.5 NaCl, 2.5 KCl, 1 MgCl$_2$, 5 HEPES, pH 7.4, for 75–90 min. Isolated oocytes were rinsed with and stored in ND96 bathing solution (ND96B) containing (in mM) 96 NaCl, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 HEPES, 50 µg/ml of both gentamicin and tetracycline (Sigma-Aldrich), pH 7.4 at 18°C. Approximately 24 h after extraction, oocytes were microinjected with 27.6 nl total volume of cRNA containing wild-type or mutant Q1 (7.5 ng/oocyte), with or without E1 or E3 (3.75 ng/oocyte). After 3–6 d, currents were recorded using Warner Instrument (OC-725) two-electrode voltage clamp (TEVC) and the data were acquired with Digidata 1322A using pClamp 9 (Axon Instruments). Electrodes were filled with 3 M KCl, 5 mM EGTA, 10 mM HEPES, pH 7.6, and had resistance between 0.2 and 1.0 MΩ. Current–voltage relationships were measured in ND96 (in mM): 96 NaCl, 2 KCl, 0.3 CaCl$_2$, 1 MgCl$_2$, 5 HEPES, pH 7.4, by holding at −80 mV and pulsing for 4 s to potentials between −100 and +40 mV in 20-mV increments.

MTS Modification Experiments
To assess extracellular exposure of introduced cysteines, accessibility to the positively charged membrane-impermeant [2-(trimethylammonium)ethyl] methanethiosulfonate (MTSET) or the negatively charged (2-sulfonatoethyl)methanethiosulfonate (MTSES; Toronto Research Chemicals) was determined by measuring changes in current amplitude at +40 mV. Since the half-life of these MTS reagents is ∼15 min in aqueous solutions (Stauffer and Karlin, 1994), a 0.5 M stock solution was dissolved in water, and aliquots snap frozen in liquid nitrogen. Aliquots were freshly diluted to 0.4–1.6 mM in ND96 recording solution immediately before perfusion, and every 5 min thereafter to maintain a relatively constant concentration for the duration of each experiment. Two different pulse protocols were used to determine if cysteine exposure was state dependent. In the open protocol, the membrane was depolarized 11% of the time, for 2 s every 18 s or for 4 s every 36 s. In the closed protocol, the membrane was held at −80 mV for

99.4% of the time, and only depolarized to +40 mV for 250 ms every 42 s or for 500 ms every 84 s. Switching to the open protocol after ~500 s allowed a measure of the extent of modification for residues that were modified slowly in the closed protocol. This was done in the presence and absence of MTSET to rule out any change in current associated with variation in pulse duration and interpulse interval. To compare each mutant, the currents were normalized using two procedures. For mutants that showed a decrease in current upon MTS modification, the data were normalized such that the current before reagent perfusion was equal to one. For mutants that showed an increase in current upon MTS modification, the baseline was defined as zero and the currents were subsequently normalized based on the full extent of modification. The normalized data were plotted versus reaction time for each MTS modification. All data fit well to a single exponential except for A226C, which required a biexponential fit to extract the fast component of modification.

For the varying pulse duration experiments, five different pulse protocols were used. In each protocol, the membrane was depolarized 11% of the total pulse time. MTSET or MTSES modification was monitored as the membrane was held at −80 mV and depolarized to +40 mV for 100 ms every 900 ms, 500 ms every 4.5 s, 1 s every 9 s, 2 s every 18 s, or 4 s every 36 s. Modification-induced current changes were monitored at the end of the shortest pulse duration used for each set of experiments. For voltage dependence experiments with I230C/E3, oocytes were held at −80 mV and pulsed for 4 s to either −100, −80, −40, 0, or 40 mV, followed by a −30 mV tail pulse. Current changes were monitored at the end of the −30 mV tail for each voltage potential studied.

RESULTS

Identification of State-dependent S4 Residues in KCNQ1 K+ Channels

To examine the influence of KCNE peptides on the position and equilibrium of the voltage sensor, we first determined whether there were residues in the S4 voltage sensor of Q1 that were accessible to an externally applied aqueous cysteine-specific modifying reagent, MTSET. We individually mutated S4 residues 226–232 to cysteine, expressed these Q1 mutants in *Xenopus* oocytes, and examined the currents elicited from a series of test depolarizations using a TEVC. The majority of the cysteine mutants (A226C, I227C, G229C, I230C, F232C) resembled wild-type Q1 (Fig. 1 C); however, charge neutralization of either of the two arginine residues by cysteine mutagenesis resulted in currents with altered gating kinetics (Fig. 2 A, Before). R228C afforded small currents that slowly activated; R231C was a constitutively conducting channel. The gating kinetics observed for these Q1 cysteine mutants were nearly identical to those observed when the arginines were mutated to alanine (Panaghie and Abbott, 2007).

We then screened these cysteine mutants for changes in current amplitude or gating kinetics when treated with MTSET. To monitor the rate of cysteine modification, we elicited a series of test pulses to 40 mV with each mutant in the presence of 400 μM MTSET. Cysteines at positions 226–228 were modified using this open protocol (vide infra), as the current increased exponentially with MTSET treatment (Fig. 2). Modification-induced current changes were also measurable for G229C and I230C when the MTSET concentration was doubled. R231C and F232C were unaffected by MTSET, which indicates that these deeper residues were either not accessible to the reagent or the modification did not induce a measurable change in current. Examination of the Q1 mutants after MTSET modification revealed that the gating kinetics of the modified channels became nearly instantaneous and were open at hyperpolarized potentials (Fig. 2 A, After). Since all modifications required MTSET treatment for longer than the half-life of the reagent (~15 min) (Stauffer and Karlin, 1994), we continuously added freshly prepared MTSET to the gravity-fed perfusion device every 5 min to maintain a relatively constant concentration (Materials and methods). Currents from wild-type Q1 expressed alone, with E1 or E3 were unchanged by MTSET (Fig. 2 B), demonstrating that changes in current observed with the mutants were due to the presence of cysteines in the S4 segment.

After identifying the S4 mutants that were measurably modifiable by MTSET, we then determined whether

TABLE I

Comparison of MTSET Modification of S4 Residues in KCNQ1

Construct	KCNQ1		KCNQ1/KCNE1		KCNQ1/KCNE3	
	k_{open}	k_{closed}	k_{open}	k_{closed}	k_{open}	k_{closed}
A226C	153 ± 10	21 ± 7[a]	11 ± 2	5.2 ± 0.3[a]	64 ± 5	23 ± 2[a]
I227C	161 ± 31	60 ± 3[a]	12 ± 3	13 ± 1	29 ± 3	26 ± 4
R228C	20 ± 1	~1	15 ± 1	~1	28 ± 3	19 ± 2[a]
G229C	1.5 ± 0.2	~0.1	0.49 ± 0.06	<0.05	8.5 ± 1.0	5.3 ± 0.6[a]
I230C	9.4 ± 1.1	~0.5	1.2 ± 0.1	<0.05	19 ± 2	15 ± 1

Data from individual exponential fits in ND96, obtained from three to seven oocytes. All MTSET modifications were fit to a single exponential, except for Q1(A226C) in the open protocol, which required a biexponential fit to extract the fast component of the reaction, as described in the Materials and methods. k_{open} and k_{closed} are the second order modification rate constants ($M^{-1}·s^{-1}$) determined using the open and closed pulse protocols. Measured values are mean ± SEM. Approximate values were calculated based on the extent of modification in the closed protocol, as described in the Materials and methods. Statistical comparison is between k_{open} and k_{closed} for each mutant K+ channel complex.
[a]P < 0.05.

these modifications occurred in a state-dependent manner. To determine whether S4 modification occurred in the resting state, we compared the rates of modification using two test pulse protocols: open and closed. In the closed protocol, the channels are held at -80 mV for the majority of the pulse duration and only briefly depolarized to ascertain MTSET modification rate; therefore, the S4 voltage sensors will primarily be in the resting state. In the open protocol, the channels are depolarized $\sim$18-fold more, which shifts the equilibrium of the S4 voltage sensors to favor the active state. Thus, state-dependent S4 residues will be modified faster in the open protocol compared with the closed whereas state-independent residues will be modified at a similar rate independent of the protocol used. The rates of modification of the S4 cysteine mutants using the open and closed protocols are compared in Fig. 2 (B and C). Modification rates for the first two residues (A226C, I227C) could be measured in both the open and closed protocols. Since the "open" protocol is only open 11% of the test pulse cycle, we expected the MTSET reactions to exhibit biexponential kinetics for the cysteine mutants that were appreciably modified in both states, as long as the rates of modification in the two states were significantly different. For A226C, the reaction rate using the open protocol could not be fit to a single exponential, consistent with different rates of modification in the resting and active states of S4. When the data were fit to two time constants, the fast component of the exponential was well fit (Table I); however, the error of the fit of the slower component was very large. To measure and accurately fit the slow component of the reaction, we used the closed protocol, which minimizes modification in the active state. Using this protocol, the reaction was fit to a single exponential (Fig. 2 B and Table I), consistent with modification occurring primarily in the resting state. Comparing the two rates showed that A226C was modified 7.5-fold faster in the open protocol. In contrast to A226C, modification of I227C using both the open and closed protocols appeared to follow a single exponential time course (Fig. 2 B). Although a two exponential fit was expected, the similar rates of modification were not resolvable by mathematical fitting. Nonetheless, these results demonstrate that A226C and I227C are accessible to the extracellular solution when the S4 is at rest, but upon depolarization the residues are modified at a slightly faster rate.

MTSET modification of the three other S4 residues (R228C, G229C, I230C) could only be measured in the open protocol, but were well fit to a single exponential (Fig. 2 B). These residues were somewhat modified by MTSET in the "closed" protocol; however, the linear rate of modification was consistent with the reaction occurring during the short test depolarizations when the S4 is in the active state. Since the time course needed to

complete the reaction in the closed protocol was not experimentally tractable (hours) with workable concentrations of MTSET, we switched to the open protocol after $\sim$500 s to determine the extent of modification in the closed protocol. Normalization of the data using this end point allowed for comparison of the data generated from the two protocols (Fig. 2 B). Based on the extent of modification, we estimate that the reaction proceeded in the closed protocol $\sim$15–20-fold slower than in the open, which closely approximates the 18-fold difference in depolarization duration between the two protocols. Thus, these three S4 mutants (R228C, G229C, I230C) are only modified when the channel is in the depolarized state.

Measuring the Rate of Voltage Sensor Equilibration in Q1/E1 K+ Channel Complexes

We next determined whether these mutant Q1 channels would assemble with E1 to produce complexes with slowly activating kinetics and remain modifiable in a state-dependent manner. Coexpression of Q1 mutants (R228C, G229C, I230C) with E1 produced channel complexes that were highly reminiscent of wild-type Q1/E1, but after MTSET modification the mutant complexes became rapidly activating and open at negative potentials, as is shown for R228C/E1 in Fig. 3 A. MTSET modification was state dependent for all three mutant Q1/E1 complexes (Table I). Fig. 3 B shows that MTSET modification of R228C/E1 occurs rapidly in the open protocol, but using the closed protocol the mutant complex was modified very slowly, consistent with the reaction occurring primarily in the active state. To estimate the rate of modification in the closed protocol, we shifted from the closed to the open protocol and followed the reaction to completion (Fig. 3 B, arrow). This observed increase in current was due to subsequent modification of unreacted cysteines in S4 and was not an artifact of changing the interpulse interval since it was only observed when MTSET was in the bath solution.

We then used these three state-dependent Q1/E1 mutant complexes to determine whether the slow gating in Q1/E1 complexes is due to S4 slowly transitioning from a resting to active state. If the slow activation observed in Q1/E1 complexes is due to a sluggish voltage sensor, this predicts that the MTSET modification rate of the cysteines in S4 will decrease with shorter pulse durations, as long as the opening of the intracellular gate itself does not alter S4 accessibility to MTSET. Conversely, if E1 has no effect on voltage sensor movement, then the modification rate should be independent of pulse duration. To experimentally test these two possibilities, the total depolarization time was kept constant (11%), but the individual pulse lengths were varied between 0.1 and 4 s. (Fig. 3 C, inset). We first examined the R228C/E1 mutant complex. Since a series of rapid, short pulses can cumulatively shift the S4
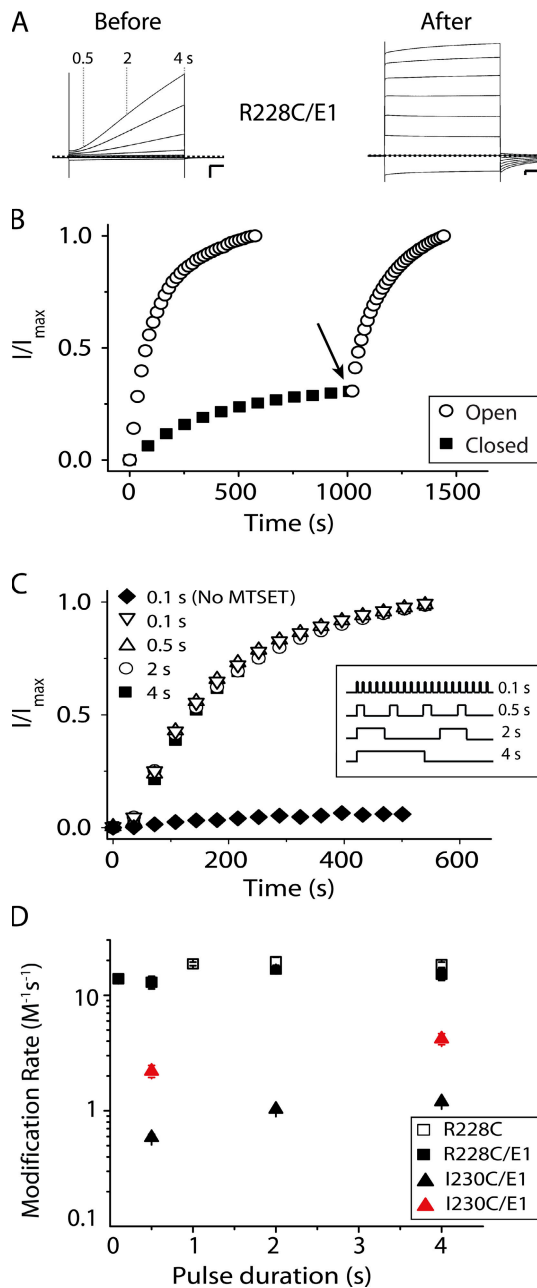
Figure 3. The S4 voltage sensor reaches equilibrium quickly in KCNQ1/KCNE1 complexes upon depolarization. (A) TEVC recordings from R228C/E1 complexes expressed in Xenopus oocytes before and after MTSET modification. Oocytes were held at −80 mV and currents were elicited from 4-s command voltages from −100 mV to +40 mV in 20-mV increments. Gray dotted lines denote the amount of current from a 40-mV depolarization at 0.5, 2, and 4 s. Scale bars represent 0.5 µA and 0.5 s. Dashed line indicates zero current. (B) Change in current for R228C/E1 monitored over time using 40-mV test pulses with continuous perfusion of 400 µM MTSET. In the "open" protocol (open circles) channels were depolarized 11% of the total time; "closed" protocol (filled squares) 0.6% of the total time. Shifting to the open protocol (arrow) after ~1,000 s shows the completion of MTSET modification. Currents from the open and closed protocols were normalized to the maximal change in current for comparison. (C) Pulse duration has no effect on the rate of MTSET modification of R228C/E1. Representative plots from the MTSET reaction

segments into the active state and give rise to an apparent increase in instantaneous conductance (Bett et al., 2006), we first determined the interpulse interval required to fully reset the voltage sensors by pulsing in the absence of MTSET (Fig. 3 C, filled diamonds). MTSET treatment of R228C/E1 with different pulse durations from 0.1 to 4 s resulted in nearly identical rates of modification (Fig. 3 C). As a comparison, we performed a similar set of pulse frequency experiments on unpartnered R228C and determined that the rate of S4 modification in homotetrameric Q1 channels also remained constant with various pulse durations (Fig. 3 D). Similar pulse duration experiments with the G229C/E1 mutant complex were not experimentally feasible due to the extremely slow modification rate (Table I). However, for I230C/E1, MTSET modification rates were modestly dependent on pulse duration. With 500-ms pulses, the rate of modification was approximately twofold slower than for 4 s. A similar result was also obtained using the negatively charged MTS reagent, MTSES (Fig. 3 D, red triangles) Thus, the examination of the state-dependent Q1/E1 complexes in pulse duration experiments shows that the voltage sensors reach equilibrium quickly when E1 is present.

Voltage Sensor Equilibrium Measurements in Q1/E3 K+ Channel Complexes

We next examined the effects of E3 on Q1's voltage sensors. Coexpression of E3 with all but one of the S4 cysteine mutants resulted in functional complexes that were constitutively conducting and possessed rapid gating kinetics similar to wild-type Q1/E3 complexes (Fig. 4 A). The one deviant, R228C/E3, appeared to be closed at hyperpolarizing potentials and the depolarization-elicited currents were small in amplitude and slowly activating. Of these mutants, five were rapidly modified by MTSET and the reactions went to completion in both the closed and open protocols (Fig. 4, A and B). Moreover, all MTSET reactions were pseudo-first order and well fit to single exponentials, indicating that the S4 residues in Q1/E3 complexes were readily accessible to the extracellular solution in both the closed and open protocols (Table I). The lack of state-dependent modification for these S4 cysteine mutants when paired with E3 are in striking contrast to when the mutants were expressed alone, where A226C showed biexponential

with R228C/E1 using 0.1, 0.5, 2, or 4 s 40-mV pulses, where the total depolarization time was kept constant (inset). The total MTSET exposure time is plotted versus normalized current at the end of the depolarization. Filled diamonds indicate the interpulse interval required to reset voltage sensors between pulses when no MTSET was added (900-ms interval for 100-ms pulse). (D) Comparison of R228C, R228C/E1, and I230C/E1 in pulse duration experiments. Black symbols represent modification by MTSET, red symbols modification by MTSES. Data were averaged from three to six oocytes ± SEM.
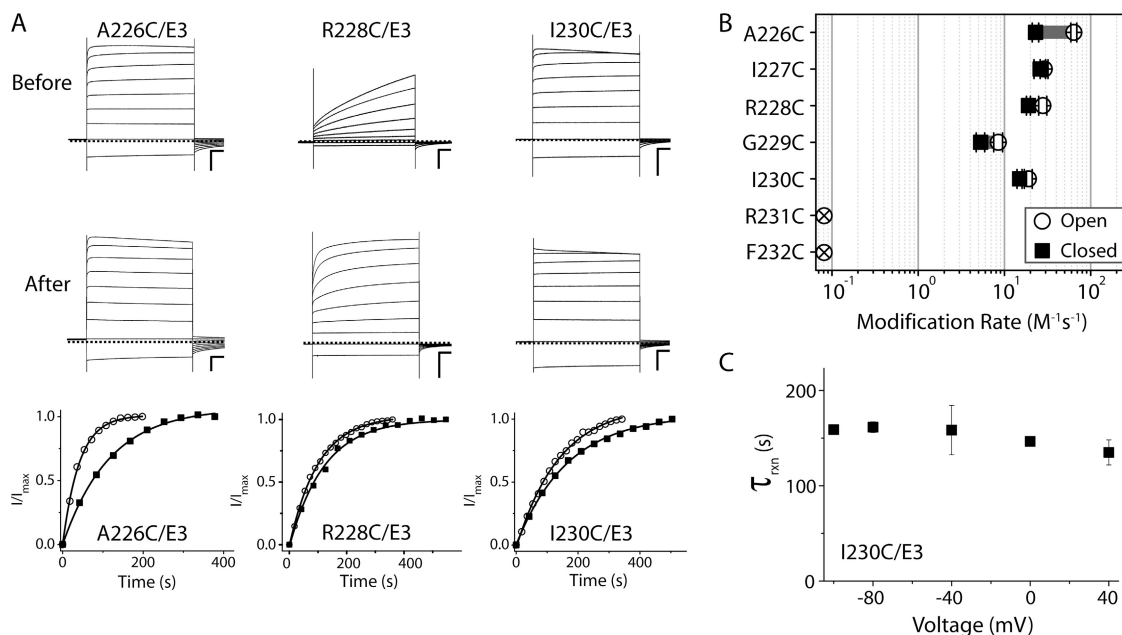
Figure 4. The equilibrium of S4 voltage sensors is shifted to favor the active state in KCNQ1/KCNE3 complexes. (A) TEVC recordings from A226C/E3, R228C/E3, and I230C/E3 complexes expressed in *Xenopus* oocytes before and after MTSET modification. Oocytes were held at −80 mV and currents were elicited from 4-s command voltages from −100 to +40 mV in 20-mV increments. Scale bars represent 0.5 μA and 0.5 s. Dashed line indicates zero current. (B) Change in current monitored over time using 40-mV test pulses with continuous perfusion of 400 μM MTSET. In the "open" protocol (open circles), the channel complexes were depolarized 11% of the total time; "closed protocol" (filled squares) 0.6% of the total time. Currents from the open and closed protocols were normalized to the maximal change in current for comparison. Curves were fit to monoexponential time courses and the reaction rates are tabulated in Table I. (C) Comparison of MTSET modification rates for Q1/E3 S4 cysteine substitutions in the open (open circles) or closed (filled squares) protocols. The gray bar gives the fold-change in rate between the open and closed protocols. X-out open circles indicate no observed change of current using open protocol. Data were averaged from three to five oocytes ± SEM. (D) The rate of MTSET reaction with I230C/E3 channel complexes is independent of voltage. Oocytes were held at −80 mV, and for each voltage, 4-s pulses were followed by a −30 mV tail pulse (100 ms), which was used to monitor the change in current upon MTSET application. The modification reaction time constant from single exponential fits is plotted for each voltage potential. Data were averaged from three to four oocytes ± SEM.

modification rates using the open protocol and R228C, G229C, and I230C were only modified upon depolarization (Fig. 2, B and C). We were initially concerned that the loss of state-dependent modification of Q1/E3 channels compared with unpartnered Q1 was due to the native extracellular cysteine in E3. Although control experiments with wild-type Q1/E3 complexes showed no measurable effect in the presence of MTSET, modification of this E3 cysteine will result in a disulfide bond, which could react with the cysteine mutants in S4 via an accelerated disulfide exchange reaction. To eliminate this possibility, we repeated the experiments with a cysteineless version of E3 and obtained similar state-independent modification of I230C's voltage sensor (unpublished data). Examination of the deepest modifiable cysteine residue (I230C) with E3 at different test pulse potentials revealed that rate of MTSET modification was independent of voltage from −100 to 40 mV (Fig. 4 C). In total, these results argue that at hyperpolarizing potentials the equilibrium of the voltage sensor in Q1/E3 complexes is shifted such that it significantly exposed to the extracellular solution.

DISCUSSION

MTSET Accessibility of Cysteine Residues in the S4 Segment of Q1 Channels

We have examined the extracellular accessibility of introduced cysteines in the S4 voltage sensors of Q1 channel complexes to indirectly assess their positions and equilibrium. Although this approach has faithfully mirrored more direct measurements of S4 whereabouts in other voltage-gated channels (with gating currents and fluorescently labeled voltage sensors), there are at least three caveats to consider. First, accessibility measurements may not exclusively report on S4 movement since other K^{+} channel rearrangements could expose S4 to the extracellularly applied reagent. Second, since modification is ascertained by measuring changes in macroscopic current, it is unclear how many modified S4 segments are required to produce the measured effect. Third, MTS-modified cysteines can undergo disulfide exchange with nearby free sulfhydryls, which may affect the rate and magnitude of the measurement.

Using MTSET as our accessibility reagent, we observed modification of cysteines introduced from residues 226–230 of the S4 in Q1 channels. The measured MTSET modification rates were slower compared with voltage sensors in other channels as well as model thiols (Larsson et al., 1996; Karlin and Akabas, 1998). Although voltage-gated channels share a common protein fold, differences in the microenvironments (steric and electrostatic) surrounding the S4 segment could explain the slow reaction rates observed with Q1. To further elucidate the influence of the Q1 protein environment on S4's range of motion, examination of the intracellular accessibility to MTSET would be particularly informative. Unfortunately, the current from Q1 channel complexes in excised macropatches rapidly decreases over time (rundown) (Loussouarn et al., 2003), preventing the use of this experimental technique.

External MTSET modification also revealed that the voltage sensors in Q1 channels are sensitive to the removal, introduction, and specific location of charges in the S4 segment. Removal of the positive charge at residues 228 and 231 by cysteine substitution ablated activation kinetics, as was previously observed with alanine mutants at these same positions (Panaghie and Abbott, 2007). Charge reintroduction by MTSET modification restored gating kinetics and increased current output for R228C. However, introduction of positive charge at previously uncharged positions resulted in channels with nearly instantaneous activation kinetics for all modifiable cysteine mutants except I230C. Thus, the charge sensitivity of the Q1 S4 segment makes the effects of MTSET modification on the voltage dependence and changes in current amplitude unpredictable. In contrast, the state dependence of MTSET modification of the S4 cysteine residues in Q1 followed a clear pattern. The more N-terminal and presumably more accessible S4 residues were measurably modified in both the open and closed states. Correspondingly, modification of the more C-terminal residues was not detected, suggesting that these residues are too buried to react with MTSET. The remaining three residues (R228C, G229C, I230C) were strongly state dependent and therefore used to examine the effects of E1 and E3 on voltage sensor equilibrium.

E1 Does Not Appreciably Slow the Equilibration Rate of the Q1 Voltage Sensor

Coexpression of E1 with the three state-dependent Q1 mutants resulted in two different rates of MTSET modification: R228C/E1 was modified at a similar rate as R228C alone whereas the modification of G229C and I230C was considerably reduced (~10-fold) in the presence of E1 (Table I). While it is tempting to compare the absolute rates of MTS modification between Q1 and Q1/E1 channel complexes to determine whether E1 slows the voltage sensors, this measurement reports on the equilibrium of the voltage sensor and not the kinetics of movement. Thus, the recent conclusion that E1 peptides slow the transition of the S4 segment to the active state based on differences in MTS modification rates was premature (Nakajo and Kubo, 2007). Moreover, the inclusion of KCNE peptides in the Q1 complex adds the potential for steric and electrostatic interactions that could substantially reduce or enhance the rates of MTS modification. Therefore, it is imperative to examine each individual complex to elucidate the effects of KCNE peptides on voltage sensor equilibration rates. Accordingly, we measured the dependence of MTSET modification rate on pulse duration in attempt to extract the kinetics of voltage sensor movement in Q1/E1 complexes.

Using two of the strongly state-dependent S4 cysteine mutants, we found that R228C/E1 was modified independent of pulse duration (as short as 100 ms) whereas I230C/E1 modified somewhat slower with the shortest depolarizations. For R228C/E1, this result implies that voltage sensors reach equilibrium in <100 ms and that the rate limiting step is the opening of the Q1/E1 activation gate. In support of this model, a recent report found that the rate of A226C/E1 modification by MTS reagents is also independent of pulse duration with pulses as short as 30 ms (Nakajo and Kubo, 2007). For I230C/E1, we did observe a twofold difference in modification rate between the 500-ms and 4-s pulse durations using both MTSET and MTSES. However, this difference does not fully account for the approximately sevenfold change in conductance observed over this time frame, and may be attributed to increased extracellular exposure of this residue induced by cytoplasmic gate opening or other delayed conformational changes. Alternatively, if E1 does partially slow voltage sensor equilibration, the lack of dependence on pulse duration for R228C/E1 (and A226C/E1) can be explained by two pairs of voltage sensors moving at different rates. To directly measure these rates, it will require either measuring gating currents or monitoring S4 motions with reporter probes. Both of these experimental approaches will be challenging since the S4 segment is charge-poor and its modification with cysteine-specific reagents (at least MTSET) typically abolishes Q1 channel gating.

E3 Shifts the Voltage Sensor Equilibrium to Favor the Active State

For Q1/E3 complexes, the entire panel of S4 cysteine mutants was modified by MTSET in the closed protocol, indicating that these residues are equally accessible to the reagent at resting and depolarizing potentials (Table I). Although the increase in reactivity for a single mutant could be attributed to local accessibility differences between Q1 and Q1/E3 channel complexes, the across the board loss of state dependence strongly argues that the voltage sensor equilibrium in Q1/E3 complexes is

shifted to favor the active state even at hyperpolarizing potentials. This result confirms that the tight linkage between voltage sensor and activation gate, which has been observed in the majority of wild-type voltage-gated channels, is maintained in Q1/E3 complexes. This differs from mutagenic investigation of activation gates and voltage sensors in other voltage-gated channels that abolish this link, uncoupling the coordinated movement of these two protein domains (Lu et al., 2002; Sukhareva et al., 2003). Since Q1/E3 complexes exhibit some voltage dependence, this would suggest that E3 does not lock the voltage sensors up, but enables voltage-independent access to the active state. A recent mutagenic investigation of KCNQ channel voltage sensors suggests that E3 converts Q1 into a leak channel because the S4 segment has a smaller net positive charge ($+3$) compared with the other members in the family (Panaghie and Abbott, 2007). For most of our cysteine modifications, adding an additional positive charge to the S4 with MTSET converted Q1 channels and Q1/E1 complexes into voltage-independent leak channels. This trend appears to contradict the requirement for a charge-poor S4 to induce a leak current. However, the resultant disulfide bonded ethyltrimethylammonium is a terrible structural mimic of arginine or lysine. Moreover, the haphazard attachment of positive charge to the S4 could also disrupt voltage sensing since the spacing of charges in voltage-sensitive channels is also highly conserved (Catterall, 1988). On the other hand, we stumbled upon one MTSET modification that supports the paucity of charge model proposed by Abbott and coworkers. Unmodified R228C/E3 complexes afforded small currents that were only measurable at positive potentials (Fig. 4 A); however, reinstating the charge at position 228 with MTSET afforded robust currents with more Q1/E3 character.

Conclusions

The discovery that E1 and E3 differently influence the motions of Q1 channels supports a bipartite model that we previously proposed for KCNE modulation of Q1 channels (Gage and Kobertz, 2004). Our model proposed that the E3 transmembrane domain was dominant in modulation and overrides the conserved C-terminal domain of KCNE peptides, whereas the E1 transmembrane domain was passive in modulation, allowing the C terminus to influence channel gating. These new data suggest that the mechanism for bipartite modulation arises from the tight coupling of the voltage sensor position to the activation gate. The E3 transmembrane domain shifts the voltage sensor equilibrium to favor the active state, resulting in a predominately open activation gate. Moreover, since E1 does not appreciably slow the rate of voltage sensor equilibration, it would allow the cytoplasmic domain of E1 to slow activation gate opening. Although these data support the bipartite model and suggest potential Q1-KCNE protein–protein interactions, future structure–function studies are needed to determine whether the modulatory effects of KCNE peptides on voltage sensors and activation gates is via a direct or allosteric mechanism.

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