TEA⁺-sensitive KCNQ1 Constructs Reveal Pore-independent Access to KCNE1 in Assembled $I_{Ks}$ Channels

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ABSTRACT $I_{Ks}$, a slowly activating delayed rectifier K⁺ current through channels formed by the assembly of two subunits KCNQ1 (KvLQT1) and KCNE1 (minK), contributes to the control of the cardiac action potential duration. Coassembly of the two subunits is essential in producing the characteristic and physiologically critical kinetics of assembled channels, but it is not yet clear where or how these subunits interact. Previous investigations of external access to the KCNE1 protein in assembled $I_{Ks}$ channels relied on occlusion of the pore by extracellular application of TEA⁺, despite the very low TEA⁺ sensitivity (estimated EC₅₀ > 100 mM) of channels encoded by coassembly of wild-type KCNQ1 with the wild type (WT) or a series of cysteine-mutated KCNE1 constructs. We have engineered a high affinity TEA⁺ binding site into the h-KCNQ1 channel by either a single (V319Y) or double (K318I, V319Y) mutation, and retested it for pore-delimited access to specific sites on coassembled KCNE1 subunits. Coexpression of either KCNQ1 construct with WT KCNE1 in Chinese hamster ovary cells does not alter the TEA⁺ sensitivity of the homomeric channels (IC₅₀ = 0.4 mM [TEA⁺]o), providing evidence that KCNE1 coassembly does not markedly alter the structure of the outer pore of the KCNQ1 channel. Coexpression of a cysteine-substituted KCNE1 (F54C) with V319Y significantly increases the sensitivity of channels to external Cd²⁺, but neither the extent of nor the kinetics of the onset of (or the recovery from) Cd²⁺ block was affected by [TEA⁺]o at 10× the IC₅₀ for channel block. These data strongly suggest that access of Cd²⁺ to the cysteine-mutated site on KCNE1 is independent of pore occlusion caused by TEA⁺ binding to the outer region of the KCNQ1/V319Y pore, and that KCNE1 does not reside within the pore region of the assembled channels.

KEY WORDS: heart potassium channels • pore • cysteine substitution • LQT-1 • subunit assembly

INTRODUCTION

$I_{Ks}$, a slowly activating delayed rectifier K⁺ current through channels formed by the assembly of two subunits KCNQ1 (KvLQT1) and KCNE1 (minK), contributes to the control of the cardiac action potentials (Kass and Davies, 1996). The uniquely slow kinetics of $I_{Ks}$ are particularly well suited to time repolarization in cardiac ventricular muscle cells, where the duration of the action potential can be several hundreds of milliseconds (Kass, 1995, 1996; Suessbrich and Busch, 1999). KCNQ1 has six hydrophobic transmembrane segments similar to other voltage-gated K⁺ channels, but KCNE1 is a 129-amino acid residue protein with a single transmembrane segment (Takumi et al., 1988; Barhanin et al., 1996; Sanguinetti et al., 1996) resembling β subunits of other channels (Abriel et al., 2000; Sanguinetti, 2000a). Coexpression of KCNQ1 with KCNE1 leads to slowly activating K⁺ currents with characteristics similar to those of the native cardiac $I_{Ks}$ that are so crucial to the maintenance of normal cardiac function. Evidence for the importance of this current to human physiology comes from studies of the long QT syndrome, which is an inherited arrhythmia that is correlated with episodes of syncope and sudden cardiac death (Suessbrich and Busch, 1999). Mutations that map to the KCNQ1 and KCNE1 genes account for >50% of reported genotyped cases of QT syndrome (Splawski et al., 1997). Some of these mutations reported in KCNE1 have been shown to modify the gating kinetics of channels expressed heterologously, suggesting that the molecular interaction between two proteins may be altered by these mutations (Splawski et al., 1997; Sesti and Goldstein, 1998). However, the molecular mechanism of the interaction between KCNQ1 and KCNE1 proteins remains to be understood.

To determine whether sites of the KCNE1 protein are exposed within the $I_{Ks}$ pore, Tai and Goldstein (1998) and Wang et al. (1996) performed scanning susceptibility analysis (Akabs et al., 1992; Stauffer and Karlin, 1994; Pascual et al., 1995). These experiments were carried out for $I_{Ks}$ channels expressed in Xenopus oocytes using the thiol-reactive transition metal Cd²⁺ and a series of mutants for which a single residue was mutated to cysteine in sequential positions along the putative transmembrane segment of KCNE1. In this work, $I_{Ks}$ channels formed with F54C or the G55C mutant human KCNE1 were sensitive to external but not internal Cd²⁺, whereas those with mutation F56C were sensitive...
to internal but not external reagent. Tai and Goldstein’s hypothesis was that there is a barrier to Cd\(^{2+}\) movement between residues G55 and F56 in the conducting pore. External Cd\(^{2+}\) access to these key sites was interpreted as pore-delimited based on competition between externally applied TEA\(^{+}\) and Cd\(^{2+}\) in channel-blocking experiments. However, these key experiments and their interpretation were limited by the very low TEA\(^{+}\) sensitivity of wild-type KCNQ1 channels.

Here, we have reinvestigated pore-delimited access of the thiol-reactive reagent (Cd\(^{2+}\)) to specific cysteine-mutated sites (F54C and G55C) on the h-KCNE1 protein by coexpressing these constructs with highly TEA\(^{+}\)-sensitive mutant h-KCNQ1 channels (K318I + V319Y; V319Y) in transiently transfected Chinese hamster ovary (CHO) cells. Externally applied TEA\(^{+}\) rapidly, reversibly, and potently blocked channels consisting of these mutants alone or coassembled with WT and mutant KCNE1. We tested for, but did not find evidence of, restrictions of Cd\(^{2+}\) access to F54C or G55C when this KCNE1 mutant was coexpressed with the TEA\(^{+}\) sensitive mutants. Our data strongly suggest that KCNE1 does not reside within the pore region and that any effects of KCNE1 assembly are likely to be due to interactions between KCNE1 and KCNQ1 that reside elsewhere, perhaps with the S4 segments themselves.

MATERIALS AND METHODS

Molecular Biology

The human cDNA clone KCNQ1 was a gift from Dr. M. Keating (Department of Human Genetics, University of Utah, Salt Lake City, UT) and subcloned into the mammalian expression vector pCDNA3.1 (Invitrogen). Mutations of both KCNQ1 and KCNE1 were performed by plaque-forming unit-based mutagenesis (QuickChange™ site-directed mutagenesis kit; Stratagene), and the mutant gene fragments were inserted into translationally silent restriction sites. All sequences were performed by the chain termination method in the DNA Sequencing Facility at Columbia University. The basic protocol uses Chinese hamster ovary (CHO) cells. Externally applied TEA\(^{+}\) rapidly, reversibly, and potently blocked channels consisting of these mutants alone or coassembled with WT and mutant KCNE1. We tested for, but did not find evidence of, restrictions of Cd\(^{2+}\) access to F54C or G55C when this KCNE1 mutant was coexpressed with the TEA\(^{+}\) sensitive mutants. Our data strongly suggest that KCNE1 does not reside within the pore region and that any effects of KCNE1 assembly are likely to be due to interactions between KCNE1 and KCNQ1 that reside elsewhere, perhaps with the S4 segments themselves.

Electrophysiology and Data Analysis

Currents were recorded using the whole-cell patch-clamp technique (Hamill et al., 1981). CHO cells were plated on small petri dishes, and cultured in an atmosphere of 5% CO\(_2\) in air. We avoided using CHO cells after 15 passages because we found this improved our transfection efficiency and cell quality. Transfected cells were plated on small petri dishes, and cultured in an incubator with 5% CO\(_2\).

RESULTS

External TEA\(^{+}\) Block of Wild-type and Mutant I\(_{Ks}\) Channels

Because it is generally assumed that KCNQ1 encodes the pore forming subunit of functional I\(_{Ks}\) channels (Sanguinetti et al., 1996), we sought to increase the TEA\(^{+}\) sensitivity of assembled I\(_{Ks}\) channels by mutating KCNQ1 as a first step in determining pore-delimited access to sites on the h-KCNE1 protein. Common residues (position 449) in the extracellular loop of the Shaker K\(^{+}\) channel outer pore region have been shown to coordinate TEA\(^{+}\) binding (MacKinnon and Yellen, 1990) and C-type inactivation (Lopez-Barneo et al., 1993). To evaluate whether this region affects the TEA\(^{+}\) sensitivity of I\(_{Ks}\) channels, we mutated two residues (K318; V319) in the KCNQ1 channel according to the equivalent positions in the Kv2.1 channel (Lipkind et al., 1995) and KCNQ2 channel (Wang et al., 1998a), which are both sensitive to extracellular TEA\(^{+}\) block in the micromolar concentration range.

We next investigated the effects of these KCNQ1 mutations on gating and sensitivity to [TEA\(^{+}\)]\(_{o}\) and without coexpression of KCNE1. In the absence of KCNE1, wild-type (WT) KCNQ1 channels were not completely blocked by >50 mM TEA\(^{+}\) as previously reported (Wang et al., 1996; Tai and Goldstein, 1998; Fig. 1, A and C, top), but the single mutant (V319Y) channel was reversibly blocked by 0.5 mM TEA\(^{+}\) (Fig. 1 B, top). At 5 mM, external TEA\(^{+}\) completely blocked the currents in both the double (K318I + V319Y) and the single mutant channels. Thus, the mutant KCNQ1 channels exhibit at least a 100-fold increase in the sensitivity to extracellular TEA\(^{+}\). Accompanying the change in TEA\(^{+}\) sensitivity were mutation-induced
changes in channel gating, which is consistent with mutation-induced inactivation of the channel (Fig. 1 B, top panel). Thus, it appears that the common residues in KCNQ1 coordinate high affinity TEA$^+$ binding and most likely C-type inactivation, as is the case in Shaker channels (MacKinnon and Yellen, 1990; Lopez-Barneo et al., 1993). Interestingly, coexpression with KCNE1 results in channels that activate and deactivate with kinetics remarkably similar to WT KCNQ1/KCNE1 (compare bottom panels, Fig. 1, A and B), but now also with greatly increased sensitivity to extracellular TEA$^+$ (Fig. 1 B, bottom panel). We compared the TEA$^+$ sensitivity of heteromultimeric (KCNQ1/KCNE1) and homomultimeric (KCNQ1) channels for the various KCNQ1 constructs in Fig. 1 C. The IC$_{50}$ values of TEA$^+$ block for K318I + V319Y and V319Y heteromultimeric channels (recorded at +60 mV) are 0.43 mM (nH 0.98) and 0.41 mM (nH 0.87), respectively. For both mutants, these IC$_{50}$ values were almost the same at +40 and +80 mV within a range between 0.4 and 0.50 mM, indicating weak voltage-dependent inhibition by TEA$^+$, which is consistent with a TEA$^+$ binding site near the outer pore of the channel. Data for K318I + V319Y KCNQ1 channels reveal the same TEA$^+$ sensitivity in the absence or presence of KCNE1.

Mutant heteromultimeric channels retain the TEA$^+$ sensitivity of the homomultimer (Fig. 1 C), as is the case for WT KCNQ1 subunits. This result provides evidence that KCNE1 coassembly does not markedly alter the structure of the outer pore of the KCNQ1 channel. Furthermore, since we measured the same TEA$^+$ sensitivity for KCNE1 expressed with either the single or double KCNQ1 mutant, we used the V319Y KCNQ1 construct as a tool for highly TEA$^+$-sensitive $I_K$ channels in the remaining experiments.

**External Cd$^{2+}$ Sensitivity of WT and Cysteine-mutated KCNE1 Constructs**

We next coexpressed the TEA$^+$-sensitive KCNQ1 construct (V319Y) with the cysteine-mutated KCNE1, and determined the external Cd$^{2+}$ sensitivity of the KCNQ1–KCNE1 channel complex. Exposure of V319Y/WT KCNE1 channels to 2 mM Cd$^{2+}$ reversibly blocked the expressed current by $<$10% (30-s exposure). This result suggests a subtle involvement of endogenous cysteines, which causes a background block of expressed channels. We next sought to determine whether or not engineering a cysteine into KCNE1 increases the Cd$^{2+}$ sensitivity of the encoded channels. We tested the Cd$^{2+}$ sensitivity of WT KCNQ1 coexpressed with mutant (F54C) KCNE1 (Fig. 2 B), and found the mutation significantly increased 2 mM Cd$^{2+}$ block approximately threefold as shown previously (Tai and Goldstein, 1998). We found a similar Cd$^{2+}$ sensitivity of V319Y KCNQ1/F54C and V319Y KCNQ1/G55C channels (Fig. 2, D and E) and, thus, concluded that the V319Y mutation by itself did not interfere with access of extracellularly applied Cd$^{2+}$ to cysteine at position 54. We also tested for, but found no evidence of, an effect of test voltage on Cd$^{2+}$ block of the expressed channels (data not shown), as had been reported for channels expressed in Xenopus oocytes (Tai and Goldstein, 1998).

Because Tai and Goldstein (1998) reported that the substitution at KCNE1 position 56 (F56C) conferred internal but not external sensitivity of expressed channels to Cd$^{2+}$, we tested for similar effects in our experiments, and confirmed this observation. Coexpression of KCNQ1 with F56C KCNE1 more than doubles the sensitivity of expressed channels to internally applied 2
mM Cd$^{2+}$ (percent rundown of currents for 13 min: WT $87.7 \pm 4.2\%$, $n = 5$; and F56C $37.8 \pm 9.5\%$, $n = 5$), without altering the sensitivity of the expressed channels to externally applied 2 mM Cd$^{2+}$ (percentage of remaining currents: F56C $90.9\%$, $n = 2$; data not shown). Thus, our data support the view that cysteine substitution over a very short region of the KCNE1 protein (residues 54–56) confers side-specific Cd$^{2+}$ sensitivity upon heteromultimeric KCNE1/KCNQ-1 channels. We next proceeded to use the V319Y KCNQ1 construct coexpressed with F54C and G55C KCNE1 mutants to determine whether or not external Cd$^{2+}$ access to these sites on KCNE1 is pore-delimited.

**Rapid Kinetics of TEA$^+$ Block**

Our rapid application system (time constant $>20$ ms) allowed us to investigate the effects of TEA$^+$ during 2-s depolarizing pulses. As shown in Fig. 3, exposure of 10 mM TEA$^+$ to V319Y $I_{Ks}$ completely blocked the expressed current within 500 ms, and the block can be washed out within 200 ms. Because of the slow kinetics of the channels, both wash in and wash out could be measured during a single depolarizing pulse. The rapid and reversible block by TEA$^+$ is consistent with interactions at the mutated site in KCNQ1 (position 319), which is equivalent to Shaker 449T (Yellen et al., 1991; Heginbotham and MacKinnon, 1992). Thus, our data suggest that the high affinity TEA$^+$ block of the mutant KCNQ1 constructs is likely to occur by occluding the channel pore, and that these constructs can be used to assay pore-delimited access to sites on either channel subunit.

**Onset of Cd$^{2+}$ Block in the Absence and Presence of TEA$^+$**

Thus, we next tested for the possibility that the interaction of Cd$^{2+}$ with the Cys at position 54 of KCNE1 is affected by TEA$^+$ block of the channels. We carried out two sets of experiments to test for this possibility. First, we measured the time course of the onset and recovery of Cd$^{2+}$ block in the absence and presence of a saturating TEA$^+$ concentration. If the pore of the $I_{Ks}$ channel is occluded completely by high concentration of TEA$^+$, and if Cys54 was located within the pore, then we reasoned that accessibility of Cd$^{2+}$ to Cys54 would be restricted by TEA$^+$ block.

The protocol we chose took advantage of the application of TEA$^+$ and Cd$^{2+}$ via the rapid solution change system illustrated in Fig. 3 and applied in Figs. 4 and 5. For these experiments, the same concentration of Cd$^{2+}$ application was applied in the absence or presence of TEA$^+$ in the same cell. In the absence of TEA$^+$, we measured the onset of and recovery from Cd$^{2+}$ block of the channels. In the absence of TEA$^+$, the application

![Figure 2](image1.png)

**Figure 2.** The effects of 2 mM of external Cd$^{2+}$ on $I_{Ks}$ channels coexpressed with wild-type or mutated (F54C) KCNE1. The figure shows typical traces recorded in response to 2-s pulses to +60 mV in the absence (○) and presence (●) of 2 mM (extracellular) Cd$^{2+}$ and then after wash out (□). (A) WT KCNQ1 + WT KCNE1. Bars represent 2 nA and 1 s. (B) WT KCNQ1 + F54C KCNE1. Bar: 2 nA. (C) V319Y KCNQ1 + WT KCNE1; (D) V319Y KCNQ1 + F54C KCNE1; and (E) V319Y KCNQ1 + G55C KCNE1. Bars: (C–E) 0.2 nA.

![Figure 3](image2.png)

**Figure 3.** Rapid application of [TEA$^+$]o (10 mM) blocks V319Y KCNQ1 + F54C KCNE1 channels. Currents were recorded by the same voltage protocol as in Fig. 2; and traces with (●) or without (○) application of external TEA$^+$ were superimposed. The duration of the rapid application of TEA$^+$ was indicated as the white bar below the traces. Bars represent 0.5 nA and 1 s.
of Cd$^{2+}$ resulted in approximately a 30% reduction in the current (also see Fig. 2 D), which could be completely reversed with wash out (Fig. 4). We repeated the experiment, but in this case first applied TEA$^+$ at a sufficiently high concentration (10 mM) to completely block the channels. It was possible to assay Cd$^{2+}$ block of the channel that had occurred in the presence of total channel block by TEA$^+$ by quickly washing out TEA$^+$ and measuring the amplitude of the remaining current (Fig. 4, A and C). Washout of TEA$^+$ revealed Cd$^{2+}$-blocked channel activity remarkably similar to that obtained in the absence of TEA$^+$ (Fig. 4, compare B and C). This and other similar experiments are summarized in Fig. 5, which confirms the observation that the magnitude Cd$^{2+}$ inhibition of the V319Y/F54C channel is significantly different from inhibition of V319Y/WT KCNE1 channels, but is not affected by the presence of 10 mM TEA$^+$. These data suggest that pore occlusion by TEA$^+$ does not prevent Cd$^{2+}$-access to the Cd$^{2+}$ sensitive site on KCNE1 (position 54).

**Influence of TEA$^+$ on the Kinetics of Cd$^{2+}$ Block**

Binding of TEA$^+$ and Cd$^{2+}$ to their respective sites of interaction is a dynamic process and possible effects of TEA$^+$ on Cd$^{2+}$ binding will be determined by the on and off rates of the two ions. Assuming pore-limited access of Cd$^{2+}$ to the F54C KCNE1 site, a simple model for Cd$^{2+}$ binding is shown in Scheme 1. In the simplest case, because of rapid TEA$^+$ kinetics, TEA$^+$ is in equilibrium (within dotted square in Scheme 1), and Cd$^{2+}$ is assumed to bind only to the fraction of channels that are not blocked by TEA$^+$ ($f_{UB}$). This fraction of channels is defined as:

$$f_{UB} = \frac{[\text{TEA}^-]}{[\text{TEA}^-] + k_{-1(\text{TEA})}/k_{+1(\text{TEA})}},$$  

where $k_{+1(\text{TEA})}$ and $k_{-1(\text{TEA})}$ represent the apparent onset rate constant (s$^{-1}$M$^{-1}$) and the apparent off rate constant for TEA$^+$ (s$^{-1}$), respectively. The kinetics of

![Figure 4](image_url)  

**Figure 4.** Typical recordings of the effects of external Cd$^{2+}$ on V319Y KCNQ1 + F54C KCNE1 channels in the absence and presence of [TEA$^-$]o (10 mM). The currents were elicited by 2 s pulses to +60 mV every 15 s from a -60-mV holding potential. (A) The experimental procedure is shown at a slow time base. The application of Cd$^{2+}$ and/or TEA$^+$ is indicated as the bars above the current traces. The time scale is shown as the time after the rupture of the membrane. The traces shown in B and C are marked by an open circle (before Cd$^{2+}$), closed circle (after Cd$^{2+}$), and an open square (wash out). Current traces from A are displayed at a faster time base for the application of Cd$^{2+}$ alone (B) and for the application of Cd$^{2+}$ in the presence of 10 mM TEA$^+$ (C). Bars represent 2 nA and 1 s.

![Figure 5](image_url)  

**Figure 5.** Summary of the effects of external Cd$^{2+}$ on TEA$^-$-sensitive constructs. The percentage of remaining current after a 30 s application of 2 mM Cd$^{2+}$ was normalized to current before Cd$^{2+}$ application. The currents were elicited by the same voltage protocol as that in Fig. 4. White column shows block of V319Y KCNQ1 + WT KCNE1 constructs without TEA$^+$ ($n = 8$). Gray column shows block of V319Y KCNQ1 + F54C KCNE1 constructs without TEA$^+$ ($n = 12$). Hatched column shows block for V319Y KCNQ1 + F54C KCNE1 constructs in the presence of 10 mM TEA$^+$ outside ($n = 6$) measuring the data according to Fig. 4 A.
Cd\(^{2+}\) block of the channel can be calculated by considering the interaction of Cd\(^{2+}\) with channels that are not blocked by TEA\(^{+}\), or \(f_{UB}\) derived above. The equation for the time constant (\(\tau\)) of Cd\(^{2+}\) block (unblock) of channels in the presence of TEA\(^{+}\) will be:

\[
1/\tau = (f_{UB} \cdot L \cdot k_{1} + k_{-1}), \quad \text{or} \quad \tau = (f_{UB} \cdot L \cdot k_{1} + k_{-1})^{-1},
\]

(2)

(3)

where \(\tau\) is the time constant in seconds, \(L\) is the concentration of Cd\(^{2+}\) in moles, \(k_{1}\) is the apparent onset rate constant (s\(^{-1}\)M\(^{-1}\)), and \(k_{-1}\) is the apparent off rate constant for Cd\(^{2+}\) (s\(^{-1}\)). To test this prediction, experimental determination of the parameters in Eq. 3 is needed. The data in Fig. 1 can be used to estimate \(f_{UB}\) as a function of [TEA]\(_{o}\), but the rate constants \(k_{1}\) and \(k_{-1}\) must be extracted from kinetic data for Cd\(^{2+}\) block.

We first estimated the association rate constant for Cd\(^{2+}\) inhibition of \(k_{a}\), assuming that the onset of Cd\(^{2+}\)-induced inhibition also obeyed pseudo–first order association kinetics. Fig. 6 A shows a typical recording for Cd\(^{2+}\) block of V319Y/F54C currents and a plot of the averaged peak currents versus time of application of Cd\(^{2+}\). The onset of block was well fit by a single-exponential decay function. On average, the onset time constant was 12.5 ± 2.2 s (\(n = 4\)) and, thus, the association rate constant \((k_{app})\) was 0.08 s\(^{-1}\). We next determined \(k_{1}\) from the wash out of Cd\(^{2+}\) block in Fig. 6 B. As in the experiment of Fig. 4, we first exposed the cell to Cd\(^{2+}\), blocked the channels, and returned it to Cd\(^{2+}\)-free solution. After washing out Cd\(^{2+}\), the currents recovered slowly to the control values with averaged time constant of 21.0 ± 1 s (\(n = 4\)). Assuming pseudo–first order dissociation kinetics, the dissociation rate constant \((k_{-1})\) is 0.05 s\(^{-1}\). Thus, the association rate constant \((k_{1} = (k_{app} - k_{-1})/L)\) and the apparent dissociation constant \((K_{d} = k_{-1}/k_{1})\) were estimated 15 M\(^{-1}\)s\(^{-1}\) and 3.3 \times 10\(^{-3}\) M, respectively.

We next began testing for evidence of overlap of TEA\(^{+}\) and Cd\(^{2+}\) binding sites in the channels by studying the influence of externally applied TEA\(^{+}\) on the kinetics of Cd\(^{2+}\) block of expressed channels. As shown in the bottom panels of Fig. 6, we found no significant effect of external TEA\(^{+}\) (2 mM) on the onset of or recovery from Cd\(^{2+}\) block of V319Y/F54C channels (onset \(\tau = 16.4 ± 2.8\) s [\(P = 0.06\) versus control; paired \(t\) test]; and offset \(\tau = 21.5 ± 0.9\) s [\(P = 0.25\) versus control; paired \(t\) test]). These data suggest that the sites for TEA\(^{+}\) and Cd\(^{2+}\) block do not overlap. We repeated these experiments testing for the effects of extracellular...
lar TEA⁺ on Cd²⁺ block of a second KCNE1 mutant (G55C), in which the mutated cysteine may be located deeper within the channel pore and, thus, more susceptible to occlusion by TEA⁺ block of the channel. However, as was the case for V391Y/F54C channels, we found no effect of TEA⁺ on the on or off kinetics of Cd²⁺ block of V319Y/G55C channels (Fig. 7).

Finally, to test the predictions of the effects of TEA⁺ on the kinetics of Cd²⁺ block more completely, we investigated the effects of a broad range of TEA⁺ concentrations on Cd²⁺ block. We measured the ratio of time constants of Cd²⁺ block in the presence (τ) and absence (τ₀) of TEA⁺, which according to Eqs. 1–3, is given by the following relationship:

\[
\frac{\tau}{\tau_0} = \frac{(L + K_d)}{(f_{UB} \cdot L + K_d)},
\]

where terms are as defined above. In the limit when \( f_{UB} = 0 \), or when all channels are blocked by TEA⁺, this reduces to \( \tau/\tau_0 = (L/K_i + 1) \).

The results of our experiments are summarized in Fig. 8. First, we confirmed that, at 10 mM [Cd²⁺], expression of V319Y KCNQ1 and F54C KCNE1 encodes Cd²⁺-sensitive channels. We have found that a 30-s application of 10 mM Cd²⁺ suppressed the currents by 70.1 ± 3.9% (n = 5), compared with 26.0 ± 9.6% (n = 3) suppression of V319Y/WT channels, indicating significant Cd²⁺ block conferred by the F54C mutation. Next, we determined the kinetics of the onset of Cd²⁺ block in the presence and absence of TEA⁺ and the experiments are summarized in Fig. 8. Fig. 8 (A and B) illustrates records and summary data from a typical experiment in which we measured the effects of 2 mM TEA⁺ on Cd²⁺ block of the channels. In the same cell, we first applied 10 mM Cd²⁺ for a fixed period (1.5 min; Fig. 8 A), and measured the effects of the cation on currents (asterisks) recorded during depolarizing pulses (Fig. 8 B). We washed out Cd²⁺ (closed circle), applied 2 mM TEA⁺ which blocked the currents by 80% (f_{UB} = 0.20) (arrowhead), and reapplied 10 mM Cd²⁺ (asterisks in Fig. 8 B, top). On average (five cells), we found the f_{UB} value was 0.18 ± 0.01. We then used this value and calculated the τ/τ₀ ratio in each cell. At 2 mM TEA⁺, we determined a mean τ/τ₀ ratio of 1.1 ± 0.1, which is much smaller than the ratio of 2.6 predicted by Eq. 4 using our measured dissociation constant (3.3 mM). Other τ/τ₀ ratios were determined with different concentrations of TEA⁺, and then summarized in Fig. 8 D. For comparison, we repeated these experiments for KCNQ1 (WT)/F54C channels over a much higher range of TEA⁺ concentrations (Fig. 1). We found that neither 50 mM TEA⁺ (f_{UB} = 0.71) nor 100 mM TEA⁺ (f_{UB} = 0.3) markedly affects the kinetics of Cd²⁺ block of expressed channels in contrast to the results of similar experiments in Xenopus oocytes (Tai and Goldstein, 1998). The simplest interpretation of these results is that TEA⁺-induced pore occlusion does not limit the access of externally applied Cd²⁺ to KCNE1 residues, Cys54 and 55 on the KCNE1–KCNQ1 channel complex expressed in CHO cells.

**DISCUSSION**

We have used the substitute cysteine accessibility method (Karlin and Akabas, 1998) to reinvestigate the
location of the KCNE1 subunit relative to the outer pore of assembled \( I_{KS} \) channels. This methodology, in which reporter cysteines are engineered into specific regions of interest in channel proteins, was used in this study to test the influence of externally applied TEA\(^+\) upon access of the thiol-reactive reagent Cd\(^{2+}\) to cysteine-substituted residues on KCNE1 that previously had been suggested to line the pore of assembled \( I_{KS} \) channels (Tai and Goldstein, 1998). In the present experiments, we expressed cysteine-substituted KCNE1 mutants with a KCNQ1 construct that also had been mutated to encode highly TEA\(^+\)-sensitive channels. Together, these constructs provided a system in which we could investigate side-specific effects of Cd\(^{2+}\) on expressed channel activity, and determine whether or not external application of TEA\(^+\) over a low concentration range altered these interactions. Our results indicate that, as previously reported (Tai and Goldstein, 1998), the distinction between external and internal access to reporter cysteines was detected over a very small range of KCNE1 residues (Tai and Goldstein, 1998). However, in contrast to previous studies, we find that access of externally applied Cd\(^{2+}\) to KCNE1 residue C54 was not prevented by TEA\(^+\) pore occlusion. These results clearly show that access of Cd\(^{2+}\) to the cysteine-substituted KCNE1 residue is independent of the pore occlusion by external TEA\(^+\), and argue against the possibility that KCNE1 lines the pore of assembled \( I_{KS} \) channels.

**Consideration of Previous Data**

Our results contradict the interpretation of previous experimental previous data, obtained primarily in *Xenopus* oocytes, which suggested that the same cysteine-substituted site (F54C, KCNE1) is exposed deep into the conducting pore (Tai and Goldstein, 1998). How may the two sets of data be reconciled? First, we confirm that intracellularly applied Cd\(^{2+}\) does not access site Cys54, but it does interact with the cysteine-mutated residue at position 56. Hence, as was shown in previous experiments by Tai and Goldstein (1998), there is a very narrow region over which the KCNE1 protein apparently is exposed to intracellular and extracellular water-filled pathways. However, neither the kinetics nor the magnitude of the Cd\(^{2+}\) block of cysteine-substituted F56C subunits expressed with V319Y KCNQ1 subunits was affected by externally applied TEA\(^+\), arguing against pore-delimited access to Cd\(^{2+}\) to this site. Both K318I + V319Y and V319Y channels were reversibly blocked by, and with, the same sensitivity to exter-
nal TEA⁺ regardless of coassembly with KCNE1, indicating that KCNE1 coassembly does not markedly alter the structure of the outer pore of KCNQ1 channel. This contrasts with WT KCNQ1 channels, which were not completely blocked by >50 mM TEA⁺ in the absence or presence of KCNE1.

Association with KCNE1 Protein Does Not Markedly Alter the Structure of the Outer Pore of KCNQ1 Channel

In the present experiments, because we studied channels expressed in CHO cells in which endogenous expression of KCNQ1 subunits is minimal (Barhanin et al., 1996; Sanguinetti et al., 1996), we were able to focus on activity of channels encoded by mutant constructs of both key subunits. In addition, comparison between the effects of Cd²⁺ block of channels assembled by mutant and wild-type KCNE1 were used to exclude the influence of 13 endogenous cysteine residues in KCNQ1 and cysteine at position 106 in KCNE1 in the effects we investigated. Furthermore, we were careful to limit the concentration range of Cd²⁺ in experiments designed to inhibit channel activity to minimize other known effects of Cd²⁺ on Iᵦₖ channel activity such as changes in surface potential (Kwok and Kass, 1993; Daleau et al., 1997; Wickenden et al., 1999).

Implications for KCNE1/KCNQ1 Assembly

How is it possible to reconcile our data with previous investigations of the location of KCNE1 relative to the KCNQ1 channel pore? In terms of structure and molecular size, it is unlikely that KCNE1 lines the deep pore in the vicinity of the K⁺ channel selectivity filter (Doyle et al., 1998). However, recent experiments in which the crystal structure of the CSA potassium channel have been analyzed in the absence (Doyle et al., 1998) and presence of subunit coassembly (Gulbis et al., 2000) indicate that a complex structural architecture that can exist in the regions of interactions between protein subunits (raising the possibility of alternate geometries) may explain these data. In case of voltage-gated Na⁺ channels, the S4 voltage-sensing region has shown to move inside a narrow cavity that is distinct from the ion conducting pore (Yang et al., 1996). Furthermore, in Shaker K⁺ channels, the possibility of extra pore crevices has been recently suggested to explain local changes in ionic strength on channel gating (Islam and Sigworth, 2000). It is possible, should similar crevices exist for the KCNE1–KCNQ1 channel complex, that KCNE1 may link intra- and extra-space across a narrow portion of the crevice.

Implications for Other Channels

KCNQ1 is the first member of a family of proteins referred to as KCNE proteins. Coassembly of KCNE1 with KCNQ1 causes marked changes in channel gating and single-channel properties (Barhanin et al., 1996; Romey et al., 1997; Splawski et al., 1997; Sesti and Goldstein, 1998; Tristani-Firouzi and Sanguinetti, 1998; Yang and Sigworth, 1998), and KCNE1 can coassemble with HERG channels and alter their properties (McDonald et al., 1997). Modulation of channel gating by other members of both the KCNQ and KCNE gene families has been demonstrated with equally impressive functional changes in the properties of the expressed channels (Abbott and Goldstein, 1998; Abbott et al., 1999; Schroeder et al., 2000) and multiple mutations in members of each gene family have been linked to human disease (Sanguinetti, 2000b). Our data suggest that the interactions between these two families of subunits do not occur entirely within the pore region of the encoded channels, but instead suggest interaction of regions with other regions, perhaps with S4 segments themselves.

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