Modulation of HERG affinity for E-4031 by $[K^+]_o$ and C-type inactivation

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Abstract Rectification of HERG is due to a rapid inactivation process that has been labeled C-type inactivation and is believed to be due to closure of the external mouth of the pore. We examined the effects of mutation of extracellular residues that remove C-type inactivation on binding of the intracellularly acting methanesulfonanilide drug E-4031. Removal of inactivation through mutation reduced drug affinity by more than an order of magnitude. Elevation of $[K^+]_o$ in the wild-type channel reduces channel affinity for E-4031. Elevation of $[K^+]_o$ also interferes with the extracellular pore mouth closure associated with C-type inactivation through a 'foot in the door' mechanism. We examined the possibility that $[K^+]_o$ elevation reduces drug binding through inhibition of C-type inactivation by comparing drug block in the wild-type and inactivation-removed mutant channels. Elevation of $[K^+]_o$ decreased affinity in both channel constructs by a roughly equal amount. These results suggest that $[K^+]_o$ alters drug binding affinity independently of its effects on C-type inactivation. They further suggest that inhibition of pore mouth closure by elevated $[K^+]_o$ does not have same effect on drug affinity as mutations removing C-type inactivation.

Key words: Potassium; Delayed rectifier; Repolarization; Cardiac

1. Introduction

In cardiac myocytes, the rapid component of the delayed rectifier K^+ channel (I_{Kr}) has been demonstrated to contribute to repolarization in cardiac muscle [1]. I_{Kr} activates more rapidly than the slow component of the delayed rectifier (I_{Ks}), shows strong inward rectification (I_{Kr}) [2,3], and is believed to have *HERG* (human ether-à-go-go related gene) as its molecular basis [4–6]. One unique feature of this channel is the specificity of its blockade by methanesulfonanilide drugs (e.g. E-4031 [1,2]), which has been exploited in the design of new antiarrhythmic drugs. Despite their selective action on this channel, little is understood about their mechanism of action.

The strong inward rectification of HERG and I_{Kr} has recently been proposed to result from a time-dependent inactivation process [2,4,5,7,8]. This inactivation is insensitive to N-terminal deletion and involves closure of the external mouth of the pore. Thus, inactivation of HERG has been attributed to a C-type mechanism [9]. However, inactivation of HERG has some properties which are not traditionally associated with C-type inactivation and may be difficult to reconcile with a localized closure of the external mouth of the pore. In particular, inactivation has intrinsic voltage sensitivity [10,11]. The voltage sensitivity of inactivation results from movement of charge associated with conformational changes in membrane spanning domains. Such conformational changes are unlikely to be confined to the external mouth of the pore, but may be manifest through alteration of properties at the intracellular side of the channel. We therefore examined the effects of a mutation in the extracellular domains of HERG, which eliminates rapid inactivation, on kinetics, $[K^+]_o$ sensitivity and binding of an intracellularly acting [12–14] methanesulfonanilide compound, E-4031. Removal of C-type inactivation in this channel greatly lowers affinity for E-4031 and alters the voltage dependence of drug binding.

2. Materials and methods

Oocytes were voltage clamped using a two-microelectrode 'bath clamp' amplifier (OC-725A, Warner Instruments Corp., Hamden, CT) as has been described in detail elsewhere [15]. Microelectrodes were fabricated from 1.5 mm OD borosilicate glass tubing (TW150F-4, WPI) using a two-stage puller (L/M-3P-A, Adams & List Associates, Ltd., Great Neck, NY), filled with 3 M KCl, and had resistances of 0.6–1.5 MΩ. During recording, oocytes were continuously perfused with control ND 96 solution (in mM: 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, pH = 7.4, adjusted with NaOH). 98 K solution contained (in mM): 98 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, pH = 7.4, adjusted with NaOH. Currents were recorded at room temperature (21–23°C) and were filtered at 2.5 kHz for the two-electrode voltage clamp recordings. Data were recorded on video tape using an A/D VCR adaptor (model PCM 4/8, Medical Systems Corporation, Greenvale, NY) and digitized using 'pClamp' software (Axon Instruments, Inc., Foster City, CA). Unless otherwise stated, raw two-electrode voltage clamp data traces were not leakage or capacitance subtracted. Defolliculated *Xenopus laevis* oocytes (stage V–VI) were injected with 50 nl cRNA solution prepared as described [15], containing up to 50 ng *HERG* cRNA made from cDNA kindly provided by M. Keating, Univ. of Utah, Salt Lake City, UT. E-4031 was the generous gift of Esai Co. Ltd., Tsukuba Research Laboratories, Japan. Confidence levels were calculated using a paired *t*-test.

3. Results

The HERG channel has a six membrane spanning domain topology similar to other voltage gated channels similar from the *Shaker* family of potassium channels (Fig. 1). Despite a long intracellular N-terminal domain, this channel has been demonstrated to inactivate by C-type inactivation, or external pore mouth closure. Mutations in regions near the extracellular mouth of the pore have been shown to be involved in C-type inactivation [9,16]. When residues at two positions indicated in Fig. 1 are mutated to cysteines, C-type inactivation is removed in the mutant channel (HERG[GS:CC]) [9]. We

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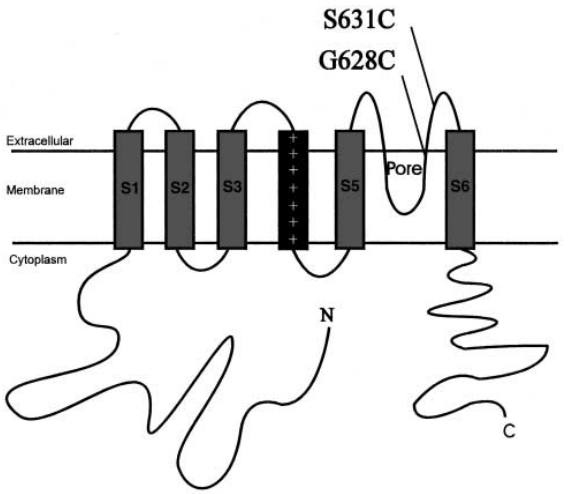

Fig. 1. Schematic diagram of the transmembrane spanning topology of the HERG channel showing the sites of the two point mutations at glycine-628 and serine-631. HERG channels show a six membrane spanning domain topology similar to the *Shaker* family of K^+ channels. S4 is thought to form the voltage sensor. The H5 loop is thought to form the outer portion of the pore. The two mutations introduced are shown. The resulting mutant construct HERG[GS:CC] lacks rapid C-type inactivation [9].

compared the properties of the WT channel with this mutant construct with respect to the $[K^+]_o$ and drug binding.

Fig. 2A shows typical wild-type HERG currents recorded in response to a series of depolarizing P1 pulses for 500 ms to potentials ranging between −80 and +50 mV followed by an 500 ms P2 pulse to −40 mV ($V_{holding} = -80$ mV) in 2 mM $[K^+]_o$. These currents show the typical inward rectification characteristic of the HERG channel. HERG[GS:CC] shows no inward rectifying behavior (Fig. 2B) due to disruption of a rapid C-type inactivation mechanism as previously demonstrated [9]. Previous studies have shown that C-type inactivation rate and steady-state C-type inactivation are altered in a destabilizing manner by elevation of $[K^+]_o$ [9–11,16]. The behavior of the WT channel and HERG[GS:CC] mutant channel in the presence of 98 mM $[K^+]_o$ is shown in Fig. 2C,D.

Previous studies have shown that C-type inactivation interferes with the binding of methanesulfonanilide drugs to the HERG channel [12–14]. This suggested that there was some interaction between intracellular binding of methanesulfonanilide drugs and C-type inactivation. In order to examine the molecular basis of this interaction, we measured the effect that the HERG[GS:CC] mutation had on the affinity of the channel for E-4031. E-4031 and the other methanesulfonanilide drugs which show specificity for I_{Kr} are reported to be open

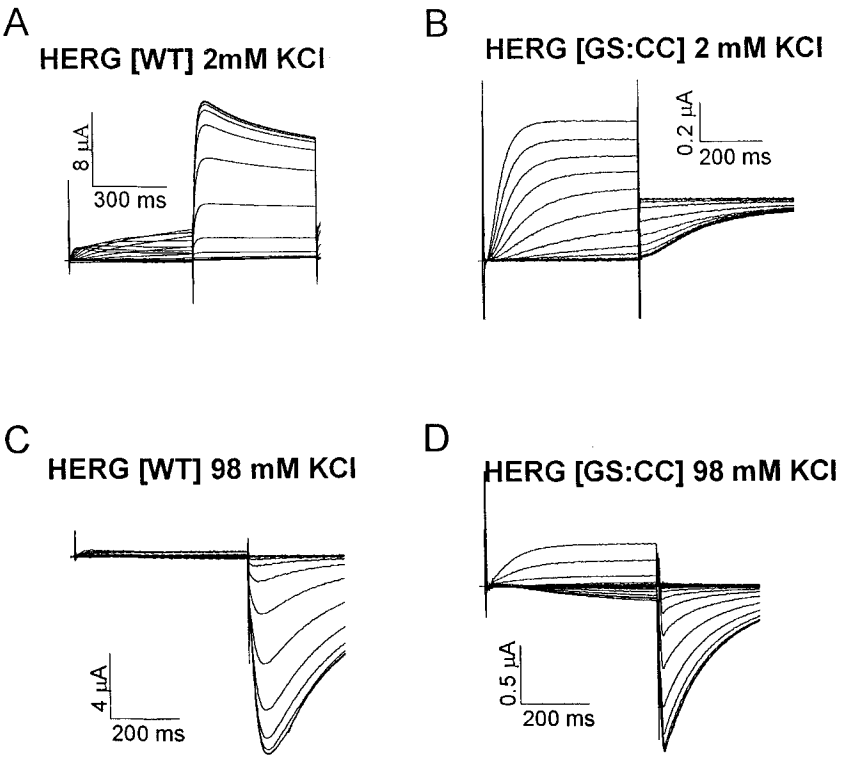

Fig. 2. Characteristics of HERG and HERG[GS:CC] mutant characteristics. A: Currents recorded from wild-type HERG expressed in *Xenopus* oocytes in the presence of 2 mM $[K^+]_o$. Oocytes were held at a clamp potential of −80 mV and stepped to potentials ranging between −80 and +50 mV for 500 ms and then returned to −40 mV for 500 ms to elicit tail currents. Note that the tail currents are larger than the fully activated currents at more positive potentials due to time-dependent inward rectification. B: Currents recorded from oocytes containing the mutations G628C and S631C (designated HERG[GS:CC]), which have previously been shown to remove rapid C-type inactivation in HERG in the presence of 2 mM $[K^+]_o$. Note that time-dependent inward rectification is abolished and that the tail currents are much smaller than the fully activated currents. Oocytes were held at a clamp potential of −80 mV and stepped to potentials ranging between −80 and +50 mV for 500 ms and then returned to 0 mV for 500 ms to elicit tail currents. C: Currents recorded from wild-type HERG in the presence of 98 mM $[K^+]_o$. Oocytes were held at a clamp potential of −80 mV and stepped to potentials ranging between −80 and +50 mV for 500 ms and then returned to −80 mV to elicit tail currents. D: Currents recorded from HERG[GS:CC] in the presence of 98 mM $[K^+]_o$ using the same protocol as in panel C. Oocytes were held at a clamp potential of −80 mV and stepped to potentials ranging between −80 and +50 mV for 500 ms and then returned to −80 mV to elicit tail currents.

channel blockers and require channel activation in order for the drug to bind [12–14]. Drug binding is very slow and requires special voltage clamp protocols for development of block [5,17]. We followed the example of Spector et al. [17] and allowed 10 min of perfusion time to allow equilibration of E-4031 across the membrane. After this wash-on period, a series of depolarizing pulses (from a holding potential of −80 mV to 0 mV for 400 ms at a frequency of 1 Hz for 1 min) was applied, to elicit steady-state block. After this preconditioning protocol, steady-state block was measured from the degree of suppression of tail currents. As seen in Fig. 3A, application of 10 μM E-4031 resulted in almost complete inhibition of the wild-type current. But 10 μM E-4031 resulted in only a partial block of the HERG[GS:CC] mutant channel (Fig. 3B). The overall dose-response curves for these two channel constructs shows that the mutation changed affinity for E-4031 by more than an order of magnitude (Fig. 4A). The K_D was shifted from 1.71 μM to 19.3 μM, demonstrating that the binding affinity for this intracellularly acting compound was changed by an order of magnitude by the HERG[GS:CC] mutation.

The change in drug affinity associated with the HERG[GS:CC] mutant suggested that C-type inactivation might itself be a determinant of drug binding properties. We tested this possibility using another manipulation that alters C-type inactivation through another mechanism, namely changing $[K^+]_o$. Changes in $[K^+]_o$ have been shown to influence drug binding to HERG [18]. C-type inactivation of HERG channels is sensitive to $[K^+]_o$ both in rate of development [9,10,16] and in equilibrium [11] at any particular potential. If C-type inactivation conformational changes are important in creating a high affinity binding site for E-4031, we would predict that elevation of $[K^+]_o$ would tend to reduce drug binding in the wild-type channel, while drug binding in the HERG[GS:CC] mutant channel would be unaffected by elevation of $[K^+]_o$. To test the effects of $[K^+]_o$ on drug affinity, we utilized a concentration of E-4031 that was near the K_D for the WT and mutant channels (1.71 μM for WT channels and 19.3 μM for mutant channels). Repeated pulses (from −80 mV to 0 mV for 400 ms, 1 Hz, 1 min) were applied until block reached steady-state in both 2 and 98 mM $[K^+]_o$. Block was measured as suppression of peak tail currents at 0 mV for 2 mM $[K^+]_o$ and −80 mV for 98 mM $[K^+]_o$. As shown in Fig. 4B the fractional inhibition of currents was $[K^+]_o$ sensitive for the WT channel with an average 32% decrease in total cur-

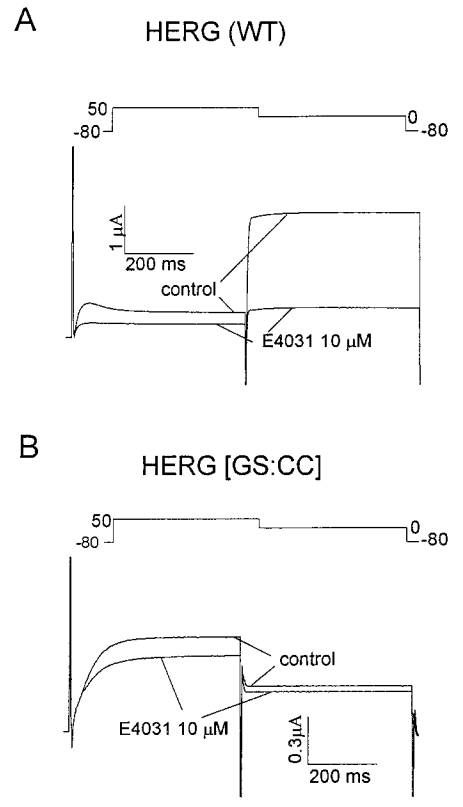

Fig. 3. Block of HERG and HERG[GS:CC] by E-4031. A: Current traces showing the effects of 10 μM E-4031 on the HERG channel. Both the outward current during a P1 pulse to +50 mV and the resulting tail currents were strongly blocked by this concentration of E-4031. B: Current traces showing the effects of the same concentration (10 μM E-4031) on the HERG[GS:CC] mutant channel. In contrast to the effect on the WT channel, this concentration of E-4031 only weakly blocked the channel.

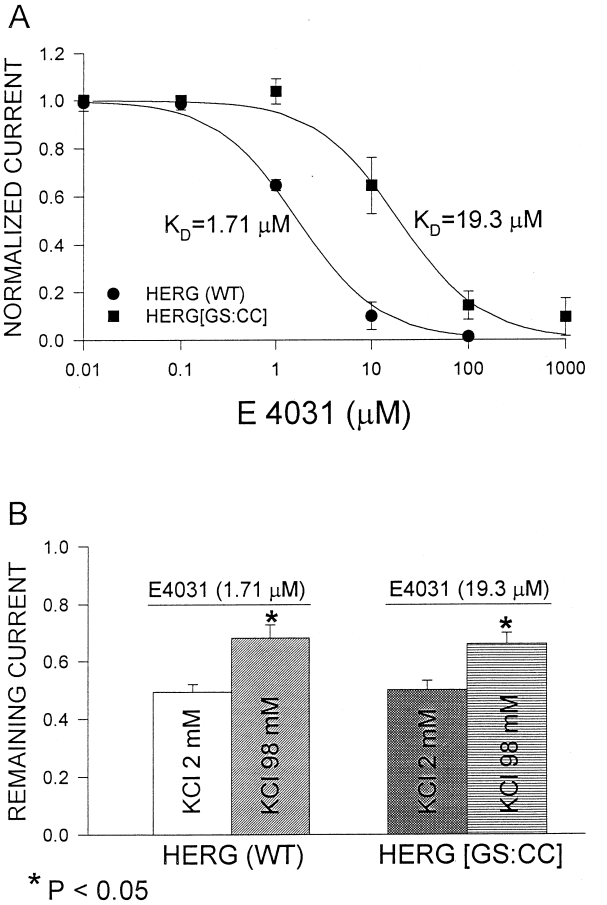

Fig. 4. Removal of C-type inactivation does not alter the $[K^+]_o$ dependence of E-4031 binding. A: Dose-response curves showing the order of magnitude decrease in affinity of the HERG channel for E-4031 following removal of C-type inactivation through mutation. B: Effects of $[K^+]_o$ on block of HERG and HERG[GS:CC]. The bar chart shows the percentage of control current in 2 mM and 98 mM $[K^+]_o$ in the presence of 1.71 μM E-4031 for the HERG channel and 19.3 μM E-4031 for the HERG[GS:CC] mutant channel (* denotes statistical difference between $[K^+]_o$ groups $P<0.05$, $n=4$, paired t-test). Note that the fractional reduction of HERG and HERG[GS:CC] current shows nearly identical sensitivity to changes in $[K^+]_o$.

rent. Surprisingly, the block of mutant channels showed a virtually identical sensitivity (34%) of block to $[K^+]_o$.

4. Discussion

Our data have shown that the HERG[GS:CC] mutation, which removes C-type inactivation through mutation of extracellularly facing residues, also strongly reduces affinity of this channel for E-4031. We also demonstrated that elevation of $[K^+]_o$ resulted in decreased affinity for E-4031 in the wild-type channel. Since elevation of $[K^+]_o$ also destabilizes C-type inactivation [9,11], there were two potential mechanisms by which $[K^+]_o$ could be modulating drug binding affinity. One mechanism was direct interaction, e.g. through electrostatic repulsion or a 'knock-off' process. The other mechanism would be indirect interaction through destabilization of the C-type inactivated conformation which favors drug binding. Had the second mechanism been important we would have expected that the HERG[GS:CC] mutant would have shown little change in affinity with elevation of $[K^+]_o$. Our results showed that the action of $[K^+]_o$ on drug binding was independent of the presence of C-type inaction, suggesting that a direct interaction was dominant.

Since both potassium and E-4031 carry a net single positive charge, electrostatic repulsion can provide a simple explanation for the effects of potassium on E-4031 affinity. The calculated affinity of E-4031 for wild-type HERG for shifts from 1.71 to 3.63 mM when switching from 2 to 98 mM $[K^+]_o$. This corresponds to a reduction in the free-energy of drug binding of 0.44 kcal/mol. If an external binding site were 100% occupied, the free energy change of binding due to the electrostatic force of repulsion between it and an intracellular positively charged drug could be calculated from the equation:

$$\Delta G_{electrostatic} = Q_1 Q_2 / 4\pi\varepsilon\varepsilon_o d \quad (1)$$

where Q_1 and Q_2 are the net charges on the permeant ion and drug, respectively, ε is the dielectric constant, ε_o is the polarizability of free space and d is the distance separating Q_1 and Q_2. If we assume that the polarizability of a channel is similar to that of water ($\sim$80 [19]) and that when bound to the channel, both drug and externally bound permeant ion are approximately one bilayer thickness apart (2.3 nm [19]) then the maximal free energy of repulsion between an externally bound permeant ion and an intracellularly bound positively charged blocker is 3.2 kcal/mol. The conductance-activity relationship of HERG macroscopic currents and the single channel conductance $[K^+]_o$ activity relationship of I_{Kr} are indicative of a strong increase in ion occupancy between 2 and 98 mM $[K^+]_o$. Therefore, the total free energy available from the binding of extracellular $[K^+]_o$ is sufficient to account for all of the observed reduction in drug sensitivity.

Consideration of available free energy can also provide insights into the role of pore mouth closure and C-type inactivation in the drug-binding process. Elevation of $[K^+]_o$ from 2 to 98 mM shifts the steady-state inactivation relation for HERG by an amount which is equivalent to 1.02 kcal/mol (calculated from [11]). This is also more than the 0.44 kcal/mol change in the free energy of drug binding observed for the change in $[K^+]_o$. The preceding calculations demonstrated that there was sufficient free energy in both the potential electrostatic interaction and in the modulation of P_{open} through C-type inactivation to account for the $[K^+]_o$ modulation of drug binding. Thus, we initially anticipated that the free energy change in drug binding to the wild-type channel due to elevation of $[K^+]_o$ could be expressed as a sum of these two effects:

$$\Delta G_{WT,K} = 0.44 \text{ kcal/mol} = \Delta G_{Direct,electrostatic} + \Delta G_{indirect,C-type} \quad (2)$$

where $\Delta G_{WT,K}$ is the change in free energy of E-4031 drug binding observed when switching from 2 to 98 mM $[K^+]_o$. $\Delta G_{Direct,electrostatic}$ is the direct contribution of $[K^+]_o$ to this reduction in affinity due to electrostatic repulsion presumably, but also potentially due to interactions such as a 'knock-off' mechanism or involving other conformational changes not directly resulting in closure of the extracellular mouth of the pore due to C-type inactivation. $\Delta G_{indirect,C-type}$ is the energetic contribution to the reduced affinity due to disruption of C-type inactivation in switching from 2 to 98 mM $[K^+]_o$. In the mutant channel, C-type inactivation is unavailable for modulation so $\Delta G_{indirect,C-type}$ should be equal to zero. Therefore, for the mutant channel Eq. 2 should simplify to:

$$\Delta G_{MT,K} = 0.39 \text{ kcal/mol} = \Delta G_{Direct,electrostatic} \quad (3)$$

where $\Delta G_{MT,K}$ is the change in free energy of E-4031 drug binding observed when switching from 2 to 98 mM $[K^+]_o$. Subject to the assumption that the mutation left $\Delta G_{Direct,electrostatic}$ unchanged, the total change in free energy of drug binding that can be attributed to changes in the development of C-type inactivation under these conditions can be estimated as the difference between Eqs. 2 and 3:

$$\Delta G_{indirect,C-type} = \Delta G_{WT,K} - \Delta G_{MT,K} = 0.44 - 0.39 \text{ kcal/mol} = 0.05 \text{ kcal/mol} \quad (4)$$

which suggests that little, if any, of the free energy of drug binding is dependent on the external mouth of the pore closing during C-type inactivation.

It has recently been suggested that many compounds show use dependence due to an ability to increase the rate of development of C-type inactivation [20]. This is proposed to occur through a mechanism by which C-type inactivation is promoted by decreased K^+ occupancy of the pore associated with block of the channel and the associated decrease in K^+ efflux through the pore [20]. This increase in the rate of development of C-type inactivation seems unlikely to be the case for E-4031 block of HERG, since such a mechanism predicts a recovery from use-dependent block that parallels the rate of recovery from inactivation. Recovery from inactivation of HERG occurs in the order of 25 ms [11] and is far too fast to explain the use-dependent behavior shown for methanesulfonanilide drugs. Thus, simple promotion of C-type inactivation is not a viable explanation for the relationship between drug binding and the HERG[GS:CC] mutation.

How, then, does the GS:CC mutation alter affinity at the binding site on the opposite side of the membrane? Direct changes in net charge on the membrane protein itself are possible but appear unlikely since the GS:CC mutation does not involve a change in charged side chains. Since these residues also lie in the permeation pathway, it is also possible

that a binding site for permeant or impermeant (e.g. divalent) ions has been disrupted. However, the similarity in the energetics of $[K^+]_o$ on drug binding to the mutant and wild-type channel does not argue strongly for changes in the permeation pathway with the GS:CC mutation. Other data are difficult to reconcile with a the GS:CC mutation altering drug affinity purely through removal of extracellular pore mouth closure. WT HERG channels show decreasing rates of development of block with stronger depolarizations [12–14], a phenomenon which has been attributed to competition with C-type inactivation [12–14]. In support of this hypothesis, we found that increasing depolarizations in mutant channels lacking C-type inactivation increased the rate of development of block in a fashion consistent with intracellular binding of a positively charged compound (data not shown). The GS:CC mutation data would seem to suggest a requirement for C-type inactivation, while other data would seem to suggest that C-type inactivation interferes with drug binding at the intracellular side. These data are difficult to reconcile with a pure extracellular pore-mouth closure mechanism.

Another possible mechanism is that the GS:CC mutation changes the ability of the channel to form a high affinity binding conformation at the intracellular side of the pore. To date, the investigations into the mechanism of C-type inactivation in *Shaker* K^+ channels have implicated only fairly small rearrangements near the extracellular mouth of the pore [21]. It is not surprising, therefore, that C-type inactivation in *Shaker* and closely related channels shows little or no voltage dependence (e.g. [22,23]) as the superficial location of the residues involved suggests that they would move through a small fraction of the total transmembrane electrical field. On the other hand, inactivation of HERG occurs with a strong voltage dependence, suggesting that a substantial transmembrane movement of charge occurs during this conformational change [11]. Such transmembrane movement is likely to alter intracellular as well as extracellular conformation.

A body of evidence is slowly emerging that strongly suggests that multiple conformational changes involving membrane spanning domains occur in association with the phenomenon of C-type inactivation. This study sheds some additional light on these conformational changes. Mutation of residues near the outer mouth of the pore strongly reduced the affinity of HERG for E-4031 at the intracellular side of the channel. However, simply stopping the development of C-type inactivation in HERG through direct intervention via the 'foot in the door' mechanism with 98 mM $[K^+]_o$ was not sufficient to alter drug binding. Thus, the GS:CC mutation may have disrupted both external pore mouth closure and formation of an intracellular binding conformation for E4031.

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