Molecular Interactions Between Two Long-QT Syndrome Gene Products, HERG and KCNE2, Rationalized by In Vitro and In Silico Analysis

Reza Mazhari, Joseph L. Greenstein, Raimond L. Winslow, Eduardo Marbán, H. Bradley Nuss

Abstract—The cardiac delayed rectifier potassium current mediates repolarization of the action potential and underlies the QT interval of the ECG. Mutations in either of the two molecular components of the rapid delayed rectifier ($I_{K,r}$), HERG and KCNE2, have been linked to heritable or acquired long-QT syndrome. Mechanisms whereby mutations of KCNE2 produce fatal cardiac arrhythmias characteristic of long-QT syndrome remain unclear. In this study, we characterize functional interactions between HERG and KCNE2 with a view to defining underlying mechanisms for action potential prolongation and long-QT syndrome. Whereas coexpression of hKCNE2 with HERG alters both kinetics and density of ionic current, incorporation of these effects into a quantitative model of the action potential predicts that only changes in current density significantly affect repolarization. Thus, the primary functional consequence of hKCNE2 on action potential morphology is through modulation of $I_{K,r}$ density, as predicted by the model. Mutations associated with long-QT syndrome that result only in modest changes of gating kinetics may be epiphenomena or may modulate action potential repolarization via interaction with alternative pore-forming potassium channel $\alpha$ subunits. (Circ Res. 2001;89:33-38.)

Key Words: delayed rectifier potassium channels ▪ Markov chains ▪ action potential ▪ arrhythmia ▪ accessory proteins

It has been shown that human ether-à-go-go–related gene (HERG)$^{1,2}$ encodes the pore-forming subunit of the rapid delayed rectifier potassium channel ($I_{K_r}$).$^{1,3}$ Abbott et al$^4$ recently showed that channels formed by coexpression of KCNE2 (encoding minK-related peptide 1, MiRP1) and the pore-forming subunit HERG resemble native cardiac $I_{K_r}$ channels more closely in their gating and unitary conductance, modulation by extracellular potassium, and inhibition by class III antiarrhythmic medications (eg, E-4031).$^4$ More importantly, they identified mutations in hKCNE2 (eg, Q9E and M54T) that were associated with acquired long-QT syndrome and ventricular fibrillation. Various mutations in HERG have also been linked with the familial form of long-QT syndrome.$^{5,6}$ Therefore, whereas lesions in either of the two molecular components of $I_{K_r}$, HERG and hKCNE2, have been linked to heritable or acquired long-QT syndrome, mechanisms whereby genetic lesions of KCNE2 produce fatal cardiac arrhythmias remain unclear. Mutant hKCNE2, when coexpressed with HERG, can result in alterations of both current density and kinetics or of kinetics alone.$^4$ This raises the question of whether altered current density or channel kinetics contributes more significantly to action potential prolongation and arrhythmia in patients with these genetic lesions. Better understanding of functional interactions between these two gene products could facilitate development of superior therapeutic approaches for particular lesions in either HERG or hKCNE2.

Our first objective, therefore, is to characterize the functional effects of hKCNE2 coexpression with HERG. Because ion channel gating models provide a quantitative description of gating behavior and give clues to channel structure,$^7$ the second objective is to develop a Markov state model of both HERG and HERG-hKCNE2 coassembly (Figure 1) and use this model to additionally elucidate mechanisms of interactions. Because mutations in both HERG and hKCNE2 have been identified to be associated with long-QT syndrome,$^{5,6}$ a third objective is to predict the consequences of HERG-hKCNE2 interactions for action potential repolarization by incorporating the newly developed Markov model into a mathematical model of the cardiac action potential.$^8$

Materials and Methods

Transfection of HEK 293 Cells

Stably transfected, G-418–resistant HEK 293 cell lines coexpressing HERG channels and green fluorescent protein (GFP) were used for the experiments.$^9$ These cells were transiently transfected with
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HERG and HERG-hKCNE2 Markov State Transition Rates

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HERG</th>
<th>HERG-hKCNE2</th>
<th>Fold ∆</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_0$</td>
<td>0.0069 · $\text{exp}(0.0272V_m)$</td>
<td>0.0171 · $\text{exp}(0.0330V_m)$</td>
<td>2.48, 1.21</td>
</tr>
<tr>
<td>$\beta_0$</td>
<td>0.0227 · $\text{exp}(-0.0413V_m)$</td>
<td>0.0397 · $\text{exp}(-0.0431V_m)$</td>
<td>1.74, 1.00</td>
</tr>
<tr>
<td>$\alpha_1$</td>
<td>0.0218 · $\text{exp}(0.0262V_m)$</td>
<td>0.0206 · $\text{exp}(0.0262V_m)$</td>
<td>0.95, 1.00</td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>0.0009 · $\text{exp}(-0.0269V_m)$</td>
<td>0.0013 · $\text{exp}(-0.0269V_m)$</td>
<td>1.44, 1.00</td>
</tr>
<tr>
<td>$\alpha_2$</td>
<td>0.0622 · $\text{exp}(0.0120V_m)$</td>
<td>0.1067 · $\text{exp}(0.0057V_m)$</td>
<td>1.71, 0.48</td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>0.0059 · $\text{exp}(-0.0435V_m)$</td>
<td>0.0065 · $\text{exp}(-0.0454V_m)$</td>
<td>1.10, 1.03</td>
</tr>
<tr>
<td>$\alpha_3$</td>
<td>1.29E-5 · $\text{exp}(2.71E-6V_m)$</td>
<td>8.04E-5 · $\text{exp}(6.98E-7V_m)$</td>
<td>6.23, 0.26</td>
</tr>
<tr>
<td>$K_a$</td>
<td>0.0266</td>
<td>0.0261</td>
<td>0.98</td>
</tr>
<tr>
<td>$K_b$</td>
<td>0.1348</td>
<td>0.1483</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Fold ∆ refers to fold change from HERG to HERG-hKCNE2 state model; first and second numbers indicate changes in A and B of $A \cdot \text{exp}(B V_m)$, respectively. Refer to Figure 1 for definition of parameters shown.

Statistical Analysis

All the results referred to as HERG are RFP-only transfection of the stable cells (n = 5), and the HERG-hKCNE2 group is RFP and hKCNE2 cotransfection (n = 6). All data shown are mean ± SEM. ANOVA was used for statistical analysis, and P < 0.05 was considered statistically significant. Solid lines in all figures represent the Markov model results unless otherwise specified.

Results

We tested whether coassembly with hKCNE2 alters activation properties of HERG and found that addition of hKCNE2 did not affect voltage-dependent activation. There was no significant difference in the half-maximal activation voltage (−9.5 ± 5.2 versus −8.6 ± 3.4 mV for HERG and HERG-hKCNE2, respectively) determined by fitting the experimental data to a Boltzmann function; there was also no significant difference in the slope factors (7.2 ± 0.3 versus 7.4 ± 0.4 mV for HERG and HERG-hKCNE2, respectively) (Figure 2A). Addition of hKCNE2, however, resulted in a change of activation kinetics (Figure 2B). Compared with HERG alone, coassembly with hKCNE2 resulted in an acceleration of activation with the largest effect at the lowest potential (0 mV) (Figure 2C, protocol 2).

Coassembly of HERG and hKCNE2 also affects deactivation kinetics (Figure 3B). Analysis of tail currents (Figure 3A, tail currents of protocol 3) shows that deactivation time constants (τ) are decreased on average by 40% (P < 0.0001). At potentials positive to the reversal potential (−82 mV), there is no significant difference in deactivation rate constants. For example, at −70 mV, deactivation time constants were 331 ± 60 mV, test pulse at 20 mV steps, test pulse for 0.2 seconds at 50 mV for 0.5 seconds. (2) Activation kinetics: incremental prepulse duration from 0.035 to 0.49 seconds at 0 to 60 mV in 2 pulse to 50 mV for 0.5 seconds. (3) Deactivation kinetics and current-voltage relation: prepulse for 2 seconds at 60 mV, test pulse at −140 to 40 mV in 10-mV steps for 1 second. (4) Inactivation kinetics, steady-state inactivation, and instantaneous current-voltage relation: prepulse to 60 mV for 0.5 seconds, pulse to −80 for 30 ms, test pulse at −80 to 60 mV in 10-mV steps for 0.2 seconds. (5) Recovery from inactivation: prepulse to 60 mV for 0.3 seconds, test pulse at −100 to 20 mV in 10-mV steps for 0.2 seconds.

Mathematical Model

The structure of the Markov model is shown in Figure 1. This model is based on previous work of Liu et al14 and Wang et al15 for $I_{K_r}$ and HERG currents, respectively. Closed-state inactivation ($C_r$) was set to 0.0203 mS/mV, test pulse at 20-mV steps, test pulse for 0.2 seconds at 50 mV for 0.5 seconds. (2) Activation kinetics: incremental prepulse duration from 0.035 to 0.49 seconds at 0 to 60 mV in 2 pulse to 50 mV for 0.5 seconds. (3) Deactivation kinetics and current-voltage relation: prepulse for 2 seconds at 60 mV, test pulse at −140 to 40 mV in 10-mV steps for 1 second. (4) Inactivation kinetics, steady-state inactivation, and instantaneous current-voltage relation: prepulse to 60 mV for 0.5 seconds, pulse to −80 for 30 ms, test pulse at −80 to 60 mV in 10-mV steps for 0.2 seconds. (5) Recovery from inactivation: prepulse to 60 mV for 0.3 seconds, test pulse at −100 to 20 mV in 10-mV steps for 0.2 seconds.

A model of the canine cardiac midmyocardial action potential was used.8 The existing $I_{K_v}$ component of the model was replaced with the newly developed Markov representation of HERG or HERG-hKCNE2. The rates in both models were adjusted for temperature to match kinetics of $I_{K_v}$ at 37°C using experimental reports of Zhou et al13 (using $Q_{10}$ of 3.3). Temperature effects were assumed to be similar for both HERG and HERG-hKCNE2 channels. Channel conductance ($G_{K_v}$) was set to 0.0203 mS/mV to match the reported $I_{K_v}$ current densities reported by Li et al16 for human cardiac myocytes, which are similar to those reported in canine. Therefore, the baseline or wild-type $I_{K_v}$ model is based on HERG-hKCNE2 kinetics (Table)

either 0.8 µg of hMiRP1 cDNA (hKCNE2) (kindly provided by Dr Steve Goldstein, Yale University, New Haven, Conn) and 0.2 µg of red fluorescent protein (RFP) (DsRed, Clontech) or 1 µg of RFP alone using Lipofectamine (Gibco BRL). GFP- and RFP-positive cells were studied within 24 to 36 hours of transfection.

Patch-Clamp Recording

Membrane currents were measured using whole-cell patch clamp.10 Cells were bathed in solution containing (in mmol/L) NaCl 140, KCl 40, MgCl$_2$ 1, HEPES 10, glucose 10, and CaCl$_2$ 2, pH 7.4 (adjusted with NaOH). Borosilicate glass pipettes were pulled and fire polished to form tip resistances of 5 to 6 MΩ when filled with pipette solution containing (in mmol/L) NaCl 140, MgCl$_2$ 1, HEPES 10, EGTA 5, and MgATP 4, pH 7.3 (adjusted with KOH). Uncompensated capacitance currents in response to small hyperpolarizing voltage steps were recorded for offline integration as a means of measuring cell capacitance. All recordings were obtained at room temperature.

Voltage protocols used in this study are as follows, with holding potential of −80 mV in all cases. (1) Voltage-dependent activation: prepulse for 3.5 seconds from −80 to 60 mV in 10-mV steps, test pulse to −50 mV for 0.5 seconds. (2) Activation kinetics: incremental prepulse duration from 0.035 to 0.49 seconds at 0 to 60 mV in 20-mV steps, test pulse for 0.2 seconds at −120 mV. (3) Deactivation kinetics and current-voltage relation: prepulse for 2 seconds at 60 mV, test pulse at −140 to 40 mV in 10-mV steps for 1 second. (4) Inactivation kinetics, steady-state inactivation, and instantaneous current-voltage relation: prepulse to 60 mV for 0.5 seconds, pulse to −80 for 30 ms, test pulse at −80 to 60 mV in 10-mV steps for 0.2 seconds. (5) Recovery from inactivation: prepulse to 60 mV for 0.3 seconds, test pulse at −100 to 20 mV in 10-mV steps for 0.2 seconds.

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time constants at all potentials ($P<0.0001$, using protocols 4 and 5). There is a 68% reduction in inactivation time constants for potentials $\leq 0$ mV and a 39% reduction for potentials $>0$ mV (Figure 4B). However, there is no significant change in the voltage dependence of steady-state inactivation (half-maximal inactivation voltage of $-34.6 \pm 5.6$ versus $-38.9 \pm 3.2$ mV and slope factor of $20.1 \pm 2.2$ versus $20.3 \pm 0.9$ mV for HERG versus HERG-hKCNE2, respectively) (Figure 4A). Therefore, coassembly of hKCNE2 and HERG affects only the rates of development and recovery from, but not the voltage dependence of, inactivation.

Coassembly of hKCNE2 and HERG results in a significant decrease of inward (40%) and outward (55%) current density (Figure 5A, $P<0.001$; using peak tail currents of protocol 3), as confirmed by a 2-fold reduction ($P<0.001$) in the slope of the instantaneous current-voltage relationship (from $2.9 \pm 0.5$ to $1.5 \pm 0.3$ pS/pF, $P<0.05$, using peak tail currents of protocol 4) (Figure 5B).

Rate constants associated with the Markov models (Figure 1) representing HERG and HERG-hKCNE2 coexpression are shown in the Table. Consistent with our experimental data, with the exception of $\alpha_o$ and $\alpha_i$ (see Figure 1), all rate constant variation resulting from coassembly is voltage independent (ie, no change in B of $A \cdot \exp(BV_m)$). Moreover, inactivation from the closed state ($C3 \rightarrow I$) is nearly voltage independent and has magnitude significantly smaller than the open to inactivated state transition rate ($O \rightarrow I$). Although addition of hKCNE2 accelerates closed-state inactivation, this rate is still significantly smaller than that of open-state inactivation. The rate of recovery from inactivation to the closed state ($I \rightarrow C3$) is significantly smaller than recovery to the open state ($I \rightarrow O$), with largest values at the most hyperpolarized potentials (ie, $V_m \leq -80$ mV). In summary, addition of hKCNE2 to HERG only alters kinetics of channel gating, with no change in voltage dependence.

Whereas the results shown thus far shed light on the functional consequences of coexpression of hKCNE2 and HERG, they do not provide an explanation as to how deletion of hKCNE2 from the channel structure, or a mutation associated with it, would affect cardiac action potential repolarization and ultimately lead to long QT in the affected patients. To address this issue, we have simulated effects of the observed changes associated with this coassembly on action potential repolarization using a computational model of the action potential by guest on May 25, 2017

Figure 2. Activation characteristics of HERG and HERG-hKCNE2 coassembly. A, Voltage-dependent activation (using protocol 1, normalized peak tail currents). B, Activation kinetics (using protocol 2) at 0 (triangles), 20 (circles), 40 (diamonds), and 60 mV (squares). C, Activation time constants ($\tau$) as a function of voltage. Filled symbols indicate HERG; open symbols, HERG-hKCNE2; and solid lines, model results. *$P<0.05$.

Figure 3. Deactivation characteristics. A, Representative normalized experimental (top) and model (bottom) tail currents (protocol 3). B, Deactivation time constants ($\tau$) (using tail currents in protocol 3, as shown in panel A). Filled symbols indicate HERG; open symbols, HERG-hKCNE2; and solid lines, model results.

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Replacing rate constants from the HERG-hKCNE2 model to the HERG model (Table) results in an 18-ms reduction in action potential duration measured at 90% repolarization (APD90) (Figure 6B). Inclusion of changes in both kinetics and conductance (increasing GKr 2-fold) results in a 62-ms reduction in APD90 (Figure 6C), whereas an increase in conductance alone produces a 40-ms reduction in APD90 (Figure 6A). It is clear from these data that presence of a loss-of-function mutation would result in a decrease in action potential duration as a result of an increase in peak IKr density in phase 3 of the action potential (Figures 6C and 6D, bottom panels). These changes were magnified at shorter cycle lengths (Figure 6E), indicating that perhaps alterations observed here may figure prominently at higher heart rates (eg, during β-adrenergic stimulation or exercise). Thus, whereas coexpression of hKCNE2 with HERG alters both the kinetics and density of ionic current, incorporation of these data into a quantitative model of the action potential predicts that changes in current density exert the greatest effect on repolarization.

We have also used the HERG-hKCNE2 model to predict effects of a point mutation associated with hKCNE2 (M54T). As reported previously, this mutation results in a 2- to 3-fold increase in deactivation rates and a modest reduction in activation slope factor compared with HERG-WT hKCNE2 coexpression. Here rate constants of the HERG-hKCNE2 Markov model (Table) were adjusted on the basis of data presented by Abbott et al to include kinetic changes associated with M54T-hKCNE2 mutation and incorporated in the action potential model (Figure 6D). These rate changes result in an increase in APD90 by 30 ms. More importantly, the prolongation of action potential was magnified at shorter cycle lengths (Figure 6E; eg, a 50-ms prolongation in APD90 at 500-ms cycle length compared with a 30-ms prolongation at 1000-ms cycle length). This finding helps to rationalize previous clinical observations in which M54T-hKCNE2 mutation resulted in prolongation of the QT interval in the ECG of the affected patient during exercise.

**Discussion**

In this study we took an integrative approach with a view to defining the underlying mechanisms by which HERG and hKCNE2 interact and the way in which this interaction affects action potential repolarization. We first characterized the functional effects of hKCNE2 coexpression with HERG and found that addition of hKCNE2 to HERG accelerates the kinetics of activation, deactivation, and inactivation, with no change in the voltage dependence of activation. Coassembly also results in a significant reduction in current density compared with channels formed by HERG alone. Whereas the alterations observed in the kinetics and current densities confirm those in the original report on HERG-KCNE2 coassembly, we did not observe any change in the voltage dependence of activation. This discrepancy may be attributable to the differences in the expression system used (oocyte expression system as opposed to HEK293 cells used here) and in the voltage protocols applied.

Because ion channel gating models provide a quantitative description of gating behavior and give clues to channel...
structure-function relationships, a Markov state model of both HERG and HERG-hKCNE2 coassembly (Figure 1) was developed and used to additionally elucidate mechanisms of interactions. Consistent with the experimental observations, optimization of parameters in these models suggested that addition of hKCNE2 to HERG only alters kinetics of channel gating, with no change in voltage dependence (Table). Therefore, the density of ionic current alter action potential repolarization. Lower panels in C and D represent action potential repolarization. A, Effects of increase in conductivity (\(G_{K,r}\)) alone (from HERG-hKCNE2 to HERG model, Table). B, Effect of changes in kinetics (\(\Delta K\)) alone (from HERG-hKCNE2 to HERG model, Table). C, Effects of changes in conductivity (\(G_{K,r}\)) and kinetics (\(\Delta K\)). D, Effects of M54T-hKCNE2 mutation on action potential repolarization. Lower panels in C and D represent \(k_{ij}\) currents corresponding to each case in these perturbations during action potential. Wild-type (HERG-hKCNE2 kinetics and \(G_{K,r}\) conductance) action potentials and \(k_{ij}\) currents are shown in thick black lines. E, Changes in APD90 relative to wild-type models (\(\Delta\)APD90) as function of cycle length for each case described in above panels (symbols match the symbols shown above each action potential trace). Negative and positive values indicate a decrease and an increase in APD90 compared with wild-type model, respectively. Cycle length of 1 second in panels A through D (arrow in panel E). Horizontal scale bar=0.1 second; vertical scale bar=0.2 pA/pF.

Figure 6. Effects of alterations in kinetics or conductivity on action potential repolarization. A, Effects of increase in conductivity alone (\(G_{K,r}\)). B, Effect of changes in kinetics (\(\Delta K\)) alone (from HERG-hKCNE2 to HERG model, Table). C, Effects of changes in conductivity (\(G_{K,r}\)) and kinetics (\(\Delta K\)). D, Effects of M54T-hKCNE2 mutation on action potential repolarization. Lower panels in C and D represent \(k_{ij}\) currents corresponding to each case in these perturbations during action potential. Wild-type (HERG-hKCNE2 kinetics and \(G_{K,r}\) conductance) action potentials and \(k_{ij}\) currents are shown in thick black lines. E, Changes in APD90 relative to wild-type models (\(\Delta\)APD90) as function of cycle length for each case described in above panels (symbols match the symbols shown above each action potential trace). Negative and positive values indicate a decrease and an increase in APD90 compared with wild-type model, respectively. Cycle length of 1 second in panels A through D (arrow in panel E). Horizontal scale bar=0.1 second; vertical scale bar=0.2 pA/pF.

actions potential (as we have illustrated here for the case of M54T-hKCNE2). Note that this result is in contrast to what is seen in LQT3 patients (SCN5A mutations, attributable to persistent inward sodium current),17,18 where action potential prolongation and early afterdepolarization are observed during longer cycle lengths (ie, lower heart rates), as opposed to shorter cycle lengths observed here.

In summary, our principal finding is that kinetic changes associated with coexpression of hKCNE2 and HERG have little effect whereas changes of channel conductance have large effects on action potential repolarization. This finding rationalizes the pathogenicity of clinical mutations, which results in a modest alteration of kinetics and larger changes of current densities, in which the affected patients are susceptible to arrhythmias and long-QT syndrome (eg, M54T-hKCNE2 and I577T-hKCNE2).4 These conclusions can be generalized to include other genes in the KCNE family (eg, S74L-KCNE1 and D76N-KCNE1).6,19,20 Furthermore, if there are mutations identified in hKCNE2 that result in a modest alteration in kinetics alone (eg, recent work of Schulze-Bahr et al21 on hKCNE2) and yet patients show susceptibility to arrhythmias, it is conceivable that these phenotypes are a result of additional, not yet fully understood interactions between these peptides (hMiRP1) and an alternative potassium alpha subunit (eg, KCNQ1).22 This is consistent with the present expanded view of protein function where each protein in the cell membrane functions as part of an extended web of interacting molecules.23 The integrative and iterative approach of combining gene expression, functional analyses, and mathematical modeling enables us to demonstrate underlying functional mechanisms not evident from each individual component alone.

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References


19. Sesti F, Goldstein SA. Single-channel characteristics of wild-type $I_{Ks}$ channels and channels formed with two minK mutants that cause long QT syndrome. J Gen Physiol. 1998;112:651–663.


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