Modification of hERG1 channel gating by Cd$^{2+}$

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Each of the four subunits in a voltage-gated potassium channel has a voltage sensor domain (VSD) that is formed by four transmembrane helical segments (S1–S4). In response to changes in membrane potential, intramembrane displacement of basic residues in S4 produces a gating current. As S4 moves through the membrane, its basic residues also form sequential electrostatic interactions with acidic residues in immobile regions of the S2 and S3 segments. Transition metal cations interact with these same acidic residues and modify channel gating. In human ether-á-go-go–related gene type 1 (hERG1) channels, Cd$^{2+}$ coordinated by D456 and D460 in S2 and D509 in S3 induces a positive shift in the voltage dependence of activation of ionic currents. Here, we characterize the effects of Cd$^{2+}$ on hERG1 gating currents in Xenopus oocytes using the cut-open Vaseline gap technique. Cd$^{2+}$ shifted the half-point for the voltage dependence of the OFF gating charge–voltage (QOFF-V) relationship with an EC$_{50}$ of 171 µM; at 0.3 mM, V$_{1/2}$ was shifted by +50 mV. Cd$^{2+}$ also induced an as of yet unrecognized small outward current (I$_{Cd-out}$) upon repolarization in a concentration- and voltage-dependent manner. We propose that Cd$^{2+}$ and Arg residues in the S4 segment compete for interaction with acidic residues in S2 and S3 segments, and that the initial inward movement of S4 associated with membrane repolarization displaces Cd$^{2+}$ in an outward direction to produce I$_{Cd-out}$. Co$^{2+}$, Zn$^{2+}$, and La$^{3+}$ at concentrations that caused $\sim$+35-mV shifts in the Q$_{OFF}$-V relationship did not induce a current similar to I$_{Cd-out}$, suggesting that the binding site for these cations or their competition with basic residues in S4 differs from Cd$^{2+}$. New Markov models of hERG1 channels were developed that describe gating currents as a noncooperative two-phase process of the VSD and can account for changes in these currents caused by extracellular Cd$^{2+}$.

INTRODUCTION

The human ether-á-go-go–related gene type 1 (hERG1) channel was originally cloned from a human hippocampal cDNA library (Warmke and Ganetzky, 1994) and was later reported to be highly expressed in many other cell types, where it can contribute to spike frequency adaptation, maintenance of K+ homeostasis, set the resting membrane, or mediate action potential repolarization (Sanguinetti, 2010). hERG1 channels are also highly expressed in the heart, where they conduct the rapid delayed rectifier K+ current, I$_{kr}$ (Sanguinetti et al., 1995; Trudeau et al., 1995), and their loss of function can cause arrhythmia. Mutations in HERG1 (KCNH2) or block of hERG1 channels causes long QT syndrome, characterized by prolonged ventricular repolarization and an increased risk of malignant arrhythmia and sudden death (Curran et al., 1995; Keating and Sanguinetti, 2001; Sanguinetti and Tristani-Firouzi, 2006).

The gating of hERG1 channels is modified by divalent cations, including the alkaline earth cations Ca$^{2+}$ and Mg$^{2+}$ (Ho et al., 1998; Johnson et al., 1999b, 2001; Lin and Papazian, 2007), and the transition metal cations Cd$^{2+}$, Co$^{2+}$, Ni$^{2+}$, and Zn$^{2+}$ (Anumonwo et al., 1999; Johnson et al., 1999a; Sanchez-Chapula and Sanguinetti, 2000; Fernandez et al., 2005). The effects of Cd$^{2+}$ on hERG1 channels have been well characterized and were first studied in isolated feline cardiomyocytes, where it was reported to shift the voltage dependence of I$_{kr}$ activation to more positive potentials and accelerate the rate of current deactivation (Follmer et al., 1992). Later studies of hERG1 channels heterologously expressed in Xenopus oocytes determined that Cd$^{2+}$ also slows the rate of activation and produces a positive shift in the voltage dependence of inactivation (Johnson et al., 1999a). Mutations of Asp residues located in the S2 (D456 and D460) and S3 (D509) domains of the hERG1 subunit attenuate the effects of extracellular Cd$^{2+}$ on hERG1 gating, and it has been proposed that together these three acidic residues form a coordination site for Cd$^{2+}$ (Fernandez et al., 2005). These acidic residues, especially D456, also participate in the coordination of Ca$^{2+}$ and Mg$^{2+}$ (Fernandez et al., 2005; Lin and Papazian, 2007). The changes in hERG1 channel gating currents that accompany the altered voltage dependence and...
kinetics of ionic currents caused by the binding of divalent cations have not been characterized.

Here, we determine the effects of Cd$^{2+}$ on the gating currents of hERG1 channels heterologously expressed in Xenopus oocytes. As expected from previous recordings of ionic currents, Cd$^{2+}$ shifted the OFF gating charge–voltage ($Q_{\text{OFF}}$–$V$) relationship to more positive potentials and accelerated the decay of OFF gating current ($I_{\text{OFF}}$). Cd$^{2+}$ also induced a transient outward current ($I_{\text{Clout}}$) upon membrane repolarization from test potentials positive to 0 mV that immediately preceded inward $I_{\text{Cl}}$. Other polyvalent metal cations, including Co$^{2+}$, Zn$^{2+}$, Ca$^{2+}$, and La$^{3+}$, caused a positive shift in the $Q_{\text{OFF}}$–$V$ relationship, but did not induce a similar transient outward current. $I_{\text{Clout}}$ was also not observed in D456C or D509C hERG1 channels treated with Cd$^{2+}$ at concentrations that shifted the voltage dependence of channel activation. We propose that $I_{\text{Clout}}$ is an ionic current conducted by Cd$^{2+}$ that is displaced from its primary coordination site by Arg residues in S4, as this segment moves inward in response to repolarization of the membrane. The effects of Cd$^{2+}$ were simulated with a new Markov model that describes hERG1 channel gating currents as a noncooperative two-phase process of the voltage sensor domain (VSD).

MATERIALS AND METHODS

Heterologous expression of hERG1 channels in oocytes

HERG1a cDNA (GenBank accession no. NM000328) in the pSP64 plasmid vector (Promega) was linearized with EcoR1, and cRNA was transcribed in vitro with an mMessage mMachine SP6 kit (Applied Biosystems). Mutations were introduced into wild-type (WT) hERG1 using the QuikChange site-directed mutagenesis kit (Agilent Technologies).

Oocytes were isolated by dissection from adult Xenopus laevis anaesthetized by immersion in 0.2% tricaine (Sigma-Aldrich) for 10–15 min. After ovarian lobes containing oocytes were removed, the small abdominal incision was sutured closed. Frogs were allowed to recover for at least 1 mo before repeating the surgical procedure to harvest additional oocytes. After a third and final harvest, frogs were anaesthetized with tricaine before pithing. Clusters of oocytes were treated with 2 mg/ml of type 2 collagenase (Worthington) to remove follicle cells. Individual stage IV or V oocytes were microinjected with 5 ng HERG1a cRNA as described previously (Stühmer, 1992) and incubated in Barth’s solution at 19°C for 2–6 d before use in voltage clamp experiments.

Voltage clamp methods

Ionic and gating currents of hERG1 channels were measured using the cut-open oocyte Vaseline gap (COVG) recording technique (Stefani and Bezanilla, 1998). The COVG chamber consists of three compartments (upper, guard, and bottom) that are isolated from one another by Vaseline seals. Microelectrodes were pulled from borosilicate glass capillary tubes to obtain resistances of 0.1–0.5 MΩ when filled with 3 M KCl and were used to record the transmembrane potential of the oocyte domus protruding into the upper compartment. Electrical access to the cytoplasm was obtained by permeabilizing the portion of the oocyte isolated in the bottom compartment with 0.3% saponin for 2 min. hERG1 channel ionic currents were blocked by adding 10 µM MK-499 (or 10 µM terfenadine in a few experiments) to the upper compartment and tetraethylammonium–methanesulfonic acid (TEA-MES) in all compartments. In addition, to reduce intracellular [K$^+$], the membrane was initially clamped to 0 mV for 30 min and the solutions in the chamber were exchanged twice.

An amplifier (CA-1B; Dagan), a data acquisition system (Digidata 1322A; MDS Analytical Technologies), and a personal computer were configured to voltage clamp an oocyte with command voltage pulses generated with PCLAMP8 software (MDS Analytical Technologies). Signals were low-pass filtered at 10 kHz and digitized at 40 kHz. Linear leak and capacitance currents were compensated by analogue circuitry and subtracted on-line by using a p/−8 protocol (Armstrong and Bezanilla, 1977). Unless indicated otherwise, the holding potential was −110 mV and the duration of depolarizing test pulses was 300 ms. All recordings were performed at room temperature (22–24°C) under no-flow conditions.

The effects of Cd$^{2+}$ on ionic currents conducted by mutant hERG1 channels were also determined using standard two-microelectrode voltage clamp techniques (Stühmer, 1992), as described previously in detail (Fernandez et al., 2005).

Solutions

Oocytes were incubated in Barth’s solution that contained (in mM): 88 NaCl, 1 KCl, 0.4 CaCl$_2$, 0.33 Ca(NO$_3$)$_2$, 1 MgSO$_4$, 2.4 NaHCO$_3$, 10 HEPES, and 1 pyruvate; pH 7.4, supplemented with 50 µg/ml gentamycin. Ionic currents were recorded using an extracellular solution in the top and guard compartments that contained (in mM): 96 NaCl, 4 KCl, 1 CaCl$_2$, 1 MgCl$_2$, and 5 HEPES; pH adjusted to 7.6 with NaOH. The same solution was used to bathe oocytes that were voltage clamped using the standard two-microelectrode voltage clamp technique. The extracellular solution in the top and guard compartments for measurement of gating currents contained (in mM): 120 TEA-MES, 2 Ca-MES, and 10 HEPES, pH 7.4. The intracellular solution in the bottom compartment contained (in mM): 120 TEA-MES, 2 EDTA, and 10 HEPES, pH 7.4. CdCl$_2$ was obtained from Sigma-Aldrich. No compensation was made for Cd$^{2+}$ binding to HEPES (Cherny and DeCoursey, 1999). RPR260243, a hERG1 channel activator (Kang et al., 2005), was provided by Sanofi-Aventis Pharmaceuticals and prepared for use by dilution of a 10-nM DMSO stock solution.

Data analysis

pCLAMP8 (MDS Analytical Technologies) and Origin 7.5 (Origin-Lab) were used for off-line data analysis. Integration of the OFF gating current ($I_{\text{OFF}}$) induced by subsequent repolarization was used to calculate the OFF intramembrane charge displacement ($Q_{\text{OFF}}$). Accurate measurement of ON intramembrane charge displacement ($Q_{\text{ON}}$) was difficult because nonlinear and/or time-dependent leak and endogenous currents activated at the more positive test potentials could not be adequately subtracted with the p/−8 pulse protocol. The charge–voltage ($Q_{\text{OFF}}$–$V$) relationship for each oocyte, defined by the plot of $Q_{\text{OFF}}$ measured at −110 mV versus test potential ($V_t$), was normalized to the calculated maximum value ($Q_{\text{OFF,max}}$) determined from fitting the data to a Boltzmann function:

$$\frac{Q_{\text{OFF}}}{Q_{\text{OFF,max}}} = \frac{1}{1 + e^{\frac{z}{RT/V_t - V_{1/2}}}}$$

where $V_{1/2}$ is the half-point of the relationship, $z$ is the effective valence, and $RT/F$ is 25.4 mV at 22°C. The onset of the extracellular Cd$^{2+}$-induced alteration of gating currents required only a few minutes to reach a steady state. However, continued exposure to solutions with [Cd$^{2+}$] > 100 µM slowly reduced the peak
In the Cd\(^{2+}\) model, the gating current was extended by a current related to Cd\(^{2+}\) movement to the transmembrane electric field. Rates associated with Cd\(^{2+}\) movement in the VSD (\(\mu_{C} \) and \(\eta_{C}\); Fig. 17) were defined as voltage dependent, as described in Eqs. 2 and 3. Parameters of the Markov models were determined by numerical fitting of feature vectors extracted from experimental and model data, \(I_{g}\) and \(I_{g\,OFF}\), respectively. The fit error \(E\) was defined as:

\[
E = \sqrt{\sum_{n=1}^{N} \left( \frac{|I_{g\,n} - f_{g\,n}|}{f_{g\,n}} \right)^{2} + (1 - Max S_{14,\,ON})^{2} + (Min S_{14,\,OFF})^{2}},
\]

with the number of features \(n\), the Euclidean norm \(\|\cdot\|_{2}\), the maximum of the state \(S_{14}\) during the test pulses Max \(S_{14,\,ON}\), and the minimum of \(S_{14}\) after the test pulses Min \(S_{14,\,OFF}\). Features included Q-V relationships and parameters from exponential fits of \(I_{g\,ON}\) and \(I_{g\,OFF}\) at different voltages (Table S1). The fit error \(E\) included functions of Max \(S_{14,\,ON}\) and Min \(S_{14,\,OFF}\) to select parameter sets that cause high state probabilities of \(S_{14}\) at activating voltages and low probability of \(S_{14}\) at deactivation, respectively. The fitting procedure was iterative and combined random perturbation and selection of parameter sets causing the smallest fit error followed by their optimization. The number of iterations was 80. In each iteration, 1,600 perturbed parameter sets were evaluated. Perturbations were initially in the range of 1–200% of the original parameter value. The perturbation range was decreased in each iteration to a final range of 99.9–100.1%. The 64 parameter sets with the smallest fit error were optimized using the steepest descent method (Press et al., 1992) and used for the parameter set perturbation in the next step. Fitting of the Cd\(^{2+}\) model was based on parameters of the standard model. The sum of \(z_{a}\) and \(z_{b}\) (charge associated with fast transitions), as well as of \(z_{a}\) and \(z_{b}\) (charge associated with slow transitions), was kept the same in the standard and Cd\(^{2+}\) models.

The simulations were performed with a variable order method for solution of ordinary differential equations based on numerical differentiation formulas (Matlab function: ode15s). An initial time step \(\Delta t\) of 10 fs was chosen. For both models, the temperature was set at 296°K. All calculations were performed in double precision. All software was developed with Matlab 7.9 (The MathWorks Inc.). Numerical fitting procedures were accelerated by using the Matlab Parallel Computing Toolbox.

**Online supplemental material**

Table S1 lists fit errors for model fitting of Q-V relationships and kinetics of \(I_{g\,ON}\) and \(I_{g\,OFF}\). Table S2 presents the initial values for channel states of the standard and Cd\(^{2+}\) models. Table S3 lists the rate constant parameters for the standard and Cd\(^{2+}\) models. The online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.201010450/DC1.

**RESULTS**

Cd\(^{2+}\) shifts the voltage dependence of hERG1 channel gating

hERG1 channel gating currents were elicited with 300-ms depolarizations to test potentials that ranged from −100 to +60 mV. Outward ON gating currents (\(I_{g\,ON}\)) were detectable for depolarizations to test potentials positive to −70 mV, and inward OFF gating currents (\(I_{g\,OFF}\)) were induced upon subsequent repolarization to the holding potential of −110 mV. An example of

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currents recorded from a control oocyte (not injected with cRNA) at a test potential of +60 mV is shown in Fig. 1 A. In this and four other oocytes examined, no gating currents were observed before or after treatment with 30 µM Cd²⁺. Fig. 1 B shows currents recorded at test potentials of −10, +10, and +60 mV in an oocyte previously injected with hERG1 cRNA. In this cell, depolarizing pulses elicited a transient outward gating current (I_g_{ON}) composed of rapid and slow components. Repolarization to −110 mV also elicited rapid and slow components of inward gating current (I_g_{OFF}). At increasingly positive test potentials, the slow component of I_g_{ON} became more apparent and the fast component of I_g_{OFF} was reduced. In addition, a very small transient outward current that preceded the large inward I_g_{OFF} was measured upon repolarization to −110 mV from the pulse to +60 mV. This tiny outward current could represent a bona fide reduction in capacitance caused by a decrease in polarizability (Sigworth, 1994) or an artifact of the p/−8 subtraction procedure (Stühmer et al., 1991). The same oocyte was then exposed to 30 µM Cd²⁺ for 10 min before gating currents were again recorded at the same test potentials (Fig. 1 C). Cd²⁺ reduced the magnitude of the slow component of I_g_{ON} at all test potentials and reduced the fast component of I_g_{ON} at +10 and +60 mV. Note that in the presence of 30 µM Cd²⁺, the magnitude and kinetics of I_g_{ON} at +60 mV nearly matched I_g_{ON} recorded at +10 mV under control conditions, consistent with a Cd²⁺-induced positive shift in the voltage dependence of channel activation. The most obvious effect of Cd²⁺ was the appearance of a large transient outward current that immediately preceded I_g_{OFF}. This Cd²⁺-induced outward current associated with membrane repolarization is hereafter referred to as I_{Cd-out}.

The Q_{OFF}-V relationship was determined for the same oocytes under control ionic conditions and in the presence of extracellular solutions containing [Cd²⁺] that was varied from 3 to 300 µM. Cd²⁺ induced a concentration-dependent positive shift of V_{1/2} and a reduction in the slope of the Q_{OFF}-V relationship (Fig. 2 A). The shift in V_{1/2} of the Q_{OFF}-V relationship measured from gating currents and the G-V relationship measured from ionic currents are plotted as a function of [Cd²⁺]e in Fig. 2 B. For both the Q_{OFF}-V and G-V plots, the maximum Cd²⁺-induced shift in V_{1/2} (ΔV_{1/2max}) was set at +90 mV, the value determined from extrapolation of the averaged ΔV_{1/2} for the G-V dataset. The EC_{50} for the Cd²⁺-induced shift in V_{1/2} for the Q_{OFF}-V relationship was 171 ± 13 µM with a Hill coefficient, n_H, of 0.53 ± 0.03. The EC_{50} for the Cd²⁺-induced shift in V_{1/2} for the G-V relationship was ~2.4 times greater (416 ± 20 µM), but the Hill coefficient was similar (n_H = 0.57 ± 0.02). The effects of Cd²⁺ on the values of V_{1/2} and z determined from fitting these data to a Boltzmann function are summarized in Fig. 2 (C and D, respectively). The [Cd²⁺]-dependent reduction in z likely results from a slowing of I_g_{ON} and incomplete charge displacement during the 300-ms test pulse for intermediate depolarizations. Thus, Cd²⁺ induced a concentration-dependent positive shift in the Q_{OFF}-V relationship for hERG1, an effect that was 2.4 times more potent than the voltage-dependent shift of the G-V relationship for ionic currents.
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For this oocyte, a normal shift in the QOFF-V relationship was observed (Fig. 4 C), as well as a large $I_{Cd-out}$ after pulsing to positive potentials (Fig. 4 D).

To facilitate viewing the effects of Cd$^{2+}$ on the multiple components of gating currents, traces for currents elicited in response to a test pulse to +40 mV before and after treatment of an oocyte with 30 µM Cd$^{2+}$ are superimposed in Fig. 3. Cd$^{2+}$ reduced the amplitude of both the rapid initial component and the slow component of $I_{g_{ON}}$, and it altered the kinetics of $I_{g_{OFF}}$ (Fig. 3 A). The initial 9 ms of $I_{g_{ON}}$ traces recorded during control conditions and after treatment of an oocyte with 30 µM Cd$^{2+}$ are shown in Fig. 3 B. The time to peak outward $I_{g_{ON}}$ was decreased from 430 to 300 µs by Cd$^{2+}$, and the magnitude of the slow component was reduced, consistent with the positive shift in the voltage dependence of gating (Fig. 2). The initial 9 ms of $I_{g_{OFF}}$ traces recorded during control conditions and after treatment of an oocyte with 30 µM Cd$^{2+}$ are shown in Fig. 3 C. Cd$^{2+}$ induced a large outward component of $I_{g_{OFF}}$ and slightly prolonged the time to peak of inward $I_{g_{OFF}}$.

Characterization of $I_{Cd-out}$

Cd$^{2+}$ did not induce $I_{Cd-out}$ in uninjected oocytes (Fig. 1 A), indicating that this current does not represent some unidentified Cd$^{2+}$-activated endogenous current or nonlinear capacitance current. Nonetheless, with a time to peak of <240 µs, the obvious concern is that $I_{Cd-out}$ represents an experimental artifact resulting from the leak and capacitance current subtraction protocol. When using p/$-8$ subtraction, we never observed an inward transient at the start of the depolarizing pulse, arguing against poor compensation as a potential problem. We also measured gating currents in oocytes where the capacitance current was compensated by analogue circuitry only, without p/$-8$ on-line leak subtraction (Fig. 4, A and B). For this oocyte, a normal shift in the QOFF-V relationship was observed (Fig. 4 C), as well as a large $I_{Cd-out}$ after pulsing to positive potentials (Fig. 4 D).

**Figure 2.** Extracellular Cd$^{2+}$ shifts the Q-V relationship for hERG1 to more positive potentials. (A) Normalized $Q_{off}$ is plotted as a function of $V_i$ and [Cd$^{2+}$]. Averaged data for control and each [Cd$^{2+}$], were fitted to a Boltzmann function (smooth curves). (B) Plot of change in $V_{1/2}$ for Q-V and G-V relationships as a function of [Cd$^{2+}$]. Data were fitted to a Hill equation to determine EC$_{50}$ and Hill coefficient ($n_H$). Δ$V_{1/2,max}$ was set at +90 mV (Fernandez et al., 2005). For Q-V relationship, EC$_{50}$ = 171 ± 13 µM; $n_H$ = 0.53 ± 0.03. For G-V relationship, EC$_{50}$ = 416 ± 20 µM; $n_H$ = 0.57 ± 0.02. The number of oocytes used to calculate the average data is indicated next to each data point. (C) [Cd$^{2+}$]-dependent effects on $V_{1/2}$ for Q-V relationship. (D) [Cd$^{2+}$]-dependent effects on effective valence, $z$, of Boltzmann function for Q-V relationship. For A, C, and D, the number of oocytes was as follows: control, 20; 3 µM Cd$^{2+}$, 8; 10 µM Cd$^{2+}$, 7; 30 µM Cd$^{2+}$, 12; 100 µM Cd$^{2+}$, 10; 300 µM Cd$^{2+}$, 14.

**Figure 3.** Cd$^{2+}$ reduces the fast and slow components of $I_{g_{ON}}$ and induces the appearance of $I_{Cd-out}$, a brief initial outward component that precedes $I_{g_{OFF}}$. (A) Superimposed traces for gating currents measured before and after treatment of an oocyte with 30 µM Cd$^{2+}$. Top panel shows pulse protocol. (B) $I_{g_{ON}}$ shown on an expanded time scale. (C) $I_{g_{OFF}}$ shown on an expanded time scale.
The time course of $I_{\text{Cd-out}}$ determined in the cell using $p/-8$ subtraction (Fig. 4 E, blue trace) coincided in time and approximate peak magnitude with the current measured without the use of the $p/-8$ subtraction protocol (Fig. 4 E, red trace). To be certain that $I_{\text{Cd-out}}$ was not an artifact arising from subtraction of a rapid component of $I_{\text{GON}}$ that activated at test potentials negative to the holding potential of $-110 \text{ mV}$, we confirmed that no $I_{GON}$ was present for pulses applied to test potentials between $-140$ and $-100 \text{ mV}$ from a holding potential of $-150 \text{ mV}$ (not depicted).

The relative magnitude of the charge associated with the outward component of $I_{\text{OFF}}$ ($Q_{\text{out}}$) under control ionic conditions and $I_{\text{Cd-out}}$ ($Q_{\text{Cd-out}}$) in the presence of Cd$^{2+}$ compared with $Q_{\text{OFF}}$ was determined for test potentials ranging from 0 to $+60 \text{ mV}$ and for $[\text{Cd}^{2+}]_e$ ranging from 3 to 300 $\mu\text{M}$. Fig. 5 (A and B) illustrates how the $I_{\text{Cd-out}}$ and inward $I_{\text{OFF}}$ were defined for this comparison, and Fig. 5 C shows the integration of the two current components. The peak outward component of $Q_{\text{OFF}}$ was defined as $Q_{\text{Cd-out}}$, and the peak of the inward component of $Q_{\text{OFF}}$ was defined as $Q_{\text{OFF}}_{\text{total}}$. Under control ionic conditions, $Q_{\text{Cd-out}}$ was very small, equivalent to $\sim 0.3\%$ of $Q_{\text{OFF}}_{\text{total}}$ for a $V_t$ of $+40 \text{ mV}$. Cd$^{2+}$ increased the relative magnitude of $Q_{\text{Cd-out}}$ at all test potentials in a concentration-dependent manner for $3$–$100 \mu\text{M Cd}^{2+}$ (Fig. 5 D). At 300 $\mu\text{M}$, the voltage dependence of $Q_{\text{Cd-out}}/Q_{\text{OFF}}_{\text{total}}$ was right-shifted and therefore reduced compared with 100 $\mu\text{M Cd}^{2+}$. Fig. 5 E is a plot of $Q_{\text{Cd-out}}/Q_{\text{OFF}}$ as a function of $V_t$, for 100 $\mu\text{M Cd}^{2+}$. The maximum value of $Q_{\text{Cd-out}}/Q_{\text{OFF}}_{\text{total}}$ was estimated to be 0.064. Thus, even at high $[\text{Cd}^{2+}]_e$, $Q_{\text{Cd-out}}$ is a very minor component of the total $Q_{\text{OFF}}$.

Finally, we determined if the increase in the magnitude of $Q_{\text{Cd-out}}$ and $Q_{\text{OFF}}$ followed a similar time course. This was accomplished by pulsing to $+60 \text{ mV}$ for a variable time and plotting the integrals of $I_{\text{Cd-out}}$ and $I_{\text{OFF}}$ as a function of pulse duration. Fig. 6 A shows control gating currents (top traces) elicited with test pulses of variable duration to $+60 \text{ mV}$. The middle traces illustrate the pulse protocol, and the bottom traces show the corresponding integrals of $I_{\text{OFF}}$. Fig. 6 B shows gating currents and their integrations after exposure of the same oocyte to 30 $\mu\text{M Cd}^{2+}$. The values of $Q_{\text{OFF}}$ were plotted as a function of pulse duration, and the data were fitted to an exponential function (Fig. 6 C). The time constant for the increase in $Q_{\text{OFF}}$ by pulse duration was 29 ms in control and was slowed to 100 ms in the presence of Cd$^{2+}$. After an initial delay of $\sim 50 \text{ ms}$, presumably caused by a masking of $I_{\text{Cd-out}}$ by the overlapping large fast inward component of $I_{\text{OFF}}$, $Q_{\text{Cd-out}}$ was increased exponentially with a time constant of 95 ms (Fig. 6 D). The average time constant for the increase of peak $Q_{\text{OFF}}$ in four oocytes was $107 \pm 14 \text{ ms}$ in the presence of Cd$^{2+}$.
Thus, the time course for increase in $Q_{Cd-out}$ as a function of pulse duration matches reasonably well with the increase in $Q_{OFF}$.

Effect of extracellular Co$^{2+}$, Zn$^{2+}$, La$^{3+}$, and elevated [Ca$^{2+}$]$_e$ on hERG1 gating currents

Similar to the effects of Cd$^{2+}$, elevation of [Ca$^{2+}$]$_e$ causes a positive shift in the G-V relationship of hERG1 (Ho et al., 1998; Johnson et al., 1999b). As expected, the elevation of [Ca$^{2+}$]$_e$ from 2 to 10 mM shifted the $Q_{OFF}$-V relationship by +26 mV; however, unexpectedly, $Q_{OFF}$-max was increased by 44% (Fig. 7 A). The increase in $Q_{OFF}$-max was reversible upon return to a lower [Ca$^{2+}$]$_e$. Unlike Cd$^{2+}$, the shift in the $Q_{OFF}$-V relationship by Ca$^{2+}$ was not associated with the appearance of a brief outward current upon repolarization to −110 mV. In fact, 10 mM Ca$^{2+}$ eliminated the small outward transient (Fig. 7, B and C), probably because it was masked by the accelerated rate of onset of inward $I_{gOFF}$.

The effect of the addition of Cd$^{2+}$ in the continued presence of 10 mM Ca$^{2+}$ is illustrated in Fig. 7 (D–G). Although no outward component of $I_{gOFF}$ is visible with 10 mM Ca$^{2+}$, the addition of 100 µM Cd$^{2+}$ induced the appearance of $I_{Cd-out}$ (Fig. 7, D and E). These changes reflect altered hERG1 gating because no rapid currents were induced in un.injected oocytes treated with these concentrations of Co$^{2+}$ or Cd$^{2+}$ (Fig. 7 F). The addition of Cd$^{2+}$ reduced $Q_{OFF}$-max to nearly the same level measured with 2 mM Ca$^{2+}$ and shifted the $V_{1/2}$ of the $Q_{OFF}$-V relationship by a further +25 mV (Fig. 7 G). Normally, 0.1 mM Cd$^{2+}$ causes a +35-mV shift in the $Q_{OFF}$-V relationship (Fig. 2). The reduced shift in $V_{1/2}$ and the reduction in $Q_{OFF}$-max caused by Cd$^{2+}$ in the continuous presence of 10 mM Ca$^{2+}$ indicate that the effects of Ca$^{2+}$ and Cd$^{2+}$ were not additive, consistent with competition for the same or overlapping binding site. The same effects of elevated [Ca$^{2+}$], and the subsequent addition of 100 µM Cd$^{2+}$ were observed in five oocytes.

Other divalent transition metal cations such as Co$^{2+}$ (Sanchez-Chapula and Sanguinetti, 2000) or Zn$^{2+}$ (Anumonwo et al., 1999), or the trivalent La$^{3+}$ (Sanchez-Chapula and Sanguinetti, 2000), also cause a positive shift in the G-V relationship of hERG1. Therefore, we characterized the effects of these three cations at concentrations chosen to match the +35-mV shift in the $Q_{OFF}$-V relationship caused by $100 \mu M$ Cd$^{2+}$ that also induced the maximal increase in $Q_{Cd-out}$. Co$^{2+}$ at 3 mM shifted $Q_{OFF}$-V by +35 mV, with only a slight change in effective valence ($z$) and without changing the peak value of $Q_{Cd-out}$ (Fig. 8 A). However, similar to Ca$^{2+}$ and unlike Cd$^{2+}$, Co$^{2+}$ eliminated the small outward transient under control conditions (Fig. 8, B and C). Zn$^{2+}$ at 1 mM shifted $Q_{OFF}$-V by +34 mV, but it also reduced $z$ for this relationship and reduced $Q_{OFF}$-max by −20% (Fig. 8 D). However, unlike Cd$^{2+}$, Ca$^{2+}$, or Co$^{2+}$, Zn$^{2+}$ did not appreciably alter the initial kinetics of $I_{gOFF}$ or alter the amplitude of its brief


outward component (Fig. 8, E and F). La\(^{3+}\) at a concentration of 10 µM caused a +40-mV shift of the \(Q_{\text{OFF}}-V\) relationship, reduced \(\tau\), and reduced \(Q_{\text{OFF-max}}\) by ~20% (Fig. 8 G), mirroring the effects of Zn\(^{2+}\). However, like Ca\(^{2+}\) and Co\(^{2+}\), the brief outward current associated with repolarization was absent in the presence of La\(^{3+}\), presumably because it was obscured by the rapid onset of inward \(I_{\text{G-Off}}\) (Fig. 8, H and I).

In summary, a transient outward current associated with repolarization was: only observed in oocytes expressing hERG1 channels; increased as a function of [Cd\(^{2+}\)], and the charge carried by this current \((Q_{\text{Cd-out}})\) increased in magnitude as a function of test pulse duration similar to \(Q_{\text{OFF}}\); and not observed in oocytes treated with Co\(^{2+}\), La\(^{3+}\), or elevated [Ca\(^{2+}\)].

\(I_{\text{Cd-out}}\) is eliminated by neutralization of acidic residues in the S2 or S3 segment

The hERG1 subunit contains two acidic residues in S2 (D456 and D460) and one in S3 (D509) that together constitute the primary components of a putative binding site for extracellular applied Cd\(^{2+}\) (Fernandez et al., 2005). Assuming that a Cd\(^{2+}\) ion bound to a coordination site is within the transmembrane electric field, its outward displacement in response to membrane repolarization could produce \(I_{\text{Cd-out}}\). Conceivably, Cd\(^{2+}\) movement could result from its unbinding from a unique coordination site, or by a change in the highly localized electrostatics of the binding site (e.g., altered interaction with one or more of the Asp residues in S2 or S3). Mutation of the key acidic residues in S2 or S3 could alter the coordination chemistry of this putative Cd\(^{2+}\) binding site and potentially alter the properties of \(I_{\text{Cd-out}}\).

We previously reported that mutation of D456, D460, or D509 to Ala shifted the voltage dependence of hERG1 channel activation to more positive potentials and greatly attenuated the effects of Cd\(^{2+}\) on the gating of ionic currents (Fernandez et al., 2005). Here, we instead mutated these residues to Cys to neutralize their negative charge while retaining their ability to coordinate Cd\(^{2+}\).

Ionic and gating currents were recorded from D456C and D509C channels, but D460C channels did not express at sufficient levels to permit recording of gating currents and were not further characterized. We first characterized the effect of the single Cys mutations on hERG1 ionic currents. The \(V_{1/2}\) of the G-V relationship for D509C ionic currents activated with 5-s test pulses was 6.6 ± 0.7 mV \((z = 1.89 ± 0.06; n = 5)\), a shift of +42 mV compared with WT hERG1 \((V_{1/2} = -35.0 ± 0.3 mV; z = 3.5 ± 0.1; n = 14)\). Cd\(^{2+}\) at 1 mM shifted the \(V_{1/2}\) for activation of D509C hERG1 channel by an additional +34.4 ± 0.3 mV \((n = 5; \text{Fig. 9 A})\), similar to the +36-mV shift produced with 0.5 mM Cd\(^{2+}\) on WT hERG1 channels under identical conditions (Fernandez et al., 2005). The \(V_{1/2}\) for activation of D456C hERG1 channel...
ionic currents was 5.1 ± 3.4 mV (z = 1.89 ± 0.11; n = 8), a shift of +40 mV compared with WT hERG1. Cd²⁺ at 1 mM shifted the V₁/₂ for activation of D456C hERG1 channels by +34.4 ± 2.2 mV (n = 5; Fig. 9 B). Thus, both D456C and D509C mutant hERG1 channels retained high sensitivity to Cd²⁺, albeit reduced by twofold compared with WT hERG1.

For WT hERG1, the \( Q_{\text{Cd-out}}/Q_{\text{OFF}} \) ratio was most prominent at 100 µM Cd²⁺ (Fig. 5 D). Considering the approximately twofold lower potency of Cd²⁺ on the ionic currents of D456C and D509C hERG1, we used 0.3 mM Cd²⁺ to maximize the chances of observing \( I_{\text{Cd-out}} \) in these mutant channels. Cd²⁺ shifted the \( Q_{\text{OFF}}-V \) relationship to more positive potentials and accelerated the onset and decay of \( I_{\text{Cd-out}} \) for D509C and D456C channels. For D509C, \( V_{1/2} \) was +49.6 ± 4.5 mV (z = 0.76 ± 0.03) during control and +86.8 ± 1.5 mV (z = 0.75 ± 0.03) in the presence of 0.3 mM Cd²⁺, a shift of +37 mV (n = 4). For D456C, \( V_{1/2} \) was +28.0 ± 1.7 mV (z = 0.79 ± 0.05) during control and +62.5 ± 2.5 mV (z = 0.85 ± 0.05) after 0.3 mM Cd²⁺, a shift of +34.5 mV (n = 3; not depicted). However, in contrast to WT hERG1 (Fig. 10 A), Cd²⁺ did not induce the appearance of \( I_{\text{Cd-out}} \) in the two mutant hERG1 channels. Examples of \( I_{\text{Cd-off}} \) activated by repolarization from a \( V_t \) ranging from +30 to +90 mV (or +20 to +80 mV), applied in 20-mV increments, are shown in Fig. 10 B for D509C and Fig. 10 C for D456C channels. These specific test potentials are +30 or +40 mV positive to the voltages used to elicit \( I_{\text{OFF}} \) for WT channels (Fig. 10 A) and represent equivalent stimuli, taking into consideration the shift in the voltage dependence of activation induced by the specific Cys substitutions. Thus, similar to WT hERG1 channels, Cd²⁺ shifted the voltage dependence of activation and accelerated the decay of \( I_{\text{OFF}} \) of D509C and D456C channels. However, unlike in WT channels, Cd²⁺ did not induce the appearance of \( I_{\text{Cd-out}} \) in these mutant channels.

Markov models of hERG1 channel gating and gating currents

For most voltage-gated ion channels, the intramembrane displacement of charge that produces a gating current was 5.1 ± 3.4 mV (z = 1.89 ± 0.11; n = 8), a shift of +40 mV compared with WT hERG1. Cd²⁺ at 1 mM shifted the \( V_{1/2} \) for activation of D456C hERG1 channels by +34.4 ± 2.2 mV (n = 5; Fig. 9 B). Thus, both D456C and D509C mutant hERG1 channels retained high sensitivity to Cd²⁺, albeit reduced by twofold compared with WT hERG1.

For WT hERG1, the \( Q_{\text{Cd-out}}/Q_{\text{OFF}} \) ratio was most prominent at 100 µM Cd²⁺ (Fig. 5 D). Considering the approximately twofold lower potency of Cd²⁺ on the ionic currents of D456C and D509C hERG1, we used 0.3 mM Cd²⁺ to maximize the chances of observing \( I_{\text{Cd-out}} \) in these mutant channels. Cd²⁺ shifted the \( Q_{\text{OFF}}-V \) relationship to more positive potentials and accelerated the onset and decay of \( I_{\text{Cd-out}} \) for D509C and D456C channels. For D509C, \( V_{1/2} \) was +49.6 ± 4.5 mV (z = 0.76 ± 0.03) during control and +86.8 ± 1.5 mV (z = 0.75 ± 0.03) in the presence of 0.3 mM Cd²⁺, a shift of +37 mV (n = 4). For D456C, \( V_{1/2} \) was +28.0 ± 1.7 mV (z = 0.79 ± 0.05) during control and +62.5 ± 2.5 mV (z = 0.85 ± 0.05) after 0.3 mM Cd²⁺, a shift of +34.5 mV (n = 3; not depicted). However, in contrast to WT hERG1 (Fig. 10 A), Cd²⁺ did not induce the appearance of \( I_{\text{Cd-out}} \) in the two mutant hERG1 channels. Examples of \( I_{\text{Cd-off}} \) activated by repolarization from a \( V_t \) ranging from +30 to +90 mV (or +20 to +80 mV), applied in 20-mV increments, are shown in Fig. 10 B for D509C and Fig. 10 C for D456C channels. These specific test potentials are +30 or +40 mV positive to the voltages used to elicit \( I_{\text{OFF}} \) for WT channels (Fig. 10 A) and represent equivalent stimuli, taking into consideration the shift in the voltage dependence of activation induced by the specific Cys substitutions. Thus, similar to WT hERG1 channels, Cd²⁺ shifted the voltage dependence of activation and accelerated the decay of \( I_{\text{OF}} \) of D509C and D456C channels. However, unlike in WT channels, Cd²⁺ did not induce the appearance of \( I_{\text{Cd-out}} \) in these mutant channels.

Markov models of hERG1 channel gating and gating currents

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current is attributed to movements of the VSD. The movements are thought to be associated with transitions between multiple closed states with little to no charge associated with transitions between a final closed state and the open state (C↔O). In contrast, previous models of hERG1 channel gating, including our own, assumed that significant charge displacement is associated with C↔O transitions and between open and inactivated states (O↔I) (Lu et al., 2001; Mazhari et al., 2001; Piper et al., 2003; Perry et al., 2007). However, as described below, the effects of a hERG1 agonist on channel gating suggest that C↔O transitions are not accompanied with a measurable component of charge. RPR260243 ((3R,4R)-4-[3-(6-methoxy-quinolin-4-yl)-3-oxo-propyl]-1-[3-(2,3,5-trifluorophenyl)-prop-2-ynyl]-piperidine-3-carboxylic acid) is a recently described hERG1 channel activator that causes a marked slowing in the rate of deactivation. At 10 µM, RPR260243 slowed the rate of deactivation of ionic currents by at least 20-fold (Kang et al., 2005; Perry et al., 2007); however, 212 hERG1 channel gating
as shown in Fig. 11 A, RPR260243 at 30 µM had no discernable effect on the kinetics or magnitude of \(I_{\text{gON}}\) or \(I_{\text{gOFF}}\) at any potential examined. It also had no effect on the \(Q_{\text{OFF}}-V\) relationship (\(n=5\); Fig. 11 B). Thus, RPR260243 dissociates channel closure, as quantified by the rate of ionic current deactivation, from the movement of intramembrane gating charge. For this reason, we chose to develop a new Markov model that describes hERG1 gating currents as intramembrane charge displacement associated solely with transitions between closed states.

The standard Markov model (in the absence of Cd\(^{2+}\)) was developed to reconstruct gating currents measured with the standard voltage clamp protocol: 300-ms pulses to voltages ranging from \(-110\) to +60 mV, applied from a holding potential of \(-110\) mV and followed by

\[
Q_{\text{OFF}} = \text{Normalized } Q_{\text{OFF}}-	ext{V relationship}
\]

\[
V_{1/2} = -18.6 \pm 0.5 \text{ mV and } z = 1.84 \pm 0.01.
\]

In the presence of RPR260243, \(V_{1/2} = -19.0 \pm 0.5 \text{ mV and } z = 1.88 \pm 0.01.\)
repolarization to $-110$ mV for 300 ms. Parameters of the standard model were determined by a numerical fitting procedure to features extracted from averaged ($n = 12$) and normalized gating currents for WT hERG1 channels (Fig. 12). A schematic of the standard Markov model for gating of WT hERG1 channels is shown in Fig. 13, where states $S_0$ to $S_{14}$ (A) define VSD movements, and the states $C_0$, $C_1$, $O$, and $I$ (B) reflect activation and

![Figure 12](image12.png)

**Figure 12.** Averaged and normalized $I_g$ of WT hERG1 channels measured using $p/-8$ leak and capacitance current subtraction. (A) $I_g$ in response to test voltages $V_t$ of $-60$, $-30$, …, $+60$ mV during 0–300 ms. The membrane voltage is clamped to $-110$ mV otherwise. (B and C) $I_{g ON}$ at 0–10 ms and 10–300 ms, respectively. (D and E) $I_{g OFF}$ at 300–310 ms and 310–600 ms, respectively.

![Figure 13](image13.png)

**Figure 13.** Schematics of Markov model for gating and ionic currents of WT hERG1 channels. The model consists of two components, one for voltage sensing with the states $S_0$ to $S_{14}$ (A) and the other for activation and inactivation with the states $C_0$, $C_1$, $O$, and $I$ (B). The coupling between the components is via the rate coefficient $\alpha_{C0,C1}$, which is a function of the states $S_0$, $S_8$, $S_{10}$, $S_{13}$, and $S_{14}$ in the component for voltage sensing.
Several variants of the standard model were studied to evaluate cooperativity and parameterization of the activation/inactivation component. Allowing for cooperativity of the fast transitions in the numerical fitting procedure had only minor effects on the fit error and yielded cooperativity factors close to 1, suggesting no cooperativity of these transitions. Previously published models (Lu et al., 2001; Perry et al., 2007) were tested for parameterization of the activation/inactivation component of the standard model (Fig. 13 B). The inclusion of these models into the standard model increased the fit error, in particular, errors associated with features of $I_{G_{OFF}}$.

The $Cd^{2+}$ model was parameterized using features extracted from averaged ($n = 10$) and normalized data measured for WT hERG1 channels in the presence of 100 µM $Cd^{2+}$ (Fig. 16). A schematic of the Markov model for the $I_g$ of hERG1 channels in the presence of extracellular $Cd^{2+}$ is presented in Fig. 17. States $S_0$ to $S_{14}$ describe configurations of the hERG1 tetramer with two $Cd^{2+}$ ions bound at sites in the resting configuration of the VSDs. States $S_{15}$ to $S_{24}$, and $S_{25}$ to $S_{30}$, are color coded and depict hERG1 channel configurations having one and two $Cd^{2+}$ ions moved inwardly from inactivation of the pore domain. Transitions between all states in the model satisfy the condition of microscopic reversibility. Features and fit errors associated with parameterization of the VSD model (Fig. 13 A) are listed in Table S1. Fig. 14 shows a comparison between these features extracted from the measured and simulated data. The total gating charge for the model was 5.1 e$_0$.

Gating currents simulated with the standard model are presented in Fig. 15. The simulations showed instantaneous peak outward current in response to test voltages followed by a plateau current (Fig. 15 B) that decayed slowly (Fig. 15 C). Repolarization to $-110$ mV caused an instantaneous inward current followed by a plateau (Fig. 15 D) and slowly decaying current (Fig. 15 E). The standard model explains the instantaneous $I_{G_{ON}}$ peak by gating current associated with the fast transition between $S_0$ and $S_1$ (Fig. 15 F). The model suggests that the plateau component of $I_{G_{ON}}$ is a mixture of decreasing and increasing currents. Simulated plateau $I_{G_{ON}}$ is primarily composed of decreasing gating currents associated with $S_1$–$S_6$, $S_2$–$S_6$, $S_3$–$S_7$, and $S_4$–$S_8$ transitions and increasing currents of $S_9$–$S_{10}$, $S_{10}$–$S_{11}$, $S_{10}$–$S_{12}$, $S_{11}$–$S_{13}$, and $S_{13}$–$S_{14}$ transitions (Fig. 15 G).
their original VSD binding sites, respectively. Transitions between all states in the Cd$^{2+}$ model satisfy the condition of microscopic reversibility. Features and fit errors for the standard and Cd$^{2+}$ models are listed in Table S1. Tables S2 and S3 list the initial parameters and rate constants for transitions between channel states for both models. In Fig. 18, we compare features extracted from measured data to features from simulated data. Gating currents simulated with the Cd$^{2+}$ model are presented in Fig. 19. The model reconstructs the decreased peak and plateau of $I_{GON}$ (Fig. 19 B), attenuated slow decay of $I_{GON}$ (Fig. 19 C) and outward current $I_{Cd-out}$ (Fig. 19 D), and accelerated slow decay of $I_{GOFF}$ for positive voltage steps. The model explains these phenomena by a current $I_{Cd}$ associated with Cd$^{2+}$ displacement from one binding site to another. $I_{Cd}$ is opposite to components of $I_g$ related to transitions between closed states (Fig. 19 F). The contribution of gating (VSD movement) and Cd$^{2+}$ currents to the outward component of $I_{GOFF}$ is further detailed in Fig. 19 G. $I_{Cd-out}$ is dominated by transitions between states with one and two Cd$^{2+}$ ions moved outward relative to their positions occupied when the VSDs are in their activated position.

**DISCUSSION**

Extracellular Cd$^{2+}$ alters hERG1 channel gating

Extracellular application of Cd$^{2+}$ caused a concentration-dependent shift of the $Q_{OFF-V}$ relationship to more positive potentials, similar to our previously reported shift in the G-V relationship for hERG1 ionic currents (Fernandez et al., 2005). The shifted $Q_{OFF-V}$ relationship was accompanied by an alteration in the kinetics of $I_{GON}$ and $I_{GOFF}$. These kinetic changes included both a slowing and reduction in the slow component of $I_{GON}$, and for $I_{GOFF}$ a slowing of the initial slow component and an acceleration of the late slow component, thus creating a crossover with the control $I_{GOFF}$. In addition, Cd$^{2+}$ induced the appearance of a large transient outward current component of $I_{GOFF}$ ("$I_{Cd-out}$"). We propose that all of these effects of Cd$^{2+}$ are mediated by their original VSD binding sites, respectively. Transitions between all states in the Cd$^{2+}$ model satisfy the condition of microscopic reversibility. Features and fit errors for the standard and Cd$^{2+}$ models are listed in Table S1. Tables S2 and S3 list the initial parameters and rate constants for transitions between channel states for both models. In Fig. 18, we compare features extracted from measured data to features from simulated data. Gating currents simulated with the Cd$^{2+}$ model are presented in Fig. 19. The model reconstructs the decreased peak and plateau of $I_{GON}$ (Fig. 19 B), attenuated slow decay of $I_{GON}$ (Fig. 19 C) and outward current $I_{Cd-out}$ (Fig. 19 D), and accelerated slow decay of $I_{GOFF}$ for positive voltage steps. The model explains these phenomena by a current $I_{Cd}$ associated with Cd$^{2+}$ displacement from one binding site to another. $I_{Cd}$ is opposite to components of $I_g$ related to transitions between closed states (Fig. 19 F). The contribution of gating (VSD movement) and Cd$^{2+}$ currents to the outward component of $I_{GOFF}$ is further detailed in Fig. 19 G. $I_{Cd-out}$ is dominated by transitions between states with one and two Cd$^{2+}$ ions moved outward relative to their positions occupied when the VSDs are in their activated position.

**Figure 15.** Modeled $I_g$ of WT hERG1 channels. (A) $I_g$ in response to the voltage-clamping protocol used in Fig. 12. (B and C) $I_{GON}$ at 0–10 ms and 10–300 ms, respectively. (D and E) $I_{GOFF}$ at 300–310 ms and 310–600 ms, respectively. (F) The initial phase (0–1 ms) of $I_{GON}$ is primarily carried by the fast transition between $S_0$ and $S_1$. (G) The slow decay of $I_{GON}$ between 2 and 10 ms is caused by a mixture of increasing and decreasing gating currents associated with the slow transitions in the gating model.
its interaction with an extracellular, accessible coordination site formed by acidic residues in the S2 (D456 and D460) and S3 (D509) segments of each hERG1 subunit. We previously found that charge neutralization of any one of these acidic residues reduced the Cd^{2+}-induced shift in the G-V relationship, and that combined mutation of all three residues nearly eliminated the effects of Cd^{2+} on hERG1 channel gating (Fernandez et al., 2005). In addition to the kinetic effects described above, prolonged incubation of oocytes with Cd^{2+} was associated with a reduction in total Q_{OFF}, an effect that may be associated with a slow accumulation of cytoplasmic Cd^{2+}.

**Origin of I_{Cd-out}**

Extracellular Cd^{2+} induced a brief outward current (I_{Cd-out}) that coincided with repolarization from test potentials positive to 0 mV to a negative holding potential and preceded the inward component of I_{G_{OFF}}. The appearance of I_{Cd-out} associated with membrane repolarization was unexpected and counterintuitive, as normally a hyperpolarizing pulse produces only an inwardly directed charge displacement. However, the magnitude of I_{Cd-out}, quantified as either peak amplitude or by its integral, was increased by 3 to 300 µM Cd^{2+} in a concentration-dependent manner.

Even in the absence of extracellular Cd^{2+}, a small outward component of I_{G_{OFF}} was observed that coincided with repolarization from positive test potentials in oocytes expressing hERG1 (Fig. 3 C). The charge associated with the outward current under control conditions amounted to only 0.3% of the total Q_{OFF}. The origin of this tiny outward current is uncertain, but it is related to overexpression of hERG1 channels because it was not observed in un.injected oocytes. The current could be an experimental artifact related to the use of the on-line p/—8 subtraction procedure if channels were not fully closed at the holding potential used for the linear leak/capacitance subtraction pulses (—110 mV in our experiments), as assumed previously for other Kv channels (Stühmer et al., 1991). However, Sigworth (1994) recorded a similar outward current that preceded inward I_{G_{OFF}} in oocytes expressing Shaker K+ channels and suggested that it might represent a decrease in capacitance due to a change in polarizability (e.g., reorientation of side chains of polar amino acids) associated with a change in channel states. In Shaker channels, the integral of this small current for a step from +40 to —100 mV was only 0.3% of the total Q_{OFF} (Sigworth, 1994), the same as we recorded for hERG1 channels with a similar step in voltage (Fig. 5 D). One possible explanation of I_{Cd-out} is that it represents an extreme (>10-fold) increase in polarization of the channel, but this seems unlikely given the absence of the current component in D456C or D509C channels treated with Cd^{2+} or in WT hERG1 channels exposed to other divalent cations.

An outward current component of I_{G_{OFF}} was not observed under control conditions for mutant D509C or D456C hERG1 channels. Moreover, I_{Cd-out} was not induced by Cd^{2+} concentrations that caused significant changes in the kinetics of inward I_{G_{OFF}} and significant rightward shifts in the voltage dependence of channel activation. One interpretation of this finding could be that inward displacement of S2 and S3 (and thus, D509 and D456) precedes S4 movement to produce the
outward component of $I_{g_{OFF}}$, and that in the presence of Cd$^{2+}$, this movement is accentuated in WT channels and absent in mutant channels where these acidic residues are neutralized. However, available evidence suggests that S2 and S3 segments of Kv channels either do not move during gating or that the S3b and S4 segments move together (and not in opposite directions) across the membrane as a rigid “paddle” structure (Jiang et al., 2003). The large intramembrane displacement of S3b/S4 proposed by the paddle model has been challenged (Ahern and Horn, 2005; Tombola et al., 2006), and a recent luminescence resonance energy transfer study estimated that although the S4 segment of Shaker moves $\sim$10 Å during gating, the S3 segment moves only $\sim$2 Å and in the opposite direction (Posson and Selvin, 2008). Neutralization of the two acidic residues in the S3 of Shaker also failed to impact the gating charge in Shaker channels (Gonzalez et al., 2005). These and other studies suggest that the S3 segment has only a minor or no role in the net intramembrane charge displacement associated with Shaker channel gating. In contrast, the acidic residue in S2 of Shaker is estimated by luminescence resonance energy transfer to move $\sim$4 Å outward during gating (Posson and Selvin, 2008) and thus contributes to the total gating charge, as suggested previously (Seoh et al., 1996). In summary, based on studies of other Kv channels, an S4-independent, accentuated inward displacement of S2 and/or S3 segments in response to repolarization is very unlikely to produce the outward component of $I_{g_{OFF}}$ either in the absence or presence of extracellular Cd$^{2+}$. Instead, we propose that $I_{Cd_{out}}$ represents an outward ionic current conducted by Cd$^{2+}$, as it is displaced in an extracellular direction from its coordination site between the S2 and S3 segments as described below.

The voltage-dependent activation gating of ion channels is associated with sequential ion pair formation between basic residues in the mobile S4 segment and specific acidic residues in the fixed S2 segments. In the closed state of Kv channels (Silverman et al., 2003) and NaChBac channels (DeCaen et al., 2008, 2009), a specific basic residue (“R1”) located in the outer region of S4 charge pairs with an acidic residue in S2 (“D1”). Membrane depolarization causes outward movement of S4 and sequential ion pair formation between D1 and different Arg residues. In the fully activated state, “R3”

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**Figure 17.** Schematics of Markov model for $I_g$ of WT hERG1 channels in the presence of extracellular Cd$^{2+}$. The model extends the description of $I_g$ in Fig. 13 A with states and transitions describing the movement of Cd$^{2+}$. States $S_0$ to $S_{14}$ (green) describe hERG1 configurations with Cd$^{2+}$ bound to a site in the VSD. States $S_{15}$ to $S_{24}$ (blue), and $S_{25}$ to $S_{30}$ (brown), are associated with hERG1 configurations having one and two Cd$^{2+}$ ions moved in the VSD, respectively.
and/or “R4” of S4 forms the final electrostatic interaction with D1. hERG1 subunits contain two additional acidic residues, D460 in S2 and D509 in S3, that are not present in the more well-studied Shaker, Kv1.2, KvAP, and NaChBac channels. As noted previously, both of these acidic residues are important components of the coordination site for Cd\textsuperscript{2+} (Fernandez et al., 2005), and based on double-mutant cycle analysis, both are energetically coupled to R531 (R3) during hERG1 channel gating (Piper et al., 2008). The importance of D456 and D509 in hERG1 gating is exemplified by the finding that neutralization of either residue causes large positive shifts in the voltage dependence of channel activation (Liu et al., 2003; Fernandez et al., 2005; Lin and Papazian, 2007; Piper et al., 2008). Thus, it is likely that in addition to the well-characterized electrostatic interactions between R1 and D1 in Kv channels, D2 (D456) and D3 (D509) of hERG1 subunits also interact with basic residues in the S4 during channel gating.

The details of hERG1 channel gating with regard to sequential ion pairing between charged residues in S4 and S2/S3 have not yet been systematically elucidated. Nonetheless, we propose a simple gating model to explain the origin of $I_{\text{Cd-out}}$. An outward displacement of Cd\textsuperscript{2+} (opposite to electrical driving force) could be driven by an appropriate change in the highly localized electrostatic energy associated with the coordination of Cd\textsuperscript{2+}. It is conceivable that only a subtle movement of S4 is required to alter the structural basis of the Cd\textsuperscript{2+} coordination site; e.g., by favoring charge pairing between an Asp in S2 or S3 with an Arg in S4 instead of with Cd\textsuperscript{2+}. A net outward current ($I_{\text{Cd-out}}$) could be produced if the initial voltage-induced change in S4 displacement produced a small inward gating current relative to a much larger outward current associated with a change in the coordination site for Cd\textsuperscript{2+}. The model, illustrated in Fig. 20, shows the orientation of S2–S4 of the VSD for a single hERG1 subunit and highlights the key charged residues in these segments. K1 (equivalent to R1 in other channels) plus R2–R4 represent the basic residues in S4, and D1–D3 represent Asp residues located near the extracellular ends of S2 and S3 as indicated.

Figure 18. Features of measured and modeled $I_g$ in the presence of extracellular Cd\textsuperscript{2+}. (A) Maximal $I_{\text{ON}}$-$V_i$ relationship. (B) Maximal $I_{\text{OFF}}$-$V_i$ relationship. Time constants-$V_i$ relationships for $I_{\text{ON}}$ (C) and $I_{\text{OFF}}$ (D). Relationship of exponential fit coefficients to $V_i$ for $I_{\text{ON}}$ (E) and $I_{\text{OFF}}$ (F).
At depolarized potentials when channels are in the open state, R531 (R3) forms an ion pair with D456 (D1), and Cd\(^{2+}\) interacts with its “depolarized” coordination site. Repolarization induces inward movement of S4 and displacement of Cd\(^{2+}\), first by R528 (R2) and then by K525 (K1). Because \(I_{\text{Cd-out}}\) is an outward current, we propose that Cd\(^{2+}\) is displaced in an outward direction compared with its “depolarized” coordination site. For example, the depolarized coordination site could be formed by D456, D460, and D509 (D1, D2, and D3), whereas the “repolarized” coordination site for Cd\(^{2+}\) might be formed primarily by the outermost Asp residues, D456 and D509.

Disruption of the normal electrostatic interactions between S4 and S2/S3 by Cd\(^{2+}\) or other divalent cations causes a positive shift in the voltage dependence of hERG1 channel activation. Neutralization of D456 or D509 by mutation to the uncharged Cys would obviously prevent their electrostatic interaction with S4. However, Cys can coordinate Cd\(^{2+}\) ions as effectively as does Asp (Rulísek and Vondrásek, 1998) and thus, it was not unexpected that Cys substitutions only modestly attenuated the Cd\(^{2+}\)-induced positive shift in the voltage dependence of activation. However, \(I_{\text{Cd-out}}\) was not observed for either mutant channel, perhaps because Cd\(^{2+}\) is situated in a slightly different molecular space when coordinated by a Cys instead of an Asp and is not displaced by movement of S4. Alternatively, inward S4 movement might displace Cd\(^{2+}\) in a lateral or inward direction relative to the transmembrane electric field when its coordination site is formed in part by a Cys rather than an Asp residue.

The other polyvalent metal cations examined in this study, Ca\(^{2+}\), Co\(^{2+}\), Zn\(^{2+}\), and La\(^{3+}\), did not induce an outward current similar to \(I_{\text{Cd-out}}\) at concentrations that caused positive shifts in the \(Q_{OFF}-V\) or \(G-V\) relationships approximately equivalent to that produced by 0.1 mM Cd\(^{2+}\).

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**Figure 19.** Modeled \(I_g\) of WT hERG1 channels in the presence of extracellular Cd\(^{2+}\). (A) \(I_g\) in response to the voltage-clamping protocol used in Fig. 12. (B and C) \(I_{\text{GON}}\) at 0–10 ms and 10–300 ms, respectively. (D and E) \(I_{\text{GOFF}}\) at 300–310 ms and 310–600 ms, respectively. (F) Gating current (\(I_g\)) caused by movement of VSD and current conducted by Cd\(^{2+}\) (\(I_{\text{Cd}}\)) for a 300-ms voltage step to +60 mV, followed by a return to the holding potential of −110 mV. The sum of these two currents produces the gating currents simulated to occur in the presence of Cd\(^{2+}\). (G) Currents underlying the initial 2 ms of \(I_{\text{GOFF}}\) (300–302 ms). Major contributors to initial \(I_{\text{GOFF}}\) are Cd\(^{2+}\) currents (\(I_{\text{Cd}}\)) related to transitions between S19–S25 and S24–S30 (black) and between S5–S15 and S14–S24 (pink). Currents related to VSD movement (red, green, and blue) have a small contribution to initial \(I_{\text{GOFF}}\) but dominate after decay of \(I_{\text{Cd}}\).
Additive, inconsistent with these cations binding to distinct sites on the channel.

RPR260243 dissociates gating currents from ionic currents of hERG1.

RPR260243 is a hERG1 channel activator. Site-directed mutagenesis was used to localize its binding site to specific residues in the S5 and S6 segments and nearby S4–S5 linker (Perry et al., 2007). The location of this binding site helps to explain its ability to markedly slow the rate of deactivation of hERG1 ionic current (Kang et al., 2005). Unexpectedly, RPR260243 had no detectable effect on the kinetics, magnitude, or voltage dependence of hERG1 gating currents. The same remarkable dissociation between effects on ionic and gating currents was recently reported for FPL 64176 on L-type CaV1.2 channels. To explain this dissociation, it was proposed that when bound by FPL 64176, CaV1.2 channels conduct Ca$^{2+}$ in the inactivated state without having any effect on the rates of VSD movement (McDonough et al., 2005). We propose that RPR260243 (a) has no effect on the rates of transitions between closed states of the hERG1 channel and thus produces no change in the kinetics or magnitude of gating currents, and (b) slows the transition of the open to the closed state of the pore domain. Thus, similar to the action of FPL 64176 on CaV1.2, RPR260243 dissociates gating charge displacement (VSD movement) from the opening and closing of the activation gate formed by the S6 bundle crossing.

Gating models

A novel feature of our Markov model for hERG1 channel gating is the decoupling of VSD movements (S0 ↔ S1,...,S13 ↔ S14 transitions) from the opening, closing, and inactivation of the pore domain (C0 ↔ C1 ↔ O ↔ I transitions). Implicit in this model is that the transitions

Figure 20. Schematic showing state-dependent positions of Cd$^{2+}$ and key charged residues in S2–S4 segments of the VSD of a single hERG1 channel subunit. According to this model, Cd$^{2+}$ is displaced in an outward direction by basic residues in the S4 segment as it moves inward in response to membrane repolarization. (Left) VSD at depolarized potentials and Cd$^{2+}$ in its “depolarized” coordination site. (Right) VSD at a negative transmembrane potential when all channels are closed and Cd$^{2+}$ located in a position defined as the “repolarized” coordination site. (Middle) A putative intermediate state of the channel. K1, K525; R2, R528; R3, R531; R4, R534; D1, D456; D2, D460; D3, D509. The yellow circle represents a Cd$^{2+}$ ion.
between states S₀ to S₁₄ describe the vast majority of charge displacement. Some previous Markov models of hERG1 included a significant amount of total charge displacement associated with the gating between the final closed state and the open and inactivated state (Lu et al., 2001; Mazhari et al., 2001; Piper et al., 2003; Perry et al., 2007). In addition, as summarized in Table I, our model assumes no cooperativity for the VSD displacement, similar to the original Shaker model (Zagotta et al., 1994), but unlike a later Shaker model (Schoppa and Sigworth, 1998) or our previous hERG model (Piper et al., 2003) that used a cooperativity factor of 1.3–1.5, respectively. Finally, the total gating charge estimated for the model was 5.1 e₀. This is 2.7-fold less than that estimated from experimental data for Shaker K⁺ channels (~13.6 e₀) (Schoppa et al., 1992; Aggarwal and MacKinnon, 1996). The total gating charge per hERG1 channel (ztotal) has not been determined. However, the e₀ value obtained from the fitting of QV relationships for hERG1 is ~2 (Fig. 2 D) versus ~7 for Shaker channels (Olcese et al., 1997), a 3.5-fold difference.

The Cd²⁺ model assumes that two out of four Cd²⁺-binding sites in the VSD hERG1 channels are occupied, and that rearrangement of the VSD facilitates displacement of Cd²⁺ from a site to another position in close proximity. During activation, slow VSD transitions precede the inward Cd²⁺ movement, which is oppositely directed to the electrical field. The structural basis of the Cd²⁺ binding site was not investigated in this study, but based on our previous work (Fernandez et al., 2005), extracellular Cd²⁺ binds to a coordination site formed by three specific acidic residues in S2/S3; inward or outward displacement of Cd²⁺ from this site in response to channel activation or deactivation, respectively, could generate I Cd. The charge displacement associated with I Cd was estimated in the model to be 0.2 e₀ for each Cd²⁺ displaced. Assuming that on average half the Cd²⁺-binding sites were occupied per channel, the charge associated with I Cdout would be equivalent to ~8% of the magnitude of the total charge modeled for the S₀<->S₁₄ transitions (5.1 e₀) for a single channel. The maximum value of the ratio Q Cdout/Q OFFtotal expressed as a percent was ~6% (Fig. 5 E). This measured value is necessarily an underestimate because Q Cdout was defined as the integral of I Cdout and thus was decreased by the overlap of the initial rapid component of inward I OFF. Assuming that half of the Cd²⁺ coordination sites (two of four sites occupied for a total Cd²⁺ charge of +4) are occupied at 100–300 µM Cd²⁺ (EC₅₀ = 171 µM), it can be estimated that the I Cdout measured after pulsing to a test potential of +60 mV could be produced by two Cd²⁺ ions traversing ~8% ([5.1e₀ × 0.06]/4 = 0.077) of the transmembrane electric field.

The standard and Cd²⁺ Markov hERG1 gating models have several limitations. First, the models do not describe ionic currents through hERG1 channels. Parameterization of the activation/inactivation component (Fig. 13 B) based on previous models of hERG1 channel currents was not possible due to their large gating currents associated with C<->O and O<->I. The finding indicates that redesign of this model component will be necessary. Such a redesign might also help with solving discrepancies between experimental and modeling data for the time constant of the fast component of I OFF at voltages >0 mV (Figs. 14 D and 18 D). Second, the presented simulations do not account for system functions of the measurement devices and signal processing. In particular, applying a low-pass filter at 10 kHz as in the measurements to the modeled I g would reduce the differences in the responses of I g to voltage steps. Third, the Cd²⁺ model was designed for reproducing measured data for [Cd²⁺]s of 100 µM that we assumed to cause two bound Cd²⁺ per hERG1 channel. The model does not reconstruct [Cd²⁺]-dependent effects. Although not a focus of the presented study, the model could be extended for this purpose, e.g., by integration with models for one, three, and four bound Cd²⁺ per hERG1 channel. Fourth, the fitting process did not yield unique solutions for parameters of the Cd²⁺ model, in particular, the Cd²⁺ movement parameters (μ₀, ζₙ, η₀, ξ₀, and z Cd).

### Table I

| Model | Transitions | α | β | z CD | c |
|-------|-------------|---|---|-----|---|
| Zagotta et al. 1994 (Shaker channel) | R₁<->R₂ | 1.120 | 0.25 | 373 | 1.6 | 1 |
| Schoppa and Sigworth 1994 (Shaker channel) | S₁<->S₂ | 4.900 | 0.08 | 960 | 0.52 | 1 |
| | S₂<->S₃ | 1.650 | 0.47 | 450 | 0.52 | 1 |
| Piper et al. 2003 (hERG1 channel) | S₁<->S₂ | 1.000 | 0.2 | 1,500 | 0.04 | 1 |
| Standard model (hERG1 channel) | fast (e.g., S₁<->S₄) | 296 | 0.43 | 2,683 | 0.076 | 1 |
| | slow (e.g., S₁<->S₄) | 73 | 0.21 | 1.9 | 0.56 | 1 |
| Cd²⁺ model (hERG1 channel) | fast (e.g., S₁<->S₄) | 563 | 0.27 | 2,329 | 0.23 | 1 |
| | slow (e.g., S₁<->S₄) | 20.9 | 0.30 | 5.7 | 0.68 | 1 |
Other parameter sets yielded similar fit errors, perhaps because the transitions between the different Ca\textsuperscript{2+}-bound levels are not exponential processes.

**Summary**

Cd\textsuperscript{2+} shifted the voltage dependence of the Q-V relationship for hERG1 channels in accordance with the positive shift of the G-V relationship for ionic currents. Cd\textsuperscript{2+} also induced the appearance of a transient outward current, \( I_{\text{Cd out}} \), upon repolarization from test potentials above 0 mV. Collectively, our present and previous (Fernandez et al., 2005) findings suggest that:

(a) at depolarized potentials, extracellular Cd\textsuperscript{2+} binds to a “depolarized” coordination site formed by acidic residues located in the S2/S3 segments of each subunit;

(b) with membrane repolarization, Cd\textsuperscript{2+} is displaced to accommodate sequential electrostatic interactions between Arg residues in S4 and acidic residues in S2 and S3 that are required for inward movement of S4;

(c) outward displacement of Cd\textsuperscript{2+} to its “repolarized” coordination site produces \( I_{\text{Cd out}} \); and

(d) inward movement of S4 does not require similar displacement of Ca\textsuperscript{2+}, Co\textsuperscript{2+}, Zn\textsuperscript{2+}, or La\textsuperscript{3+} (or Cd\textsuperscript{2+} when bound to D456C or D509C hERG1 channels), or if they are displaced, these cations move in a lateral or inward direction relative to the cell membrane and thus do not produce an outward current.

New Markov models of hERG1 channel gating were formulated. Gating currents are described as noncooperative transitions between multiple closed states of the channel with no charge displacement associated with transitions to or from the open or inactivated states. The finding that RPR260243 markedly slows deactivation of ionic currents without having any effect of gating currents provided experimental support for this model.

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