A Novel Extracellular Calcium Sensing Mechanism in Voltage-Gated Potassium Ion Channels

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Potassium (K^+) channels influence neurotransmitter release, burst firing rate activity, pacing, and critical dampening of neuronal circuits. Internal and external factors that further modify K^+ channel function permit fine-tuning of neuronal circuits. Human *ether-à-go-go*-related gene (HERG) K^+ channels are unusually sensitive to external calcium concentration ($[Ca^{2+}]_o$). Small changes in $[Ca^{2+}]_o$ shift the voltage dependence of channel activation to more positive membrane potentials, an effect that cannot be explained by nonspecific surface charge screening or channel pore block. The HERG–calcium concentration–response relationship spans the physiological range for $[Ca^{2+}]_o$. The modulatory actions of calcium are attributable to differences in the Ca^{2+} affinity between rested and activated channels. Adjacent extracellular, negatively charged amino ac-

ids (E518 and E519) near the S4 voltage sensor influence both channel gating and Ca^{2+} dependence. Neutralization of these charges had distinct effects on channel gating and calcium sensitivity. A change in the degree of energetic coupling between these amino acids on transition from closed to activated channel states reveals movement in this region during channel gating and defines a molecular mechanism for protein state-dependent ligand interactions. The results suggest a novel extracellular $[Ca^{2+}]_o$ sensing mechanism coupled to allosteric changes in channel gating and a mechanism for fine-tuning cell repolarization.

Key words: human *ether-à-go-go*-related gene; potassium channel gating; HERG; calcium; allosteric; Monod–Wyman–Changeux

Plasticity and adaptive behavior in circuits of the nervous system depend on the dynamic properties of ion channels. Potassium channels modulate neuronal excitability and, in turn, are modulated by both intracellular and extracellular factors. The human *ether-à-go-go*-related gene (HERG) potassium channel has recently been discovered to play important roles in excitable tissues. Originally identified in hippocampus (Warmke and Ganetzky, 1994), HERG channels also play a role in myocardial repolarization (Sanguinetti et al., 1995). ERG channels have been implicated in neural crest cell development (Arcangeli et al., 1997), neoplastic cell survival (Bianchi et al., 1998), and neural spike frequency adaptation (Chiesa et al., 1997). Furthermore, the *Drosophila erg* is encoded at the seizure locus, originally defined by temperature-sensitive paralytic mutations in flies (Titus et al., 1997; X. J. Wang et al., 1997).

HERG channels (Warmke and Ganetzky, 1994; Trudeau et al., 1995) are six transmembrane (6 TM) family voltage-gated potassium channels and contain the typical motifs identified in these channels (S5-P-loop-S6, charged S4 voltage sensor, etc.). Unlike many other 6 TM K^+ channels (*Drosophila Shaker*, *Shal*, *Shab*, *Shaw*, vertebrate Kv channels), HERG channels are highly sensitive to modest changes in extracellular divalent cation concentrations (Ho et al., 1998; Johnson et al., 1999; Po et al., 1999) around the physiological set point (1–3 mM). This sensitivity is much greater and qualitatively distinct from that in other voltage-

gated delayed rectifier potassium channels (Frankenhaeuser and Hodgkin, 1957; Gilly and Armstrong, 1982; Johnson et al., 1999; Po et al., 1999) and cannot be explained by nonspecific surface charge screening or pore block. Changes in extracellular Ca^{2+} appear to discretely modify the voltage dependence of activation of HERG with little effect on channel inactivation (Johnson et al., 1999), suggesting that the voltage dependence of HERG K^+ channel inactivation is distinct from the voltage dependence of activation gating as noted before (Spector et al., 1996; S. Wang et al., 1996, 1997; J. Wang et al., 1998; Zou et al., 1998). This specific interaction with one aspect of channel gating is intriguing and suggests interactions of Ca^{2+} with amino acids in regions of the channel involved in the voltage dependence of channel opening.

Interestingly, there are two nonconserved negatively charged amino acids (glutamic acids) located in the HERG extracellular loop between S3 and S4. These extracellular facing negative charges are immediately adjacent to the S4 voltage sensor and are good candidates for an interaction with Ca^{2+} or with some of the positive charges in the S4, leading to Ca^{2+} titratable changes in gating. The purpose of this investigation was to develop a better understanding of the molecular basis of modifications of HERG function by Ca^{2+} through analysis of the gating and $[Ca^{2+}]_o$ interactions in wild-type (WT) and HERG mutant channels in which these negative charges were neutralized.

MATERIALS AND METHODS

cDNA constructs and site-directed mutagenesis. The human *ether-à-go-go*-related gene (HERG) cDNA was obtained from Dr. Mark Keating (University of Utah) and ligated into the pSI mammalian expression plasmid (Promega, Madison, WI). Dr. Richard Horn (Jefferson Medical College) kindly provided the CD8 antigen gene in the EBO-pcD Leu2 vector. CD8, a human T lymphocyte surface antigen, was cotransfected with the channel construct to allow visual identification of transfected cells (Jurman et al., 1994). Mutagenesis was performed by the overlap-extension recombinant PCR technique using VENT® DNA polymerase

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(New England Biolabs, Beverly, MA). All constructs were assembled in the HERG pSI construct. Briefly, two separate PCR reactions were performed to generate overlapping products containing a desired point mutation in the overlap region. The first round PCR products were purified with QIAquick purification columns (Qiagen, Hilden, Germany) to remove primers, and the two products were denatured and annealed to one another. Finally, the complete region was amplified in a second round of PCR with a nested set of primers. The PCR cassette was digested then and subcloned into the full-length cDNA using flanking restriction sites. Multiple clones were selected and analyzed by restriction digestion, and DNA sequencing of the entire region was amplified by PCR. For each mutant, two independent correctly assembled and fully sequenced clones were tested electrophysiologically.

Electrophysiology and solutions. HERG channel function was studied with the whole-cell patch-clamp technique by methods identical to those in Johnson et al. (1999). Cells were patch clamped 36–60 hr after transfection, and all experiments were performed at room temperature (23–25°C). The intracellular recording solution for all experiments contained (in mM): 110 KCl, 5 K₂ATP, 5 K₄BAPTA, 2 MgCl₂, 10 HEPES, pH 7.2. The extracellular recording solution used for the experiments in Figure 1A contained (in mM): 145 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, pH 7.35. The base extracellular solution for all other figures contained (in mM): 145 NaCl, 4 KCl, 10 HEPES, 10 glucose. For solutions of Ca²⁺ concentrations from 100 μM to 10 mM, the appropriate amount of 1 M CaCl₂ was added to a 2× concentrated form of the above base solution before addition of NaOH to bring the pH to 7.35 and the final dilution to 1× with deionized water. For Ca²⁺ concentrations from 3 to 30 μM, 1 mM H-ethylenediamine-*N,N',N'*-triacetic acid was added to the base solution to buffer the Ca²⁺ concentration. The free Ca²⁺ concentrations were calculated assuming 25 μM contaminant Ca²⁺ using MaxChelator Sliders version 1.0 software (Chris Patton, Stanford University, www.stanford.edu/~cpatton/maxc.html) using the Martell and Smith constants.

In figures with raw data, the zero current level is indicated by the bottom of the current calibration bar (vertical) in each figure. Leak correction was not used in any of the raw current traces shown. In a few cases, moderate linear leak correction was used offline using Clampfit. Cell capacitance and series resistance were compensated using the analog controls on the Axopatch 200.

Chinese hamster ovary K1 (CHO-K1) cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in HAMS F-12 media (Life Technologies, Grand Island, NY) supplemented with 1 mM L-glutamine and 10% heat-inactivated fetal bovine serum (Life Technologies) in a humidified, 5% CO₂ incubator at 37°C. CHO-K1 cells were cotransfected with the HERG and CD8 plasmids in a ratio of 4:1. Transfection was accomplished using the Lipofectamine transfection reagents and method (Life Technologies).

Data analysis. Data were analyzed and plotted using pClamp 6.03, Origin 5.0 (Microcal Software, Northampton, MA), SigmaPlot 4.0 (SPSS Inc., Chicago, IL), and TableCurve 3D 3.0 (SPSS Inc.) software. The midpoints ($V_{1/2}$) of channel activation were determined by fitting the data with a Boltzmann function of the form:

$$I/I_{\text{Max}} = 1/(1 + \exp([V - V_{1/2}]/k_v)), \quad (1)$$

where I_{Max} is the limiting amplitude, $V_{1/2}$ is the membrane potential where $I/I_{\text{Max}} = 0.5$, and k_v is the slope factor. Concentration-effect data were fit with the Hill equation:

$$I/I_{\text{Max}} = y_0 + (A[Ca^{2+}]^b)/((K_D)^b + [Ca^{2+}]^b), \quad (2)$$

where I is current, I_{Max} is the maximal current, y_0 is the current at a given voltage in the absence of Ca²⁺, A is an amplitude term, K_D is the apparent Ca²⁺ dissociation constant, and b is the Hill coefficient.

A voltage-dependent Monod-Wyman-Changeux (MWC) model of allosteric protein function (Monod et al., 1965; Galzi et al., 1996; Cox et al., 1997; Changeux and Edelstein, 1998) was used to analyze the data. The model is described by Equation 3 below:

$$G/G_{\text{Max}} = \frac{1}{1 + \frac{1 + \frac{[Ca^{2+}]^4}{K_c}}{1 + \frac{[Ca^{2+}]^4}{K_a}} L_0 e^{-(QFV/RT)}}. \quad (3)$$

F is the Faraday constant in coulombs per mole; T is temperature in degrees Kelvin; R is the gas constant in Joules per degree (Kelvin) per mole. V is membrane potential (volts). There are four free parameters in the equation: Q , K_c , K_a , and L_0 . The apparent gating charge, Q , that moves through the membrane electrical field during the transitions from closed to activated determines the voltage dependence, i.e., the steepness of the sigmoid curves in Figures 4 and 6. K_c is the Ca²⁺ dissociation constant for the closed channel. K_a is the Ca²⁺ dissociation constant for the activated (open) channel. The degree of separation between the voltage-dependent activation curves in low and high Ca²⁺ is determined by the relative affinities of the closed and activated states (K_c and K_a). A large difference between K_c and K_a results in a larger separation between the voltage-dependent activation curves. L_0 is the closed–open equilibrium constant in the absence of calcium, and it determines the lower limit of the midpoints of the voltage-dependent activation curves. L_0 determines the relative position of the family of voltage-dependent activation curves on the voltage axis (see Fig. 4) or the baseline of the $V_{1/2}$ versus Ca²⁺ concentration relationship (see Fig. 5). Global fitting (Balsler et al., 1990) of the allosteric MWC model to HERG currents simultaneously across multiple voltages and Ca²⁺ concentrations was performed with TableCurve 3D software using an iterative 64-bit Levenburg–Marquardt fitting algorithm. It should be noted that although the model is helpful for quantifying and interpreting the effect of the mutations and Ca²⁺, the primary conclusions are not dependent on the model.

Thermodynamic cycle analysis. Channel alterations that energetically affect ligand binding or gating can be analyzed formally by evaluating the contributions of individual perturbations (Carter et al., 1984; Sali et al., 1991; Hidalgo and MacKinnon, 1995). For example, alterations in binding caused by a mutation can be defined in terms of perturbations in binding energies:

$$\Delta\Delta G = -RT \ln[K_{\text{mutant}}/K_{\text{WT}}], \quad (4)$$

where K_{mutant} and K_{WT} are the equilibrium binding constants for mutant and wild-type channels, respectively. If different amino acids contribute to the binding but they contribute independently, then altering them should independently affect the outcome. The diagram below illustrates the method used for these calculations:

$$\begin{array}{ccc} \text{E518:E519} & \text{X1} & \text{518A:E519} \\ \text{Y2} \Big| & & \Big| \text{Y1} \\ \text{E518:519A} & \text{X2} & \text{518A:519A} \end{array}$$

The **bold lettering** indicates a wild-type residue. The upper left construct is the fully WT channel, and the bottom right is the EEAA double mutant channel. $X1$ is the dissociation constant for the WT **E518:E519** channel divided by that of the single mutant 518A:**E519** and represents the change in free energy between the WT and E518A single mutant. $Y1$ is the dissociation constant for the single mutant 518A:**E519** divided by that of the double mutant 518A:519A and reflects the change in free energy between the E518A single mutant and the EEAA double mutant channel. These calculations were then extended to generate $X2$ and $Y2$. The null hypothesis is that, if there is no energetic coupling between the two residues, the change in free energy between the WT and EEAA channel will be independent of which residue was mutated first, generating an energetic coupling coefficient (Ω) of unity. Ω is described by the equation (Carter et al., 1984):

$$\Omega = X1 * Y1 = X2 * Y2 = \frac{K_D(\text{wt:wt}) * K_D(\text{mut:mut})}{K_D(\text{wt:mut}) * K_D(\text{mut:wt})}. \quad (5)$$

In all cases, Ω values calculated by $X1/X2$ were identical to those calculated by $Y2/Y1$.

RESULTS

Direct observation of HERG inactivation: effect of extracellular Ca²⁺ on channel gating

The HERG K⁺ channel was originally described as an inwardly rectifying channel based on the current–voltage relationship (Trudeau et al., 1995). Subsequent studies indicated that this apparent rectification results from inactivation gating (Schönherr and Heinemann, 1996; Smith et al., 1996; Spector et al., 1996). In this regard, HERG K⁺ channels seemed to be atypical, com-

Figure 1. Direct observation of HERG channel inactivation and the effects of Ca^{2+} to slow the rate of channel activation. *A*, Activation and inactivation of HERG K⁺ currents measured during voltage-clamp steps to membrane potentials between +180 and +30 mV in 10 mV increments for 50 msec followed by a step to −50 mV for 100 msec. These recordings show rapid activation of HERG K⁺ currents followed by subsequent inactivation resulting in transient outward currents. On stepping to −50 mV, a large slow tail current is observed. The rate of activation (initial rising phase) is highly voltage dependent in this potential range, whereas the inactivation (decay) is not. The membrane potential was held at −80 mV before and after the test steps. *B*, Increases in the extracellular Ca^{2+} concentration caused a slowing of channel activation with no effect on inactivation, resulting in a truncation of the peak transient outward currents. The rate of tail current decay at −50 mV was also increased. Seven K⁺ current traces from the same cell, each in a different extracellular $[\text{Ca}^{2+}]$ (3 μM , 10 μM , 30 μM , 300 μM , 1 mM, 3 mM, and 10 mM), are superimposed. The membrane potential was held at −80 mV before stepping to +70 mV for 2 sec, then repolarized to −50 mV to measure tail currents. Note the two time scale bars corresponding to before and after the break in the current record. After ~250 msec at +70 mV, the steady state current level was the same in all $[\text{Ca}^{2+}]$; even the current in 10 mM Ca^{2+} had fully activated.

pared with other six transmembrane family K⁺ channels that are either delayed rectifiers or A-type channels with rapid channel activation followed by somewhat slower inactivation. Figure 1*A* illustrates HERG K⁺ currents measured during brief (50 msec) steps between +30 and +180 mV. At these membrane potentials,

the rate of channel activation became rapid relative to inactivation, and the inactivation process was directly observed. Although the rate of activation remained highly voltage-dependent, the rate of inactivation was nearly constant in this voltage range.

Changes in extracellular Ca^{2+} modify the voltage dependence of opening of this channel with little effect on channel inactivation (Johnson et al., 1999). Figure 1*B* demonstrates this directly. Superimposed K⁺ current tracings from a single cell are shown in different extracellular Ca^{2+} concentrations ranging from 3 μM to 10 mM. In the lowest concentrations of Ca^{2+} , the voltage step to +70 mV elicited a rapidly activating current that quickly inactivated like an A-type K⁺ channel. As extracellular Ca^{2+} was increased, channel opening was delayed, but the rate of inactivation remained unchanged. The result was a truncation of the observed current peak. Note that marked modulation occurred between 0.3 and 3 mM. The specific interaction of Ca^{2+} with the voltage dependence of channel activation, but not inactivation, led us to investigate the role of amino acids in the vicinity of the channel voltage sensor in determining channel Ca^{2+} sensitivity.

Extracellular negatively charged amino acids and the effect of $[\text{Ca}^{2+}]_o$

Ca^{2+} typically interacts with negatively charged amino acid side chains of proteins (Cowan, 1993; Eismann et al., 1994; Schreiber and Salkoff, 1997). This is attributable to the fact that ionized Ca^{2+} has a full outer electron orbital, giving it a noble gas-like unreactive chemistry. As a result, Ca^{2+} tends to interact with other molecules largely through electrostatic attraction and repulsion (Cowan, 1993). Likely candidates for Ca^{2+} interaction are extracellularly exposed negatively charged amino acids such as glutamate and aspartate. Because Ca^{2+} does not permeate HERG channels and the intracellular solution always contained a high affinity Ca^{2+} chelator (5 mM BAPTA), the hydrophilic extracellular loops of the channel are candidate regions for Ca^{2+} interaction. The S4 transmembrane segment has been identified as a voltage sensor of channels in the six transmembrane voltage-gated K⁺ channel family (Papazian et al., 1987; Tempel et al., 1987; Perozo et al., 1994; Mannuzzu et al., 1996). The extracellular S3–S4 linker of HERG contains only two acidic amino acids, adjacent glutamates at positions 518 and 519, and they are located just above the S4 transmembrane segment. These negatively charged amino acids were mutated individually and together to probe their role in the response to extracellular Ca^{2+} . The phenotypes of the WT and mutant channels are shown in Figure 2. K⁺ current from cells expressing WT, E518A, E519A, or the double mutant (change of E518 and E519 to alanines: EEAA) are shown at numerous test potentials. Each mutation significantly altered the gating of HERG channels in control extracellular solutions. Interestingly, the E518A mutant appeared very similar to the WT channel in elevated $[\text{Ca}^{2+}]_o$, e.g., removal of the negative charge caused a decrease in outward current during depolarizations and enhanced the rates of tail current decay. Thus, removal of this negative charge seemed to resemble addition of positive charges (Ca^{2+}) in the WT channel. In all three cases (E518A, E519A, and EEAA), outward currents during depolarizations were decreased, and the rates of decay of tail currents were increased. Further evaluation of these mutant channels compared with WT revealed unique differences (described below). First, we evaluated other channel properties to determine whether inactivation or permeation properties were altered.

Figure 2. Comparison of HERG K⁺ currents in WT and S3–S4 charge neutralization mutants. Superimposed families of K⁺ current traces were recorded using the voltage-clamp protocol shown for each channel construct. Cells were held at -80 mV before stepping to test potentials between $+70$ and -60 mV for 2 sec. The membrane potential was then stepped to -50 mV for 2 sec to record tail currents. All records were made in the presence of 3 mM extracellular Ca²⁺.

Effect of negatively charged amino acids, mutations, or Ca²⁺ on inactivation

Our previous study demonstrated a significant effect of Ca²⁺ on HERG channel activation gating in the absence of an effect on inactivation (Johnson et al., 1999). Therefore, we wished to determine whether these amino acids that appeared to influence channel activation gating had an effect on channel inactivation. Despite the distinct current phenotypes of the mutant channels, their inactivation was not affected. Neutralization of E518, E519, or both together did not change the HERG channel inactivation time constants measured between 0 and $+100$ mV ($p > 0.2$; $n = 4$ –8) (Fig. 3). Thus, HERG inactivation gating is insensitive both to external Ca²⁺ and mutation of E518 and E519, in direct contrast to activation gating.

Figure 3. S3–S4 charge neutralization did not affect inactivation. The voltage-clamp protocol is shown at the top. A representative family of current traces is shown in the middle panel. The step to $+60$ mV was 2 sec long; the scale bar to the right applies only to the current trace to the right of the break in the record. Time constants were obtained by fitting an exponential function to the decaying current during the third voltage step. Error bars indicate the SEM for 4–8 cells for each mutant and at each membrane potential.

Mutations did not change K⁺ selectivity

In contrast to changes in activation gating, we observed no effects on the ion permeation properties, suggesting that other channel properties were not affected by these changes. The ionic selectivity of all the mutant channels was normal, because there was no change in the K⁺ current reversal potential measured in 4 mM extracellular K⁺: WT, -83 ± 0.9 mV, $n = 10$; E518A, -81 ± 0.3 mV, $n = 9$; E519A, -82 ± 0.3 mV, $n = 10$; EEAA, -82 ± 0.6 mV, $n = 10$ (NS; $p > 0.2$).

Effect of negatively charged amino acids or Ca²⁺ on channel activation

In Figure 4, voltage–activation curves are shown to compare the shifts in the voltage dependence caused by changes in [Ca²⁺]_o in the WT and mutant HERG channels. Increasing Ca²⁺ caused progressive shifts of channel voltage-dependent activation relationships to more depolarized potentials in all channels, but the magnitude of the Ca²⁺ response and the relative position of the voltage dependence curves differed among the WT and charge-neutralized mutant channels. Note the differences in degree of separation of the curves in high and low Ca²⁺ and the relative

Figure 4. Effect of extracellular Ca^{2+} on the voltage dependence of activation of WT and mutant HERG channels. Voltage-dependent activation curves were determined from peak tail current amplitudes measured at -50 mV with a 2 sec step to the test potential (as in Fig. 2). Error bars indicate SEM ($n = 3$ –15 for each concentration). Smooth curves correspond to the predicted values based on the three-dimensional fit of the voltage-dependent Monod–Wyman–Changeux model (see Fig. 6, Eq. 3).

shifts caused by change in Ca^{2+} among the different channels. There did not appear to be changes in the slope of the relationships, but the entire family of curves in a given channel (WT vs each mutant) was variously translated along the voltage axis. The degree to which elevation of Ca^{2+} was able to shift the curves was different among the channels as well. Initially it seemed paradoxical that channels with such similar and modest changes in amino acid sequence could behave so differently. A channel with the simple removal of a negative charge behaved very differently than when the adjacent charge was neutralized. These distinctions were very nicely accounted for by considering the allosteric nature of the channels. The solid curves through the data were generated by the allosteric gating model described below.

The Ca^{2+} -independent voltage dependence of the channels (i.e., that measured in very low calcium: $3 \mu\text{M}$ Ca^{2+}) differed among the channels as well. This can be seen more clearly in Figure 5 in which the half maximal voltages of channel activation

Figure 5. Ca^{2+} dependence of the half maximal voltage ($V_{1/2}$) of activation of WT and mutant HERG channels obtained from the curves in Figure 4. Error bars indicate SEM. Smooth curves correspond to the predicted values based on the three-dimensional fit of the voltage-dependent Monod–Wyman–Changeux model (see Fig. 6).

($V_{1/2}$) measured from data in Figure 4 are plotted as a function of the Ca^{2+} concentration. The double mutant (EEAA) and the E518A single mutant had a minimal $V_{1/2}$ of approximately $+30$ mV more depolarized than that of the WT or E519A mutant channels ($V_{1/2}$ in $3 \mu\text{M}$ $[\text{Ca}^{2+}]_o$: WT, -44 ± 6 mV; E518A, -17 ± 3 mV; E519A, -48 ± 5 mV; EEAA, -17 ± 8 mV). For all channels, the $V_{1/2}$ – $[\text{Ca}^{2+}]$ curve saturated before reaching the lowest tested $[\text{Ca}^{2+}]$ ($3 \mu\text{M}$). Therefore, the minimal $V_{1/2}$ can be considered Ca^{2+} independent. Neutralization of glutamate 518, but not 519, resulted in channels with an altered Ca^{2+} -independent voltage dependence. Channels in which E519 was neutralized had an altered Ca^{2+} response, but their Ca^{2+} -independent voltage dependence was like that of WT channels.

The Ca^{2+} responses of the WT and mutant channels for currents measured at membrane potentials between -60 and $+30$ mV were fit with a Hill equation (Eq. 2; same data as in Fig. 4 analyzed as a function of $[\text{Ca}^{2+}]_o$; data not shown). The fitted parameters (K_D and Hill coefficient, b) were highly voltage dependent. The Hill coefficients from these fits varied from a minimum near one to a maximum greater than three depending on the membrane potential, suggesting multiple binding sites and cooperativity. Thus, a single Ca^{2+} binding site seems unlikely for several reasons. First, the Hill coefficient ($b > 1$, Eq. 2) values indicate increased complexity and suggest multiple Ca^{2+} binding sites. Secondly, HERG, like other six transmembrane domain voltage-gated K⁺ channels, is believed to form rotationally symmetric homotetrameric channels when expressed alone in heterologous systems. If all four subunits cooperated to create a single Ca^{2+} binding site on the extracellular face of the channel, this site would necessarily be centrally located and would therefore likely be in the channel pore. However, we know that Ca^{2+} does not block HERG channels as would be expected for a binding site in the pore lumen (Johnson et al., 1999). In the absence of a single centrally located Ca^{2+} binding site, a likely possibility for a homotetrameric channel is that each subunit has its own Ca^{2+}

site(s). The similarities to hanatoxin modification of Kv2.1 and omega-Aga-IVA modification of calcium channels are also consistent with four Ca²⁺ sites and certainly more than one (Swartz and MacKinnon, 1995, 1997a,b; Li-Smerin and Swartz, 1998; Winterfield and Swartz, 2000).

The Hill fit-derived parameters must be interpreted cautiously, and the apparent voltage dependence of the Hill-derived K_D s can be interpreted in several ways. First, this nonglobal analysis (i.e., fitting only a single independent variable at a time) neglects relevant information about the system, for example membrane potential. Although the Ca²⁺ response is obtained at several membrane potentials, each fit is independent and not constrained by data at other membrane potentials. The Hill fit analysis also lumps all interactions (possibly multiple K_D s) into a single K_D . In contrast, the global fit (discussed below) is constrained by information obtained across all membrane potentials and calcium concentrations. The voltage-dependent apparent K_D values from the Hill fit analysis suggest several possible interpretations. First, the accessibility of the ligand to a single binding site of constant affinity could change as a function of membrane potential, because of the voltage-dependent channel conformational changes that must occur for channel function, i.e., the binding site is partially guarded. This would cause an apparent change in the affinity, but cannot account for the Hill coefficients that differ from unity. Another possible explanation for the voltage-dependent K_D is that Ca²⁺ binding is influenced by the electric field through a direct effect on the positively charged ligand. In the Hill equation analysis, the fitted K_D s ranged from $\leq 10^{-4}$ M at -50 mV to 5×10^{-3} M at $+25$ mV. These changes are consistent with a positively charged ligand binding with higher affinity at negative membrane potentials. This cannot readily explain the voltage-dependent changes in Hill slopes either.

In addition and not mutually exclusive, these observations could indicate that the affinity of the binding site for Ca²⁺ changes as a function of membrane potential because of a change in the protein conformation. For example, different channel conformations (closed or activated) may have distinct Ca²⁺ affinities. The data indicate that the resting closed channels bind Ca²⁺ with a higher affinity than the activated channel. Membrane potential determines the fraction of channels that are in the resting or activated states and the apparent K_D increases at more depolarized potentials in which more channels are activated.

The voltage dependence of the Hill fits led us to consider an allosteric model for the Ca²⁺-channel interactions. Many multisubunit proteins including ion channels exhibit cooperativity, and conformational changes that confer differential ligand affinity have been described for many allosteric proteins (Monod et al., 1965; Hille, 1977; Hondeghem and Katzung, 1977; Marks and Jones, 1992; Galzi et al., 1996; Cox et al., 1997; Changeux and Edelstein, 1998; Horrigan and Aldrich, 1999; Rothberg and Magleby, 1999).

Allosteric modulation of gating by Ca²⁺

To refine the analysis of the Ca²⁺-HERG interaction, we considered voltage-dependent allosteric models of channel-gating and Ca²⁺ interactions. Because HERG is a voltage-gated channel (Sanguinetti et al., 1995; Trudeau et al., 1995), numerous states must be considered to account for channel voltage dependence (Patlak, 1991; Bezanilla et al., 1994; S. Wang et al., 1997). These channels are homotetramers when expressed heterologously. The four-fold symmetry of the channel naturally leads to

at least five states to describe channels with 0–4 voltage sensors activated. Channel inactivation requires additional states. If only a single Ca²⁺ binds to each homotetrameric channel, then all four subunits must create a single Ca²⁺ binding site on the extracellular face of the channel. This site would necessarily be centrally located, as with tetraethylammonium (TEA) and certain toxins (Hurst et al., 1992), and would therefore likely be in the channel pore. However, Ca²⁺ does not block HERG channels (Johnson et al., 1999). The radial symmetry of the channel, the apparent cooperativity seen in the Ca²⁺ concentration–response curve Hill fits (data not shown), and the unlikelihood of a single central Ca²⁺ binding site suggest that each subunit has its own Ca²⁺ site(s). Minimally, to accommodate five voltage-dependent states and five calcium-liganded states requires a 25 state model. To include Ca²⁺ and voltage dependence and the allosteric changes from closed to activated channel conformations requires at least a two-tiered 50 state allosteric model (Rothberg and Magleby, 1999). Nonindependent voltage sensors or Ca²⁺ binding would lead to even larger models (Horrigan et al., 1999).

As the complexity of these models increases, the number of free parameters increases, and confidence in the meaning of individual parameters decreases. Results shown below and in our previous work (Johnson et al., 1999) indicate that the inactivated states cannot be distinguished from open states with regard to Ca²⁺; hence, combining open and inactivated states that are occupied during depolarizations can be justified. Because the primary goal of this work was to understand the Ca²⁺ modulation of HERG, we can further simplify the analysis by combining the voltage sensing steps into a single concerted transition. There is reasonable evidence that in other voltage-gated K⁺ channels (Bezanilla, 2000), Ca²⁺-activated K⁺ channels (Cox et al., 1997; Horrigan et al., 1999; Rothberg and Magleby, 1999), and Ca²⁺ channels (Marks and Jones, 1992) a final cooperative step is indeed responsible for channels leaving the closed state.

Assuming four binding sites for the tetrameric channel, there are at least five types of states corresponding to the channel with 0–4 Ca²⁺ bound. The open to inactivated transitions of HERG are Ca²⁺ insensitive (Johnson et al., 1999), which permitted us to combine the open and inactivated states (referred to as activated). Making the assumption that only the voltage-dependent conformation of the channel (closed or activated) determines the affinity for Ca²⁺ and that each subunit binds Ca²⁺ independently from the others, the degree of Ca²⁺ binding then biases the fundamental voltage-dependent transition. The result is the simplified two-tiered, 10 state MWC model [see Cox et al. (1997) for in-depth discussion of this approach shown below in scheme 1 and described in Eq. 3] if Ca²⁺ only distinguishes closed and activated states (i.e., all closed state dissociation constants, K_c , are equivalent, as are all activated state dissociation constants, K_a). In this model, the receptor (here the HERG channel) protein exists in two possible conformations, tense or relaxed, corresponding in this case to the closed or activated channel (Monod et al., 1965; Changeux and Edelstein, 1998).

This voltage-dependent allosteric model (see Eq. 3) was fit to all of the data simultaneously as a function of the known membrane potentials and Ca²⁺ concentrations. Figure 6 shows the voltage- and Ca²⁺-response surface that was fitted to these data sets. The shaded gray bands indicate the height along the vertical axis. Round symbols show the mean data points, and the vertical lines on the symbols show the distance of the data points from the fitted plane, illustrating the quality of the fit.

This simple model fit the observed data remarkably well. The

Figure 6. Ca²⁺-membrane voltage-response surface. Three-dimensional regression fits of the voltage-dependent Monod-Wyman-Changeaux model to relative K⁺ currents for WT and mutant HERG measured in different Ca²⁺ concentrations and at different membrane potentials. The mean voltage-activation data for all Ca²⁺ concentrations are plotted as a function of membrane potential in a three-dimensional format. The vertical error bars on the symbols indicate the distance of the mean data point from the fitted plane.

smooth curves in Figures 4 and 5, as well as the shaded gray surface in Figure 6, are all from the model fit. The fitted parameters are shown in Figure 7. The sensitivity of the channels to changes in membrane potential was similar in all channels and at all Ca²⁺ concentrations, as reflected by the fitted Q (apparent gating charge), which was the same for all the channels (mean $\pm$ SE; WT = $2.3 \pm 0.05 e^-$, E518A = $2.5 \pm 0.04 e^-$, E519A = $2.4 \pm 0.05 e^-$, EEAA = $2.5 \pm 0.002 e^-$). The constancy of Q among the different channels suggests that the E518 and E519 do not constitute part of the gating charge and are not directly responsible for voltage sensing. The change in free energy required to move the channels from closed to activated states in the absence of Ca²⁺ and voltage (L_0) differed considerably among the channels. This is apparent not only from fitted L_0 values (Fig. 7), but also by the increased voltage threshold for channel activation in the E518A and EEAA mutants visible in Figures 4 and 5. In contrast, the E519A mutant had an L_0 similar to that of the wild-type

Figure 7. Fitted parameters of the voltage-dependent MWC model for WT and mutant HERGs. L_0 is the equilibrium constant for the allosteric transition between the closed and activated states in the absence of Ca²⁺ at 0 mV. Q is the equivalent gating charge in e^- . K_c is the Ca²⁺ dissociation constant for the closed channel. K_a is the Ca²⁺ dissociation constant for the activated channel. The parameter uncertainties (SDs) are shown as error bars and were determined from the covariance matrix.

channel. Removal of the negative charge at position 518 had little effect on the Ca²⁺ dissociation constant of the closed channel (*K_c*; Fig. 7), but channels bearing an E519A mutation had a *K_c* twice that of the WT or E518A mutant, indicating that neutralization of this negative charge decreased the affinity of the closed channel for Ca²⁺.

We found that the E518A mutant had a reduced *K_a*, indicating an enhanced affinity of activated channels for Ca²⁺, despite the fact that *K_a* was not reduced in the EEAA double mutant. This observation suggests that the 518 and 519 amino acids are not entirely independent with respect to their influence on activated channel Ca²⁺ binding. To quantify the degree of energetic coupling between these amino acids, we used thermodynamic mutant cycle analysis (see Scheme 1 and Eq. 4) (Carter et al., 1984; Sali et al., 1991; Hidalgo and MacKinnon, 1995; Schreiber and Fersht, 1995; Horowitz, 1996; Ranganathan et al., 1996; Frisch et al., 1997). The distinct Ca²⁺ dissociation constants for the closed (*K_c*) and activated (*K_a*) states and the equilibrium constant (*L₀*) for the distribution among closed and activated states for each channel were used to estimate the energetic interactions between these charged amino acids in the different conformational states. Using these parameters, a coupling coefficient (Ω) was calculated (Eq. 4). An Ω of unity indicates independence and a lack of energetic coupling. Deviations of Ω from unity indicate energetic coupling between the amino acids. The Ω values were: *L₀*, 21.2; *K_c*, 1.1; *K_a*, 2.4. The large Ω for the *L₀* indicates that there is energetic coupling between these amino acids in determining the equilibrium change in free energy between the closed and activated states, although clearly E518 was more critical in determining the *L₀* value (Fig. 7). The coupling coefficient for the closed channel Ca²⁺ dissociation constant was near unity, indicating a lack of coupling between these residues in the closed states. In contrast, Ω for activated channels was 2.4, indicating that an interaction between these amino acids influences Ca²⁺ binding on channel opening. This Ω value is relatively small, but it is noteworthy because these adjacent residues did not display coupling in the closed state. This implies a change in the microenvironment around E518 and E519 between the closed and activated channels. The negative charge at position 518 influences Ca²⁺ association when the channel is in the activated state but not when the channel is closed.

DISCUSSION

Our data revealed a novel mechanism for extracellular calcium modulation of an important ion channel and indicated that the affinity of HERG channels for Ca²⁺ is dependent on its conformational state. Significant changes in gating occur with very modest changes in [Ca²⁺]_o because the modulation occurs in the middle of the normal extracellular range of calcium concentrations. This suggests that rather modest fluctuations in [Ca²⁺]_o in clefts or restricted spaces can have marked changes on this repolarizing K⁺ current.

We find that negatively charged amino acids in the S3–S4 linker critically bias normal voltage and Ca²⁺ dependence of the channel. Ca²⁺ is well known as an intracellular second messenger, but recently it has also been found to be an important first messenger extracellularly (Brown, 1999). Extracellular Ca²⁺ receptors are involved in regulation of serum Ca²⁺. These receptors, like HERG, have millimolar affinity for Ca²⁺. Even small changes in extracellular Ca²⁺ can have large effects on HERG current density under conditions resembling those *in vivo* (Johnson et al., 1999). A low-affinity interaction between Ca²⁺ and

extracellular sites on proteins like HERG and the Ca²⁺ receptor is mandatory if physiological fluctuations in Ca²⁺ are to dynamically modify protein function. At normal mammalian serum Ca²⁺ concentrations of ~1.3 mM (Brown, 1999), a high-affinity site, like an EF hand motif, would be continuously occupied, preventing modulation.

Our results indicate that the glutamate at position 518 is critical in determining the normal voltage dependence of HERG channels. In contrast, the glutamate at position 519 has little effect on the intrinsic voltage dependence of the channel, but instead is involved in determining the response of the channel to extracellular Ca²⁺. What is the physical explanation for these effects? The diagram in Figure 8 shows one possibility. Transmembrane domains of a single channel subunit emphasizing the positively charged S4 transmembrane voltage sensor domain are represented. In the diagram, activation of the voltage sensor is shown as a movement of the S4 as recent studies indicate (Larsson et al., 1996; Cha and Bezanilla, 1997, 1998; Cha et al., 1999; Bezanilla, 2000; Horn, 2000). The negative charge of a glutamate (E518) in the extracellular loop connecting S3 and S4 electrostatically interacts with positive charge(s) of the S4 voltage sensor, encouraging (attracting) the activating motion of the S4. Removing the negative charge at position 518 (E518A mutation) increases the energy required for activation and thus shifts the voltage dependence of activation to more positive potentials. Removing the negative charge at position 518 (E518A mutation) also increases the affinity of the channel for Ca²⁺ during channel activation (opening) relative to the WT channel, suggesting that the Ca²⁺ binding motif is better able to associate stably with Ca²⁺. The negative charge of a glutamate (E519) participates in the association of Ca²⁺ with the channel protein, and thus the E519A mutation alters the Ca²⁺ response. A conformational change in the S3–S4 loop during channel activation gating (opening) interferes with the association of Ca²⁺ with the channel protein, lowering the affinity.

It should be noted that we have no direct proof that either of the two glutamic acid residues that we investigated directly interacts with Ca²⁺. They may merely influence its effect on the channel, perhaps through the surrounding electrostatic field projected from the protein surface or through other allosteric mechanisms. Our data do show that the actions are fairly specific and that these amino acids are involved. Calcium ions are typically coordinated by multiple negatively charged amino acids, and mutation of one of the coordinate sites is highly disruptive of high-affinity interactions. The interaction seen here is rather low affinity, unlike typical Ca²⁺ coordination sites, for example in calmodulin, so perhaps there is no such coordination, but simply an electrostatic interaction. This could explain both the lower affinity and the modest disruption caused by the mutations, but it requires other interaction sites.

Interestingly, many peptide toxins also modify voltage-gated ion channels by altering voltage dependence through an association with the S3–S4 extracellular domain. The voltage dependences of Na⁺, Ca²⁺, and K⁺ channel gating are modified by differences in this region or by binding of toxins to the S3–S4 linker domains (Rogers et al., 1996; Dib-Hajj et al., 1997; McDonough et al., 1997; Cestele et al., 1998). Splice variants of α 1A (Bourinet et al., 1999) and α 1B (Hans et al., 1999; Lin et al., 1999) Ca²⁺ channel subunits that differ in this region are distinguishable by their altered voltage dependence of gating.

Other K⁺ channels are also sensitive to changes in the extracellular residues near the voltage sensor (Elinder and Århem,

Figure 8. *Top*, Amino acid alignment of S3–S4 segments of several voltage-gated potassium channels. Standard single letter amino acid abbreviations are used. Glutamate 518 (E518) in HERG is indicated by an arrow. *Kv1.1*, *Kv1.5*, *Kv2.1*, *Kv3.1*, and *Kv4.3* indicate the human channels corresponding to KCNA1, KCNA5, KCNB1, KCNC1, and KCND3, respectively. *eag* is *Drosophila ether-à-go-go*, and HERG corresponds to KCNH2. *Bottom*, Diagram of a plausible physical explanation for the interactions between negative charges in S3–S4, positive charges in S4, and extracellular Ca²⁺. Ca²⁺ interacts at the surface of the channel at a site near the S4 voltage sensor by attraction to the negative charge of glutamate 519. The data suggest that E519 may participate in the Ca²⁺ binding site. The negative charge of glutamate 518 (E518) attracts or stabilizes positive charges in S4 and promotes or facilitates channel opening. Thus, E518 affects the Ca²⁺-independent voltage-dependence of the channel by electrostatic interaction with the voltage sensor. Adapted from Papazian and Bezanilla (1999) for HERG channels.

1999). Tang et al. (2000) characterized the effects of Mg²⁺ on *Drosophila ether-à-go-go* K⁺ channel (*eag*). They found that Mg²⁺ slowed activation *eag*-gating kinetics. Splice variants of bovine *eag* that differ in the length of the S3–S4 loop show distinct Mg²⁺ sensitivities (Frings et al., 1998). *Drosophila eag* contains a DRED (Asp-Arg-Glu-Asp) motif at amino acids 333–337 at the C-terminal end of S3. Tang et al. (2000) deleted these charged amino acids and found a depolarizing shift in the voltage–activation curves consistent with removal of a negative surface charge, but gating was still modified by Mg²⁺. They concluded that the DRED sequence does not constitute an Mg²⁺ binding site. HERG channels do not possess DRED amino acids at analogous positions. Interestingly, mutation of leucine at position 342 to histidine (L342H) in S3–S4 eliminated the modulation by Mg²⁺. It is unlikely that leucine is directly involved in Mg²⁺ binding, although a mutation at this position may perturb Mg²⁺ binding or the coupling of the Mg²⁺ effect to the voltage sensor. Alternatively, addition of a charged histidine may prevent access to an Mg²⁺ site by introduction of a local positive charge. More recently, Silverman et al. (2000) provided evidence that aspartic acids at positions 278 and 327 in *eag* constitute an Mg²⁺ binding site. Mutations of these negatively charged amino acids to ala-

nines greatly altered the response to Mg²⁺, although no concentration dependence was defined. As such it is not possible to compare the binding affinities or energetics. The composition of the S3–S4 linker affects the voltage sensitivity of Shaker K⁺ channel gating (Mathur et al., 1997), and natural toxins affect K⁺ channel gating by binding near this site (Swartz and MacKinnon, 1997a,b), all consistent with our observations on HERG channels that S3–S4 is a critical modulatory domain. Together, these observations of interactions between extracellular effectors and channel S3–S4 regions near the voltage sensor reveal the general importance of this key modulatory domain (Li-Smerin and Swartz, 1998; Winterfield and Swartz, 2000).

We conclude that extracellular Ca²⁺ is an allosteric modulator of the HERG K⁺ channel. Ca²⁺ associates with closed channels (occupied at negative membrane potentials) with higher affinity than with activated channels (occupied at less negative membrane potentials), thereby stabilizing the closed state. Two glutamates in the S3–S4 linker domain, E518 and E519, are critical for normal HERG function. Neutralization of E518 shifts the voltage dependence of channel opening to more depolarized membrane potentials promoting closed states, possibly by removing a negative charge that is normally attracting the S4 positive charge.

Neutralization of E518 does not affect the affinity of the closed channel for Ca²⁺. Neutralization of E519 does not appear to affect the membrane potential sensed by the voltage sensor but does decrease the affinity of the closed channel for Ca²⁺. The E518 and E519 residues are energetically coupled in the activated channel but not in the closed channel. These results emphasize the importance of the S3–S4 linker in channel function and present a mechanism of Ca²⁺ action on this physiologically important ion channel.

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