Negative Charges in the Transmembrane Domains of the HERG K Channel Are Involved in the Activation- and Deactivation-gating Processes

JIE LIU, MEI ZHANG, MIN JIANG, and GEA-NY TSENG
Department of Physiology, Virginia Commonwealth University, Richmond, VA 23298

ABSTRACT The transmembrane domains of HERG (S1–S3) contain six negative charges: three are conserved in all voltage-gated K channels (D456 and D466 in S2, D501 in S3) and three are unique to the EAG family (D411 in S1, D460 in S2, and D509 in S3). We infer the functional role of these aspartates by studying how substituting them with cysteine, one at a time, affects the channel function. D456C is not functional, suggesting that this negative charge may play a critical role in channel protein folding during biogenesis, as has been shown for its counterpart in the Shaker channel. Data from the other five functional mutants suggest that D411 can stabilize the HERG channel in the closed state, while D460 and D509 have the opposite effect. D466 and D501 both may contribute to voltage-sensing during the activation process. On the other hand, all five aspartates work in a concerted fashion in contributing to the slow deactivation process of the HERG channel. Accessibility tests of the introduced thiol groups to extracellular MTS reagents indicate that water-filled crevices penetrate deep into the HERG protein core, reaching the cytoplasmic halves of S1 and S2. At these deep locations, accessibility of 411C and 466C to the extracellular aqueous phase is voltage dependent, suggesting that conformational changes occur in S1 and S2 or the surrounding crevices during gating. Increasing extracellular [H+] accelerates HERG deactivation. This effect is suppressed by substituting the aspartates with cysteine, suggesting that protonation of these aspartates may contribute to the signaling pathway whereby external [H+] influences conformational changes in the channel’s cytoplasmic domains (where deactivation takes place). There is no evidence for a metal ion binding site coordinated by negative charges in the transmembrane domains of HERG, as the one described for the EAG channel.

KEY WORDS: mutagenesis • Xenopus oocytes • structure-function relationship • voltage-gated K channel

INTRODUCTION

HERG is encoded by the human ether-a-go-go–related gene (HERG)* and functions as the pore-forming component of the rapid delayed rectifier K(\(I_{Kr}\)) channel in the heart (Sanguinetti et al., 1995). The focus of this study is the negatively charged residues in the transmembrane domains (S1, S2 and S3) of HERG. Fig. 1A shows an alignment of amino acid sequences of these domains, along with those of S4, for HERG and two voltage-gated K(Kv) channels (EAG and Shaker), for which the functional roles of the negative charges in the transmembrane domains have been studied (Papazian et al., 2002). There are three negative charges well conserved in all Kv channels known so far (Papazian et al., 2002). They are denoted as −2, −4, and −5 in Fig. 1. The functional role for these conserved negative charges in the Shaker channel has been thoroughly studied by Papazian and her colleagues (Seoh et al., 1996; Tiwari-Woodruff et al., 1997, 2000; Papazian et al., 2002). E283 (−2) can form a specific salt-bridge with R368 (+3) or R371 (+4) in the S4 domain during biogenesis. This helps ensure a proper folding of the Shaker channel protein, which can mature and reach the cell surface (Tiwari-Woodruff et al., 1997). Once the channel is in the cell-surface membrane, these negative charges engage in the gating processes in several ways. They may face water-filled crevices that penetrate into the protein core. By shaping the local electrical field in the crevices surrounding the major voltage sensor, S4, these negative charges can indirectly affect the channel’s gating process (Iblas and Sigworth, 2001). E283 may facilitate channel activation by forming a salt-bridge with R368 in an intermediate state during activation transitions, and with R371 when the channel reaches the final open state, thus stabilizing the channel in the activated states (Seoh et al., 1996; Tiwari-Woodruff et al., 2000). E293 may move relative to the membrane electrical field during gating, thereby directly contributing to the channel’s gating charge (Seoh et al., 1996).

The EAG channel has more negative charges in the transmembrane domains (Papazian et al., 2002, Silverman et al., 2000). They are marked by −1, −3, and −6

*Abbreviations used in this paper: HERG, human ether-a-go-go-related gene; MTS, methanethiosulfonate.
in Fig. 1. The EAG channel has a unique gating property among Kv channels: prepulse hyperpolarization can delay EAG activation not only by increasing the transition time among closed states (“Cole-Moore shift,” as is generally observed for Kv channels; Cole and Moore, 1960), but also by slowing the final opening transition (Tang et al., 2000). Increasing the extracellular Mg$^{2+}$ concentration ([Mg$^{2+}$]o) can accentuate the slowing effect of prepulse hyperpolarization (Terlau et al., 1996; Tang et al., 2000). It has been shown that negative charges −3 and −6 can coordinate a divalent cation binding site within the EAG channel (Silverman et al., 2000). It is further suggested that Mg$^{2+}$ binding to this cation-binding site limits S4’s outward movement during membrane depolarization, thus slowing channel activation (Schonherr et al., 2002). Whether the negative charge in S1 (−1 in Fig. 1) plays any role in EAG channel function has not been examined.

As its name implies, HERG is related to the EAG channel (Warmke and Ganetzky, 1994). Despite the homology in amino acid sequence between HERG and EAG channels (e.g., Fig. 1 A), there are distinct differences in their gating behavior. The rate of HERG activation and deactivation is much slower (Sanguinetti et al., 1995; Robertson et al., 1996; Herzberg et al., 1998). HERG shares the conserved as well as the extra negative charges in the transmembrane domains (Fig. 1 A). What is the role of these negative charges in its function? Besides the question about whether −1 has any functional role, there are three more reasons to ask this question. First, extracellular acidosis (changing pHo from 7.5 to 6.5) has a selective effect on the rate of HERG deactivation (Jiang et al., 1999). The mechanism is not clear, because deactivation in the HERG channel, as in other Kv channels, is mediated by conformational changes in the cytoplasmic domains (S4–S5 linker and cytoplasmic half of S6) (Lu et al., 2002; Tristani-Firouzi et al., 2002). The slow rate of deactivation in the HERG channel is further due to an interaction between the NH1-terminal “PAS” domain (aa 2–138) (Morais Cabral et al., 1998) and the S4-S5 linker (Wang et al., 1998), which stabilizes the channel in the open state. However, there is a strong possibility that extracellular acidosis may transmit the signal to cytoplasmic domains by protonating carboxylate side chains in the transmembrane domains of HERG. Second, the effects of extracellular Mg$^{2+}$ on HERG are distinctly different from those on the EAG channels (Ho et al., 1998; Tang et al., 2000). Elevating [Mg$^{2+}$], in the range of 1–10 mM slows EAG activation without any effect on the voltage dependence of activation or the rate of deactivation (Tang et al., 2000). On the other hand, changing [Mg$^{2+}$], in the same range causes a prominent positive shift in the voltage-dependent activation and a marked acceleration of deactivation in HERG (Ho et al., 1998). This raises the question as to whether Mg$^{2+}$ affects the HERG channel
by the same mechanism, i.e., binding to a metal ion binding site in the transmembrane domain, as has been described for the EAG channel. Third, the charge distribution along the S4 domain in HERG differs from that in the Shaker channel. According to the proposed S4 alignment (Gandhi and Iscoff, 2002), HERG lacks the first positive charge (+1) in S4. Furthermore, the charge distribution around the S4 surface after +5 in HERG is very different from that in Shaker. These differences may entail a pattern of interaction between the S4 and the negative charges in HERG different from that described for the Shaker channel (highlighted in Fig. 1 C).

We ask three questions in this study: (a) How does neutralizing the negative charges in S1–S3 of HERG affect the channel function? (b) Are these aspartate side chains accessible to the extracellular solution? (c) Are these negative charges involved in HERG channel modulation by extracellular H+ and divalent cations? The answer to the second question bears on the answer to the third one. We choose to substitute these aspartate residues with cysteine. The side chain volume of cysteine is 108.5 Å3, not very different from that of aspartate side chain (111.1 Å3). More importantly, the cysteine side chain is highly reactive to methanethiosulfonate (MTS) reagents. This allows us to probe the side chain accessibility by applying membrane impermeable MTS reagents to the bath solution. These mutants are designated by D (for aspartate), position number, and C (for cysteine). Among the six mutants created, D456C (−2 in S2, Fig. 1) does not produce functional channels despite repeated attempts. This suggests that the negative charge at 456 may play a critical role in protein folding during biogenesis of the HERG channel (Tiwari-Woodruff et al., 1997). This mutant is not included in the current study.

**Materials and Methods**

**Mutagenesis**

HERG in a vector, pGH19 (a gift from Dr. Gail A. Robertson, University of Wisconsin-Madison), was subcloned into the KpnI/XbaI site of pAlterMax. Mutagenesis was performed using the oligonucleotide-directed method and a commercial kit (Alter site II in vitro Mutagenesis System; Promega). Mutations were confirmed by direct DNA sequencing around the mutation sites. Two independent colonies were picked and used for cRNA transcription and oocyte expression. No differences in the channel phenotype were noticed between two colonies from the same mutant. For transcription, plasmids were linearized by NotI and transcribed using T7 RNA polymerase using a commercial kit (Message Machine; Ambion). The quality and quantity of transcribed cRNA products were evaluated using densitometry (ChemiImager model 4400; α-Innotech Corp).

**Oocyte Preparation**

Oocytes were isolated as described before (Tseng-Crank et al., 1990), incubated in an ND96-based medium (composition given below), and supplemented with 10% horse serum and penicillin/streptomycin) at 16°C. 5–12 h after isolation, each oocyte was injected with 40 nl of cRNA solution (containing cRNA of 4–18 ng), using a Drummond digital microdispenser. Oocytes were incubated in the above medium at 16°C, and studied 2–4 d after cRNA injection.

**Voltage Clamp Experiments**

Before recording, oocytes expressing cysteine-substituted mutants were pretreated with DTT: incubation in freshly made DTT solution (5 mM in the incubation medium described above) at room temperature for 2–4 h, followed by three washes in 3 ml of DTT-free incubation medium. Membrane currents were recorded from whole oocytes using the “2-cushion pipette” voltage clamp method (Schreibmayer et al., 1994). Both current-passing and voltage-recording pipettes had tip resistance 0.1–0.3 MΩ. During recordings, the oocyte was continuously superfused with a low-Cl ND96 solution to reduce interference from endogenous Cl− channels. Voltage clamp was done at room temperature (24–26°C) with OC-725B or OC-725C amplifier (Warner Instruments). Voltage clamp protocol generation and data acquisition were controlled by pClamp5.5 via a 12-bit D/A and A/D converter (DMA, Axon Instruments, Inc.). Current data were low-pass filtered at 1 kHz (Frequency Devices) and stored on disks for off-line analysis.

**Voltage Clamp Protocols and Data Analysis**

To study voltage dependence of activation, the following protocol is used: from Vh = −80 mV, 1-s test pulses to Vt ranging from −70 to +60−120 mV (depending on the voltage range of channel activation) in 10-mV increments are applied once every 15 s. The peak amplitudes of tail currents are normalized by the maximal tail current following Vt to +60 mV. This gives an estimate of the “fraction of channels activated” at the end of the 1-s test pulses. Its voltage dependence is analyzed by fitting the data with a simple Boltzmann function:

\[
\text{fraction activated } = \frac{1}{1 + \exp[z(V_{0.5} - V_f)/F/RT]},
\]

where \(V_{0.5}\) and \(z\) are the half-maximum activation voltage and apparent gating charge, respectively. F is Faraday constant, R is gas constant, and T is absolute temperature (F/RT = 0.04 mV−1). The same protocol and data analysis are applied to all functional mutant channels except D411C. The activation curve of D411C requires an empirical double Boltzmann function for a good fit:

\[
\text{fraction activated } = A_1/\left[1 + \exp[z_1(V_1 - V_f)/F/RT]\right] + A_2/\left[1 + \exp[z_2(V_2 - V_f)/F/RT]\right],
\]

where \(A_1, V_1, z_1, A_2, V_2, z_2\) are the fraction, half-maximum activation voltage, and apparent gating charge for the \(1\)-th Boltzmann component.

The time constant of activation (\(\tau_a\)) and its voltage dependence are studied using an “envelope test” protocol: from \(V_h = −80\) mV, test pulses to \(V_t\) ranging from −20 to 60 mV in 20-mV increments for varying durations are applied once every 15 s. The time course of growth of peak tail current amplitude can be well fit with a single exponential function with a time delay:

\[
I_{tail} = I_{max} \times \left[1 - \exp\left\{-\left(t - t_{delay}\right)/\tau_a\right\}\right],
\]

where \(I_{tail}\), \(I_{max}\), \(t_{delay}\), and \(\tau_a\) are the peak tail current amplitude after a test pulse of duration \(t\), the asymptotic value of \(I_{tail}\) test.
pulse duration, time delay, and τ of activation, respectively. To estimate the voltage dependence of the opening transition, the following equation is used (Ko is the reciprocal of τo, see Scheme I in **RESULTS**):

$$K_o(V) = K_o(0) \times \exp(z_VVF/RT),$$

(4)

where $K_o(V)$ and $K_o(0)$ are rate constants of channel opening at test pulse voltages of V and 0 mV, respectively, and $z_V$ is the apparent gating charge for channel opening. The value of $z_V$ is obtained by a linear regression analysis of relationship between ln($K_o(V)$) and V in an appropriate V range (e.g., −40 to 40 mV for WT in Fig. 2 B). Since channels need to make transitions between closed states before opening, $1/\tau_o$ does not approximate $K_o$ until the voltage is positive enough so that the degree of activation has plateaued. In Fig. 2 B, the voltage dependence of $\tau_o$ appears to decrease in this voltage range, suggesting that $K_o$ is not very voltage dependent and that our fit of the data overestimates the charge associated with the opening transition. Therefore, $z_V$ probably reflects the charge associated with closed state transitions.

To construct the fully activated current-voltage ($I_{a-V}$) relationship, the following protocol is used: from $V_h$, −80 mV, 0.2-s conditioning pulses to +60 mV are applied to fully activate the channels once every 15 s, followed by repolarization to $V_r$ +50 to −120 mV in 10-mV increments. The peak tail current amplitudes are normalized by the maximum outward tail current at $V_r$ −50 or −60 mV, and averaged over cells.

To estimate the time constant of the major (fast) component of deactivation ($\tau_d$) in the voltage range below the threshold of channel activation, a voltage clamp protocol similar to that used to construct the $I_{a-V}$ relationship is used. Time course of decay of tail current in a voltage range negative to the activation threshold is fit with a double-exponential function:

$$I_{tail}(t) = I_{fast} \times \exp(-t/\tau_{fast}) + I_{slow} \times \exp(-t/\tau_{slow}) + I_{SS},$$

(5)

where $I_{tail}(t)$ is tail current amplitude at time t after the beginning of repolarization, $I_{fast}$, $I_{slow}$ and $I_{SS}$ are the fast, slow, and steady-state components of tail current, $\tau_{fast}$ (= $\tau_d$) and $\tau_{slow}$ are time constants of the two time-dependent current component.

To study the rate of C-type inactivation in HERG and its mutants, a three-pulse voltage clamp protocol is used: from $V_h$ −80 mV, depolarization to +40 mV for 1 s is applied to activate then inactivate the channels. This is followed by a 10-ms step to −80 mV, during which channels recover from inactivation without appreciable deactivation. Then a third pulse to $V_h$ ranging from +40 to −20 mV is applied during which the channels (recovered from inactivation and still in the activated state) reactivate. The decay phase of current during $V_h$ (excluding the capacitive transient during the first 2 ms) can be well fit with a single exponential function, which allows the determination of time constant of inactivation ($\tau_d$).

To study the voltage dependence of accessibility of introduced cysteine side chains to extracellular MTSET, the following general voltage clamp protocol is used: currents are elicited by repetitive 1-s pulses from $V_h$ −80 to +20 mV once every 30 s. Three pulses are applied before MTSET to confirm current stability (pulses 1–3). Then the membrane voltage is held at $V_h$ of either −80, −20, or +20 mV for 10 min, during which the oocyte is superfused with MTSET solution (1 mM) for 5 min, followed by a 5-min wash. Four or more pulses are then resumed to test for MTSET effects (pulses 4–7).

In the voltage clamp protocols described for the construction of activation curve and $I_{a-V}$ relationship, a 10-ms prepulse from $V_h$ −80 mV to −100 mV precedes every depolarization pulse. Currents jump during these prepulse is used for leak-subtraction.

The following software is used for data analysis: pClamp6 or 8, EXCEL (Microsoft), SigmaPlot, SigmaStat, and PeakFit (SPSS).

**Cysteine Side Chain Modification**

MTSET or MTSES (Toronto Research Chemicals, Inc.) powder was dissolved in deionized water at 0.1 M shortly before experiments. The stock solution was stored on ice and used within 2 h. After control data were obtained, the MTSET or MTSES stock solution was diluted with bath solution to 1 or 10 mM and applied to the oocyte immediately.

**Solutions**

The ND96 solution had the following composition (in mM): NaCl 96, KCl 2, CaCl2 1.8, MgCl2 1, HEPES 5, Na-pyruvate 2.5, pH 7.5. The low-Cl ND96 used during voltage clamp experiments were made with Cl− ions in ND96 replaced by methanesulfonate. In some experiments, [K] in low-Cl ND96 was raised to 98 mM. In this case, Na+ and Na-pyruvate were omitted to maintain the osmolality. Solutions with pH 6.5 and 8.5 were titrated with methanesulfonic acid or NaOH to the desired pH. Solution of 10 mM (Ga2+) was made by adding CaCl2 without compensation for osmolality.

**RESULTS**

We replace aspartate residues in S1, S2, and S3 of HERG (D411, D456, D460, D466, D501, and D509, Fig. 1) by cysteine, one at a time. The mutants produce functional channels except D456C. For the functional mutants, we study the effects of cysteine substitution on the channel function, accessibility of the introduced thiol side chains to extracellular hydrophilic MTS reagents, and the effects of mutations on channel modulation by extracellular H+ and Ca2+ ions. The voltage clamp protocols and methods of data analysis used to characterize channel function are illustrated in Fig. 2, using wild-type (WT) HERG as an example. The voltage dependence of activation is characterized by a “1-s isochronal” activation curve (Fig. 2 A), which can be well fit with a simple Boltzmann function for WT and mutant channels except D411C (Fig. 3 B). We use two parameters generated from curve fitting (half-maximum activation voltage, $V_{0.5}$, and apparent gating charge for channel activation, $z_g$) to compare the voltage dependencies of channel activation.

The rate of activation is measured using an “envelope test” protocol (Fig. 2 B). Because the C-type inactivation in the HERG channel is extremely fast relative to channel activation, the activation rate cannot be directly measured from the time course of current during depolarization. On the other hand, upon repolarization the channels rapidly recover from C-type inactivation before deactivation sets in. Therefore, the growth of peak tail current amplitude after increasing durations of depolarization can be used to trace the time course of channel activation. This time course can be well described by a single exponential function with a
short delay (Fig. 2 B, inset, and Eq. 3 in MATERIALS AND METHODS). The time delay (to account for the initial sigmoidal phase of current activation) is much shorter than the single exponential rising component (Fig. 2 B, legend). This suggests that the channel spends relatively little time in transitions among closed states (C_n to C_{n-1}, Scheme I), but more time in the final opening step (C_n to O):

relatively fast transitions \[ K_0(V) \]

Scheme I

where \( K_0(V) \) is the rate constant of transition from \( C_n \) to \( O \) at voltage \( V \). Note that the inactivated state is omitted in this simplified gating scheme. This is justified because the envelope test protocol negates influence from channel inactivation on the measurement. \( K_0(V) \) is estimated by the reciprocal of \( \tau_{a}(V) \), the time constant of activation at voltage \( V \), obtained by curve fitting. The relationship between \( \tau_{a}(V) \) and depolarization voltage \( (V_\text{r}) \) is plotted on a semilogarithmic scale in Fig. 2 B. It is clear that this relationship is largely linear over the \( V_\text{r} \) range of \(-40 \) to \(+40 \) mV. The exponential relationship between \( \tau_{a}(V) \) and \( V_\text{r} \) indicates a voltage-dependent opening transition, thus:

\[
K_0(V) = K_0(0) \exp(z_a V_\text{r} / RT),
\]

where \( K_0(0) \) is the rate constant of opening transition at 0 mV, and \( z_a \) is the apparent gating charge involved in channel opening. A linear regression analysis on data shown in Fig. 2 B gives an estimate of \( K_0(0) \) and \( z_a \) of 1.4 s\(^{-1}\) and 0.83 for the WT HERG.

The fully activated current-voltage (I_f\_a-V) relationship shown as in Fig. 2 C can be used to deduce the pore-domain function: K\(^+\) selectivity and C-type inactivation (Liu et al., 2002). The reversal potential (E_{rev}) of the I_{f-a-V} relationship for WT HERG is \(-102.2 \pm 0.7 \) mV. This is very close to the estimated E_K (\(-105 \) mV), suggesting a strong K\(^+\) selectivity. The negative slope of the I_{f-a-V} relationship between \(-60 \) and \(+30 \) mV reflects a decrease in channel conductance due to C-type inacti-
vation. The kinetics of C-type inactivation is further analyzed by the three-pulse protocol (Fig. 2 E) and the summary data are presented in Fig. 2 F.

The time course of HERG deactivation can be well described by a double-exponential function. The fast phase of deactivation is the major component in the voltage range negative to the threshold of channel activation (accounting for ~60% of the tail current at \( V_r = -80 \text{ mV}, \) and ~100% at \( -100 \text{ mV} \) and more negative \( V_r \)). Therefore, we used these time constants to compare the rate of channel closing in the negative voltage range (Fig. 2 D).

Voltage clamp protocols and data analysis similar to those described for WT HERG are used to characterize the mutant channels’ function. Where modifications are necessary, due to mutation-induced alterations in voltage dependence and/or kinetics of gating, they are specified in the figures. For a clear comparison, the WT data in Fig. 2 are shown again in the following figures reporting mutant channels’ function.

**Effects of Replacing Aspartates in HERG’s Transmembrane Domains with Cysteine on the Channel Function**

**D411C in the intracellular half of S1 domain.** The gating behavior of D411C differs from that of WT HERG in three aspects. First, the voltage dependence of D411C activation manifests two apparent Boltzmann components, with ~50% of the channels activated in a more negative voltage range than that of the WT channels, while the remaining 50% superimposed with the WT activation curve at voltages > 0 mV (Fig. 3 B). Second, the rate of D411C activation is much faster than that of WT. This is indicated by the early peak outward current followed by a decay in the D411C current traces during membrane depolarization (Fig. 3 A, but more clearly seen in Fig. 3 C, inset). The decay phase is due to the same fast C-type inactivation process as in the WT channel (Fig. 3 D). Since the rate of D411C activation becomes much faster, a large part of the C-type inactivation process occurs after channel activation, creating the early peak followed by a decay phase. Upon membrane repolarization, D411C quickly recovers from this C-type inactivation, as is the case for the WT channel. Therefore, the envelope test can be used to deduce the rate of C-type inactivation, as is the case for the WT channel.

**D460C in the middle of S2 domain.** Fig. 4 shows that the gating behavior of D460C differs from that of WT HERG in three aspects. First, the activation curve of
The voltage dependence of D466C activation is shifted in the positive direction. Furthermore, the rate of D466V activation is slower than that of WT in the Vt range /H11022 0 mV (Fig. 5 C), and the rate of D466C deactivation is faster than that of WT (Fig. 5 E). These changes are similar to those described for D460C. However, the effect of mutation on the voltage dependence of activation differs between the two. The voltage dependence is not altered by D460C. On the other hand, in D466C the voltage sensitivity of activation gating is reduced: the zg value is reduced to 1.48 (from 2.70 in WT), and the za value is reduced to 0.48 (from 0.83 in WT). Despite the decrease in za, the Ko value is the same as that of WT (Fig. 5 C). D466C maintains a strong and fast C-type inactivation and K*/H11001 selectivity, similar to those of the WT channel (Fig. 5 D).

**D501C in the intracellular half of S3 domain.** D501C currents are small when recorded under the same conditions as used in the other mutant channels ([K]/o = 2 mM). Increasing the amount of D501C cRNA injected also enhances the oocyte endogenous Cl− current (Fig. 6 A). Relative to the WT channel, the D501C activation curve is shifted in the depolarizing direction (Fig. 6 B). The rate of D501C activation is measured in 98 mM [K]/o (to boost the current amplitude). The envelope tests on the WT channel is also done in 98 mM [K]/o for comparison. Fig. 6 C shows that the τo of D501C activation is shorter than that of WT in the Vt range > 0 mV, but the two datasets crossover with each other at 40 mV. Linear regression analysis gives an estimate of K(0) and za of 0.81 s−1 and 1.0 for WT, and 2.28 s−1 and 0.16 for D501C. Therefore, the D501C mutation markedly reduces the voltage sensitivity of the opening transition, although the rate constant of opening transition at 0 mV is higher than that of WT. Fig. 6 E shows D501C deactivation rate measured in 2 mM [K]/o. It is much faster than that of the WT channel. Because of the interference of oocyte Cl− current, leak-subtraction cannot be used in the construction of the If-a-V relationship for D501C (Fig. 6 D). The resulting Ica-V curve shows a plateau phase in the Vr range of −40 to +30 mV. Since the I-V relationship of oocyte endogenous currents may have a positive slope in this voltage range, we suggest that D501C has at least some residual C-type inactivation, that offsets the positive slope of oocyte endogenous currents and creates the plateau phase. The apparent Erev suggested by the Ica-V relationship of D501C is slightly less negative than that of WT (~90 mV, Fig. 6 D). Again, this can be explained by the interference of
The Journal of General Physiology

Transmembrane Domain Negative Charges and HERG Gating

The data points are superimposed on lines calculated from Eq. 4, with $z_a$ values of 1.0 and 0.16 for WT and D501C, respectively. The D501C data in main graphs are averaged from four to seven cells each. Calibration bars correspond to 1 $\mu$A and 0.2 s.

**Figure 7.** Comparison of gating behavior between D501C and WT recorded in 98 mM $[K]_o$. (A) Activation curves. For WT, $V_{0.5} = -25.4 \pm 2.2$ mV, $z_g = 2.79 \pm 0.03$. For D501C, $V_{0.5} = -2.8 \pm 0.6$ mV, $z_g = 1.45 \pm 0.05$. (B) Comparison of $\tau_a$ values between D501C and WT. (Inset) WT and D501C tail currents and curve fitting at $-80$ mV. Data are summarized from three to four cells each. Calibration bars correspond to 1 $\mu$A and 0.2 s.

D509C in the extracellular half of S3 domain. Relative to the WT channel, the D509C activation curve is shifted in the positive direction in a parallel fashion (Fig. 8 B). The rate of D509C activation is much slower than that of WT, but the voltage sensitivity remains the same (Fig. 8 C). D509C maintains a strong and fast C-type inactivation process and $K^+$ selectivity (Fig. 8 D). Finally, the rate of D509C deactivation is faster than that of WT (Fig. 8 E). These changes in the channel gating behavior induced by the D509C mutation are similar to those described for D460C (Fig. 4).

Accessibility of Introduced Thiol Side Chains to Extracellular MTS

To further probe the structure-function relationship of HERG involving these aspartate residues, we test the accessibility of the introduced cysteine side chains to extracellular MTS reagents. An important assumption here is that the accessibility of thiol side chains in the cysteine-substituted mutants reflects the accessibility of the native side chains in the WT channel, i.e., cysteine substitution does not grossly perturb the channel conformation. Although the mutations all visibly alter the voltage dependence and kinetics of channel gating, conformational changes responsible for these alterations in gating are likely to be subtle. First, all mutants
retain a high $K^+$ selectivity and the C-type inactivation process, indicating an intact outer mouth and pore function, as well as a proper communication between the voltage-sensing domain and the pore domains (which is essential for the C-type inactivation process; Gandhi et al., 2000; Liu et al., 2002). Second, mutation-induced changes in the free energy involved in the gating processes are small (calculated based on the shift of the activation curve). They amount to 1–3 kcal/mol, about the energy of one or two H-bonds in aqueous environment. Therefore, the mutant channels are sufficiently similar to the WT channel to justify the assumption stated above. In some cases, we test the effects of both MTSET and MTSES. Both reagents can increase the side chain volume of a modified cysteine. However, MTSET adds a positive charge while MTSES adds a negative charge to the side chain. Therefore, the resulting changes in channel function may yield some information about the underlying mechanism: a steric effect (similar between MTSET and MTSES) or a charge effect (different between the two). WT-HERG has 12 native cysteines, 5 of them are potentially accessible to external MTS (C445 and C449 in the extracellular S1-S2 linker, C555 and C566 in the S5 domain, and C643 in the S6 domain). However, external MTSET or MTSES has no effects on WT-HERG, indicating that the native cysteines are either not accessible to extracellular MTS reagents, or modification of them by MTS has no impact on channel function (Liu et al., 2002). Thus, the observed effects on cysteine-substituted HERG mutants are likely due to MTS modification of the introduced cysteine side chains.

411C is accessible to MTSET in a voltage-dependent manner. Data for D411C are shown in Fig. 9. MTSET (1 mM) is applied for 5 min when the membrane voltage is held at $-80$ mV (most channels in the closed state) or $-20$ mV (channels in activated or inactivated state). MTSET is washed out for 5 min, and the effect on D411C current amplitude is evaluated. MTSET reduces the D411C current amplitude, and the effect can be seen only when it is applied during sustained depolarization at $V_h = -20$ mV, but not at $V_h = -80$ mV. However, once the $V_h$ is returned to $-80$ mV (when D411C channels are allowed to close), the MTSET effect gradually disappears. Thus, MTSET modification of 411C is voltage-dependent and unstable.

Thiol side chain accessibility along the S2 domain (460C and 466C). Thiol side chain at position 460 is accessible to extracellular MTSET and MTSES, and the thiol side chain at position 466 is accessible to extracellular MTSET. However, external MTSET or MTSES has no effects on WT-HERG, indicating that the native cysteine is either not accessible to extracellular MTS reagents, or modification of them by MTS has no impact on channel function (Liu et al., 2002). Thus, the observed effects on cysteine-substituted HERG mutants are likely due to MTS modification of the introduced cysteine side chains.

Figure 8. D509C channel function. The format is the same as that of Fig. 3. In B, the D509C activation curve has $V_{1/2} = 22.7 \pm 1.4$ mV and $z_f = 1.88 \pm 0.04$. In C, the D509C data points are superimposed on a line calculated from Eq. 4, with $z_o = 1.15$. The D509C data in main graphs are averaged from 9 to 10 cells each. Calibration bars correspond to 1 nA and 0.2 s (except panel C, for which the time calibration bar is 0.5 s).

Figure 9. Testing the accessibility of thiol side chain at position 411 to extracellular MTSET. (A) Left, experimental protocol. Right, superimposed tail current traces (at $-80$ mV, gray shade in inset) recorded from two experiments. The $V_h$ and pulse numbers are marked. (B) Summary data. The peak amplitudes of tail currents are normalized by the control value just before MTSET application, and plotted against pulse numbers. Data points from different experiments are denoted by different symbols as denoted in the inset ($n = 6$ for $V_h = -20$ mV, and $n = 4$ for $V_h = -80$ mV). Calibration bars correspond to 1 nA and 0.1 s.
Transmembrane Domain Negative Charges and HERG Gating

...tion in D460C current amplitude (n = 15). MTSES modification causes a modest increase (by 27 ± 3%, n = 6, Fig. 10 C), and prevents further modification by MTSET (unpublished data). Thus, the 460 side chain is in a relatively wide open crevice around the S2, while side chain charge can impact on current amplitude. Further down the S2 helix, thiol side chain at position 466 is still accessible to extracellular MTSET. However, MTSET modification of 466C is stronger when applied at V_h = -80 mV (channels in closed state) than at V_h = 20 mV (channels in activated/inactivated state) (Fig. 11 C vs. Fig. 10 C). Thus, the water-filled crevice around S2 narrows around 466.

Thiol side chain accessibility along the S3 domain (509C and 501C). MTSET modification of 509C causes little changes in the current amplitude, but there is a marked acceleration of channel deactivation (Fig. 12 A). The thiol group at position 509 is in a wide open space where side chain charge has a great impact on the channel deactivation rate.

These findings indicate that 509 is in a wide open space where side chain charge has a great impact on the channel deactivation rate.

Effects of Extracellular H+ on HERG: Role of Protonating Aspartate Residues in the Transmembrane Domains

As shown previously, the most prominent effect of changing pH_o on HERG is the alteration of deactiva-
tion rate (Jiang et al., 1999). This is illustrated in Fig. 13, top left. For WT-HERG, changing pHo from 7.5 to 6.5 markedly accelerates deactivation, whereas changing pHo from 7.5 to 8.5 has the opposite (although more modest) effect. These effects are greatly reduced in D411C, and totally abolished in the other four mutants (Fig. 13). Similar findings are obtained in four to six cells each. These data are consistent with the notion that these aspartate residues are critically involved in pHo modulation of the deactivation rate in HERG. However, removing any one of them, leaving the other four still negatively charged, greatly attenuates or abolishes the pHo effect, as if all five negative charges are required for the manifestation of the pHo effect.

During these experiments, we also notice that D411C has a unique response to changing pHo. As shown in Fig. 14, top, changing pHo, in the range of 6.5 to 8.5 has little effect on the voltage dependence of activation in the WT channel. On the other hand, although changing pHo from 7.5 to 8.5 has little effect on the activation curve of D411C, changing pHo from 7.5 to 6.5 causes a marked positive shift. This shift seems to be selective for the Boltzmann component in the negative voltage range, such that the activation curve of D411C in pHo 6.5 can be well described by a single Boltzmann function. The activation curve of D411C is almost superimposable with the activation curve of WT-HERG at pHo 6.5. The implications of these findings, and those shown in Fig. 13, will be addressed in the discussion.

Does D460 Contribute to a Metal Ion Binding Site in HERG?

The effects of elevating [Mg]o on HERG are essentially the same as those of elevating [Ca]o (Ho et al., 1998). We choose to study the effects of Ca2+, instead of Mg2+, because the mechanism of Ca2+ action on HERG has been more thoroughly studied (Johnson et al., 1999, 2001). Fig. 15 shows that for WT-HERG, elevating [Ca]o from 1.8 to 10 mM causes a prominent positive shift in the activation curve (Fig. 15 B), and an acceleration of deactivation (Fig. 15 A, left, and C). Elevating [Ca2+]o also causes a modest slowing of activation and a biphasic change in the current amplitude (Fig. 15 A, left, and C). Elevating [Ca2+]o also causes a modest slowing of activation and a biphasic change in the current amplitude (Fig. 15 A, left, and C). Elevating [Ca2+]o also causes a modest slowing of activation and a biphasic change in the current amplitude (Fig. 15 A, left, and C). Elevating [Ca2+]o also causes a modest slowing of activation and a biphasic change in the current amplitude (Fig. 15 A, left, and C). Elevating [Ca2+]o also causes a modest slowing of activation and a biphasic change in the current amplitude (Fig. 15 A, left, and C). Elevating [Ca2+]o also causes a modest slowing of activation and a biphasic change in the current amplitude (Fig. 15 A, left, and C). Elevating [Ca2+]o also causes a modest slowing of activation and a biphasic change in the current amplitude (Fig. 15 A, left, and C).
neutralizing the ‘D278-equivalent’ in HERG (D460C) does not alter the effects of Ca$^{2+}$ on HERG channel function, including the positive shift in the activation curve (Fig. 15 B), acceleration of deactivation (Fig. 15 A, right, and C), and the biphasic change in the current amplitude (Fig. 15 A, right). Therefore, D460 in HERG and D278 in EAG are not functionally equivalent.

DISCUSSION

Cysteine substitutions of the aspartate residues in S1–S3 of HERG have distinct effects on the channel function. The phenotypes of the 5 mutants studied here (D411C, D460C, D466C, D501C, and D509C) can be summarized as the following. First, there are three patterns of changes in the voltage dependence of activation. For D411C in the S1 domain, 50% of the channels are activated in a more negative voltage range than the WT channel, and the rate of activation is markedly accelerated. For D460C and D509C in the extracellular half of S2 and S3, respectively, the activation curve is shifted in the positive direction and the rate of activation is slowed, but there is little change in the voltage dependence of the activation gating process. For D466C and D501C that are in the intracellular half of S2 and S3, the activation curve is shifted in the positive direction. Furthermore, there is a marked reduction of voltage sensitivity in the activation-gating process. Second, all the mutant channels manifest an accelerated deactivation process. In contrast to the marked changes in the activation and deactivation processes, all the mutants retain the ability to C-type inactivate and to select for K$^{+}$ ions as the charge carrier, indicating that the pore-domain function and the communication between the pore-domain and the voltage-sensing domain are maintained.

Role of Transmembrane Domain Aspartate Residues in the Activation Process of HERG

Two aspects of the activation process are characterized: activation curve and rate of activation. Two parameters are generated from fitting the activation curve with the Boltzmann function: $V_{0.5}$ (half-maximum activation voltage) and $z_{g}$ (apparent gating charge for channel activation). The ideal way to measure the gating charge is to record gating currents (Bezanilla, 2000). However, due to the slow rate of HERG activation, recording gating currents from the HERG channel is difficult (Smith and Yellen, 2002). Therefore, although $z_{g}$ most likely underestimates the number of gating charges involved in HERG activation (Bezanilla, 2000), it nevertheless helps us compare the mutant channels in their voltage sensitivity of activation. A concern is whether changes in the position or steepness of the activation curve seen in the mutant channels may have contribution from mutation-induced alterations in the inactivation process, because some of the channels may not have recovered from inactivation at the peak of the tail current. Although we cannot absolutely exclude this possibility, we believe this contribution is not significant. We show that the rate of inactivation is not altered by the mutations, suggesting that the inactivation process is little affected.

The time course of activation of WT and mutant channels can be well described by a single exponential function with a time delay. Two parameters are obtained by a linear regression of the relationship between ln(1/$\tau_{c}(V)$) and $V_{t}$: $K_{e}(0)$ (rate constant of opening transition at 0 mV) and $z_{a}$ (gating charge involved in channel opening). Mutation-induced changes in the $K_{e}(0)$ value will indicate how the mutation affects the activation energy involved in the transition from the last closed state ($C_{n}$ in Scheme I) to a transition state, and changes in $z_{a}$ will reveal whether the amount of gating charge involved in this transition is altered.

D466 and D501 may be directly involved in voltage sensing during HERG activation. For both D466C and D501C,
Importantly, the $z_a$ value is markedly reduced, although the $K_v(0)$ value is not altered (in D466C, Fig. 5 C) or even increased (in D501C, Fig. 6 C). Therefore, removing the negative charge at either 466 or 501 reduces the amount of gating charge involved in channel opening, suggesting that in the WT channel aspartates at these two locations deep in the cytoplasmic half of S2 and S3 may contribute to voltage sensing during activation. This may occur through a state-dependent interaction between D466 and/or D501 with a positive charge in the S4, or from a movement of the negative charge(s) relative to the membrane electrical field during gating.

For D466, either scenario is consistent with results from the accessibility test: the thiol side chain at 466 is accessible to extracellular MTSET preferentially in the closed state. It has been suggested that the “D466-equivalent” in the Shaker channel, E293, directly contributes to the channel’s gating charge because removing the negative charge at 293 can reduce the number of gating charges by ~50% (Seoh et al., 1996).

D460 and D509 increase the stability of HERG in the activated state relative to the closed state. D460C and D509C manifest similar changes in the activation gating process: a parallel shift of the activation curve in the positive direction, and a slowing of channel opening without alteration in the voltage sensitivity (reduction in $K_v(0)$, without change in $z_a$). These suggest that cysteine substitution of either aspartate increases the activation energy involved in the transition from the closed state to the transition state during activation, but the amount of gating charge involved in channel activation remains the same. The accessibility tests indicate that thiol side chains at both positions are in a relatively wide open space, accessible to extracellular MTS reagents at both $V_h = -80$ mV (channels in the closed state) and $+20$ mV (channels in activated/inactivated state). This is consistent with the opposite effects of MTSET and MTSES on the two mutants. MTSET modification of 460C decreases the current amplitude, whereas MTSES modification causes a modest increase. MTSET modification of 509C accelerates the rate of channel deactivation, whereas MTSES modification has the opposite effect, and neither alters the 509C current amplitude. Therefore, the effects of MTS modification at both locations are due to the added charge, but not due to the added side chain volume. The mechanism by which D460 and D509 influence the channel’s activated gating process is not clear.

D411 in the S1 domain stabilizes HERG in the closed state. The D411C mutation shifts the voltage dependence of activation in the negative direction and accelerates the rate of channel opening. These observations can be explained by proposing that the negative charge at position 411 can pair with a nearby positive charge when the WT channel is in the closed state. Membrane depolarization induces conformational changes in the S1 domain, or in its relation to the surroundings. These changes need to disrupt the ion pairing before the channel can reach the open state. Therefore, removing the negative charge at 411 can destabilize the channel in the closed state relative to the transition state during activation. This scenario is consistent with two other observations reported here. First, changing $pH_o$ from 7.5 to 6.5 shifts the activation curves of WT and D411C channels so that they superimpose at $pH_o$ 6.5. This in-
crease in [H⁺] may protonate the aspartate side chain at 411, abolishing its stabilizing effect on the closed state and negating the difference between WT and D411C. A corollary of this inference is that in WT-HERG changing pHₐ from 7.5 to 6.5 may favor channel opening by protonating D411. This will offset the positive shift in the voltage dependence of channel activation associated with proton effect on the external surface potential. The dual effect of extracellular protons on HERG gating may explain why changing pHₐ from 7.5 to 6.5 has such a small effect on V₀.₅ of HERG activation (positive shift by ~5 mV, Fig. 14), whereas a similar change in pHₐ can cause a much larger effect on other voltage-gated channels (e.g., V₀.₅ positive shift by ~25 mV in Kv1.5; Zhang et al., 2003). Second, the thiol side chain at 411 is accessible to extracellular MTSET when the membrane voltage is held at ~20 mV (channels in activated/inactivated states), but not at ~80 mV (channels in the closed state). This state dependence of accessibility indicates that during the activation process, there are conformational changes in S1 or in its surroundings at the level of 411, and these changes may cause the 411 side chain to switch from pairing with a nearby residue (not reactive to MTSET) to facing a water-filled crevice (reactive to MTSET).

**How Are the Aspartate Residues Involved in the Deactivation Process of HERG?**

The deactivation process in the HERG channel is much more complex than a simple reversal of the S4 movement. In WT-HERG, the deactivation rate is a function of the voltage and duration of the previous depolarization pulse (Viloria et al., 2000). This can be explained by a time-dependent interaction between the NH₂-terminal PAS domain and the gating machinery, likely the S4-S5 linker (Wang et al., 1998; Viloria et al., 2000). Upon membrane depolarization, the PAS domain can bind to the S4-S5 linker only when the channel reaches the open state. Upon repolarization, the channel cannot close until the PAS domain has dissociated from the S4-S5 linker. Therefore, the deactivation rate will be slower after a stronger or a longer depolarization pulse (more PAS binding to the S4-S5 linker), until the channels are fully activated (maximal PAS binding).

All five mutants accelerate the deactivation rate. Do these five aspartate residues contribute to the deactivation process in an additive manner (e.g., each making an independent contribution to the stability of the WT channel in the open state), or in a concerted manner (e.g., all five working together to stabilize the WT channel in the open state)? We can get some clues from the pHₐ experiments: neutralizing any one of these negative charges, leaving the other four intact, can largely prevent the pHₐ effects on the deactivation process. We propose that there may be a “master-switch” in the HERG channel that keeps the deactivation rate slow. Turning off this switch will set in motion a fast deactivation process. All five aspartates need to be present simultaneously to prevent the master-switch from turning off. What domain(s) may constitute this master-switch? The interaction between the PAS domain and the S4-S5 linker is a good candidate. This is consistent with our previous observation that deleting a large part of the NH₂-terminal region from the HERG channel, including the PAS domain, creates a phenotype similar to that of the mutants reported here: the deactivation rate is markedly accelerated and the response of deactivation rate to changes in pHₐ is markedly reduced (Jiang et al., 1999).

**Is There a Metal Ion Binding Site in the Transmembrane Domains of HERG?**

It has been shown that −3 in S2 (D278) and −6 in S3 (D327) together form a metal ion binding site in the transmembrane domain of the EAG channel, and Mg²⁺ slows EAG activation by binding to this site (Silverman et al., 2000; Papazian et al., 2002; Schonherr et al., 2002). Can this be generalized to HERG? Although the effects of Mg²⁺ on HERG are different from those on EAG, this may result from the intrinsic differences in the gating behavior between the two channels, but not due to a difference in mechanism. A more direct test is to neutralize the “D278-equivalent” in the HERG channel (D460). If this aspartate contributes to a metal ion binding site in HERG, as in the EAG channel, then removing the negative charge should abolish the effects of Mg²⁺ or Ca²⁺ (which affects HERG in the same fashion, and likely by the same mechanism) on HERG.

Our data show that D460C does not alter the effects of Ca²⁺ on HERG. Therefore, there is no evidence for a metal ion binding site in the HERG channel, equivalent to that described for the EAG channels. The effects of Mg²⁺ and Ca²⁺ on the HERG channel can be explained by one or a combination of the following mechanisms. First, extracellular divalent cations can bind to negative surface charges on the HERG protein and alter the membrane electrical field sensed by S4 (Johnson et al., 1999). E518 and E519 in the extracellular S3-S4 linker may constitute the external divalent cation binding site in HERG (Johnson et al., 2001). Second, extracellular divalent cations can enter and block the HERG pore in a voltage- and time-dependent fashion (Ho et al., 1998). This can better explain the biphasic effect of Ca²⁺ on the current amplitude: Ca²⁺ decreases the peak tail current amplitude by blocking the pore at negative voltages. It unblocks at positive voltages, but at the same time interferes with the C-type inactivation process, thus causing an increase in the test pulse current amplitude late during depolarization.
Moving relative to the membrane electrical field either due to a rotation of S2 and/or S3, or due to changes in the crevice around them. (b) There may be a rotation of S1, or the surrounding crevice, at the level of D411. (c) D456 may form an ion pair with S4’s positive charge. And (d) binding of the NH2-terminal PAS domain to the cytoplasmic S4-S5 linker serves as a “master-switch” that controls the rate of channel deactivation.

**Structural Implications of our Findings**

Fig. 16 summarizes what we can conclude (and propose) from our findings. (a) Our data suggest that water-filled crevices penetrate deep into the HERG protein core, reaching the level of 411 around S1 and the level of 466 around S2. (b) Accessibility of side chains at these two positions to extracellular aqueous phase is voltage dependent: 411 side chain is accessible at Vh = 20 mV, whereas 466 side chain is accessible preferably at Vh = –80 mV. Therefore, during alterations in the membrane potential, conformational changes occur in S1 and S2, or in the crevices around them. (c) Side chains at 460 and 509 are in a relatively wide open space readily accessible to the extracellular aqueous phase. A kink in the S3 domain, caused by the proline at position 507 (Fig. 1) (Li-Smerin and Swartz, 2001), may help create this wide crevice between S2 and S3. (d) D411 may form an ion pair with a nearby positive charge when the HERG channel is in the closed state. (e) D466 and D501 are intimately involved in the voltage-sensing machinery in HERG. Given the possibility that the S2 domain may be surrounded by water-filled crevices on a large portion of its surface and carries a modest density of negative charges along its length, it is not unlikely that S2 may move in response to a change in the membrane voltage and that D466 may be a direct contributor to the HERG channel’s gating charge. (f) D456 may play a critical role in channel protein folding and maturation, similar to the role of E283 in the Shaker channel. (g) To account for our data, we hypothesize that there is a “master-switch” in HERG that maintains a slow rate of deactivation. Turning off this master-switch will set the channel in a fast deactivation mode. To keep the master-switch in the ON position requires the simultaneous presence of all five aspartate residues (D411, D460, D466, D501, and D509). A candidate for this master-switch is the NH2-terminal PAS domain.

This study is supported by HL 46451 from the National Heart, Lung, and Blood Institute, National Institutes of Health, and a Grant-in-Aid Award from the American Heart Association/Mid-Atlantic affiliate (G.-N. Tseng).

Lawrence G. Palmer served as editor.

Submitted: 6 January 2003
Revised: 28 April 2003
Accepted: 29 April 2003

**REFERENCES**

Bezanilla, F. 2000. The voltage sensor in voltage-dependent ion channels. *Physiol. Rev.* 80:555–592.

Cole, K.S., and J.W. Moore. 1960. Potassium ion current in the squid giant axon: dynamic characteristic. *Biophys. J.* 1:1–14.

Gandhi, C.S., and E.Y. Isacoff. 2002. Molecular models of voltage sensing. *J. Gen. Physiol.* 120:455–463.

Gandhi, C.S., E. Loots, and E.Y. Isacoff. 2000. Reconstructing voltage-sensor-pore interaction from a fluorescence scan of a voltage-gated K+ channel. *Neuron.* 27:585–595.

Herzberg, I.M., M.C. Trudeau, and G.A. Robertson. 1998. Transfer of rapid inactivation and sensitivity to the class III antarrhythmic drug E-4031 from HERG to M-eag channels. *J. Physiol.* 511:3–14.

Ho, W.-K., I. Kim, C.O. Lee, and Y.E. Earm. 1998. Voltage-dependent blockade of HERG expressed in *Xenopus* oocytes by external Ca2+ and Mg2+.

*J. Physiol.* 507:631–638.

Islas, L.D., and F.J. Sigworth. 2001. Electrostatics and the gating pore of Shaker potassium channels. *J. Gen. Physiol.* 117:69–89.

Jiang, M., W. Dun, and G.-N. Tseng. 1999. Mechanism for the effects of extracellular acidification on hERG channel function. *Am. J. Physiol.* 277:H1283–H1292.

Johnson, J.P., J.R. Balser, and P.B. Bennett. 2001. A novel extracellular calcium sensing mechanism in voltage-gated potassium ion channels. *J. Neurosci.* 21:4143–4153.

Johnson, J.P., F.M. Mullins, and P.B. Bennett. 1999. Human ether-a-go-go-related gene K+ channel gating probed with extracellular Ca2+ Evidence for two distinct voltage sensors. *J. Gen. Physiol.* 113:565–580.

Li-Smerin, Y., and K.J. Swartz. 2001. Helical structure of the COOH
terminus of S3 and its contribution to the gating modifier toxin receptor in voltage-gated ion channels. *J. Gen. Physiol.* 117:205–217.

Liu, J., M. Zhang, M. Jiang, and G.-N. Tseng. 2002. Structural and functional role of the extracellular S5-P linker in the HERG potassium channel. *J. Gen. Physiol.* 120:723–737.

Lu, Z., A.M. Klem, and Y. Ramu. 2002. Coupling between voltage sensors and activation gate in voltage-gated K+ channels. *J. Gen. Physiol.* 120:663–676.

Morais Cabral, J.H., A. Lee, S.L. Cohen, B.T. Chait, M. Li, and R. MacKinnon. 1998. Crystal structure and functional analysis of the HERG potassium channel N-terminus: an eukaryotic PAS domain. *Cell.* 95:649–655.

Papazian, D.M., W.R. Silverman, M.-C.A. Lin, S.K. Tiwari-Woodruff, and C.-Y. Tang. 2002. Structural organization of the voltage sensor in voltage-dependent potassium channels. *Novartis Foundation Symposium.* 245:178–192.

Robertson, G.A., J.W. Warmke, and B. Ganetzky. 1996. Potassium currents expressed from *Drosophila* and mouse eag cDNAs in *Xenopus* oocytes. *Neuropharmacology.* 35:841–850.

Sanguinetti, M.C., C. Jiang, M.E. Curran, and M.T. Keating. 1995. A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the *I_{Kr}* potassium channel. *Cell.* 81:299–307.

Schonherr, R., L.M. Mannuzzu, E.Y. Isacoff, and S.H. Heinemann. 2002. Conformational switch between slow and fast gating modes: allosteric regulation of voltage sensor mobility in the EAG K+ channel. *Neuron.* 35:935–949.

Schreibmayer, W., H.A. Lester, and N. Dascal. 1994. Voltage-clamping of *Xenopus laevis* oocytes utilizing agarose-cushion electrodes. *Pflugers Arch.* 426:653–658.

Seoh, S.-A., D. Sigg, D.M. Papazian, and F. Bezanilla. 1996. Voltage-sensing residues in the S2 and S4 segments of the *Shaker* K+ channel. *Neuron.* 16:1159–1167.

Silverman, W.R., C.-Y. Tang, A.F. Mock, K.-B. Huh, and D.M. Papazian. 2000. Mg2+ modulates voltage-dependent activation in Ether-a-go-go potassium channels by binding between transmembrane segments S2 and S3. *J. Gen. Physiol.* 116:668–677.

Smith, P.L., and G. Yellen. 2002. Fast and slow voltage sensor movements in HERG potassium channels. *J. Gen. Physiol.* 119:275–293.

Terlau, H., J. Ludvig, R. Steffan, O. Pongs, W. Stuhmer, and S.H. Heinemann. 1996. Extracellular Mg2+ modulates slow gating transitions and the opening of *Drosophila* ether-a-go-go potassium channels. *J. Gen. Physiol.* 115:319–337.

Tristani-Firouzi, M., J. Chen, and M.C. Sanguinetti. 2002. Interactions between S4-S5 linker and S6 transmembrane segment mediate folding of shaker K+ channel subunits. *Biophys. J.* 72:1489–1500.