Cooperative Interactions Between R531 and Acidic Residues in the Voltage Sensing Module of hERG1 Channels

David R. Piper^{1, 2}, Jason Rupp¹, Frank B. Sachse^{1,3}, Michael C. Sanguinetti^{1,2} and Martin Tristani-Firouzi^{1,4}

¹Nora Eccles Harrison Cardiovascular Research and Training Institute, ²Department of Physiology, ³Bioengineering Department and ⁴Division of Pediatric Cardiology, University of Utah, Salt Lake City

Key Words

Potassium channel • Inactivation gating • Activation gating • Site-directed mutagenesis • Double mutant cycle analysis

Abstract

HERG1 K⁺ channels are critical for modulating the duration of the cardiac action potential. The role of hERG1 channels in maintaining electrical stability in the heart derives from their unusual gating properties: slow activation and fast inactivation. HERG1 channel inactivation is intrinsically voltage sensitive and is not coupled to activation in the same way as in the Shaker family of K⁺ channels. We recently proposed that the S4 transmembrane domain functions as the primary voltage sensor for hERG1 activation and inactivation and that distinct regions of S4 contribute to each gating process. In this study, we tested the hypothesis that S4 rearrangements underlying activation and inactivation gating may be associated with distinct cooperative interactions between a key residue in the S4 domain (R531) and acidic residues in neighboring regions (S1 – S3 domains) of the voltage sensing module. Using double-mutant cycle analysis, we found that R531 was energetically coupled to all acidic residues in S1–S3 during activation, but was coupled

only to acidic residues near the extracellular portion of S2 and S3 (D456, D460 and D509) during inactivation. We propose that hERG1 activation involves a cooperative conformational change involving the entire voltage sensing module, while inactivation may involve a more limited interaction between R531 and D456, D460 and D509.

Copyright © 2008 S. Karger AG, Basel

Introduction

Human *ether-a'-go-go*-related gene (hERG) channels are primarily expressed in the brain and heart, and abnormally expressed in various tumors [1, 2]. In the heart, hERG1 channels conduct the rapid delayed rectifier K⁺ current (I_{Kr}), which is an important mediator of cardiac action potential repolarization [3, 4]. Reduced hERG1 function, either by disease-causing mutations or channel block by commonly prescribed medications, predisposes to ventricular arrhythmias and sudden death [5]. The critical role of hERG1 channels in the maintenance of normal cardiac electrical activity stems from its unusual gating properties. Slow activation and fast inactivation combine to produce inward rectification, a property that

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2008 S. Karger AG, Basel
1015-8987/08/0213-0037\$24.50/0

Accessible online at:
www.karger.com/cpb

M. Tristani-Firouzi
Pediatric Cardiology, Suite 1500 PCMC
100 N. Medical Drive, University of Utah School of Medicine
Salt Lake City, UT 84113 (USA)
E-Mail mfirouzi@cvrti.utah.edu

limits K⁺ efflux during the plateau phase of the cardiac action potential [5].

HERG1 channel inactivation is intrinsically voltage sensitive and is not coupled to activation in the same way as in the Shaker family of K⁺ channels. First, the time constants of hERG1 inactivation remain voltage dependent at potentials where the channel is fully activated [6-8]. Second, the midpoint of steady-state hERG1 inactivation is more negative than activation. In addition, pore mutations and external cations differentially influence the voltage dependence of activation and inactivation [9-13]. These observations suggest that distinct voltage-sensing mechanisms might underlie activation and inactivation gating in hERG1 channels.

We recently performed a substituted alanine scan of the hERG1 S4 domain and flanking S3-S4 and S4-S5 linkers to determine the comparative roles of single residues in the primary voltage sensor during activation and inactivation gating [14]. Mutations that perturbed activation were scattered throughout the scanned region, with the exception of one helical face of S4. Mutations that perturbed inactivation localized to the helical face of S4 that was mostly devoid of activation-perturbing residues. These findings suggested that the S4 functions as the voltage sensor for activation and inactivation, but that a limited region of S4 contributes to inactivation. Thus, a large repositioning (e.g., translocation and twisting) [15-17] of the entire S4 activates the channel. This is followed by a small conformational change involving a subset of S4 residues that leads to inactivation. These movements in S4 are facilitated by electrostatic interactions between the specific residues in the voltage sensor module. Basic residues in S4 are proposed to interact with acidic residues in S2 and S3 to facilitate channel biosynthesis and to stabilize the open or closed state of *ether-a'-go-go* (EAG) and Shaker channels [18-20]. The recently solved crystal structure of the Kv1.2 channel revealed a salt bridge between R303 in S4 and E226 in S2 in the open conformation [16, 21]. The homologous residues in hERG1 are R531 and D456. R531A was identified as the most perturbing mutant in our alanine scanning mutagenesis study and the only mutation that impacted charge movement, channel opening and inactivation [14]. Substituted tryptophan, glutamine and cysteine scans of S4 basic residues in hERG1 also identified R531 as the most crucial for normal activation gating [22-24].

In this study, we used double-mutant cycle analysis to test the hypothesis that activation and inactivation gating of hERG1 includes distinct cooperative interactions between R531 and acidic residues in the neighboring

transmembrane domains (S1, S2, S3) of the voltage sensing module.

Materials and Methods

Molecular biology and oocyte preparation

Site-directed mutagenesis [25] of WT and mutant *HERG1*, *in vitro* synthesis of cRNA, isolation and injection of *Xenopus laevis* oocytes were performed as previously described [26, 27]. Oocytes were injected with 2-10 ng of cRNA and incubated at 19°C for 2-3 days before use in voltage clamp experiments.

Electrophysiology

To study channel activation, ionic currents were measured with a GeneClamp 500B (Molecular Devices, Sunnyvale, CA) using the two microelectrode voltage clamp (TEVC) technique [27]. Currents were filtered at 1 kHz with an eight-pole Bessel filter, and then digitized at 2 kHz. To study channel inactivation, ionic currents were measured with a CA-1B amplifier (Dagan Corp., Minneapolis, MN) using the cut-open vaseline gap (COVG) voltage clamp technique [28]. We used the more tedious COVG technique to study inactivation as the rapid clamp speed allows us to more accurately resolve the time course of inactivation. In addition, the p/-8 protocol (see below) eliminates the capacitance transient allowing direct measurement of the peak recovered current. Measurements of the voltage dependence for activation were identical when determined using the TEVC or COVG. For analysis of inactivation, currents were filtered at 5 kHz with an eight-pole Bessel filter, and then digitized at 20 kHz. Voltage commands were generated with a personal computer using pCLAMP8 software and a DigiData 1322 analog to digital interface (Molecular Devices, Sunnyvale, CA).

Microelectrodes were pulled from borosilicate glass capillary tubes (World Precision Instruments, Sarasota, FL) to obtain resistances of 0.7-2.0 MΩ for use in TEVC and 0.1-0.5 MΩ for use in COVG when filled with 3 M KCl. All recordings were performed at 22-24°C. For TEVC recordings, the extracellular solution contained (in mM): 96 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, pH 7.6. The recording chamber was perfused at a rate of ~1 ml/min. The same extracellular solution was used for the top and guard chambers in COVG recordings. The intracellular solution in the bottom COVG chamber contained (in mM): 120 K-glutamate, 10 HEPES, pH 7.0. For COVG recordings, the oocyte was permeabilized by adding 0.1-0.3% saponin in 120 K-glutamate, 10 HEPES (pH 7.0) to the lower chamber for 2 min. Linear leak and capacitance currents were compensated by analog circuitry and subtracted on-line using a p/-8 protocol [29] from a holding potential (HP) of -90 mV.

Data analysis

To quantify the energetic perturbation produced by individual mutations, we calculated the free energy difference (ΔG) between the closed-open and open-inactivated transitions in WT channels and compared these values to the energy difference induced by Ala-substitution. The difference in ΔG between WT and mutant channels ($\Delta\Delta G$) reflects the

perturbation in a gating transition produced by the Ala-substitution. To determine the closed-open channel equilibrium, the relative conductance-voltage (g_{rel} -V) relationship was determined by an analysis of deactivating ionic currents at -70 mV. The decaying component of tail currents was fit to a bi-exponential function and the fit was extrapolated back to the moment of repolarization to estimate peak values. Normalized values of tail current magnitude (I_{rel}) were calculated as the fraction of maximal tail current and plotted versus test potential. The resulting g_{rel} -V relationship was fitted to a Boltzmann function, Equation 1:

$$I_{\text{rel}} = \frac{I}{I_{\text{max}}} = \frac{1}{1 + e^{-zF/RT(V_t - V_{0.5})}} \quad (\text{Eqn. 1})$$

where I_{max} is the maximum tail current, V_t is the test potential, $V_{0.5}$ is the voltage required to elicit $0.5 \cdot I_{\text{max}}$, z is the effective valence, F is Faraday's constant, R is the gas constant, and T is room temperature in °K.

The open-inactivated equilibrium (I_{inact} -V) was determined using a three pulse protocol [30]. First, hERG1 channels were activated and inactivated by a 300 ms prepulse to +40 or +80 mV (V_{pre}), then channels were recovered from inactivation by a 10 ms hyperpolarizing interpulse (V_{inter}) that was varied from -110 to +40 mV. Finally, the magnitude of the current conducted by channels that recovered from inactivation in response to V_{inter} was determined by a test pulse (V_{test} = +40 or +80 mV). V_{pre} and V_{test} were +40 mV for WT and mutants with a $V_{0.5}$ for activation similar to WT (D411A, D466A, D466A-R531A, D509A-R531A). For all other mutant channels, V_{pre} and V_{test} were set to +80 mV. Data was not corrected for the amount of current that deactivated during the hyperpolarizing interpulse. However, this error was minimized by excluding voltages where deactivation reduced the recovered current to greater than 15%. I_{inact} -V curves were fitted with a Boltzmann function to estimate $V_{0.5}$ and z for recovery from inactivation.

$V_{0.5}$ and z values from the Boltzmann fitting were used to estimate the ΔG for activation and inactivation using Equations 2 and 3:

$$\Delta G = zF V_{0.5} = 0.02306 z V_{0.5} \quad (\text{Eqn. 2})$$

Where the conversion value 0.02306 kcal/(coulomb*mV*mol) incorporates the value of F .

$$S_{E,\Delta G} = \Delta G \sqrt{\left(\frac{z_{S.E.}}{z}\right)^2 + \left(\frac{V_{0.5S.E.}}{V_{0.5}}\right)^2} \quad (\text{Eqn. 3})$$

Where S_E refers to standard error. Perturbations in the ΔG ($\Delta\Delta G$) were estimated for each mutation by Equation 4 and the standard error (S_E) calculated by equation 5:

$$\Delta\Delta G = \Delta G_{\text{MUT}} - \Delta G_{\text{WT}} \quad (\text{Eqn. 4})$$

$$S_{E,\Delta\Delta G} = \sqrt{S_{E,\Delta G, \text{WT}}^2 + S_{E,\Delta G, \text{MUT}}^2} \quad (\text{Eqn. 5})$$

The free energy of coupling ($\Delta\Delta G_{\text{coupling}}$) was calculated as follows:

$$\Delta\Delta G_{\text{coupling}} = (\Delta G_{\text{MUT1}} - \Delta G_{\text{WT}}) - (\Delta G_{\text{MUT1-2}} - \Delta G_{\text{MUT2}}) \quad (\text{Eqn. 6})$$

$$S_{E,\Delta\Delta G} = \sqrt{S_{E,\Delta G, \text{MUT1}}^2 + S_{E,\Delta G, \text{MUT1-2}}^2} \quad (\text{Eqn. 7})$$

Energetic independence (no coupling between the two mutated residues) was defined as $|\Delta\Delta G_{\text{coupling}}|$ less than or equal to its standard error. Energetic coupling between the two mutated residues was defined as $|\Delta\Delta G_{\text{coupling}}| >$ the standard error of its measurement [31-33].

Data are expressed as mean $\pm S_E$ (n = number of oocytes). Data plots, Boltzmann fits and $\Delta\Delta G$ calculations and were performed using Origin 7.0 (OriginLabs Corporation, Northampton, MA).

Limitations of free energy perturbation analysis

Equilibrium analysis is based on the assumption that complex gating processes can be simplified into transitions between two states (e.g., closed vs. open; open vs. inactivated). While multiple channel states are known to exist, the equilibrium distributions measured here (g_{rel} -V, I_{inact} -V) are well fit by single Boltzmann functions. Moreover, simplified two state models are commonly used to analyze the effect of mutations on steady-state gating properties [34-39]. We chose to estimate ΔG in order to consider the impact of the mutation on both the steepness (z) and midpoint of the distribution ($V_{0.5}$). Our analysis identifies only those residues that experience a change in local energetics in the transition from one state to another. Thus, we are unable to distinguish between a residue that remains relatively static from one that moves into a new, but energetically similar environment. In addition, a mutation may reciprocally alter z and $V_{0.5}$ such that there is no net change in calculated $\Delta\Delta G$ and yet may still impact the kinetics of gating transitions. Finally, substitution of amino acids other than Ala would be expected to cause greater or lesser changes in free energy. However, Ala substitution is commonly used to probe the effect of removing the normal WT function of a residue while preserving the native α -helical structure, usually without introducing a novel interaction.

Results

Effects of neutralizing acidic S1 - S3 amino acids on activation parameters

The topology of a single hERG1 channel subunit is depicted in Fig. 1, together with a sequence alignment of the S1, S2, S3 and S4 domains of hERG1, EAG and Kv1.2 channels. Similar to EAG, six Asp residues are located in the hERG1 S1 - S3 transmembrane domains (Fig. 1). Each Asp residue was individually substituted with Ala and the effects on activation gating were determined. Ala substitution was tolerated at every position with the

Fig. 1. Top panel, Cartoon of hERG1 channel topology. Relative positions of acidic and basic amino acids in the S1 – S4 voltage sensing domain are denoted by (-) and (+) symbols. Bottom panel, Amino acid sequence alignment of hERG1, bovine EAG (bEAG) and rat Kv1.2 channel voltage sensing domain using AlignX software (Invitrogen, Carlsbad, CA). The alignment is similar to that previously reported [22].

Fig. 2. Effects of neutralizing acidic amino acids in S1 - S3 on activation of hERG1 channels. A, Current traces elicited by 2-s step depolarizations from a HP of -80 mV to + 40 mV (WT, D411A, D466A) or +80 mV (D456A, D460A, D509A, R531A), applied in 20 mV increments. Tail currents were elicited by repolarization to -70 mV. For all currents, vertical and horizontal scale bars represent 0.5 μ A and 1 s, respectively. B, Bar graph depicting $\Delta\Delta G$ for channel activation of channels containing the indicated point mutations. D411A (n = 10), D456A (n = 23), D460A (n = 15), D466A (n = 8), D509A (n = 10) and R531A (n = 10).

exception of D501. D501A channels expressed at levels insufficient to accurately quantify activation and inactivation and, therefore, were excluded from further analysis. In a previous report, D501C channels were also noted to express at markedly low levels [22]. Ionic current traces for WT and the remaining Ala substitutions are shown in Fig. 2A. The effects of neutralizing S1 – S3 Asp residues on activation parameters are listed in Table 1. D411A, located near the intracellular end of S1, shifted the g_{rel} -V relationship in the hyperpolarized direction, resulting in a $\Delta\Delta G$ value of -2.2 ± 0.3 kcal* mol^{-1} (Fig. 2B). Thus, mutation of D411 favors the open state of the channel. D456A, D460A and D509A caused depolarizing shifts in the g_{rel} -V relationship (Table 1) and produced positive values in $\Delta\Delta G$ (Fig. 2B), implying that the presence of acidic residues in these positions normally stabilize the open channel conformation. While D466A did not alter the closed-open equilibrium as measured by $\Delta\Delta G$ (-0.03 ± 0.14 kcal* mol^{-1} n = 8), this mutation did

affect the kinetics of channel opening and closing, as noted by the more rapid rate of activation and deactivation (Fig. 2B). As previously reported [14], R531A caused the greatest shift in the g_{rel} -V relationship with a $\Delta\Delta G$ value of 4.3 ± 0.2 kcal* mol^{-1} (n = 10).

Effects of neutralizing acidic S1 - S3 amino acids on inactivation parameters

The voltage dependence of recovery from inactivation was assessed using a triple-pulse protocol as described in Methods and illustrated in Fig. 3A. Representative currents recorded during pulses to V_{inter} when channels recovered from inactivation and V_{test} when channels inactivated are shown in Fig. 3B for WT and two mutant channels, D411A and D456A. The effects of neutralizing S1 – S3 Asp residues on inactivation parameters are listed in Table 1. Charge neutralization of S1 – S3 Asp residues perturbed inactivation to a variable degree, with D456A having the greatest effect and D411A

Mutation	$V_{0.5}$ (mV)	z (e_0)	ΔG (kcal/mol)	n
WT	-17.7 ± 1.2	3.21 ± 0.10	-1.32 ± 0.10	21
D411A	-44.1 ± 0.8	3.42 ± 0.23	-3.48 ± 0.24	10
D456A	10.3 ± 1.2	2.78 ± 0.09	0.65 ± 0.08	23
D460A	3.25 ± 0.9	2.63 ± 0.12	0.20 ± 0.06	15
D466A	-27.3 ± 0.8	2.13 ± 0.14	-1.34 ± 0.10	8
D509A	17.6 ± 1.0	2.54 ± 0.06	1.03 ± 0.06	10
R531A	41.0 ± 1.8	3.03 ± 0.11	2.87 ± 0.16	11
D411A:R531A	25.0 ± 1.5	4.12 ± 0.22	2.37 ± 0.19	7
D456A:R531A	43.8 ± 1.2	2.99 ± 0.06	3.02 ± 0.10	23
D460A:R531A	19.6 ± 1.1	2.47 ± 0.08	1.12 ± 0.07	11
D466A:R531A	-15.6 ± 1.6	1.66 ± 0.06	-0.60 ± 0.07	12
D509A:R531A	-13.9 ± 0.7	2.87 ± 0.13	-0.92 ± 0.06	8

Table 1. Activation parameters for hERG channels derived from g_{rel} -V relationships fitted with Boltzmann functions.

the least effect (Table 1 and Fig. 3C). Similar to our previous alanine substitution scan of the voltage sensor [14], mutation-induced perturbations were greater for activation than for inactivation gating.

Double-mutant cycle analysis of activation and inactivation gating

We used a double-mutant cycle analysis approach to determine whether the coupling of R531 to acidic residues in S1 - S3 differs during activation compared to inactivation. Double-mutant cycle analysis quantifies the independence or dependence of two mutations on a particular gating transition as illustrated by the thermodynamic box in Fig. 4A. If mutation 1 (M1) and mutation 2 (M2) alter channel function independently, then the effect of M1 will be the same irrespective of whether the mutation is made in the background of WT or M2. Thus, $\Delta zFV_{0.5}$ [WT→M1] = $\Delta zFV_{0.5}$ [M2→M1-M2]. If M1 and M2 are energetically coupled then the effect of M1 will be different depending upon whether the background channel is WT or M2 and therefore $\Delta zFV_{0.5}$ [WT→M1] $\neq$ $\Delta zFV_{0.5}$ [M2→M1-M2].

The effects of combining R531A with neutralization of acidic S1 – S3 residues on g_{rel} -V relationships are depicted in Table 2. Examples of currents conducted by these double mutant channels are depicted in Fig. 4B and the averaged g_{rel} -V relationships for channels with single and double mutations are plotted in Fig. 5. Perturbations in $\Delta\Delta G$ for activation caused by the double mutants were not equal to the sum of the perturbations caused by the single mutants (Fig. 4C). The values of $\Delta\Delta G_{coupling}$ for activation varied between $|1.9 - 5.6 \text{ kcal} \cdot \text{mol}^{-1}|$ (Fig. 4D). Thus, during channel opening R531 was energetically coupled to all of the S1 – S3 acidic residues tested.

Mutation	$V_{0.5}$ (mV)	z (e_0)	ΔG (kcal/mol)	n
WT	-87.2 ± 0.9	1.09 ± 0.02	-2.20 ± 0.05	10
D411A	-74.1 ± 2.3	1.11 ± 0.02	-1.89 ± 0.07	5
D456A	-48.13 ± 3.4	1.20 ± 0.04	-1.33 ± 0.10	12
D460A	-76.7 ± 2.2	0.91 ± 0.01	-1.62 ± 0.05	9
D466A	-76.1 ± 2.9	1.00 ± 0.03	-1.75 ± 0.08	14
D509A	-76.8 ± 3.5	0.89 ± 0.03	-1.56 ± 0.09	11
R531A	-83.8 ± 4.1	0.86 ± 0.03	-1.67 ± 0.10	6
D411A:R531A	-66.6 ± 3.2	0.85 ± 0.02	-1.31 ± 0.07	6
D456A:R531A	-80.0 ± 3.4	0.84 ± 0.03	-1.55 ± 0.08	10
D460A:R531A	-97.9 ± 2.6	0.88 ± 0.03	-1.99 ± 0.08	5
D466A:R531A	-73.5 ± 5.6	0.78 ± 0.05	-1.33 ± 0.14	5
D509A:R531A	-98.4 ± 0.9	1.16 ± 0.04	-2.64 ± 0.09	6

Table 2. Inactivation parameters of hERG channels derived from I_{inact} -V relationships fitted with Boltzmann functions.

Fig. 3. Effects of neutralizing acidic amino acids in S1 - S3 on hERG1 channel inactivation. A, WT hERG1 current elicited by the indicated voltage pulse protocol used to determine inactivation parameters (see Methods for details). B, Magnified view of current during recovery from inactivation and onset of inactivation (dotted box in panel A) for WT, D411A and D456A hERG1 channels. Current during V_{inter} was elicited by hyperpolarizing pulses between -110 (-100 mV for D456) and 10 mV, applied in 20 mV increments. For C, Bar graph depicting effect of Ala substitution on $\Delta\Delta G$ for channel inactivation of channels containing the indicated point mutations. D411A (n = 5), D456A (n = 12), D460A (n = 9), D466A (n = 14), D509A (n = 11) and R531A (n = 5).

Fig. 4. Double mutant cycle analysis of hERG1 channel activation. A, The equilibrium between closed and open (or open and inactivated) states of WT, single mutant (M1 or M2), and double-mutant (M1-M2) channels are related by a thermodynamic box. B, Representative currents of double mutant hERG1 channels elicited by the voltage pulse protocol as illustrated in Fig. 2A. For all current traces, vertical and horizontal scale bars represent 0.5 μ A and 1 sec, respectively. C, Bar graph depicting effect of double Ala substitution on $\Delta\Delta G$ for channel activation for D411A-R531A (n = 7), D456A-R531A (n = 23), D460A-R531A (n = 11), D466A-R531A (n = 12) and D509A-R531A (n = 8). The small filled squares represent the mean value $\pm$ SEM for the sum of $\Delta\Delta G$ for individual Ala substitutions. D, Bar graph depicting coupling energies for activation ($\Delta\Delta G_{\text{coupling}}$).

Fig. 5. g_{rel} -voltage relationships for single and double mutant hERG1 channels. N = same as listed in Figure 4 C legend.

The results of double-mutant cycle analysis for inactivation gating are summarized in Fig. 6 and Table 2. The averaged I_{inact} -V relationships for channels with single

and double mutations are plotted in Fig. 7. D411 and D460 are located near the intracellular end of S1 and S2, respectively. Mutation of either one of these residues to

Fig. 6. Double mutant cycle analysis of hERG1 channel inactivation. A, Bar graph depicting effect of double Ala substitution on $\Delta\Delta G$ for channel inactivation for channels with indicated double mutations. D411A-R531A ($n = 6$), D456A-R531A ($n = 10$), D460A-R531A ($n = 5$), D466A-R531A ($n = 5$) and D509A-R531A ($n = 6$). The small filled squares represents the mean value $\pm$ SEM for the sum of $\Delta\Delta G$ for individual Ala substitutions. B, Bar graph depicting coupling energies for inactivation ($\Delta\Delta G_{\text{coupling}}$).

Fig. 7. Inactivation-voltage relationships for single and double mutant hERG1 channels. N = same as listed in Fig. 6 A legend.

Ala in the WT or R531A background resulted in nearly equal energy perturbations (Fig. 6A), such that the coupling energies were zero within the error of measurement (-0.06 ± 0.14 and 0.10 ± 0.14 kcal* mol^{-1} , D411 and D460, respectively; Fig. 6B). For D456, D460 and D509, the energetic perturbation caused by mutation to Ala in the WT background was not the same as in the

R531A background (Fig. 6A). Coupling energies for inactivation gating varied from 0.7 – 1.6 kcal* mol^{-1} for these residues (Fig. 6B). These results demonstrate that R531 was energetically coupled only to D456, D460 and D509 in the inactivation pathway. Thus, R531 coupled to all 5 acidic residues in domains S1 – S3 during activation and to only 3 acidic residues during inactivation.

Fig. 8. Ribbon diagram of open state homology model of hERG1 channel subunit based on Kv1.2 crystal structure. Proposed position of R531 (red) in S4 near D456 in S2 (yellow) D460 in S2 (green) and D509 in S3 (blue). D466 in S2 is depicted in grey. The homology model of Kv1.2 [21] and ribbon diagram were produced using InsightII Molecular Modeling Software (Accelrys Inc, San Diego, CA).

Discussion

In this study, we focused on the role of a key residue (R531) in hERG1 channel activation and inactivation by characterizing potential cooperative interactions with negatively charged residues in domains S1 - S3. Our hypothesis was that activation and inactivation are functionally coupled to S4 movement, but that distinct interactions between charged residues in the voltage sensor module contribute to the different voltage dependencies of these two processes. Our previous alanine scanning mutagenesis of S4 identified R531 as the most critical voltage sensor residue for hERG1 gating. Of the 32 amino acids mutated to Ala, only R531A perturbed charge movement, channel opening and inactivation [14]. Likewise, other studies of the S4 domain in hERG1 also found that R531 mutations caused the greatest perturbation in channel opening [22-24].

Double-mutant cycle analysis is widely used to determine the degree to which residues in different regions of a protein are functionally coupled to a particular process, such as enzymatic activity, binding affinity or ion channel gating. If the change in free energy due to mutation of residue 1 is independent of residue 2, then the coupling energy will be zero, within the error of its measurement. Non-zero $\Delta\Delta G_{\text{coupling}}$ values represent non-independence or coupling [31-33].

As in our previous study [14], the mutation-induced $\Delta\Delta G$ for activation gating were much larger than inactivation gating, consistent with the effective valence of their voltage dependencies. hERG1 inactivation is weakly voltage-dependent ($z \sim 1e_0$) compared to activation ($z \sim 3e_0$), resulting in smaller calculated values of mutation-

induced $\Delta\Delta G$. As an example, a hERG1 mutation that causes +80 mV shift in $V_{0.5}$ for inactivation (without altering z), yields a $\Delta\Delta G$ of 2, while an identical shift in $V_{0.5}$ for activation yields $\Delta\Delta G$ of 6. In our previous S4 mutagenesis scan, we used cluster analysis to define threshold $\Delta\Delta G$ values of 0.5 and 1 kcal $\cdot$ mol $^{-1}$ to represent a significant mutation-induced perturbation in inactivation and activation, respectively [14]. In the present study, the smaller numbers of mutated residues preclude cluster analysis. Irrespective of the magnitude of the individual perturbations in ΔG , we were primarily interested in functional coupling between R531 and the negatively charged residues in S1 - S3.

Double-mutant cycle analysis revealed that R531 was energetically coupled to all 5 acidic residues tested in the S1 - S3 domains during channel opening. Perturbations induced by the double mutations were not a simple sum of the perturbations induced by the single mutations. This does not imply that R531 physically interacts with all Asp residues in the neighboring transmembrane domains during the transition(s) into the open state. Energetic coupling does not require that the coupled residues are adjacent in the tertiary structure. Large scale, concerted allosteric conformational changes are possible between distant regions of the protein [39]. Movement of S4 (and thus R531) during the activation process may require subtle repositioning of the S1, S2 and S3 domains [16].

Our double mutant cycle analysis does not provide direct evidence for a physical interaction between R531 and acidic residues in S1 - S3. However, based on previous studies from several Kv channels (Shaker, EAG, Kv1.2 and hERG1), R531 likely interacts with D456 to

stabilize the open channel state. Papazian and colleagues have extensively studied electrostatic interactions between basic residues in S4 and acidic residues in S2 and S3. They first demonstrated that an electrostatic interaction between E283 in S2 and R371 in S4 in Shaker (corresponding to D456 and R531 in hERG1, respectively) is required for normal channel biogenesis [40]. Subsequently, they proposed that the homologous residues stabilize the open channel state in EAG [20, 41]. These specific interactions were supported by the recently solved crystal structure of Kv1.2, demonstrating a salt bridge between R303 and E226 (corresponding to R531 and D456 in hERG1, respectively) in the open conformation [21]. Moreover, D456, D460 and D509 in hERG1 form a coordination site for external Cd^{2+} implying that these residues are located close to one another [42]. A homology model of hERG1 based on the Kv1.2 crystal structure illustrates the proximity of R531 to this negatively charged pocket in the open state (Fig. 8). Thus, the observed energetic coupling between R531 and D456, D460 and D509 in channel opening may result from repacking of these aspartates with respect to R531.

Double mutant cycle analysis revealed that R531 was coupled to 5 acidic S1 – S3 residues (D411, D456, D460, D466 and D509) during activation gating. In contrast, R531 was not coupled to D411 and D466 (the two acidic residues located toward the intracellular portion of the membrane) during inactivation gating. If R531 is positioned near D456, D460 and D509 in the open state as suggested by the homology model (Fig. 8), then a subtle repositioning of R531 with respect to these residues may be associated with the transition into the inactivated state. This implies that the S4 functions as the voltage sensor for both activation and inactivation, with inactivation coupled to activation. That is, the S4 voltage sensor must achieve its activated position in order for channels to open and inactivate. Indeed, all published measures of hERG1

inactivation involve a depolarizing pre-pulse that causes maximal gating charge displacement. We previously proposed that the voltage dependence of hERG1 inactivation might involve a small rigid body movement of S4 [14]. Our current findings suggest that such a movement may involve cooperative interactions between R531 and D456, D460 and D509. The mechanism of transduction of voltage sensing to channel inactivation was not addressed in the current study, but it has been proposed that this process may involve rearrangements in the linker between S5 and the pore helix (S5-P linker) [43-46].

Zhang et al. [47] recently used a double mutant cycle analysis approach to study cooperative rearrangements within the voltage sensing module of hERG1. They focused on interactions between the most N-terminal (K525, R528) and C-terminal (K538) basic S4 residues and the acidic residues in S1 – S3. K538 was energetically coupled to D411 and this interaction was proposed to stabilize the closed channel state. Both K525 and R528 were energetically coupled to D456. D456 interacted with K525 during early activation transitions and with R528 during later transitions in the activation pathway [47]. Our investigation of R531 complements the findings of Zhang et al. and extends the analysis into the inactivation pathway.

Acknowledgements

We thank Kam Hoe Ng for expert technical assistance. This work was supported by NHLBI/NIH grants HL65299 (M.C.S) and HL075536 (M.T-F) and by an American Heart Association Postdoctoral Fellowship award (D.R.P). We gratefully acknowledge the support of the Richard A. and Nora Eccles Harrison Endowment and the Nora Eccles Treadwell Foundation

References

- 1 Bianchi L, Wible B, Arcangeli A, Taglialatela M, Morra F, Castaldo P, Crociani O, Rosati B, Faravelli L, Olivetto M, Wanke E: *Herg* encodes a K^+ current highly conserved in tumors of different histogenesis: A selective advantage for cancer cells. *Cancer Research* 1998;58:815-822.
- 2 Warmke JW, Ganetzky B: A family of potassium channel genes related to *eag* in *Drosophila* and mammals. *Proc Natl Acad Sci USA* 1994;91:3438-3442.
- 3 Sanguinetti MC, Jiang C, Curran ME, Keating MT: A mechanistic link between an inherited and an acquired cardiac arrhythmia: *Herg* encodes the i_{Kr} potassium channel. *Cell* 1995;81:299-307.
- 4 Trudeau M, Warmke JW, Ganetzky B, Robertson GA: *Herg*, a human inward rectifier in the voltage-gated potassium channel family. *Science* 1995;269:92-95.
- 5 Sanguinetti MC, Tristani-Firouzi M: *Herg* potassium channels and cardiac arrhythmia. *Nature* 2006;440:463-469.

- 6 Rasmusson RL, Morales MJ, Wang S, Liu S, Campbell DL, Brahmajothi MV, Strauss HC: Inactivation of voltage-gated cardiac K^+ channels. *Circ Res* 1998;82:739-750.
- 7 Spector PS, Curran ME, Zou A, Keating MT, Sanguinetti MC: Fast inactivation causes rectification of the i_{Kr} channel. *J Gen Physiol* 1996;107:611-619.
- 8 Zhang M, Liu J, Tseng GN: Gating charges in the activation and inactivation processes of the herg channel. *J Gen Physiol* 2004;124:703-718.
- 9 Johnson JP, Jr., Balser JR, Bennett PB: Enhancement of herg K^+ currents by Ca^{2+} destabilization of the inactivated state. *Biophys J* 1999;77:2534-2541.
- 10 Johnson JP, Mullins FM, Bennett PB: Human ether-a-go-go-related gene K^+ channel gating probed with extracellular Ca^{2+} . Evidence for two distinct voltage sensors. *J Gen Physiol* 1999;113:565-580.
- 11 Sanchez-Chapula JA, Sanguinetti MC: Altered gating of herg potassium channels by cobalt and lanthanum. *Pflugers Arch* 2000;440:264-274.
- 12 Schonherr R, Heinemann SH: Molecular determinants for activation and inactivation of herg, a human inward rectifier potassium channel. *J Physiol* 1996;493.3:635-642.
- 13 Zou A, Xu QP, Sanguinetti MC: A mutation in the pore region of herg K^+ channels reduces rectification by shifting the voltage dependence of inactivation. *J Physiol* 1998;509:129-138.
- 14 Piper DR, Hinz WA, Talluri CK, Sanguinetti MC, Tristani-Firouzi M: Regional specificity of human ether-a'-go-go-related gene channel activation and inactivation gating. *J Biol Chem* 2005;280:7206-7217.
- 15 Durell SR, Hao Y, Guy HR: Structural models of the transmembrane region of voltage-gated and other K^+ channels in open, closed, and inactivated conformations. *Journal of Structural Biology* 1998;121:263-284.
- 16 Pathak MM, Yarov-Yarovoy V, Agarwal G, Roux B, Barth P, Kohout S, Tombola F, Isacoff EY: Closing in on the resting state of the shaker K^+ channel. *Neuron* 2007;56:124-140.
- 17 Tombola F, Pathak MM, Isacoff EY: How does voltage open an ion channel? Annual review of cell and developmental biology 2006;22:23-52.
- 18 Seoh S-A, Sigg D, Papazian DM, Bezanilla F: Voltage-sensing residues in the s2 and s4 segments of the *shaker* K^+ channel. *Neuron* 1996;16:1159-1167.
- 19 Tiwari-Woodruff SK, Schulteis CT, Mock AF, Papazian DM: Electrostatic interactions between transmembrane segments mediate folding of shaker K^+ channel subunits. *Biophys J* 1997;72:1489-1500.
- 20 Silverman WR, Roux B, Papazian DM: Structural basis of two-stage voltage-dependent activation in K^+ channels. *Proc Natl Acad Sci U S A* 2003;100:2935-2940.
- 21 Long SB, Campbell EB, Mackinnon R: Crystal structure of a mammalian voltage-dependent shaker family K^+ channel. *Science* 2005;309:897-903.
- 22 Liu J, Zhang M, Jiang M, Tseng GN: Negative charges in the transmembrane domains of the herg K channel are involved in the activation- and deactivation-gating processes. *J Gen Physiol* 2003;121:599-614.
- 23 Subbiah RN, Clarke CE, Smith DJ, Zhao J, Campbell TJ, Vandenberg JI: Molecular basis of slow activation of the human ether-a-go-go related gene potassium channel. *J Physiol* 2004;558:417-431.
- 24 Subbiah RN, Kondo M, Campbell TJ, Vandenberg JI: Tryptophan scanning mutagenesis of the herg K^+ channel: The s4 domain is loosely packed and likely to be lipid exposed. *J Physiol* 2005;569:367-379.
- 25 Sarkar G, Sommer SS: The "Megaprimer" Method of site-directed mutagenesis. *BioTechniques* 1990;8:404-407.
- 26 Goldin AL: Expression of ion channels by injection of mrna into xenopus oocytes. *Methods in Cell Biology* 1991;36:487-509.
- 27 Stuhmer W: Electrophysiological recording from xenopus oocytes. *Methods Enzymol* 1992;207:319-339.
- 28 Stefani E, Bezanilla F: Cut-open oocyte voltage-clamp technique; in Conn PN (ed) *Methods in enzymology*. San Diego, Academic Press, 1998, vol 293, pp 300-318.
- 29 Armstrong CM, Bezanilla F: Inactivation of the sodium channel. II. Gating current experiments. *J Gen Physiol* 1977;70:567-590.
- 30 Smith PL, Baukowitz T, Yellen G: The inward rectification mechanism of the herg cardiac potassium channel. *Nature* 1996;379:833-836.
- 31 Horovitz A: Double-mutant cycles: A powerful tool for analyzing protein structure and function. *Fold Des* 1996;1:R121-126.
- 32 Horovitz A: Non-additivity in protein-protein interactions. *Journal of molecular biology* 1987;196:733-735.
- 33 Horovitz A, Fersht AR: Strategy for analysing the co-operativity of intramolecular interactions in peptides and proteins. *Journal of molecular biology* 1990;214:613-617.
- 34 Hong KH, Miller C: The lipid-protein interface of a shaker K^+ channel. *J Gen Physiol* 2000;115:51-58.
- 35 Choe S, Stevens CF, Sullivan JM: Three distinct structural environments of a transmembrane domain in the inwardly rectifying potassium channel romkl defined by perturbation. *Proc Natl Acad Sci U S A* 1995;92:12046-12049.
- 36 Monks SA, Needleman DJ, Miller C: Helical structure and packing orientation of the s2 segment in the shaker K^+ channel. *J Gen Physiol* 1999;113:415-423.
- 37 Li-Smerin Y, Hackos DH, Swartz KJ: A-helical structural elements within the voltage-sensing domains of a K^+ channel. *J Gen, Physiol* 2000;115:33-49.
- 38 Li-Smerin Y, Hackos DH, Swartz KJ: A localized interaction surface for voltage-sensing domains on the pore domain of a K^+ channel. *Neuron* 2000;25:411-423.
- 39 Yifrach O, MacKinnon R: Energetics of pore opening in a voltage-gated K^+ channel. *Cell* 2002;111:231-239.
- 40 Papazian DM, Shao XM, Sech S-A, Mock AF, Huang Y, Wainstock DH: Electrostatic interactions of s4 voltage sensor in shaker K^+ channel. *Neuron* 1995;14:1293-1301.
- 41 Silverman WR, Tang CY, Mock AF, Huh KB, Papazian DM: Mg^{2+} modulates voltage-dependent activation in ether-a-go-go potassium channels by binding between transmembrane segments s2 and s3. *J Gen Physiol* 2000;116:663-678.
- 42 Fernandez D, Ghanta A, Kinard KI, Sanguinetti MC: Molecular mapping of a site for Ca^{2+} -induced modification of human ether-a-go-go-related gene (herg) channel activation. *J Physiol* 2005;567:737-755.
- 43 Clarke CE, Hill AP, Zhao J, Kondo M, Subbiah RN, Campbell TJ, Vandenberg JI: Effect of s5p alpha-helix charge mutants on inactivation of herg K^+ channels. *J Physiol* 2006;573:291-304.
- 44 Jiang M, Zhang M, Maslennikov IV, Liu J, Wu DM, Korolkova YV, Arseniev AS, Grishin EV, Tseng GN: Dynamic conformational changes of extracellular s5-p linkers in the herg channel. *J Physiol* 2005;569:75-89.
- 45 Liu J, Zhang M, Jiang M, Tseng GN: Structural and functional role of the extracellular s5-p linker in the herg potassium channel. *J Gen Physiol* 2002;120:723-737.
- 46 Torres AM, Bansal PS, Sunde M, Clarke CE, Bursill JA, Smith DJ, Bauskin A, Breit SN, Campbell TJ, Alewood PF, Kuchel PW, Vandenberg JI: Structure of the herg K^+ channel s5p extracellular linker: Role of an amphipathic alpha-helix in c-type inactivation. *J Biol Chem* 2003;278:42136-42148.
- 47 Zhang M, Liu J, Jiang M, Wu DM, Sonawane K, Guy HR, Tseng GN: Interactions between charged residues in the transmembrane segments of the voltage-sensing domain in the herg channel. *J Membr Biol* 2005;207:169-181.