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kinetics. The mechanism by which the eag domain regulates gating remains unclear. Recent evidence suggests the intracellular loop between the S4 and S5 transmembrane domains (S4-S5 linker) may be important in regulating both activation and deactivation, and that modulation of gating by the eag domain may act via the S4-S5 linker. Here we sought to investigate the role of the S4-S5 linker using site-directed mutagenesis and a combination of electrophysiology and Förster Resonance Energy Transfer (FRET). We found that channels with alanine mutations in the S4-S5 linker exhibited altered gating. All the S4-S5 mutant channels caused an acceleration of deactivation kinetics, except for S543A which had significantly slowed deactivation. Co-expressing an eag domain gene fragment (N-eag) with S4-S5 mutant channels which additionally lacked a native eag domain (Δeag) failed to restore slow deactivation kinetics to the mutant channels. FRET analysis revealed that eag domains tagged with a CFP were in close proximity to each of the S4-S5 mutant channels tagged with a Citrine. Replacement of the entire S4-S5 linker with alanines (hERG [S4-S5]Ala) produced channels with altered gating, including fast deactivation and a far left-shifted steady-state activation curve. Co-expression of hERG Δeag[S4-S5]Ala channels with N-eag did not alter channel gating; however, FRET analysis revealed that N-eag was in close proximity to the mutant channels. Together, these findings suggest that an intact S4-S5 linker is necessary to transduce eag domain-dependent regulation of gating, but it is not required for the eag domain to bind to the channel.

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S4-S5 Linker Flexibility Stabilizes hERG Channel Closed States

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The hERG cardiac K^+ channel is characterized by unusually slow activation and deactivation gating kinetics compared to *Shaker*-like voltage-gated K^+ (Kv) channels. In *Shaker*-like Kv channels movement of the voltage sensor upon depolarization is mechanically transduced by the α-helical S4-S5 linker to S6 activation gate opening. Given the unique gating properties of hERG channels, the details of voltage sensor coupling with the S6 activation gate are of significant interest. We have recently shown that substitution of a glycine residue (G546) within the S4-S5 linker with a leucine residue destabilizes the closed state (left-shifts the $V_{1/2}$ of activation by ~50 mV and accelerates the rate of channel opening) and suggested that flexibility of the linker may be a key determinant of the closed-open equilibrium in hERG channels. Here, we have investigated this further by re-introducing glycine residues within the S4-S5 linker (from position 539 to 552) in the G546L background to determine whether flexibility introduced at different positions within the helix restores WT-like gating. We found a cluster of sites in the N-terminal portion of the S4-S5 linker (D540, R541, Y542, E544) that, when replaced with a glycine residue, rescued the −50 mV shift caused by the G546L mutation restoring a WT-like voltage dependence of activation. None of these mutations affected the voltage dependence of activation in the WT background. All other mutations tested did not rescue WT function and presented the destabilized closed state phenotype that is characteristic of the G546L mutation. These results suggest that flexibility of the N-terminal S4-S5 linker contributes to stabilization of hERG channels in the closed state and that the native glycine, G546, affords this flexibility in WT channels.

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A Proline Scan Approach to Investigate the Activation Gate of hERG Channels

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In *Shaker* channels, the activation gate is formed at the bundle crossing by the convergence of the inner S6 helices near a conserved proline-valine-proline (PVP) motif, which introduces a kink in the helices that allows for electromechanical coupling with voltage sensor motions via the S4-S5 linker. Human ether-a-go-go related gene (hERG) channels lack the PVP motif and the location of the intracellular pore gate and how it is coupled to S4 movement is less clear. Here, we performed a proline scan of the inner S6 helix, from I655 to Y667, to determine the position of the gate. The rationale was that proline-induced S6 disruption would impede gate function when a proline was engineered above, but not below, the native gate region. We discovered that proximal substitutions (I655P-Q664P) impeded gate closure trapping channels in the open state, while distal substitutions (R665P, L666P and Y667P) preserved wild type-like gating. That proline substitutions below Q664 preserved channel gating, while residues above disrupted gate function, strongly suggests that the position of the intracellular gate is formed at Q664. These data are consistent with previous homology model-based predictions[1]. Interestingly, in V659P channels the gate was trapped open, but upon strong hyperpolarization channels slowly activated into a distinct voltage-dependent open state, reminiscent of the well-studied hERG mutation, D540K. The presence of voltage-dependent gating in this mutant suggests that the trapped open phenotype is due to uncoupling of gate closure from voltage sensor gating, rather than an immobilization of sensor movement. Moreover, the activation of V659P channels upon hyperpolarization suggests a 'down' configuration of the voltage sensor that is distinct from that occupied at −80 mV and that leads to hyperpolarization-activated pore opening, as in HCN channels.

[1] Wynia-Smith et. al., J. Gen. Physiol. 132:507-520, 2008.

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Examining the Regulation of Voltage-Dependent S4 Movement in hERG Potassium Channels

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Unlike many Kv channels, hERG channel activation and deactivation are slow and are apparently limited by slow movement of the S4 voltage sensor. In fast-activating Shaker channels, a putative gating charge transfer centre formed in part by F290 has been proposed to interact with S4 charges (e.g. R1, K5) and, thus, modulate voltage-dependent gating. F290 is conserved in hERG (F463), but the relevant residues in the hERG S4 are reversed (K1, R5) and there is an extra positive charge adjacent to R5 (K6). We have examined whether hERG channels possess a transfer centre similar to that in Shaker and if these S4 charge differences underlie slow gating in hERG channels. Of five hERG F463 hydrophobic substitutions tested, only F463W and F463Y shifted the G-V relationship to more depolarized potentials and dramatically (>20x) slowed channel activation. With the S4 residue reversals (i.e. K1, R5) taken into account, this closed state stabilization suggests a role for F463 that is similar to that described for F290 in Shaker. Also consistent with results from Shaker, the hERG K1R mutation left-shifted the G-V relationship and stabilized the open state. In contrast to a predicted stabilization of the open state, R5K caused a moderate right-shift of the G-V and closed-state stabilization. Intriguingly, the neighbouring K6 residue was more important than R5 in hERG voltage sensing, since K6R dramatically slowed and right-shifted activation. Taken together, these data suggest a role for F463 in mediating the closed-open equilibrium, similar to that proposed for F290 in Shaker channels. Furthermore, the hERG channel open state appears to be stabilized by the unique configuration of R5 and K6 side chains. Both charges play important roles, but that of K6 appears more critical at stabilizing the activated state of the channel.

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Measurement of hERG Ion Channel Currents in Lipid Bilayer

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hERG channels (human ether-à -go-go related gene, Kv11.1) play an important role conducting potassium ions in the cardiac delayed rectifier current, IKr, during the repolarization phase of the cardiac action potential. We have measured hERG channels in droplet interface bilayers using membrane preparations made from eukaryotic cells expressing hERG. We find single channel conductance and reversal potentials consistent with previously published patch clamp studies as well as the sensitivity of the measured currents to astemizole, a potassium channel blocker, and E-4031, a hERG specific blocker. This sensitivity is dosage dependent, with IC50 values measured, 91 nM and 12.4 nM for astemizole and E-4031, respectively.

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Functional Analysis of Concatenated Heterotetrameric hERG1 Channels Reveals Requirement for Binding to Four Identical Sites to Achieve Full Activation by hERG1 Agonists

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Recently, several small molecule activators of hERG1 K^+ channels have been discovered, including PD-118057 (PD) and ICA-105574 (ICA). The putative binding sites for these two activators overlap and are located in a hydrophobic pocket between the S5 and S6 segments of adjacent hERG1 subunits. Thus, a homotetrameric hERG1 channel has potentially four identical binding sites. However, it is unclear whether drug binding to more than one site is required for full channel activation. Concatenated hERG1 tetramers containing wild-type and mutant subunits, heterologously expressed in *Xenopus* oocytes, were employed to determine the binding stoichiometry of hERG1 agonists. We previously found single mutation (L646E in S6 or F557L in S5) abolished the effects of PD and ICA, respectively. Concatenated tetramers were therefore constructed to contain a variable number (0 to 4) of the mutant subunits.