The Most Recent, Catalytically Fit HDV Ribozyme Exhibits Minimal Global and Small-Scale Conformational Change upon Cleavage
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Historically, all small catalytic RNAs have been shown to undergo global conformational changes upon phosphodiester cleavage. However, the most recent of numerous hepatitis delta virus (HDV) ribozyme crystal structures has challenged this trend, as this crystal structure suggests that the precursor structure is already product-like in conformation. To further investigate this unusual observation, we have extensively characterized the solution behavior of several three-stranded versions of the HDV ribozyme from the recent crystal structure. Fluorescence gel shift assays show that varying lengths of the 5’ overhang sequence adjacent to the active site result in the same degree of cleavage, whereas noncleavable substrates exhibit significantly more heterogeneity. Complete steady state and time-resolved FRET assays demonstrated that the length of the 5’ overhang sequence adjacent to the cleavage site affects the rates of conformational change upon substrate binding and cleavage. Molecular dynamics (MD) simulations were also performed to gain insight into the atomic behavior and catalytic relevance of the HDV ribozyme from the Chen et al crystal structure. These simulations showed that the magnesium ion resolved near the scissile phosphate results in favorable catalytic geometry compared to simulations neutralized with sodium. Our experimental results demonstrate that, despite previously published results, all forms of the HDV ribozyme undergo significant global conformational changes upon self-cleavage, and our simulations show that C75 is poised to act as a general acid during cleavage.

Deletion of the Amino-Terminus Uncovers an Inactivated State in EAG1 Channels
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ether-a-go-go (Eag) family channels are voltage-gated K⁺ channels that are important in cardiac and neural function and have a well-documented role in disease. One of eight members of the mammalian Eag family, EAG1 channels expression is confined to the central nervous system of the healthy adult. EAG1 channels are also expressed in cancer cells and are implicated in tumor progression and regulation of the cell cycle. Most members of the Eag channel family exhibit voltage-dependent inactivation, yet wild-type EAG1 channels exhibit only voltage-gated activation and deactivation, with no apparent inactivation. Here we report that deletion of the entire intracellular amino-terminal domain uncovers an inactivated channel gating state at depolarizing potentials. We characterized this inactivated state in excised patches from Xenopus oocytes expressing mutant EAG1 channels, and recorded their currents in the inside-out configuration of the patch clamp technique. Similar to wild-type channels, EAG1 with a deleted amino-terminal begins to activate around ~80 mV, however, these channels also inactivate at potentials higher than ~40 mV. The rate of inactivation becomes faster with increasingly depolarizing potentials. Additionally, the kinetics of the EAG1 channels with a deleted amino-terminal are quite slow. Other members of the Eag family of channels exhibit C-type inactivation, which results from collapse of the selectivity filter. To determine whether the inactivation found in this EAG1 mutant was also C-type, we mutated a pore-lining residue known to alter C-type inactivation in both Shaker and hERG1 channels. As a second approach, we asked whether triethylammonium applied to the external surface of the channel altered the rate of inactivation. Instead, we hypothesize that inactivation in the EAG1 channel lacking their N-terminal affects voltage-dependent transitions near the open state. Sensitivity analysis of our HERG model suggests that the effects may be more broad. Manipulation of only the early states failed to optimize the rate of deactivation, but optimization of only the final voltage dependent steps fails to recapitulate the combination of shifted steady-state activation and slope factor for the HERG1b isoform. In conclusion, our data indicate that the N-terminal interacts primarily through modification of voltage-sensitive transitions.
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 S4A3 which had significantly slowed deactivation. Co-expressing an eag domain 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 potassium (K+ or 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 a-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 V1/2 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 releases 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 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 electrophysiological coupling with voltage sensor motions via the S4-S5 linker. Human ether-a-go-go related gene (hERG) channels lack the PVP motif 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 close similarity in gating suggests 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. Interestingly, 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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hERG1 channels (human ether-á-go-go related gene, Kv1.4) 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 used to delineate 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.