597-Pos  Board B377
Reconstitution and Measurement of Ion Channel Ensembles in Droplet Bilayers
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Droplet bilayers have been used recently to reconstitute ion channels from cellular membrane preparations. Frequently large currents are observed resulting from ion channel ensembles which are blockable by the same pharmacological compounds that inhibit cellular ion channel currents measured with patch clamp. We have used droplet bilayers to reconstitute hERG, TRPV1, and Kv 7.1 channels from membrane preparations of HEK cells. We present our measurements of these preparations which yielded ion channel currents pA - nA in magnitude with ion selectivity, voltage dependent and time dependent conductance, and dose-dependent drug inhibition. We will also present preliminary results of our work measuring these membrane preparations in bilayer arrays, which have potential applications for sensing and pharmaceutical screening.

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The Conformation of KcsA’s Selectivity Filter Influences the Opening of its Activation Gate
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Our knowledge about structural-function relationships in K⁺ channels has grown exponentially, mostly because of the use of the prokaryotic version of these membrane proteins. This is especially true for the KcsA K⁺ channel, a two transmembrane, proton-gated K⁺ of these membrane proteins. This is especially true for the KcsA K⁺ channels, for which the structures, for different kinetic states, are known1,2. This minimal version of a K⁺ channel faithfully mimics the most important functional features of its eukaryotic and more complex relatives, the Kv channels. Previously, it has been shown that in KcsA, C-type inactivation is allosterically coupled to activation gating, as well as in other K⁺ channels. This means that the conformational changes associated to the opening of the activation gate (AG) propagate to the channel’s selectivity filter (SF), triggering its collapse conformation. It has been shown before that the more open is the channel’s AG, the deeper inactvates. By extension, the structural conformation of the SF should influence the AG opening. Thus to investigate this possibility, we have systematically studied the opening of the channel’s AG by spectroscopic approaches, in conditions that relieve or promote C-inactivation. Our results suggest that the conformational state of the channel’s SF indeed strongly influence the AG opening.

Reference:

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Engineering hERG Channel Inner Cavity within KcsA Structure
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To provide some structural insights about the molecular basis of the inactivation gating and drug binding properties of the hERG channel, we have reasoned that a chimeric approach in which we recreate the cavity of the hERG channel in to the KcsA structure could potentially lend a structural framework to begin understand inactivation gating and pharmacology of the hERG channel from a structural point of view. Recently, we have made an important advance toward this goal by expressing, purifying and crystallizing a KcsA-hERG chimera that contains the entire inner cavity of the hERG channel. A comprehensive biochemical, functional and crystallographic study will be presented.

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PIP2 Modifies the Free Energy of the Kv1.2 Voltage-Sensor Activation
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Application of phosphatidyl-4,5-bisphosphate (PIP2) to the voltage-gated potassium channel Kv1.2 causes the loss-of-function effect, manifested by positive-shifting the activation voltage dependence. This loss-of-function effect was attributed to the multiple sat bridges formation between positive residues of the resting voltage sensor and a negative headgroup of PIP2. In this work, we uncover the free energy surfaces underlying the entire activation path of the Kv1.2 voltage-sensor embedded into the bare zwitterionic bilayer (palmitoyl-oleoyl-glycerophosphocholine, POPC) and into the zwitterionic bilayer with several PI(2)P lipids (POPC/PI(2)P). Comparison between these free energy surfaces reveals that PI(2)P modifies both, the relative stability of the Kv1.2 voltage-sensor states and the free energy barriers separating them. We posit that these modifications induce the loss-of-function effect observed experimentally.

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K⁺-Dependent Selectivity and External Calcium Block of Shab Potassium Channels
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Potassium channels allow the selective flux of K⁺ excluding the smaller, and more abundant in the extracellular solution, Na⁺ ions. Here we show that Shab is a typical K⁺ channel that excludes Na⁺ under bi-ionic, Nao/Ki or Nao/Rbi, conditions. However, when internal K⁺ is replaced by Cs⁺ (Nao/ CsI), stable inward Na⁺ and outward Cs⁺ currents are observed. These currents show that Shab selectivity is not accounted for by protein structural elements alone, as stated in the snug-fit model of selectivity. Additionally, here we report the block of Shab channels by external Ca²⁺ ions, and compare the effect that internal K⁺ replacement exerts on both Ca²⁺ and TEA blockage. Our observations indicate that Ca²⁺ blocks the channels in a site located near to the external TEA binding site, and that this pore region changes conformation under conditions that allow Na⁺ permeation. Based on our observations and the structural information derived from the NaK bacterial channel, we hypothesize that Ca²⁺ is probably coordinated by main chain carbonyls of the first K⁺-binding site of the pore.

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Time-Dependent Voltage Sensor Relaxation in hERG Channels
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Upon membrane depolarization the voltage sensors (S4) of Kv channels undergo conformational changes that lead to pore opening. Recent studies in Shaker have shown that prolonged depolarization reconfigures S4 into a stable relaxed state that results in a hyperpolarizing shift of the voltage dependence of S4 return and subsequent pore closure compared to that of S4 activation and pore opening. A similar mode-shift has been demonstrated in hERG channels. The voltage-dependence of hERG ionic current deactivation is shifted by ~30 mV relative to that of activation which parallels the shifts of voltage-dependent S4 movement from voltage clamp fluorimetry (VCF) and gating current recordings. In this way, S4 relaxation may contribute to the characteristic slow deactivation gating of the channel. Here we report VCF recordings of S4 movement in I663P channels, in which the pore gate is trapped open and therefore functionally isolated from the voltage sensor, which show a similar ~25 mV mode-shift between S4 activation and deactivation. These data demonstrate that the mode-shift of hERG ionic current is due to voltage sensor relaxation, which is an intrinsic property of the voltage sensor. The time-dependence of hERG S4 relaxation has not yet been fully characterized. We measured the time-dependence of relaxation by applying depolarizing steps of increasing duration and observing the progressive slowing of S4 return. Initial VCF data from ~60 mV steps up to 2 s in duration suggest that entry of S4 into the relaxed state in hERG channels occurs with a $\tau = 271 \pm 106$ ms (n=4). This suggests that voltage sensor relaxation in hERG channels occurs within a physiologically relevant time course that may modify cardiac action potential duration.


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Block of HERG by Extracellular Calcium and other Divalent Ions
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Reduction of the current carried by the cardiac potassium channel HERG can lead to Long QT syndrome, an arrhythmia characterized by a rapid heart rate and reduced cardiac output, which can, in certain situations, be fatal. The effect of extracellular electrolytes on the biophysical properties of the HERG channel have been studied in some detail. In particular, increases in extracellular calcium, magnesium and hydrogen have been shown to slow channel
activation, increase channel deactivation, and shift the G-V curve to more positive voltages. A number of reports have also shown that hydrogen can reduce hERG current by a mechanism that does not involve an effect on channel deactivation and which likely involves pore block. We have shown previously that reducing extracellular potassium results in a significant increase in hERG current reduction by a number of extracellular divalent ions, including calcium, magnesium, cobalt, manganese, and hydrogen. We show here that current reduction of WT hERG by extracellular calcium is voltage dependent and that current reduction of the hERG mutant G628C/S631A is significantly reduced compared to WT at all voltages tested. In addition current reduction of the hERG mutant S631A shows altered voltage dependence compared to WT hERG. These results suggest that calcium and other divalent ions can block hERG and may interact with the outer pore of the hERG channel.

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External Protons Destabilize the Relaxed State of the hERG Channel Voltage Sensor

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Voltage sensor relaxation may contribute to the slow deactivation kinetics of hERG channels. Relaxation stabilizes the voltage sensor in the activated state resulting in a mode-shift, where the voltage-dependence of channel deactivation is shifted ~30 mV compared with that of channel activation. Voltage clamp fluorimetry (VCF) reports of voltage sensor movement show a similar shift in the voltage-dependence of S4 return compared with its activation. However, the mechanism underlying voltage sensor relaxation in hERG is unknown. In Shaker channels, voltage sensor relaxation has been shown to be sensitive to the length and composition of the S3-S4 linker. Here, we assessed the role of S3-S4 linker length and composition in voltage sensor relaxation in hERG channels using two constructs: a chimera with the 9 residue hERG S3-S4 linker substituted with the 31 residue Shaker S3-S4 linker; the other with the 9 residues of the hERG S3-S4 linker mutated to glycine residues. In both cases, the robust shifts in the voltage-dependence of ionic current deactivation relative to that of activation were preserved (~35 and ~29 mV in the 31 residue Shaker linker construct (n=4) and the 9 glycine construct (n=5), respectively), suggesting that length and composition of the S3-S4 linker are not important mediators of hERG channel relaxation. Instead, we demonstrate that the ionic current mode-shift is reduced by external protons in a pH-dependent manner with a pK of 5.5 (n=5). Furthermore, VCF recordings of voltage sensor movement show that acidic pH directly inhibits voltage sensor relaxation. These data suggest that external protons modify voltage sensor relaxation by destabilizing the relaxed conformation and reveal a mechanism by which acidic pH may accelerate hERG channel deactivation gating kinetics.


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Novel Voltage Protocols for Determining hERG Channel Kinetics

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Drug interactions with cardiac ion channels can cause disturbances to normal electrical activity in the heart. In particular, hERG channel block has been linked with QT prolongation and increased pro-arrhythmic risk. Consequently, hERG channel screening, quantifying drug block at different drug concentrations, forms a routine part of cardiovascular safety assessment. Recent initiatives led by the US Food and Drug Administration (FDA) aim to reform the current safety assessment guidelines and in silico approaches are envisaged to form an integral part of the new regime (Sager et al., Am Heart J., 2014.).

Mathematical ion channel representations, embedded within action potential models, can now be constructed through fitting to patch clamp data that are acquired by applying a series of voltage-step protocols. But different models exhibit disparate behaviour under non-standard protocols; including physiologically relevant action potential clamps, or clamps mimicking arrhythmogenic behaviour. We demonstrate the influence the choice of hERG channel representation can have on action potentials simulated from a mathematical ventricular myocyte model when predicting drug-induced effects; indicating the necessity of including accurate descriptions of hERG channel kinetics.

We present novel rapidly fluctuating voltage-clamp protocols, designed mathematically to enable a robust model of hERG channel kinetics to be determined, on a cell-specific basis. The protocols have been tested in manual patch clamp experiments, using hERG-1a transfected HEK 293 cells. The approach will be extended to determine compound-specific models describing hERG channel kinetics in the presence of different drugs, which may enhance the predictive ability of in silico approaches used for cardiovascular safety assessment.

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Stoichiometry of a hERG1 Agonist on Channel Gating

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Human ether-a-go-go-related gene 1 (hERG1) K+ channels mediate repolarization of action potentials in cardiomyocytes. A refined mechanistic understanding of the action of hERG1 agonists may assist development of novel compounds for treatment of arrhythmia associated with acquired or congenital long QT syndrome. RPR-260243 [3(3R,4R)-4-[3-(6-methoxyquinolin-4-yl)-3-oxo-propyl]-3-[3-(2,3,5 trifluorophenyl)-prop-2-ynyl]-piperidine-1-carboxylic acid] (RPR) profoundly slows deactivation and modestly attenuates fast C-type inactivation of hERG1 channels without affecting its kinetics. A Markov model of channel gating incorporating two open states reproduced these experimental findings. A binding site for RPR has been localized to a hydrophobic pocket between two adjacent hERG1 subunits. Additionally, one homotetrameric hERG1 channel contains four identical RPR binding sites. In this study, the stoichiometric basis of RPR-altered hERG1 channel currents was investigated. Concatenated hERG1 tetramers incorporating a variable number of wild-type subunits and mutant subunits (L553A to prevent RPR binding) were constructed and heterologously expressed in Xenopus oocytes. RPR slowed the rate of hERG1 channel deactivation as a function of the number of wild-type subunits (i.e., accessible RPR binding sites) contained within a tetramer. Occupancy of all four available binding sites was required to attain maximum effect. In contrast, occupancy of only three binding sites was sufficient to achieve the maximum shift (+24 mV) in the voltage required for half-maximal inactivation. The distinct subunit stoichiometries associated with RPR-induced changes in hERG1 deactivation and inactivation reflects the different structural basis of the activation (S6 bundle crossing) and inactivation (selectivity filter) gates.

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Molecular Origins of State-Dependent Herg1 Blockade by Dofetilide

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Dofetilide is an antiarrhythmic agent used for the maintenance and protection of sinus rhythm from atrial fibrillations and flutters in individuals. However, dofetilide is better known for its arrhythmogenic activity and blockade of IKr currents and is limited in clinical use. An inadvertent drug blockade of hERG channel predisposes to drug-induced Long-QT2 syndrome in humans. It is well established that dofetilide reversibly binds with moderate-to-high affinity to an open state of hERG1 reversibly but irreversibly blocks open-inactivated state. Here, we used a combination of MD simulations and free energy simulations to unravel key determinants of state-dependent blockade of hERG1. We found that inactivation of the channel and formation of open-inactivate state of hERG1 leads to narrowly localized high-affinity binding pocket for dofetilide. The corresponding Potential of Mean Force (PMF) for drug dissociation from open state of the channel is markedly different. It shows shallow but broad minima. To place findings from atomistic simulations to cellular context we performed kinetic modeling of IKr currents using Markov-net models developed previously. The results were compared to electrophysiological recordings of dofetilide binding to WT and 656C mutants of hERG1 channel.

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The Role of Met-691 in Heme-Dependent Regulation Supports the Presence of a Cytochrome-C-Like Structure in Human BK Channels

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We have recently proposed that the linker region between RCK domains in the cytoplasmic portion of BK channels consists of a Cytochrome-c-like...