Other K Channels

655-Pos Board B424
The Sensing Sites for the Membrane Inner Lipids Regulate the Activation Gating of the KcsA Potassium Channel
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The activity of the ion channels are influenced by membrane lipid compositions and specific lipid molecules are indispensable for maintaining channel activities. For the KcsA potassium channel, the presence of anionic phospholipids such as phosphatidylglycerol (PG) is prerequisite for the channel activity. Previously we demonstrated by means of single-channel current recordings in the asymmetric lipid bilayer that the PG molecule on the inner leaflet, rather than the outer leaflet, renders the KcsA channel highly active. The fluorescent method revealed that the helix-bundle gate is kept open in the PG liposome but not much in the liposomes made of neutral or cationic phospholipids. To elucidate the underlying mechanism of the interaction between anionic lipids on the inner leaflet and the activation gate, charge-neutralizing mutations to positively charged residues were introduced. Several amino acid residues lying at the inner boundary of the membrane were found to be sensitive to the PG effect on the gating. Mechanism underlying lipid-mediated regulation of the activation gating of the KcsA channel will be discussed.

656-Pos Board B425
Dynamics of Conformational Transitions during KcsA Gating from EPR and Solution NMR Experiments
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Despite its apparent simplicity, the potassium channel KcsA possesses a relatively complex gating cycle. Previous studies show conformational changes at the inner gate with an increase in distance from the central axis of symmetry when activated. These changes, coupled to rearrangements at the selectivity filter, are hypothesized to underlie activation, inactivation and modal gating. Defining the dynamic properties of the conformational wave linking the inner gate and selectivity filter is a critical step in understanding the mechanism of gating and permeation in K^+ channels.
Here, we have used CW and DEER Electron Paramagnetic Resonance to measure the opening of the inner gate and NMR to investigate the dynamics of the backbone of KcsA in a pH dependent manner. KcsA G116C was spin-labeled and reconstituted into liposomes or DDM micelles for EPR studies. NMR studies used wild type KcsA Δ125 in micelles (from pH7 to pH3 in 0.5 unit increments) with 2H/15N labeling and a TROSY pulse sequence. Pilot experiments investigating dynamics used a CPMG pulse sequence at pH7 and pH3.
The extent of conformational dynamics from the inner gate ranged from 10.0 Å (closed, pH7) to 23.7 Å (open, pH3) with pH activation. Distance changes were abrupt near the pKa of activation (pH 3.9). Importantly, an observed increase in the width of the population distribution, and hence dynamics, was also observed at this point. Preliminary NMR experiments confirm that this region possesses distinct chemical shifts from a closed versus an open, inactivated state. Ongoing experiments target the process of backbone dynamics at different regions of KcsA. New pulse sequence schemes and kinetic gating mutants of KcsA are being studied to isolate the multiple dynamics components that define KcsA modal gating.

657-Pos Board B426
The KcsA Channel Cytoplasmic Domain Effects on the Inactivation Gating
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The KcsA channel is a representative potassium channel that is activated by changes in pH. Recently, we found that the cytoplasmic domain (CPD) acts as a pH-sensor by observing significant confirmation changes there in response to pH. These changes can influence the opening and closing of the KcsA channel. A selectivity filter region within the KcsA channel is also known to regulate gating by forming activation and inactivation states. However, it is not clear how the CPD and the selectivity filter region coordinate. We therefore made a mutant channel that has the wild-type filter and a CPD with all its negative charges neutralized. We found that this mutant had high activity independent of pH and no inactivation gating, indicating removal of negative charges in the CPD causes removal of inactivation. These results suggest that the CPD primarily regulates activation gating, and conformational changes of the CPD effects on the inactivation gating.

658-Pos Board B427
Thermal Effects and Free-Energy Barrier Differences in the Ion Selectivity Mechanism of KcsA
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The ion selectivity mechanism has been studied for decades, but still lacked details on how thermal fluctuations of water, ions and carbonyl oxygen atoms of the KcsA selectivity filter (KSF) interplay. Here, we apply a step-wise pulling protocol to address the thermal effects and evaluate free-energy profiles based on Jarzynski's Equality. To pull an ion (K^+ or Na^+) from the vestibule ($z = 0$) through KSF, we use a harmonic potential, which couples an ion's z-coordinate with the center of the potential λ. Our protocol uses 24 discrete pulling steps with increments of 1.0 from $\lambda = 0$ Å, and requires relaxation times τ at each step. The relaxation time permits collection of thermal fluctuations to identify stable positions, and to calculate work distributions, which are used to compute free-energy profiles. From the minima of the free-energy profiles, the most stable positions are identified as $<z_{Na^+}> = 5.5$ Å, below KSF, and $<z_{K^+}> = 7.1$ Å, inside KSF, with respect to the center-of-mass of the system, $z = 0$. These positions clearly indicate that KSF selects K^+ more preferentially than Na^+. The corresponding free-energy minima are -7.0 and -7.8 ± 1.0 kcal/mol for Na^+ and K^+, respectively. Beyond these positions, the ions experience different free-energy barriers. With increasing relaxation times τ, the first free-energy-barrier difference between the ions converges to 3.7 kcal/mol at $\tau \sim 9$-11 ns, representing a higher free-energy barrier for Na^+ than for K^+, as the ions further enter KSF. These differences in free-energy minimum positions and barriers are due to (1) the distinguishable flexibility and coordination of the carbonyl oxygen atoms for different binding to the ions, and (2) significantly smaller thermal fluctuations of the first water shell for Na^+ than for K^+.

659-Pos Board B428
Understanding the Molecular Gates of KirBac3.1
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Potassium currents across cell membranes are an essential part of electrical signalling in all cells. KirBac channels are prokaryotic homologs to mammalian Kir channels and superposition of the recently solved structures show that the fold and key features are almost indistinguishable. Potassium currents are proposed to be switched on and off using two molecular gates in the permeation pathway, in the inner helix bundle crossing and in the selectivity filter. Previous studies on KirBac3.1 channels used flux assays to highlight activatory mutants that open these molecular gates. However, there is a lack of single channel data from KirBac3.1 channels and gating is not clearly understood. Here we showed that the single channels currents from recombinant KirBac3.1 channels have two types of gating, a slow gating mode and a high frequency flicker mode. Based on structural information and previously studied mutants we identified mutants that target the slow gating or the high frequency flicker mode. Initial experiments show that the mutant S129R, which is located near the inner helix bundle crossing, affected the slow gating and the mutant F88L, which is located near the selectivity filter reduced the frequency flicker mode of the inward currents. These results provide evidence that there is a molecular gate at the selectivity filter, which is important in the flicker gating seen in KirBac potassium channels, and a molecular gate at the helix bundle crossing that is important for slow gating.

660-Pos Board B429
Activation Gating of KcsA: New Insights into Cooperativity and Energy Landscape from Essential Dynamics Simulations
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Recent crystal structures of the bacterial potassium channel KcsA have shed light on the intermediate and open conformation of the channel (Cuello et al. 2010). Although crystal structures provide excellent insights into different conformations of proteins, they feature only snapshots of dynamical proteins. Therefore, the transition steps and the mechanisms of activation gating are still unknown. In order to elucidate the activation gating dynamics KcsA, we applied essential dynamics (ED) simulations. The successful simulation of activation gating allowed us to calculate the underlying energy landscape by umbrella sampling. The energy profile revealed three energy wells that are separated by two barriers. We identified structural rearrangements of F114 which correspond the first energy barrier. The change in rotameric state of F114 is crucial for initial pore opening. Regarding the second energy barrier, we observed large conformational changes of the TM2 helices which represent the main final activation gate opening. Cooperativity of activation gating was studied by

applying our ED protocol to different number of subunits (SUs). Our simulations showed that the movement of a single SU is not sufficient to open the activation gate. But by moving three SUs by ED simulations, the activation gate opened to the same extent as in the four SUs ED simulation protocol. These finding is in line with fluorescence detection studies, which showed that the SUs act cooperatively during gating (Blunck et al. 2008).

661-Pos Board B430

Gating Motions of KirBac1.1 Cytoplasmic Domain with Respect to Transmembrane Domain Revealed by FRET

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KirBac1.1 is a bacterial inward rectifier potassium (Kir) channel, which, contrary to its eukaryotic homologues, is strongly inhibited by phosphatidylinositol-4,5-bisphosphate (PIP2). The most recent crystal structures of eukaryotic Kir2.2 in complex with PIP2 indicate that the TM-CD linker forms a short a-helix in the presence of PIP2. As a result, the cytoplasmic domain of Kir2.2 moves about 6 angstrom towards the membrane surface. However, the 'KKR' motif in the TM-CD linker of Kir2.2, which directly interacts with PIP2, is absent in KirBac1.1 and the question arises: how does KirBac1.1 cytoplasmic domain move in response to PIP2 inhibition? In the present work, we have made KirBac1.1 tandem tetramer constructs and purified KirBac1.1 tandem proteins successfully. Reconstituted liposome flux assays indicate that the KirBac1.1 tandem protein remains functional, and retains sensitivity to PIP2 inhibition. We introduced two cysteine into the KirBac1.1 tandem tetramer, one at the extracellular loop of subunit 1 (G85 or T120) and one in the cytoplasmic domain of subunit 2 (A273). We labeled these cysteines with a FRET dye pair (Alexa-Fluor 488 and DABCYL) and measured FRET efficiencies in protein samples reconstituted into liposomes, in the absence and presence of PIP2. Our results indicate that the KirBac1.1 cytoplasmic domain moves ~2-3 angstrom away from the transmembrane domain in the presence of PIP2 - opposite the direction suggested from eukaryotic Kir2.2 crystal structures in the presence and absence of PIP2. Reversed PIP2-dependent motions of the cytoplasmic domain with respect to the transmembrane domain between prokaryotic and eukaryotic Kir channels may explain their differential response to PIP2 modulation.

662-Pos Board B431

Simulations of the Helix Bundle Crossing Gate Opening in Kir Channels

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Inwardly rectifying K^+ (Kir) channels are gated by the phospholipid PIP_2. Along the ion permeation pathway, three relatively narrow regions (the selectivity filter -SF, the inner helix bundle crossing (HBC), and the intracellular G-loop) may serve as gates to control ion permeation. A crystal structure of a Kir3.1 chimera [Nishida et al., 2007] captured the cytosolic G-loop gate in ''closed/constricted'' or ''open/dilated'' conformations. 100 ns Molecular Dynamics (MD) simulations studying the PIP_2-driven Kir channel activation of the Kir3.1 chimera led us to propose a molecular mechanism of the G-loop gate opening [Meng et al., 2012]. However, opening of the HBC gate was not observed throughout this simulation. Mutagenesis and single-channel recording studies in our lab showed that a proline mutation on the inner helix of the Kir3.4 channel dramatically increased the open probability of the channel [Jin et al., 2002]. We introduced the corresponding M170P mutation on the Kir3.1 chimera structure and ran 100 ns long simulations of four mutant channel systems: dilated and constricted M170P Kir3.1 chimera in the presence (holo) and absence (apo) of PIP_2, using the GROMACS program [Hess et al., 2008]. Three potassium ions present in the SF passed through the HBC gate in the system of the holo dilated M170P Kir3.1 chimera within the 100 ns simulation time. Minimal distance measurements indicated that the HBC gate was able to open only when PIP_2 was present and the G-loop gate was stabilized in the open state. Principal component analysis revealed coupled conformational changes in the Slide helix, DE- and LM-loops, possibly related to the opening of the HBC gate. Moreover, unique residue interactions within the transmembrane domains were observed in the dilated holo system. Predictions of these models are being tested experimentally.

663-Pos Board B432

Native Currents in Hepatocytes with Characteristic Properties of Kir2 Channels

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We investigated the electrophysiological properties of parenchymal liver cells (hepatocytes) by using the perforated patch-clamp technique with Amphotericin B. We found that dissociated mouse hepatocytes exhibited native currents with characteristic properties of inward rectifier potassium (Kir) channels, subfamily 2, which has not been previously reported. Currents were constitutively active and stable for longer than 30 min during recording, with a current density of -16.2 ± 1.7 pA/pF ($V_m = -114$ mV, current at $[K^+]_{out} = 5$ mM minus current at $[K^+]_{out} = 0$ mM, n=14 cells). Currents exhibited strong and ''steep'' inward rectification, with essentially no outward current at voltages more positive than -20 mV, as typically seen with Kir2 channels. The reversal potential approximated the predicted E_K (-81.8 ± 0.4 *vs.* -84.4 mV, $[K^+]_{in} = 134$ mM and $[K^+]_{out} = 5$ mM). Varying $[K^+]_{out}$ over a range of 5 to 144 mM showed that E_{rev} was strongly dependent on $[K^+]_{out}$, with a Nernstian slope of 58.8 mV/decade, demonstrating that the currents are highly selective for K^+. Currents were fully blocked by external Ba^{2+}, with $K_{1/2} = 2.7 \pm 0.2$ μM ($V_m = -94$ mV, $[K^+]_{out} = 60$ mM, n=6 cells). This $K_{1/2}$ value is in close agreement with published data on Ba^{2+} block of homomeric Kir2.1 channels (Liu GX et al. *J Physiol* 2001). The currents were not significantly inhibited by acidification of the bath solution or pipette solution, which argues against a contribution of pH-sensitive Kir2 subunits such as Kir2.2 or Kir2.3. We thus hypothesize that the molecular identity of the observed currents is homomeric Kir2.1. Additional experiments are underway to test this hypothesis and to elucidate the physiological role of native Kir2 channels in hepatocytes.

664-Pos Board B433

Reduced PIP2 Binding to KCNJ2 (M307I) Channels is Linked to Type 1 Andersen-Tawil Syndrome

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Background: Inward rectifier potassium channels play a key role in setting and maintaining the resting membrane potential and regulating excitability in various tissues. Kir2.x subfamily members mediate the cardiac inward rectifier potassium current (IK1). KCNJ2 encodes Kir2.1, the pore-forming alpha subunit responsible for cardiac IK1, and the mutations in this gene are associated with type 1 Andersen-Tawil Syndrome (ATS1). A Kir2.1 missense mutation, M307I, has been identified in a Korean family with ATS1. We found that the ATS1-associated M307I mutation is a loss-of-function mutation in KCNJ2 that mediates a dominant-negative effect on wild-type (WT) channels. M307I is located in the intracellular C-terminal domain in a region known to be associated with putative phosphatidylinositol 4,5-bisphosphate (PIP2) binding and channel trafficking. Here we explored the mechanisms underlying the dominant-negative effect of the mutation.

Methods and Results: Human Kir2.1 was subcloned into pFlag-CMV vector and pFlag-Kir2.1-M307I was generated by site-direct mutagenesis. The Flag-Kir2.1-WT and Flag-Kir2.1-M307I were expressed in HEK293 cells and affinity purified. PIP2-binding was assessed using a Lipid-bead-protein pull-down assay with cell lysate and Protein-lipid overlay assay with purified proteins. The electrophysiological data showed that the M307I mutant channel significantly reduces whole cell current densities when co-expressed with Kir2.1-WT channels. Immunofluorescence (IF) staining assays reveal that M307I channels exhibit normal membrane trafficking. PIP2 binding assays show that Flag-Kir2.1-M307I channels exhibit dramatically decreased binding to PIP2 compared to WT channels.

Conclusions: M307I is an ATS1-associated, loss-of-function missense mutation in KCNJ2 that mediates a dominant-negative effect on WT channels by reducing PIP2 binding to the channel.

665-Pos Board B434

Targeting of $K_{ir}2.1$ and Downregulation of Inward Rectifier K^+ Current by miR-212

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Downregulation of inwardly rectifying K^+ channels contributes to an increased risk of cardiac arrhythmia in heart failure and to impaired cerebral arterial dilation in chronic alcohol consumption. The downregulation mechanism is unknown, although post-transcriptional regulation of gene expression by microRNAs is a strong possibility that has not been fully investigated. miR-212 is markedly augmented in heart failure and in chronic alcoholism, and is predicted by bioinformatic algorithms to target $K_{ir}2.1$, the predominant inward rectifier K^+ channel expressed in heart and arterial smooth muscle. We developed a fluorescence-based assay for identifying microRNA targets, using mCherry red