states. By our new method, 'cryo-positive stain EM', the microtubule-bound stalks, as well as the ring-like heads and curved tails, were clearly observed. In the no nucleotide state (with apyrase; the post-powerstroke state), the majority of dynein images showed two rings superimposed, suggesting close association of the two heads. When ATP and vanadate were added (the ADP●Vi, pre-powerstroke state), one of the heads moved with respect to the other. There was no detectable difference in the orientation of the stalks between the two nucleotide states; the stalks always pointed at the same angle towards the minus end of the microtubule to which they were bound (Ueno et al., 2008). The results disagree with models in which the stalk rotates on the microtubule and acts as a lever arm to amplify structural changes.
Rotation of the tail relative to the head would change the distance between the stalk tip and the tail-microtubule attachment point. In fact, some ADP●Vi images clearly showed the tail in a more extended state. Based on these results, we propose a new model, in which dynein pulls a microtubule by shortening the distance between its head/stalk and the tail-microtubule attachment, without rotating the stalk.

Platform AZ: Voltage-gated K Channels-Gating

3229-Plat
Thermodynamic Properties of Ionic Currents in *shaker* K^+ Channel Heterotetramers with Different Stoichiometries of ILT Mutations and/or Quadruple Gating Charge Neutralization
Dominique G. Gagnon, Francisco Bezanilla.
University of Chicago, Chicago, IL, USA.
The concurrent substitutions V369I, I372L, and S376T (ILT) in the S4 segment N-terminal end of voltage-dependent *Shaker* K^+ channels uncouple gating currents from ionic conduction (Smith-Maxwell *et al.*, 1998). This ILT mutation is believed to affect the last concerted step for channel activation. We aimed at determining the effect of temperature on steady-state and kinetics of activation of heterotetramers with different stoichiometries of ILT mutations. In addition, we also studied the single voltage sensor *Shaker* containing the ILT mutation in its voltage sensor. The heterotetrameric channels were encoded by concatemerized cDNA of *Shaker* zH4 Δ(6-46), expressed and studied in *Xenopus* oocytes using cut-open oocyte voltage-clamp under temperature ranging from 5-20 °C. The concatemeric channels $4wt_{ILT}$, $3wt_{ILT}$/wt, $2wt_{ILT}$/2wt, wt_{ILT}/3wt and wt_{ILT}/3mut (mut=R362Q/R365Q/R368N/R371Q) were studied. For all constructs, the amplitude of the ionic currents decreased by about 50-75% and the weighted time constants of activation increased by ten to fifteen fold when going from 19 to 5 °C, in a reversible manner. We also measured the steady-state voltage dependence of the conductance to estimate the entropic change during the final transition. The enthalpic and entropic components estimated from the temperature dependence of kinetics of activation for the different constructs provide essential information about the nature of the conformational changes and interactions between subunits. This information is used to refine the proposed model of independent voltage sensors followed by a concerted opening step.
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3230-Plat
A New Approach to the Structural Investigation of the Voltage-Sensitive Domain of Voltage-Gated Cation Channels as a Function of the Transmembrane Voltage
Jing Liu[1], Venkata Krishnan[1], Joseph Strzalka[2], Andrey Tronin[1], J. Kent Blasie[1].
[1]University of Pennsylvania, Philadelphia, PA, USA, [2]Advanced Photon Source, Argonne National Laboratory, Argonne, IL, USA.
Two fundamentally different approaches, self-assembly from solution and directed-assembly at the water-gas interface, are effective in the vectorial immobilization of the expressed voltage-sensitive domain of the KvAP channel on a suitably alkylated surface of silicon. The solvation of the immobilized protein was subsequently exchanged to form a phospholipid bilayer by incubating in a phospholipid-detergent solution in the presence of BioBeads. The formation of the protein monolayer and the vectorial-orientation of the protein molecules therein were investigated via interferometric X-ray reflectivity. The electron density profile of the tethered protein monolayer is consistent with the profile computed from the crystal structure, irrespective of the preparation procedure. Formation of lipid bilayer will require confirmation via neutron reflectivity using deuterated phospholipid. This approach enables an investigation of the structure of the VSD itself, as well as within the intact Kv-channel, as a function of the applied transmembrane voltage via a number of time-resolved techniques.

3231-Plat
The Importance of Ion Binding for Potassium Channel Inactivation and Recovery
Albert C. Pan[1], Sudha Chakrapani[1], Vishwanath Jogini[2], Luis G. Cuello[3], Doris M. Cortes[3], Eduardo Perozo[1], Benoit Roux[1].
[1]University of Chicago, Chicago, IL, USA, [2]D. E. Shaw Research, Hyderabad, India, [3]Texas Tech University, Lubbock, TX, USA.
Potassium channels control the flow of ions across cell membranes with gating mechanisms involving conformational changes at the intracellular gate and the selectivity filter. Opening of the intracellular gate via external stimuli (activation) results in a transient period of conduction before the selectivity filter undergoes a conformational change, which constricts the permeation pathway (inactivation). When the applied stimulus is removed and the lower gate closes (deactivation), the filter slowly resets to a conductive conformation (recovery from inactivation). Using the KcsA channel as a prototypical model system to examine these issues, a combination of computer simulation (all-atom free energy and potential of mean force computations as well as transition pathway determination using the string method with swarms-of-trajectories) and experiment (electrophysiology and X-ray crystallography) is used to provide new insight into the microscopic mechanism that underlies inactivation and recovery from inactivation. An ion binding event is revealed as a crucial step in resetting the inactive filter during the recovery from inactivation.

3232-Plat
Charge Reversion to Charge Carrying Positions of S4 in Voltage Gated Shaker K-Channels
Vivian González-Pérez[1,2], Katherine Stack[1], **David Naranjo**[1].
[1]Universidad de Valparaíso and CINV, Valparaíso, Chile, [2]Programa de Doctorado en Ciencias mención Neurociencia, Valparaíso, Chile.
Voltage gated Shaker potassium channels increases open probability (Po) by 20-fold with a ~6 mV depolarization. Such high voltage sensitivity is mostly due to the electrophoretic transmembrane relocation of four arginines residues in each of their four voltage sensing protein domains (VSD). These arginines movement across the electric field make possible channel opening upon membrane depolarization. We tested if the positions occupied by the voltage sensing arginines could carry acidic residues. We mutated three of these positions to aspartate: Arg362, Arg365, and Arg368 on an N-type inactivation removed background Shaker channel. All mutations were introduced with the use of the QuikChange kit and the mutation was verified by sequencing. Heterologous expressed in Xenopus oocytes, all mutants showed levels of expression comparable to that of the native channel. To determine voltage sensitivity of these charge reverting modified channels, we measured the voltage dependency of Po at voltages negative enough to observe only sporadic single channel openings in membrane patches containing hundreds of channels. From the exponential relation between Po and voltage we estimated the effective valence of opening in the range of Po ~10-6. All charge mutants showed an effective valence ~50% of that of the native Shaker. These results together with the comparable level of channel expression in oocytes are consistent with the idea the voltage sensitive positions in S4 are not specific for basic residues.
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3233-Plat
Sequential Electrostatic Interactions between E160 in S2 and Arginines in S4 During Voltage Dependent Activation of Kv7.1 Channels
Dick Wu, Kelli Delaloye, Mark Zaydman, Jianmin Cui.
Washington University, Saint Louis, MO, USA.
The fourth transmembrane segment of Kv channels, S4, contains a series of positively charged residues that imparts voltage sensitivity to the channel. Because the insertion of a highly charged peptide into a hydrophobic lipid environment is energetically unfavorable, electrostatic interactions with countercharges in the protein and phospholipids are required to lower this energy barrier. However, once the protein has been inserted into the membrane, what further role do these interactions play? In functional channels, electrostatic interactions are assumed to stabilize voltage sensor movement from a resting to an activated conformation. Although this assumption is at the crux of many models of voltage dependent gating, experimental evidence specifically examining these interactions in functional channels is incomplete. Here, we demonstrate in Kv7.1 channels that the first glutamate in S2, E160 (E1), form state dependent electrostatic interactions with arginines in S4. We used charged MTS reagents to directly probe the environment around E1 after mutating E1 to cysteine. We found that $MTSES^-$ but not $MTSET^+$ modifies E1C, suggesting a positively charged environment around E1. Mutations neutralizing or reversing the charge of the first or fourth arginine in S4 (R1 or R4) change the polarity of the environment around E1C such that $MTSET^+$ modifies E1C in the

presence of these secondary mutations. Therefore, R1 and R4 both contribute to the positive electrostatic environment around E1. Moreover, $MTSET^+$ modification of E1C with R1E could only occur at hyperpolarizing voltages but not at depolarizing voltages, suggesting that R1 is proximal to E1 only at the resting state but moves distally at the activated state. Overall, our data is consistent with a mechanism where arginines interact sequentially with E1 as S4 moves from a resting to an activated conformation.

3234-Plat
A Single S4 Arginine is Sufficient for Voltage Sensitivity in the Hv1 Proton Channel
Ingrid Carvacho[1,2], I. Scott Ramsey[3], David E Clapham[1,2].
[1]Children's Hospital Boston, Boston, MA, USA, [2]Harvard Medical School, Boston, MA, USA, [3]Virginia Commonwealth University, School of Medicine, Richmond, VA, USA.
Voltage-gated proton conductances (G_{vH+}) are found in a variety of cell types (e.g. alveolar epithelia and phagocytes) where they mediate an H^+-selective transmembrane ion efflux that alkalinizes the cell and provides charge compensation for NADPH oxidase. The voltage sensor domain (VSD) protein Hv1 is required for native G_{vH+} and sufficient to reconstitute the hallmark biophysical features of G_{vH+} in heterologous expression systems. Conserved Arg residues in the S4 transmembrane helix of VSD proteins are believed to constitute the primary voltage sensing elements. Voltage-dependent conformational rearrangements of S4 thus drive channel gating. The Hv1 contains three putative voltage-sensing arginines (R205, R208 and R211) in S4. In order to examine the contribution of S4 Arg residues to voltage-dependent activation in Hv1, we mutated each to Ala and measured expressed H^+ currents in voltage-clamped 293T cells. The effect of single mutations on the apparent threshold for voltage-dependent activation (V_{THR}) with symmetrical [H^+] ranged from negligible (R205A) to +77 mV (R211A). In order to determine the minimal number of S4 arginines that are required for channel opening, we constructed double mutations in S4 (R205A-R208A, R208A-R211A and R205A-R208A). Mutations bearing a single Arg in either position 208 or 211 generated measurable H^+ currents with dramatically shifted V_{THR} values (>+90 mV). A unique biophysical feature of G_{vH+} is the coupling of voltage and pH gradient sensing: voltage-dependent activation shifts ~40mV per pH unit change in the H^+ gradient ($\Delta pH = pH_{OUT} - pH_{IN}$). Interestingly, the slope of the $V_{THR}/\Delta pH$ relation was similar to *wt* Hv1 for all of the mutations tested. Our results demonstrate that a single S4 Arg is sufficient for voltage and ΔpH sensing in Hv1 and suggest that S4 arginines differentially contribute to the voltage sensing mechanism.

3235-Plat
Probing Energetic Contributions of Aromatic Residues at the Intracellular Gate of Shaker Potassium Channels
Stephan A. Pless, Ana Niciforovic, Christopher A. Ahern.
University of British Columbia, Vancouver, BC, Canada.
Voltage-gated potassium channels contribute to cellular excitability by selectively gating the transmembrane passage of potassium ions. The intracellular bundle crossing of these channels is populated by aromatic phenylalanine and tyrosine residues that have been proposed to form a hydrophobic seal in the closed conformation of the channel. One such residue, Phe481, has resisted previous study by traditional site-directed mutagenesis because non-aromatic substitutions at this site fail to produce functional channels. We therefore expressed Shaker potassium channels carrying subtly altered phenylalanine residues with one, two or three added fluorine atoms to their aromatic ring, a manipulation which serves to serially reduce the negative electrostatic potential on the face of the aromatic while leaving the size and hydrophobicity of the side-chain virtually unperturbed. These unnatural phenylalanine derivatives were introduced at either of two positions, Phe481 and Phe484, near the bundle crossing with the in vivo nonsense suppression method to directly study the contribution of the electrostatic component of the side-chain to channel gating. In each case, the fluorinated phenylalanine side-chains were well tolerated, producing potassium channels with normal voltage-dependent activation and robust expression, albeit reduced when compared to wild-type channels. Serial fluorination at Phe481 lead to a stepwise left-shift of the conductance-voltage relationship (~6 mV for each added fluorine atom) and exponential fits showed channel deactivation slowed significantly at −60 mV, with time constants of ~3 ms and ~160 ms, for wt and the tri-fluorinated phenylalanine derivative, respectively. Conversely, serial fluorination at Phe484 had no effect on the voltage-dependence of activation or the time course of deactivation. Taken together, these results suggest that an electrostatic component of Phe481, but not Phe484, serves to stabilise the closed state of Shaker potassium channels.

3236-Plat
Strict Structural Requirements for Cholesterol to Inhibit BK Channels Point to Specific Steroid-Protein Interactions
Anna N. Bukiya[1], Aditya K. Singh[1], Jitendra D. Belani[2], Scott Rychnovsky[2], Alejandro M. Dopico[1].
[1]University of Tennessee HSC, Memphis, TN, USA, [2]University of California, Irvine, CA, USA.
Cholesterol decreases large conductance, voltage/calcium-gated potassium (BK) channel activity (NPo), an action that solely requires the channel pore-forming (α) subunit and a minimum phospholipid environment (Crowley et al., 2003). This cholesterol action is attributed to cholesterol-induced tight packing of bilayer lipids (Chang et al., 1995). Cholesterol modulation of ion channels *via* direct protein-steroid interactions, however, is increasingly recognized (Epshtein et al., 2009). Cholesterol analogs have been widely used to distinguish between lipid bilayer-mediated and specific protein recognition mechanisms. Thus, we probed cholesterol analogs on BK α subunits cloned from rat cerebral artery myocytes ("cbv1"; AY330293) after channel reconstitution into 3:1 (w/w) POPE:POPS bilayers. Cholesterol (33 mol%) decreased cbv1 NPo by ≈25%. In contrast, 5-cholenic acid-3β-ol, having a carboxyl group at the lateral chain end, failed to decrease NPo, underscoring the importance of a hydrophobic chain for sterol insertion into the bilayer hydrophobic core and channel inhibition. Coprostanol and cholestanol having the A/B junction in *cis* and *trans,* respectively, also decreased NPo (≤25%). In contrast, cholesterol, coprostanol and cholestanol epimers, having the C3-hydroxyl group in α-configuration, failed to decrease NPo. Therefore, a β-conformation in the hydroxyl is necessary for these monohydroxy-sterols to inhibit BK channels, strongly suggesting specific, steroid-protein interactions. Moreover, we probed the cbv1 channel with enantiomeric cholesterol (*ent*-cholesterol), which has physico-chemical properties similar to those of cholesterol yet can be differentially sensed by protein sites, as demonstrated by the lack of viability of *C. Elegans* when only *ent*-cholesterol is present (Crowder et al., 2001). Remarkably, *ent*-cholesterol repeatedly failed to reduce cbv1 NPo, buttressing the idea that cholesterol inhibition of BK channels requires steroid recognition by protein site(s), likely present in the cbv1 subunit itself.
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Platform BA: Emerging Single Molecule Techniques II

3237-Plat
High-Resolution smFRET in a Microfluidic Gas Exchange Platform
Yann Gambin[1], Edward A. Lemke[2], Virginia Vandelinder[3], Alexander Groisman[3], Ashok A. Deniz[1].
[1]The Scripps Research Institute, San Diego, CA, USA, [2]EMBL, Structural and Computational Biology Unit, Heidelberg, Germany, [3]University of California, San Diego, San Diego, CA, USA.
Single-molecule Förster Resonance Energy Transfer (smFRET) is a powerful technique providing new insights in the physics and chemistry of bio-molecules. The smFRET signal quality depends on the photon-flux that can be harvested from the fluorescent dyes. Mere use of high excitation intensities produces counter-productive results, mainly because oxygen-mediated photobleaching of the fluorophores rapidly destroys useful FRET signals. Here, we developed a microfluidic device that ensures a large decrease in the oxygen content of buffers in-situ, allowing us to substantially reduce photobleaching even at high laser powers and obtain dramatic enhancement of signal for several dye-pairs. The principle of the deoxygenation is simple: the microchannels carrying the samples are flanked by large channels ventilated by nitrogen; oxygen is removed by molecular diffusion through porous walls. The device combines this deoxygenation with on-chip mixing and generation of dual-component triplet quenchers (increasing the burst brightness), and also has laminar-flow mixing for kinetic studies.
The increased photon flux obtained in the flow device leads to resolution improvements in two key dimensions: (i) it reduces considerably the time (by a factor of ~ 10 from the typical 500μs) needed to collect high-quality FRET signal, thus providing higher time-resolution, and (ii) it allows the use of higher thresholds, which significantly reduces the width of the smFRET distributions and allows a better resolution of molecular subpopulations. In contrast with a popular enzymatic oxygen scavenger, the method can be used for de-oxygenation in denaturing conditions, hence enabling studies of protein folding. Overall, the platform combines multiple enabling features that can accommodate a range of equilibrium and kinetic and biochemical experiments, along with