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.
Support: AA11560 and HL077424 (AMD), UTHSC Neuroscience Postdoctoral Fellowship (AB).

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