inactivation process in WT channels. To investigate the structural changes that underlie the unusual behaviour of this mutant, we performed a series of MD simulations of the pore domain of WT hKv1.5 and T480A. Analysis of the trajectories shows that T480A affects the stability and flexibility of the filter region and the surrounding pore loop. These results show that residue T480 (located outside the pore region that determines the integrity of the selectivity filter) affects the stability of the filter and influences C-type inactivation.

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Free Energy Landscape for the Inactivation of the KcsA Potassium Channel

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The potassium ion channel, KcsA, gates the passage of ions through cell membranes in response to a change in pH. Recent experimental results have demonstrated the existence of two gates in KcsA: an intracellular gate and a gate at the selectivity filter. Lowering the pH opens the intracellular gate allowing ions to pass. After a period of time, however, the channel inactivates by constricting the selectivity filter and impeding the flow of ions even though the bottom gate remains open. We have used path-based molecular dynamics simulations to probe the detailed mechanism of this phenomenon by finding dynamical pathways to inactivation in KcsA. We have computed free energies and rates of inactivation that agree with recent experimental results. We also provide a molecular rationalization for the coupling between the opening and closing of the lower gate and the inactivation of the selectivity filter.

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Influence Of The Kcsa C-terminal Domain In The Coupling Between Activation And Inactivation Gates

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Truncation of KcsA C-terminal domain (CTD) been reported to impair ion channel activity 1. However, we have shown that a KcsA lacking the CTD (KcsA-ΔCTD) is capable of catalyzing pH-dependent rubidium influxes 2.To investigate the functional and structural roles of KcsA CTD in channel gating, we have studied pH-dependent structural changes of the activation gate by EPR and Fluorescence spectroscopy in full length (FL) and ΔCTD KcsA. Proton-dependent macroscopic currents of KcsA-ΔCTD inactivated faster and deeper when compared to the FL channel. Additionally, single channel analysis showed that at steady state KcsA-ΔCTD has an open probability Po not higher than ~ 0.001, about one order of magnitude lower than FL-KcsA. Recently, by solving a family of KcsA-ΔCTD open structures we have proposed the mechanism by which the activation gate is allosterically coupled to the selectivity filter. As a result, we have hypothesized that a larger opening at the activation gate in KcsA-ΔCTD is directly correlated with an enhancement in the rate of inactivation. Distances estimated by fluorescence resonance energy transference (FRET) indicates that KcsA-ΔCTD activation gate opened to a larger extent than that in FL-KcsA, thus strengthening the coupling between activation and the collapse of the selectivity filter. Our x-ray structures of closed and open FL-KcsA in addition to the KcsA-ΔCTD in the open conformation are in agreement with a mechanistic model where the larger the opening at the activation gate the deeper inactivation at the selectivity filter.

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On the Structure-Function correlates of Ion Occupancy and modulation of C-type Inactivation

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KcsA, a proton activated K channel, has served as an archetypical K pore providing molecular insights into understanding selectivity, ion-permeation, gating and pore-blocking. A recent set of crystal structures describing the mechanism of C-type inactivation in this channel now allows for an understanding this mechanism at atomistic level. Our results show that KcsA inactivation is strongly coupled to the opening of the activation gates and is modulated by the amount and direction of current passing through the channels. As also implicated by studies in eukaryotic channels, C-type inactivation in KcsA involves an intimate interplay between the selectivity-filter region and permeant-ions. This study attempts at further understanding this close association between the ion and filter by correlating high resolution structures with macroscopic and single- channel functional data. We have obtained several KcsA crystal structures of the closed and the open mutant channel, in the presence of different permeant ions (K+, Rb+, Cs+ and NH4+) and blockers (Ba2+ and TEA). These structures reveal different ion occupancies depending upon the nature of the permeant ion, blocker and the extent of channel opening. Analyzed in the light of extensive functional evidence, these results uncover several important features of the interplay between ion interactions and the evolution of C-type inactivation.

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Nanoplasmonic Fluorescence Enhancement Applied to Study of Ion Channels

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A metal nanoparticle can act as an antenna capable of increasing both the excitation rate and quantum yield of a fluorophore in close proximity to the nanoparticle. These properties thus enhance the fluorescence yielding an increase in brightness and decrease of the photobleaching rate - a highly useful tool in fluorescence studies of membrane proteins from the macroscopic to single molecule level. We have applied this technique to image purified membrane proteins in supported bilayer. The biological sample is purified KcsA K-channels labeled with TMR-6-M in the bundle crossing and reconstituted as proteoliposomes. We have also studied the membrane fluorophore DiI C18 in supported bilayer as a control. Among the many approaches towards fabrication of effective nanoparticles, we have synthesized spherical silver nanoparticles of ~100 nm diameter coated with a thin SiO2 outer layer (Ag@SiO2). We have explored different size particles and various SiO2 thicknesses to find experimental conditions for optimal fluorescence enhancement. The SiO2 layer provides protection from chemical attack, acts as a spacer layer to avoid direct metal-fluorophore quenching, and allows surface functionalization. We have conjugated silica-coated silver particles to glass coverslips via polylysine (PL) in order to achieve a high-density silver nanoparticle monolayer. We record fluorescence from the labeled ion channels in an inverted TIRF microscope configuration imaged with a high-speed EMCCD camera. KcsA proteoliposomes are added to an Ag@SiO2-PL coverslip surface to rupture as supported bilayer patches for single molecule imaging. DiI liposomes were used in the same way. The KcsA-TMR and DiI samples show enhancement of at least 4-fold and 10-fold, respectively, compared to the same sample without nanoparticles. These results demonstrate the utility of this technique in fluorescence studies of ion channels or other membrane proteins. Supported by NIH 1R21MH078822 & 1F31NS054532.

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KcsA Gating Explored with Quaternary-Ammonium Blockers

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The bacterial potassium channel KcsA, an archetypal K^+ channel pore, is proposed to close at an intracellular constriction. The inner helices form a bundle crossing that separates the intracellular solution from a large, hydrated internal vestibule within the pore domain (Doyle et al. Science, 1998). This vestibule has been shown to be the receptor-site for open-channel blockers such as quaternary-ammonium ions in KcsA and other voltage-gated potassium channels (Armstrong and Hille, JGP, 1972; Holmgren et al. JGP, 1997; Zhou et al. Nature, 2001; Lenaeus et al. NSMB, 2005; Yohannan et al. JMB, 2007). Since KcsA is gated by intracellular protons, it is predicted that pH will dramatically alter the accessibility of channel blockers to the vestibule. We are exploring the state-dependence of channel block by quaternary-ammonium ions using steady-state single channel recording of the non-inactivating KcsA E71A channel (Cordero-Morales et al. NSMB, 2006). Preliminary results indicate a profound state-dependence, with TBA blocking kinetics and percent block changing dramatically as a function of channel open probability. We will compare these results with blocking data for a pH-insensitive KcsA mutant we previously reported (Thompson et al. PNAS, 2008). These results demonstrate that the pH-sensor of KcsA operates to gate ion access to the vestibule.

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Stability And Conductance Assessment Of A Putative Low-k+ Inactivated State Of The Kcsa Channel

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Potassium channels constitute a large family of proteins, notably involved in the regulation of the activity of excitable cells. The channels partly exert that function by varying their conductance through a mechanism known as C-type inactivation: Shortly after the activation of K+ channels, their selectivity filter stop conducting ions at a rate that depends on various stimuli. This

inactivation process plays a critical role in controlling the length and frequency of cardiac action potentials, as well as the firing patterns in neurons. The molecular process underlying the C-type inactivation mechanism remains unexplained despite the accumulation of experimental evidences showing the key role played by the channels' selectivity filter and some neighboring residues. It's been recently shown that the prokaryotic KcsA channel undergoes C-type inactivation like its eukaryotic counterparts (Gao et al., PNAS, 102:17630 (2005)), establishing KcsA as a perfect prototypic model to study the structural basis of the inactivation mechanism. An X-ray structure of the KcsA channel obtained in presence of low K+ concentration (Zhou et al., Nature 414:43 (2001), pdb code 1K4D) has since then been postulated to correspond to the C-type inactivated state of the channel. While the structural analysis of this static conformation suggests that pore lining amide hydrogens would prevent the permeation of ions, uncertainties remain about its stability under physiological conditions and its ion occupancy state. These questions are of primary importance to better understand the relevance of this structure to the physiological regulation of ion permeation in K+ channels. Using molecular dynamics simulations and free energy calculations, we investigated on the stability, selectivity, and conductance of the selectivity filter of KcsA in this putative inactivated state.

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Ancillary subunits and S3b amino acid substitutions alter the affinity of HpTx2 for Kv4.3

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HpTx2 is an ICK gating modifier toxin that selectively inhibits Kv4 channels, making it an excellent probe for understanding Kv4 structure and function. To characterize the molecular determinants of interaction, we performed alanine scanning of Kv4.3 S3b linker. HpTx2-Kv4.3 interaction had a $K_d = 2.4$ μM. Two alanine mutants in Kv4.3 caused a dramatic increase in K_d: 6.4 μM for V276A and 25 μM for L275A. LV275AA nearly eliminated toxin interaction. Unlike HaTx and other well-characterized ICK toxins, HpTx2 binding does not require a charged amino acid. To determine if the identity of the amino acids altered HpTx2 specificity, we constructed LV275IF. This mutation decreased the K_d to 0.54 μM, suggesting that the hydrophobic character of the binding site is the most important property for interaction with HpTx2. Two of the alanine mutants, N280A and D282A caused stronger interaction of HpTx2 with Kv4.3; the K_d for Kv4.3 [N280A] was 0.26 μM. We propose that these mutations either remove a steric barrier to HpTx2 occupation of S3b, or that removal of these polar side chains increases toxin affinity by increasing the hydrophobic character of the binding site. To understand Kv4.3-based transient outward currents in native tissues, we tested the affinity of HpTx2 for Kv4.3 co-expressed with KChIP2b. The toxin's K_d for Kv4.3+KChIP2b was 0.95 μM. HpTx2 binds to the closed state of Kv4.3, which is stabilized by KChIP2b. We propose the increased affinity is due to the increased stabilization of the closed state. These data show that HpTx2 binding to Kv4.3 has aspects in common with other ICK gating modifier toxins, but that the interventions that increase toxin affinity suggests flexibility toward channel binding that belies its unusual specificity for Kv4 channels.

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HpTx2 Interaction With Kv4.3 and Kv4.1 Reveals Differences in Gating Modification

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HpTx2 is an ICK gating modifier toxin specific for Kv4 channels. The K_d of HpTx2 for two similar channels, Kv4.3 and Kv4.1, was 2.4 μM and 7.1 μM, respectively. HpTx2 inhibition of Kv4.3 is highly voltage dependent, shifting the current-voltage relationship to more depolarized potentials; consistent with the classic behavior of gating modifier toxins. However, modification of Kv4.1 gating is much less voltage dependent. Site directed mutagenesis of the S3b interaction site of Kv4.3 and Kv4.1 shows that the same two conserved bulky hydrophobic amino acids are required for HpTx2 interaction with each channel. While the interior of the binding site is conserved between Kv4.3 and Kv4.1, three amino acids adjacent to the binding site are not conserved. Swapping these amino acids between Kv4.3 and Kv4.1 swaps the phenotypic response to toxin, while having minimal effect on gating properties of the channels. We modeled the activation gating of Kv4.3 and Kv4.1 and incorporated the effects of HpTx2 into the kinetic parameters of activation. The model is similar for both channels; it has four voltage-dependent transitions between the closed states followed by voltage-independent transition to an open state. Voltage-dependent transitions in Kv4.3 are more strongly affected by toxin. In Kv4.1, the voltage-independent transition from the closed pre-open to open state is most affected by HpTx2. Therefore, a higher proportion of toxin-bound Kv4.1 channels are in the closed pre-open state, compared to toxin-bound Kv4.3 channels. This decreases the voltage dependence of toxin-bound Kv4.1 opening. The model closely recapitulates our experimental data. These data show that amino acids near the HpTx2 binding site play a role in the kinetics of Kv4 channel activation gating.

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A Potassium Channel Blocking Toxin Isolated From The Venom Of The Scorpion *Centruroides suffusus suffusus*

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Voltage-gated potassium channels are widespread among various cell types playing roles in numerous cellular functions by controlling the membrane potential. Pharmacological manipulation of these channels carries the potential of selective interference with such well-defined cellular functions if molecules that bind to them with high affinity and specificity are available. Scorpion venoms have long been known for containing small peptide toxins bearing these properties. We are particularly interested in peptides blocking T cell Kv1.3 channels whose normal operation is essential for T cell mediated immune responses.

We screened HPLC purified peptide fractions from *Centruroides suffusus suffusus* scorpion venom for Kv1.3 channel blocking potency. Screening resulted in one biologically active peptide component, which was purified to homogeneity and named Css20. The toxin's amino acid sequence was completed by Edman degradation and mass spectrometry analysis. It contains 38 amino acid residues with a molecular weight of 4,000.3 Da, tightly folded by three disulfide bridges.

We have found that Css20 preferentially blocked the currents of the voltage-gated K^+ channels Kv1.2 and Kv1.3. Its blocking potency was tested on six other potassium channels and a cardiac sodium channel, but the toxin proved ineffective at 10 nM concentration. Dose-response curves of Css20 yielded an IC50 of 1.3 and 7.2 nM for Kv1.2 and Kv1.3 channels, respectively. Interestingly, despite the similar affinities for the two channels the association and dissociation rates of the toxin were much slower for Kv1.2, implying that different interactions may be involved in binding to the two channel types; an implication further supported by *in silico* docking analyses. Based on the primary structure of Css20, the systematic nomenclature proposed for this toxin is α-KTx 2.13.

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Differential Effects of Isoflurane on Mutant Cardiac IKs Channels

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The slow delayed-rectifier potassium (IKs) channel mediates repolarization of the cardiac action potential, and its dysfunction can lead to the long QT syndrome (LQTS). The IKs channel consists of two subunits, the pore-forming α-subunit, KCNQ1, and the auxiliary β-subunit, KCNE1. Volatile anesthetics have significant inhibitory effects on the IKs current, but their underlying molecular mechanism remains to be determined. We have previously shown that F340 was a critical residue involved in this interaction, similar to the observation reported for other putative IKs antagonists. In order to further elucidate this mechanism, we investigated the effects of a volatile anesthetic, isoflurane, on mutant constructs of the IKs channel. Two KCNQ1 mutants, A341C and A344C, were constructed by site-directed mutagenesis. These two residues were chosen due to their vicinity to F340, and to their roles in inherited LQTS. Whole-cell current was recorded from transiently transfected HEK-293 cells in the absence and presence of isoflurane (0.52 ± 0.01 mM). Isoflurane inhibited wild-type- (WT) KCNQ1 by 62.1 ± 1.9% (mean ± SEM, n=9). This inhibition was significantly attenuated when WT-KCNQ1 was cotransfected with WT-KCNE1 (40.7 ± 4.5%; n=9). In contrast, isoflurane inhibited the mutant A344C current by 25.5 ± 2.0% (n=13), but this inhibition was not affected by the cotransfection with WT-KCNE1 (26.3 ± 3.2%, n=13). The A341C mutant alone did not express any current. However, when A341C was cotransfected with WT-KCNE1, an IKs-like current was elicited that was inhibited by isoflurane by 63.1 ± 2.9% (n =11). These results show that in addition to F340, the A341 and A344 residues also contribute to the anesthetic binding environment. Furthermore, the differential impact of KCNE1 on the isoflurane effects on WT-KCNQ1 and the mutant constructs suggests a complex role for the β-subunit in modulating the pharmacology of the IKs channel.