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The Effect of D_2O on the Inactivation Kinetics and Recovery from Slow Inactivation of Shaker-IR K^+ Channels

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In the absence of N-type inactivation Shaker potassium channels display slow (C-type) inactivation which is stabilized by a multipoint hydrogen-bond network behind at the selectivity filter in eukaryotic K_V channels. The selectivity filter is sterically locked in the inactive conformation by buried water molecules which prevent recovery from inactivation. To further test the critical role of these „structural water'' molecules in the conformational stability of the selectivity filter we studied the effects of substituting water for heavy water (deuterium oxide, D_2O in the solutions) on various gating transitions in Shaker-IR channels having the following mutations and allowing slow inactivation at various rates: T449A, T449A/I470A and T449K/I470C. All channels were transiently expressed in tsA_201 cells and ionic currents were recorded in excised inside-out patches. 2.0-s-long depolarizing pulses from a holding potential of -120 mV to $+50$ mV were applied to measure the current through the channels. The extracellular application of D_2O dramatically slowed entry into the inactivated state, the inactivation time constats were $\tau_{i,D2O} = 189 \pm 48$ ms (n=5) (T449A, $\tau_{i,H2O} = 72 \pm 13$ (n=5)), $\tau_{i,D2O} = 418 \pm 63$ ms (n=4) (T449A/I470A, $\tau_{i,H2O} = 247 \pm 90$ (n=5)), $\tau_{i,D2O} = 60 \pm 21$ ms (n=6) (T449K/I470C, $\tau_{i,H2O} = 31 \pm 15$ ms (n=10)), respectively. In contrast, applying D_2O from the intracellular side did not change the inactivation kinetics. The kinetics of recovery from slow inactivation was also slowed down in the D_2O environment. Our macroscopic current measurements confirm that water molecules may have access to the region behind the selctivity filter only from the external solution and the binding of these 'hidden' molecules slows the development of inactivation and that of the recovery process.

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Non-Canonical Start Codons Reinitiate Translation in N-Terminal Truncated Kv Channels

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Genetic incorporation of unnatural amino acids (UAA) is a powerful tool to investigate protein structure and function. Through nonsense suppression of stop codons, UAAs are incorporated into proteins using chemically charged tRNAs or orthogonal tRNA/tRNA synthetase (tRNA/RS) pairs. Previously, we successfully incorporated a fluorescent UAA into the voltage gated-potassium (Kv) channel. The nonsense stop codons were located either central or C-terminal, and no leak expression was detected in the absence of the UAA, reflecting orthogonality of the tRNA/RS pair used. Here, we show that when stop codons are introduced in the near N-terminal region of the same Kv channel, a considerable amount of leak expression results from translation reinitiation. Since reinitiation only can occur when the distance between the first methionine and the inserted stop codon is short, it explains the position-dependent leak expression. We demonstrate that reinitiation occurs at several non-canonical (non-AUG) start codons, and leak expression was decreased from 25% to less than 1% through silent mutations of start codons. When using UAAs, it is crucial to have control of the protein translation process in order to get reliable results, and we emphasize that caution should be taken when UAAs are incorporated in the N-terminal region of any protein. This is particularly important if the N-terminal has a functional role where truncation generates a modulated phenotype which could be wrongly interpreted to be caused by the inserted UAA. To avoid reinitiation-mediated leak expression, we therefore recommend removing both canonical and non-canonical start codons.

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Activation Gate Region Influences Stoichiometry of Heteromeric Shaker Family Channels

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Shaker family voltage-gated K^+ channel subunits can typically form functional homotetrameric channels or heterotetrameric channels with closely related subunits. Assembly is governed by cytoplasmic T1 domain contacts that are compatible within, but not across, gene subfamilies (Shaker, Shab, Shal and Shaw). Shaker family regulatory subunits differ in that they can only function in heteromeric channels because of T1 self-incompatibility. Mammalian regulatory subunits (Kv6, Kv8, Kv9) are members of the Shab family and modify the functional properties of Kv2.1 and Kv2.2. Kv2.1 and Kv9.3 form functional channels in a 3:1 stoichiometry. Using a combination of TIRF microscopy and electrophysiology, we show here that Kv2.1subunits also exclusively form 3:1 heteromers with Kv6.4. These findings raise the question of how stoichiometry is determined: it is unclear how T1 domain compatibility rules alone could distinguish between formation of 3:1 and 2:2 heteromers. We hypothesized that the activation gate region of the inner pore may be important in refining the stoichiometry of regulatory subunit-containing heteromers because it forms a large contact interface between subunits. Additionally, the sequence of this region in the regulatory subunits has drifted from the highly conserved Shab family consensus. Reverting the gate sequence in Kv6.4 to the Shab family consensus allowed the formation of functional 2:2 heteromers between Kv6.4 and Kv2.1 that could be detected by electrophysiology and TIRF microscopy. Drift of the gate sequence consensus can be observed in all verified regulatory subunits, including these mammalian Shab channels and evolutionarily separate regulatory subunit expansions in the Shaker, Shal and Shaw subfamilies in cnidarians. We suggest that the evolution of T1 self-incompatibility is a key event in the evolution of the regulatory subunit phenotype, but that drift of the activation gate interface plays an important role in fixing heteromeric channel stoichiometry.

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Ion Permeation in Potassium Channels Involves Direct Coulomb Knock-On between Ions

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Potassium channels are essential elements in almost all cell types. They are, at the same time, highly selective for potassium and conduct potassium ions across membranes with great efficiency. By analyzing more than 1,300 potassium permeation events recorded in Computational Electrophysiology (CompEl) [1] atomistic molecular dynamics simulations under physiological voltages, we observed that Coulomb repulsion between neighboring potassium ions in the selectivity filter is the key to high-efficiency potassium flux. This is in contrast to previous models which assumed that alternate occupancy of the selectivity filter binding sites with potassium and water underpins potassium conduction. The mechanism we observed relies on the formation of ion pairs in the selectivity filter. These ion pairs form over very broad potassium concentration ranges in our simulations from 10-400 mM. Reinvestigation of crystallographic data on several potassium channels showed that the crystal data are consistent with ions occupying adjacent filter binding sites. The rate of potassium throughput across the filter recorded in our simulations depends on the rate with which incoming ions arrive at the filter. Taken together, the mechanism we propose therefore explains the efficiency, diffusion control, and linear concentration dependence of potassium ion conduction across potassium channels in a simple new model.

[1] C. Kutzner et al., Biophys. J. 101, 809-817 (2011).

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Dynamics of the KcsA Selectivity Filter Probed using Intrinsic Tyrosine Fluorescence

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KcsA is activated by intracellular protons through a large conformational change in the inner helical-bundle gate. The selectivity filter and surrounding structures play a crucial role in ion conduction as an inactivation gate. To monitor the conformational dynamics of the selectivity filter under various experimental conditions we engineered a KcsA construct devoid of tryptophan and tyrosine residues with the exception of residue Y78, locatd in the middle of the selectivity filter. Fluorescence lifetimes indicate that the microenvironment polarity of Y78-KcsA decreases upon gating, irrespective of the type of ion (K^+, Rb^+, $NH_4{}^+$, Cs^+) present in the selectivity filter. Further, time-resolved anisotropy measurements indicate that the dynamics associated with the segmental mobility of the selectivity filter is significantly restricted, and this gating-driven motional restriction is more pronounced in High K^+ and Rb^+ conditions. Red Edge Excitation Shift (REES) measurements of Y78-KcsA show slow water relaxation dynamics under conditions that stabilize conductive and non-conductive filter conformations, suggesting the presence of bound/restricted water molecules in both filter conformations. Interestingly, opening the lower gate appears to further restrict the water