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Dynamic Coupling of Voltage Sensor and Gate Involved in Closed-State Inactivation of Kv4.2 Channels

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Voltage-gated potassium channels related to the *Shal*-gene of *Drosophila* (Kv4 channels) mediate a subthreshold-activating current (I_{SA}) which controls dendritic excitation and the backpropagation of action potentials in neurons. Kv4 channels also exhibit a prominent low-voltage-induced closed-state inactivation, but the underlying mechanism is poorly understood. We examined a structural model, in which uncoupling of the voltage sensor from the cytoplasmic gate mediates inactivation in Kv4.2 channels. Support for such a model comes from our finding that chimeric swapping of S4-S5 linker and distal S6 sequences between N-terminally truncated Kv4.2Δ2-40 and *Shaker*IR channels slowed inactivation of the former and induced a form of fast macroscopic inactivation in the latter under two-electrode voltage-clamp in *Xenopus* oocytes. We performed a Kv4.2 alanine scanning-mutagenesis in the S4-S5 linker, the initial part of S5, and the distal part of S6 and functionally characterized these mutants. In a large fraction of the mutants (> 80%) normal channel function was preserved, but the mutations influenced the likelihood of the channel to enter the closed-inactivated state. Depending on the site of mutation, the onset kinetics of low-voltage inactivation and/or the kinetics of recovery from inactivation were accelerated or slowed and the voltage dependence of steady-state inactivation was shifted positive or negative. In some mutants these inactivation parameters remained unaffected. Double-mutant cycle analysis based on kinetic and steady-state parameters of low-voltage inactivation revealed that residues known to be critical for voltage-dependent gate-opening, including Glu 323 and Val 404, are also critical for Kv4.2 closed-state inactivation. Selective redox modulation of corresponding double-cysteine mutants by dithiothreitol (DTT) tert-butyl hydroperoxide (*tb*HO_2) supported the idea that these residues are involved in a dynamic coupling, which mediates both transient activation and closed-state inactivation in Kv4.2 channels.

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The Role of ILT Mutations in Individual Subunits

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The concurrent mutation of three hydrophobic residues (V369I, I372L, and S376T) in the N-terminus of the S4 segment of *Shaker* K^+ channel slows down the kinetic of channel activation by a ~6-fold factor and shifts the conductance-voltage (G-V) curve to 115 mV (Smith-Maxwell *et al.*, 1998). We sought to determine the effect of the ILT mutations in heterotetrameric concatemerized channels with different stoichiometries to gain insights into the mechanisms of action of the ILT mutations. The channels were expressed in *Xenopus* oocytes and studied using cut-open oocyte voltage clamp and patch clamp techniques. The G-V curve of a concatemer with the ILT mutations in only one subunit (wtILT/3wt) is centered at ~8 mV compared with ~115 mV for the homotetramers *Shaker* ILT, while the homotetramer concatemerized *Shaker* zH4 Δ(6-46) (4wt) as a $V_{1/2}$ of −20 mV. The slope of the G-V curve is reduced. The kinetics of activation and deactivation are similar between 4wt and wtILT/3wt. The ILT mutations were introduced on the single wild type voltage sensor of a heterotetramer having three subunits with the four gating charges neutralized (wtILT/3mut; mut = R362Q/R365Q/R368N/R371Q). The G-V of the wtILT/3mut heterotetramer is right shifted ($V_{1/2}$ ~43 mV) compared with the wtILT/3wt and its slope is even more reduced. The number of closed states estimated from the Cole-Moore shift with an exponential function raised to a power n was slightly higher for wtILT/3wt than that of 4wt [$9.5 \pm 2(9)$ vs. $7.5 \pm 1.5(8)$, P=0.05]. However, wt/3mut and wtILT/3mut have the same estimates [n=$1.58 \pm 0.4(5)$ vs. $1.8 \pm 0.7(7)$, P=0.5]. wtILT/3wt has the same single channel conductance than *Shaker* ILT. We conclude that a single ILT wild type subunit is not sufficient for fully modifying the concerted step as in homotetramers. (Support: NIHGM30376 and NSERC fellowship to DGG)

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Gating currents from neuronal K_v7 channels

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The Kv7 family of voltage-gated K+ channels consists of five members (Kv7.1- Kv7.5), each showing specific tissue distribution and physiological role. Mutations in Kv7.1 cause the long QT syndrome, whereas Kv7.4 mutations underlie a rare form of slowly progressive deafness (DFNA2); finally, mutations in Kv7.2 and Kv7.3 genes have been identified in families affected by Benign Familial Neonatal Seizures. Disease-causing mutations often affect residues in the voltage-sensing domain of Kv7 subunits. In the present study, we have characterized the ionic and gating current of homomeric neuronally-expressed Kv7 channels (Kv7.2-Kv7.5) using the cut-open oocyte voltage clamp. The ionic and gating current were recorded at 18°C and also at 28°C to speed up gating charge movement. Increasing the recording temperature from 18°C to 28°C caused an accelerated activation/deactivation kinetics of the ionic currents in all homomeric Kv7 channels (the Q10 for the activation kinetics at 0 mV was 3.8, 4.1, 8.3, and 2.8 for Kv7.2, Kv7.3, Kv7.4 and Kv7.5). Moreover, currents carried by Kv7.4 channels (and less so Kv7.2) also showed a significant increase in their maximal value. Gating currents were only resolved in Kv7.4 and Kv7.5 channels, possibly because of an higher membrane channel density; ON gating charges at saturated potential (+40mV) amounted to 1.34 ± 0.34 (Kv7.4) and 0.79 ± 0.20 nC (Kv7.5). At 28°C, Kv7.4 gating currents had the following salient properties: 1) similar time integral of ON and OFF, indicating charge conservation; 2) a left-shift in the V1/2 of the ON gating charge when compared to ionic currents; 3) a rising phase in the OFF gating charge after depolarizations to values >0 mV. These results represent the first description of Kv7.4 and Kv7.5 gating currents and may help to clarify the molecular consequence of disease-causing mutations affecting channel gating. Supported by NIH GM30376 and Telethon GGP07125.

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Contributions of the central hydrophobic residue in the PXP motif of Voltage-Dependent K+ Channels to S6 flexibility and Gating Properties

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Voltage-gated K+ channels are composed of four subunits each of which contains a voltage-sensing domain (S1-S4) and a pore domain (S5-P loop-S6). The exact molecular mechanisms underlying the opening and closing of the channel pore are still unclear, although evidence suggests that this process involves pivoting bending of the inner pore-lining S6 segments at the ''helix-bundle crossing''. Shaker-like (Kv1.1) channels contain a highly conserved Pro-Val-Pro (PVP) motif at the base of S6 that produces a kink in the S6 helices and provides a flexible element thought to be essential for channel gating. The role of proline-induced kinks in transmembrane helices is well known, but the role of the small hydrophobic valine between these prolines, is unknown, and interestingly, Shab-like (Kv2.1) channels possess a an isoleucine at this position (PIP). Here we show that the exact nature of this central hydrophobic residue within the PXP motif confers unique functional properties to Kv1 channels, including changes in activation and deactivation kinetics, voltage-dependent properties and open probabilities, but that single-channel conductance and cell expression levels are not affected. In support of these functional changes, molecular dynamic simulations demonstrate that valine and isoleucine contribute differently to S6 flexibility within this motif. These results therefore indicate that the nature of the central hydrophobic residue in the PXP motif is an important functional determininat of Kv channel gating by contributing, at least in part, to the relative flexibility of this motif.

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A P-helix Mutant In A *Shaker*-type Kv Channel Converts The Inactivated State Into A Conducting One

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Voltage-gated potassium (Kv) channels are tetramers of α-subunits, each composed out of six membrane-spanning helices (S1-S6) with a pore loop between S5 and S6 that forms the channel's selectivity filter. The activation gate that seals off the ion conducting pore in a closed channel is controlled by the transmembrane potential. After channel activation most Kv channels display C-type inactivation, a process that is believed to involve reorientations of the selectivity filter and results in a non-conducting channel although the channel gate is open. hKv1.5 (a *Shaker*-type Kv channel) displays such C-type inactivation. Here we report that an alanine substitution for residue T480 that is located at the end of the pore-helix prevents hKv1.5 channels from entering the inactivated state. The mutant T480A had an isochronal activation curve similar to Wild Type hKv1.5 when determined with 250ms depolarizing steps. Longer depolarizations (~5 seconds) caused WT channels to inactivate (~58%) displaying an inactivation curve with a midpoint of -23.2 ± 1.2 mV and a slope factor of about 4. However, T480A did not inactivate and such long depolarizations caused an additional (slow) activation at more negative potentials thus generating an isochronal activation curve with properties that were similar to the

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