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Hydration and the Electric Field in the Voltage Sensing Domain of the Kv1.2 Channel: Quantum Calculations show S4 Doesn't Move, but Water and Protons Move

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Quantum calculations on the VSD of Kv1.2 (3Lut pdb coordinates) show several water molecules move into the VSD when the sign of the electric field goes from positive intracellularly to negative (closed). The protein backbone remains essentially immobile; S4 does not move vertically with respect to the other transmembrane segments, but may have minimal horizontal motion (parallel to the membrane surface, were the membrane included in the calculation); side chain rearrangements, however, change some intramolecular distances. We have calculated the dipole moments of the optimized structures for several cases, as well as the structures of the water clusters (these calculations:18 water molecules, 373 atoms from the protein, from the 2nd to the 4th arginine in S4, and the complementary sections of S1, S2, and S3). Rotating two water molecules in the cluster (closed conformation) sufficed for a significant change in dipole (in most calculations, counting the dipole for the entire system; dipole changes with state as well, $\leq$5 D for the open configuration, approximately an order of magnitude more when closed, suggesting dipole shift is part of the sensing mechanism). Several energy minima were determined; the closed configurations were several kT lower in energy than open configurations. The water behavior resembled a phase change, with finite ΔV (volume) in its overall shift in structure with changes in electric field; there is more than one energy minimum, but the change in water density is unambiguous, although the water is not ordered in either case. Gating is coupled to water via a proton shift in the lower section of the VSD (see our other abstract).

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Water and a Proton Shift between a Tyrosine and a Glutamate are Two Keys to Gating in Kv1.2; A Hypothesis Based on Quantum Calculations: The Sensor is Dynamic, Based on Hydrogen Bond Rearrangements, Principally in Water Rotational Degrees of Freedom, Plus a Proton Pathway

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An apparent proton pathway in the Kv1.2 K^+ channel exists starting with a tyrosine (Y266, 3Lut pdb numbering) and continuing through a glutamate (E226); amino acids that would constitute several more steps can be identified, on to the H310 at the intracellular end of S4. Quantum calculations to date include four cases in which a proton shifts between Y266 and E226, the first step in the pathway, depending on electric field,. Energy differences with H^+ transfer are small, $\approx$5 kT (open), 2 kT (closed). The calculations are, so far, approximate, but good enough to show that H^+ transfer is possible, and the proton may ''flicker'' between the two positions. We hypothesize that proton transfer proceeds intracellularly on closing, down to H310 (the complete proposed pathway, with distances between groups in the path, can be identified); H310 affects the PVPV gate such that, with the proton at the intracellular end of this path, the gate closes. The proposed proton pathway structure resembles that in bacteriorhodopsin, where the proton transfer has been established. The water movement discussed in our other abstract appears to push a water molecule into position to assist in the first step transfer, based on calculations completed so far.

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Structural Insights of the Calcium Mediated Reorganization of the Calmodulin/Kv7.2 Channel Complex

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Calmodulin (CaM), a bi-partite protein, binding to the A-B module, a bi-partite target, affect the function of Kv7.2 subunits, which are the main component of the non-inactivating K^+ M-current, a key controller of neuronal excitability. Simultaneous binding to helix A and B is crucial for trafficking to the plasma membrane, and mediates Ca^{2+}-dependent inhibition of channel function. We have resolved the [CaM]/[AB-Kv7.2] complex by NMR and characterized the influence of Ca^{2+} on the structure. The results suggest a reorientation of A-B helices triggered by Ca^{2+}. How this structural change may propagate to affect gating will be discussed.

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Biochemical Analysis of the Regulation of Kv7 Channels by PIP2 and Calmodulin

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Kv7 (M-type, KCNQ) channels produce an outward potassium current (M-current) and play dominant roles in control of neuronal excitability due to their threshold of activation at sub-threshold potentials. Kv7 channels have been identified as therapeutic targets to reduce neuronal excitability in certain brain disorders, such as epilepsy and chronic pain. We seek to elucidate the molecular mechanisms of Kv7-channel regulation by second-messengers used in their regulation by Gq/11-coupled receptors, and the structural determinants of the channels that are involved. Activation of Gq/11-mediated signals results in the hydrolysis of the membrane-bound lipid, PIP2 (phosphatidylinositol 4,5-bisphosphate), and an increase in calcium-bound calmodulin (Ca2+/CaM) that is associated with M-current suppression and enhanced neuronal excitability. The structure of Kv7 channels consists of a 6-transmembrane-spanning region with an extended cytoplasmic carboxy-terminus containing a proximal ''regulatory domain'' with two alpha helices and two regions enriched in basic amino acids that are thought to be the principal sites of PIP2- and CaM-mediated binding. Recently, we examined the affinity of PIP2 to this regulatory domain spanning from the S6-TMD to the end of the B-helix domain (KCNQ-RD). By monitoring the shift in intrinsic protein fluorescence, we observed a change in the conformation of KCNQ-RD by the presence of the water-soluble analog, diC8-PIP2. Fluorescence polarization assays showed a reduction of anisotropy of a fluorescently labeled PIP2 analog with increasing KCNQ-RD titrations. These results support that PIP2 directly binds the KCNQ-RD. Since CaM also binds in this region, we hypothesize that the interactions of CaM and PIP2 with Kv7 channels are allosterically or sterically coupled, such that the binding of one molecule affects the affinity of binding of the other, or affects the efficacy of its action in modulating channel gating.

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Centrin 4 is a Binding Partner of Rat EAG1 K^+ Channels

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The *ether-à-go-go* (Eag) potassium (K^+) channel belongs to the superfamily of voltage-gated K^+ channel. In mammals, the expression of Eag channels is neuron-specific but their neurophysiological role remains obscure. We have applied the yeast two-hybrid screening system to identify rat Eag1 (rEag1)-interacting proteins from a rat brain cDNA library. One of the clones we identified was centrin 4, which is an EF-hand calcium-binding protein with four putative calciumion-binding sites. Data from *in vitro* yeast two-hybrid and GST pull-down assays suggested that the direct association with centrin was mediated by the carboxyl-termini of rEag1. Co-precipitation of the two proteins was confirmed in both heterologous HEK293T cells and brain lysates. Centrin 4 and calmodulin are closely related four-EF-hand calcium-binding proteins. The binding affinity of calmodulin to rEag1 was notably enhanced in the presence of calcium. In contrast, the interaction between centrin 4 and rEag1 appeared to be calcium-independent. We therefore propose that centrin 4 may serve as a constitutive binding-partner of rEag1.

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Gating Current Models Computed with Consistent Interactions

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Gating currents of the voltage sensor involve back-and forth movements of positively charged arginines through the hydrophobic plug of the gating pore. Transient movements of the permanent charge of the arginines induce structural changes and polarization charge nearby. The moving permanent charge induces current flow everywhere. Everything interacts with everything else in this structural model so everything must interact with everything else in the mathematics, as everything does in the structure. Energy variational methods *EnVarA* are used to compute gating currents in which all movements of charge and mass satisfy conservation laws of current and mass. Conservation laws are partial differential equations in space and time. Ordinary differential equations cannot capture such interactions with one set of parameters. Indeed, they may inadvertently violate conservation of current. Conservation of current is particularly important since small violations (<0.01%) quickly (microseconds) produce

forces that destroy molecules. Our model reproduces signature properties of gating current: (1) equality of ON and OFF charge (2) saturating voltage dependence and (3) many (but not all) details of the shape of charge movement as a function of voltage, time, and solution composition. The model computes gating current flowing in the baths produced by arginines moving in the voltage sensor. The movement of arginines induces current flow everywhere producing 'capacitive' pile ups at the ends of the channel. Such pile-ups at charged interfaces are well studied in measurements and theories of physical chemistry but they are not typically included in models of gating current or ion channels. The pile-ups of charge change local electric fields, and they store charge in series with the charge storage of the arginines of the voltage sensor. Implications are being investigated.

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A Molecular Substrate for Long QT in HIV Patients: Tat Protein Reduces IKR in Human Induced Pluripotent Stem Cells-Derived Cardiomyocytes

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Compared to the general population, individuals with HIV have a 4.5 fold higher risk of sudden cardiac death, and up to 20% of them present with a long QT syndrome (LQTS). Notably, torsades de pointes arrhythmias have been described even in the absence of drug therapy, suggesting a rather direct implication of HIV in LQTS. The HIV-encoded Tat protein appears as a potential candidate for inducing ventricular arrhythmias, since it was shown to prolong the action potential (AP) of guinea-pig ventricular cardiomyocytes by reducing IKr. Tat protein serves as a transactivator of transcription required for HIV replication. Tat is released from infected cells and is found circulating in the blood of HIV-infected patients.

The aim of this study was to evaluate the human induced pluripotent stem cells-derived cardiomyocytes (hiPS-CMs) as a model to study the cellular mechanisms involved in the Tat-dependent alteration of cardiac electrical activity in human.

Our data show that Tat incubation reduces IKr in hiPS-CMs while it does not alter IhERG in transfected COS-7 and HEK293 cellular models. hERG protein expression was reduced only in Tat-incubated hiPS-CMs; such reduction likely contributes to IKr reduction. Ventricular AP durations (APD70 and APD90) were significantly increased in hiPS-CMs incubated with Tat compared to buffer incubation. In addition, most Tat-treated cells showed a higher APD90 dispersion which resulted from AP duration and amplitude alternans. Alternans were exacerbated at faster stimulation rates.

This work highlights that Tat-treated hiPS-CMs recapitulate alterations of the cardiomyocyte electrical activity, consistent with the arrhythmias observed in HIV patients. hiPS-CMs represent a relevant model for further investigations of the cellular mechanisms involved in AIDS, and, more generally, in cardiac non-genetic diseases.

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Characterization of a Fast Voltage-Sensing Protein using Voltage-Clamp Fluorometry

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Voltage-sensing domains in voltage-activated ion channels and other voltage-sensing proteins contain well-conserved S4 helices containing basic residues capable of sensing changes in membrane potential to trigger opening or closing of ion-selective pores or phosphatase activity in voltage-sensitive phosphatases. Here we report the discovery of a protein which contains a voltage-sensing domain capable of rapid and slow rearrangements in response to changes in voltage. In place of a pore domain, this voltage sensor has large N- and C-termini predicted to contain structured and disordered domains for interaction with intracellular proteins. Our working hypothesis is that this protein, which we name Coupled Voltage Sensor (CVS), functions as a voltage sensor that couples to intracellular signaling pathways (as yet undefined). Our goal in the present experiments was to demonstrate that CVS undergoes voltage-dependent conformation changes and to characterize those using site-specific voltage-clamp fluorometry. We identified several positions at the external end of S4 where introduced and fluorophore-labeled Cys residues produce changes in fluorescence as a function of membrane potential. Several positions give complex fluorescence responses, starting with a rapid fluorescence increase (dequenching), followed by slower fluorescence decrease (quenching). Interestingly the rapid component is as fast as the clamp speed when using the cut-open voltage clamp technique (tau ~ 200 μs). To exclude the possibility of Stark effect we examined the effects of a series of soluble quenchers and observed enhanced quenching with membrane depolarization, consistent with an outward movement of the S4 helix. Overall, our results support a model in which CVS undergoes two temporally distinct conformation rearrangements in response to membrane depolarization.

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Patch-Clamp Fluorometry based Determination of Relative Ion Permeability for HCN Channels

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Determining the relative permeability of different ions is a classical topic for channel biophysics. Two traditional methods have been used are: 1) single channel recording method, and 2) the reversal potential measurement based on the Goldman-Hodgkin-Katz (GHK) equation. Both methods have been used extensively, but certain limitations such as the extremely small single channel conductance prevent the broad application of either method to every type of channels. For the GHK/reversal potential method, when the differential ion concentrations across the cell membrane lead to changes in channel gating property or local biophysical parameters such as pH, the interpretation of the results could be complicated. For the determination of the relative permeability of ammonium ($NH4^+$) compared to another ion, such as potassium (K), the GHK/reversal potential method may not produce accurate results because $NH4^+$ in mill molar range produces a change in local pH on other side of the membrane. The hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are known to be sensitive to different levels of pH. Here we have proposed a novel method for determining the relative permeability of different ions based on the technique of patch-clamp fluorometry (PCF), which enables simultaneous measurements of macroscopic current amplitude and fluorescence intensity. The slope of the current amplitude vs. fluorescence intensity is directly correlated to the relative ionic permeability. We applied this PCF-based method to the EGFP-tagged HCN channels and determined the ionic selectivity of K, Na^+, and NH^{4+}. The PK/PNa was found to be 2.92, the PK/PNa in the absence of any potassium ions was found to be 39, and the PK/PNH4 was found to be 15. Our results were consistent with previous reports. The PCF-based approach holds the potential as an alternative approach for determining the relative ion permeability.

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Distance-Resolving Voltage Clamp Fluorometry (drVCF) Quantifies Intramolecular Transitions in the Human BK and *Ci*-VSP Voltage Sensors Under Physiologically-Relevant Conditions

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The BK channel voltage-sensing domain (VSD) exhibits important differences from the canonical VSD model: an additional helix (S0), a decentralized distribution of voltage-sensing charges throughout S2-S3-S4 and cooperative S2/S4 activation transitions. To understand the voltage-evoked conformational rearrangements of this unique voltage sensor, and address the unmet need of quantifying short-range intramolecular distances in conducting channels under physiological conditions, we have been developing a new theoretical and experimental framework: distance-resolving Voltage Clamp Fluorometry (drVCF).

drVCF exploits state-dependent collisional quenching of site-specifically conjugated tetramethylrhodamine fluorophores of different length by tryptophan. Firstly, voltage-evoked, Trp-dependent fluorescence deflections of each label are acquired from the VSD of conducting human BK channels expressed in *Xenopus* oocytes using VCF-enabled cut-open oocyte voltage clamp. The labeling site/tryptophan distance in the resting and active states is determined by fitting VCF data to quenching probability density over distance, generated by fluorophore molecular dynamics simulations. Simultaneous fitting of data from multiple fluorophores results in well-constrained solutions. Confidence intervals are established using bootstrap resampling.

At rest, S0, S1 and S2 are approximately equidistant from S4: S0-S4 mean=12.5Å; 95% CI [12.4-12.8Å]; S1-S4=11.9Å [10.4-12.9Å]; S2-S4=11.5Å [8.8-12.3Å]. Upon VSD activation, S4 diverges from S0 (22.0Å [21.4-23.1Å]) and S1 (18.4Å [17.7-20.0Å]), while the S2-S4 distance exceeds 24Å. Using this information, we constructed a structural model for the BK voltage sensor in the resting and active states. Applying drVCF in *Ci*-VSP, we resolved that positions 137 (S1) and 212 (S4) are 19-24Å apart in the active state, as in the atomic structure (22Å: Li *et al.*, 2014; PDB#4G7V). Thus, the new drVCF approach complements FRET-based techniques for distance measurements of short-range transitions under physiologically-relevant conditions.