1642-Pos

Identification of Regulatory Phosphorylation Sites in Slack and Slick Sodium Activated Potassium (K_{Na}) Channels

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Na^+-activated potassium channels (K_{Na} channels), which are encoded by the *Slack* and *Slick* genes contribute to neuronal adaptation during sustained stimulation, and, in auditory brainstem neurons, may regulate the accuracy of timing of action potentials. These channels have been found to be modulated very potently by activation of protein kinase C (PKC) and by receptors linked to activation of this kinase. Activators of PKC increase the amplitude of Slack-B currents and slow their rate of activation, and in contrast, activation of PKC decreases the amplitude of Slick currents. Heteromeric Slack/Slick channels are regulated by PKC to a greater extent than either Slack-B or Slick heteromers (90% decrease in amplitude). Previous experiments using Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) have identified three serine residues in Slack-B that are phosphorylated under basal conditions, but are not within consensus sites for PKC. In order to study the mechanisms of regulation of Slack and Slick channels by phosphorylation, we have begun to identify the specific residues that undergo phosphorylation by protein kinase C. Consensus sequence analysis predicts that there are 13 potential sites of possible PKC phosphorylation in the Slack-B sequence. We have constructed individual site mutants for each of these sites in which the serine/threonines have been mutated to alanines to prevent phosphorylation at these residues. These mutants were expressed in *Xenopus* oocytes and their response to a PKC-activating phorbol ester (TPA) was characterized by two-electrode whole cell clamp electrophysiology. Of the 13 consensus site mutants, only one generated currents that matched wild-type Slack-B currents in their amplitude and kinetic behavior, but completely failed to respond to application of TPA, suggesting that the phosphorylation state of a single residue regulates Slack-B current amplitude and rate of activation.

1643-Pos

Voltage Sensor Activation Facilitates Magnesium-Gated Channel Opening in BK Channels

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Under physiological conditions, Mg^{2+} is an intracellular activator of Ca^{2+}- and voltage-activated potassium (BK) channels. To investigate gating by Mg^{2+} acting through its low affinity site located under each S4 voltage sensor (Yang et al. 2007, 2008; Horrigan & Ma 2008), we studied a BK channel in which the high affinity Ca^{2+} sites in both the RCK1 domain and the calcium bowl were disabled by mutation. Using single-channel recording from inside-out patches to measure channel activation after voltage steps from −100 mV to +100 mV, we found that 10 mM Mg^{2+} reduces the latency to first opening after the voltage step and increases channel activation through an increase in the number of openings per burst and mean open duration. This suggests that Mg^{2+} can bind to both closed and open states of the channel, but it is not clear whether the closed-state binding occurs at the negative or positive potential. Therefore we recorded single-channel activity in macro-patches held at a constant −50 or −100 mV. At −50 mV, when the voltage sensors are occasionally activated, 10 mM Mg^{2+} decreases the duration of the closed intervals between bursts of activity and increases burst duration through an increase in the number of openings per burst and mean open duration. At −100 mV, when the voltages sensors are mainly deactivated, 10 mM Mg^{2+} has little effect on the closed intervals between bursts or mean open duration, and most of the bursts are unitary, consisting of a single brief opening. The above data are consistent with a model in which Mg^{2+} can bind to the BK channel in the closed conformation when the voltage sensors are activated. The bound Mg^{2+} then facilitates opening. Supported by NIH grant AR32805.

1644-Pos

Zn2+ Activates Human Large-Conductance Ca2+-Activated K+ Channel

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Zinc plays a crucial role as an integral structural and catalytic factor in many transcription factors and enzymes and this metal ion (Zn2+) is also increasingly recognized as a potential intracellular signaling molecule. However, the direct Zn2+ sensors especially those membrane localized are not fully established. Using inside-out patch clamp recording, we observed that heterologously expressed human large-conductance and voltage-and Ca2+-activated K+ (BKCa, Slo1 BK, or KCa1.1) channels were quickly and reversibly activated by intracellular Zn2+. Zn2+ did not affect the unitary conductance but significantly increased the channel open probability. Macroscopic current measurements showed that Zn2+-mediated activation of the channel was accompanied by a leftward shift of the conductance-voltage (G-V) curve by up to 75 mV. The effect of Zn2+ was antagonized by high concentrations of intracellular H+ or Ca2+, indicating the three factors activate BK channels via a common mechanism. Mutagenesis experiments showed that mutation of H365 to arginine, a critical residue for the channel activation by H+ and Ca2+, fully abolished the effect of Zn2+. In addition, mutation of two other nearby acidic residues D367 or E399 to alanine also partially impaired the effect of Zn2+. Collectively, our results suggest that a novel multifunctional structure located in the regulator of conductnace for K+ (RCK)1 domain is involved in Zn2+ coordination and activation of the Slo1 BK channel and indicate a potential role of Slo1 BK channel in Zn2+ signaling in both physiological condition and hypoxic/ischemic diseases in which [Zn2+]i is significantly increased. (Supported in part by NIH)

1645-Pos

Molecular Determinants For hSlo1 Bk Channel Activation by Dihydroabietic Acid Derivatives

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Large-conductance calcium- and voltage-activated potassium (BK) channels are key players in vasoregulation and in the control of neurotransmitter release. Use of an alternative exon9 results in a neuronal variant of BK channels (e9alt) with altered amino acid sequence extending from the middle of S6 to a cytosolic linker connecting to the RCK gating ring (C-linker of 14 residues). hSlo1 wild-type (e9), splice variant and mutant channels were heterologously expressed in HEK 293 cells and analyzed in inside-out patches. Channels were characterized with respect to activation by transmembrane voltage, intracellular calcium and dihydroabietic acid derivatives (DHAAs). diCl-DHAA, triCl-DHAA and Cym04 (10 μM, Cui et al. (2008) Bioorg Med Chem Lett 18:6386-6389) were found to activate e9 channels via shifting their voltage dependence by up to ~40 mV to hyperpolarized potentials in the virtual absence of calcium. In contrast, e9alt channels did not exhibit a left-shift in activation. The most active compound, Cym04, was used to identify the molecular determinants for BK activation by DHAAs by mutagenesis. Differences between e9 and e9alt in calcium- and voltage-dependent gating are brought about by residues within S6 and in the C-linker. In contrast, molecular determinants for activation by Cym04 reside in the C-linker: of the 10 residues differing between e9 and e9alt, amino-acid exchanges G327L, K330N, S337N and S340G had a high impact on channel activation by Cym04. Mutation S340G almost completely abolished BK activation by Cym04 without markedly changing calcium- and voltage-dependent gating. The linker connecting the RCK gating ring and the pore of BK channels therefore represents a candidate region for binding DHAA-type, isoform-specific BK channel activators.

1646-Pos

An Epilepsy-Associated Mutation Enhances BK Channel Activity by Altering the Coupling of Calcium Sensing to Gate Activation

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Voltage and Ca^{2+} activated BK channels modulate neuronal activities. Previous studies found that Ca^{2+} binding sites and the activation gate are spatially separated, but how Ca^{2+} binding couples to gate opening is not clear. We address this question by studying how a mutation in BK channels, which is associated with generalized epilepsy and paroxysmal dyskinesia (D434G in hSlo, the epilepsy mutation), enhances Ca^{2+} sensitivity. The epilepsy mutation is located in a structural domain (the AC region) that connects the S6 transmembrane segment with the cytoplasmic domain and close to a putative Ca^{2+} binding site. Mutagenesis studies show that the AC region is important in the coupling between Ca^{2+} binding and gate opening. We found that discrete locations in the AC region that are distant from the epilepsy mutation alter the functional effects of the mutation. Thus, the epilepsy mutation enhances Ca^{2+} sensitivity by an allosteric mechanism affecting the coupling between Ca^{2+} binding and gate opening. Interestingly, mutating the Asp residue to

amino acids other than Gly does not result in the same phenotype as the epilepsy mutation, suggesting that a change in protein flexibility is involved in the effects of the mutation. Consistent with this result, altering the flexibility of the channel protein by changing viscosity of the intracellular solution also modulates Ca^{2+} sensitivity; and the epilepsy mutation reduces such modulation, possibly because it has already altered the flexibility. These results are consistent with a model that the peptide loop where the putative Ca^{2+} binding site and the epilepsy mutation are located acts as a spring-hinge for the conformational change of the AC region during channel opening; Ca^{2+} binding and the epilepsy mutation affect channel gating by altering the function of this spring-hinge.

1647-Pos

Regulation of Drk1 Channels by Carbon Monoxide and Carbon Monoxide-Releasing Molecule-2

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Carbon monoxide is a poisonous gas that is also syntethized in several tissues in the body, where it acts as a signaling molecule. CO has been shown to regulate ion channels such as voltage-dependent calcium channels (VDCC) and large-conductance calcium-activated potassium (BK) channels, a mechanism which is important for oxygen sensing in the carotid body. The Kv2.1 channel has been implicated with oxygen sensing in the pulmonary arteries and the ductus arteriosus, a process which may be regulated by carbon monoxide. We therefore investigated the effects of carbon monoxide and tricarbonyl dichlororuthenium dimmer (CORM-2), a widely used carbon monoxide releasing molecule, on the Kv2.1 channel. We found that, unlike with VDCC and BK channels, CORM-2 does not have the same effect as carbon monoxide: CO does not have an effect on the channel, while CORM-2 acts as an intracellular allosteric inhibitor of channel function. CORM-2 slows channel activation and deactivation kinetics by reducing the voltage-dependence of the rate constants. It also reduces overall open probability without affecting steady-state voltage-dependence. Manganese-decacarbonyl, another carbon-releasing molecule also does not have an effect on the Kv2.1, while ruthenium red seems to have biphasic effects, one mimicking the action of CORM-2 on the channel and the other representing voltage-dependent pore block, which suggests that CORM-2's actions on the Kv2.1 are independent from its CO-releasing properties.

1648-Pos

Mechanism of Kv1 Channel Redox Modulation by Kvβ

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The *Shaker* type voltage-dependent K^+ channels (Kv1) are expressed in a wide variety of cells and essential to regulating membrane potential and cellular excitability. All Kv1 channels assemble with cytoplasmic β subunits (Kvβ) to form a stable complex. Kvβ is an aldo-keto reductase that utilizes NADPH as a cofactor, and certain Kvβs have an N-terminal segment that blocks the channel by the N-type inactivation mechanism. The enzymatic activity and channel inactivation are functionally coupled: when the Kvβ1-bound NADPH is oxidized, the N-type inactivation is inhibited and, as a result, current increases. To understand the molecular basis of the coupling, we first focused on the N-terminal segment of Kvβ1 that induces channel inactivation. We have identified a stretch of amino acid residues from the N-terminus that are required for redox modulation, which we define as the Redox Regulation Sequence (RRS). Based on our studies, we found that it is likely that the RRS binds directly to the aldo-keto reductase core of Kvβ. To test this hypothesis, and to eventually construct a mechanism for redox modulation, we have started to identify regions on the AKR core that may serve as the ''receptor site'' for the RRS. Initial mutational studies have identified a candidate receptor site, and structural and biochemical studies will further examine how the physical interaction is achieved, and how the interaction is dependent on the redox state.

1649-Pos

Quantifying the Absolute Number of Voltage Gated EGFP Tagged Ion Channels by Fluorescence Intensity Measurements

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In the past, much work has been done to evaluate the gating charge, q, of single voltage gated K+ or Na+ channels. A figure of about 13 e is generally accepted for K+ channels. For Na+ channels, the figure of q estimated by various methods, is less well established and ranges from 5 to 15 e. Recently, we determined the gating charge of single Na+ channels indirectly to be about 6 e by applying our newly determined ratio of q K+ /q Na+ $= 2.5 \pm 0.4$ (Gamal El-Din et al. 2008) and assuming 13 e for q K+. Hereby we used the total fluorescence intensity of EGFP tagged channels expressed in X. laevis oocytes as a measure for the relative number of ion channels and correlated it to the total gating charge, Q.
Currently, we develop a more direct method to estimate the total number of ion channels per oocyte. Analysis of microscopic images of oocytes has been done according to Gamal El-Din et al. (2008). In addition, calibration of EGFP fluorescence intensity with EGFP solutions was done in a hemocytometer. Additional refinements for several correction factors have been tested to obtain reliable absolute numbers of ion channels: Especially the attenuation of the fluorescence from fluorophores at circumferential areas of the oocyte compared to those from frontal areas was taken into account. To obtain a measure of the attenuation factor we used oocytes homogeneously labeled with an extrinsically fluorescent dye and compared the total fluorescence intensity, extrapolated from cirumferential areas, with those extrapolated from frontal areas. Transfer of the attenuation factor of extrinsically labeled oocytes to the instrinsically EGFP labeled oocytes is being discussed and labeling profiles are shown.

1650-Pos

Voltage Clamping a Supported Bilayer

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Supported bilayer has many advantages over conventional black lipid bilayer. A supported bilayer is highly stable and can be made with a large surface area. Membrane proteins can be incorporated in very large numbers without disruption of the bilayer, thereby allowing robust macroscopic recordings. A very important advantage is that incorporated proteins are immobile. This lack of mobility is essential for the study of conformational changes with single molecule fluorescence. We are interested in studying gating charge movement and conformational changes in voltage dependent membrane proteins such as a voltage dependent K+ channel. For this purpose, we have developed an essentially electrostatic voltage clamp system for a supported bilayer that allows measurement of intramembrane transient currents but not DC currents. The supported bilayer is formed by liposome fusion on top of a semiconductor substrate that serves as the reference electrode. Electrolyte solution is present above the bilayer where an Ag/AgCl electrode serves as the active electrode. The electrode pair is connected to conventional voltage clamp electronics that imposes the voltage and measures the current. We verified supported bilayer formation by the decrease of the total capacitance. Furthermore, we have verified that a voltage is imposed across the bilayer by using voltage dependent fluorescent membrane probes. The electrolyte/supported bilayer/electrode system is essentially linear across a voltage range of −300 to +300 mV. We have seen that direct incorporation of the voltage dependent protein KvAP into the supported bilayer modifies the kinetics of the transient currents as well as the voltage dependence of charge movement. This method opens the possibility for studies of simultaneous gating charge movement electrically and voltage dependent conformational change spectroscopically in purified membrane proteins.
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1651-Pos

Validation of Automated Patch-Clamp Instrumentation Competency for Herg Channel Liability Detection in Lead Optimization Programs

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Avoidance of HERG Channel liability is an established regulatory requirement given its capabilities in predicting acquired Long-QT Syndrome, a cardiopathology which may lead to life threatening arrhythmias (Torsade de Points). The sensitivity and precision of patch-clamp electrophysiology, the gold standard in HERG safety pharmacology, have been expanded into the high throughput necessities of the drug discovery industry. This report presents results obtained in the early detection of possible HERG liabilities within