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Voltage-gated K Channels - Gating II

2480-Pos Board B450

Gate Opening Remotely Controls the Interaction between the Voltage Sensor and the Cytosolic Domain in BK Channels

Huanghe Yang, Jingyi Shi, Jianmin Cui.
Washington University, St. Louis, MO, USA.

In voltage- and ligand-gated ion channels the voltage sensor domain (VSD) and the ligand sensor change conformation upon stimulation, which then triggers the activation gate to open. However, the retrograde control of the conformation of the sensors by the activation gate has not been well studied. Recently, we reported that Mg2+ binds to the interface between the cytosolic domain and the membrane-spanning VSD of BK type Ca2+-activated K+ channels and activates the voltage sensor through an electrostatic interaction (Yang et al., 2007; Yang et al., 2008). Here we show that the interaction between Mg2+ and the voltage sensor is controlled by the opening of the activation gate. A mutation (F315A) in the middle of the pore-lining S6 segment altered channel opening such that the channels did not open even though the voltage sensor was fully active. The lock of the channel at the closed conformation also abolished the electrostatic interaction between Mg2+ and the VSD. 100 μm [Ca2+]i opened the activation gate of the mutant channel and reestablished the electrostatic interaction. Therefore, the activation gate and the sensory domains in BK channels are allosterically coupled and undergo concerted movements during channel gating.

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2481-Pos Board B451

Cooperativity Between Voltage-sensing Domains in the Human BK Channel Revealed by Voltage-clamp Fluorometry.

Antonios Pantazis, Vadym Gudzenko, Nicoletta Savalli, Azadeh Kohanteb, Daniel Sigg, Riccardo Olcese.
UCLA, Dept. of Anesthesiology, Los Angeles, CA, USA.

Like other members of the voltage-gated K+ channel superfamily, BK channels are thought to derive voltage sensitivity from charge-possessing transmembrane segments S2-S4. Particularly in BK, S2 is thought to have a direct role in voltage sensing (Ma et al., JGP2006). We combined cut-open oocyte voltage-clamp with fluorometry, after labeling unique cysteines introduced in cysteine-less BK channels (hSlo) with TMRM (Savalli et al., PNAS2006 and JGP2007), to resolve voltage-dependent conformational rearrangements near the extracellular side of S2. The intensity of fluorescence emission (ΔF) was strongly voltage-dependent (FV_{half}=-92 ± 2.7mV, Fz=0.95 ± 0.07, n=8), reporting protein rearrangements. To investigate voltage sensor function, we targeted two putative voltage-sensing residues: D153 (S2) and R213 (the single voltage-sensing residue in S4 -Ma et al., JGP2006). Neutralizing D153 in S2-labeled channels abolished voltage-evoked fluorescence deflections, strongly supporting the role of D153 in voltage activation of S2. Neutralizing R213 in the S4-labeled channel gave rise to a detectable but weakly voltage-dependent ΔF (Fz<0.2, n=2), perhaps arising from the S3 charge (D186, Ma et al., 2006). Cooperativity amongst voltage sensing transmembrane segments was evaluated by investigating ionic currents and ΔF from an S4 charge mutant labeled in the S2 and *vice versa*. In both cases, protein rearrangements were detected, albeit less voltage-dependent (Fz=0.21-0.23, n=5-6 respectively). These findings revealed that strong S2-S4 cooperativity underlies voltage sensing in the intact channel. In contrast to fluorescence experiments, the change in voltage sensitivity of ionic currents was smaller (Gz=0.70-0.86), supporting the view that the pore has intrinsic voltage dependence. An allosteric model of gating composed of two types of voltage sensing tetramers surrounding a single pore was used to provide a global fit of the experimental results.

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Structural and Functional Analysis of the Purified Cytosolic C-Terminus of the Human BK Channel

Anoosh Javaherian[1], Taleh Yusifov[1], Debora Nicoll[2], Chris Gandhi[3], Riccardo Olcese[1].
[1]UCLA, Dept. of Anesthesiology, Los Angeles, CA, USA, [2]UCLA, Dept. of Physiology, Los Angeles, CA, USA, [3]Caltech-Howard Hughes Medical Institute Research Laboratory, Pasadena, CA, USA.

The large-conductance voltage- and Ca^{2+}-activated K^+ channels (BK) are activated by membrane depolarizations and intracellular Ca^{2+}. Two consecutive intracellular RCK (Regulators of K^+ Conductance) domains, RCK1 and RCK2, are responsible for calcium sensitivity and together form the majority of the C-terminus.

Information about the structural and functional properties of the individual RCK1 and RCK2 domains has been reported by our group (Yusifov, et. al. PNAS 2008). However, the characterization of the full C-terminus remains unresolved. Here we report the initial structural and functional characterization of a high-purity protein expressed and purified from *E. coli* corresponding to the human (hSlo) BK channel C-terminus. The expressed C-terminus includes 684aa, starting from the S6-RCK1 linker and encompassing RCK1, RCK2, and the interconnecting 92aa RCK1-RCK2 linker.

The calcium-binding activity of the C-terminus (10ug), loaded on a nitrocellulose membrane, was probed by dot blot analysis of $^{45}Ca^{2+}$-binding. The C-terminus displayed a strong calcium-binding property when compared to Albumin.

The organization of the secondary structure of the C-terminus was investigated using Circular Dichroism (CD) spectroscopy. Far-UV CD spectra (190-260nm) of the C-terminus, analyzed with CONTIN/LL algorithm from the CDpro suite (SMP56 protein reference set), gave a secondary structure consisting of 29% α-helix, 20% β-strand, 22% turn, and 29% unordered.

The quaternary structure of C-terminus was investigated using size-exclusion chromatography with a Superdex 200 10/300 column. The C-terminus eluted in a single peak at a molecular weight of 330kDa corresponding to the theoretical tetrameric C-terminus complex (310kDa). In denaturating condition (SDS-gel electrophoresis), the C-terminus migrated as a monomeric 74kDa band (expected 77.6kDa).

In conclusion, we have successfully purified the functional human BK channel C-terminus domain, which allows for further investigation of the properties of the mammalian BK ''Gating ring,'' encompassing eight RCK domains.

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Calcium Sensing Properties of the RCK1 Domain of the Human BK Channel: Effects of the D362/367A Mutation

Taleh Yusifov[1], Anoosh Javaherian[1], Debora Nicoll[2], Chris Gandhi[3], Riccardo Olcese[1].
[1]UCLA, Dept. of Anesthesiology, Los Angeles, CA, USA, [2]UCLA, Dept. of Physiology, Los Angeles, CA, USA, [3]Caltech-Howard Hughes Medical Institute Research Laboratory, Pasadena, CA, USA.

Recent studies suggest that Ca^{2+}-dependent activation of the large-conductance voltage- and Ca^{2+}-activated K^+ (BK) channel is controlled by two RCK (Regulators of K^+ Conductance) domains located in the C-terminus, encompassing high affinity Ca^{2+}-sites D362/367 and M513 in RCK1, and a Ca^{2+}-bowl (D894-898) in RCK2 (Schreiber, et al, 1999, Lingle et al. 2002, Bao et al. 2004). Previously, we characterized Ca^{2+}-induced conformational changes and the role of the Ca^{2+} bowl in the hSloRCK2 domain using solution-based analysis (Yusifov et al., PNAS 2008).

Using a similar approach, we are now investigating the Ca^{2+}-dependent properties of the WT and D362/367A mutations of a purified protein corresponding to the amino acid sequence (322*IIE¼ DPL*667) that forms the human BK channel's RCK1 domain.

The calcium-binding activity of purified RCK1 (10ug), loaded on a nitrocellulose membrane, was directly probed by dot blot analysis of $^{45}Ca^{2+}$-binding. Albumin and Troponin were used as negative and positive controls, respectively. RCK1 showed remarkable calcium-binding ability when compared to Albumin.

Circular Dichroism (CD) analysis of the WT-RCK1 revealed a calcium-dependent spectral change, corresponding to an increased β-strand content of ~9% as the free [Ca^{2+}] was increased from 0.015 to 31.2μM. This change was paralleled by a similar decrease in α-helix content, while the turns and unordered fractions remained practically unchanged.

On the other hand, the CD spectra of RCK1-D362/367A mutant displays ~7-8% increased beta content, similar to the calcium-bound form of WT-RCK1. The Far-UV CD spectra obtained of hSloRCK1-D362/367A mutant in increasing free Ca^{2+} displayed no changes, suggesting a lack of substantial Ca^{2+}-dependent structural changes. Based on these findings, we propose that the D362/367A mutation in hSloRCK1 may lead to a conformational state of hSloRCK1 that is unable to translate Ca^{2+}-binding to channel gating.

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Cholesterol-ethanol Interactions On Vascular Myocyte BK Channels: Contribution To Alcohol-induced Cerebrovascular Constriction

Thirumalini Vaithianathan, Anna Bukiya, Jianxi Liu, Alejandro M. Dopico.
University Of Tennessee Health Science Center, Memphis, TN, USA.

The activity of large conductance, calcium- and voltage-gated potassium (BK) channels critically limits the degree of vascular smooth muscle contraction, favoring cerebrovascular dilation (Brayden and Nelson, 1992). Ethanol at levels reached in circulation after moderate binge drinking (50 mM) inhibits the activity of cerebrovascular myocyte BK channels, leading to endothelium-

independent cerebrovascular constriction (Liu et al., 2004). On the other hand, high cholesterol levels, which inhibit vascular smooth muscle BK channels (Bolotina et al., 1989), decrease vascular compliance, favoring vasoconstriction (Bukiya et al., 2008). Synergistic inhibition of cerebrovascular BK channels by cholesterol and ethanol would certainly have a profound negative impact on vascular compliance and dilation. Remarkably, such synergism on channel function has not been studied. Thus, we cloned BK subunits (channel-forming cbv1 and accessory, smooth muscle-abundant β1) from rat resistance-size cerebral arteries, reconstituted the channel complex into 1-palmitoyl-2-oleoyl phosphatidylethanolamine/1-palmitoyl-2-oleoyl phosphatidylserine (POPE/POPS) bilayers, and studied cholesterol modulation of ethanol action on channel steady-state activity (NP_o). Acute exposure to 50 mM ethanol mildly yet significantly decreased BK NPo (−4 ± 0.8 % from control) without modifying channel unitary conductance. In the same bilayer type, incorporation of cholesterol at levels found in cell membranes (15 % w/w) also reduced BK NPo (-8.78 ± 5.2 % from control). Remarkably, 50 mM EtOH added to the cholesterol-containing bilayer resulted in a robust decrease in BK NP_o (−36 ± 8.4 % from control). These data unveil a multiplicative inhibition of BK channel activity by alcohol and cholesterol. The kinetic and biophysical mechanisms of such synergism are currently being investigated.

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Regulation Of The Slo2.2 Channel By Na+ Ions And Phosphatidylinositol 4,5 Bisphosphate

Zhe Zhang[1], Avia Rosenhouse-Dantsker[2], Scott K. Adney[1], Diomedes E. Logothetis[1].
[1]Physiology and Biophysics department, School of Medicine, Virginia Commonwealth University, Richmond, VA, USA, [2]Department of Medicine, University of illonis at Chicago, Chicago, IL, USA.

A sodium coordination loop has been shown to bind sodium and switch the sensitivity of Kir channels to phosphatidylinositol-4,5-bisphosphate (PIP2) (Rosenhouse-Dantsker et al., Nat. Chem. Biol. 2008 4:624-631). The large conductance potassium channel Slo2.2 (Slack) is activated by intracellular Na+ and is regulated by Gq-coupled receptor stimulation. Here we investigated whether the molecular switching induced by Na+ in Kir channels operated also in Slack channels. First, by using polylysine and PIP2 in the inside-out patch configuration, we demonstrated that the Slack channel activity can be regulated by PIP2. Second, we screened the intracellular domains of Slack for potential Na+ sites and found that a coordination site similar to the one found in Kir channels controls the sensitivity of Slack channels to Na+. Mutation of an Aspartate located in the RCK2 domain of Slack decreased Na+ sensitivity by 4-5 fold, while it had no influence on Cl- sensitivity. Our preliminary results suggest that the Slack channel shares with Kir channels a similar mechanism of Na+ activation that is likely to modulate its sensitivity to PIP2.

2486-Pos Board B456

Role of Charged Residues in the S1-S4 Domains of Slo2.1 K+ Channels

Li Dai, Michael Sanguinetti.
University of Utah, Salt Lake City, UT, USA.

Slo2.1 is a weakly voltage-dependent, large conductance K^+ channel activated by intracellular Na^+. Unlike the typical Kv channel, the S4 transmembrane domain of human Slo2.1 contains two basic residues (K174, R186) whose charge is partially offset by two acidic residues (E178, D183). The N-terminal residue of the putative S45 linker contains a single charged residue, R190. In addition, Slo2.1 has two basic residues in S1 (R80, K70) and a single acidic residue each in S2 (E118) and S3 (E143). We examined the effects of mutation of individual charged residues to Ala. Human Slo2.1 channels were expressed in *Xenopus* oocytes and whole cell currents were measured using the two electrode voltage clamp technique. In normal extracellular solution, Slo2.1 channels were closed, but could be activated by bathing oocytes in a K^+-free solution for 10-15 minutes (to increase $[Na^+]_i$) or by exposure to 1 mM niflumic acid (NFA). The $V_{1/2}$ for activation of wild-type Slo2.1 channels activated by NFA was −5 mV; effective valence, $z = 0.56$. Point mutations of the charged residues in S1-S4 induced relatively small changes in voltage dependence of activation (max $\Delta\Delta G$=0.25). R190E Slo2.1 channels were constitutively active (current not enhanced by NFA) and the $V_{1/2}$ was shifted to −63 mV; $z = 0.55$. Introduction of a second site mutation (R190E/D183K) reverted channels to wild-type gating mode (closed under control conditions, but activated 10-fold by NFA). Thus, an electrostatic interaction between D183 in S4 and R190 in the S45 linker may stabilize the closed state of Slo2.1 channels.

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Amino-termini Isoforms Of Slack K(Na) Channel Differentially Influence The Rate Of Neuronal Adaptation.

Maile R. Brown[1], Jack Kronengold[1], Arin Bhattacharjee[2], Leonard K. Kaczmarek[1].
[1]Yale University, New Haven, CT, USA, [2]SUNY Buffalo, Buffalo, NY, USA.

The rates of activation and unitary properties of Na+-activated K+ currents, K(Na) currents have been found to vary substantially in different types of neurons. One class of K(Na) channels are encoded by the Slack gene. We have now determined that alternative RNA splicing gives rise to at least five different transcripts for Slack, one class of K channels which produce Slack channels that differ in their predicted cytoplasmic amino-termini and in their kinetic properties. Two of these, termed Slack-A channels, contain an amino-terminus domain closely resembling that of another class of K(Na) channels encoded by the Slick gene. Neuronal expression of Slack-A channels and of the previously described Slack isoform, now called Slack-B, are driven by independent promoters. Slack-A mRNAs were enriched in the brainstem and olfactory bulb and detected at significant levels in four different brain regions. Slack-A channels activate rapidly upon depolarization and, in single channel recordings in Xenopus oocytes, are characterized by multiple subconductance states with only brief transient openings to the fully open state. In contrast, Slack-B channels activate slowly over hundreds of milliseconds, with openings to the fully open state that are ~6 fold longer than those for Slack-A channels. In numerical simulations, neurons in which outward currents are dominated by a Slack-A-like conductance adapt very rapidly to repeated or maintained stimulation over a wide range of stimulus strengths. In contrast, Slack-B currents promote rhythmic firing during maintained stimulation, and allow adaptation rate to vary with stimulus strength. Our data suggest that alternative promoters of the Slack gene differentially modulate the properties of neurons. Supported by NIH Grants NS61479 and DC01919.

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Single channel studies of heteromer formation between Slick and Slack K(Na) subunits

Jack Kronengold[1], Maile R. Brown[1], Haijun Chen[2], Lawrence B. Salkoff[3], Leonard K. Kaczmarek[1].
[1]Yale University School of Medicine, New Haven, CT, USA, [2]State University of New York at Albany, Albany, NY, USA, [3]Washington University in St. Louis Medical School, St. Louis, MO, USA.

Slack (Slo 2.2) and Slick (Slo 2.1) encode sodium-activated K^+ channels (K_{Na}). Native K_{Na} currents may enhance the phase locking of action potential firing at high frequencies, adaptation to prolonged stimulation and are believed to protect both excitable and non-excitable cells from hypoxic injury. Slack and Slick resemble native K_{Na} channels in their Na^+ sensitivity and large unitary conductances (140 and 180 pS in 130 mM KCl, respectively). Two alternative isoforms have been described for the Slack gene; Slack-A and Slack-B. The Slick channel differs from Slack in its opposite regulation by PKC, the presence of an ATP binding site, sensitivity to intracellular Na^+ and Cl^- ions and channel kinetics. We have obtained direct electrophysiological evidence for Slick and Slack-B heteromer formation at the single channel level by constructing a Slick* Q276E, Y279E (Slick*EE) mutant and coinjecting it with Slack-B in Xenopus oocytes. Introducing these negatively charged residues in the inner pore S6 helix resulted in a dramatic increase in the unitary conductance of the Slick*EE homomeric channel from 140 pS to ~ 450 pS (140 mM KCl). In a 1:1 cRNA injection of Slick*EE and Slack-B we identifed conductances of ~330 pS. In contrast, we found no evidence for heteromer formation between Slick and Slack-A at the single channel level. These findings support previous studies demonstrating that Slick and Slack-B, but not Slack-A, subunits can be co-immunoprecipitated from rat brain and from co-transfected HEK cells.

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Soluble β-amyloid oligomers alter biophysical properties of Kv1.3 channels

Maria I. Lioudyno[1], Yuri Sokolov[1], J. Ashot Kozak[2], Michael D. Cahalan[1], James E. Hall[1].
[1]University of California, Irvine, Irvine, CA, USA, [2]Wright State University, Dayton, OH, USA.

The aggregation of amyloid peptides in brain tissue is a hallmark of Alzheimer's disease. Amyloid beta (Aβ) exists in several assembly states, which may play different physiological or pathophysiological roles. The effects of Aβ on voltage-dependent ion channels in neurons and microglia were implicated in early stages of neurodegeneration. We tested the effect of soluble oligomers (Aβ1-42) of amyloid precursor protein (APP) on voltage-dependent potassium channels Kv1.3. Potassium current was measured during whole-cell recording from L929 cells, stably expressing Kv1.3. Acute application of Aβ1-42 reversibly reduced peak current amplitudes and affected kinetics of current activation, inactivation and deactivation in a voltage- and a dose-dependent manner. The time constant of K^+ current activation during