Our previous studies revealed a key role for the Ca^{2+}-activated K^+ (BK) channel in determining the spontaneous contractions of detrusor smooth muscle (DSM) in mammals. Using tissue baths and isometric smooth muscle tension recordings, we examined the contribution of BK channels in the neurogenic contractions of DSM strips isolated from rats and Guinea pigs. Neurogenic contractions were induced by increasing electrical field stimulation (EFS) frequencies (0.5-50 Hz) under stimuli with constant amplitude (20 V), pulse width (0.75 ms), and duration (3 s). These EFS-induced contractions were abolished by the Na^+ channel inhibitor, tetrodotoxin (1 ∈ 1/4M), indicating their neurogenic origin. Blocking the BK channel with its specific inhibitor, iberiotoxin (200 nM), caused a statistically significant increase in the EFS-induced contraction amplitude at all stimulation frequencies in both species. We further dissected the BK channel contribution to the cholinergic and purinergic neurogenic contractions using specific neurotransmitter receptor inhibitors. Atropine (1 ∈ 1/4M) was used to block the cholinergic component and a combination of suramin (10 ∈ 1/4M), and α,β-meth-ATP (10 ∈ 1/4M), was used to block the purinergic component of the EFS-induced neurogenic contractions. In both species, blocking the BK channel caused a statistically significant increase in both cholinergic and purinergic components of the EFS-induced neurogenic contraction amplitude. However, the BK channel contributed more to the purinergic component. With a blocked BK channel, the maximum response of the purinergic component was doubled and achieved at lower frequencies of stimulation. Time controls were performed for each experimental series to confirm the stability of the preparations. The data indicate that the BK channels function to oppose neurogenic contractions in rat and Guinea pig DSM. Supported by NIH DK084284 & DK070909.

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The Putative Ca2+-Acting Site in ANO1

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Ca^{2+}-activated Cl- channels (CaCC) mediate numerous physiological functions including vectorial Cl- movements across epithelia. Anoctamin1/TMEM16A (Ano1) confers Ca^{2+}-activated Cl^- currents. ANO1 having TMs with a putative pore region between TM5 and TM6 shows similar biophysical as well as pharmacological properties as those known for endogenous CaCCs. More importantly, ANO1 is activated by intracellular Ca2+ with EC50 of 2.6 microM at −60 mV. Furthermore, ANO1 activation is also voltage dependent as known for endogenous CaCCs. Because functional importance lies on its activation by intracellular Ca2+, the Ca2+ action site in ANO1 was determined with mutagesis. However, unlike other Ca2+-activated channels such as BK channels, there is no consensus sites for Ca2+ binding except one region that shows weak sequence homology with the Ca2+ action site in BK channel. This region contains many negatively charged amino acids. When we deleted 14 amino acids including the highly negatively charged region, the mutant ANO1 was rarely activated by intracellular Ca2+ with right shift of G-V curves, indicating that this region is important for Ca2+ action. With various mutants in this region, we can localize a sensitive site for Ca2+ response. However, when negatively charged amino acids were replaced by alanine, this mutant showed a comparable sensitivity to Ca2+. Judging from the experimental results of chimera studies with other ANOs, we can conclude that Ca2+ action on this site is essential for its activation.

This work was supproted by the WCU program of the Ministry of Education and Science ans Technology of Korea.

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Heme-Driven Conformational Changes in the Human Slo1 BK_{Ca} Channel Gating Ring

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Large-conductance voltage- and Ca^{2+}-activated K^+ channels (BK_{Ca}) are regulated by signaling molecules acting on two intracellular Regulator of K^+ Conductance (RCK) domains which assemble into a gating ring superstructure. The discovery of heme as a BK_{Ca} channel modulator has unveiled novel aspects of its physiological function (Tang et al., *Nature* 2003). A recent study (Horrigan et al., *JGP* 2005) suggests that heme induces conformational changes within the BK_{Ca} gating ring altering its interaction with the voltage sensor.

Here, we report direct evidence of heme-induced conformational transitions in the purified human BK_{Ca} gating ring, expressed and purified from *E. coli* as reported previously. Its assembly into physiologically-relevant homotetramers (~310kDa) was confirmed by size-exclusion chromatography. Heme-binding properties of the gating ring were studied under physiologically-relevant conditions using steady-state and time-resolved fluorescence spectroscopy in combination with biochemical methods. Heme binding to BK_{Ca} gating ring was detected using UV-visible absorption spectroscopy, which revealed a strong peak at 420nm (Soret band) and a peak at 550nm (α/β-band), characteristic of heme-protein complex formation. The gating ring's Tryptophan fluorescence decreased by up to $44 \pm 1.5\%$ with the addition of heme in a dose-dependent manner ($K_{0.5}$=211 ± 24.0nM, n=2.7 ± 0.10). In contrast, addition of protoporphyrin IX (900 nM), a heme analog lacking Fe, did not affect the Tryptophan fluorescence intensity. Time-correlated single-photon counting (TCSPC) spectroscopy resolved heme-induced structural alterations of the gating ring. Heme binding reduced the average excited-state Tryptophan lifetime from ∉″$_{avg}$=2.6ns (no heme) to ∉″$_{avg}$=1.6ns ([heme]=687nM), revealing an altered conformational state. Effects of heme and Ca^{2+} on fluorescence lifetime were not additive as, after saturating Ca^{2+}-induced effect were reached (35∈ 1/4M), addition of saturating [heme]=687nM produced minimal effect (∉″$_{avg}$=1.5ns). In summary, we demonstrate heme-induced conformational transitions in the human BK_{Ca} gating ring, likely correlated to the modulation of channel activity.

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MthK Gating Explored with a High Affinity Quaternary-Ammonium Blocker

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The bacterial potassium channel MthK is activated by Ca^{2+} binding to intracellular RCK-domains. The channel is homologous to the K^+ conductive pore and C-terminal Ca^{2+} binding domains of the eukaryotic BK channel but lacks the N-terminal voltage-sensing domain. A low resolution x-ray crystal structure showed the MthK pore in a proposed open conformation, consistent with the presence of Ca^{2+} in the crystals (Jiang et al. Nature, 2002). It is widely assumed that the closed state of the channel involves an intracellular constriction of pore-helices as seen in the KcsA crystal structure (Doyle et al. Science, 1998; Jiang et al. Nature, 2002). However, several ligand-gated K^+ channels, including the BK channel, have been proposed to use the selectivity-filter near the extracellular side and not an intracellular constriction as the conduction gate (Wilkens and Aldrich, JGP, 2006). We tested the presence of an intracellular gate in MthK by using a quaternary-ammonium (QA) blocker, bbTBA. QA blockers bind within the aqueous vestibulethat lies between the proposed intracellular gate and the selectivity-filter 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). We measured the bbTBA binding affinity to the open channel and have studied the state-dependence of channel block using single-channel recording in artificial bilayers. Preliminary data indicate the channel can close while the blocker remains bound. Further experiments involving macroscopic recordings may distinguish whether the blocker is trapped inside the closed channel or has state-independent binding.

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Mechanism Underlying pH-Modulation of Ca^{2+}-Dependent Gating in the MthK Channel

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MthK is a Ca^{2+}-gated K^+ channel whose activity is modulated by cytoplasmic pH. To determine possible mechanisms underlying the channel's pH sensitivity, we recorded current through MthK channels, which were purified from E.coli membranes, reconstituted into liposomes and then incorporated into planar lipid bilayers. Each bilayer recording was obtained at up to six different [Ca^{2+}] (ranging from nominally 0 to 30 mM) at a given pH, in which the solution bathing the cytoplasmic side of the channels was replaced via a perfusion system to ensure complete solution exchanges. We observed a steep relation between [Ca^{2+}] and open probability (Po), with a mean Hill coefficient (n_H) of 9.9 ± 0.9. Neither the maximal Po (0.93 ± 0.005) nor n_H changed

significantly as a function of pH over pH ranging from 6.5 to 9.0, suggesting that H^+ does not alter either functional coupling or cooperativity in Ca^{2+}-dependent gating. In addition, channel openings were not observed in the nominal absence of Ca^{2+} at pH up to 9.0. However, increasing pH decreased the EC_{50} for Ca^{2+} activation by ~4.7-fold per 10-fold increase in $[H^+]$, displaying a linear relation between $\log(EC_{50})$ and pH over the entire range of pH studied (6.5 to 9.0). Together, these results suggest that H^+-binding does not directly modulate either the allosteric coupling between Ca^{2+}-binding and channel opening or the channel's closed-open equibrium. We may account for the pH modulation by assuming that increasing pH yields a relative energetic stabilization of the Ca^{2+}-bound states over unliganded states of the channel.

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Identification of Divalent Cation Coordinating Residues in a K^+ Channel RCK Domain by NMR Spectroscopy

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TvoK is a prokaryotic K^+ channel whose gating is modulated by divalent cation-binding to a carboxy-terminal RCK domain. To gain insight toward mechanisms underlying divalent cation binding and subsequent conformational changes, we measured chemical shift perturbations upon ligand binding in the soluble cytoplasmic RCK domain of TvoK using heteronuclear NMR spectroscopy.Uniformly ^{15}N-labeled, highly deuterated TvoK RCK domain was overexpressed in *E.coli* and purified by affinity and gel-filtration chromatography. ^{15}N-HSQC spectra showed well-dispersed crosspeaks corresponding to >85% of the 238 predicted backbone NH groups. Five-point titration experiments using 0 to 100 $\in$ 1/4M Mn^{2+} identified 12 residues that surrounded a putative divalent cation binding site, as indicated by spectral line-broadening due to the paramagnetic relaxation enhancement effect of Mn^{2+} (Mn-PRE). Partial resonance assignments, made through a combination of HNCA experiments and residue-specific ^{15}N-labeling, identify D192 and E226 as key residues in divalent cation coordination, as indicated by high sensitivity to Mn-PRE ($K_{app} < 10 \in 1/4M$). Further resonance assignments will identify remaining residues that lie within ~15 Å of the binding site. These experiments may reveal differences between the structural and chemical properties of the TvoK binding site and the Ca^{2+}-selective binding site of the MthK RCK domain, which may underlie differential selectivities of MthK and TvoK RCK domains for divalent cations.

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Voltage-Dependent Motions Reported from the N-Terminal Region in Human Slo1 BK_{Ca} Channels: S0 and Voltage Sensor Operation

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The transmembrane region of large-conductance, voltage- and Ca^{2+}-activated K^+ (BK_{Ca}) channel α subunits (Slo1) possesses a unique topological feature when compared with those of other voltage-activated proteins: S0, an additional transmembrane segment that renders their short N-terminus extracellular (Wallner *et al.*, 1996, *PNAS*). S0 mediates the interaction between pore-forming α and auxilliary β subunits, which facilitate channel activation and, in the case of β2 and β3, induce fast inactivation. Recent findings have proposed that S0 is flanked by transmembrane voltage-sensing domains S2-S4 (Liu *et al.*, 2008, *JGP*). We used cut-open oocyte voltage-clamp fluorometry to explore the role of S0 in the function of the BK voltage sensor. By substituting unique Cysteines at positions 17-19 (20 thought to be at the extracellular tip of S0) of hSlo1 M4, and labeling them with the environment-sensitive fluorophore TMRM, we have resolved voltage-dependent conformational rearrangements, with $V_{half} = -79 \pm 2.9$ mV and $z = 0.84 \pm 0.046\ e^0$. Intriguingly, channel activation in these Cysteine mutants exhibited a facilitated voltage dependence of ionic conductance ($\in'' V_{half} = -30$ mV) compared to pseudo-WT channels. We provide direct evidence that the N-terminus influences the operation of the voltage-sensing S2 and S4 transmembrane domains: mutation R20A induced pronounced shifts in the activations of voltage-sensing segments S2 and S4 ($\in'' V_{half} = 46$ mV for S2, 66mV for S4) and a 35% reduction in the effective charge (z) of both. These results strongly indicate that the N-terminal region plays a significant role in the voltage sensor operation of human BK_{Ca} channels.

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Charge Substitution of a Deep-Pore Residue Suggests Structural Rearrangements During BK Channel Activation

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During scanning mutagenesis of the S6 region of BK channels, we found a deep pore residue (M314 in hSlo) with interesting gating phenotypes. At pH7, the histidine substitution at this position (M314H) gave rise to currents with significantly slower deactivation kinetics than the wild-type BK. Deactivation became even slower with intracellular pH6 and pH5. To test whether this observation is an effect due to side-chain charges, we made three other charge-substituting mutants, M314K, M314E and M314D. For M314K, deactivation kinetics of the currents at pH7 was similar to that of M314H at pH5. This is consistent with the fact that the lysine (K) side-chain has a more basic pKa than that of histidine (H). For both M314H and M314K, increasing proton concentration (lowering pH) made the deactivation slower while reducing proton concentration (increasing pH) made the deactivation faster. For M314E, deactivation kinetics of the currents was fast at pH5 and became slower as the intracellular proton concentration was reduced to pH6, 7, 8, 9 and 10. The M314D channels could stay open at negative potentials in 0 Ca2+, at pH7, 8, 9 and 10. The most effective way to close the M314D channels was to increase the intracellular proton concentration to pH5. The results from all the charge-substituted mutants (M314H, M314K, M314E and M314D) support the idea that neutralizing the side-chain at the 314 position helped the channels close while charging the side-chain made it difficult for the channels to close. We propose that the charged form of the substituted residues may prefer the polar environment of the pore and stabilize the open state. The 314 residue may become more exposed to the pore as the channels transition from the closed to the open conformation.

662-Pos

Contribution of S3-S4 Extracellular Loop Residues to Block of Kca2 Channels by Apamin

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Small conductance Ca^{2+}-activated K^+ ($K_{Ca}2$) channels are widely distributed within the CNS and peripheral tissues. The cloning of these channels revealed three subtypes, each displaying a different sensitivity to block by the $K_{Ca}2$ selective toxin apamin. Mutation studies of $hK_{Ca}2.1$ and $rK_{Ca}2.2$ have indicated the importance of particular residues in both the channel pore and S3-S4 extracellular loop for block by apamin.

It has been reported that mutation of threonine (T)216 within the S3-S4 loop of $hK_{Ca}2.1$ to the corresponding serine (S) in $rK_{Ca}2.2$ resulted in a current that was more sensitive to block by apamin (Nolting et al, 2007; JBC 282, 3478). We have further investigated the residues in this extracellular loop region that contribute to block by apamin, d-tubocurarine (dTC) and tetraethylammonium (TEA). Block of expressed $K_{Ca}2$ channel current was assessed using outside-out macropatches, with current activated by 1 μM intracellular Ca^{2+}. Mutation S245 to T, to mirror the previously reported mutation of $hK_{Ca}2.1$(T216S), resulted in a reduction in the sensitivity to apamin and no change in the sensitivity to TEA and dTC. Double point mutation of the loop YA246/7 of $rK_{Ca}2.2$ to the corresponding LV of the apamin-insensitive $rK_{Ca}2.1$, abolished block by apamin, reduced sensitivity to dTC sensitivity, but did not affect sensitivity to TEA. In contrast, generation of $K_{Ca}2.2$(Y246L) reduced sensitivity to block by apamin, but did not change sensitivity to dTC. These data suggest that additional residues within the S3-S4 extracellular loop contribute to the high sensitivity to block by apamin exhibited by $K_{Ca}2.2$.

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A Molecular Model for the Bkca Channel and the Location of B1 in the B1/A Subunit Complex

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We describe two approaches for the construction of a 3D molecular model for BK. First, we used a LRET technique in which an encoded **L**anthanide **B**inding **T**ag (**LBT**) that binds Tb^{3+} plays the role of the donors and SulphoRodhamine Methanothiosulfonate (TMSR) attached to Charibdotoxin (ChTX)) plays the role of the acceptor. The data obtained allowed us to determine the distances from the center of symmetry of the channel to the external aspect of S0, S1, S2, S3-S4 linker in the α subunit. The distances from the channel center of symmetry to TM1, TM2 and three positions in the β1 loop were obtained using the same methodology, by inserting LBT's in the β1 subunit. Second, molecular