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Diverse Effects of a Benzofuroindole on Different K^+ Channels and Localization of Its Receptor on BK_{Ca} Channel

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We reported previously that the activity of the large-conductance calcium-activated potassium channels (BK_{Ca} channel) could be strongly potentiated by certain derivatives of benzofuroindole scaffold when treated from extracellular side of the membrane (Gormemis *et al*., 2005; Ha *et al.*, 2006). In order to localize the receptor site on the BK_{Ca} channel, we surveyed the effects of CTBIC, the most potent benzofuroindole compound, on various K^+ channels. While the compound increase the activity of voltage-gated K^+ channels, $K_V1.5$ and HERG, CTBIC did not affect the activity of inward rectifier K^+ channel, Kir3.1, significantly. Intriguingly enough, the same compound greatly *decreased* the activity of SK2, a different subclass of Ca^{2+}-activated K^+ channel. In addition, the affinity of charybdotoxin, a peptide pore-blocker, was reduced by the co-treatment with CTBIC, whereas that of tetraethylammonium, a small pore-blocking quaternary ammonium, was not altered. Guided by these results, we performed mutagenesis studies on the outer vestibule of the BK_{Ca} channel to localize the residues that affect the binding of CTBIC. We identified three residues in the loop that connects with the pore-forming region of the channel, which was strongly affected by alanine substitution. Our results suggest that the turret region of the BK_{Ca} channel may play a critical role in the modulation of the channel activity and may thus represent a therapeutic target site of K^+ channels.

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NS8593-Mediated Negative Gating Modulation Depends on Residues in the Inner Pore Vestibule of Kca2 Channels

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The identification of NS8593 has provided a selective and novel means for modulating the activity of the small-conductance calcium-activated potassium channels $K_{Ca}2.1$-2.3. Acting as a negative gating modulator, NS8593 shifts the apparent calcium dependence of channel gating to higher calcium concentrations. It has been assumed that the binding site for NS8593 was located in the C-terminal region, similar to that of some positive gating modulators (e.g. EBIO and CyPPA, but not GW542573X). However, by employing a progressive chimera approach, (where all critical constructs were tested for normal Ca^{2+}-sensitivity in inside-out patches) we were able to localize the site-of-action to the pore. For example, when we transferred the C-terminus from the NS8593-insensitive intermediate-conductance $K_{Ca}3.1$ channel to $K_{Ca}2.3$ the chimeric channel remained as sensitive to NS8593 as WT-$K_{Ca}2.3$. In contrast, when we transferred the $K_{Ca}2.3$ pore, $K_{Ca}3.1$ became sensitive to NS8593. Subsequently, by using site-directed mutagenesis we identified two residues in the inner vestibule of $K_{Ca}2.3$ (Ser-507 and Ala-532) that mediate the activity of NS8593. By mutating these residues to the corresponding residues in $K_{Ca}3.1$ (Thr-250 and Val-275), we were able to make $K_{Ca}2.3$ insensitive. Conversely, replacement of these two residues was sufficient to render $K_{Ca}3.1$ sensitive to NS8593. The positions of these residues, Ser-507 in the pore-loop near the selectivity filter and Ala-532 in an adjacent position in the S6 segment, are within in the region predicted to contain the channel gate. Based on these results, we propose that NS8593 mediated gating modulation of $K_{Ca}2.3$ occurs at a position deep within the inner pore vestibule.

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Structural Determinant of Altered Current Expression, Activation Kinetics and Beta-Subunit Interaction of the Neuronal X1 Splice Variant of the Rat BK Channel

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We have identified and cloned a splice variant of the rat BK channel called X1 (Poulsen et al., 2009, Biochimica et Biophysica Acta. 1788(380-389)) which is exclusively expressed in brain or nervous tissue, which has not previously been functionally characterized. The X1 variant is different from the insertless variant Zero by having an eight amino acid insert in the extracellular loop between S1 and S2, a four amino acid insert between C-terminal S8 and S9 (SS1) and 27 amino acids between S9 and S10. Another variant Slo27, widely expressed in brain and some vascular tissues, also contains the 27 residues between S9 and S10 but only a 3 residue insert between S8 and S9 (SS2). When expressed in *Xenopus* oocytes, the X1 variant shows less current expression than Slo27 or Zero and an apparently faster activation speed. We attempted to dissect the underlying mechanism by generating constructs lacking one of the insert sequences. Deletion of the eight amino acids between S1 and S2 resulted in higher current expression similar to Slo27 or Zero while retaining the fast activation speed. Deletion of the four S8-S9 residues resulted in low current expression but still fast activation. Thus the eight residue insert seems to suppress channel surface expression or channel gating at low calcium concentrations, while the structural determinator of fast activation speed is less clear.
We also co-expressed the X1 variant with beta 2, which is present in nervous tissue also. Beta 2 co-expression reduced current expression further and slowed channel activation but showed no signs of inactivation (at low calcium), which is a key feature of beta 2 when co-expressed with Zero or Slo27.

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Acceleration of Cutaneous Wound Healing by Suppression of Large Conductance Ca^{2+}-Activated K^+ Channels

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Many kinds of K^+ channels are involved in the regulation of cell migration and proliferation, which are required for the processes of wound healing. However, the role of K^+ channels on cutaneous wound healing has not yet been reported. Here, we demonstrate that inhibition of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels expressed in human epidermal keratinocyte facilitate cutaneous wound healing by activating both cell migration and proliferation. In the group treated with 25 mM KCl, *in vivo* wound healing was facilitated more rapidly than that in control group. *In vitro* assay of wound healing showed that 25 mM KCl significantly increased wound closure in keratinocytes after creation of linear wound with ~200 ∈ 1/4m wide defect. KCl (25 mM) promoted processes of cell migration and proliferation. BK_{Ca} and two-pore domain K^+ channels were recorded in the keratinocytes by using patch-clamp technique. The BK_{Ca} channel, among these K^+ channels, is the most frequently observed in cell-attached mode. NS1619, a BK_{Ca} channel opener, inhibited the proliferation and migration of keratinocytes in a dose- and time-dependent manner. Charybdotoxin and iberiotoxin, BK_{Ca} channel blockers, facilitated both cell proliferation and migration by $10 \pm 7\%$ and $30 \pm 4\%$, respectively. Cutaneous wound healing was also facilitated by siRNA against BK_{Ca} (BK_{Ca}/siRNA). The migration and proliferation were more enhanced by cotransfection with BK_{Ca}/siRNA and TASK-1/siRNA. BK_{Ca} channel blockers activated PKC and ERK in a time-dependent manner. These results show that BK_{Ca} and TASK-1 channels regulate proliferation and migration of human epidermal keratinocytes by activation of PKC-ERK pathway and indicate that BK_{Ca} channel could be a molecular target for regulation of cell proliferation and migration.

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An Unconventional Role in Store-Independent Constitutive Calcium Signaling by the Secretory Pathway Calcium - Atpases in Mammary Tumors

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Constitutive calcium signaling in cancer cells drives tumor proliferation and metastasis. Secretory Pathway Ca^{2+}-ATPases (SPCA) were highly upregulated in breast cancer derived cell lines and human breast tumors. Depletion of SPCA in human breast adenocarcinoma cells attenuated basal Ca^{2+} levels and downstream cell proliferation, anchorage-independent growth and tumor formation in mice. Contrary to its known role in Golgi Ca^{2+} sequestration, SPCA overexpression increased cytosolic Ca^{2+} by activation of the store-operated Ca^{2+} channels. However, SPCA mediated Ca^{2+} influx was independent of Ca^{2+} stores or sensors and not dependent on its transport ATPase activity, revealing a new signaling paradigm.

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Role Of Ca^{2+}-Activated K^+ Channel in the Neurogenic Contractions Induced by Electrical Field Stimulation in Detrusor Smooth Muscle Isolated from Rats and Guinea Pigs

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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.

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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