determine the in situ K_d of furaptra and a subsequent measure of intra-SR [Ca], thus allowing the establishment of the steady-state SR Ca pump efficiency in cardiac myocytes.

Voltage-gated K Channels - I

507-Pos C-type Inactivation From The Perspective Of Permeant Ions

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Board B355

KcsA is widely accepted to enclose a prototype of K^+-selective pathway and has provided a structural-framework to understand selectivity, ion-permeation, gating and pore-blocking. Recently identified C-type inactivation in KcsA furthers strengthens its role as an archetypical pore of Kv channels. In an attempt to tease-out the fine details of the inactivation mechanism in KcsA, we have carried out a systematic analysis of macroscopic and single-channel currents under varying conditions of pH, voltage, permeant-ions and blockers. We find that KcsA inactivation is modulated by voltage and recovery occurs predominantly via closure of the lower activation-gate. Inactivation in KcsA is not entirely a property of the open-conducting channel but also occurs from partially "activated"-closed-states with rates progressively increasing from the farthest closed-state to the open-state suggesting a strong coupling between activation and inactivation. Recovery experiments demonstrate that when preceded by high-throughput ion-permeation, inactivation occurs faster and to a much deeper extent (compared to without ion-conduction in the absence of a driving force). Further, inactivation rates also vary as a function of the amount of current passing through the channel; these results reveal a greater tendency of the filter to collapse while the ions transition from one binding-site to the next. This collapse, as suggested in previous studies, likely involves a constriction at the external binding-site and is favored under low external K^+ where inactivation is significantly faster. Consistent with this idea, ions with long residence-time in the filter (Rb^+, Cs^+, Ba^{2+}) dramatically slow-down inactivation, a property closely reflecting the "foot-in-the-door" effect observed in K_v channels. As also implicated by studies in eukaryotic channels, C-type inactivation in KcsA appears to involve an intimate interplay between the selectivity-filter region and permeant-ions. We analyze these findings in the light of new high-resolution structural information on the inactivated-states.

508-Pos A Multi-point Hydrogen Bond Network Driving Kcsa C-type Inactivation

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Board B356

The prokaryotic proton-gated potassium channel KcsA undergoes a time dependent slow inactivation process, which is modulated by transmembrane voltage but not by pH. Previous results have suggested that the number and strength of hydrogen bonds between residues in the pore helix and external vestibule determine the rate and extent of the C-type inactivation. The interaction between Glu71 and Asp80 is one of the key driving forces that promote filter instability through a compression of the selectivity filter parallel to the permeation pathway, which energetically biases it towards the inactivated conformation. High resolution KcsA structures suggest that the selectivity filter is also stabilized by an interaction between Trp67 and Asp80, a partnership conserved in most potassium channels. Using patch clamp experiments, EPR spectroscopy and X-ray crystallography, we have studied the effect of amino acid substitutions at position 67, in an attempt to establish the role of this residue in the inactivation gating at the selectivity filter of KcsA. Substitution of Trp67 to phenylalanine decreases the rate and extent of inactivation as determined by macroscopic and single channel currents. Thus, as in the Glu71-Asp80 interaction, disrupting the interaction between residues Trp67 and Asp80 unmasks the presence of another critical hydrogen bond at the selectivity filter of KcsA. Indeed, sequence alignment of several prokaryotic potassium channels suggests tyrosine as the natural amino acid substitution at this position. Furthermore, substitution to tyrosine (W67Y) recovered the inactivation phenotype presumably by reestablishing the 67 and Asp80 interaction through a hydrogen bond. Taken together, the present results suggest that in addition to the Glu71 and Asp80 carboxyl-carboxylate interaction, the hydrogen bond between Trp67 and Asp80 at the KcsA selectivity filter and its adjacent pore helix constitutes another key interaction that determines the rate and extent of C-type inactivation.

509-Pos Gating-related Conformational Changes in the Outer Vestibule of KcsA: A Functional and Spectroscopic Analysis

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Board B357

The potassium channel KcsA is gated by proton and modulated by the transmembrane voltage. It is well established that there is a large conformational change in the lower gate of KcsA upon change in pH. In addition, it has been shown that the selectivity filter also play a crucial role as a second gate in ion conduction. The high sequence similarity between KcsA and eukaryotic potassium channels at the p-loop region makes it a suitable model system for studying the conformation changes associated with the gating. In this study, we have monitored the conformational changes in the outer vestibule of KcsA during gating using electrophysiological and spectroscopic (EPR and fluorescence) measurements. We generated several cysteine mutants corresponding to the outer vestibule of full-length KcsA using wild type and E71A mutant backgrounds (representing the inactivated and non-inactivated states of KcsA), to understand the gating-related conformational changes. Our EPR mobility

results show that wild-type KcsA shows very subtle changes in the conformation of the outer vestibule upon channel opening and inactivating. These changes seem to be maximized in the non-inactivating form of KcsA. This is supported by liposome-patch macroscopic electrophysiological measurements. This structural change could be attributed to the changes in the selectivity filter when the channel undergoes a change from non-conductive to conductive state, and might be related to the uncoupling of the lower gate and the selectivity filter in the non-inactivating condition.

510-Pos Insights into the Mechanism of pH-sensing of KcsA K+ Channels

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Board B358

The bacterial potassium channel KcsA is gated by intracellular protons, opening at a narrow pH window of 3.5–4.0, and completely closing at pH >5. The molecular basis of pH sensing remains unknown, but is likely controlled by proton binding sites at or near the intracellular gate. We have investigated the role that N and C-terminal glutamate and histidine residues play in proton sensing. Glutamate (solution pKa = 4.25) is a prime candidate for the pH sensor. At pH< 4.0 the glutamate carboxylate is protonated and thus uncharged. At pH>4.0, glutamate deprotonates (becomes negatively charged), altering the local electrostatic landscape. N-terminal histidines (solution pKa=6.0) can be positively charged and are proximal to C-terminal glutamates. Using site directed mutagenesis we investigated the role these residues play in proton gating using the inactivation-removed E71A KcsA. Glutamates were mutated to glutamine and alanine. Histidines were mutated to arginine and alanine. Purified KcsA protein was incorporated into liposomes, and then used in planar lipid bilayer recordings. We found that these mutations dramatically altered the pH sensitivity of the channel, allowing it to stay open at pH as high as 6.5. These results suggest that the glutamate and histidine residues constitute part of the proton sensing mechanism of KcsA channels by contributing to the open/closed equilibrium of the intracellular gate through an electrostatic mechanism and/or hydrogen bonding networks.

511-Pos Lipid Dependence of KcsA Inactivation

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Board B359

Ion permeation through the bacterial potassium channel KcsA can be blocked by two gates. One is represented by the cytosolic ends of the transmembrane helices and the other by the selectivity filter. The conformational dynamics of the selectivity filter rely on the reorientation of the carboxylic moiety of Glu71. We now have tested the hypothesis that these dynamics are sensitive to the local electrostatic environment by reconstitution of the wild type channel and the E71A mutant into solvent free planar lipid bilayers of different compositions. The dependence of channel lifetime and open probability on lipid charge confirms that the interaction between neighbouring helices is, at least in part, determined by the lipid environment.

512-Pos Global Twisting Motion Of KcsA Potassium Channel Upon Gating Detected By The Diffracted X-ray Tracking Method

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Board B360

We detected motions of the KcsA channel upon gating in a single molecular level with the diffracted X-ray tracking (DXT) method. In this method KcsA channels were fixed on a glass plate in different orientations and a gold nanocrystal with the size of 20–25 nm was attached to the end of the cytoplasmic domain. Synchrotron white X-rays were irradiated normal to the glass plate and a diffraction spot from the attached nanocrystal was recorded in video rate. When channels were fixed in upright position, the spot moved radially when the channel bent, and the spot moved circumferentially when the channel twisted around longitudinal axis. At neutral pH where the channel kept closed, the restricted Brownian motion was detected and the range of the channel motions was within 3 degrees. At acidic pH where the channels underwent gating, the channel exhibited vigorous twisting motions. The range of the twisting motion exceeded 20 degrees, while the bend motion was within 5 degrees. The motions were also observed from the channel fixed in two different orientations: erect orientation and sideways laid orientation. Patterns of motions viewing from different viewpoints gave consistent dynamic picture that channel molecule twists around the longitudinal axis upon gating. To detect motion of the gate directly the cytoplasmic domain was deleted and the C-terminal end of the M2 helix was labeled by the nanocrystal. The twisting motions were also detected and slightly enhanced. In the presence of tetrabuthylammonium (TBA) the twisting motion was stopped for the full-length and CP domain-deleted channels. Therefore we conclude that the KcsA channels exhibit global twisting motion upon gating and there is a tight mechanical coupling between the transmembrane and cytoplasmic domains.

513-Pos Direct observation of KcsA reconstituted in lipid bilayers by Atomic Force Microscopy

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Board B361

Atomic Force Microscopy (AFM) offers several advantages over other methods of ion channel structure investigation. The protein is reconstituted in a lipid bilayer, and thus is likely to resemble its native state more closely. Potentially AFM could be used to observe conformational changes, enabling different states of the channel to be imaged. Furthermore AFM can be operated in aqueous buffer at ambient temperatures (unlike the low temperature conditions required for X-ray and cryoEM) in conditions closer to physiological. *Streptomyces lividans* KcsA is a 160aa membrane protein that oligomerizes to form a tetrameric potassium channel. KcsA is a valuable channel to study as the crystal structure of the closed state has been solved.

We used high-resolution, AM-mode AFM imaging to study the cytoplasmic and extracellular surface structures of single KcsA channels. KcsA was purified from E coli and reconstituted into liposomes with different lipid compositions. The liposomes were adsorbed onto freshly cleaved mica and imaged in KCl and NaCl buffers at pH 7. We were able to distinguish the extracellular and cytoplasmic faces of single KcsA by their different lateral and height profiles.

Under certain conditions, KcsA reconstituted in POPE:POPC (3:1) membranes was observed to form clusters, ~50nm diameter, with long-range hexagonal order analogous to small 2D crystals. In POPE:POPG (3:1) membranes, disordered clusters of 3–4 molecules were observed. This suggests that non-anionic (POPC) lipids preferentially facilitate ordered clustering. Previous AFM images of K channels have used 2D crystals. This is the first example of K channels that have been imaged by AFM AM-mode in lipid membranes.

514-Pos Importance of C-Terminal Residues to Selectivity and Conductance of *Streptomyces lividans* KcsA

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Board B362

The *Streptomyces lividans* channel, KcsA, is a supramolecular complex in which four polypeptides surround a conductive polyphosphate (polyP) chain. The end unit of the polyP has a pK_2 ~7, consequently, at physiological pH the end unit bears a divalent negative charge and displays a strong preference for divalent cations. In wild-type KcsA, the C-terminal residues that surround the end unit of polyP are basic arginines (RR) which reduce the effective negative charge of polyP, thus making the channel selective for K^+ over Mg^{2+}. Replacing a single arginine with a neutral residue (VR or NR) does not alter channel selectivity but decreases open time indicating an increase in block by Mg^{2+}. However, when both arginines are replaced with neutral residues (VL or NN), the channel becomes sensitive to intracellular pH; Mg^{2+} is preferred over K^+ at pH >7 and K^+ over Mg^{2+} at pH <7. When arginines (pI= 10.8) are replaced with lysines (pI=9.8), the mutant KcsA channels (KK) have a lower open time and begin to lose selectivity for K^+ over Mg^{2+} at pH >9. KcsA mutants with one terminal lysine (NK) have a very low open time and also a lower conductance magnitude. The NK channels begin to lose selectivity for K^+ over Mg^{2+} at pH >8. The results show the importance of the C-terminal arginines in maintaining KcsA selectivity and conductance at physiological pH and during local changes in pH.

515-Pos Determining The Coupling Between Subunits In Kcsa Using Fluorescence Spectroscopy

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Board B363

Ion permeation through K^+ channels has been studied intensively in the KcsA channel. It is generally agreed that the helical bundle crossing formed by the four TM2 helices of the tetrameric KcsA channel have to open in order for ion conduction to occur. It is still unknown, whether the opening of the lower pore region happens independently for each subunit or whether all TM2 open simultaneously, a question that is also discussed for voltage-gated ion channels. Here, we address this problem using single molecule fluorescence spectroscopy.

We have shown previously (*Blunck et al., JGP 2006*), that we can monitor the opening of KcsA optically by fluorescently labeling it at the C-terminus of TM2. We introduced purified and labeled KcsA channels in supported bilayer and imaged the time course of fluorescence. We found single fluorescent channels that bleached in up to four distinct photobleaching steps. Since we have four cysteines per channel, each channel should contain (dependent on bleaching and labeling efficiency) up to four fluorophores. Thus, we interpreted that each fluorophore represents a single subunit. Fluctuations of the fluorescence prior to photobleaching, which were interpreted as movements of the subunits (*Blunck et al., JGP 2006*), showed the correct pH dependence of KcsA. We frequently found simultaneous gating of several subunits suggesting a coupling between them. The fluorescence traces were further analyzed with Hidden Markov Models (using QUB), which convoluted bleaching and gating of the channels. Although the coupling constant still varies widely due to the short observation times, these results cannot be explained with independently acting subunits.

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516-Pos Water In Potassium Channels: Quantum Calculations On Selectivity And Gating

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Board B364

1)**SELECTIVITY**: QM calculations on K^+ and Na^+ in the cavity and lowest selectivity filter position in KcsA show large differences in solvation of the ions. A local minimum exists for K^+ but not Na^+ in the cavity center; K^+ is surrounded by 8 water molecules (water at two different distances). The minimum just below the selectivity filter shows K^+ with one water, the Na^+ with five, with solvation from carbonyls and, for K^+, the four hydroxyls of thr-75, compared to one T75 for Na^+. The Na^+ is slightly displaced from the center of the path, coming closer to one domain. In the lowest selectivity filter position, there are additional differences in (co)solvation. Results are in qualitative agreement with Lockless et al[1].

2) **GATING**: Preliminary results on an aqueous path from $K_v1.2$ (pdb coordinates 2A79) with amino acids which line the path, taken from S3, S4, S5, S6, show a water wire forming; this would allow protons to pass, as in our gating model proposed earlier[2–4]. We include a phosphate ion in the calculation at the intracellular end, and it behaves much as expected, complexing with two arginines[5], possibly blocking access to the cleft. The result also suggests a possible mechanism by which moving protons could unlock S6 to open the channel.

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517-Pos The Carboxyl-terminal Domain Determines Selectivity Filter Gating In KcsA Channels

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Board B365

The bacterial KcsA potassium channel consists of a small extracellular domain, a transmembrane domain housing the selectivity filter, and an extended intracellular domain. The selectivity filter not only is responsible for ion selectivity, but it also functions as a gating mechanism that causes channel inactivation after intracellular H^+ activates the channel. In this study, we examined whether the intracellular domain of KcsA plays a role in selectivity filter gating. We expressed KcsA in COS-1 cells and recorded the macroscopic current using an inside-out patch-clamp configuration. With K^+ as the charge carrier, the wild-type KcsA current exhibited fast activation followed by inactivation when intracellular pH was lowered below 4.5. E71 is a residue in the pore helix near the selectivity filter; an E71A mutation almost completely retarded the inactivation in reconstituted KcsA channels (Cordero-Morales et al., *Nat. Struct. Mol. Biol. 13*:311–318, 2006). However, under our experimental conditions, mutation of E71 to either A or V resulted in a subtle change in both the magnitude and time course of inactivation, whereas mutation of E71Q almost completely silenced the channel activity. In contrast, a C-terminal deletion (Δ120) significantly retarded channel inactivation and accelerated recovery from inactivation, in addition to causing the alkaline shift in the pH-activation curve that was reported previously. E71V combined with the Δ120 deletion further attenuated the inactivation. Surprisingly, with Rb^+ as the charge carrier, inactivation of the channels carrying the Δ120 deletion was dramatically potentiated and accelerated, whereas recovery from inactivation was slow and composed of a large irreversible component. However, inactivation of the wild-type channels was similar regardless of whether K^+ or Rb^+ was the charge carrier. Collectively, these results suggest that the C-terminal domain structure is crucial to the gating process that occurs at the selectivity filter in KcsA channels.

518-Pos Characterization of the C-terminal Domain of a Potassium Channel from *Streptomyces lividans* (KcsA)

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Board B366

KcsA, a potassium channel from *Streptomyces lividans*, is a good model to study the general working mechanism of potassium channels. This channel is pH responsive *in vitro* and its C-terminal domain (CTD) is implicated as a modulator. We investigated the nature of the CTD's involvement in the opening of KcsA by studying the channel's biochemical and electrophysiological properties with/without its CTD. We also investigated the pH dependent aggregation property of a recombinant CTD fragment. Our results show that the CTD destabilizes KcsA in the condition that it is supposed to be open. A detailed mechanism will be presented.

519-Pos Second Open State of wt *h*Kv1.3 Channels Revealed by a Slowly Deactivating Mutant A386C Channel and its almost Voltage-independent Verapamil Block

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Board B367

The voltage-gated K^+ channel Kv1.3 is a member of the *Shaker*-related K^+ channel family and verapamil is a potent blocker of

currents through Kv1.3 channels. The block of verapamil in the wt channel is state-dependent and earlier measurements demonstrated that verapamil blocked the open- and inactivated-state of the channel but not the closed state. In addition, verapamil block was weakly voltage-dependent at positive potentials but the off-rate for verapamil was strongly voltage-dependent at potentials more negative than −60 mV (Roebe *et al.*, 2000, *Br J Pharmacol* ***131***:1275). The strong voltage-dependence at negative potentials, however, could also be due to the closing of the channel and not to the voltage seen by verapamil. To distinguish these two possibilities we used a mutant channel (A386C, located in the pore helix) showing a strong reduction of deactivation. We performed electrophysiological experiments and determined the affinity of verapamil for the mutant at +40 mV (K_d ~8 μM) and at -120 mV (K_d ~11 μM). This result indicated that verapamil block is hardly voltage-dependent. Therefore we conclude that at hyperpolarizing potentials following a depolarization the wt Kv1.3 channel undergoes at least two different conformational changes before finally closing. The first conformational change pushes verapamil out of its binding site but still allows ions to pass and the second conformational change finally closes the channel. The result also gives evidence for a second open state of the channel that is not blocked by verapamil.

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520-Pos A New Mode Of Regulation Of N-type Inactivation In A *Caenorhabditis Elegans* Voltage-gated Potassium Channel

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Board B368

N-type inactivation in voltage-gated K^+ (Kv) channels is a widespread means to modulate neuronal excitability and signaling. Here we show a novel mechanism of N-type inactivation in a *Caenorhabditis elegans* Kv channel. The N-terminal sequence of KVS-1 contains a domain of twenty-two amino acids_that resembles the inactivation ball in A-type channels_which is preceded by a domain of eighteen amino acids. Wild type KVS-1 currents can be described as A-type, however their kinetics are significantly (~ 5-fold) slower. When the putative inactivation ball is deleted, the current becomes non-inactivating. Inactivation is restored in non inactivating channels by diffusion of the missing inactivation domain in the cytoplasm. Deletion of the domain in front the ball speeds inactivation kinetics ~5-fold. We conclude that KVS-1 is the first example of a novel type of Kv channel simultaneously possessing an N-inactivating ball preceded by a N-Inactivation_Regulatory_Domain (NIRD) that acts to slow down inactivation through steric mechanisms.

521-Pos Kv4 Inactivation Suppressing (KIS) Domains are Present on Specific KChIP2 and KChIP3 Splice Variants

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Board B369

Subthreshold A-type, transient K^+ current (I_{SA}) actively regulates the membrane excitability of neuronal cells, affecting action potential firing properties and retrograde propagation into the dendritic arbors. Kv channel-interacting proteins (KChIPs) are constitutive components of the Kv4-based channel complexes that underlie I_{SA}. An isoform of KChIP4 (KChIP4a) exhibits the unusual property of dramatically suppressing Kv4 inactivation through the actions of its N-terminal KIS domain (Holmqvist *et al.*, 2002). In addition, association with KChIP4a alone or mixed with other KChIP subunits promote the retention Kv4.2 channels in the perinuclear endoplasmic reticulum (Shibata et al., 2003). Here, we report that previously uncharacterized isoforms of KChIP2 (KChIP2x) and KChIP3 (KChIP3x; KChIP3b) also produce marked slowing of Kv4 inactivation kinetics, with differing effects on other channel properties. Expressed in oocytes and recorded using two-electrode voltage clamp, Kv4.2 currents in the presence of KChIP2x or KChIP3x inactivate dramatically slower than Kv4.2 expressed alone, similar to KChIP4a coexpression (half-inactivation times, +50 mV: Kv4.2 = ∼48 ms, +KChIP4a = ∼424 ms, +KChIP2x = ∼441 ms, +KChIP3x = ∼1101 ms). Like KChIP4a, both KChIP2x and KChIP3x contain a long stretch of hydrophobic residues within their variable N-terminal regions. Consistent with the presence of a KIS domain, deletion at the distal N-terminus of KChIP3x (KChIP3x/ΔN2-10) greatly reduces its inactivation slowing effect. However, unlike KChIP4a, KChIP3x and KChIP2x produced a dramatic rightward shift (∼16 mV) in steady-state inactivation and significantly accelerated the recovery from inactivation of Kv4.2 channels. Together with RT-PCR and EST cloning data showing KChIP2x and KChIP3x expression in rat brain, our results suggest that inactivation-suppressing KChIP isoforms are more commonly expressed than previously thought and likely play a major role in modulating I_{SA} functional properties.

522-Pos Does DPP10a-mediated Fast Inactivation Involve An N-type-like Mechanism?

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Board B370

We have demonstrated that coassembly of Kv4.2 α-subunits and auxiliary subunits (KChIP and DPP10a) reconstitutes the rapidly inactivating I_{SA} found in cortical neurons (Jerng et al., 2007). The DPP10a-mediated fast inactivation is dominant over the modulatory effects of other KChIP and DPP10 isoforms, yet the molecular mechanism remains unknown.

Kv4 channels reportedly experience two types of inactivation: closed-state inactivation resulting from desensitization to voltage and inactivation by internal pore occlusion (N-type-like). The relative importance of these mechanisms depends on specific auxiliary subunits. Here, we employed molecular and electrophysiological approaches to probe the molecular mechanism of DPP10a-

mediated fast inactivation. Closed-state inactivation is accelerated by elevated external K^+; however, coexpression of DPP10a with Kv4.2 lacking N-type-like inactivation (Kv4.2/ΔN2-40) generated fast inactivation insensitive to 98 mM external K^+. A mutation that slows Kv4.2 channel closing (C320S) is associated with dramatically slower inactivation; nevertheless, DPP10a introduced marked fast inactivation when coexpressed with Kv4.2/ΔN2-40/C320S channels. These results suggest that DPP10a-mediated fast inactivation occurs independently of the closed-state mechanism. Deletion of either first 5 or 20 residues of DPP10a (DPP10a/ΔN2-5, DPP10a/ΔN2-20) eliminated fast inactivation without altering other effects of the subunit such as accelerated activation and recovery from inactivation. Thus, the N-terminal residues 2–5 are required for fast inactivation. Furthermore, and according to the expectations of N-type-like mechanism, 5 mM TEA applied to Kv4.2/ΔN2-40 + DPP10a channels in inside-out patches resulted in slowing of inactivation. Currently, additional experiments are underway to further probe this mechanism.

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523-Pos HMJ-53A Accelerates Slow Inactivation Gating Of Delayed Rectifier K^+ Channels In Mouse Neuroblastoma N2A Cells

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Board B371

Delayed rectifier K^+ channels are voltage-gated K^+ (Kv) channels which are important in repolarization of excitable cells such as neurons. Delayed rectifier K^+ channel gating exhibits slow inactivation during depolarization. The molecular mechanism involved in such slow inactivation remains obscure, but evidence has suggested that it involves a restriction of the outer channel pore surrounding the selectivity filter. Pharmacological tools probing this slow inactivation process are scarce. In this work we reported that bath application of HMJ-53A (30 μM), a novel compound, could drastically speed up the slow decay (decay $\tau = 1677 \pm 120$ ms and 85.6 ± 7.7 ms, respectively, in the absence and presence of HMJ-53A) of delayed rectifier K^+ currents in neuroblastoma N2A cells. HMJ-53A also significantly left-shifted the steady-state inactivation curve by 12 mV. HMJ-53A, however, did not affect voltage-dependence and kinetics of channel activation. Intracellular application of this drug through patch pipette dialysis was ineffective at all in accelerating the slow current decay, suggesting that HMJ-53A acted extracellularly. Blockade of currents by HMJ-53A did not require an open state of channels. In addition, the inactivation time constants in the presence of HMJ-53A were independent of the degree of depolarization, suggesting that this drug did not appear to directly occlude the outer channel pore during depolarization (channel opening). Taken together, our results suggest that HMJ-53A selectively affected (accelerated) the slow inactivation gating process of delayed rectifier K^+ channels, and could thus be a potential probe for the inactivation gate.

524-Pos Allosteric Mechanisms Dominate Coupling between N- and C-type Inactivation in Kv1.4 Voltage-gated Potassium Ion Channels

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Board B372

Voltage-gated Kv1.4 channels produce a rapidly inactivating transient outward current. Kv1.4 channels exhibit two distinct inactivation mechanisms. Fast "N-type" inactivation is well characterized, and operates by a "ball and chain" mechanism. Slower C-type inactivation is not so well defined, but involves conformational changes of the pore resulting in a block of current. N- and C-type inactivation are coupled, with the presence of N-type inactivation speeding the rate of development of C-type inactivation.

We developed a model of potassium ion binding and permeation through the pore. The model consists of three binding sites equally spaced at 33, 67 and 100% (outside to inside the cell) across the pore, with a linear drop of electrical field across the pore. The low energy well at the intracellular side of the pore represents a drug-binding site which is readily occupied by intracellular potassium. Repulsion between adjacent ions (other than the intracellular binding site) was modelled as a factor of 41.2, with the result that it was 1700 times more difficult for an ion to occupy a site when the neighboring site was already occupied by another ion.

This model reproduces the conductance activity relationship of Kv1.4 channels. In the context of inactivation/channel block, movement of potassium from the intracellular side of the channel to the outside cannot change occupancy of the external site when physiological levels of extracellular potassium are present, which suggests coupling between N- and C-type inactivation is primarily via an allosteric mechanism. Changes in occupancy of the pore contribute only a small fixed energy in the presence of extracellular potassium. Our study suggests that N-type inactivation and, by similarity, open pore drug binding, interact with C-type inactivation primarily through an allosteric mechanism.

525-Pos Slow Inactivation of Shab K channels

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Board B373

A recent study, about the effect of the membrane potential on the irreversible drop of the Shab conductance in 0 K^+ solutions, has suggested that the slow inactivation of Shab channels may not completely block the communication (the exchange of ions) between the pore and the extracellular solution. Therefore, in order to gain insight on this point, we have started a study of the slow inactivation of Shab channels. Here we show that Shab inactivation presents several properties that differ from those of the extensively studied C-type inactivation of Shaker channels: In standard Na_{out}/K_{in} solutions, inactivation kinetics is best described by the sum of two exponential components, with Ts $\approx$ 1 and 3 sec, respectively.

Raising the external [K^+] eliminates the sow kinetic component, and thus speeds inactivation. In addition to K+, external TEA also eliminates the slow kinetic component. Recovery from inactivation is markedly voltage dependent, and it is not affected by the external [K^+]. On the other hand, preliminary observations indicate that external Ba^{++} is able to bind to the pore of inactivated channels, suggesting that the communication between the pore of inactivated channels and the external solution is not completely occluded.

526-Pos Dynamics of Slow Inactivation in *Shaker* Potassium Channels. Insights from Fluorination of a Pore Aromatic, Foot-in-the-Door, and Single Channels

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Board B374

Slow inactivation of Kv1 channels involves conformational changes near the selectivity filter. We report three manifestations of such changes in Δ6–46 *Shaker* channels. First, subtle modifications of an aromatic side-chain at position 449, just external to the selectivity filter, have dramatic effects on slow inactivation. Using *in vivo* nonsense suppression, we serially fluorinated the Phe449 aromatic ring, a manipulation that withdraws π electrons from the aromatic face with little or no effect on the shape, charge, or hydrophobicity of the side-chain. While channels expressing Phe449 inactivate slowly ($\tau \approx 13$ s at +20 mV), progressive fluorination of Phe449 monotonically increased the rate of inactivation ($\tau \approx 90$ ms for the trifluorinated derivative). Second, the V438A mutation, located in the pore helix near the bottom of the selectivity filter, also speeds inactivation in channels containing Phe449 ($\tau \approx 175$ ms). Mutations of the aligned residue in KcsA, Glu71, are known to affect both slow inactivation and the structure of the pore region. In the *Shaker* double mutant V438A/T449F, channel block by extracellular tetraethylammonium (TEA) slows inactivation exactly as predicted by the canonical foot-in-the-door model. Surprisingly, the foot-in-the-door effect was not only absent in single mutant T449F channels containing the native Val438, but the slow inactivation time course is *accelerated* with added TEA, suggesting that the blocker promotes the collapse of the outer vestibule. Third, single channel analysis reveals that V438A/T449F channels have three distinct conductance levels and gating behaviors, of which the middle conductance level is similar to that of single mutant T449F channels lacking the V438A mutation. These disparate results suggest that this region of the potassium channel is highly labile, with consequences on the kinetics of slow inactivation.

527-Pos Role of S4 Positively Charged Residues in the Regulation of Kv4.3 Inactivation and Recovery

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Board B375

The molecular and biophysical mechanisms by which Kv4 voltage-sensitive K+ channels inactivate and recover from inactivation are presently unresolved. There is a general consensus, however, that Shaker-like N- and P/C-type mechanisms are likely not involved. Kv4 channels also display prominent inactivation from pre-activated closed-states (closed-state inactivation, CSI), a process which appears to be absent in Shaker channels. As in Shaker, voltage sensitivity in Kv4 is thought to be conferred by positively charged residues localized to the fourth transmembrane segment (S4) of the voltage-sensing domain. To investigate the role of S4 positive charge in Kv4.3 gating transitions, we analyzed the effects of charge elimination at each positively charged arginine (R) residue by mutation to the uncharged residue alanine (A). We first demonstrate that R290A, R293A, R296A, and R302A mutants each alter basic activation characteristics consistent with positive charge removal. We then provide strong evidence that recovery from inactivation is coupled to deactivation, show that the precise location of the arginine residues within S4 plays an important role in the degree of development of closed-state inactivation and recovery from CSI, and demonstrate that development of CSI can be sequentially uncoupled from activation by R296A, specifically. Taken together, these results extend our current understanding of Kv4.3 gating transitions.

528-Pos Kv1.5 R487 mutants do not share the inactivation properties of the analogous *Shaker*IR T449 mutants

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Board B376

P/C-type inactivation in Kv1.5 differs from that in *Shaker*IR (*Sh*IR) channels insofar as the extent of inactivation following a 10 s depolarization is much less, and neither the extent nor the rate of inactivation is affected by changing $[K^+]_o$ between 0 and 140 mM (pH 7.4) These disparities prompted us to determine if Kv1.5 R487 mutations mimic the changes of inactivation reported for the analogous *Sh*IR T449 mutants. Analyses of mutant Kv1.5 macroscopic currents expressed in a fibroblast cell line (*ltk-*) confirmed that the residue at position 487 can dramatically influence the inactivation rate. In terms of the relative rates of inactivation, the sequence for Kv1.5 R487X was (slowest to fastest and measured at 50 mV with 3.5 mM K^+_o): R (*wt*) $\leq$ V < A < S < K $\ll$ Q < E. The sequence for *Sh*IR T449X, reported by others, is: V $\ll$ T (*wt*) < A < K = E = Q < S. Except for the valine mutant, the qualitative outcome of a given mutation is similar in Kv1.5 and *Sh*IR, however there are a number of quantitative differences. For example, whereas τ_{inact} in *Sh*IR T449S is 5 ms, in Kv1.5 R487S it is 870 ms, a difference of more than two orders of magnitude. As in *Sh*IR, the fast inactivating Kv1.5 mutants exhibit a partial (R487K) or complete (R487Q, R487E) collapse of current upon removing external K^+. In contrast to *wt* Kv1.5, increasing $[K^+]_o$ in the fast inactivating Kv1.5 mutants slows the inactivation rate. These results indicate that

extrapolation of structure/function relations between these related channels is not straightforward and point to a significant influence of other channel regions on the molecular movements that underlie the P/C-type inactivation process.

529-Pos Endocytic Recycling Of KCa2.3 And KCa3.1 In HEK And Endothelial Cells

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Board B377

We previously demonstrated that the time constant for total protein degradation of KCa2.3 and KCa3.1 are widely disparate, being >36 hrs and 5 hrs, respectively. Based on this, we have begun to address the question of how these channels are retrieved from the plasma membrane and degraded. We inserted the biotin ligase acceptor peptide sequence into the second extracellular loop of both KCa2.3 and KCa3.1 such that these channels could be rapidly labeled at the cell surface and their endocytosis monitored by immunofluorescence. We demonstrate that KCa3.1 is completely endocytosed from the plasma membrane of both HEK and human microvascular endothelial cells (hMEC) in less than 1 hr and completely degraded in approximately 5 hrs. In contrast, KCa2.3 is still clearly localized at the plasma membrane even after 5 hrs in both HEK and hMEC cells. Following endocytosis both KCa2.3 and KCa3.1 are localized in a perinuclear endosomal compartment. Based on these observations, we determined whether these channels are cleared from the membrane in a single pass or are recycled back to the plasma membrane. Both KCa3.1 and KCa2.3 were accumulated in recycling endosomes in the presence of the dominant negative RME-1 in both HEK and hMEC cells. Also, following immunoisolation of Rab11 containing vesicles both KCa3.1 and KCa2.3 were identified upon immunoblot. These results confirm that both KCa2.3 and KCa3.1 enter recycling endosomes. Our data demonstrate that KCa2.3 and KCa3.1 have significantly different plasma membrane half-lives, although both channels enter recycling endosomes prior to being targeted for degradation.

530-Pos Role Of Derlin-1 And p97 (Cdc48) In The Degradation Of KCa2.3 And KCa3.1

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Board B378

KCNN gene family members (KCa2.x and KCa3.1) have conserved arginines in S4 as well as a conserved glutamic acid in S3. These charged amino acids are critical in the biogenesis of voltage-gated K^+ channels and we previously demonstrated that this function is conserved in KCa2.x and KCa3.1. Mutation of these charged amino acids results in a rapid, lactacystin-dependent degradation of KCa2.3 and KCa3.1. Wild type KCa3.1 is also sensitive to lactacystin, suggesting a fraction of this channel is similarly targeted to the proteosome. Steady-state protein levels for KCa3.1 and KCa2.3 as well as the S3 mutations E112A (KCa3.1) and E370A (KCa2.3) were increased by the ubiquitin E1 ligase inhibitor, UBEI-41, confirming a role for ubiquitination in their degradation. Many misfolded proteins are translocated out of the ER by Derlin-1 after which they are extracted from Derlin-1 by the AAA ATPase, p97 and shuttled to the proteosome for degradation. We demonstrate that knockdown of Derlin-1, using an shRNA, results in an increase in wild type KCa3.1 and KCa2.3 as well as their respective glutamic acid S3 mutations. Co-IP studies confirm a direct association of these channels with Derlin-1. To evaluate the role of p97 in the degradation of these channels we utilized a dominant negative mutation in which the ATPase function has been abrogated, p97QQ. Co-transfection of p97QQ with either KCa2.3, KCa3.1 or their respective S3 glutamic acid mutants resulted in an increased steady-state protein expression level compared to wild type p97. This result confirms that p97 is required to target these channels for proteosomal degradation. This is the first demonstration of a role for Derlin-1 and p97 (Cdc48) in the degradation of misfolded K^+ channels.

531-Pos Novel Activators of Small and Intermediate-conductance Calcium-activated Potassium Channels Based on the Neuroprotectant Riluzole

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Board B379

Calmodulin-gated small-conductance (KCa2.1-2.3) and intermediate-conductance (KCa3.1) calcium-activated potassium channels are critically involved in modulating calcium signaling cascades and membrane potential in both excitable and non-excitable cells. Activators of these channels constitute both useful pharmacological tools as well as potential novel drugs for diseases such as ataxia, epilepsy, hypertension, incontinence and cystic fibrosis. We here used the neuroprotectant riluzole (6-trifluoromethoxybenzothiazol-2-amine) as a template for the design of potent KCa2/3 channel activators that could potentially be used for *in vivo* studies. Riluzole itself increases KCa2/3 currents maximally 30-fold at 250 nM intracellular calcium and activates KCa2 channels with EC_{50}s of 12 to 21 microM and KCa3.1 with an EC_{50} of 2 microM. Our structure activity studies revealed that an intact benzothiazole ring system with a 2-amino group and sulfur at position 1 is essential for KCa2/3 activation. While replacement of the 6-position trifluoromethoxy group with other functional groups in various positions had little effect, introduction of annelated aliphatic and aromatic ring systems increased KCa2/3 activating activity 2-10 fold. The two most potent compounds of our benzothiazole library, SKA-20 (anthra[2,1-d]thiazol-2-amine) and SKA-31 (naphtho[1,2-d]thiazol-2-amine) activate KCa2.1 with EC_{50}s of 430 nM and 2.9 microM, KCa2.2 with EC_{50}s of 1.9 microM, KCa2.3 with EC_{50}s of 520 nM and 2.5 microM, and KCa3.1 with EC_{50}s of 115 nM and 260 nM. In contrast to riluzole, SKA-31 and SKA-20 exhibit

increased selectivity over sodium and calcium channels. We are currently in the process of evaluating the effect of SKA-31 and SKA-20 on the medium afterhyperpolarization in hippocampal neurons and on endothelium mediated dilation of carotid arteries (EDHF response).

532-Pos Structure and Divalent Cation Modulation of a Potassium Channel RCK Domain

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Board B380

TvoK is a prokaryotic Ca^{2+}-gated K^+ channel that displays low-affinity activation by several divalent cations, including Ca^{2+}, Mg^{2+}, Mn^{2+} and Ni^{2+} similar to the low-affinity divalent activation observed in eukaryotic BK channels (Parfenova *et al.*, *J. Biol. Chem. 282*:24302–24309, 2007). Biochemical studies using analytical gel filtration chromatography, crosslinking and native gel electrophoresis illustrate that the TvoK channel's ligand-binding RCK domain forms an octameric assembly in solution. We performed structural analysis of this RCK domain in solution using small-angle X-ray scattering, and found that the TvoK RCK domain forms an octameric "gating ring", similar to the gating ring of MthK in both shape and dimension (Ye *et al.*, *Cell126*:1161–1173, 2006). To identify residues that may determine channel activation properties by different divalent cations, we analyzed the ability of Ca^{2+}, Mg^{2+}, Mn^{2+} and Ni^{2+} to impart resistance of the TvoK RCK assembly to denaturation by urea, and analyzed the effects of mutations at putative divalent-coordinating residues. We found that the Mg^{2+} binding site in TvoK may be determined in part by Arg-228; mutating this residue to Glu decreases the apparent affinity for Mg^{2+} in imparting resistance to denaturation. Using NMR techniques, we are additionally analyzing the structural changes involved in ligand binding, and putative residues involved in coordination of Mn^{2+}. Because of its regulation by divalent cations, studying the TvoK RCK domain may provide insight toward ligand binding mechanisms of BK and other related channels.

533-Pos Stoichiometry of hSloC911A Subunits In the Heteromeric Channel and Resistance to Oxidation

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Board B381

hSlo channels are modulated by high glucose- and H_2O_2-mediated oxidation and C911 is a key molecular target as the hSloC911A mutant is resistant to the such effects. To determine the number of hSloC911A subunits needed to confer resistance to oxidation in the tetrameric channel, we constructed the hSloY294V-C911A double mutant channel. The Y294V mutant is insensitive to inhibition by extracellular TEA and the single channel current amplitude is determined by the Y294V subunit stoichiometry in the heteromeric channel. Hence, there are four levels of single channel current amplitudes in the presence of external TEA when hSloY294V and wild-type channels are coexpressed. Using this strategy, we coexpressed hSloY294V-C911A and wild-type channels in HEK293 cells. Inside-out recordings (at +60 mV) were performed with symmetrical K^+ (140 mM), 2 mM TEA in the pipette solution, and 200 nM Ca^{2+} in the bath solution. Membrane patches that contained one channel were analyzed. Stoichiometry of the double mutant subunit in the heteromeric channel is inferred from the single channel current amplitude (3.5 pA, 7 pA, 10.5 pA, and 14 pA for heteromeric channels that contained one, two, three, and four double mutant channel subunits respectively) and channel sensitivity to H_2O_2 is determined by single channel Po before and after exposure to 4 mM H_2O_2. Channels containing one mutant subunit were sensitive to H_2O_2 which reduced the relative Po to 54±14% that at baseline (n=3, *$p<0.05$). In contrast, channels that contained two, three, or four mutant subunits were resistant to H_2O_2 with relative Po maintained at 113±32% (n=3), 138±44% (n=5), and 114±14% (n=4) to those at baseline respectively. These results indicate that the presence of two out of four subunits that contain C911A is adequate to protect hSlo from H_2O_2 modulation.

534-Pos Identification of Large Conductance Calcium-activated Potassium Channel Subunits from Cholinergic Canine Intracardiac Neurons

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Board B382

Isolated canine intracardiac neurons display outward potassium currents characterized by a transient and a sustained component. Electrophysiological and pharmacological studies suggest that these currents result from activation of large conductance calcium-dependent potassium (BK) channels. We conducted immunohistochemical studies to elucidate the molecular identity of these BK channels and the neurochemical nature of the neurons expressing them. We identified cholinergic neurons with a monoclonal antibody against the vesicular acetylcholine transporter (VAChT). We found that all principal neurons showing positive staining for the neuronal marker PGP9.5 were positive for VAChT staining (n=293 neurons). A separate set of experiments showed that 89.5% of the cells positive for VAChT were positive for staining against the α subunit of the BK channel (179/200 neurons). Immunostaining experiments using monoclonal antibodies against the BK channel regulatory subunits, β2, β3, and β4 revealed that β4 is the predominant BK channel regulatory subunit in these neurons. Single channel activity, recorded in 10μM, 3μM and 0.3μM internal calcium gave $V_{1/2}$ activation values of 35.7±11mV (n=6), 29.8±2.2mV (n=7) and 70±15mV (n=8), respectively. Our immunohistochemical studies suggest that canine intracardiac ganglia are mainly composed of cholinergic neurons and that BKα subunits in these neurons are most likely associated to β4 subunits. Consequently, the BK current

inactivation observed in these neurons is not due to the presence of associated inactivating subunits such as β2 or β3. Interestingly, the $V_{1/2}$ activation values obtained are shifted toward more hyperpolarized potential compared to what has been reported for the heterologous coexpression of the BKα and β4 subunits. Thus, biophysical properties of native BK channels from intracardiac cholinergic neurons cannot be completely explained by the sole interaction between BKα and β4 subunits.

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535-Pos Estrogen Alters Expression Levels and Splicing Pattern of Human BK Channel Alpha Subunit

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Board B383

Studies in animal models have shown that estrogen has a protective role on cardiovascular function. However, in humans the long-term protective role of estrogen on vascular function is less clear. Estrogen regulation of vascular smooth muscle tone may be mediated via BK (MaxiK, Slo) channels through a non-genomic pathway of estrogen action. However, this mechanism may not completely explain the long-term effects of estrogen observed on cardiovascular physiology. Here, we present evidence of a genomic regulation of human BK channel alpha subunit (hSlo) expression and splicing by estrogen. In isolated primary culture of human coronary and aortic smooth muscle cells estrogen treatment increased transcript levels of hSlo. Sequence analysis of the promoter-regulatory regions of the gene revealed the presence of multiple half sites of estrogen response element (ERE) that can bind estrogen receptor alpha. In addition, multiple Sp1 transcription factor binding sites that can assist or mediate estrogen action were detected. In a heterologous system hSlo promoter could be stimulated with estrogen in a saturating dose-dependent manner. We also monitored changes in splicing patterns after estrogen stimulation of coronary smooth muscle cells. Two splice inserts namely, STREX and SV29 (contained in hBr5) were upregulated when compared to the constitutive mRNA expression levels. Thus, estrogen via a genomic mechanism modulates hSlo expression that may contribute to effects observed in the vascular system after long-term estrogen treatments

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536-Pos Statistical Mechanical Evaluation of an Allosteric Three-Gate Four-subunit Model of the BK Channel

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Board B384

BK channels are opened by two main stimuli: membrane depolarization and elevation in cytoplasmic [Ca^{2+}]. It appears that two high affinity Ca^{2+} binding sites exist within the cytoplasmic C-terminus. It has been recently proposed that each binding site is located within functional domains known as RCK1 and RCK2 (Yusifov et al Bioph Meet 2007, 1312-pos). Voltage and Ca^{2+} sensors are separate domains that operate independently in inducing channel opening. However, experimental and theoretical evidence suggests that some degree of cooperativity exists between voltage and Ca^{2+} sensors of the channel. To date, the most comprehensively studied model of calcium and voltage activation of the BK channel possesses a single Ca-binding site (Horrigan and Aldrich, 2002). We propose a physiologically meaningful allosteric model of BK activation that contains three regulatory gating structures: the S4 voltage-sensitive gate and two Ca^{2+} sensors represented by the RCK1 and RCK2 domains. As in the Horrigan and Aldrich model, the channel opens and closes in concerted fashion involving all four subunits analogous to the Monod-Wyman-Changeux allosteric model, but the complete set of allosteric interactions among the three gates evokes the Koshland-Nemethy-Filmer sequential model. We used an energetics approach to derive the partition function of this rather large gating scheme. The model was used to perform a global, simultaneous fitting of equilibrium conductance and S4-labeled fluorescence vs. voltage for three BK channels (WT, neutralized RCK1 Ca^{2+} sensor (D362A/D367A) and neutralized RCK2 Ca^{2+} sensor (Ca bowl, D894-898N). The results indicate that mutation of RCK1, which is closely located downstream the pore gate, greatly affects the interaction energy with the pore while mutation of RCK2 seems to affect mainly its interaction with the S4 voltage sensor.

Voltage-gated K Channels - II

540-Pos Comparing the Functional Expression of BK Channels Using Heterologous Expression in Different Cell Backgrounds

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Board B385

Modified insect baculoviruses containing mammalian gene expression cassettes (BacMam viruses) were used to transiently express BKα, BKαβ1 and BKαβ4 ion channels in modified CHO and human osteosacroma (U-2 os) cell backgrounds. We compared the transient BacMam expression of the BK channel complexes with those same channels expressed in stable CHO-K1 cell lines. Functional expression was determined by direct measurement of ionic currents using an Ionworks platform or a fluorescent readout assay measuring thallium influx using FLIPR. There was a positive correlation between the magnitude of outward current and the amount of BKα BacMam virus added to the culture medium with apparent saturation reached at a multiplicity of ~100 viral particles per cell. Resistance to iberiotoxin served as a marker of BKαβ4 expression while slowing of the activation time course was a marker for BKαβ1 expression. We co-transduced BKα with either BK β1 or BKβ4