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Negative charges in the loop between the A and B box of the T1 domain are involved in Kv2.1 and Kv2.1/Kv6.3 channel tetramerization

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Voltage-gated potassium (Kv) channels are tetramers of four α-subunits. Formation of homo- or heterotetramers is determined by their subfamily specific N-terminal T1 domain. This domain contains two regions designated A and B box, which display a pattern of conservation within and between the Kv subfamilies assumed to determine subfamily specific channel assembly. However, the linker between both boxes is not conserved and its function is uncertain. Here we report that negatively charged residues in this linker are involved in Kv2.1 channel assembly. Mutating these negative charges to arginines caused at least a 15-fold down regulation in current density. To investigate if this was due to a trafficking or a tetramerization deficiency we used FRET analysis to determine the amount of unpaired channel subunits in the plasma membrane versus the ER. Using N-terminal CFP and YFP constructs we observed a 2.5 to >10-fold decline in the FRET efficiency in the ER for these charge reversal substitutions, indicating that there were more unpaired CFP-labeled subunits and that the reduction in current is most likely due to deficient tetramerization. These negative charges are also present in Kv6.3, an electrically silent subunit that fails to form functional homotetramers but selectively co-assembles with the Kv2.x subunits and generates heterotetrameric channels in which Kv6.3 modulates the Kv2.x currents. Mutating this charge cluster in Kv6.3 resulted likewise in a tetramerization defect with Kv2.1 as shown by the reduced FRET efficiencies and the lack of modulating Kv6.3 effects. These results indicate that besides the T1 domain's A and B boxes, the A-B linker is involved in channel tetramerization and could be one of the determinants for the selective co-assembly of Kv6.3 with Kv2.x channels.

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Structural Insights into KChIP4a Modulation of Kv4.3 Inactivation

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Dynamic inactivation in Kv4 A-type K^+ current is critical in regulating neuronal excitability by shaping action potential waveform and duration. Multifunctional auxiliary KChIPs1-4 subunits that share a high homology in the C-terminal core regions exhibit distinctive modulation on inactivation and surface expression of pore-forming Kv4 subunits. How structural differences of KChIPs in determining their functional diversity remain unknown.

Here, we describe a crystal structure of KChIP4a resolved at 3.0 Å resolution. The KChIP4a structure shows the distinct N-terminal α-helices that differentiate it from other KChIPs. The N-terminal residues of KChIP4a form a long α-helix followed by a short rigid coil that binds to a well-defined hydrophobic pocket formed by the conserved structural components. Structural comparison indicates that the hydrophobic pocket of KChIP4a is similar to that of KChIP1. This hydrophobic pocket has recently been shown to be the same type of binding groove that is recognized by the Kv4.3 N-terminus in KChIP1(ref. 1-2). The core KChIP4a (without its N-terminal α-helices) reveals that the hydrophobic groove can sequester the N-terminus of Kv4.3. Structural observations suggest that N-termini of both Kv4.3 and KChIP4a can competitively interact with the same hydrophobic pocket of KChIP4a. Biochemical experiments showed that competitive binding of Kv4.3 N-terminal peptide to the hydrophobic groove of core KChIP4a causes the release of its N-terminus that suppresses the inactivation of Kv4.3 channels. Electrophysiology confirmed that the N-terminal first α-helix peptide of KChIP4a either by itself or fused to N-terminal truncated Kv4.3 can confer the slow inactivation. We propose that N-terminal binding of Kv4.3 to the core KChIP4a mobilizes the KChIP4a N-terminus which may serve as a slow inactivation gate.

References:
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Divergent PIP_2 Sensitivity Confers Differential Muscarinic Agonist Efficacy For Suppression Of Kv7 K^+ Channels

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Kv7 channels encode the M-current, a non-inactivating current that controls neuronal excitability. Stimulation of M_1 muscarinic receptors suppresses M-current via depletion of membrane phosphatidylinositol 4, 5-bisphosphate (PIP_2). We have suggested Kv7 channels to be directly regulated by PIP_2 binding at a carboxy-terminal inter-helix domain and divergent PIP_2 apparent affinity to underlie the differences in gating of these channels (Hernandez et al., *J Gen Physiol.* 132: 361-81, 2008). Taking advantage of the differences in PIP_2 affinities for different Kv7 subunits and the biophysical differences in current amplitude and unitary conductance displayed by the Kv7.3 (A315T) pore mutant suggested to "unlock" the channels into a conductive conformation (Zaika et al., *Biophys J.* Sep 12, 2008), we sought to determine the relationship between receptor-induced modulation and PIP_2 apparent affinity. Using perforated patch clamp, we studied CHO cells in which cloned Kv7 channels were co-expressed with M_1 muscarinic receptors. Heteromeric Kv7.2/7.3 currents were depressed by the muscarinic agonist oxotremorine-M (oxo-M) in a concentration-dependent manner with a maximal inhibition of about 80% and an EC_{50} of 0.5 ± 0.1 μM, similar to modulation of native M current. However, oxo-M induced a much weaker suppression of homomeric Kv7.3 (A315T) currents, manifested by a significantly reduced maximal inhibition (~40%) and a small shift of the dose response curve to the right (EC_{50} = 1.0 ± 0.3 μM). We hypothesize that channels with high PIP_2 apparent affinity, like Kv7.3, are more saturated at tonic levels of membrane PIP_2, and less-sensitive to inhibition by M_1 receptor-induced membrane PIP_2 depletion than channels with low PIP_2 apparent affinity, like Kv7.2/7.3 heteromers, that are not saturated by tonic levels of PIP_2 in the membrane and are thus more sensitive to M_1 receptor-induced PIP_2 depletions.

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Two Distinct Molecular Mechanisms Underlie pH Sensitivity of the Human Potassium Leak Channel $K_{2P}2.1$

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The mammalian $K_{2P}2.1$ potassium channel (TREK-1, KCNK2) is highly expressed in excitable tissues, where it plays a key role in the cellular mechanisms of neuroprotection, anaesthesia, pain perception and depression. Here, we report that external acidification, within the physiological range, strongly inhibits the human $K_{2P}2.1$ channel in two distinct time scales. We have identified two histidine residues (*i.e.,* H87 and H141), located in the first external loop of the channel, which govern the fast response of the channel to external pH (in the time scale of seconds). We demonstrate that these residues are within physical proximity to glutamate 84, homologous to *Shaker* E418, KcsA E51 and KCNK0 E28 residues, all previously reported to stabilize the outer pore gate in the open conformation by forming hydrogen bonds with pore-adjacent residues. We thus propose a novel mechanism for pH sensing in which protonation of H141 and H87 generates a local positive charge that serves to draw E84 away from its natural interactions, facilitating the collapse of the selectivity filter region, a mechanism which resembles C-type gating of voltage dependent potassium channels. In accordance with this proposed mechanism, the proton-mediated effect was inhibited by external potassium ions, modified the channel's ion selectivity and was enhanced by a mutation, S164Y, known to accelerate C-type gating. In addition, we show that the slow regulatory effect (in the time scale of minutes) is mediated by proton-sensitive G-protein coupled receptors (GPCRs), which activated phospholipase C via the G_q pathway. We demonstrate that three residues within the C-terminal of $K_{2P}2.1$ mediate the channel's response to the observed GPCRs activation by acidic pH.

Taken together, our results highlight the physiological importance of human $K_{2P}2.1$ channels as sensors of extracellular pH in the central nervous system.

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How Do Mutations in the L0-linker of ABCC8 Produce Neonatal Diabetes?

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Numerous mutations in ABCC8 (SUR1), the neuroendocrine-type regulatory subunit of K_{ATP} channels, cause Neonatal Diabetes (ND). Many of these mutations compromising insulin release cluster around the first ND mutation identified in the SUR1 L0-linker, L213R (NEJM 355:456). Here we show the ND-SUR1 increased the on-cell open channel probability, Po, not the density of Kir6.2-based channels. Single-channel kinetics analyses demonstrate that the increase in the Po is due to a ligand-independent stabilization of the active (burst) state. A similar diabetogenic effect was reproduced by deletions in the Kir6.2 N-terminus (BBRC 255:231; Cell 100:645) that partners with the L0-linker controlling the "slow" (inter-burst) gating transitions (JBC 278:41577). These findings illustrate the physiologic significance of our original model of SUR1/Kir6.2 coupling (JBC 278:41577) where the L0-linker plays a key role in controlling the maximal Po.

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Comparison between the Functions of TRPP and Other Structurally-related Channels and their Links to Membrane Trafficking

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TRPP and other structurally-related proteins belong to the same subfamily of TRP channels. Despite the similarities in their membrane topology, their cellular distribution is different and mutations in the genes encoding them cause

different types of diseases. These channels are nonselective and permeable to Na+, Ca++ and other cations and they are modulated by changes in the extracellular and intracellular concentrations of these cations. Our studies also show that the translocation of these proteins to the cell membranes is stimulated by defined chemical chaperones, proteasome inhibitors and modulators of ion homeostasis. They play a role in membrane trafficking and their translocation between different subcellular compartments is modulated by hormones involved in the homeostasis of Na+ and other ions. Cells expressing mutated TRPP and related proteins show abnormal trafficking of subcellular membrane vesicles. Such abnormalities may explain the symptomatology and the pathogenesis of the disorders caused by the mutations of the genes encoding these proteins.

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Binding of Syntaxin 1A to the C-terminus of hERG Channels Affects Channel Trafficking and Inactivation

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The SNARE protein, syntaxin 1A (STX1A), functionally regulates cardiac ion channels, including the human ether-à-go-go related gene (hERG) which encodes the pore-forming voltage-gated K^+ channel underlying I_{Kr} in the heart. The primary mode for STX1A-dependent inhibition of hERG channel function is trafficking impairment which can be rescued by reduced temperature or the high-affinity channel blocker, E4031. A secondary mode is achieved by the production of a hyperpolarizing shift in the voltage dependence of steady-state inactivation. Here we report the STX1A binding region on hERG channels. GST pulldown and coimmunoprecipitation demonstrates that the cytosolic SNARE motif-containing STX1A-H3 domain preferentially binds to hERG. This cytosolic domain is attached to the TM region by a short inflexible linker and is 60 residues in length. Use of N- (hERG-Δ2-16 and hERG-Δ2-354) and C-terminal truncation mutations (hERG-Δ1120, hERG-Δ1045, hERG-Δ1000, hERG-Δ960, hERG-Δ899, hERG-Δ860-899, hERG-Δ860, and hERG-Δ814) demonstrates that STX1A binds to all truncation mutations tested. The hERG C-terminus begins at approximately residue 670 immediately adjacent to the cytosolic portion of the S6 helices. Therefore, we deduce that the STX1A-H3 domain interacts with the C-terminus of hERG channels between residues 670 and 814. Functional analysis of C-terminal truncation mutations demonstrates that STX1A inhibits the trafficking of truncations up to hERG-Δ1000, but has no effect on hERG-Δ960 while enhancing trafficking and function of hERG-Δ899. We infer that STX1A-interaction disrupts normal protein folding thereby inhibiting channel trafficking, and may alter the movement of S6 helices affecting the hERG channel inactivation gate, shifting the voltage-sensitivity of channel inactivation. SNARE protein-mediated regulation of cardiac ion channels represents a novel biological mechanism that may have universally intrinsic implications for normal and diseased heart function.
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905-Pos Board B784

Rapidly Inducible Protein Modification Using Rapamycin-mediated Complementation Of Tobacco Etch Virus (TEV) Protease

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Traditional modalities of protein knock down rely on transcriptional or translation suppression and are thus hampered by slow endogenous protein turnover rates and compensatory mechanisms. Directly targeting proteins, rather than synthetic pathways, facilitates more rapid depletion of the targeted protein. Tobacco etch virus protease (TEVp), a potyvirus nuclear inclusion protein, cleaves at a canonical sequence, ENLYFQ/G, with sufficient specificity to be useful for targeting specific proteins. Starting with a TEVp mutant (S219V) deficient in autocatalysis (Kapust et al., *Protein Eng.* 14:993, 2001), we have humanized the codon bias and introduced mutations (van den Berg et al., *J. Biotechnology* 121:291, 2006) to increase solubility. Expression of the modified TEVp was well tolerated by Hela cells and resulted in efficient cleavage, as determined by Förster resonance energy transfer, of a co-expressed reporter consisting of fluorescent proteins (Cerulean and Venus) fused with a linker containing a TEVp cleavage sequence. Splitting the TEVp at residue 119 (Wehr et al., *Nat. Methods* 3:985, 2006) and fusing the fragments to FKBP12 and the c-terminus of FRB resulted in inducible enzyme activity following addition of rapamycin to living cells. Cleavage at 37°C proceeded exponentially with a time constant of approximately 100 minutes. In an effort to accelerate cleavage, the two halves of the complementation system were fused to form circular permutated unitary constructs. However, these constructs demonstrated constitutive activity without rapamycin. Fusing one half of TEVp to the reporter protein (cleavage in *cis*) also resulted in non-regulated activity. Current efforts are aimed at targeting one half of TEVp to the plasma membrane and recruiting the other half from the cytoplasm upon addition of rapamycin. Targeted protein cleavage was monitored by measuring $K_V3.4$ inactivation of a reporter construct consisting of EGFP-TEVP site-$K_V3.4$.

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Resolving the Structural-Functional Interaction Between HIV-1 Vpu and TASK Channels by FLIM/FRET

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An established membrane potential is fundamental to the survival of a cell. The majority of cellular and physiological processes foremost require orchestration of various ion channel activities and interactions in a spatially and temporally interdependent fashion. Previously we found that Vpu, a single transmembrane protein encoded by HIV-1, could interact with the assembly of endogenous K_{2P} channels that set resting potentials. This Vpu function reflects its viroporin structure, conferring the propensity to oligomerize into homomeric channels or promiscuously with homologous endogenous channel subunits. By interfering with normal assembly of background K^+ channels, Vpu suppresses the stabilizing K^+ conductance and dissipates the transmembrane voltage constraint on viral particle discharge. In this report, we measured membrane potential changes with respect to TASK-Vpu interaction in single cells by implementing a FRET pair with potential-tracking imaging. Our combined measurements from FLIM/FRET and confocal microscopy showed a structural-functional coupling between the two physical parameters (protein-protein interaction; membrane potential), and confirmed the previous experimental results obtained by independent means.

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The Positive Effect Of STREX On BK Channels

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The large conductance voltage and calcium sensitive potassium channel (BK) is encoded by a single gene KCNMA1. The inclusion of a stress regulated exon (STREX) at a splice site (C2) in the intracellular carboxyl (C) terminus of BK confers differing properties to the channel. STREX insertion generates a putative polybasic region. In a number of other channels, polybasic regions are suggested to interact with negatively charged phospholipids, such as phosphoinositides, to control ion channel gating and membrane targeting. We hypothesised that the polybasic region including STREX may serve as a membrane targeting domain of the STREX BK channel C-terminus. To test this, a GFP-tagged carboxyl terminal construct spanning the S6 transmembrane domain to the COOH end region of the intracellular carboxyl terminus was constructed (S6-COOH-STX) and transiently transfected into HEK293cells. The construct localised at the plasma membrane and this was abolished when the STREX insert was deleted. To test whether the polybasic region is important for plasma membrane targeting, two approaches were taken: Firstly, site directed mutagenesis to change selected positive residues into neutral (alanine) residues, also abolished membrane targeting of S6-COOH-STX to the plasma membrane. Secondly, to discern whether the polybasic region may interact with negatively charged phosphoinositides at the plasma membrane, the S6-COOH-STX construct was co-transfected with a 5′phosphatase, IPP. Cells co-expressing IPP displayed significantly reduced plasma membrane targeting of S6-COOH-STX, however a phosphatase null mutant of IPP did not effect plasma membrane expression. These data suggest that the polybasic region generated by inclusion of the STREX insert is an important determinant of STREX domain interaction with the plasma membrane. The functional role of the polybasic region and the interaction of phospholipids on BK channel calcium and voltage sensitivity were elucidated using patch clamp electrophysiology and high throughput dyes.

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Regulation Of Kcnq2/3 Channels By The Transcriptional Repressor REST In Nociception

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Understanding how excitability of sensory neurons is regulated is an important goal since this excitability underlies pain transmission and unfortunately almost everyone will suffer from inflammatory pain at some point in their life. Recent studies have identified expression of M-type K^+ channels (encoded by KCNQ genes) in damage-sensing (nociceptive) sensory neurons, where they are thought to control excitability. Accordingly, receptor-induced inhibition of M-current in these neurons has been shown to contribute to peripheral sensitisation and inflammatory pain. Here we describe a new mechanism for downregulation of KCNQ channel expression in nociceptors. We identified binding sites for the transcriptional repressor REST within both the KCNQ2 and