relaxation is seemingly an intrinsic property of VSDs, we sought to identify regions of the domain involved in the triggering of relaxation. For this, we focused our study on the loop connecting the S3 and S4 segments (S3-S4 loop). We argue that, like the S4-S5 linker coupling the VSD to the pore domain, the S4 segment can do electromechanical work on the rest of the VSD using the S3-S4 loop as ''coupling element''. Several studies have proposed that the S3-S4 is helically structured. Hence, we hypothesized that the movement of the S4 segments can be readily transmitted to the S3 segment and the rest of the VSD causing relaxation through a ''rigid'' S3-S4 loop. Conversely, a ''flexible'' S3-S4 would be able to absorb the movement of the S4 segment, dissipating mechanical energy and diminishing relaxation. To test this idea, we determined amplitude and timing of relaxation by performing cut-open voltage-clamp recordings of potassium currents from ShakerIR S3-S4 loop proline-to-alanine mutants. We reason that, since prolines disrupt helical structure, introduction of a ''helix-friendly'' alanine will increase the rigidity of the loop. Consistently, our data indicate that the proline-to-alanine mutations increase the magnitude of relaxation. Hence, we proposed that S3-S4 loop's prolines act as ''hinges'' conferring flexibility to this region. Similar results were obtained with glycine-to-cysteine S3-S4 loop mutants of Ci-VSP, suggesting that the S3-S4 segments may have similar function. In summary, we conclude that the S3-S4 helix seemingly acts as a mechanical stress dissipater in VSDs.

2388-Pos Board B407

Structural Analysis of S4-S5 Linker Architecture in Depolarization and Hyperpolarization activated K^+ Channels

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MVP, the hyperpolarization-activated potassium channel from the archaeon M. jannaschii, provides a unique opportunity for investigating electromechanical coupling, or how the voltage-sensing domain (VSD) couples to the pore domain, in voltage-gated potassium (Kv) channels. In contrast to depolarization-activated Kv channels, the pore domain of MVP opens when the S4 helix moves inwards at hyperpolarizing potentials, and closes when the S4 helix moves outwards at depolarizing potentials. Because of this inverse relationship between the pore domain and VSD, MVP is in a closed conformation in the absence of a membrane potential whereas depolarization-activated channels, such as KvAP, are in an open conformation. We have taken advantage of this property to compare the local structure and dynamics of the S4-S5 linker of these two channels using site-directed spin labeling and electron paramagnetic resonance (EPR) spectroscopy. Structural and biochemical studies of depolarization-activated channels suggest that the movement of the S4 is transmitted to the pore domain via the S4-S5 linker. The S4-S5 linker is proposed to rest against the S6 helix as a rigid helical lever to hold the pore closed at hyperpolarizing potentials. However, directed mutagenesis in the S4-S5 linker region in MVP testing properties such as length, helicity, and sequence dependence have suggested that MVP may not be using the S4-S5 linker as a helical lever to couple the VSD and pore domain. using EPR, we present a comparison of the local structure and dynamics of this region in MVP and KvAP from recombinant protein samples reconstituted in liposomes. We expect these results will inform the mechanism for electromechanical coupling in MVP and add to the overall understanding of electromechanical coupling in Kv channels.

Mechanosensitive Channels

2389-Pos Board B408

Inflammatory Signals Enhance piezo2-Mediated Mechanosensitive Currents

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Heightened nociceptor function caused by inflammatory mediators such as bradykinin (BK) contributes to increased pain sensitivity (hyperalgesia) to noxious mechanical and thermal stimuli. Although it is known that sensitization of the heat transducer TRPV1 largely subserves thermal hyperalgesia, the cellular mechanisms underlying mechanical hyperalgesia have been elusive. The role of the mechanically activated (MA) channel piezo2 present in mammalian sensory neurons is unknown. We tested the hypothesis that piezo2 activity is enhanced by BK, an algogenic peptide that induces mechanical hyperalgesia within minutes. We elicited piezo2-mediated currents by poking transiently transfected HEK293T cells with a blunt glass probe. Piezo2 current amplitude increases and inactivation is slowed by activation of exogenous bradykinin receptor B2. The area under the curve was enhanced 6.5-fold by BK compared with vehicle. Inclusion of GDPbetaS in the pipette occluded both effects on amplitude and inactivation. The protein kinase A (PKA) and protein kinase C (PKC) agonists 8-BrcAMP and PMA, respectively, enhanced piezo2 activity. No enhancement of piezo2-mediated currents was observed during exposure to the PLC activator m-3M3FBS (25 microM), suggesting phosphatidylinositol-PLC pathways do not increase piezo2 activity; inhibition of store release by thapsigargin had no effect on BK-induced changes. The PKA inhibitor H-89 and PKC inhibitor BIM I applied together effectively abrogated the BK-induced changes. Finally, piezo2-dependent MA currents in a class of native sensory neurons are enhanced 8-fold by BK via a PKA and PKC dependent mechanism, similarly to that observed in HEK cells. Thus, piezo2 sensitization may contribute to PKA- and PKC-mediated mechanical hyperalgesia. The mechanism described here differs from another model of mechanical hyperalgesia (epinephrine-induced PKCε-mediated) in which the downstream effector of cAMP is EPAC rather than PKA, since the BK effects observed here require PKA activity, and neither PI-PLC nor phospholipase D appears to be involved.

2390-Pos Board B409

Positively Charged Residues on GsMTx4 are Crucial for Inhibition of the Mechanosensitive Ion Channel Piezo1

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GsMTx4 is the only known peptide inhibitor of mechanosensitive channels. It is a gating modifier and presumably acts by modifying the arrangement of lipid molecules in the channel-lipid interface. Like other ICK peptide toxins GsMTx4 has a hydrophobic face which presumably facilitates partitioning of the molecule to the water/membrane interface of the bilayer. Prior studies also show that charged lysine residues may further influence the positioning of the peptide in the bilayer through interactions with the headgroups of anionic lipids. Coarse-grained simulations of GsMTx4 in the lipid bilayer suggest the existence of two binding modes: a shallow-binding mode and a deep-binding mode. The deep-binding mode in particular involves interactions between positively charged residues on the peptide and carbonyl atoms of the headgroups of inner-leaflet lipids. To provide insight into these interactions for GsMTx4 function, we investigated if reversing the charge by mutating lysine residues to glutamate affected the activity of GsMTx4. Each lysine residue in GsMTx4 was individually changed to glutamate to generate six mutants: K8E, K15E, K20E, K22E, K25E and K28E. When tested on outside-out patches from HEK cells transfected with mPiezo1 cDNA, all KtoE mutants showed reduced ability to inhibit the channel. In addition to reduced activity, K15E peptide altered the inactivation rate of the mPiezo1 currents. Even at saturating concentrations, K28E mutation reduced the activity of the peptide to 23% of that of the wild-type peptide. This study shows that charge reversing mutations to lysine residues diminish the efficacy of GsMTx4, and this phenomenon may involve disruption of peptide partitioning into the membrane and/or peptide-induced bending of the membrane bilayer.

2391-Pos Board B410

Piezo1 Mutations Identified in Xerocytosis alter the Inactivation Rate

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Piezo1 is a cation selective channel in eukaryotes, and Xerocytosis is a disease of red cells that has been mapped to mutations in the gene encoding Piezo1. We introduced these mutations into a cloned human Pieoz1 and measured their effects on the channel kinetics. Substituting arginine for methionine at position 2225 (M2225R), or a hisitidine for arginine at position 2455 (R2455H) slowed inactivation. This behavior is true in whole cell recordings and cell-attached and outside-out current patches. At position 2455, introduction of lysine, a conservative amino acid change for arginine, we were unable to restore inactivation, indicating that a charge movement at that location in the membrane field is not involved in voltage-dependent inactivation. Co-transfection of cells with Piezo1 and MscL allowed us to estimate the relative stress sensitivity, equivalent to the mean area change, of both channels in a single patch. Surprisingly, the slope sensitivities of both channels were similar, indicating that the dimensional changes for opening Piezo1 is close to that of MscL at 6.5 nm2. We also demonstrate that the inhibitor of mechanical channels, GsMTx4, also blocks currents from mutant channels. The biophysics of Piezo dysfunction shows that the channel stays open longer than in the wild type and likely affects red cell volume regulation by allowing an excess influx of calcium.

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