whether Cav1.2 channels are mechanosensitive and the possible role of integrins in this process, patch clamp methods were used to investigate the properties of either native or heterologously expressed Cav1.2 cannels by stretch of single cells plated onto a flexible substrate. Thin silicone membranes were coated with either fibronectin (FN) or poly-l-lysine (PLL) to assess integrin-dependent and -independent responses, respectively, and stretched using two blunt micropipettes driven in equal and opposite directions by piezoelectric translators. Graded stretch to 130% of resting cell length induced graded increases in Cav1.2 current (up to 63%) in HEK 293 cells expressing the neuronal channel isoform (Cav1.2c). The increase in current was ~2-fold greater for cells adhering to FN than for cells on PLL. On FN, 130% longitudinal stretch of primary VSM cells induced ~50% increases in Cav1.2 current. However, the magnitude of stretch-activated Cav1.2 current was the same on FN or PLL for cells expressing a Cav1.2 construct containing two C-terminal mutations (Y2122F/S1901A) to prevent phosphorylation by PKA and c-Src, or for cells expressing a Cav1.2 construct with the C-terminus truncated. Our results suggest that the Cav1.2 channel can be potentiated by membrane stretch, with one component due to intrinsic mechanosensitivity of the channel and a second component due to signaling through an integrin-dependent process.

1682-Pos

Dissecting the Molecular Mechanism of How Force Activates Yeast TRP Channel TRPY1

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Though clear for the prokaryotic mechanosensitive (MS) MscL and MscS, how mechanical force activates eukaryotic MS channels remains poorly understood. Several members of transient receptor potential (TRP) channel family are apparently involved in animal mechanosensations and are therefore promising MS-channel candidates. The yeast homolog, TRPY1, expressed in the yeast vacuolar membrane, can clearly be gated by hyper-osmotic shock *in vivo* and by directly stretching excised vacuolar membrane patches under patch clamp. Here, we investigated the structure-function relationship of TRPY1 by mutagenesis, aiming at dissecting how force is sensed by TRPY1 and how force opens the channel gate. TRP channels share general organization with well-studied voltage-gated potassium channels, being tetramers with each subunit consisting of six transmembrane helices (S1-S6) and N- and C-terminal cytoplamic domains. We found that the C-terminal cytoplasmic domains of TRPY1 harbor Ca^{2+} binding motifs, which confer TRPY1 Ca^{2+} activation. We demonstrated that the Ca^{2+} activation and the force activation are synergistic and the two gating mechanisms act in parallel. We proposed that force is perceived by the transmembrane domains. Our further in-depth analyses showed that strategic insertions of long peptide linkers before S4-S5 linker and after S6 can surprisingly yield functional channels with largely intact mechanosensitivity, highlighting the crucial roles of the pore module in TRPY1 mechanosensitivity. Together with our detailed scanning mutagenesis, we will discuss possible molecular mechanisms on how force activates TRPY1.

1683-Pos

Inactivation of the Bacterial Mechanosensitive Channel MscL Involves Flexible Transmembrane Helices and a 'Dry' Gate

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MscL, a mechanosensitive channel of large conductance, is an emergency release valve residing in the cytoplasmic membrane of E. coli. Under osmotic shock, when membrane tension approaches the lytic limit of ~10 mN/m, MscL opens a 3 nm pore relieving osmotic stress. The conformational transition in the pentameric complex was previously envisioned as a tilting iris-like motion of tightly coupled pairs of the centrally located TM1 and peripheral TM2 helices. Wetting the hydrophobic constriction formed by the rings of L19 and V23 was identified as is the rate-limiting step over a barrier of more than 50 kT. While adaptation of MscL was reported, the channel was generally considered non-inactivating. Special pressure protocols involving prolonged conditioning steps and short saturating test pulses revealed that after a 30 s exposure to half-saturating pressure (p0.5) in spheroplast patches, about 20% of MscL population reversibly inactivates. The channels return to the resting state within 1 s upon pressure release. Introduction of a flexible double glycine motif (A91G/I92G) in TM2 dramatically increased the rate of inactivation resulting in a 90% silent channel population after a 10 s step to p0.5. Single-channel traces revealed a split of concerted 70 pA opening transitions into a staircase of irregular ~7 pA substates in the double glycine mutant. The additional hydrophilic substitution in the constriction (V23T/A91G/I92G) pre-hydrates the pore, reduces p0.5 by ~55% and, while generating multiple substates, completely abolishes inactivation. In extrapolated-motion simulations TM2 kinks at A91G/I92G and its C-terminal end separates from TM1. We propose that in WT MscL TM1s and TM2s are tightly coupled, whereas in the double glycine mutant the unsupported TM1s may reform the tight hydrophobic seal independent of the positions of TM2s thus creating a tension-insensitive non-conductive state.

1684-Pos

Caveolin and Cholesterol Control of Mechanosensitive Channels in Muscle

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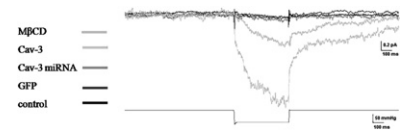
In Duchene muscular dystrophy the loss of the cytoskeletal element dystrophin modifies the localization of sarcolemma components and the stress distribution to those components. There is a change in the distribution of caveolae and dysregulation of mechanosensitive ion channels (MSCs) both of which may contribute to the elevated Ca2+ levels present in dystrophic myotubes. Caveolae are curved cholesterol rich membrane structures associated with dystrophin that contain many signaling molecules. We have shown that overexpression of Caveolin-3 in mouse myotubes increases the incidence of MSCs in patches and average MSC current. However, it has no effect on their kinetics of activation. Cholesterol depletion by MβCD produces an even larger increase in MSC incidence and average patch current, and also significantly decreases the relaxation rate of the membrane as observed by membrane capacitance changes. We have used miRNA against TRP channels (TRPC1, TRPC4, TRPC6, TRPV2) that have been reported to mechanosensitive in various systems to try and determine the identity of the channel revealed by cholesterol depletion. Average patch current and immunofluorescence staining has been used to determine the level of knockdown of the individual TRP channel subunits.



1685-Pos

Clustering and Functional Interaction of MscL Channels

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Mechanosensitive channels allow bacteria to respond to osmotic stress by opening a nanometer size pore in the cellular membrane. While the underlying mechanism has been studied intensively on the basis of individual channels, the work described here sheds light on the behavior of an ensemble of mechanosensitive channels of large conductance (MscL) in the membrane. Evaluating the spatial distribution of MscL channels in the bilayer using patch clamp, fluorescence, neutron scattering and reflection techniques, as well as atomic force microscopy and mathematical modeling, MscL was found to form clusters under a wide range of conditions. Within the cluster, MscL is closely packed, but still active and mechanosensitive. The channel activity, however, is modulated by the presence of neighbouring proteins, indicating functional protein-protein interactions. Collectively, the results demonstrate a potential functional role for self-assembly of MscL in the membrane.

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1686-Pos

A Kinetic Characterization of the Human Erythrocyte Mechano-Activated K^+ Channel Inactivation Process

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Human erythrocyte (hRBC) shows a live span of 120 days, as these cells lack nucleus and organelles a question arises: which is the subjacent molecular process to this tightly controlled programme cell death (the biological clock)? It has been proposed that the increased Ca^{2+} concentration characteristic of the senescent cells is due to a mechanical stress at the microcirculation level. Using the Patch Clamp Technique, we had characterized a mechano-activated K^+

channel which shows a sigmoid dependence of *Po* on applied pressure, a mean conductance of 17pS, and is Ca^{2+} modulated (*140mM*KCl, *10mM*NaCl, *1mM*$CaCl_2$, *pH*7.0)(HEMKCA)(1,2), and had proposed a new hypothesis for the senescent process of hRBC with this channel as the responsible for the molecular clock. This channel presented an inactivation process producing an exponential decay of *Po* (τ=4.55 ± 1.95min). Here we present a complete kinetically characterization of this inactivation process: intriguingly, this process seemed to begin just when a voltage step is applied and ionic current started, suggesting that the activation process is necessary but not sufficient to allow the inactivation development. We had characterized the burst mode activity of this channel (17.43 ± 17.15 events/burst, 264.27 ± 291.3ms burst duration and 15.67 ± 7.1ms intraburst interval). The decay in *Po* produced by the inactivation seemed to be the effect of decay in the number of events per burst, probably related to burst duration decay, with no effect in intrabursts variables like intraburst intervals and intraburst mean duration. We present a complete kinetic model for this channel with two independent kinetic branches: one for the non-inactivated mode and the other for the inactivation pathway. This inactivation mechanism is presented as a molecular damper (security system) in our new hypothesis for the hRBC senescence.

(1)(2005) Biophys. J.88(1):593
(2)(2008) Biophys. J.91(1):1101
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1687-Pos

Deletion Analysis of the Mechanosensitive TREK-1 Channel

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TREK-1, the first functionally characterized mechanosensitive K channel from the two-pore family (K2P) is involved in protective regulation of resting potential in CNS neurons and many other tissues. The structural basis of TREK-1 sensitivity to stretch and other factors such as arachidonic acid (AA) and anesthetics remains unknown. Attempts to use existing K channel structures as templates for TREK-1 modeling have identified several motifs that are not present in canonical K channels, which include divergent cytoplasmic N- and C-termini, and a characteristic 50-residue extracellular loop in the first homologous repeat. To characterize functional roles of these domains, we analyzed TREK-1 deletion and cysteine mutants in patch-clamp experiments. In response to steps of suction, the control TREK-1-EGFP fusion protein expressed in HEK-293 cells produced transient currents in cell-attached patches and non-inactivating sustained currents upon patch excision. Responses in both configurations were augmented by AA. Deletion of the extracellular loop (Δ76-124) reduced functional surface expression of channels and increased background activity, but the activation by tension augmented by AA was fully retained. Further deletion of the C-terminal end (Δ76-124, Δ334-411) produced no additional effect. In an attempt to generate cysteine-free version of the channel, we additionally mutated two cysteines in the transmembrane domain. C219A did not compromise channel activity, whereas C159A/S was essentially inactive. Experiments in the presence of mercaptoethanol suggested that none of these cysteines form functionally-important disulfides. The functional deletion mutant without C219 is now topologically closer to other K channels and makes an amenable system for homology modeling and testing by disulfide cross-linking.

1688-Pos

Hot Or Hot? Differentiating the Effects of Heat and Capsaicin on the TRPV1 Channel

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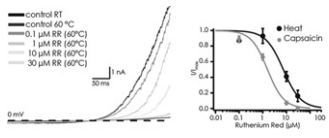
The temperature-sensitive ion channel family is receiving increasing attention as a potential contributor to pain states. In particular, ion channels activated by noxious heat, for example the TRPV1 channel, could provide a novel target for the treatment of chronic pain.

Using an automated patch clamp system, TRPV1 receptors expressed in CHO cells were activated using either noxious heat or the ligand capsaicin. Antagonists can be used to block either the heat- or capsaicin-activated TRPV1 response. Since blocking the heat response of TRPV1 can have undesirable effects on core body temperature in animals and humans, being able to discriminate between antagonising the ligand-activated vs. the heat-activated response of TRPV1 channels may be important for discovering novel compounds with a reduced side-effect profile. Data will be shown of TRPV1 currents activated by increasing temperature and block of the heat-activated response by ruthenium red. Data will also be presented showing different antagonist profiles for ligand- and heat-activation of TRPV1.



1689-Pos

Heat and Capsaicin Activate TRPV1 Channels to Different Open States:

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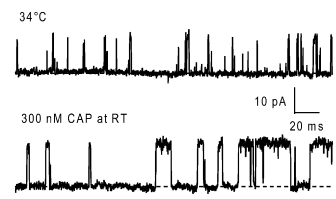
Both heat and capsaicin can strongly activate TRPV1 channels. However, the underlying mechanism by which the activation gate responds to these two distinct stimuli is unknown.

We use single-channel recordings of the wildtype and mutant TRPV1 channels with large unitary conductance to compare channel activation driven by each of the gating modalities. As shown in the figure, the heat-induced openings (top trace) exhibit much shorter open times than those of ligand-induced channel openings (bottom trace). In addition, we observe that at saturating concentrations capsaicin can only partially open TRPV1 channels. Raising temperature in the presence of capsaicin leads to an increase in open probability.



These results demonstrate that heat and capsaicin gate the channel to different open states, suggesting that they do not share the same activation pathway.

1690-Pos

Study Heat-Induced TRP Channel Activation Using Near-Infrared Laser As a Heat Source

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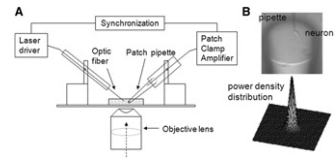
Rapid and reliable temperature change is required for the study of temperature-dependent channel gating mechanisms. We use continuous near-infrared (CNI) laser as a heat source to rapidly increase the local temperature at a cell or a cell-free patch membrane containing temperature-sensitive TRP channels. The photothermal effects of laser irradiation can be characterized by transient changes in fluorophore emission, liquid junction potential, and function of membrane proteins. The CNI laser-based method is advantageous over perfusion-based heating methods mainly in speed but also in its reproducible temperature raising profile and minimal interference with perfused solutions containing channel agonists/antagonists.



1691-Pos

The Proximal C-Terminal Region of TRPV1 Controls Phosphoinositide Selectivity

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The lipid messenger PIP2 is a critical modulator of multiple membrane proteins, including TRPV1 ion channels. We have previously reported that PIP2 is an important cofactor for activation of TRPV1. A critical question to elucidate the molecular mechanism of activation is where in the channel is the binding site for PIP2. Here we report that a single amino-acid mutation, located in a region of the C-terminus proximal to the transmembrane domains of TRPV1, inverts selectivity of these channels for phosphoinositides by making PI(4)P a stronger activator than PIP2. An *in vitro* FRET-based binding assay shows this proximal site is capable of binding PIP2. In addition, the distal C-terminal region, previously proposed as a candidate site for PIP2 binding, is not required for PIP2 regulation. We also addressed a recent report suggesting that an integral membrane protein called Pirt acts as the PIP2 sensor for regulation of TRPV1. Pirt expression did not appear to alter TRPV1 apparent affinity for PIP2. In summary, these results implicate the C-terminus proximal site as a PIP2 interacting domain. More importantly, PIP2 binding to this proximal site is central to TRPV1 activation.