proteins) in lipid environments by applying site-directed fluorescence labelling along with patch-clamping, confocal microscopy and Förster Resonance Energy Transfer (FRET) analysis.
A number of single cysteine mutants of each protein, labelled randomly with donor and acceptor fluorophores, and reconstituted into artificial liposomes are imaged as unilammellar blisters, under conditions similar to patch-clamp studies. FRET efficiencies can be calculated (either as an average for each liposome or by a pixel-by-pixel counting method), both before and after channel activation, achieved by addition of lysophosphatidylcholine (LPC). Using a Monte-Carlo simulation scheme the efficiencies can be correlated to the radius of the channel. Changes in FRET efficiencies are also correlated with channel functionality measured by patch-clamp experiments. We thus observe that for MscL, transition to the open state occurs via helical rearrangements throughout the protein that increase the overall channel diameter by 15 Å to accommodate a large 28 Å open channel pore. In case of MscS, preliminary results using fluorescence spectroscopy indicate changes in channel diameter of about 10-15 Å.
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Open Channel Structure of MscL from Spectroscopy and Simulation
Ben Corry[1], Annette C. Hurst[2], Prithwish Pal[1], Paul Rigby[1], Boris Martinac[2].
[1]University of Western Australia, Perth, Australia, [2]University of Queensland, Brisbane, Australia.
Mechanosensitive channels open in response to membrane bilayer deformations occurring in physiological processes such as touch, hearing, blood pressure regulation or osmoregulation. Here, we have determined the likely structure of the open state of the mechanosensitive channel of large conductance from *E. coli* (MscL) in a natural environment using a combination of patch-clamp studies, FRET spectroscopy, EPR data, molecular and Brownian dynamics simulation. Structural rearrangements of the protein are measured while controlling the state of the pore by modifying lipid bilayer morphology. FRET efficiency changes can be related to distance changes using a Monte Carlo analysis program in conjunction with detailed orientational analysis. These measurements are used as restraints in all atom molecular simulations in order to determine the likely structure of the open state of the pore. Finally, the width of the pore is confirmed by calculating its likely conductance and using additional experimental measurements.
Transition to the open state occurs via large rearrangements throughout the protein that create a wide pore nearly 30 Å in diameter. The motion of the transmembrane helices, however, is less dramatic than previously proposed thus minimising the structural change required within each channel subunit. Both transmembrane helices are found to line part of the pore. The N terminal helix is found to lie along the face of the membrane where it can act to sense membrane tension and directly transfer this to the pore lining helices. The strain in the helix that this creates can then act to restore the pore to the closed state once membrane tension is ceased.
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The Peptide GsMTx4 Inhibits the TREK-1 Channel from the Intracellular Side
Philip A. Gottlieb, Frederick Sachs.
SUNY at BUffalo, Buffalo, NY, USA.
GsMTx4, the only known specific inhibitor for mechanically gated ion channels, is 34 amino acid long and adopts an ICK motif. The peptide when applied extracellularly inhibits nonselective MSCs. Understanding the relationship peptide's structure and mechanism using mutagenesis has been slowed due to a lack of a specific target. In this work we show that GsMTx4 can also inhibit TREK-1, a well characterized mechanical channel, but only from the intracellular side. Application of the peptide to the inner leaflet of transfected cells with TREK-1 produced a greater than 90% inhibition in the 2-5 μM range. GsMTx4 does not inhibit TREK-1 on the extra-cellular side and the enantiomeric form of GsMTx4 is equally effective. There are two observable responses; GsMTx4 decreases the magnitude of the mean channel activity produced by external stimuli from a pressure clamp and GsMTx4 also decreases the background activity. The latter is expected since the gigaseal adhesion energy activates channels at rest and Gsmtx4 blocks those channels. We have developed a 3 state model (close-open- inactivated) which allows us to extract the binding and unbinding kinetic parameters for the liganded and unliganded states.

1307-Pos Board B151
Effects Of GsMTx4 On Bacterial Mechanosensitive Channels In Situ
Kishore Kamaraju[1], Philip Gottlieb[2], Frederick Sachs[2], Sergei Sukharev[1].
[1]University of Maryland, College Park, MD, USA, [2]University of Buffalo, Buffalo, NY, USA.
The GsMTx4 toxin from Grammostola spatulata spider is a 34-residue inhibitory cysteine knot peptide which selectively blocks several types of mammalian mechanosensitive (MS) channels with essentially no effect on voltage-gated and other channels. Here we report that the effects of GsMTx4 on the bacterial mechanosensitive channels MscS and MscL studied in giant E. coli spheroplasts are distinct from those known for eukaryotic MS channels. Presented to excised patches from the cytoplasmic side, GsMTx4 (up to 20 υM) shifts activation curves for MscS and MscL to the left effectively sensitizing both channels to tension. The sensitization of MscS by the toxin was comparable under ramp or pulse stimulation. We found that GsMTx4 increases gating hysteresis for MscS observed with ascending and descending ramps of pressure, which can be ascribed to the markedly decreased closing rate in the presence of toxin. Desensitization of MscS manifested as a right-shift of activation curves under prolonged exposure to sub-threshold pressure steps, was not affected by the toxin. While slow closing rate complicated assessment of the inactivation rate with the toxin, we found that the time of recovery from inactivation increased four fold in the presence of 5 υM GsMTx4. We discuss the data in terms of possible stabilization of the protein-lipid boundary for the expanded (open and inactivated) conformations of MscS by the toxin interacting with the protein rim and annular lipids.

1308-Pos Board B152
Hypoxia Activates a Ca2+-permeable Channel Activity Sensitive To Carbon Monoxide And To Grammastola Spatulata Mechanotoxin IV (gsmtx-4) In Human And Mouse Sickle Erythrocytes
David H. Vandorpe[1], Chang Xu[1], Boris E. Shmukler[1], Leo E. Otterbein[1], Marie Trudel[2], Frederick Sachs[3], Philip A. Gottlieb[3], Carlo Brugnara[4], Seth L. Alper[1].
[1]Beth Israel Deaconess Medical Center, Boston, MA, USA, [2]Institut de Recherches Cliniques de Montreal, Montreal, QC, Canada, [3]Department of Physiology and Biophysics, SUNY Buffalo, Buffalo, NY, USA, [4]Children's Hospital, Boston, MA, USA.
Deoxygenation of sickle erythrocytes activates a cation permeability (Psickle) leading to elevated $[Ca^{2+}]_i$ and subsequent K_{Ca} channel activation. The resulting erythrocyte volume decrease is believed to accelerate deoxygenation-induced HbSS polymerization. Deoxygenation-activated currents with some properties of Psickle have been recorded from sickle erythrocytes in whole cell configuration. We now show by cell-attached patch clamp of human sickle erythrocytes and of erythrocytes from two mouse models of sickle disease that deoxygenation activates Ca^{2+}- and cation-permeable channel activity sensitive to inhibition by *Grammastola spatulata* mechanotoxin-4 (GsMTx-4), dipyridamole, DIDS, and carbon monoxide. Deoxygenation increases cytosolic $[Ca^{2+}]$ with similar pharmacological properties. These responses to deoxygenation are absent from normal human and normal mouse erythrocytes. Deoxygenation-induced $[Ca^{2+}]_i$ in mouse sickle cells did not require IK1/KCNN4 activity. These data constitute the first evidence in sickle erythrocytes for deoxygenation-induced single channel activity with permeability to Ca^{2+}, and strongly suggest that channel activation requires HbS polymerization.

1309-Pos Board B153
Tension-dependency Of The Mammalian Mechanosensitive Channel TREK-1
Grigory Maksaev, Sergei Sukharev.
University of Maryland College Park, College Park, MD, USA.
The lateral (in-plane) expansion associated with opening of a tension-sensitive membrane channel is analogous to the gating charge in voltage-activation. However, unlike transmembrane potential, membrane tension acting on mechano-activated channels is not easy to control. Not only the poorly defined curvature of the patch membrane poses the problem but also the presence cortical cytoskeleton complicates the mechanical response of the membrane to pipette pressure. In this work we combine high-speed pressure clamp and DIC videomicroscopy to quantify the responses of GFP-labeled stretch-activated TREK-1 channels expressed in HEK 293T cells. Recordings were made with large pipettes (BN=7, ~3 μm diameter) with the tips bent to be oriented in the focal plane. 100 ms pressure pulses evoked transient current responses and the patch curvature changes were monitored in several consecutive video frames. Dose-response curves recorded in cell-attached patches were markedly right shifted ($\gamma_{1/2} = 9$ dyn/cm) compared to the curves taken in apparently cytoskeleton-free blebs ($\gamma_{1/2} = 4$ dyn/cm). Fitted with the Boltzmann model $P_o/P_c = \exp[-(\Delta E - \gamma\Delta A)/kT]$, the curves from cell-attached patches revealed $\Delta A = 3$ nm^2 and $\Delta E = 7$ kT versus $\Delta A = 4$ nm^2 and $\Delta E = 5$ kT for blebs. Large inside-out patches excised from intact membrane exhibited parameters of TREK activation similar to those observed in cell-attached patches. We conclude that the cytoskeleton indeed bears a part of tension developed in a cell membrane in response to a transversal pressure gradient. The activation area for TREK-1 obtained in