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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1305-Pos Board B149
Open Channel Structure of MscL from Spectroscopy and Simulation
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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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1306-Pos Board B150
The Peptide GsMTx4 Inhibits the TREK-1 Channel from the Intracellular Side
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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
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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
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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
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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

bleb patches appears to be larger than proposed previously (Honore et al., 2006, PNAS **103**:6859).

1310-Pos Board B154

Rapid And Efficient Co-reconstitution Of Bacterial Mechanosensitive Ion Channels Of Small And Large Conductance Into Liposomes

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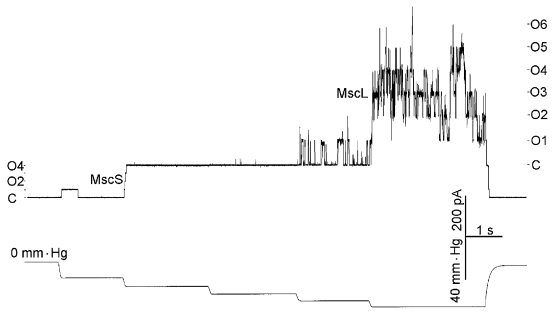
Bacterial mechanosensitive (MS) channels protect bacterial cells from osmotic shock, acting as emergency relief valves (1,2). *E. coli* has three such channels, the MS channel of large conductance (MscL), the MS channel of small conductance (MscS) and the MS channel of mini conductance (MscM). Both MscL and MscS have been extensively studied using the patch-clamp technique in giant spheroplasts (3,4). However, only MscL incorporates efficiently in liposomes (2,5). Here we report the first example of co-reconstitution of both MscS and MscL into azolectin liposomes. We also report reconstitution efficiencies of both proteins into liposomes of different lipid composition and using different incorporation methods.



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1311-Pos Board B155

Modulating The Conductance And Ionic Preference Of MscL, A Biological Nanovalve

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The bacterial mechanosensitive channel of large conductance, MscL, has many properties that are ideal for use as a nanosensor. Previous studies have shown that the pore size is huge (>30Å), it can be translated *in vitro* or synthetically synthesized, and it can spontaneously assemble into a functional complex. In addition, the modality of the channel can be changed; studies have shown that the sensor can be engineered to be sensitive to light, pH, and post-translational chemical modification as well as modulated by different heavy metals and redox. Hence, many studies have suggested its potential in nano-technological applications such as nano-scaled sensors in microchips and drug delivery systems. On the other hand, its large conductance may actually be limiting in some microchip applications, and even though some molecules pass through the MscL nanovalve, modifying its ionic preference could have advantages for vesicular release of charged compounds. Here we demonstrate that MscL can be molecular engineered to have altered conductance or ionic preference to better serve specific purposes. We found that constricting the cytoplasmic loops between the pore and a C-terminal cytoplasmic helical bundle of the channel by bridging cysteines, or coordinating heavy metals with histidines, can decrease the channel conductance as much as 50%; in both instances, the change is reversible. In addition, we found that the ionic preference of the channel can be modified by altering residues near the pore; changing the ionic preference of MscL towards anionic alters the permeability of spermidine, a polycationic organic compound. In summary, our results demonstrate that the conductance and ionic preference of the MscL nanovalve can be modified, and thus designed for specific applications. Keywords: MscL; channel conductance; ionic preference; Nanotechnology.

1312-Pos Board B156

Adaptive Behavior of Bacterial Mechanosensitive Channels in Excised Patches is Coupled to Membrane Mechanics

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MscS, a tension-driven osmolyte release valve residing in the inner membrane of E. coli, exhibits complex adaptive behavior, whereas MscL, its functional counterpart, was considered non-adaptive. When a membrane patch is held under a constant non-saturating pressure gradient, MscS exhibits desensitization (mode-shifting) manifested as a reversible closure followed by complete inactivation. Attempts to utilize MscL as a non-adaptive 'reference' channel revealed that a prolonged exposure of patches to sub-threshold tensions right-shifts activation curves for both MscS and MscL with similar magnitudes and time courses. MscS channels were also found to retain a 'memory' of prior desensitization, returning to the mode-shifted state after being fully opened by a saturating pressure pulse. When recorded in the whole-spheroplast mode under positive pipette pressure, MscS shows no desensitization whereas some inactivation still occurs. We thus link desensitization observed specifically in excised patches with mechanical relaxation of the inner leaflet not attached to the glass pipette, which may create a distribution of tension less favorable for opening. To further characterize and separate the processes of desensitization and inactivation in MscS we applied multi-pulse pressure protocols to excised patches. The results indicated that membrane tension slows reversible closure (desensitization) but speeds up inactivation and strongly impedes the process of recovery from inactivation. These dependencies indicate that the MscS channel contracts in the plane of the membrane when it reversibly desensitizes, but further expands when it inactivates. This calls for models with a more compact gate formed by the TM3 helices in both closed and inactivated states. In contrast, the peripheral transmembrane helices (TM1-TM2) can assume different conformations to confer a larger in-plane area for the inactivated state.

1313-Pos Board B157

Gating Of Bacterial Cyclic Nucleotide Gated (bCNG) Channels In Response To Membrane Tension

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We have identified, cloned, and characterized a new family of bacterial cyclic nucleotide gated (bCNG) ion channels. While we have demonstrated that these channels gate in response to cyclic adenosine monophosphate (cAMP), all bCNG channels exhibit significant homology to the pore lining helix (TM3) of the mechanosensitive channel of small conductance (MscS). This homology suggests that these channels might gate in response to mechanical stress in addition to ligand binding. To test this hypothesis, we have explored the ability of bCNG channels to rescue MscL/MscS/MscK null *E. coli* from osmotic downshock. While some homologues, such as bCNG from *Synechococcus sp. PCC 6803,* show rescue similar to that observed for wild-type *E. coli* MscS, other homologues, such as bCNG from *M. loti,* do not exhibit osmotic rescue. In the bCNG channel family, the numbers of transmembrane domains varies from two to six, with high homology between pore lining helices. Our current data implies that bCNG channels with greater homology to MscS are more likely to be mechanosensitive.

1314-Pos Board B158

Caveolae Act As Membrane Reserves Which May Limit $I_{Cl,swell}$ Activation During Cardiac Myocyte Swelling

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The channel responsible for swelling-activated chloride current ($I_{Cl,swell}$) is a mechanosensor which responds to changes in membrane tension during cell swelling, and regulates cell volume. It has been proposed that the $I_{Cl,swell}$ channel (or elements that regulate this) are dependent on caveolae, and we have previously shown that disrupting caveolae increases the rate of hyposmotic cardiac myocyte swelling. Here we test the hypothesis that the role of caveolae as a membrane reserve limits activation of $I_{Cl,swell}$. Rat ventricular myocytes were treated with methyl- β-cyclodextrin (MBCD) to disrupt caveolae and exposed to 0.02T solution (until cell lysis) or 0.64T solution for 10-15 min (swelling). Maximum cell volume achieved prior to lysis was calculated from a video image. Swollen cells (0.64T) were fixed for electron microscopy, and the negative inotropic response to swelling used as an index of $I_{Cl,swell}$