functional mammalian isoforms have been discovered so far with different functional and pharmacological properties. In our study, eight subtypes of the voltage gated sodium channel were tested in parallel on the automated patch clamp system QPatch HT. The new clone screening feature developed for QPatch 16 and QPatch HT allows running up to eight different cell lines (clones or subtypes) at the same time, thus ensuring that the exact same conditions (temperature, Ringer's, pH etc.) are applied for each of the cell lines tested. $Na_v1.1$, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7 and 1.8 were tested together in a range of experiments on QPatch HT. All but $Na_v1.2$ and $Na_v1.8$ were co-expressed with the β1 subunit. Three types of experiments were designed to explore 1) TTX sensitivity, 2) IV-relationship for activation and inactivation, and 3) recovery from inactivation, for the entire panel of Na_v channel subtypes in a single experiment. It was shown that QPatch experiments using the cell clone screening feature together with the QPatch Assay Software data analysis package, enables the experimenter to obtain IC_{50} values for TTX, IV-relationships and time constants for recovery from inactivation which are very similar to manual patch clamp data, for all Nav subtypes, thus successfully distinguishing one subtype from another.

Mechanosensitive Channels

1300-Pos Board B144

Analysis of Gating Process Associated with Water Permeation of the E-coli Mechanosensitive Channel MscL Using Molecular Dynamics Simulations

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The bacterial mechanosensitive channel of large conductance MscL is constituted of homopentamer of a subunit with two transmembrane inner and outer α-helices, and its 3D structure of the closed state has been resolved. The major issue of MscL is to understand the gating mechanism driven by tension in the membrane. Although several models for the opening process have been proposed with Molecular Dynamics (MD) simulations, as they do not include MscL-lipid interactions, it remains unclear which amino acids sense membrane tension and how the sensed force induces channel opening. We performed MD simulations for the mechano-gating of MscL embedded in the lipid bilayer. Upon tension generation in the bilayer, Phe78 in the outer helix was dragged by lipids, leading to a tilting of the helices. Among amino acids in the outer helix facing the bilayer, Phe78 at the water-lipid interface showed the strongest interaction with lipids, thus may work as a major tension sensor. Neighboring inner helices cross each other in the inner leaflet, forming the most constricted part of the pore. As tension increases, the crossings move toward the cytoplasm associated with an expansion of the constricted part. During the movement, a hydrophobic water block environment around the constricted part was broken followed by water penetration and permeation. We modeled G22N mutant, known to have an ability to permeate ions without increasing membrane tension, and performed 5 ns equilibrium simulations. We analyzed movements of water molecules around the block and found that the asparagine substitution resulted in spontaneous water flow due to a hydrophilic side chain of asparagine, leading to partial channel opening. Thus a change in the environment around the most constricted part from hydrophobic to hydrophilic promotes MscL opening.

1301-Pos Board B145

MscL Gating In Liposomes

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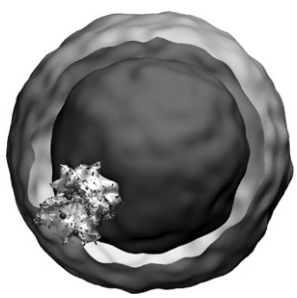
Mechano-sensitive channels function as safety valves of a cell by controlling the permeability of the plasma membrane. They are non-selective and respond rapidly to sudden changes in the tension of the membrane. During a hypo-osmotic shock mechano-sensitive channels sense the growing turgor pressure and start to gate, thereby releasing the tension and preventing the rupture of the cell membrane.



Mechano-sensitive channel of large conductance (MscL) forms a large, non-selective channel when activated. The crystal structure of MscL in its deactive state extracted from Mycobacterium tuberculosis has given insights into the possible activation mechanism of MscL and has enabled the tentative mapping of the closed-open transition pathway by molecular dynamics simulations. Nonetheless, the role and importance of e.g. membrane curvature, cytoplasmic helix-bundle and directional ion flux are still unclear.

Liposomes, i.e. tiny lipid vesicles, offer unprecedented possibilities to study the effects of membrane curvature and directional ion flux on MscL gating. We have studied in near-atomistic detail liposome embedded Tb-MscL using the recently developed MARTINI coarse-grained model for biomolecules. Various pressure gradients were inflicted across the liposomal membrane to map the tension-activation response of MscL and to obtain fully activated channels capable of rapid release of liposomal stress.

1302-Pos Board B146

Multi-scale Modelling Of Tb-Mscl Gating In Its Native Environment

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Here we explore the possibility of combining Molecular Dynamics (MD) simulation together with Monte-Carlo simulation in order to predict the gating pathway for a tension-gated pore protein, the Mechanosensitive Channel of Large Conductance from Mycobacterium Tuberculosis (Tb-MscL). To mimic its native environment, we embed the channel protein in a native-like lipid membrane, itself first equilibrated by MD, and the whole system is then equilibrated using MD, followed by rigid cluster decomposition by FIRST software and Monte-Carlo simulation of channel opening using FRODA software. Our goal was to explore in a more atomistic level protein-lipid interactions that were explored by continuum models, and uncover the role played by various components of the protein-membrane system during the channel gating. Our results suggest that protein-lipid interactions are necessary in order to produce an asymmetric motion of channel subunits, which was observed in previous experimental studies.

1303-Pos Board B147

Spandex Proteins: Mechanosensitive Closed-closed Transitions Suitable for Osmoprotector and for Tension Damper Functions in Large Membrane Proteins

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Large membrane proteins are potentially more expansible than bilayers. We therefore term membrane proteins with stochastic tension-sensitive transitions between closed states "spandex proteins" and ask what design features might allow spandex proteins to act as tension relievers. Spandex is modeled with two states (contracted, expanded). Its barrier state location strongly impacts the timescale of the expansion transition. Expansion depends on spandex concentration, with the apparent midpoint tension shifting to larger tensions as the membrane density of spandex increases.

In a cell, there are two ways spandex might be advantageous. In the case of an abrupt tension increase, spandex expansion could reduce bilayer tension enough to prevent unnecessary opening of osmotic valve channels. To achieve this safely, a protein that expands at a very precise tension before the channels can open is required. This requires a large spandex protein, whose barrier is located close to the expanded state, ensuring that if tension is high, the spandex will react rapidly.

Secondly, spandex proteins could be used to maintain a steady bilayer tension. However, a single species of spandex could not be both a good partner for osmotic valves and a good tension damper. For reliable tension damping, the spandex tension midpoint must equal the target tension. To ensure the spandex reacted rapidly to tension fluctuations, its barrier would need to be located halfway between the contracted and expanded states. Also, the larger the change in area of the protein, the more precisely the target tension will be maintained in the bilayer. The concentration needed depends on the strain amplitude that is to be dealt with. We discuss possible interactions among the tension sensitive closed-closed and closed-open transitions of different bacterial membrane proteins. *NSERC funded.*

1304-Pos Board B148

A Site-directed FRET Confocal Microscopy Approach for Studying Conformational Changes in the Mechanosensitive Ion-channels, MscL and MscS

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Bacterial mechanosensitive channels act as safety valves that protect cells from hypo-osmotic shock by opening under membrane tension to relieve pressure within the cell. Although the crystal structures of two such ion channels - the mechanosensitive channels of large (MscL) and small (MscS) conductance - are known, the mechanism by which bilayer deformations are transduced into channel opening is still being worked out. Here we describe a method to study conformational changes associated with the channel opening (of both

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.
Supported by the Australian Research Council and National Health and Medical Research Council.

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.
Supported by the Australian Research Council.

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