changes induced were large but two intracellular Cl induced the largest conformational change, repelling the side chain of E148 against the external channel wall. This distortion produced a pathway that had an area 2.4 times bigger than the one seen with COO^- and no Cl. We anticipate that this larger pathway will allow Cl conduction easily. Our results imply that the combine actions of Cl and protonation of the E148 lateral chain are necessary to open the pore. Finally, the energy barriers that Cl faces during conduction strongly depend on structure, relative orientation, and chemical composition of the pore entryway.
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2428-Pos Board B398
Cooperative Ion Binding and Transport Mediated by a CLC-Type H^+/Cl^- Exchanger
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CLC-ec1 is a prokaryotic CLC-type Cl^-/H^+ transporter of known structure that stoichiometrically exchanges two Cl^- for one H^+. The crystal structures show that Cl^- binds to three sites (S_{ext}, S_{cen} and S_{in}) that define a pathway through the protein. Recently we used Isothermal Titration Calorimetry (ITC) to show that Cl^- binding to CLC-ec1 is cooperative: the affinity of Cl^- increases with the number of simultaneously occupied sites, despite their close spatial proximity. Here we sought to independently confirm and validate this surprising result. We used saturation equilibrium dialysis to directly determine the affinity of $^{36}Cl^-$ to WT and mutant variants of CLC-ec1 with altered ion occupancy. Our results qualitatively and quantitatively recapitulate the ITC conclusions. We found that $^{36}Cl^-$ binds to the Y445A mutant, in which only S_{in} is occupied with a $K_d > 20$ mM, and to the WT, where Cl^- can bind to both S_{in} and S_{cen}, with a $K_d \sim 3$ mM. Finally, the E148A mutant, where all three sites can be simultaneously occupied, is the tightest binder with a Kd ~ 190 μM. These binding affinities are in reasonable quantitative agreement with those determined with ITC.
To investigate the functional role of Cl^- binding in the transport cycle of CLC-ec1 we determined the Cl^- dependence of the transport rate of CLC-ec1 by varying $[Cl^-]_{ex}$ in the "Cl^- dump assay". We found that the turnover rate has a K_m of ~0.5 mM, a value similar to the K_d determined through the binding measurements. In conclusion we show here that Cl^- binds to CLC-ec1 cooperatively and that Cl^- binding is an important step in the transport cycle.

2429-Pos Board B399
Fluorine-NMR Reveals Conformational Differences Between ClC-ec1 Operating In Transporter And "Channel-like" Modes
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Despite its name, the CLC "chloride channel" family consists of both Cl^-/H^+ antiporters as well as chloride channels. This scenario presents a unique opportunity to investigate the molecular similarities and differences underlying these mechanisms. The crystal structure of the *E. coli* homolog ClC-ec1 provides an ideal framework for such an investigation, but this static picture alone cannot depict the protein movements that must occur during ion transport. In the present study we employ solution-state fluorine-NMR to monitor conformational changes in ClC-ec1 operating in three different transport modes. While ClC-ec1 normally behaves as a Cl^-/H^+ antiporter, it can be converted by point mutations into either a proton-independent chloride transporter or a chloride "channel-like" protein. In the case of wild-type ClC-ec1 (antiporter mode), we observe changes in the ^{19}F NMR spectrum upon shifting from a pH at which there is little activity to a pH that promotes high activity. We show that much of this spectral change is due to structural changes occurring at the dimeric interface. The pH-dependent changes persist when the protein is converted into a proton-independent transporter, but are eliminated in the ClC-ec1 channel-like mutant. This indicates that the channel-like protein does not rely on the same series of conformational changes that occur during coupled or uncoupled transporter activity. These results demonstrate the usefulness of ^{19}F NMR for studying CLC conformational changes and will be a springboard for future studies of CLC protein dynamics.

2430-Pos Board B400
Dynamics of Phosphate Transport by the Anion-specific Outer Membrane Protein OprP
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The outer membrane protein P (OprP) from *Pseudomonas aeruginosa* forms a water-filled channel which has an enhanced selectivity for anions, especially phosphates. The structure of this homotrimeric protein (PDB code 2O4V) reveals three positively charged loops (L3, L5, and T7) which are folded into the lumen and are suggested to funnel anions into the pore. Steered molecular dynamics (SMD) simulations have been performed to better understand the mechanism of the phosphate transport. In these SMD simulations an external force was applied to pull a phosphate anion from the extracellular to periplasmic side and *vice versa*. The SMD results have been supplemented by unbiased molecular dynamics (MD) simulations. The SMD force profiles and the phosphate trajectories reveal energy wells close to the L5, L3, and T7 regions. The dominant wells are identified at the L3 (or constriction) region, while the others are at the extracellular L5 and periplasmic T7 regions. Both the SMD and MD simulations suggest that favourable interactions with the side chains of positively charged amino acids contribute to the phosphate-protein binding site. The results of our studies suggest a full possible pathway for phosphate transport.

2431-Pos Board B401
The Regulation of Volume-Regulated Outwardly Rectifying Anion Channels by Membrane Phosphatidylinositides in Mouse Ventricular Cells
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Volume-regulated outwardly rectifying anion channel (VRAC) plays an important role in cell-volume regulation. We examined the effect of phosphatidylinositol 3,4,5-trisphosphate (PIP3) and phosphatidylinositol 4,5-bisphosphate (PIP2) on the VRAC current activated by hypotonic solution, in mouse ventricular cells. The VRAC current was inhibited strongly by intracellular application of LY294002 (a PI3 kinase inhibitor) or anti-PIP3 antibody (PIP3-Ab), and less strongly by anti-PIP2 antibody (PIP2-Ab). Intracellular application of PIP3 or PIP2 influenced neither the basal background current in isotonic solution nor the VRAC current in hypotonic solution. However, PIP3, but not PIP2, restored the VRAC current suppressed by LY294002 or PIP2-Ab. These results suggest that that PI3K-mediated PIP3 production is essential to activate the VRAC current. Furthermore, we found that an α1-adrenergic receptor (α1R) agonist, phenylephrine (PE), inhibited the VRAC current. This inhibition didn't occur in the presence of prazosin, an α1R antagonist, or when the cells were dialyzed with anti-Gq/11 antibody. U-73122, a PLC inhibitor, prevented the PE-induced inhibition of VRAC current, whereas several PKC inhibitors were without effect. Since PE unaffected the VRAC current in cells dialyzed with PIP2, PE-induced inhibition of the VRAC current may be related to PIP2 depletion. In addition, the reduction of VRAC current was also found in cells from STZ-induced insulin-deficient diabetic mice. In these cells, the attenuated VRAC current was restored by incubating the cells with insulin or by dialyzing the cells with PIP3. PIP2 loading could not restore the current. These findings suggested that an impairment of the insulin-dependent PI3K-PIP3 pathway is responsible for the attenuation of VRAC currents in STZ-diabetic cells. Taken together, we propose that VRAC in mouse ventricular cells is regulated by PIP3 and/or its down stream signaling pathways.

2432-Pos Board B402
Regulation of swelling-activated Cl channel in HEK 293 cells by extracellular low pH
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Using voltage-clamped HEK 293 cells that were exposed to hypotonic solution, we measured the effects of low pH on the slowly (~100s) swelling-activated Cl-current. The channel showed mild outward rectification during ramp-clamps, had a reversal potential (-21.7 ± 2.9 mV) close to the predicted E_{Cl} (-19.1mV), and was reversibly inhibited by DIDS. Changing extracellular pH from 7.4 to 6.0 significantly reduced the current and accelerated its inactivation measured over 200 ms at +80 mV: In cells with minimal Ca^{2+}-buffers (0.1mM EGTA), challenging with hypotonic solution at pH 6.0 reduced the initial and final currents by 49% and 55%, respectively (compared to pH 7.4 control values). Interestingly, in highly Ca^{2+}-buffered cells (10 mM BAPTA), the decay of the current at pH 6.0 was significantly faster with 49.1 % initial and 74.7% final suppression. We also found that the current was reduced by 75% by 5 μM U-73122 (an inhibitor of phospholipase C) and by 30% by 20 μM Farnesyl thiotriazole (a PKC activator). High intracellular Mg^{2+} (10.7 mM) nearly abolished activation of the current suppressing its slope conductance from 7.0 ± 0.2 to 2.1 ± 0.3 nS at +80 mV and from 4.4 ± 0.2 to 0.33 ± 0.1 nS at -80 mV ($p<0.001$). Extracellular Mg^{2+} (10 mM) had no significant effect on the current. Intracellular cAMP (200 μm) delayed, but did not prevent, the activation of the current. Extracellular cAMP suppressed 75% of the current.
These data suggest that the kinetics of the inactivation of the proton-regulated chloride channel depend on the intracellular buffering capacity for Ca^{2+} and that the magnitude of the current is regulated by PIP_2, PKC, and cAMP signaling pathways and by intracellular Mg^{2+}.

2433-Pos Board B403
Endogenous Acidification of Central Inhibitory Synapses
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In the brain, extracellular pH is rigidly maintained to ensure proper CNS function. To assess pH fluctuation at central synapses, we recorded miniature

GABA inhibitory post-synaptic currents (mIPSCs) from cultured cerebellar granule cells under varying pH and proton buffering conditions. We found an inverse relationship between extracellular pH and mIPSC amplitude and charge transfer, resulting in over a 100% increase in size of events recorded at pH6.8 vs. pH8.0. Acidification also slowed the kinetics of rise time and fast component of decay, while speeding the slow decay component. We find that lowering the pH buffering capacity of the extracellular solution from 24 to 3mM HEPES at pH7.4, results in a similar enhancement of mIPSC size, mimicking changes in kinetics induced by acidification. The effects of diminished buffering capacity on mIPSC were negated by lowering extracellular pH to 6.8. To probe these effects with physiological buffers, we measured mIPSCs using 24mM of bicarbonate and compared them with those recorded in 24mM bicarbonate supplemented with 10mM HEPES . We found that physiological concentrations of bicarbonate produced mIPSCs that were similar in size and kinetics to those found with 3mM HEPES and were similarly altered with addition of HEPES, confirming the physiological relevance of our findings. To determine the possible contribution of Na+/H+ exchanger to synaptic acidification we inhibited the exchanger with amiloride (20μM), and in a parallel set of experiments replaced extracellular sodium with lithium. Both of these treatments caused changes in mIPSCs that mirrored increased buffering capacity, and the effects were negated by acidification to pH6.8 or by increasing HEPES buffering capacity to 24mM. We conclude that GABAergic synaptic pH in vivo may be quite labile and subject to rapid and pronounced acidification from the Na+/H+ exchanger with the net effect of enhancing synaptic transmission.

2434-Pos Board B404

Voltage-dependent Gating Of Wt And D177a Eaat4-associated Anion Channels

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Excitatory amino acid transporters (EAATs) are not only secondary-active glutamate transporters, but function also anion-selective channels. Ryan and Vandenberg (JBC, 279: 20742-20751, 2004) recently demonstrated that mutations in the interlinker between transmembrane domain 2 and 3 of EAAT1 affect selectivity of EAAT anion channels suggesting that this domain forms part of the anion-selective pore. We here study the effect of a point mutation within this region, D117A, on anion channels associated with another EAAT isoform, EAAT4. WT and D117A EAAT4 were expressed in tsA201 cells and studied through whole-cell patch-clamping under a variety of conditions. WT EAAT4 anion channels conduct anions over the whole voltage range and exhibit two types of voltage-dependent gating, one activated by membrane hyperpolarisation, and another one activated during membrane depolarisation. Glutamate shifts depolarisation- and hyperpolarisation-induced gating to more negative potentials in a dose-dependent fashion. At saturating glutamate concentrations, both gates are active in a physiological voltage range. Only in the presence, but not in the absence of glutamate, gating of WT anion channels also depends on anion concentrations on both membrane sites. External anions shift the activation curve of both gating processes to more negative potentials, whereas increasing concentration of internal anions have the opposite effects. D117A has dramatic effects on permeation, gating and glutamate dependence of EAAT4 anion channels. The amplitude of D117A EAAT4 anion currents is not affected by glutamate. At symmetric anion concentrations, D117A EAAT4 anion channels are strictly outwardly rectifying, in clear contrast to WT EAAT4 that effectively conduct anions in both directions. Moreover, D117A EAAT4 channels exhibit only a single gating process, activated by membrane depolarization. Gating of D117A EAAT4 is not affected by glutamate. Our results suggest a crucial role of D117 for the function of EAAT anion channels.

Ca-Activated Channels

2435-Pos Board B405

Impaired Ca^{2+}-Dependent Activation of Large Conductance Ca^{2+}-Activated K^+ Channels in the Coronary Artery Smooth Muscle Cells of Zucker Diabetic Fatty Rats

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The vascular large conductance Ca^{2+}-activated K^+ (BK) channel plays an important role in the regulation of vasoreactivity and vital organ perfusion in response to changes in intracellular metabolic state and Ca^{2+} homeostasis. Vascular BK channel functions are impaired in diabetes mellitus but the underlying molecular mechanisms have not been examined in detail. In this study, we examined and compared the activities and kinetics of BK channels in coronary arterial smooth muscle cells from Lean control and Zucker Diabetic Fatty (ZDF) rats using single channel recording techniques. We found that BK channels in ZDF rats have impaired Ca^{2+} sensitivity, including an increase in the free Ca^{2+} concentration at half-maximal effect on channel activation, reduced steepness of Ca^{2+} dose-dependent curve, altered Ca^{2+}-dependent gating properties with decreased maximal open probability, reduced mean open time, and prolonged mean closed time durations. In the presence of 1 μM free Ca^{2+}, voltage-dependent activation of BK channels was altered in ZDF rats with a 48 mV depolarizing shift in $V_{1/2}$ compared to Lean control. However, the equivalent charge z was not changed and in 0 μM free Ca^{2+}, there was no $V_{1/2}$ shift in ZDF BK channels, suggesting that the impaired voltage-dependent changes were secondary to Ca^{2+}-dependent changes in channel gating properties. In addition, the BK channel β subunit-mediated activation by dehydrosoyasaponin-1 (DHS-1) was lost in cells from ZDF rats. Immunoblotting analysis confirmed that there was a 2.1-fold decrease in BK channel β_1 subunit expression in ZDF rats, compared with that in Lean rats. These abnormalities in BK channel gating lead to increase in the energy barrier for channel activation, and may contribute to the development of vascular dysfunction and complications in type 2 diabetes mellitus.

2436-Pos Board B406

Regulation Of BK Channels By FK506 Binding Protein 12.6 In Vascular Smooth Muscle Cells

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Big-conductance, calcium-activated potassium (BK) channels are important for numerous physiological responses, including relaxation of vascular smooth muscle cells (SMCs). The activity of BK channels can be regulated by several signaling molecules. Here we provide biochemical evidence showing that FK506 binding protein 12.6 (FKBP12.6), an endogenous molecule known to regulate ryanodine receptors/calcium release channels, is physically associated with the BK channel α subunits in mouse cerebral arteries. Inside-out single channels recordings show that application of FK506 to remove FKBP12.6 significantly decreases the open probability of BK channels in freshly isolated mouse cerebral artery SMCs. The effect of FK506 is concentration-dependent. Similar to chemical removal of FKBP12.6 with FK506 exposure, genetic removal of FKBP12.6 with gene deletion produces an inhibitory effect on the activity of single BK channels as well. FKBP12.6 gene deletion also reduces the sensitivity of BK channels to voltage and calcium. Consistent with these results, agonist-evoked vasoconstriction is augmented in isolated arteries from FKBP2.6 gene deletion mice. Moreover, blood pressure is higher in FKBP12.6 gene deletion mice than control mice. In conclusion, our findings for the first time demonstrate that FKBP12.6 is associated with BK channels and regulates the channel functions, which may play an important role in controlling vascular tone and blood pressure.

2437-Pos Board B407

Role of ESCRT Proteins in Controlling the Lysosomal Degradation of KCa3.1 in HEK and Endothelial Cells

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In a previous study we have shown that KCa3.1 is rapidly internalized from the plasma membrane and has a short half-life in HEK293 and endothelial HMEC-1 cells (Biophys. J. 2008 94: 529). The aim of the present work was to investigate the molecular mechanisms controlling this fast degradation of KCa3.1. Using the Biotin-acceptor-KCa3.1 construct, recently engineered in our lab, the channel was fluorescently labeled at the cell surface and the cells were incubated at 37°C for different periods of time. The fate of the endocytosed channels was addressed by confocal microscopy.

After 5 h incubation at 37 °C, almost all protein was degraded, as demonstrated by a very low fluorescence level inside the cells. However, when the same treatment was applied in the presence of lysosomal proteases inhibitors leupeptin/pepstatin, we observed an accumulation of the channel inside the cells, suggesting that lysosomes are involved in KCa3.1 degradation.

Next, we addressed the possible role of the endosomal sorting complex required for transport (ESCRT) components in this process. We have investigated the role of TSG101 (a member of ESCRT-I complex) and SKD1/VPS4 (ESCRT-III). Cells were doubly transfected with Biotin-KCa3.1 and either the wild type construct or a dominant negative form of SKD1/VPS4 (E235Q) and TSG101, respectively. For $SKD1^{E235Q}$ and mutant TSG101 cells, we observed a lack of channel degradation, as compared to control cells.

These results show for the first time the role of ESCRT family proteins in targeting KCa3.1 for lysosomal degradation in HEK and HMEC-1 cells.

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2438-Pos Board B408

Biochemical Evidence of Slo1 Protein Internal Myristoylation: Involvement of a Hydroxyester Chemical Bond

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