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.
Supported by CONACyT grants 45928 (RG) and 79897 (JA).

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