

whereas the reactivity of T338C was much less sensitive to the presence of the blocker. Finally we used a homology model of CFTR to estimate the relative positions of 337 and 338 in relation to the anion-selective pore. The model predicts that, whereas 338 is positioned such that it would be expected to directly line the pore, 337 could be partially occluded by other helices. Further, a molecular dynamics simulation suggests that movement of the transmembrane helices would significantly alter occlusion of F337C and have much less effect on T338C. These results demonstrate that by combining a quantitative analysis of the reactivity of substituted cysteines with an atomic-scale CFTR model we can begin to reveal the architecture of the CFTR anion-selective pore. Supported by NIH, CF Foundation and American Lung Association.

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On the Mechanism of CFTR Inhibition by CFTRinh-172

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CFTR is inhibited with high potency and selectivity by thiazolidinone, CFTRinh-172. It was reported that CFTRinh-172 decreased the P_o by increasing the mean closed time (τ_c) without changing the mean open time (τ_o). These findings lead to the conclusion that CFTRinh-172 acts on the closed state. However, our data show that CFTRinh-172 not only increases τ_c , but also decreases τ_o by ~40% ([CFTRinh-172] = 5 μ M). For wild type (WT)-CFTR, the dose response relationship of CFTRinh-172 shows a K_i of 1.433 ± 0.235 μ M. Interestingly, G551D-CFTR, which manifests a τ_c ~100 fold longer than that of WT-CFTR, demonstrates a similar degree of inhibition as WT-CFTR. In contrast, manipulating the channel open time dramatically affects the degree of inhibition. For example, 1 μ M CFTRinh-172 causes 49% inhibition of the ATP-induced current, but the same concentration of CFTRinh-172 inhibits 82% of the current elicited by N^6 -phenylethyl-ATP, a high affinity ATP analogue that opens CFTR with a longer open time. The K_i of CFTRinh-172 for WT-CFTR locked open by ATP and PPi was estimated to be 5-10 nM. This drastic shift of the dose-response relationship is also seen with E1371S CFTR, a hydrolysis-deficient mutant. Due to impaired hydrolysis at NBD2, the current relaxation of E1371S mutant upon ATP withdrawal is very slow, 0.012 ± 0.002 s⁻¹. In the presence of 5 μ M CFTRinh-172, the rate of current relaxation of E1371S increases to 0.289 ± 0.096 s⁻¹. This result indicates that the inhibitor acts after the channel is open. The rate of the recovery from inhibition is 0.0227 ± 0.0038 s⁻¹ for WT-CFTR, but 0.0036 ± 0.0020 s⁻¹ for WT-CFTR locked open by PPi, and 0.0022 ± 0.0004 s⁻¹ for E1371S. We conclude that the open state of the channel is favorable for CFTRinh-172 action and that the longer the open state, the higher the affinity for CFTRinh-172.

2419-Pos Board B389

Functional Study of CBS Domain Interaction during Common Gating of CLC-0 Chloride Channel

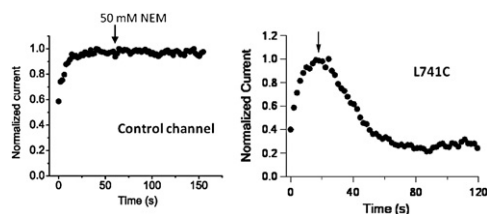
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A pair of tightly interacting cystathionine β -synthetase (CBS) domains serves important regulatory functions in various protein families. Two CBS domains (CBS1 and CBS2) exist in the C-terminal of all CLC channels and appear to mediate most of the cytoplasmic inter-subunit interactions. Previous study in our lab indicates that common gating of CLC-0 is associated with a large conformational change in the C-terminal. How interaction between CBS domains affects common gating remains elusive.

Based on three recently reported crystal structures of the C-terminal of CLC channels (CLC-0, CLC-5 and CLC-Ka), we identified a set of residues that likely contribute to CBS interaction. Mutations at most of these positions affected the gating kinetics as well as the equilibrium of common gating. Preliminary data indicate that cysteine mutations at these positions can be modified by thiol-reactive reagents. Furthermore, the kinetics of common gating changed after cysteine modification. These results indicate that CBS domains do play an important role in common gating.

The figure shows the time course of NEM modification of L741C (right); the mutation was made in the control channel (left) background that lacked native reactive cysteines.



2420-Pos Board B390

Deuterium Isotope Effects On Fast Gating Of The Chloride Channel Clc-0

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Gating of the *Torpedo* Cl⁻ channel CLC-0 is modulated by intracellular and extracellular pH, but the mechanism responsible for this regulation has remained so far elusive. Using inside-out patch clamp measurements we studied the dependence of the fast gate on pH_{int} and $[Cl^-]_{int}$. Only the closing rate, but not the opening rate showed a strong dependence on these intracellular factors. Using mutagenesis we excluded several candidate residues as mediators of the pH_{int} dependence. We propose a model in which a proton generated by the dissociation of an intrapore water molecule protonates E166 leading to channel opening. Deuterium isotope effects confirm that proton transfer is rate limiting for gate opening and that channel closure depends mostly on $[OH^-]$. The model is in natural agreement with the finding that only the closing rate constant, but not the opening rate constant, depends on pH_{int} and $[Cl^-]_{int}$.

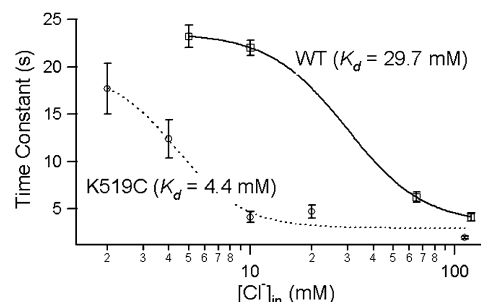
2421-Pos Board B391

R-helix Movement during Common Gating Affects Cl Binding in the CLC-0 Channel Pore

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Common (slow) gating of CLC-0 generates long silent periods in single-channel recordings and contributes significantly to regulation of Cl⁻ permeation. Our previous study suggests that movement of the pore-forming R-helix is directly coupled to common gating. We now report that R-helix movement appears to directly interfere with Cl⁻ binding in the pore. Binding of Cl⁻ to the pore facilitates common gate opening, while removing Cl⁻ slows down common gate opening. Mutations in R-helix that strongly affect common gating also appreciably shift the Cl⁻ dependence of channel open rate, apparently by altering the binding affinity of Cl⁻ in the pore. In this way, the common gating mechanism of CLC-0 is reminiscent of the fast gating, which also involves the control of chloride binding to the pore.



2422-Pos Board B392

Skeletal Muscle Chloride Channel, a Biophysical Sensor of Dystrophic Progression in Mdx Mouse, is a Potential Target of Pro-inflammatory Mediators

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A decrease in resting chloride channel conductance (gCl) characterizes myofibers of dystrophic mdx mouse in relation to both spontaneous degeneration, as in diaphragm, or exercise-induced damage as in fast-twitch EDL muscle (De Luca et al., *J. Pharmacol. Exp. Ther.* 2003). The molecular mechanism underlying gCl impairment might involve change in CLC-1 channel expression/turnover and/or function. Considering the role of inflammation in dystrophic damage, we tested if pro-inflammatory mediators may have CLC-1 channel as a target, through phosphorylating/dephosphorylating pathways. Two microelectrodes current clamp recordings were used to measure resting gCl in EDL and diaphragm muscle fibers from adult wild-type (wt) and mdx mice. In line with previous evidences, the *in vitro* application of phorbol dibutyrate (50 μ M) reduced gCl of wt EDL myofibers from 2610 ± 240 μ S/cm² (n=30) to 1265 ± 180 μ S/cm² (n=15). The application of TNF-alpha (1-30 ng/ml), a cytokine highly expressed in dystrophic muscle, to wt EDL fibers reduced gCl in a concentration-dependent manner with a maximal significant 20% decrease at 10-30 ng/ml. Angiotensin-II (10-100nM), possibly involved in muscle degeneration and oxidative stress, produced a concentration-dependent decrease of gCl in wt EDL myofibers, with a 40 % decrease at 100 nM. The PKC-inhibitor chelerythrine (1 μ M) contrasted the effect of either phorbol ester, TNF-alpha or angiotensin-II. The application of 3.3 nM IGF-1 to diaphragm and EDL muscle fibers from exercised mdx mice, significantly counteracted the 40% impairment of gCl. Okadaic acid (0.25 μ M) fully prevented

IGF-1 effect, supporting the involvement of a serine-threonine phosphatase in IGF-1 activity (De Luca et al., *Br. J. Pharmacol.* 1998). Angiotensin receptor antagonists and other tools are in use to gain further insight in the mechanisms involved in inflammation-sensitive CIC-1 impairment in muscular dystrophy (Telethon-Italy GGP05130).

2423-Pos Board B393

Evaluation of the Effects of Statin and Fibrate Treatment on Rat Skeletal Muscle: Biophysics, Genetic and Proteomic Studies

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Resting chloride conductance (gCl) sustained by the CIC-1 chloride channel have a crucial role in muscle physiology by maintaining the sarcolemma electrical stability. Its reduction can produce myotonia-like symptoms. CIC-1 channel has been shown to be a target of therapeutic molecules such as statins and fibrates. We previously demonstrated that lipophilic statins and fibrates affect skeletal muscle function by reducing resting chloride conductance (gCl) (Pierno et al, *Br J Pharmacol* 149:909, 2006). Here we studied the time course changes of fluvastatin (20 mg/kg) and fenofibrate (60 mg/kg) effects on Extensor Digitorum Longus (EDL) muscle gCl measured by two-microelectrode current clamp method. The gCl decreased in a time-dependent manner, being significantly lower after 1 week ($2464 \pm 66 \mu\text{S}/\text{cm}^2$, $n=46$ and $2510 \pm 53 \mu\text{S}/\text{cm}^2$, $n=50$ in fluvastatin and fenofibrate treated rats, with respect to $2706 \pm 83 \mu\text{S}/\text{cm}^2$, $n=27$ of control). To investigate the causes of gCl reduction we analyzed the CIC-1 gene expression by real-time quantitative PCR. The results showed a marked decrease in CIC-1 mRNA expression in both fluvastatin and fenofibrate chronically treated animals which contributes to gCl reduction. To study the involvement of other proteins essential for muscle function we analysed the proteomic map of EDL muscle from rat treated with fluvastatin (20mg/kg), atorvastatin (10mg/kg), fenofibrate (60mg/kg) and with combined fluvastatin (5mg/kg) plus fenofibrate (30mg/kg) by two-dimensional gel electrophoresis (Gelfi et al, *J Proteome Res* 5:1344, 2006). Fluorescent stained proteomic map showing ~500 spots were obtained and 40, 74, 60 and 76 differently expressed proteins were found in the above mentioned treated groups, with respect to control. The identification of each spot will allow to identify the protein targets of the myopathic process.

2424-Pos Board B394

Anomalous Mole Fraction Effect in CIC-2 Chloride Channel Pore

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The Cl^- pathway of *E. Coli* $\text{ClC H}^+/\text{Cl}^-$ exchanger has three binding sites that can be occupied by Cl^- , however SeCN^- (an anion similar to SCN^-) occupies only one site. This pathway serves as a model for pore structure of CIC channels whose gating is coupled to permeation. Thus CIC channels may gate differently in the presence of SCN^- . We analyzed the relationship between gating and pore properties by performing SCN^- mole-fraction experiments in CIC-2 channels expressed in HEK cells. Internal and external solutions with different SCN^- fractions were prepared mixing $[\text{Cl}^-] + [\text{SCN}^-] = 140 \text{ mM}$. Replacing 100% Cl^- with SCN^- on both sides of the membrane shifted to the left the voltage (V_m) vs channel P_0 curve by $\sim 18 \text{ mV}$ without changing the slope. In contrast, the protopore gate P_p vs V_m curve was shallower and shifted rightward ($+5 \text{ mV}$). Extracellular SCN^- mole-fractions produced negative shifts on reversal potential (E_R) values which are not described by GHK equation with a constant $P_{\text{SCN}}/P_{\text{Cl}}$ ratio. The $P_{\text{SCN}}/P_{\text{Cl}}$ ratios increased from 1.4 to 2.5 when the mole fraction increased, suggesting extracellular SCN^- enters the pore better than Cl^- . In addition, the slope of P_0 vs V_m curve was steeper and the mid-point voltage ($V_{0.5}$) did not change. Intracellular SCN^- mole fractions produced nonlinear negative shifts on E_R suggesting that from this side Cl^- enters the pore better than SCN^- . The slope of the P_0 vs V_m curve was shallower and $V_{0.5}$ and slope conductance vs mole fraction displayed the classical anomalous behaviour. Interestingly, the protopore gate located in the Cl^- pathway also displays the same behaviour. Our data show that the CIC-2 channel has indeed a multi-ion pore and that SCN^- enters this pore preferably from the extracellular side.

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2425-Pos Board B395

Pharmacological Characterization of GaTx2, a Peptide Inhibitor of CIC-2 Chloride Channels

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CIC-2 chloride channels are voltage-gated ion channels that are expressed in neuronal and epithelial cells where they are critical mediators for the passive diffusion of Cl^- across the plasma membrane. Although CIC-2 is nearly ubiquitously expressed in mammalian cells, many details regarding channel biophysics and the physiological role that CIC-2 channels play remain undefined. We recently isolated the first peptide toxin active against CIC channels: Georgia anion toxin 2 (GaTx2), a 3.2 kDa peptide composed of 29 residues with three disulfide bonds. Here, we describe the basic pharmacological features of the inhibitory activity of GaTx2 against CIC-2, including affinity, mechanism of inhibition, and specificity. Using two-electrode voltage-clamp, we created a dose-response curve for inhibition of CIC-2 by GaTx2 at $V_m = -100 \text{ mV}$, and calculated a K_D of $22 \pm 10 \text{ pM}$. This value was very similar to the value obtained from dose-response curves created from multi-channel patches, which gave a K_D of $12 \pm 5 \text{ pM}$. Additionally, from TEVC recordings we measured $k_{\text{on}} = 43 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, and $k_{\text{off}} = 0.0034 \text{ s}^{-1}$, which is consistent with rate constants for other peptide inhibitors. Single channel recording showed that the latency to first opening is increased nearly 8 fold in the presence of 20 pM GaTx2. Also, outside-out macropatches revealed that GaTx2 is unable to inhibit open CIC-2 channels; thus, this toxin may act as a gating modifier. Finally, we found that GaTx2 is specific for CIC-2, being unable to inhibit other CIC channels or transporters, other major classes of Cl^- channels, or voltage-dependent K^+ channels. This high affinity, highly specific inhibitor of CIC-2 will provide an excellent tool for studies designed to understand the function and regulation of this channel, and will help define its physiological role(s).

2426-Pos Board B396

Slow Gating in CIC Chloride Channels: Normal Mode Analysis

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All-atom NMA is used to explore possible mechanisms for slow gating in CIC Cl^- channels. As the "double-barreled" architecture is well established throughout the CIC family, both channels and transporters (Miller, 2006. *Nature*. 440:484), we use the high-resolution (2.5 Å) X-ray structure of an *E. coli* CIC transporter (pdb entry 1OTS) as a template, describe it with the CHARMM22 force field and carry out standard all-mode NMA. The slowest, intrinsic motions encoded in the structure are determined by protein shape. Perturbing the system in either direction along the 7th all-atom NM leads to slow relative swinging of the subunits, perpendicular to the membrane plane. The in-plane swivel axis lies at the subunit interface, near the protein's center. The intracellular interfacial domain is the region most affected. Here the two halves of the protein oscillate, separating and then nearly touching. The R and A helices execute large scale swaying, alternately increasing and decreasing their cytoplasmic ends' separations, motion in agreement with FRET experiments (Bykova et al., 2006. *Nat. Struct. Biol.* 13:1115). The ion-occupied intracellular pores behave as almost rigid units. As the subunits separate, the intracellular pore tilt relative to the membrane plane changes notably. In contrast, the extracellular portion of the subunit interface is significantly less affected, although small interfacial structural changes are clearly observable. Those extracellular regions structurally affected by the subunits' slow sway are localized near the extracellular Cl^- pathways. As the subunits separate, these regions compress, possibly shutting the extracellular pores. As they approach, the extracellular regions near the Cl^- conduction pathways relax, possibly opening them.

2427-Pos Board B397

Ab Initio Calculations Of Structural Rearrangements and Energetic of Glutamate¹⁴⁸ Site Chain of the Ec-CIC H⁺/Cl⁻ Exchanger

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$\text{ClC H}^+/\text{Cl}^-$ exchangers are homodimers with three Cl^- -binding sites within each monomer. Previous studies suggest that transport cycles are triggered by protonation of the lateral chain of a glutamate residue (E148) located in the middle of the Cl^- pathway. In this work, we performed *ab initio* density functional theory calculations using the ultra soft pseudopotential approach in order to gain insight about microscopic movements induced by H and/or Cl during transport activity. For these computations the 16 amino-acids that line the Cl^- pathway were spatially aligned according to the X-ray structure. We found that the orientation of the unprotonated E148 carboxy group was influenced when Cl was in close proximity. Attaching a single H to this COO^- group displaced the lateral chain (with respect to the unprotonated structure) towards the extracellular side and led to the formation of a sizable hole in the entryway. When Cl was placed at 2 Å from the protonated COO^- group (COOH) (in both intra- and extra-cellular positions) the conformational