1662-Pos
Alteration of Extracellular pH Dependence of CLC-5 By Modifications of Two Conserved Charged Residues
Giovanni Zifarelli, **Michael Pusch**.
Istituto di Biofisica, Genoa, Italy.
The endosomal Cl(-) / H(+) antiporter, ClC-5, is inhibited by low extracellular pH. This could be caused by the saturation of a titratable residue which is directly involved in proton translocation towards the extracellular solution; alternatively, the reduction of currents at low pH could reflect an unspecific electrostatic effect of some titratable residue on proton and / or chloride movement within the external vestibule. To gain insight into the mechanism of the pH dependence we investigated the possible involvement of two highly conserved charged residues (D76 and K210) which are located in the extracellular vestibule. Mutants D76G and D76H drastically altered pH dependence. Preliminary experiments indicated that also K210C slightly altered the pH dependence. Interestingly, the K210C mutant could be modified by pCMBS (p-chloromercuriphenylsulfonic acid): application of 100 μM pCMBS led to a partial and irreversible inhibition of K210C mediated currents (but not WT ClC-5 currents). Our results favor the idea that neither D76 nor K210 are directly involved in proton translocation. However, further experiments, exploiting the possibility to modify K210C with various cysteine modifying reagents, are necessary to draw such a firm conclusion.

1663-Pos
Role of the Q-R Linker in H^+/Cl^- Coupling in a CLC Exchanger
Christopher Hills, **Sophie Bruce**, Alessio Accardi.
University of Iowa, Iowa City, IA, USA.
Members of the CLC family function as Cl^- channels or as H^+/Cl^- exchangers. Despite this mechanistic divide the two CLC sub-classes share many functional and structural traits. CLC-ec1, a transporter, can be transformed into a Cl^--selective pore by simultaneously removing the intra- and extra-cellular gates. This entailed mutating two residues, E148 and Y445, which are conserved in both CLC channels and transporters. While the extracellular gate regulates channel gating, the intracellular one does not. We hypothesized that in the channels the intracellular gate's position is shifted so that it does not regulate ion passage anymore.
The linker connecting helices R and Q is 1-2 amino-acids shorter in the channels than in the transporters. We investigated whether shortening this linker disrupts the intracellular gate of CLC-ec1 while preserving Y445. To this end we deleted two residues, G441 and K442, in the Q-R linker. This deletion, ΔGK, functionally mirrors the gate-removed Y445A mutant: H^+ transport is nearly abolished with slight effects on Cl^- transport. We then tested the effects of single-residue deletions. The ΔG mutant is similar to the WT while the ΔK deletion impairs H^+ transport like the ΔGK deletion. K442 however is not involved in H^+ transport: Alanine or Methionine substitutions have no significant effects.
Lastly, we incorporated in CLC-ec1 increasing portions of the CLC-0 channel. Introduction of the CLC-0 Q-R linker into CLC-ec1 recapitulates the functional effects of the ΔGK and ΔK mutations. Introduction into CLC-ec1 of up to the full helix R from CLC-0 led to no further effects on H^+ transport.
In conclusion we found that the length of the Q-R linker is critical for H^+/Cl^- coupling in CLC-ec1 while the chemical properties of the residues in the linker do not seem to matter.

1664-Pos
Proton Block of Human CLC-5 Cl^-/H^+ Exchanger
Alessandra Picollo, Mattia Malvezzi, Alessio Accardi.
University of Iowa, Iowa City, IA, USA.
CLC-5 is a human Cl^-/H^+ exchanger of the CLC family. It is expressed in intracellular compartments such as endosomes, where it regulates their acidification and is indirectly involved in the re-uptake of small molecular weight proteins in renal proximal tubule. Mutations in the CLC-5 gene lead to Dent's disease, a human disorder characterized by low molecular-mass proteinuria, kidney stones and renal failure. CLC-5 currents are strongly outwardly rectifying suggesting that the rates in the transport cycle are highly asymmetrical so that Cl^- influx and H^+ efflux is permitted while the converse is not. Additionally CLC-5 currents are strongly inhibited by low external pH. It has been proposed that the rectification arises from the voltage dependence of a H^+ transport step and that the pH dependence is a result of a reduction in the total driving force for turnover. We studied the extracellular pH dependence of CLC-5 currents between pH 9 and 4.5. We found that the pH dependence is not solely due to changes in the driving force. Rather, we found that the degree of current inhibition is voltage dependent: at less positive potentials H^+ inhibition becomes stronger. This is consistent with a direct modulation of CLC-5 activity by H^+ at a single site located ~70% deep into the electric field. We propose that this site is E211, the extracellular gating glutamate. In the presence of extracellular SCN^- H^+ transport is abolished but both rectification and H^+ inhibition remain largely unaltered. This suggests that H+ transport determines neither the pH dependence nor rectification. We propose that the extracellular pH inhibition of CLC-5 currents arises from H^+ trying to drive the transport cycle in the non-permissive direction. In other words, inhibition of the CLC-5 currents by extracellular H+ is a consequence of its rectification.

1665-Pos
A Monomeric Variant of the CLC-ec1 Transporter
Janice L. Robertson, Ludmila Kolmakova-Partensky, Christopher Miller.
HHMI/Brandeis University, Waltham, MA, USA.
CLC-ec1, a prototypical Cl-/H+ exchanger of the CLC family, is a homodimer in which each subunit is thought to act as a fully functional transporter. Why then is this protein a dimer and what are the factors driving dimer formation? In this study, we address these questions by destabilizing the CLC-ec1 subunit-subunit interface. We substituted tryptophan residues to create steric mismatches at the interface that would also favor lipid interactions. A single mutation, I422W, was sufficient to destabilize the CLC-ec1 complex resulting in a shift from dimer to monomer position on gel-filtration chromatography. Glutaraldehyde cross-linking of I422W in both detergent and phosphatidyl-choline/phosphatidyl-glycerol liposomes showed that the mutant remained a monomer compared to the wild-type protein; however dimer formation was observable at high concentrations. The I422W mutant was functional as measured by 36Cl- uptake, passive Cl- efflux and Cl- driven H+ pumping in E. coli phospholipids. A Poisson-counting method demonstrated that I422W re-dimerizes in these phosphatidyl-ethanolamine-rich liposomes, even at very low protein/lipid ratio. Altogether, these findings suggest that we have purified an isolated monomeric variant of CLC-ec1 that demonstrates shifted dimerization energetics depending on the lipid environment. We continue to search for mutations that will more severely destabilize the dimer, in attempts to obtain a transport-competent, monomeric CLC exchanger.

1666-Pos
Analysis of the CLC-1 ATP Binding Site by Structure-Guided Mutagenesis
Pang-Yen Tseng, Tsung-Yu Chen.
Center for Neuroscience, University of California, Davis, Davis, CA, USA.
The CLC-1 chloride channel is abundantly expressed on the cell membrane of skeletal muscles, and thus plays an important role in controlling the muscle excitability. We previously showed that ATP can modulate the common gating of CLC-1, presumably by binding to a potential binding site at the C-terminal cytoplasmic region of the channel. The X-ray crystallographic study by others revealed the structure of the ATP-binding site, and two residues, Y617 and D727, were identified to be important for the ATP binding. Based on the CLC-5 C-terminal structure, we identify that besides V634 and E865 of CLC-1 (corresponding to Y617 and D727 of CLC-5), V613 and E860 are also important for the ATP modulation of the CLC-1 common gating. Mutating V634 to either alanine or aspartate abolishes the ATP modulation effect. Surprisingly, mutations of V634 to aromatic amino acids increase the ATP affinity: V634F and V634Y mutations decrease the apparent K1/2 by ~30- and ~13-fold, respectively. Mutations of E865 to alanine and serine abolish the ATP modulation, while a charge-conserved mutation, E865D, greatly decreases the efficacy of the ATP effect_the maximal shift of the V1/2 of the common gate P-V curve is ~10-20 % of that in the WT channel. Mutations of V860 and V613 to various amino acids also greatly reduce the efficacy of the ATP modulation. Double mutant cycle analyses show that V634 and V613 are strongly energetically coupled, with a coupling coefficient (Ω) of ~25, while V634 and E865 may be only weakly coupled with a Ω value of ~1.6. These results support the idea that ATP likely binds directly to the C-terminus of CLC-1 to modulate the common gating of the channel.

1667-Pos
Substrate-Driven Conformational Changes in CLC-ec1 Observed by Fluorine NMR
Shelley Elvington, Corey Liu, Merritt Maduke.
Stanford University, Stanford, CA, USA.
The CLC "Cl^- channel" family consists of both Cl^-/H^+ antiporters and Cl^- channels. This scenario presents a unique opportunity to investigate the molecular similarities and differences underlying these mechanisms. Although gating in CLC channels is known to involve large, cooperative conformational changes between protein subunits, it has been hypothesized that conformational changes in the antiporters may be confined to small movements localized near the Cl^- permeation pathway. However, to date few studies have directly addressed this issue, and therefore little is known about the molecular movements that underlie CLC-mediated antiport. The crystal structure of the *E. coli* antiporter ClC-ec1 provides an invaluable molecular framework for such studies,

but this static picture alone cannot depict the protein movements that must occur during ion transport. To address this issue, we have utilized fluorine NMR to monitor substrate-induced conformational changes in ClC-ec1. We show that substrate-driven conformational change is not constrained to the Cl^- permeation pathway alone, and that the ClC-ec1 subunit interface participates in protein movement. Furthermore, removal of the protein's H^+ transport ability does not eliminate the H^+-dependent protein movement observed in the intact antiporter. Finally, we observe that a ClC-ec1 ''channel-like'' mutant is not subject to the same substrate-dependent conformational changes that occur in the ClC-ec1 transporters. Together, these results provide new insight into conformational change in ClC-ec1 and lay an essential foundation for future studies on ClC-ec1 protein dynamics.

1668-Pos

Cardioprotective Role For the CLC-3 Chloride Channel in the *mdx* Mouse Model of Duchenne Muscular Dystrophy

Nathanael Heyman, Ryan Wuebbles, Linda Ye, Dayue Duan, Joe Hume, Dean Burkin.
University of Nevada, Reno, NV, USA.

Duchenne Muscular Dystrophy (DMD) is the most common human X-linked disease affecting 1/3,500 male births. DMD patients suffer from severe, progressive muscle wasting with clinical symptoms first detected between 2 to 5 years of age. As the disease progresses patients are confined to a wheelchair in their early teens and die in their early twenties from cardiopulmonary failure. There is currently no effective treatment or cure for DMD. DMD patients and *mdx* mice (the mouse model for DMD) have mutations in the dystrophin gene that result in an absence of the dystrophin protein. Dystrophin is a 427 kDa protein located under the sarcolemma on the inner cytoplasmic membrane of skeletal and cardiac muscle cells and provides structural and functional integrity to muscle. Although therapies have been developed that target skeletal muscle disease, increasing evidence suggests correcting the cardiomyopathy is critical to the survival of DMD patients. As the cardiomyopathy progresses, the hearts of DMD patients and *mdx* mice exhibit arrhythmias, conduction abnormalities and left ventricular dilation. Recent studies have shown that the chloride ion channel ClC-3, a candidate protein responsible for volume-regulated chloride channels in heart, plays a critical cardioprotective role against the development of hypertrophy and failure (*J Mol. Cell. Cardiol.* 2009 Jul 15. [Epub ahead of print]).Using transgenic mice including heart specific ClC-3 knockout *mdx* mice, echocardiography and electrophysiology, we analyzed the role of ClC-3 in modulating cardiac disease progression in dystrophic mice. Our preliminary results indicate ClC-3 may be a major modifier of cardiac disease progression in *mdx* mice and targeting ClC-3 expression or function may provide a novel therapeutic approach for the treatment of dilated cardiomyopathy in DMD (supported by 3P20RR015581 from the National Center for Research Resources).

1669-Pos

Blocking Kinetics of CFTR Channel by Aromatic Carboxylic Acid Positional Isomers Characterised using a Novel Amplitude Distribution Analysis Method

Ying-Chun Yu[1], **Yoshiro Sohma[1,2]**, Yumi Nakamura[1], Tomoka Furukawa[1,3], Yohei Matsuzaki[1,4], Seiko Kawano[5], Tzyh-Chang Hwang[2], Masato Yasui[1].
[1]Dept. of Pharmacology, Keio University School of Medicine, Tokyo, Japan, [2]Dalton Cardiovascular Research Center, University of Missouri-Columbia, Columbia, MO, USA, [3]Dept. of Biomolecular Engineering, Tokyo Institute of Technology, Tokyo, Japan, [4]Dept. of Pediatrics, Keio University School of Medicine, Tokyo, Japan, [5]Dept. of Health and Nutrition Sciences, Komazawa Women's University Faculty of Human Health, Tokyo, Japan.

To investigate the pore structure of the cystic fibrosis transmembrane conductance regulator (CFTR) channel, we performed a systematic pore probing on CFTR channel pore with a series of small aromatic carboxylic acids, including their positional isomers, e.g., 9-anthracene carboxylic acid (9-AC) and 1-anthracene carboxylic acid (1-AC).

Small compounds presumably interacting the channel protein with a few points are sensitive to structural changes of the binding site. However such low affinity blockers show fast - intermediate blocking kinetics which give us the overall affinity, but not on- and off- rates separately. To overcome this problem, we developed an iterative simulation method to estimate the on- and off- rate constants in the 9-AC or 1-AC block from the single channel amplitude distribution.

The newly developed Amplitude Distribution Analysis (ADA) program first generated a single-channel current according to the given kinetic scheme and added a Gaussian noise to the currents for mimicking the background noise. The simulated currents were low-pass filtered and digitized at the same frequencies as those in the experiments and binned into an amplitude histogram. Then the program repeats a direct likelihood comparison between the simulated and experimental current amplitude distributions to find the best fitted values for the blocking kinetic parameters.

The ADA program showed that the off-rate of 1-AC block is 3-fold slower than that of 9-AC and the on-rate of 1-AC is ~3-fold faster than that of 9-AC. The voltage-dependences of on- and off- rates of 1-AC are similar to those of 9-AC, respectively. These suggest that 1-AC and 9-AC block CFTR channel by binding to a common binding site which should be modeled by a combination of a positive charge tightly surrounded by hydrophobic residues.

1670-Pos

Effects of Aromatic Carboxylic Acids on Genistein- and Curcumin- Potentiated G551D-CFTR

Yumi Nakamura[1], Akiko Hanyuda[1], Ying-Chun Yu[1], Tomoka Furukawa[1,2], Yohei Matsuzaki[1,3], Yoichiro Abe[1], Silvia Bompadore[4], Tzyh-Chang Hwang[4], Masato Yasui[1], Yoshiro Sohma[1,4].
[1]Dept. of Pharmacology, Keio University School of Medicine, Tokyo, Japan, [2]Department of Biomolecular Engineering, Tokyo Institute of Technology, Tokyo, Japan, [3]Dept. of Pediatrics, Keio University School of Medicine, Tokyo, Japan, [4]Dalton Cardiovascular Research Center, University of Missouri-Columbia, Columbia, MO, USA.

The Cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel plays an important role in salt and water transport across epithelia and defective function due to mutations in the CFTR gene cause cystic fibrosis (CF). The glycine-to-aspartate missense mutation at position 551 (G551D) is the third most common CF-associated mutation and G551D-CFTR is characterized by a very low open probability despite of its normal trafficking to the plasma membrane.

Numerous small molecules have been shown to increase the activity of G551D-CFTR presumably by binding to the CFTR protein. Among the many G551D-CFTR potentiators, a bioflavonoid found in legumes, genistein is perhaps the most extensively studied. More recently it was reported that a component of the spice turmeric, curcumin strongly activated G551D-CFTR. However, The mechanism through which these compounds increase G551D-CFTR activity is still unclear. On the other hand, we have previously reported that anthracene-9-carboxylate (9-AC) showed an inhibitory effect and an potentiation effect on CFTR channels by binding to two chemically distinct sites for each effect (Ai *et al,* 2004).

In this study, we made a functional probing on genistein-potentiated G551D-CFTR and curcumin-potentiated G551D-CFTR using 9-AC and its positional isomer, anthracene-1-carboxylate (1-AC). In wild type- (WT-) CFTR, 9-AC induced a large voltage-independent enhancement and a voltage-dependent inhibition in the whole-cell (WC) current. 1-AC induced a smaller enhancement and a larger voltage-dependent block in WT-CFTR WC currents in compared with 9-AC. In the other hand, both 9-AC and 1-AC induced only a weak voltage-dependent inhibitions in genistein-potentiated G551D-CFTR WC currents whereas, in curcumin-potentiated G551D-CFTR WC currents, 9-AC and 1-AC induced similar effects to those in WT-CFTR. These suggest that curcumin and genistein potentiated G551D-CFTR via different mechanisms. We speculate that genistein-potentiated and curcumin-potentiated G551D-CFTRs might have different protein conformations.

1671-Pos

Optimization of the NBD1 Site Improves the Function of G551D-CFTR Channels

Ming-Feng Tsai, Kangyang Jih, Min Li, Tzyh-Chang Hwang.
Missouri University, Columbia, MO, USA.

CFTR chloride channels comprise two nucleotide binding domains (NBD1 and NBD2). Mutations (for example, G551D) that impair CFTR function result in the lethal genetic disease cystic fibrosis (CF). It's established that the opening and closing of CFTR are mainly controlled by ATP binding and hydrolysis respectively in NBD2 while ATP binding at NBD1 can modulate the stability of the open state. Using a non-hydrolytic ligand MgPPi, we locked open CFTR channels with mutations at NBD1. We found that two W401 mutations significantly increased the lock-open time (W401F: 72 ± 3s; W401Y: 51 ± 6s; WT: 27 ± 2s). These gain-of-function mutations are unexpected since the W401-equivalent residue Y1219 in NBD2 can be effectively replaced by tryptophan but not phenylalanine. As the ATP molecule bound in NBD1 may interact with NBD2's signature sequence (LSHGH), we extended our study to this region. We found that reverting the histidine residue 1348 to the canonical glycine (H1348G) similarly stabilized the lock-open state ($\tau = 67 \pm 8$s). Once W401F, H1348G, or W401F/H1348G mutations were incorporated into G551D channels which no longer respond to ATP, the mutant channels become ATP-responsive with basal activity increased by ~10-fold for W401F/G551D-, ~4-fold for H1348G/5G551D-, and ~25-fold for W401F/H1348G/G551D-channels. The increased