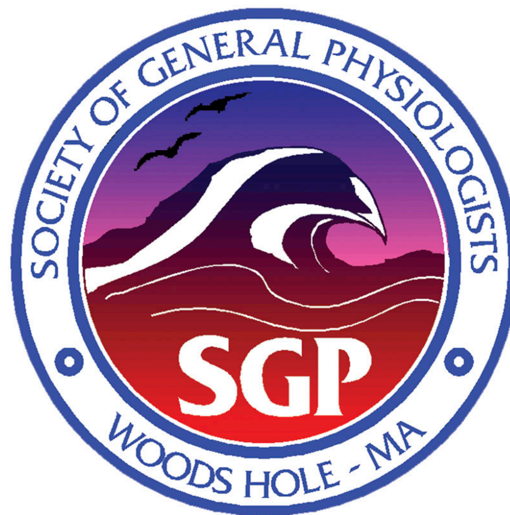
ABSTRACTS OF PAPERS AT THE SIXTY-SEVENTH

ANNUAL MEETING OF THE SOCIETY

OF GENERAL PHYSIOLOGISTS

The Enigmatic Chloride Ion:

Transport, Regulation, and Roles in Physiology



Marine Biological Laboratory

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1. Molecular Identification of the G_o-coupled Photopigment of Ciliary Photoreceptors of *PECTEN*. OSCAR ARENAS,[1] MARÍA DEL PILAR GOMEZ,[1,3] and ENRICO NASI,[2,3] [1]*Departamento de Biología and* [2]*Instituto de Genética, Universidad Nacional de Colombia, Bogotá;* [3]*Marine Biological Laboratory, Woods Hole, MA*

The two canonical animal photoreceptor classes, ciliary and microvillar, use fundamentally different light-transduction schemes: their photopigments couple to G_t versus G_q proteins, respectively, to either mobilize cyclic nucleotides versus triggering a lipid signaling cascade. A third lineage of photoreceptors has been described, which exhibit a ciliary morphology but operate via mechanisms that diverge radically from those of rods and cones: photostimulation recruits a G_o, ultimately triggering an increase in a cGMP-dependent, K-selective conductance. Little is known about the photopigments implicated. As a result of molecular searches, a group of putative "G_o-coupled opsins" has recently emerged in diverse phyla, including mollusks, echinoderms, and chordates; however, the cells in which these photopigment candidates express have not been identified, and functional properties remain unknown. Conversely, only in one invertebrate species, *Pecten irradians*, has the light response of ciliary photoreceptors been physiologically characterized, but the photopigment had not been identified molecularly. We used the transcriptome of *Pecten* retina to guide the cloning by PCR and RACE extensions of a G_o-coupled opsin orthologue. In situ hybridization shows selective transcription in the distal retina. Antibodies against a peptide from the predicted protein identify in Western blots a single band of the expected molecular mass, and distinctly label ciliary photoreceptors in retina sections. The coding sequence of this transcript has been inserted in a fusion construct with GFP or with monomeric RFP into plasmid vectors, and heterologous expression has been induced in HEK, N2A, and CL-4 cells. This paves the way for a detailed characterization of a new class of light-sensing molecules and their signaling mechanisms.

2. Dynamics Outside the Ion Pathway Are Required for Transport in a CLC-type Cl^-/H^+ Exchanger. DANIEL BASILIO,[1] KRISTIN NOACK,[1] ALESSANDRA PICOLLO,[1] and ALESSIO ACCARDI,[1,2,3] [1]*Department of Anesthesiology,* [2]*Department of Physiology and Biophysics, and* [3]*Department of Biochemistry, Weill Medical College of Cornell University, New York, NY 10065*

The CLC proteins catalyze transport of chloride ions (Cl^-) through cellular membranes ubiquitously. Although some CLCs are ion channels, others are H^+-coupled secondary active transporters. The exchange mechanism of the CLCs is unclear. All proposed models postulate that the only conformational changes taking place during transport are the movements of a conserved glutamate's side chain in and out of the Cl^- permeation pathway. This hypothesis is supported by structural and functional work. However, others have suggested that regions distal to the Cl^- pathway might also be involved in transport. To test whether transport entails only local or also global rearrangements, we constrained the movement of helices J, O, and Q, which do not line the Cl^- or H^+ pathways in CLC-ec1, a CLC prokaryotic homologue. If exchange involves the relative movement of these helices, then these constraints should reduce the transport rate. In a cys-less background, we introduced pairs of cysteines at different locations in this four-helix bundle and Hg^{2+}-cross-linked them. All unreacted proteins mediate Cl^-/H^+ exchange at rates comparable to that of the WT. Reaction with Hg^{2+} results in a striking pattern: constraining residues facing the extracellular side has no effect, whereas targeting residues deeper into the protein induces progressively a more drastic reduction of activity. Finally, constraints placed close to the intracellular side results in greatly diminished transporters. This reduction is not caused by cross-linked–induced distortion of the Cl^- pathway because: (a) the Cl^--binding affinity is largely preserved measured by isothermal calorimetry, (b) the Cl^- transport through a cross-linked doubly ungated mutant is not affected, and (c) the crystal structure of the Cl^- binding site of the cross-linked protein preserved its native structure. Here, we propose that the transport cycle in CLC-ec1 entails the movement of these helices outside of the Cl^- transport pathway.

3. Activation of ClC-2 Chloride Channel by Permeant Anions. ALEJANDRA CASTRO-CHONG,[1] CARMEN Y. HERNÁNDEZ-CARBALLO,[1] JOSÉ J. DE JESÚS-PÉREZ,[1] RU-CHI SHIEH,[3] PATRICIA PÉREZ-CORNEJO,[2] JOSÉ A. DE SANTIAGO-CASTILLO,[4] and JORGE ARREOLA,[1] [1]*Instituto de Física and* [2]*Department of Physiology and Biophysics, School of Medicine, Universidad Autónoma de San Luis Potosí, San Luis Potosí, SLP 78290, México;* [3]*Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, R.O.C.;* [4]*Instituto de Física y Matemáticas, Universidad Michoacana de San Nicolás de Hidalgo, Cd. Universitaria, Morelia, Michoacán, Mich. 58040, México*

Coupling anion permeation to the gating process seems to stem the voltage dependence of the widely distributed ClC-2 chloride (Cl^-) channel. We previously proposed that voltage-dependent gating comes from a voltage-dependent occupancy of the channel pore by intracellular permeant anion, and that extracellular H^+ stabilizes the open conformation (Sanchez-Rodriguez et al. 2012. *J. Physiol.* 590:4239–4253). If so, then ClC-2 would be open by high intracellular chloride concentration ($[Cl^-]_i$) at low extracellular $[H^+]$ that precludes protonation of the fast gate, and, permeant anions would induce opening of ClC-2. To test this idea, we recorded rat ClC-2 currents from inside-out patches from *Xenopus laevis* oocytes. Currents were absent in a

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low $[Cl^-]_i$; however, as $[Cl^-]_i$ was increased, a slowly activating current was recorded at negative voltages. The IV relation showed that strong inward rectification and macroscopic conductance increased in a sigmoideal fashion as the cytosolic $[Cl^-]_i$ was increased, while $[H^+]_O = 10^{-10}$ M. Similarly, mouse ClC-2 was activated by hyperpolarizations in HEK cells dialyzed with 25% SCN^- + 75% Cl^-, while $[H^+]_O = [H^+]_i = 10^{-10}$ M. To test for activation by permeant anions, we first determined the selectivity sequence from the permeability ratios. When foreign anions were applied from outside, the sequence was $SCN^- > Cl^- > Br^- > Ace \geq I^- > F^-$, in contrast to when applied from inside the sequence: $SCN^- > Br^- \geq Cl^- > I^- \geq F^- > Glu \geq Ace$. Although Ace, Glu, and F^- did not pass ClC-2 pore, voltage-dependent gating of mouse ClC-2 was observed with all anions when applied from the extracellular side. However, when applied from the intracellular side, only permeant anions SCN^-, Cl^-, Br^-, and I^- allowed gating. These data support the hypothesis that pore occupancy by permeant anions is responsible for voltage-dependent gating in ClC-2.

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4. A Presynaptic Chloride Conductance Activated by Serotonin Autoreceptors Mediates Autoinhibition in Serotonergic Neurons. MONTSERRAT G. CERCÓS,[1] FRANCISCO F. DE-MIGUEL,[2] and CITLALI TRUETA[1], *[1]Instituto Nacional de Psiquiatría Ramón de la Fuente and [2]Instituto de Fisiología Celular, Universidad Nacional Autónoma de México*

We studied how the activation of presynaptic chloride channels by serotonergic autoreceptors autoinhibits excitability upon serotonin release. Identified leech Retzius neurons, cultured either singly or forming synapses onto pressure-sensitive neurons, allow accurate recordings of synaptic events using intracellular microelectrodes. Action potentials were followed by a slow hyperpolarization with a 85.4 ± 5.2–ms rise time and a 252 ± 17.4–ms half-decay time. These inhibitory postpotentials were produced by chloride entry, as they were inverted by inverting the transmembrane chloride gradient when filling the microelectrodes with potassium chloride. Inhibitory postpotentials were mimicked by iontophoretic application of serotonin; they were reversibly abolished in the absence of extracellular calcium to block serotonin release and absent in neurons incubated with reserpine (1 µM) for 7 days to deplete serotonin. This suggested that autoinhibition was produced by released serotonin activating chloride channel–coupled autoreceptors. Serotonin release during stimulation trains at 10- or 30-Hz induced failures in 23 ± 6% or 47 ± 2% of the action potentials, respectively. These failures were reversibly abolished by the serotonergic antagonist methysergide (140 µM). Reserpine-treated neurons had only 5 ± 4% of failures during trains at 10 Hz, and failures increased to 35 ± 4% by iontophoretic application of serotonin. In neuron pairs forming synapses, the action potential failures correlated with reductions in postsynaptic currents. Model simulations predicted that the autoinhibitory chloride conductance reduces the amplitude of action potentials arriving at the presynaptic endings. Our results suggest that activation of presynaptic chloride channel–coupled autoreceptors by released serotonin autoinhibits subsequent serotonin release in an activity-dependent manner.

5. A Novel Role for Chloride Channels in Ciliogenesis and Cell Polarization: The Ano1 Nimbus. CHELSEY CHANDLER RUPPERSBURG, YUANYUAN CUI, and H. CRISS HARTZELL, *Department of Cell Biology, Emory University School of Medicine, Atlanta, GA 30322*

Although Cl^- channels play an important role in vectorial transepithelial transport, whether they function in establishing epithelial cell polarity is unknown. We provide evidence that the Cl^- channel Anoctamin 1 (Ano1) may organize functional membrane microdomains important in cell polarization and ciliogenesis through an apically localized ring structure we call the Ano1 Nimbus. Ano1, also called TMEM16A, is an anion-selective ion channel that is gated open by increases in intracellular Ca^{2+} to allow apical Cl^- efflux that drives epithelial fluid secretion. Ano1 also participates in development and cancer cell metastasis. Ano1 was identified in a screen for genes expressed in the zone of polarizing activity of the embryonic mouse limb bud, and loss of Ano1 causes severe developmental abnormalities as Ano1 knockout mice die quickly after birth as a result of tracheomalacia (Rock et al. 2007. *Gene Exp. Patterns.* 8:19–26; Rock et al. 2008. *Dev. Biol.* 321:141–149). To identify protein partners of Ano1, we performed SILAC proteomic analysis that revealed a high stoichiometric association of Ano1 with proteins involved in cytoskeletal organization, membrane trafficking, polarization, and primary cilium formation (Perez-Cornejo et al. 2012. *PNAS.* 109:10376–10381). We have uncovered fascinating associations between Ano1, the small GTPase Cdc42, IQGAP1, the ERM (Ezrin, Radixin, Moesin) proteins, and other proteins involved in establishment of polarity. These proteins are arranged with Ano1 and acetylated microtubules into an apical ring structure we call the Ano1 Nimbus. In cells that have not yet formed a primary cilium, a network of acetylated tubulin feeds into the Ano1 Nimbus over an area devoid of F-actin, suggesting a role of this structure in organizing the cytoskeleton. Our observations implicate a Cl^- channel in cell polarity and ciliogenesis and raise exciting questions about the relationship of Cl^- channels to the organization of the underlying cytoskeleton and apical membrane subdomains important in primary cilium formation.

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6. Modulations of CLC-1 by Intracellular Nucleotides and pH. **TSUNG-YU CHEN**, *Center for Neuroscience and Department of Neurology, University of California, Davis, Davis, CA 95618*

The CLC-1 Cl^- channel is expressed abundantly in the surface membrane of skeletal muscles and plays important roles for the skeletal muscle physiology. It has been found that CLC-1 can be inhibited by intracellular nucleotides such as ATP, ADP, and AMP, and the inhibition effect is pH dependent—the more acidic in the intracellular side, the larger the inhibition. This inhibition mechanism is physiologically important for reverting muscle fatigue resulting from accumulation of extracellular K^+ ions. Guided by the structural information of the C terminus of CLC molecules, we mutated four residues (V613, V634, V860, and E865) lining the potential ATP-binding pocket formed by the cystathionine β-synthase (CBS) domains. Mutations of these four residues all affected the ATP interaction with CLC-1, mostly by reducing the ATP effect. For V634, however, although mutations to most amino acids reduced the ATP effect, introducing aromatic amino acids at this position increased the apparent ATP affinity, suggesting that the side chain of this residue may contact the adenine ring of ATP. We also generated double mutants and showed that ATP interacts with the ATP-binding pocket using mutant cycle analysis approaches. In addition to ATP, ADP, and AMP, other structurally related molecules also inhibit CLC-1, apparently through the same mechanism. The presence of NAD or NADH in the cytoplasmic side of the channel inhibited CLC-1, and this inhibition was also potentiated by low intracellular pH. Another related molecule, NADPH, was not effective. Modeling the binding of NAD suggested that it is the ribose-nucleoside end that interacts with the deep part of the ATP-binding pocket. Although the biophysical basis of NAD/NADH effect on CLC-1 is known, the physiological role of this inhibition in cells requires further investigation.

7. Monitoring Substrate-driven Structural Changes in a CLC Chloride–Proton Antiporter with Double Electron–Electron Resonance Spectroscopy. RICKY C. CHENG,[1] PHILIP CHANG,[1] RICHARD A. STEIN,[2] KRISTIN TRONE,[1] HASSANE S. MCHAOURAB,[2] and MERRITT C. MADUKE,[1] *[1]Department of Molecular and Cellular Physiology, Stanford University, CA; [2]Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, TN*

ClC-ec1, a prokaryotic Cl^-/H^+ antiporter in the CLC family, has been crystallized under many conditions. Yet, aside from local structural differences at the chloride-binding site, only one major conformation is observed. This failure of x-ray crystallography to reveal the unknown conformational states in the transport cycle motivates the use of alternative approaches. Using ^{19}F-NMR, a previous study demonstrated that a tyrosine residue >20 Å from the chloride–transport pathway undergoes antiport-specific structural changes (Elvington et al. 2009. *EMBO J.* 28:3090–3102). To further investigate possible protein movements during the antiport cycle, we introduced nitroxide paramagnetic spin labels to single-cysteine mutants of the ClC-ec1 homodimer via site-directed spin labeling and monitored structural changes using electron–electron resonance (DEER) spectroscopy. Preliminary results show significant substrate-dependent changes in intersubunit distances near Cl^- and H^+ access pathways and suggest that the antiport cycle involves structural changes beyond local movement at the chloride-binding site.

8. Permeant Anions Affect Gating of the TMEM16B/Anoctamin2 Calcium-activated Chloride Channel. O. LIJO CHERIAN,[1] GIULIA BETTO,[1] VALENTINA CENEDESE,[1] SIMONE PIFFERI,[1] ANNA BOCCACCIO,[2] FULVIO CELSI,[1] MONICA MAZZOLINI,[1] and ANNA MENINI,[1] *[1]SISSA, International School for Advanced Studies, 34136 Trieste, Italy; [2]Istituto di Biofisica, CNR, Genova, Italy*

Calcium-activated chloride channels are involved in different physiological processes, and at least two members of the TMEM16/anoctamin family, TMEM16A/anoctamin1 and TMEM16B/anoctamin2, have been identified as responsible for a calcium-activated chloride current in diverse tissues. We expressed TMEM16B in HEK 293T cells and measured the effect of external anions on channel gating by using the whole-cell and excised-patch techniques in the presence of various intracellular Ca^{2+} concentrations. When external Cl^- was replaced by the more permeant anion thiocyanate (SCN^-) in the presence of low Ca^{2+} concentrations, we observed a reduction in outward rectification and a large increase in conductance. Moreover, in the presence of SCN^-, the voltage dependence of steady-state activation (G-V relation) was shifted to more negative potentials. Furthermore, when extracellular Cl^- concentration was reduced by substitution with a less permeant anion, the G-V relation was shifted to more positive potentials. These results show that, similarly to other calcium-activated chloride channels, TMEM16B is controlled by a complex interplay among calcium concentration, membrane voltage, and permeant anions. We are currently performing site-directed mutagenesis experiments with the aim of identifying residues that could influence the interactions between gating and permeation in TMEM16B.

9. Three Charged Amino Acids in the Outer Vestibule of CFTR Stabilize the Open Pore Architecture. GUIYING CUI,[1] CHRISTOPHER KUANG,[1] KAZI S. RAHMAN,[2] CHENGYU Z. PRINCE,[1] and NAEL A. MCCARTY,[1] *[1]Department of Pediatrics, Emory+Children's Center for Cystic*

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Cystic fibrosis transmembrane conductance regulator (CFTR) carries six extracellular loops (ECL1–6). ECL4 bears N-linked oligosaccharide chains, whereas the functions of the other ECLs remain unknown. Several amino acids of ECL1 have been identified as sites of cystic fibrosis (CF) disease mutation, including R117C/G/P/H/L, D110H/Y/N/E, and E116K/Q. It has been reported that R117H-CFTR has reduced channel amplitude, whereas D110H-, E116K-, and R117C/L/P-CFTR possibly impair channel stability. We asked whether these amino acids are directly involved in ion conduction and permeation of CFTR or contribute to stabilizing the outer vestibule architecture. We used cRNA-injected oocytes combined with electrophysiological technique to probe the possible function of these amino acids. We found that: (a) charge-altering mutants R117A-, D110R-, and E116R-CFTR exhibited multiple open states with significantly shortened burst durations compared with WT-CFTR, whereas charge-retaining mutants R117K-, D110E-, and E116D-CFTR exhibited mainly the full open state similar to WT-CFTR; (b) R117A-, D110R-, and E116R-CFTR unlike WT-CFTR failed to be locked into the open state by AMP-PNP; (c) the function of R117C-, D110C-, and E116C-CFTR did not exhibit detectable modification by extracellular $MTSES^-$ or $MTSET^+$; (d) R117C-, D110C-, and E116C-CFTR were weakly blocked by GlyH-101 compared with WT-CFTR, whereas T338A- and R352A-CFTR were strongly blocked and R334C- and R334A-CFTR were resistant to block (R334, T338, and R352 are amino acids in TM6, the most important TM in determining ion permeation in CFTR); (e) double mutant R117E/E116R, R117E/D110R, and triple mutant R117E/E116R/D110R behaved similarly as charge-swapping single mutants R117A, E116R, and D110R, whereas double mutants R104E/E116R, R117E/E1126R, and D110R/K892E exhibited channel behavior closer to WT-CFTR with longer mean burst duration and stable full open state; (f) bifunctional linker MTS-2-MTS locked R104C/E116C, R117C/E1126C(ECL6), and D110C/K892C (ECL4) into open states. Two cysteines at positions 104 and 116 formed a disulfide bond when channels were in the open state. The data so far suggest that: (a) R117, D110, and E116 are not involved in ion conduction and permeation of CFTR directly; (b) the three charged amino acids contribute to stabilizing the CFTR channel pore but do not interact with each other locally to maintain their function; (c) three salt bridges, R104-E116, R117-E1126, and D110-K892 stabilize the outer vestibule architecture of CFTR. Collectively, the data show that amino acids of different ECLs interact with each other and are necessary for CFTR channel function.

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10. Function of Intracellular Anion Transporters during Stomata Movement. **ALEXIS DE ANGELI**, *Institute of Plant Biology, Molecular Plant Physiology, University of Zürich*

Plants use a large variety of anions for different physiological needs. Inorganic anions can be absorbed from the soil (e.g., nitrate, chloride, sulfate, and phosphate), whereas organic anions originate from photosynthetic products (e.g., malate, citrate, oxalate, and fumarate). In plant physiology, anions can serve as osmotica and/or substrates for biosynthesis of organic compounds. For instance, chloride is mainly involved in osmotic homeostasis, whereas nitrate serves as both an osmoticum and a precursor in amino acid biosynthesis. Because plants are sessile organisms in a variable environment, it is essential for them to adjust the intracellular osmotic pressure. Stomata are pores on the surface of leaves delimited by two guard cells that control their aperture. The tight regulation of the stomata aperture is fundamental to allow gas exchange for efficient photosynthesis and to limit water loss. The opening and closure of the stomata pore depends on the ability of the guard cells to accumulate and release solutes in response to stimuli. During stomata opening inorganic (chloride and nitrate) and organic (malate) anions are accumulated, whereas during closure they are released. In plants, the central vacuole is the intracellular organelle in which solutes are being accumulated; thus, it represents a primary constituent in regulating stomata movement. However, the knowledge of vacuolar anion transporters and their regulation in guard cells is still limited. This talk will focus on the two families of anion channels and transporters, ALMTs and CLCs, which have been identified in guard cell vacuoles. Recent findings on their regulation and role in stomata movement will be presented.

11. New Animal Models of Cystic Fibrosis. **JOHN F. ENGELHARDT**, *Department of Anatomy and Cell Biology and Center for Gene Therapy, University of Iowa, Iowa City, IA*

For decades, the cystic fibrosis (CF) field has been dependent on CF mouse models to study disease pathogenesis and CFTR functions at the organ level. However, CF mice fail to model the human lung and pancreatic disease phenotypes. Importantly, pathology in these two organs has a significant impact on the health of CF patients. The reasons why mice fail to model CF lung and pancreatic disease are presently unclear. However, alternative chloride channels in these organs are thought to potentially compensate for the lack of CFTR. More recently, alternative CF animal models have been generated in the pig, ferret, rabbit, and rat. Differences in the cell biology of the lung between these five CF animal models may influence the ability of a particular species to acquire CF lung disease. These species-specific differences include the type of

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secretory cells found in the airway epithelium and the abundance of submucosal glands in the cartilaginous airways. Two of these models (pig and ferret) have been shown to acquire disease phenotypes in the lung, pancreas, gallbladder, and intestine that closely replicate disease in human patients. However, it is clearly evident that the severity of disease and the timing of disease onset in these organs are variable across species (human, pig, and ferret). This presentation will focus on CF lung and pancreatic disease (including CF-related diabetes) and the role that larger-animal models are playing in dissecting CF disease pathophysiology and in vivo CFTR functions.

12. Gated Access of Engineered Cysteines in CFTR's Sixth Transmembrane Segment Reveals the Location of the Gate. XIAOLONG GAO and TZYH-CHANG HWANG, *Dalton Cardiovascular Research Center, University of Missouri-Columbia, Columbia, MO 65211*

Ample evidence has suggested that the CFTR channel evolves from a primordial ABC exporter by degrading its intracellular gate while maintaining many of the conserved gating motions including dimerization of the nucleotide-binding domains and translational and rotational movements of its transmembrane segments. Although five transmembrane segments, TM1, TM3, TM6, TM9, and TM12, have been shown to line the pore, the location of CFTR's gate remains unknown. Data from substituted cysteine accessibility methods (SCAM) studies suggested that the CFTR pore may assume an hourglass-like architecture with two vestibules flanking a narrow region consisting of just four to five amino acids. To determine which part(s) of TMs constitute the gate that controls the flow of chloride ions, we used channel permeant probe, $[Au(CN)_2]^-$, to modify engineered cysteines in TM6 or TM12 and assessed if the modification exhibits any state dependence. When a cysteine is placed at position 344 (TM6) or 1148 (TM12), both localized intracellularly to the restrictive region in the pore, we found that the engineered cysteine can be accessed in both the open and closed states by internally applied $[Au(CN)_2]^-$, an observation consistent with previous results using bulky MTS reagents. In contrast, the cysteine introduced into the external vestibule (position 337 or 338 in TM6) was modified by internally applied $[Au(CN)_2]^-$ at a much faster rate in the presence of ATP than that in the absence of ATP, suggesting that the gate is located between positions 338 and 344. Experiments with externally applied $[Au(CN)_2]^-$ are currently in progress.

13. Chloride Intracellular Channel (CLIC) Proteins Are Conserved in Prokaryotes and Play a Role in Cardio Protection from Ischemia-Reperfusion Injury. SHUBHA GURURAJA RAO,[1] JEAN CHRISOSTOME BOPASSA,[2] and HARPREET SINGH,[1] [1]*Department of Pharmacology and Physiology, Drexel University College of Medicine, Philadelphia, PA;* [2]*Department of Anesthesiology, University of California, Los Angeles, Los Angeles, CA*

Chloride intracellular channel (CLIC) proteins are a unique class of intracellular channels present in soluble as well as nonsoluble forms, found in eukaryotes but not reported in prokaryotes. Additionally, in absence of specific antibodies, cellular distribution of native CLICs is not well established. Here, we report the identification of a related prokaryotic gene, stringent starvation protein (sspA), in the genome of *Burkholderiales bacterium* as well as *Neisseria meningitidis*. Similar to metazoan CLIC proteins, sspA shares structural homology with glutathione *S*-transferase (GST) and lacks GST activity. On analysis with Kyte-Doolittle hydrophobicity plot, we found that sspA has at least one transmembrane domain in the N-terminus region, and amino acids lining the pore-forming region of CLICs are conserved, implying that sspA can also form functional ion channels. To investigate the localization of native CLIC proteins, we labeled CLIC1 and CLIC4 using specific antibodies targeted against their C termini along with organelle markers. We found that metazoan CLIC1 and CLIC4 are present in the mitochondria of murine primary hippocampal and cortical neurons, cardiomyocytes, and Percoll-purified cardiac mitochondria. Because mitochondria are known to play a predominant role in cardio protection, we tested whether CLICs are involved in cardio protection from ischemia-reperfusion injury, using IAA94, a blocker of CLIC channels. Isolated hearts from male mice were preconditioned (with 30 µM IAA94), followed by 18 min of global ischemia and 60 min of reperfusion. Preconditioning with IAA94 reduced left ventricular–developed pressure by threefold at the end of 60 min of reperfusion. Similarly, the heart rate pressure product, the maximum rates of rise (dP/dt max) and fall (dP/dt min) measured at the end of 60 min of reperfusion, showed 7, 11, and sevenfold reductions with IAA94. These findings indicate that CLIC proteins are evolutionarily conserved, and blocking these channels results in cardio-deleterious effects.

14. Roles of the Anoctamin 6 Ion Channel in Cell Migration, Cell Volume Regulation, and Apoptosis. ELSE K. HOFFMANN, *Department of Biology, University of Copenhagen, Denmark*

Anoctamin 6 (ANO6) was shown by our group to be a calcium-activated anion channel with delayed calcium activation of an outwardly rectifying current. The cellular function of ANO6 is still unclear and under debate. We investigated the role of ANO6 and ANO1 channels in cell migration in Ehrlich-Lettre Ascites (ELA) cells using miRNA and stably knocked down of the expression of the channels. ANO1 KD clones were affected in their directional migration, whereas ANO6 KD clones showed a 40% reduced migration rate. Thus,

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ANO1 channels have a function in directional migration and ANO6 in determining the speed of migration. We conclude that ANO1 and ANO6 channels have a distinct impact on the steering and motor mechanisms, respectively, of migrating ELA cells (Jacobsen et al. *Eur. J. Physiol.* In press). In addition, we investigated ANO6's possible role in cell volume regulation to see if ANO6 is identical to the volume-regulated anion channel (VRAC) or the volume-sensitive organic osmolyte and anion channel (VSOAC). We found that knockdown of ANO6 in Ehrlich ascites tumor cells (EATC) significantly reduced regulatory volume decrease. This reduction was dependent on extracellular calcium. Neither overexpression of ANO6 nor of ANO1 affected VRAC currents in EATC and HEK293 cells, whereas KD of ANO6 in EATC and ELA significantly increased VRAC currents. KD of ANO6 in ELA cells did not affect the efflux of the organic osmolyte taurine under hypotonic conditions. In sum, our data show that ANO6 is different from both VRAC and VSOAC, but may interact with VRAC. Finally, we studied ANO6's potential involvement in apoptosis. We found that KD of ANO6 in ELA cells decreased cisplatin-induced caspase-3 activity compared with wild type. Thus, ANO6 is involved in apoptosis, possibly by contributing to apoptotic volume decrease (Juul et al. *Am. J. Physiol.* Submitted).

15. CFTR: An Ion Channel Evolved from ABC Exporter. **TZYH-CHANG HWANG**, *Dalton Cardiovascular Research Center, Interdisciplinary Neuroscience Program, University of Missouri, Columbia, MO*

Cystic fibrosis, the most common fatal genetic disease in Caucasian populations, is caused by loss-of-function mutations of the CFTR gene that encodes a phosphorylation-activated but ATP-gated anion channel. Interestingly, CFTR harbors all the structural elements of an ABC exporter, which moves substrates uphill across the membrane using ATP hydrolysis as the energy source. Although the cargos for CFTR and ABC exporters differ immensely, their transmembrane domains (TMDs) conform to a "6 + 6" topological fold, with each TMD bearing six transmembrane helices (TMs) and the last TM (TM6 and TM12) in each TMD fused to a nucleotide-binding domain (NBD). Biochemical and electrophysiological studies have shown that CFTR's two NBDs, like those found in other ABC transporters, dimerize in a head-to-tail configuration upon ATP binding, and this conformational change in NBDs is coupled to gate opening in TMDs. We used substituted cysteine accessibility methods (SCAM) to investigate the structure/function role of CFTR's TMDs in gating and permeation. Our data support the "degraded transporter hypothesis": CFTR uses evolutionarily conserved TMDs to enact the function of a gated pore by disbanding its cytoplasmic gate. Specifically, we found: (a) CFTR's TM6 and TM12 play a major role in forming the pore; (b) the reaction pattern supports the notion that these two segments assume a secondary structure of an α helix; (c) cysteines introduced into the cytoplasmic half of either segment are accessible in both open and closed states; (d) comparing the modification rates at multiple positions reveals an alternate pattern that is consistent with a rotational movement of the helix during gating; (e) the cytoplasmic entrance of the pore becomes narrower upon gate opening, as extremely bulky probes can only get into the pore in a closed state. Lately, we also completed SCAM studies on CFTR's TM1 and found that the four positions in TM1 identified as pore-lining all exhibited strong state-dependent accessibility as if they could be accessed from the cytoplasmic side of the channel only in the open state. These seemingly perplexing results actually support our hypothesis, as TM1 in other ABC exporters only form the substrate translocation pathway in the "outward-facing" conformation that is analogous to CFTR's open state. Ongoing work to identify the location of the gate in TMDs will be discussed at the meeting.

16. Distribution of Chloride Channels between Different Compartments of Frog, *Rana temporaria* Skeletal Muscle Fibers. IGOR V. KUBASOV,[1] RUBEN S. ARUTUNIAN,[1] and MAXIM DOBRETSOV,[2] [1]*I.M. Sechenov Institute of Evolutionary Physiology and Biochemistry, Saint-Petersburg, Russia;* [2]*Department of Anesthesiology and Department of Neurobiology and Developmental Sciences, University of Arkansas for Medical Sciences, Little Rock, AR*

Distribution of Cl^- channels between different compartments of skeletal muscle fiber remains a controversial question. To address this issue, the effect of the chloride channel blocker, 9-anthracenecarboxylic acid (9-AC), on frog Sartorius muscle fiber action potentials (APs) was studied using a novel extracellular, narrow-tipped pipette focal current recording technique. Depending on the position of the pipette on the surface of the muscle fiber, two types of AP waveforms were recorded. Bi-phasic type 1 APs (T1AP) have been recorded from the center of sarcomere, whereas tri-phasic type 2 (T2) APs represented typical response recorded at the border of a sarcomere (in the Z-line region). The addition of 9-AC (100 µM) to either the bath or pipette solution led to a gradual decrease in amplitude and an increase in duration of the second, negative phase of T1 AP. In contrast with the above, bath application of the chloride channel blocker during the recording of T2 responses resulted in the appearance of multiple (from one to four) peaks within the second phase of T2 responses. Local application of 9-AC diluted in the intra-pipette solution did not reproduce this effect of the bath-applied drug, and it did not change any other characteristic of the T2 waveform. These data can be explained by suggesting that: (a) extracellularly recorded T1 and T2 APs represent electrical activity of

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primarily superficial sarcolemma and t-tubular membrane of the muscle fiber, respectively; and (b) in frog skeletal muscle fiber, density of chloride channels sensitive to 9-AC is substantially higher in sarcolemma than that in t-tubular membrane.

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17. State-dependent but Dephosphorylation-independent Rundown of CFTR. PEI-LUN KUO,[1] CHUN-KUANG TSAI,[1] HSIN-TUAN HUANG,[1] and TZYH-CHANG HWANG,[2] [1]*Department of Medicine, National Yang-Ming University, Taiwan;* [2]*Department of Medical Pharmacology and Physiology, and Dalton Cardiovascular Research Center, University of Missouri-Columbia, Columbia, MO 65211*

CFTR, a phosphorylation-activated and ATP-gated chloride channel, evolves from the exporter member of the ABC protein family. After fully activated by PKA and ATP in excised inside-out patches, CFTR channel activity often decreases over time once PKA is removed. Such "rundown" phenomenon is often attributed to dephosphorylation by membrane-associated protein phosphatases. Here, we report two types of rundown that cannot be accounted for by dephosphorylation. First, we found that upon sudden ATP applications to patches containing ΔR-CFTR, whose activity is completely independent of phosphorylation, the current rises in two distinct phases: a rapid increase (within 1 second) followed by a slow rising phase (several seconds to minutes), suggesting the existence of at least two groups of channels with different responsiveness to ATP. The slow phase of current rise represents a gradual recovery of the channel from the poorly responsive state (defined as reversible rundown). However, once the channels recover from rundown, their activities remain fairly stable in the continued presence of ATP, and a brief (e.g., 5-second) washout of ATP followed by the reapplication of ATP results in only a fast monophasic current increase, indicating that this newly discovered rundown takes place slowly but more readily in the closed state. Indeed, when the washout duration is prolonged, the slow current rising phase reappears, and its magnitude is proportional to the washout time. Interestingly, the time constant for the recovery phase is also increased as the washout time is prolonged, indicating the presence of multiple rundown states, a proposition supported by microscopic recordings. In addition to this fully recoverable rundown, we observed an irreversible rundown that also preferentially occurs in the absence of ATP. Experiments with wild-type CFTR in patches with minimal membrane-bound phosphatase activity showed similar results. Thus, our studies demonstrate a state-dependent but phosphorylation-independent rundown of CFTR.

18. Development of a Gating-sensitive Fluorescent CFTR Probe. EMILY LANGRON and PAOLA VERGANI, *Department of Neuroscience Physiology and Pharmacology, University College London, UK*

Cystic fibrosis (CF) is a debilitating disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which codes for the CFTR anion channel.

YFP H148Q/I152L is a GFP derivative, whose fluorescence is quenched in the presence of negative ions. This modified YFP has been used to screen compound libraries for CFTR potentiators by coexpressing with CFTR in recombinant cells and recording fluorescence (Galietta et al. 2001. *Am. J. Physiol. Cell. Physiol.* 281: C1734–C1742). YFP H148Q/I152L has a higher affinity for I^- than for Cl^-. CFTR activation in the presence of external I^- buffer results in quenching of YFP fluorescence as I^- enters the cell. The rate of quenching provides a quantitative measure of CFTR activation.

The aim of this work is to use a random transposon-based insertion protocol to construct a library of CFTR-YFPH148Q/I152L fusion proteins (Mealer et al. 2008. *Method. Cell. Biol.* 85:23–44) in which YFP is inserted within the CFTR coding sequence. The library would be screened to identify fusion constructs in which gating and trafficking of CFTR is minimally affected. Such a construct could be used as a fluorescent probe to monitor CFTR function for gene therapy work, and for basic research investigating cellular physiology in CF.

In addition, we aim to use the identified fusion protein to develop an improved screen for ΔF508-CFTR potentiators. In the existing assay, I^- influx and Cl^- efflux gradually change the cytosolic $[I^-]/[Cl^-]$. Tethering each fluorophore to a CFTR channel is likely to result in immediate maximum quenching of the signal upon respective channel opening. Fluorescence could thus be closely linked to CFTR conformation. The assay could be configured to compare quenching in the absence and presence of a specific CFTR inhibitor. Thus, rapid, reversible quenching of a small signal could provide a very specific readout for CFTR activity.

Supported by the Cystic Fibrosis Trust UK.

19. Electrophysiological Phenotype of Heterologously Expressed Anoctamin 6 in HEK296 Cells. ERIK HVIID LARSEN, *Department of Biology, University of Copenhagen, Denmark*

In many cell types, members of the Anoctamin family are essential constituents of Ca^{2+}-activated Cl^- channels (CaCC). We have investigated electrophysiological properties of mouse ANO6 (Grubb et al. 2013. *J. Gen. Physiol.* 141:585–600). With a delay of several minutes after cytosolic exposure to 250 µM of

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free Ca^{2+}, heterologously expressed GFP-tagged plasma membrane–located ANO6 generated an outwardly rectifying steady-state $I_{Cl,Ca}$. Ca^{2+}-activated membrane currents were governed by an EC_{50} of 100 µM of free $[Ca^{2+}]_{cell}$. With $NMDG^+$ in bath, ANO6 exhibited anion selectivity as indicated by: (a) rectification of instantaneous membrane currents (I^{inst}) predicted by the GHK Cl^- current equation; (b) Eisenman type 1 anion selectivity sequence of $P_{SCN} > P_I > P_{Br} > P_{Cl} > P_{Asp}$; and (c) abolishment of ANO6-associated $I_{Cl,Ca}$ in a mutant of the putative pore region (R592E). In two other mutants (K616E and R636E), $I_{Cl,Ca}$ was retained, whereas the mutant K616E had a lower relative permeability to iodide, and the mutant R636E had a significantly altered anion selectivity sequence of $P_{SCN} = P_I = P_{Br} = P_{Cl} > P_{Asp}$. Thus, ANO6 constitutes a Ca^{2+}-activated anion channel or a pore-forming subunit of an anion channel with properties distinct from ANO1 that, as we could confirm, generates instantly activated, submicromolar Ca^{2+}–sensitive currents. Independent of ANO6 construct, replacing $NMDG^+$ in bath by Na^+ displaced the reversal potential of I^{inst} from E_{Cl} (−31 mV) toward E_{Na} (+58 mV), resulting in $I^{inst} = GHK\text{-}I_{Cl} + GHK\text{-}I_{Na}$, and a stimulation of P_{Cl}. Including all constructs, within the range of P_{Cl} from 10^{-7} to 25×10^{-7} cm/s, the two permeabilities were correlated according to $P_{Na} = 0.334 \times P_{Cl}$ (± SD = 0.028; n = 15 cells). Judging by the anion selectivity series obtained, we find it very unlikely that Na^+ shares TM pore with Cl^-.

20. Probing Interactions of CFTR with SLC5A8, a Putative Thyroid Iodide Transporter. YONGHAI LI, SUHASINI GANTA, and PEYING FONG, *Department of Anatomy and Physiology, Kansas State University College of Veterinary Medicine, Manhattan, KS 66506*

The cystic fibrosis transmembrane conductance regulator (CFTR) mediates thyroid anion secretion (Li et al. 2010. *Exp. Physiol.* 95:1132–1144). Surprisingly, thyroid epithelial cells expressing the F508 folding mutation respond robustly to forskolin (fsk) despite low expression (total ~9% of wt CFTR; surface ~4% of total, under both basal and stimulated conditions; Li et al. 2012. *Exp. Physiol.* 97:115–124). Thus, extremely low surface levels of F508-CFTR (and CFTR) sustain cAMP-stimulated I_{sc}, hinting at a novel interaction with another thyroid transporter. Mucosal addition of GlyH101, a CFTR inhibitor, only partially blocks fsk-stimulated I_{sc}. Complete block was achieved by subsequent addition of ibuprofen, an inhibitor of SLC5A8 (Coady et al. 2004. *J. Physiol.* 557:719–731). SLC5A8 is a putative iodide transporter that also transports sodium and monocarboxylic acids (Rodriguez et al. 2002. *J. Clin. Exp. Endocrinol. Metab.* 87:3500–3503; Coady et al. 2004. *J. Physiol.* 557:719–731). Interestingly, its carboxyl terminus bears the identical PDZ domain-binding tripeptide as CFTR's: TRL. Immunoprecipitated (IPd) fractions of thyroid lysates with anti-CFTR (M3A7) revealed strong reactivity with anti-SLC5A8. Similarly, M3A7-IPd fractions from COS-7 cells coexpressing wt-hCFTR and HA-tagged wt-hSLC5A8 show anti-hSLC5A8 reactivity, as well as signal for endogenous PDZK1. M3A7-IPd fractions from cells coexpressing HA-hSLC5A8-ΔTRL with wt-hCFTR showed reduced anti-hSLC5A8 reactivity. In both groups of coexpressing lysates, anti–HA-IPd fractions reacted with both anti-CFTR and anti-PDZK1. Comparable levels of CFTR were detected in IPd fractions from cells coexpressing either HA-wt-SLC5A8 or HA-hSLC5A8-ΔTRL with hCFTR, but paradoxically, PDZK1 levels were higher in the latter.

These findings suggest that SLC5A8 associates with CFTR via its PDZ domain-binding motif and likely additional, as yet unidentified, mechanisms. Furthermore, the SLC5A8 PDZ domain–binding motif may regulate CFTR–PDZK1 interactions and may bear special impact on thyroid function.

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21.* A Model for Chloride–Proton Exchange in CLC Transporters. **RODERICK MACKINNON**, ERNEST CAMPBELL, and LIANG FENG, *The Rockefeller University and Howard Hughes Medical Institute, New York, NY 10065*

By catalyzing the diffusion of Cl^- ions across plasma and intracellular membranes, CLC transporters and channels underlie important physiological mechanisms in kidney, muscle, bone, and other organs. In my talk I will present a model for Cl^-/H^+ exchange in the transporter members of the CLC family. The model is derived from atomic structures of the *Escherichia coli* and *Cyanidioschyzon merolae* (red algae) CLC transporters determined in our laboratory using x-ray crystallography. The structures capture three unique configurations of Cl^- ions and associated conformations of a glutamate residue side chain within the transport pathway. The states defined by these structures give rise to a plausible kinetic model that accounts for the well-known 2:1 Cl^-/H^+ exchange stoichiometry. I will discuss the principles of this model and demonstrate that it can account for the functional activity of CLC transporters documented under a variety of physical conditions.

22. Ca^{2+}-dependent Phospholipid Scrambling by a Reconstituted TMEM16 Ion Channel. MATTIA MALVEZZI,[1,2] MADHAVAN N. CHALAT,[3] RADMILA JANJUSEVIC,[3] ALESSANDRA PICOLLO,[1] HIROYUKI TERASHIMA,[1] ANANT K. MENON,[3] and **ALESSIO ACCARDI**,[1,2,3] [1]*Department of Anesthesiology,* [2]*Department of Physiology and Biophysics, and* [3]*Department of Biochemistry, Weill Cornell Medical College, New York, NY 10065*

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Phospholipid scramblases collapse the plasma membrane lipid asymmetry, externalizing phosphatidylserine to trigger blood coagulation and mark apoptotic cells. Despite their importance in cell physiology, the molecular identity of the scramblases has eluded researchers for decades. TMEM16F, a member of the TMEM16 family of Ca^{2+}-gated channels, has been shown to be involved in lipid scrambling. The function of TMEM16F remains controversial, as it has also been reported to be a Ca^{2+}-dependent cation channel and three different Cl^- channels. Two other members of the TMEM16 family, TMEM16 A and B, are Ca^{2+}-activated Cl^- channels, and they do not seem to be involved in lipid scrambling. Whether TMEM16F, and possibly other members of the family, are phospholipid scramblases or ion channels that regulate scrambling activity remains unclear.

To differentiate between these hypotheses, we expressed, purified, and reconstituted several TMEM16 family members and discovered that purified afTMEM16, from *Aspergillus fumigatus*, is a dual-function protein: it is a Ca^{2+}-gated channel, with characteristics of other TMEM16 homologues, and a Ca^{2+}-dependent scramblase, with the expected properties of mammalian phospholipid scramblases. Remarkably, we find that a single Ca^{2+} site, conserved among the TMEM16 homologues, regulates separate transmembrane pathways for ions and lipids. Two other purified TMEM16 channel homologues do not mediate scrambling, suggesting that the family diverged into channels and channel/scramblases.

Our results demonstrate for the first time that a member of the TMEM16 family is simultaneously a Ca^{2+}-dependent ion channel and a Ca^{2+}-dependent lipid scramblase. We propose that the spatial separation of the ion and lipid pathways underlies the evolutionary divergence of the TMEM16 family, and that other homologues, such as TMEM16F, might also be dual-function channel/scramblases.

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23. Involvement of Anion-permeable Aquaporin-6 in Osmotic Lysis of Parotid Secretory Granules. MIWAKO MATSUKI-FUKUSHIMA,[1] MASATAKA MURAKAMI,[2] JUNKO FUJITA-YOSHIGAKI,[1] OSAMU KATSUMATA-KATO,[1] MEGUMI YOKOYAMA,[1] and HIROSHI SUGIYA,[3] *[1]Nihon University School of Dentistry at Matsudo, Chiba, Japan; [2]National Institute for Physiological Sciences, Aichi, Japan; [3]Nihon University College of Bioresource Sciences, Kanagawa, Japan*

In secretory granules and vesicles, membrane transporters have been predicted to permeate water, ions, or small solutes to promote content maturation or exocytosis. We found that aquaporin-6 (AQP6), a water channel protein, which permeates anions dependent on acid or Hg^{2+}, is localized in rat parotid secretory granules (Matsuki-Fukushima et al. 2008. *Cell Tissue Res.* 332:73–80). Because the localization of AQP6 in other organs is restricted to cytosolic vesicles, the native function(s) of AQP6 in vivo has not been well determined. To analyze the role of AQP6 in secretory granule membranes, we used Hg^{2+}, which is known to activate AQP6, and investigated the characteristics of anion permeability. Anion permeability of secretory granule membrane was determined by using "osmotic lysis" of granules in iso-osmotic salt solution. Osmotic lysis induced by Hg^{2+} (Hg-lysis) was examined by measuring the time-dependent changes in the optical density of the secretory granule suspension at 540 nm at 37°C using a spectrophotometer. In the presence of 0.5–2.0 µM Hg^{2+}, the concentrations that activates AQP6, granule lysis was facilitated. The Hg-lysis was completely blocked by β-mercaptoethanol, which disrupts Hg^{2+} binding to cysteine residue, or by removal of chloride ions from the iso-osmotic solution. An anion channel blocker, DIDS, which does not affect AQP6, distinguished DIDS-insensitive and -sensitive Hg-lysis. These results suggest that Hg-lysis requires anion permeability through the protein transporter. A series of substitution experiments gave the following sequence of anion permeability: $NO_3^- > Br^- > I^- > Cl^-$. Finally, Hg-lysis was facilitated at acidic but not alkaline pH. The high permeability for NO_3^- and the acidic pH sensitivity were similar to the channel properties of AQP6. These data suggest that AQP6 functions as an Hg^{2+}-sensitive anion channel in rat parotid secretory granules.

24. The Role of ClC-7 in Late Endosome/Lysosome Acidification and Amyloid Degradation by Microglia. **FREDERICK R. MAXFIELD**, AMITABHA MAJUMDAR, DANA CRUZ, ESTIBALIZ CAPETILLO-ZARATE, and GUNNAR K. GOURAS, *Weill Cornell Medical College, New York, NY 10065*

Incomplete late endosome/lysosome acidification in microglia inhibits the degradation of fibrillar forms of Alzheimer's amyloid β peptide (fAβ). We found that in primary microglia, a chloride transporter, ClC-7, is not delivered efficiently to lysosomes, causing incomplete lysosomal acidification. ClC-7 protein appears to be degraded by an endoplasmic reticulum–associated degradation pathway. Activation of microglia with macrophage colony–stimulating factor induces trafficking of ClC-7 to lysosomes, leading to lysosomal acidification and increased fAβ degradation. These findings suggest a novel mechanism of lysosomal pH regulation in activated microglia that is required for fAβ degradation.

25. Turning a Transporter into an Open Pore: The CFTR Chloride Channel. **NAEL A. MCCARTY**, *Emory University, Atlanta, GA*

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Mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator protein (CFTR) cause cystic fibrosis, the most common life-shortening genetic disease among Caucasians. CFTR is a member of the ancient ATP-binding cassette (ABC) transporter superfamily. Most ABC proteins are transporters that operate via an alternating access mechanism, except for CFTR, which is best known for its activity as a chloride channel. The structure of CFTR is not known in detail, nor is the mechanism by which this ABC transporter superfamily protein is able to function as a chloride ion channel. In an effort to better understand how the conformational changes that underlie transporter activity in other ABC proteins were converted into conformations supporting channel activity in CFTR, we created new closed- and open-state homology models of CFTR, and performed targeted molecular dynamics simulations of the conformational transitions in a channel opening event. The simulations indicate a conformational wave that starts at the nucleotide-binding domains and ends with the formation of an open conduction pathway, stabilized by many residue–residue interactions. Changes in side-chain interactions are observed in all major domains of the protein, and experimental confirmation was obtained for a novel intra-protein salt bridge that breaks near the end of the transition. The models and simulation provide greater understanding of the mechanism of ATP-dependent gating in this disease-relevant ion channel.

Supported by NIH-DK056481.

26. A Fluoride-specific Ion Channel with Unprecedented Function and Architecture. **CHRISTOPHER MILLER**, *Brandeis University and Howard Hughes Medical Institute, Waltham, MA 02454*

Until last year, the F^- ion was absent from the literature of membrane biology – an ion hiding in plain sight since the very beginnings of membrane transport as a field of study. It is now known that many bacteria, archea, and unicellular eukaryotes export F^- to keep cytosolic concentrations of this anion below toxic levels (10–100 µM). They use F^- exporter proteins from either of two recently discovered, phylogenetically unrelated superfamilies: an F^--selective clade of F^-/H^+ exchangers from the CLC anion transporter family, and the "Fluc" family of small membrane proteins, previously unannotated in the database, that form ion channels. These Flucs are extremely unusual among ion channels, in several ways. Their F^-/Cl^- selectivity of >10,000 is huge, and their architecture has not been seen before in ion channels–dimeric, in which the two subunits are of opposite transmembrane topology.

27. Mechanism of Phosphorylation-dependent Long-Range Intra-Protein Signaling in a CLC Anion Channel. HIROAKI MIYAZAKI,[1] TOSHIKI YAMADA,[1] ANGELA PARTON,[1] REBECCA MORRISON,[1] SUNGHOON KIM,[2] ALBERT H. BETH,[2] and KEVIN STRANGE,[1] *[1]Boylan Center for Cellular and Molecular Physiology, Mount Desert Island Biological Laboratory, Salisbury Cove, ME 04672; [2]Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN 37232*

CLC anion transport proteins are regulated by phosphorylation, adenosine ligands, accessory proteins, and extracellular Ca^{2+}. However, the signaling and structure/function mechanisms that mediate this regulation are poorly understood. CLH-3b is a *C. elegans* CLC anion channel and member of the CLC-1/2/Ka/Kb subfamily. The channel is expressed in the worm oocyte and is activated by meiotic cell cycle progression and cell swelling. Channel inactivation is brought about by GCK-3 kinase–mediated phosphorylation of S742 and S747. These serine residues are located on an ~176–amino acid intrinsically disordered region linking the two cytoplasmic cystathionine-β-synthase (CBS) domains, CBS1 and CBS2, which dimerize to form a Bateman domain. Much of the inter-CBS linker is dispensable for channel regulation. However, deletion of a 14–amino acid activation domain encompassing S742 and S747 inhibits channel activity to the same extent and in the same manner as GCK-3–mediated phosphorylation. The crystal structure of CmCLC demonstrated that CBS2 interfaces extensively with an intracellular loop connecting membrane helices H and I, the C terminus of helix D, and a short linker connecting helix R to CBS1. Point mutagenesis of this interface identified two highly conserved aromatic amino acid residues located in the H-I loop and the first α helix (α1) of CBS2. Mutation of either residue to alanine rendered CLH-3b insensitive to GCK-3 inhibition. In silico analysis of the disordered inter-CBS linker suggests that the activation domain mediates intra-channel protein–protein interactions. We propose that the dephosphorylated activation domain normally interacts with the CBS1/CBS2 Bateman domain to maintain CLH-3b in an activated conformation. Phosphorylation or deletion of the activation domain disrupts this interaction, leading to a Bateman domain conformational change that is transduced to channel membrane domains through a conserved signal transduction module comprising the H-I loop and CBS2 α1.

28. Anion Channels Involved in Cell Survival-Death Switching. **Y. OKADA**,[1] K. SATO-NUMATA,[1] T. SHIMIZU,[2] H. SAKAI,[2] T. AKITA,[1] and T. OKADA,[1] *[1]National Institute for Physiological Science, Okazaki 444-8787, Japan; [2]Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan*

Cell volume regulation is essential for survival of animal cells. The regulatory volume decrease (RVD) and increase (RVI) are accomplished by water movement driven by KCl efflux and NaCl influx, respectively,

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mediated by volume-related anion and cation channels. Persistent cell shrinkage and swelling are major hallmarks of apoptotic and necrotic cell death, respectively, and caused by the apoptotic volume decrease (AVD) and necrotic volume increase (NVI) coupled to impairment of RVI and RVD, respectively. Because the AVD and NVI processes also involve anion channel activities, it is likely that volume-related anion channels (such as volume-sensitive outwardly rectifying anion channel [VSOR], acid-sensitive outwardly rectifying anion channel [ASOR], and CFTR) play roles in the cell survival-death switching. In fact, our studies in vitro showed that apoptotic and necrotic cell death is induced or rescued by controlling activities of these volume-related anion channels under a variety of conditions including apoptotic stimulation, excitotoxicity, acidotoxicity, lactacidosis, and hypoxia-reoxygenation. Also, ischemia-reperfusion–induced neuronal apoptosis and cardiac necrosis in vivo were found to be rescued by controlling volume-related anion channel activities. Although TMEM16F (ANO6) and ClC-3 were proposed as the molecular candidates for VSOR and ASOR, respectively, our recent studies showed that they function as Ca^{2+}-activated and Ca^{2+}-independent outwardly rectifying chloride channels, respectively, distinct from VSOR and ASOR.

29. Purification and Functional Reconstitution of the TMEM16A Ca^{2+}-activated Cl^- Channel. ALESSANDRA PICOLLO,[1] HIROYUKI TERASHIMA,[1] and ALESSIO ACCARDI,[1,2,3] [1]*Department of Anesthesiology,* [2]*Physiology and Biophysics and Biochemistry, and* [3]*Department of Biochemistry, Weill Cornell Medical College, New York, NY 10065*

Ca^{2+}-activated Cl^- channels (CaCCs) play numerous physiological roles, ranging from electrolyte secretion in epithelia and glands to muscle contraction, olfactory transduction, and nociception. Despite their physiological importance, the molecular identity of these proteins remained elusive until recent work showed that TMEM16A and B, two members of the TMEM16 family of membrane proteins, are critical components of CaCCs. Heterologous expression of these two genes in oocytes and cells results in currents closely resembling native CaCCs. It is, however, unknown whether these proteins alone are necessary and sufficient to form functional Ca^{2+}-activated Cl^- channels, or whether association to other subunits is required. Recent studies suggest that association of TMEM16A with calmodulin is required for Ca^{2+} sensitivity.

To investigate whether TMEM16A alone forms functional CaCCs or if association to other partner proteins is required, we expressed, purified, and reconstituted TMEM16A in proteoliposomes. We found that purified TMEM16A mediates Ca^{2+}-dependent Cl^- fluxes with an apparent Km of ~210 nM, a value comparable to that measured in patch-clamp experiments. Channel opening is also promoted by the application of high positive voltages, consistent with electrophysiological measurements. Channel activity is diminished by CaCC inhibitors such as niflumic acid, NPPB, NPA, and DIDS, with K1/2's comparable to those measured for native and heterologous CaCCs. Mutating two conserved glutamates in the TM5–6 intracellular loops abolishes Ca^{2+} sensitivity of the fluxes in a manner similar to what was reported for heterologously expressed TMEM16A. Finally, neither on gel filtration nor on pulldown experiments could we detect a direct interaction between purified TMEM16A and calmodulin.

In conclusion, our results demonstrate that the purified TMEM16A protein recapitulates all the fundamental biophysical and pharmacological properties of native and heterologously expressed CaCC currents, indicating that association with other partner proteins, such as calmodulin, is not strictly required for function.

30. Hydrophobic Gating of Chloride and Other Chaotropic Anions through Facultative Membrane Oligomers. ALBERTO RIVETTA and CLIFFORD SLAYMAN, *Department of Cellular and Molecular Physiology, Yale School of Medicine, New Haven, CT 06520*

The discovery—a decade ago—of sequence data that suggested that potassium active-transport proteins in yeasts and related organisms should aggregate as tetramers within their native membranes (Durell and Guy. 1999. *Biophys. J.* 77:789–807) coincided with the discovery of large, K^+-independent currents mediated by those same proteins in *Saccharomyces* (TRK family proteins; Bihler et al. 1999. *FEBS Lett.* 447:115–120). Those currents, monitored by whole-cell patch recording, were shown to increase steeply as extracellular pH was lowered from 7.5 to 4.5 to increase exponentially with membrane voltage clamped increasingly negative to −200 mV, and to depend rather precisely on the chloride concentration within the patch pipette (Kuroda et al. 2004. *J. Membr. Biol.* 198:177–192; Rivetta et al. 2005. *Biophys. J.* 89:2412–2426). Other chaotropic anions: esp. Br^-, I^-, NO_3^-, NO_2^-, and SCN^- freely replace Cl^-, and thiocyanate seems to exclude chloride conduction. However, phosphate and small organic anions (e.g., formate, gluconate, and acetate) are not conducted. This kind of selectivity, taken together with the orientation of hydrophobic amino acids in the originally postulated oligomer, strongly implicates a permeation process similar to that occurring in (pentameric) anionic ligand-gated ion channels. We have been able to show, in addition, that chaotropic-ion currents through yeast TRK proteins are suppressed by extracellular osmolytes (cryoprotectants, osmoprotectants, compatible solutes). This finding calls attention to the functional instability of water in narrow hydrophobic channels, and suggests that the fundamental process underlying chaotropic-ion flow through fungal TRK proteins is hydrophobic gating.

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31. The Single-Molecule Regimen of Membrane Proteins in Liposomes. JANICE L. ROBERTSON,[1,2] LARRY FRIEDMAN,[2] MIKE RIGNEY,[2,3] LUDMILA KOLMAKOVA-PARTENSKY,[2,3] JEFF GELLES,[2] and CHRISTOPHER MILLER,[2,3] [1]*Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA;* [2]*Biochemistry and Biophysics and* [3]*Howard Hughes Medical Institute, Brandeis University, Waltham, MA*

Studying membrane proteins on a single-molecule level provides a direct window to observe how these machines work. Of course, single-molecule studies of ion channels have been performed for decades by way of electrophysiological recordings and have allowed the unique, in-depth characterization of these proteins. Now, we can obtain dynamic, structural information about conformational states of all types of membrane proteins using fluorescence microscopy and Forster resonance energy transfer (FRET). Experiments of this sort are now being done but typically examine the protein in detergent micelles. For many questions, studying channels and transporters in membranes is critical, and although it is possible to carry out microscopy on proteins in supported membranes, formation of the bilayer can be complicated and often limits the lipid conditions. Another method is to examine the protein in liposomes, the benefits being that it is quick, easy, and reconstitution is robust under different lipid compositions. To make this approach useful for single-molecule studies, we must establish the conditions in which we have a single protein molecule per liposome. Reconstitution statistics follows a Poisson distribution, which can be measured as a function of protein to lipid density and liposome size. Here, we measure the size distributions of liposomes formed with *E. coli* polar lipids by cryo-electron microscopy and fluorescence intensity of Alexa Fluor 488–labeled PE by total internal reflection fluorescence microscopy. The protein occupancy per liposome is measured as a function of protein density, by photo-bleaching analysis of the Cl^-/H^+ transporter CLC-ec1 labeled with Cyanine-5. The results provide a foundation for future single-molecule studies of membrane proteins, such as the identification of conformational changes as well as the determination of protein stoichiometry with respect to studying the thermodynamics of protein association in membranes.

32. Slc26 Proteins as Cl^- Transporters and Cl^- Channels: Transport, Regulation, and Disease Models. **MICHAEL F. ROMERO**,[1,2,3,4] JULIAN A.T. DOW,[5] MIN-HWANG CHANG,[1] TAKU HIRATA,[1,3] and AN-PING CHEN,[1] [1]*Physiology and Biomedical Engineering,* [2]*Nephrology and Hypertension,* [3]*O'Brien Urology Research Center, and* [4]*Mayo Translational PKD Center, Mayo Clinic School of Medicine, Rochester, MN;* [5]*Institute for Molecular, Cell and Systems Biology, University Glasgow, Glasgow, UK*

SLC26 proteins function as anion exchangers, channels, and sensors. Slc26 anion transporters play crucial roles in transepithelial Cl^- absorption/secretion as well as HCO_3^- absorption/secretion. Human mutations in SLC26 proteins lead to several diseases, including CLD (chloride-rich diarhea), deafness, cartilage problems (DTDST), and cystic fibrosis phenotypes. Our investigations have focused on Slc26a6 and Slc26a9 in several species. The Slc26 family is the first family of proteins to function as electrogenic Cl^-–HCO_3^- exchangers, an activity not previously realized in tissues. Slc26a6 functions as an electrogenic Cl^-–$\underline{n}HCO_3^-$ exchanger (Xie et al. 2002. *Am. J. Physiol. Renal Physiol.* 283:F826–838) while conversely Slc26a9 electrogenic $\underline{n}Cl^-$–HCO_3^- exchanger and Cl^- channel (Romero et al. 2006. *Novartis Foundation Symposium.* 273:126–147; Chang et al. 2009. *J. Membr. Biol.* 128:125–140).

Slc26a6 also exchanges Cl^- for sulfate, oxalate (ox^{2-}), and formate. A Slc26a6 homologue exists in *Drosophila* (CG5485; dPrestin), which has all the same transport properties (Hirata et al. 2012. *Am. J. Physiol. Renal Physiol.* 303:F1555–1562; Hirata et al. 2012. *J. Insect Physiology.* 58:563–569). Feeding *Drosophila* oxalate or adding oxalate to renal tubules elicits the formation of calcium oxalate (CaOx) crystals, which are diminished with dPrestin knockdown (Hirata et al. 2012. *Am. J. Physiol. Renal Physiol.* 303:F1555–1562). This *Drosophila* model of CaOx kidney stones is being used to examine signaling regulation, interacting genes, and therapeutics that might diminish CaOx stone formation.

Slc26a9 has three discrete physiological modes (Chang et al. 2009. *J. Membr. Biol.* 128:125–140): $\underline{n}Cl^-$–HCO_3^- exchanger, Cl^- channel, and Na^+/anion cotransporter. The purified Slc26a9 STAS domain (a9STAS) binds purified (R)CFTR. When Slc26a9 and (R)CFTR fragments are coexpressed in *Xenopus* oocytes, both Slc26a9-mediated nCl^-–HCO_3^- exchange and Cl^- currents are almost fully inhibited. Deletion of the a9STAS domain removes Cl^- channel function. Sun et al. (2012. *Nat. Gen.* 44:562–569) have recently found that allelic variations in SLC26A9 are associated with some intestinal phenotypes in cyctic fibrosis patients. We then characterized the function and localization of SLC26A9-nonsynonymous cSNPs (Chen et al. 2012. *Hum. Mutat.* 33:1275–1284): Y70N (cytoplasmic N terminus) displays higher channel and enhanced nCl^-–HCO_3^- exchange activity, T127N (TM3) has altered halide currents, whereas V622L (STAS domain) and V744M (STAS adjacent) show decreased plasma membrane expression. Recently, we found the variable loop within SLC26A9-STAS domain functions as an autoinhibitor for Cl^- transport activity. Our results provide a framework to understand SLC26A9 transport modalities and structure–function relationships.

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33. Dual Topology Architecture of a Microbial Fluroide Channel. RANDY B. STOCKBRIDGE and CHRISTOPHER MILLER, *Brandeis University and Howard Hughes Medical Institute, Waltham, MA 02454*

Microorganisms have evolved an array of protective responses to the ubiquitous environmental xenobiotic F^-. Here, we describe a novel riboswitch-associated F^- channel, which we call Fluc (previously curated as an unknown function gene, *crcB*), that is present in prokaryotes, archaea, and unicellular eukaryotes including fungi and protozoa, as well as in green plants, and whose expression provides microorganisms with resistance against F^- toxicity. Fluc is phyogenetically unrelated to any protein of known function. It has four transmembrane helical spans and functions as a dimer. Experimental evidence, including chemical cross-linking and fused constructs, suggests that the Fluc dimer is constructed with an unusual antiparallel architecture that invokes the inverted repeats of many modern-day membrane transport proteins. Here, we further test the topology of single channels in planar lipid bilayers by using pore-blocking “monobody” inhibitors derived from a library of randomly mutagenized fibronectin-3 protein domains. These monobodies bind Fluc channels in the 10–100-nm range with high specificity. Here, we characterize the binding constants, stoichiometry, and blocking behavior for three of these monobodies and finally test whether the monobodies are able to bind Fluc channels at two epitopes on opposite sides of the bilayer, indicating a symmetrical antiparallel architecture.

34. Mechanisms of Phosphorylation-dependent Regulation and Long-Range Intraprotein Signaling in a CLC Anion Channel. **KEVIN STRANGE**, *Boylan Center for Cellular and Molecular Physiology, Mount Desert Island Biological Laboratory, Bar Harbor, ME*

CLH-3b is a *C. elegans* CLC-1/2/Ka/Kb anion channel subfamily member. The channel is expressed in the worm oocyte and is activated by meiotic cell cycle progression and cell swelling. Activation requires dephosphorylation by type 1 serine/threonine phosphatases. The SPAK/OSR1 kinase homologue GCK-3 phosphorylates and inactivates CLH-3b. The CLH-3b cytoplasmic C terminus includes two conserved cystathionine-β-synthase (CBS) domains, termed CBS1 and CBS2, which dimerize to form a Bateman domain. An ∼176–amino acid inter-CBS linker connects CBS1 and CBS2. In silico and NMR analyses demonstrate that the linker is an intrinsically disordered region (IDR) lacking rigid 3-D structure. GCK-3 binds to the linker and phosphorylates two downstream serine residues, S742 and S747. Both residues must be concomitantly phosphorylated to inactivate the channel. Mutagenesis studies on the cytoplasmic N and C termini demonstrate that only the CBS domains and a stretch of 14 amino acids in the inter-CBS linker termed the “activation domain” are required for phosphorylation-dependent regulation. Deletion of the activation domain, which comprises both regulatory phosphorylation sites, inactivates CLH-3b to the same extent and in the same manner as phosphorylation. A newly identified and likely conserved CLC “signal transduction domain” comprising the first α helix of CBS2 and a short intracellular loop connecting membrane helices H and I (H-I loop) mediates phosphorylation-dependent intraprotein signaling that regulates channel activity. Helices H and I form part of the interface between the two subunits that comprise functional homodimeric CLC channels. Recent studies in other CLCs suggest that the subunit interface may mediate the poorly understood CLC common gating process. Substituted cysteine accessibility studies indicate that phosphorylation induces significant conformational changes in the CLH-3b subunit interface. Interface mutations known to disrupt common gating in other CLCs also disrupt phosphorylation-dependent channel regulation. Our results suggest that the unphosphorylated activation domain interacts with the Bateman domain. Phosphorylation disrupts this interaction and induces a Bateman domain conformational change that is transduced via the H-I loop into conformational changes at the subunit interface that inactivate CLH-3b via closure of the common gate.

35. On the Mechanism of Glutamate-dependent Extreme Acid Resistance of Enteric Bacteria. MING-FENG TSAI, CAROLE WILLIAMS, and CHRISTOPHER MILLER, *Brandeis University and Howard Hughes Medical Institute, Waltham, MA 02454*

To survive the harsh gastric environment before entering into the human intestine, enteric bacteria must invoke extreme acid-resistance systems. The potent glutamate-dependent system, known to protect *E. coli* incubated at pH 2.5 for several hours, requires the presence of a glutamate (Glu)-GABA antiporter GadC and a 1 H^+/2 Cl^- antiporter CLC. How do these transporters work together to produce acid resistance? Here, we developed an oriented GadC-reconstituted liposome system capable of holding a three-unit pH gradient (outside: pH 2.2; inside: pH 5.0) to investigate the transport behavior of GadC in a stomach-like environment, where GadC substrates are present in multiple protonation forms (outside: Glu^-, Glu^0, Glu^+; inside: $GABA^0$, $GABA^+$). By assessing the electrogenicity of Glu-GABA antiport, we demonstrate that GadC selectively imports Glu^0 while expelling $GABA^+$. In acid-resisting bacteria, the imported Glu^0 would bring into cytosol a H^+, which is consumed by Glu decarboxylases to covert Glu to GABA. $GABA^+$, which carries an intracellular H^+, is then exported by GadC, resulting in next extrusion of 1 H^+ in each GadC turnover to counter proton invasion into bacteria during acid shock. Glu^0/$GABA^+$ exchange

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is electropositive and therefore suppressed by negative membrane potentials. This fact implies that *E. coli*, with a large negative resting membrane potential, might need a mechanism to quickly depolarize the membrane upon acid exposure to stimulate H^+ pumping by GadC. Indeed, polarizing the membrane of *E. coli* transformed with KcsA, a K^+ channel, by reducing $[K^+]$ in the acid-shock medium drastically lowers cell survival rate. We postulate that the role of CLC in Glu-dependent acid resistance is to depolarize bacterial membrane through the 1 H^+/2 Cl^- antiport activity. Supporting this hypothesis, introducing KcsA into CLC knockout *E. coli* cells rescues the diminished acid survival with an extracellular K^+ condition that clamps bacterial membrane potential near neutrality.

36. Role of CFTR-mediated Cl^- Secretion in Innate Immunity in the Lungs. STEVEN WONG, JAMES SHI, CHRISTIAN SCHWARZER, ZHU FU, MARK GRABINER, AVA BROZOVICH, RYAN ARANT, EHUD ISACOFF, ISABELLA MAIELLARO, ALDEBARAN HOFER, BEATE ILLEK, and TERRY E. MACHEN, *Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720, and Department of Surgery, Brigham and Women's Hospital Harvard Medical School, Boston, MA 02115*

Flagellin from bacteria triggers innate immune responses in airway epithelia, including NF-κB signaling and secretion of cytokines. This work used nonCF, CF, and CFTR-corrected airway epithelia to test CFTR in flagellin-stimulated Cl^- secretion and the roles of cAMP and/or Ca^{2+} signaling. We used Ussing chambers to measure Cl^- secretion (I_{Cl}), fura-2 imaging to measure cytosolic $[Ca^{2+}]$ (Ca_{cyto}), ER-targeted cameleon D1 (K_D (Ca^{2+}) = 160 μM) to measure Ca_{er}, TIRF imaging to measure Stim1-GFP activation, and cytosol-targeted EPAC H30 to measure $cAMP_{cyto}$. Flagellin (10^{-6} g/ml) addition to nonCF (Calu-3) or CFTR-corrected CF (CFBE41o-) cells increased I_{Cl} by 10–40 μA/cm^2 within 30 min. The cAMP agonist forskolin (1 μM) elicited faster (2 min) and larger (100–200 μA/cm^2) increases in I_{Cl} in Calu-3 and CFTR-corrected CFBE41o- cells. CFTRinh172 blocked I_{Cl}. Neither flagellin nor forskolin stimulated I_{Cl} in CF (CFBE41o-) cells. Flagellin did not affect Ca_{cyto} but caused slow, partial reductions of Ca_{er} in Calu-3 cells and often activated Stim1-GFP. Flagellin caused slow, small increases in $cAMP_{cyto}$ (20% of forskolin). Flagellin caused larger increases in both $cAMP_{cyto}$ and I_{Cl} in the presence of a phosphodiesterase inhibitor (IBMX, 0.5 mM). Flagellin caused smaller increases in I_{Cl} in cells treated with PKA blocker RpcAMPS. It is concluded that flagellin → TLR5 → cAMP → PKA → CFTR → Cl secretion. Flagellin decreased Ca_{er} so slowly that Ca^{2+} was pumped out of the cell and Ca_{cyto} did not change. Decreases in Ca_{er} may be important because the resulting activation of Stim1 could activate store-operated cAMP production. Flagellin-activated Cl^- and osmotically obliged fluid secretion are expected to facilitate mucociliary clearance of bacteria. In cystic fibrosis when CFTR is inactive, reduced mucociliary clearance will lead to increased bacterial retention. CFTR may play a similar role in the lung airways during many inflammatory events.

Supported by NIH and CFRI.

37. Regulatory Phosphorylation Induces Conformational Changes at the Subunit Interface of a CLC Anion Channel. TOSHIKI YAMADA,[1] MANASI P. BHATE,[2] and KEVIN STRANGE,[1] *[1]Boylan Center for Cellular and Molecular Physiology, Mount Desert Island Biological Laboratory, Salisbury Cove, ME 04672; [2]Department of Chemistry, Columbia University, New York, NY 10027*

CLC anion channels are homodimers. Each subunit consists of 18 α-helical domains (designated "A-R") and forms an independently gated pore that is opened and closed by movements of a conserved glutamate residue. A poorly understood regulatory mechanism termed "common gating" "closes" both pores simultaneously and thus must involve relatively large quarternary conformational changes between channel subunits. CLH-3b is a *C. elegans* CLC-1/2/Ka/Kb channel subfamily member that is activated by meiotic cell cycle progression and cell swelling. Channel inhibition occurs by GCK-3–mediated phosphorylation of two serine residues located on the cytoplasmic C terminus linker connecting two cystathionine-β-synthase (CBS) domains, CBS1 and CBS2. These serine residues are part of a 14–amino acid activation domain that is required to maintain the channel in an activated state. Phosphorylation or deletion of the activation domain inactivates CLH-3b. Two conserved aromatic amino acid residues located on the intracellular loop connecting membrane helices H and I and the first α helix, α1, of CBS2 are required for transducing phosphorylation changes into changes in channel activity. Helices H and I form part of the interface between CLC channel subunits. Using a cysteine-less CLH-3b mutant, we demonstrate that the sulfhydryl reagent reactivity of substituted cysteines at the subunit interface changes dramatically during GCK-3–mediated channel inhibition and that these changes are prevented by mutation of the H-I loop/CBS2 α1 signal transduction domain. We also show that GCK-3 modifies Zn^{2+} inhibition, which is thought to be mediated by the common gating process. These and other results suggest that phosphorylation of the cytoplasmic C terminus inhibits CLH-3b by inducing subunit interface conformation changes that "close" the common gate. Our findings have important implications for understanding CLC regulation by diverse signaling mechanisms and for understanding the structure/function relationships that mediate long-range intraprotein communication in this important family of Cl^- transport proteins.

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38. Gating Mechanisms for the Hyperpolarization-activated Mutants of CLC-0. YAWEI YU, WEI-PING YU, and TSUNG-YU CHEN, *Center for Neuroscience and Department of Neurology, University of California, Davis, Davis, CA 95618*

CLC membrane proteins play important roles in various physiological processes such as regulating the excitability of skeletal muscles, facilitating the electrolyte transport across epithelial cells, and controlling the pH in intracellular compartments. Members in the CLC family include Cl^- channels and secondary active transporters that counter-transport Cl^- and H^+ across the membrane. Despite their different functional properties, CLC channels and transporters share a similar structural architecture. Experiments have also suggested that CLC channels may consist of Cl^-/H^+ antiporter activities coupled to the gating, which may be revealed by the extracellular or intracellular Cl^- and/or H^+ regulations of CLC channel gating. To explore this possibility, we examined the extracellular and intracellular pH and Cl^- effects on the hyperpolarization-activated mutants of CLC-0, in which the voltage dependence of channel opening is reversed compared with that of the WT channel. Similar to the WT CLC-0, extracellular pH and intracellular Cl^- shifted the P_o-V curve in these mutants, and these two effects interacted with each other. However, effects of intracellular pH and extracellular Cl^- on the gating of these mutant channels were minimal. These results may suggest that the coupled transports of inward Cl^- and outward H^+ movements are lost while the outward Cl^- and inward H^+ movements are still retained in these hyperpolarization-activated channel mutants. Interestingly, when a mutation was made on Y512 at the background of these hyperpolarization-activated mutations, the voltage dependence of the channel could be restored back to that of the WT channel. Given the importance of Cl^- at the central Cl^--binding site (S_{cen}) for Cl^-/H^+-coupled transport shown in bacterial CLC molecules, the restoration of voltage dependence by mutations of Y512 may be caused by an alteration of Cl^- occupancy in the pore.

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