Speaker Abstracts

1. CLC Chloride Channels: Structure, Function, and Dysfunction THOMAS J. JENTSCH, *Zentrum für Molekulare Neurobiologie, Universität Hamburg, Falkenried 94, D-20246 Hamburg, Germany* (Sponsor: Criss Hartzell)

The CLC gene family, originally identified by expression cloning of ClC-0 from *Torpedo*, is the only known gene family of voltage-gated Cl^- channels and has members in all phylae. The crystal structure of bacterial CLC proteins, which was recently elucidated by Dutzler et al., confirmed the double-barreled structure that was previously supported by single-channel recordings and mutagenesis of ClC-0. It provided fascinating insights into the anion-dependent gating mechanism. The bacterial structure was used to guide us in the recent characterization of an inhibitor binding site in ClC-1. Only recently binding partners were identified, e.g., barttin, a β-subunit of ClC-K channels that is necessary for surface expression.

CLC channels have broad physiological functions, as evident from human disease and mouse models. ClC-1 is important for muscle excitability, its disruption leading to myotonia in mice and man. In the case of the ubiquitously expressed ClC-2, there may be species differences: ClC-2 KO mice display male infertility and blindness, but there is no change in seizure threshold. By contrast, heterozygous loss of ClC-2 function was recently suggested to lead to epilepsy. ClC-K/barttin channels are essential for transepithelial transport in certain renal and inner ear epithelia, as concluded from different forms of Bartter's syndrome.

The remaining mammalian CLC channels, as well as the single yeast CLC, perform their function primarily in intracellular vesicles. Their main role may consist in shunting the electrical current of the H^+-ATPase. ClC-3 is not a swelling-activated plasma membrane channel, but resides in endosomes and synaptic vesicles. Its disruption results in profound neurodegeneration. ClC-5 disruption results in a defect in renal endocytosis in mice and men, because it is essential for endosomal acidification. Finally, ClC-7 is a lysosomal channel whose disruption leads to osteopetrosis and neurodegeneration in mice and man. More surprises are likely to come.

2. Structural Basis for Ion Conduction and Gating in ClC Chloride Channels R. DUTZLER,[1,2] E.B. CAMPBELL,[1] and R. MACKINNON,[1] [1]*HHMI and Laboratory of Molecular Neurobiology and Biophysics, The Rockefeller University, New York, NY 10021;* [2]*Institute of Biochemistry, University of Zürich, Zürich, Switzerland*

Members of the ClC family of voltage-gated chloride channels are found from bacteria to mammals with a considerable degree of conservation in the membrane-inserted, pore-forming region. We have solved the crystal structures of the ClC channels of *E. coli* and *S. typhimurium* at 3.5- and 3.0-Å resolution, respectively (Dutzler et al. 2002. *Nature.* 415:287–294) and the structure of the *E. coli* ClC channel in complex with a monoclonal Fab fragment bound to the channel at 2.5-Å resolution (Dutzler et al. *Science.* 2003. 300:108–112). The channels provide a structural framework for the entire family. The ClC channels are homodimeric proteins with an overall rhombus-like shape. Each ClC dimer has two pores, each contained within a single subunit. The ClC subunit consists of two roughly repeated halves that span the membrane with opposite orientations. This antiparallel architecture defines a chloride selectivity filter within the 15-Å neck of an hourglass shaped pore. Three Cl^- binding sites within the selectivity filter stabilize ions by interactions with α-helix dipoles and by chemical interactions with nitrogen atoms and hydroxyl groups of residues in the protein. The Cl^- binding site nearest the extracellular solution can be occupied either by a Cl^- ion or by a glutamate carboxyl group. Mutations of this glutamate residue in Torpedo ray ClC

channels alter gating in electrophysiological assays. These findings reveal a form of gating in which the glutamate carboxyl group closes the pore by mimicking a Cl^- ion. (Supported by grants from the National Institutes of Health [R. Mackinnon]. R. Mackinnon is an investigator of the Howard Hughes Medical Institute.)

3. Identification of an Inhibitor Binding Site in ClC-1 Reveals Conservation of Chloride Channel Structure RAÚL ESTÉVEZ,[1] BJÖRN C. SCHROEDER,[1] ALESSIO ACCARDI,[2] THOMAS J. JENTSCH,[1] and MICHAEL PUSCH,[1,2] [1]*Zentrum für Molekulare Neurobiologie Hamburg, ZMNH, Hamburg, Germany;* [2]*Instituto di Biofisica, Genova, Italy* (Sponsor: Peying Fong)

Several small organic acid molecules block the muscle channel ClC-1 with an affinity in the 10–50 μM range. Exploiting the difference in inhibition by 9-anthracene carboxylic acid (9-AC) between ClC-0, ClC-1, and ClC-$2\Delta_{16\text{-}61}$, we identified a serine between helices O and P as crucial for 9-AC binding. Changing S537 in ClC-1 to threonine reduced 9-AC affinity ~40-fold, whereas the converse mutation in ClC-$2\Delta_{16\text{-}61}$ (T518S) increased its affinity ~8-fold. In contrast, ClC-0_{T471S} remained 9-AC insensitive but showed, at −140 mV, an ~10-fold increased apparent affinity for p-chlorophenoxyacetic acid (CPA), another Cl^- channel inhibitor. Based on the crystal structure of bacterial CLCs, we performed extensive mutagenesis of ClC-1 to identify further residues affecting inhibitor binding. They surround a partially hydrophobic pocket close to the chloride binding site that is accessible from the cytoplasm, consistent with the observed intracellular block by 9-AC and CPA. Mutations in presumably Cl^--coordinating residues yield additional insights into the structure and function of ClC-1. Our work shows that the structure of bacterial CLCs can be extrapolated with fidelity to mammalian Cl^- channels. (Supported by grants from Telethon Italy [1079] [M. Pusch], MIUR Italy [FIRB RBAU01PJMS] [M. Pusch], Deutsche Forschungsgemeinschaft [T.J. Jentsch], and by the Prix Louis Jeantet de Médecine to T.J. Jentsch and a Marie Curie fellowship from the European Union for R. Estévez.)

4. Measuring Neuronal Chloride Signaling with Clomeleon, a Ratiometric, Genetically Encoded Indicator G.J. AUGUSTINE, R. DUNBAR, K. BERGLUND, L.S. LOO, G.-P. FENG, T. SCHAFER, W. SCHLEICH, and T. KUNER, *Department of Neurobiology, Duke University Medical Center, Durham, NC, and Department of Cell Physiology, Max-Planck-Institute for Medical Research, Heidelberg, Germany*

Chloride-ion fluxes are primarily responsible for synaptic inhibition in the brain. To image inhibitory synaptic processes, we have generated transgenic mice expressing Clomeleon, a genetically encoded, ratiometric optical indicator for $[Cl^-]$ under the control of a neuronal promoter (thy1). We have generated eight mouse lines that express Clomeleon in many brain areas, including cortex, hippocampus, cerebellum, thalamus, brain stem, and retina. The pattern of Clomeleon expression is different in each line, allowing measurement of $[Cl^-]$ in a diverse spectrum of neuronal types. Ratiometric imaging was used to determine resting $[Cl^-]_i$ of a variety of neurons in acute brain slices. After application of the ionophores tributyltin and nigericin, Clomeleon tracked $[Cl^-]$ of the external solution. This indicates that Clomeleon is functional after chronic expression in mice. To quantify developmental changes in $[Cl^-]_i$ in cortical pyramidal cells, slices were taken from animals ranging in age from P1 to P180. $[Cl^-]_i$ in these neurons decreased exponentially during development, starting at ~35 mM at P1, declining to ~7 mM at P15, and reaching a steady-state of ~4 mM at P30. These results are consistent with electrophysiological observations that inhibitory synapses switch from excitation to inhibition in early postnatal weeks. Measurements in pyramidal neurons of the amygdala show pronounced rises in $[Cl^-]_i$ in response to activation of $GABA_A$ receptors by the inhibitory neurotransmitter, GABA, which is consistent with the known fluxes of Cl^- through these receptors. In cerebellar slices, electrical stimulation of interneurons produced rises in $[Cl^-]_i$ in postsynaptic granule cells, as expected from the role of GABA receptors in this inhibitory circuit. In summary, Clomeleon mice are a powerful tool for studying neuronal Cl^- signaling in vitro and should also permit imaging of brain inhibitory circuitry in vivo.

5. Genetics and Regulation of Anion Exchange SETH L. ALPER, *Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA*

Transmembrane exchange of Cl^- for HCO_3^- controls pH, volume, and $[Cl^-]$ inside and outside cells. Anion exchangers are encoded by the *SLC4* and *SLC26* gene families. The *SLC4* family includes the Na^+-independent, electroneutral anion exchangers AE1, AE2, and AE3, electrogenic and electroneutral Na^+-bicarbonate cotransporters, and Na^+-dependent anion exchangers. All polypeptides in this gene family encode a long NH_2-terminal cytoplasmic domain, a transmembrane domain spanning the lipid bilayer 12–14 times, and a short COOH-terminal cytoplasmic tail. The Cl^-/HCO_3^- exchange function of erythroid AE1 (eAE1, band 3), the major intrinsic membrane protein of the red cell, increases the blood-carrying capacity for CO_2 from tissue capillaries to the lungs for expiration. The

NH_2 terminally truncated kAE1 variant is crucial for urinary acidification by the Type A–intercalated cell of the collecting duct. eAE1 is a homo-oligomer in the membrane and in detergent solution. The NH_2-terminal cytoplasmic domain of eAE1 interacts with multiple cytoskeletal proteins and with glycolytic enzymes. The transmembrane domain suffices to mediate Cl^-/Cl^- exchange, but bicarbonate transport requires carbonic anhydrase 2 (CA2) binding to the COOH-terminal cytoplasmic tail of at least one subunit within an AE1 homodimer.

Polymorphisms in the ectoplasmic loops of eAE1 encode minor blood group antigens. Mutations distributed throughout eAE1 protein cause the dominant erythroid conditions spherocytic anemia and ovalocytosis, without impairment of urinary acidification. Two distinct sets of AE1 mutations cause recessive and dominant distal renal tubular acidosis (dRTA). Recessive dRTA mutations are associated with near-normal eAE1 abundance and function, but renal loss-of-function is attributed to the absence in intercalated cells of the red cell-specific AE1-binding protein glycophorin A. In contrast, dominant dRTA AE1 mutants exhibit minimal dysfunction in red cells and *Xenopus* oocytes, but in these kAE1 mutants in epithelial cells can exhibit dominant negative phenotypes for surface trafficking and, perhaps, polarized targeting.

SLC4 Cl^-/HCO_3^- exchangers differ in their acute regulatory properties, and the emerging molecular bases for these differences will be summarized.

SLC4 homologs in nonmammalian organisms will be introduced.

The phylogenetically more ancient SLC26 family includes anion exchangers of broad specificity for monovalent and divalent anions. DTD/SLC26A2 mutations cause chondrodysplasias, DRA/SLC26A3 mutations cause congenital chloride diarrhea. Pendrin/SLC26A4 mutations cause congenital deafness with variably penetrant goiter. Pendrin is also expressed in the apical membrane of Type B–intercalated cells, and isolated perfused cortical-collecting ducts from bicarbonate-loaded knockout mice exhibit impaired upregulation of bicarbonate secretion. SLC26 anion exchangers of epithelial cell apical membranes can be regulated by CFTR activity, and their secondary dysfunction likely contributes to the pathology of cystic fibrosis. (Supported by National Institutes of Health grant DK43495.)

6. Regulation of the Na-K-Cl Cotransporter by Intracellular Chloride BIFF FORBUSH, IGNACIO GIMÉNEZ, BRIAN DOWD, RACHEL DARMAN, and ANDREAS FLEMMER, *Department of Cellular and Molecular Physiology, Yale University, New Haven, CT; Mt. Desert Island Biological Laboratory, Salsbury Cove, ME*

The Na-K-Cl cotransporter (NKCC) functions in concert with other membrane transporters and channels to maintain intracellular [Cl] and cell volume. NKCC is strongly activated by a decrease in cell [Cl] or by changes in cell volume. This regulation is mediated by means of phosphorylation of ~8 residues in the NH_2 terminus of NKCC1. We have identified three of these and confirmed their importance by mutagenesis–T_{189} (in shark) is required for activation of NKCC1, whereas T184 and T202 clearly play modulatory roles. We find that the corresponding residues in the renal isoform, NKCC2, are also necessary for the full response to hypertonicity and for cotransporter regulation by vasopressin. In addition, we see that trafficking of NKCC2 to the apical membrane accompanies activation in the renal epithelium. The NH_2 terminus of the transporter also includes binding sites for its regulatory phosphatase and kinase. NKCC1, but not NKCC2, has a PP1 binding site that we have found to enhance the regulatory inhibition of this phoshatase, and both NKCC1 and NKCC2 have a binding site for the kinases, PASK/OSR1. In recent experiments we have found that when overexpressed in HEK cells or oocytes, a dominant negative PASK mutant (DN-PASK) almost completely abolishes the ability of NKCC1 and NKCC2 to be up-regulated by decreasing Cl or changes in cell volume. This is fully overcome by inhibition of PP1 with calyculin A, demonstrating that DN-PASK interferes with NKCC phosphorylation. We also see that the phosphorylation of PASK is increased fivefold on incubation of HEK cells in low-Cl medium, indicating that PASK is a key link in the Cl-sensing pathway. (Supported by DK-47661 and DK17433.)

7. K-Cl Cotransporter Pathophysiology THOMAS BOETTGER, *Martin-Luther-University of Halle-Wittenberg, Centre of Medical Basic Research, Magdeburger Str. 18, D-06112 Halle/Saale, Germany*

The K-Cl cotransporters (KCC1-4) belong to a family of cation-chloride cotransporters with the well-known constituents NKCC1, NKCC2, and NCC, mutations of which cause deafness in mice, human Bartter's syndrome, and Gitleman's syndrome, respectively.

KCCs have roles in the regulation of intracellular chloride concentration, in transepithelial transport, and in cell volume regulation. Depending on extra- and intracellular concentrations of the electroneutrally transported ions, these cotransporters work close to the electrochemical equilibrium, hence can cotransport K^+ and Cl^- in both directions over the membrane. Now, based on the analysis of human mutations and of mu-

tant mice, we have insights into the physiological role of these transporters. The neuron-specific isoform KCC2 is essential for setting the intraneuronal chloride concentration. The increased Cl^- concentration in neurons of KCC2 KO mice results in an excitatory action of the otherwise inhibitory GABA and glycine receptors that are ligand gated chloride channels (Hübner et al. 2001. *Neuron.* 30:515–524). Mutation of KCC3 results in a human peripheral neuropathy associated with loss of the corpus callosum (ACCPN), which is an inherited neuropathological disease. A mouse model confirms the findings of the human syndrome (Howard et al. 2002. *Nat. Genet.* 32:384–392). Loss of KCC4 in mouse results in deafness and renal metabolic acidosis. As a part of the K^+-recycling pathway in the inner ear KCC4 protein may mediate the uptake of K^+ within the organ of Corti. In the cortical-collecting duct of the kidney KCC4 is essential for the recycling of Cl^- at the basolateral membrane of the α-intercalated cells that are crucial for H+ secretion in the kidney (Boettger et al. 2002. *Nature.* 416:874–878).

8. CLH-3, a ClC Anion Channel Activated during Oocyte Meiotic Maturation in *C. elegans* K. STRANGE, *Departments of Anesthesiology, Molecular Physiology and Biophysics, and Pharmacology, Vanderbilt University Medical Center, Nashville, TN*

Members of the ClC superfamily of voltage-gated Cl^- channels have been found in organisms as diverse as bacteria and humans. Despite their widespread expression and likely physiological importance, the function and regulation of most ClCs are incompletely understood. The nematode *C. elegans* offers significant experimental advantages for characterizing ClC biology. *C. elegans* oocytes express an inwardly rectifying Cl^- current with properties similar to those of mammalian ClC-2. RNA-mediated gene interference (RNA_i), single oocyte RT-PCR, heterologous expression, and immunofluorescence demonstrated that the oocyte channel is encoded by *clh-3*, one of six *C. elegans* ClC genes. Membrane expression of CLH-3 begins when oocytes are formed in the gonad and is lost rapidly around the time of fertilization. CLH-3 is activated by swelling, but plays no role in oocyte volume homeostasis. The physiological signal for activation of the channel is oocyte meiotic cell-cycle progression, a process termed meiotic maturation. CLH-3 is constitutively activated in maturing oocytes. Oocyte maturation induces ovulatory contractions of electrically coupled gonadal sheath cells. *clh-3* RNA_i disrupts the timing of sheath contractions, suggesting that the channel modulates ovulation via oocyte-sheath cell intercellular signaling pathways. CLH-3 may function to synchronize meiotic maturation with ovulation and fertilization. Activation of CLH-3 in response to cell swelling and cell cycle progression is mediated by serine/threonine dephosphorylation events brought about by the PP-1 type phosphatases CeGLC7α/β. Recently, we identified four proteins by yeast two-hybrid analyses that interact with the COOH terminus of CLH-3 and may regulate channel activity. These proteins include a STE20-like kinase, a putative kinase regulatory protein, a putative ubiquitin ligase, and a potential scaffolding protein. We are currently utilizing molecular biology, reverse genetics, protein chemistry, and patch-clamp electrophysiology to define how CLH-3 senses oocyte volume changes and cell-cycle progression and to identify the signaling pathways that regulate channel activity. (Supported by National Institutes of Health grants DK51610, DK61168, DK58212.)

9. Regulation of Cellular Nitrate by ClC Channels in Plants HELENE BARBIER-BRYGOO, MARION VINAUGER-DOUARD, ANNE MARMAGNE, CARINE ALCON, BARBARA BERNASCONI, ANDEOL FALCON DE LONGEVIALLE, LIONEL GISSOT, AUDE LEZIE, MICHELE ALLOT, JEAN-MARIE FRACHISSE, SEBASTIEN THOMINE, and GENEVIEVE EPHRITIKHINE, *Institut des Sciences du Végétal, CNRS UPR2355, 91198 Gif sur Yvette, France*

Anion channels are well documented in various tissues, cell types, and membranes of algae and higher plants, and current evidences support their central role in cell signaling, osmotic processes, nutrient uptake and metal tolerance (for review see Barbier-Brygoo et al. 2000. *Biochim. Biophys. Acta.* 1465:199–218). In contrast to animals for which different classes of anion channel proteins have been identified, data on the molecular structure of such channels in plants have only started to emerge. In the entirely sequenced genome of the model plant *Arabidopsis thaliana*, seven genes belonging to the CLC family have been identified (*AtCLC-a* to *-g*). Our group is currently developing functional analyses for all members of this family.

Semiquantitative PCR analyses showed that AtCLC genes are broadly expressed all over the plant, but display differential regulations by various effectors (anions, salicylic acid, sucrose). Transient expression of AtCLC::GFP (green fluorescent protein) fusion proteins in plant cells revealed that AtCLC-a is targeted to the vacuolar membrane, whereas AtCLC-e localizes in the chloroplast envelope, and AtCLC-f in Golgi vesicles. AtCLC-f was able to functionally complement the yeast *gef-1* mutant, suggesting that the protein displays a

CLC-type function. Phenotype analyses of knock-out mutants revealed that both *clca-1* and *clce-1* mutants display hypersensitivity to salicylic acid and reduced nitrate content (up to 50%) in root and shoot tissues. These data suggest that AtCLC-a and AtCLC-e channels might play a general role in the regulation of the cellular status of nitrate, a central element of plant cell metabolism. Ongoing work on all AtCLC proteins, including analyses of new mutants, studies of expression patterns at the tissular, cellular, and subcellular levels, together with the search for protein partners will provide new insights into the roles of these channels throughout plant development. (Supported by CNRS and Génoplante AF2001065 grants.)

10. Chloride-dependent Copper Incorporation into the Yeast Multicopper Oxidase Fet3p JERRY KAPLAN and SANDRA DAVIS KAPLAN, *Department of Pathology, School of Medicine, University of Utah, Salt Lake City, UT 94132*

The budding yeast *Saccharomyces cerevisiae* accumulates iron through a high-affinity iron transport system comprised of the multicopper oxidase Fet3p and the transmembrane permease Ftr1p. The multicopper oxidase Fet3p obtains its copper in a trans-Golgi vesicle. Genetic experiments have shown that Gef1p, a member of the family of voltage-gated chloride channels, is required for the copper loading of apoFet3p. Experiments in vitro demonstrate that chloride is essential for the copper loading of isolated apoFet3p. Fet3p contains four coppers whose properties are defined by the amino acids that ligate the copper. HoloFet3p contains one type-I copper, one type-II copper and two type-III coppers. A construct of Fet3p lacking the transmembrane domain is secreted from cells as holoFet3p containing four atoms of copper/molecule. Fet3p secreted from cells lacking the trans-Golgi copper transporter Ccc2p contains only one copper/molecule. Addition of copper in vitro in the absence of chloride results in the presence of an additional copper atom/molecule. Addition of chloride permits the formation of a holoFet3p containing four atoms of copper/molecule. Generation of site-specific mutants of Fet3p in combination with in vitro copper loading has identified both chloride-dependent and chloride-independent binding sites. The copper loading of type-I and -II copper sites is chloride dependent, whereas copper loading of at least one of the type-III coppers is chloride independent.

We took advantage of secreted Fet3p to determine if the lack of Gef1p affects copper loading because of the requirement of chloride for metallation or because Gef1p is required for ion homeostasis, as the absence of chloride would affect vesicular pH and copper concentration. Secreted Fet3p obtained from cells lacking Gef1p is missing both chloride-dependent and -independent coppers. This result demonstrates that while chloride is required for the direct metallation of apoFet3p, lack of chloride affects vesicular ion homeostasis. (Supported by a grant from the National Institutes of Health [NIDDK-DK 30534].)

11. Chloride Concentration in Endosomes and Golgi A.S. VERKMAN and N.D. SONAWANE, *Departments of Medicine and Physiology, Cardiovascular Research Institute, University of California, San Francisco, CA*

We developed ratioable fluorescent indicators to measure chloride concentration in organellar compartments using the acridinium chromophore. The indicators are long-wavelength, chloride sensitive (0 to >100 mM), and pH insensitive. Fluorophores were linked to dextran to target to fluid-phase endosomes, to transferrin for early/recycling endosomes, to macroglobulin for late endosomes, to cholera toxin B subunit for Golgi, and to lipids for macropinosomes containing DNA lipopolexes. Also, pH-sensitive fluorescent indicators were synthesized for comparative measurements. Chloride in transferrin-labeled early/recycling endosomes was 20 mM just after internalization, increasing to 41 mM over ~10 min in parallel to a drop in pH from 6.91 to 6.05. The low chloride just after internalization was prevented by reducing the interior-negative Donnan potential. Chloride in macroglobulin-labeled endosomes, which enter a late compartment, increased from 28 to 58 mM at 1–45 min after internalization while pH decreased from 6.85 to 5.20. Chloride accumulation was prevented by bafilomycin but restored by valinomycin. A chloride channel inhibitor slowed endosomal acidification and chloride accumulation by ~2.5-fold. Chloride was 49 mM and pH was 6.42 in Golgi; Golgi chloride accumulation and acidification were reversed by bafilomycin. The data provide evidence that chloride is the principal counter-ion accompanying endosomal and Golgi acidification, and that an interior-negative Donnan potential is responsible for low endosomal chloride early after internalization. Reduced chloride and volume in early endosomes may permit endosomal acidification and chloride accumulation without lysis. Studies in macropinosomes containing DNA lipoplexes provide direct evidence in support of the proton-sponge hypothesis for nonviral gene delivery. Lipids containing buffered animes (PEI but not polylysine) cause slowed acidification in lipoplex-containing macropinosomes with increased chloride accumulation and volume. Our data provide the first

information about the value and determinants of organellar chloride concentration. Direct measurements of organellar chloride concentration should be useful be useful in defining the cellular physiology of organellar chloride channels.

12. Role of ClC-3 as an Intracellular Ion Channel STEVEN A. WEINMAN and XINHUA LI, *Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, TX 77555-0641*

ClC-3 is a ubiquitously expressed chloride channel that, along with its close family members ClC-4, and ClC-5, constitutes one branch of the ClC family. While ClC-3 has been detected at the plasma membrane, its primary site of localization is intracellular. Heterologous overexpression of ClC-3 in multiple different cell types results in the formation of enlarged vesicular structures that have the properties of late endosomes or lysosomes. These structures are specific for ClC-3 and are not formed by expression of either ClC-4 or ClC-5. The ClC-3–associated intracellular vesicles have high chloride permeability and acidification and expansion of these vesicles depends on active proton pumping and ClC-3 chloride channel activity. ClC-3 thus functions to facilitate acidification of an endosomal/lysosomal compartment. The intracellular distribution of ClC-3 is determined by the interaction of localization motifs on the NH^2- and COOH-terminal cytosolic domains with specific adaptor proteins of the AP family. Three different splice variants of ClC-3 have been identified, a short form (ClC-3S), a long form containing an additional 58 aa NH^2-terminal domain (ClC-3L or ClC-3A) and a variant of the long form with an alternate COOH-terminal domain (ClC-3B). Several tyrosine motifs are present in the NH^2- and COOH-terminal cytosolic segments of ClC-3 and these interact with the μ subunits of AP-1, AP-2, and AP-4 to determine channel distribution between multiple sites of the secretory and endocytic pathways. (Supported by National Institutes of Health grant DK42917.)

13. ClC-3 and Volume-regulated Cl^- Channels: The Minority Report J.R. HUME, S. YAMAMOTO-MIZUMA, G.-X. WANG, C.M. SATTERWHITE, D. DUAN, F. BRITTON, I. YAMBOLIEV, W. HATTON, and B. HOROWITZ, *Center of Biomedical Research Excellence, Departments of Pharmacology, Physiology and Cell Biology, University of Nevada School of Medicine, Reno, NV 89557-0270*

ClC-3, a ubiquitously expressed member of the ClC Cl^- channel superfamily, has been proposed as a molecular candidate for volume-sensitive osmolyte and anion channels (VSOACs) in some cells. However, the reported presence of native VSOACs in at least two cell types from transgenic ClC-3 disrupted (*Clcn3*$^{-/-}$) mice (Strobrawa et al. 2001. *Neuron.* 29:185–196), has cast doubt on this proposed role for ClC-3. We have taken three new approaches to reexamine the possible role of endogenous ClC-3 as a molecular candidate responsible for some types of native VSOACs. First, two newly developed anti–ClC-3 antibodies (Ab) are shown to block native VSOACs when dialyzed intracellularly in cardiac and smooth muscle myocytes. Second, a variety of new evidence will be presented linking endogenous xClC-3 (*Xenopus* isoform) to native VSOACs in *Xenopus* oocytes. Third, a comparison of several properties of native VSOACs in cardiac and pulmonary arterial smooth muscle cells from *Clcn3*$^{+/+}$ and *Clcn*$^{-/-}$ mice (Dickerson et al. 2002. *Brain Res.* 958:227–250) reveals that in response to loss of expression of endogenous ClC-3-mediated VSOACs, another protein may be upregulated that gives rise to a novel VSOAC subtype in cardiac and smooth muscle cells in *Clcn3*$^{-/-}$ mice. These latter findings emphasize that caution needs to be exercised in attempts to interpret the phenotypic consequences of conventional *Clcn3* gene inactivation. We conclude that endogenous ClC-3 remains a viable molecular candidate responsible for VSOAC subtypes normally expressed in cardiac and smooth muscle cells, and *Xenopus* oocytes. (National Institutes of Health grants HL 49254 and NCRR P20 RR15581.)

14. Interaction of the Yeast CLC Channel with an ATPase Protein JUTTA METZ, ANDREA WAECHTER, and BLANCHE SCHWAPPACH, *Zentrum für Molekulare Biologie, Universität Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany* (Sponsor: Criss Hartzell)

In contrast to cation channels, little is known about accessory proteins involved in the diversity of CLC chloride channel function and subcellular localization. As revealed by the recently solved three-dimensional structure of a prokaryotic CLC channel (Dutzler et al. 2002. *Nature.* 414:287–294) CLC channels exist as a dimer. The cytosolic COOH terminus of most archeal and eukaryotic CLC proteins contains two copies of a conserved CBS domain (cystathione beta synthetase; Bateman. 1997. *Trends Biochem. Sci.* 22:12–13; Ponting. 1997. *J. Mol. Med.* 75:160–163), whereas most bacterial CLC homologues lack the long COOH terminus with the CBS domains. The occurrence of such CBS domains in a number of functionally and structurally unrelated proteins has remained enigmatic. Since they mediate homomultimeric interactions in some enzymes they have been suggested to function as protein–protein interaction modules.

We have employed a modified version of the yeast two-hybrid system to detect interaction partners that recognize the multimeric COOH terminus of the yeast CLC homologue Gef1p. Yeast contains one single gene for a CLC channel, which is involved in ion homeostasis of late Golgi and prevacuolar compartments. We have identified a small dimeric ATPase protein that interacts with the yeast CLC channel. The CBS domains of Gef1p contribute to the interaction. Characterization of the respective knockout strains shows that the ATPase is not required for the role of Gef1p in high-affinity iron uptake. The results rather suggest that the Gef1p–ATPase complex is involved in toxic-cation resistance in yeast. Subcellular fractionation and microscopy demonstrate that the ATPase localizes to the endoplasmic reticulum. Our data implies that chloride transport to the lumenal phase may be regulated by the proteolytic processing of the channel (see accompanying abstract by A. Waechter and B. Schwappach) as well as by the interaction with the ATPase.

15. Neurotransmitter-gated Chloride Channels in Postsynaptic Membranes HEINRICH BETZ, BODO LAUBE, RUDI SCHEMM, JOANNA GRUDZINSKA, MATTHIAS KNEUSSEL, JENS FUHRMANN, and GREG O'SULLIVAN, *Department of Neurochemistry, Max-Planck-Institute for Brain Research, D-60528 Frankfurt, Germany*

Glycine and GABA inhibit neuronal firing by activating ligand-gated chloride channels in the postsynaptic membrane. Different subtypes of glycine and GABA-A receptors result from the regulated assembly of distinct subunit combinations. This process occurs at the ER, from which the receptors are transported to the plasma membrane via the secretory pathway, with ER exit depending on specific export sequences. Subsequent localization of the receptors at developing synapses requires anchoring of the plasma membrane–inserted proteins at selected sites of nerve terminal contact. Formation of a submembraneous scaffold of the receptor-associated protein gephyrin has been found to be essential for the clustering of glycine and GABA-A receptors at developing postsynaptic sites. Surface receptor numbers may be controlled by ubiquitination, a modification that has been found to precede internalization of glycine receptors in *Xenopus* oocytes. The mechanisms and interacting proteins implicated in the regulation of the intracellular transport, synaptic localization, and surface numbers of these neuronal chloride channel proteins will be discussed with particular consideration of recent structural data obtained by X-ray crystallography and homology modelling.

16. CFTR Chloride Channel Regulation by Intramolecular and Intermolecular Interactions KEVIN L. KIRK, *Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, AL*

The CFTR chloride channel is a major regulator of salt and water transport across epithelial tissues. Mutations in the cftr gene cause several human disorders including cystic fibrosis (CF). CFTR is an ABC transporter (albeit the only known ion channel in this transporter superfamily) and, like other ABC transporters, this protein has two membrane-spanning domains that form the translocation pathway and two nucleotide binding domains (NBDs) that control the gating of this pathway. In addition to these minimal components of an ABC transporter, CFTR has two fairly long cytoplasmic tails and a central regulatory (R) domain. These unique regions provide additional layers of channel regulation (e.g., the R domain contains multiple phosphorylation sites) as well as binding sites for interacting proteins that influence the intracellular location and/or functional activity of CFTR. However, the benefits of this extra stuff on the CFTR polypeptide come with a cost; namely, mutations in these regions can cause disease (e.g., CF). These general points will be illustrated by discussing our recent studies of a small piece of the CFTR puzzle, the amino terminal cytoplasmic tail (N-tail). Specific topics to be discussed will be: (a) the modulation of channel gating by a helical subdomain in the N-tail; a functional property that appears to be mediated by intramolecular interactions with components of the core gating machinery (R domain and NBDs); (b) the role of the N-tail as a binding site for other proteins (e.g., SNARE proteins) that may influence CFTR location and/or function via intermolecular interactions; (c) the essential role of this cytoplasmic tail in channel biosynthesis and maturation; and (d) the evidence that mutations in this region of the CFTR polypeptide can cause disease either by disrupting channel biosynthesis or by reducing channel activity.

17. Bartter Syndrome with Sensorineural Deafness Is Caused by Mutations in the Gene (*BSND*) for a Novel β-subunit (Barttin) of the CLC-KB and CLC-KA Chloride Channels FRIEDHELM HILDEBRANDT, *Departments of Pediatrics and Human Genetics, University of Michigan, Ann Arbor, MI*

Severe antenatal Bartter syndrome (BS) is an autosomal recessive salt-losing nephropathy. Mutations in the genes for the Na/K/2Cl cotransporter (NKCC2), the apical inwardly rectifying ATP-regulated potassium channel ROMK, and the basolateral chloride channel CLC-Kb have been identified as responsible for BS

types 1, 2, and 3, respectively. All three genes are expressed in the medullary thick ascending limb of Henle's loop (mTAL). Disease gene identification has led to new insights into renal salt handling, diuretic action, and blood pressure regulation. A gene locus for a fourth variant (BSND or BS type 4), associated with sensorineural deafness and end-stage renal failure, was mapped to chromosome 1p31.

We identified by positional cloning a novel gene (*BSND*) as causative for BSND (Birkenhaeger et al. 2001. *Nat. Genet.* 29:310). *BSND* is unrelated to any known genes. It is most prominently expressed in kidney. The presence of two NH_2-terminal putative transmembrane domains in the gene product indicated a potential role as a putative transport protein or regulator of transport.

Functional studies performed by the group of T. Jentsch showed that the gene product Barttin acts as an essential β-subunit for ClC-Kb and ClC-Ka chloride channels, thus representing the first known β-subunit for a ClC chloride channel (Estevez et al. 2001. *Nature.* 414:558–561 and 502). Barttin colocalizes with ClC-Kb/-Ka to basolateral membranes of renal tubules and of potassium-secreting epithelia of the inner ear. Disease-causing mutations in either ClC-Kb or barttin compromise currents through these heteromeric channels.

ClC-Kb (the gene of which is mutated in BS type 3) and not ClC-Ka, are expressed in mTAL. In marginal cells of the inner ear, both ClC-Kb and ClC-Ka, are expressed and may partially substitute for each other's function. The finding that barttin acts as an essential β-subunit for both channels might explain why *BSND* mutations lead to sensorineural deafness in addition to the BS phenotype. Barttin might represent a potential target for diuretic agents.

18. The Role of GABA Receptors in Human Epilepsy ROBYN H. WALLACE, *Center of Genomics and Bioinformatics, Department of Anatomy and Neurobiology, University of Tennessee Health Science Center, Memphis, TN*

GABA is the primary inhibitory neurotransmitter in the central nervous system. The GABA(A) receptor, a ligand-gated chloride ion channel, is the major postsynaptic receptor for GABA. Recent findings have shown that mutations in GABA(A) receptors are associated with generalized epilepsies and febrile seizures. Four different mutations in the γ-2 subunit (GABRG2) and one in the α-1 subunit (GABRA1) have been described. One of the mutations, located in the benzodiazepine binding domain of GABRG2, abolished in vitro sensitivity to diazepam, raising the possibility that endozepines do in fact exist and have a physiological role in preventing seizures (Wallace et al. 2001. *Nat. Genet.* 28:49–52). This mutation did not alter the amplitude of GABA-activated currents. This is in contrast to the other epilepsy-associated mutations in GABA(A) receptor subunits, which all result in reduction of current amplitude in response to GABA. Two of the mutations resulted in truncation of the GABRG2 protein. One of these truncating mutations introduces a premature stop codon in the intracellular loop between the third and fourth transmembrane domains of the GABRG2 subunit, and fluorescent-microscopy studies have shown that receptors containing the truncated GABRG2 protein are retained in the lumen of the endoplasmic reticulum (Harkin et al. 2002. *Am. J. Hum. Genet.* 70:530–536). The most recently described mutation is a single amino acid substitution in the third transmembrane domain of the GABRA1 receptor (Cossette et al. 2002. *Nat. Genet.* 31:184–189). Identifying these GABA receptor mutations has provided insight into chloride channel function and shown how disruption of this process can result in human disease. Further studies in animal models expressing the GABA receptor mutations will aid in our understanding of chloride channel function and epilepsy.

19. Glycine Receptors and Startle Disease PETER R. SCHOFIELD, *Garvan Institute of Medical Research, 384 Victoria Street, Sydney 2010, Australia*

Human startle disease or hyperekplexia is caused by mutations within the glycine receptor (GlyR) α1 subunit. 11 different disease-causing mutations have been identified and most flank the M2 transmembrane domain of the receptor. Expression of the R271L and R271Q mutations results in receptors with impaired glycine responses in which the agonists β-alanine and taurine behave as competitive antagonists. The distribution of single channel conductances is also determined by R271. Dominant K276E or Y279C mutations result in GlyRs which have marked reductions in glycine sensitivity and fully transform the agonists β-alanine and taurine into competitive antagonists, whereas the recessive mutation I244N converts the actions of β-alanine and taurine into partial agonists. The Q266H mutation, located in the M2 domain, also partially disrupts channel function by a reduction in the open channel lifetimes. Alanine-scanning mutagenesis of the M2-M3 and M1-M2 loops revealed additional residues that disrupt signal transduction. These naturally occurring and induced mutations demonstrate that both the extra- and intracellular domains form part of an allosteric switch responsible for inducing a conformational change in the receptor upon the binding of an agonist. This con-

formational change has been further demonstrated by cysteine substitution mutagenesis that showed that the surface accessibility of residues in the M2-M3 loop was increased in the channel open state. Recently, a GlyR β subunit compound mutation, comprising a missense mutation (G229D) and a splice site mutation (IVS5+5G>A), has been identified that results in hyperekplexia. Electrophysiological analysis showed reduced agonist sensitivity, suggesting that β subunits may also play a functional role in ligand activation. Our understanding of the mechanism of action of the GlyR and other ligand-gated ion channel receptors has been greatly aided by the characterization of naturally occurring and induced mutations. (Supported by NHMRC.)

20. The ClC-7 Chloride Channel as a Regulator of Bone Resorption in Mice and Man UWE KORNAK,[1,4,5] DAGMAR KASPER,[1] ANSGAR SCHULZ,[3] GÜNTER DELLING,[2] MARIE-CHRISTINE DE VERNEJOUL,[4] and THOMAS J. JENTSCH,[1] [1]*Center for Molecular Neurobiology Hamburg (ZMNH), University Hamburg, Germany;* [2]*Department for Osteopathology, Institute for Pathology, University Hamburg, Germany;* [3]*Children's Hospital, University Ulm, Germany;* [4]*Inserm U349, Hôpital Lariboisière, Paris, France;* [5]*Max Planck Institute for Molecular Genetics, Berlin, Germany*

ClC-7 is a ubiquitously expressed chloride channel that is mainly localized in late endosomes and lysosomes. *Clcn7*$^{-/-}$ mice show a severe osteopetrosis (marble bone disease) that becomes apparent shortly after birth (Kornak et al. 2001. *Cell.* 104:204–215). Although osteoclasts are present in normal numbers, they fail to resorb bone. In osteoclasts, ClC-7 is highly expressed in the ruffled membrane that is formed by the fusion of H^+-ATPase containing late endosomal vesicles. We could show that chloride ions conducted by ClC-7 provide the necessary countercharge to allow the H^+-ATPase to efficiently pump large amounts of protons into the resorption lacuna. The murine phenotype closely resembles human infantile malignant osteopetrosis. A screening for mutations in the human gene, *CLCN7*, was performed in 18 patients suffering from this disease. Two patients were compound heterozygous for a nonsense mutation and two different missense mutations. Two of these mutations lead to a complete loss of the ClC-7 protein in cultured patient fibroblasts corresponding to the loss of ClC-7 in *Clcn7*$^{-/-}$ mice. Recently, mutations in *CLCN7* were found to cause also the much milder autosomal dominant form of osteopetrosis (ADOII) (Cleiren et al. 2001. *Hum. Mol. Gen.* 10:2861–2867). Investigation of primary osteoclasts from several ADOII patients revealed that ClC-7 levels and subcellular localization are not detectably altered. Nevertheless, their resorptive activity is diminished. This implies that dominant mutations most likely perturb the electrophysiological function of ClC-7 and that changes in the chloride conductance of the ruffled membrane are able to regulate bone resorption and bone density.

21. Chloride Channels and Sickle-cell Anemia PALLE CHRISTOPHERSEN, *NeuroSearch, Pederstrupvej 93, DK 2750 Ballerup, Denmark*

The sickle mutation (S) is a glutamate to valine exchange in position 6 of the β-chain of human hemoglobin (Hb). Homozygotes have sickle-cell disease characterized by anemia and frequent ischaemic crises. Blood from patients contain morphologically abnormal red blood cells (RBCs), including the classical sickle shape reversibly formed by deoxygenation. Dehydration strongly promotes sickling, since the rate of HbS polymerization is proportional to a high-power function of the Hb-concentration. A population of dehydrated, irreversibly sickled RBC are supposed to be a main player in the precipitation of vaso-occlusive crises. RBC dehydration occurs as a consequence K^+ and Cl^- loss, at least partly, via the Ca^{2+}-activated K^+-channels (hIK, Gárdos channel) in parallel with the red cell Cl^- conductance (g_{Cl}, molecular identity unclear). The question was if g_{Cl} blockers would ameliorate dehydration of sickle cells in vitro and in vivo and if a block might represent a nontoxic, antisickling strategy to the treatment of sickle cell patients. Using a prototype compound, NS1652, it was shown that prevention of salt loss and dehydration of sickle cells was possible in vitro. In order to evaluate the concept of g_{Cl} block in vivo, SAD-mice (a transgenic sickle cell model) was treated for 3 wk with NS3623, an in vivo bio-available and stable analogue of NS1652. When administered P.O. in doses between 20 and 200 mg/kg/d, the erythrocyte volume and the total concentrations of cellular Na^+ and K^+ increased significantly and the mean intracellular Hb concentration decreased. Morphologically, a change from highly sickled to well hydrated nonsickled forms was seen. A left-shift of the RBC density distribution was observed and the fraction >1.1 g/cm^3 disappeared after 21 d of treatment. The experiments demonstrate the feasibility of in vivo improvement of sickle cell hydration and reduced sickling by a g_{Cl} inhibitor.

22. Molecular Physiology of Calcium-activated Chloride Channels H. CRISS HARTZELL, ZHIQIANG QU, RAYMOND WEI, WESLEY MANN, and RODOLPHE

FISCHMEISTER, *Department of Cell Biology, Emory University School of Medicine, Atlanta, GA 30322*

Calcium-activated chloride channels serve a diverse variety of important physiological functions, including chloride and fluid secretion from epithelial cells, action potential repolarization in some neurons and cardiac muscle, olfactory transduction, and fast block to polyspermy in Anurans (Fuller. 2002. *Curr. Top. Membr.* 53:1–441). Unfortunately, the molecular identity of calcium-activated chloride channels remains unresolved. Two different families of molecules have been proposed to form calcium-activated chloride channels when expressed in heterologous systems: the CLCA family and bestrophins (Sun et al. 2002. *Proc. Natl. Acad. Sci.* 99:4008–4013). We identified bestrophin as a putative chloride channel from database searches using a degenerate M2 domain from ligand-gated anion channels. Vertebrate bestrophins are ~500 amino acid proteins that fall into four different subfamilies. The first ~300 amino acids are very highly conserved among species and subfamilies, while the COOH terminus is quite variable. We cloned two subfamily-2 bestrophins from *Xenopus laevis* and expressed them heterologously in HEK-293 cells. Using antibodies raised against unique COOH-terminal peptides, the *Xenopus* bestrophins were found to be located at the cell surface. We predict that bestrophins have six transmembrane domains with the NH_2 and COOH termini oriented cytosolically. The invariant RFP sequence following the fourth transmembrane domain is hypothesized to be involved in anion selectivity. *Xenopus* bestrophins expressed in HEK-293 cells induce calcium-activated chloride currents. The EC_{50} for activation of the current by calcium is ~250 nM. The currents have linear current-voltage relationships and are time- and voltage-independent at all [Ca]. In this regard, the bestrophin-induced currents differ from calcium-activated chloride currents in *Xenopus* oocytes and epithelial cells. In this presentation, the role of bestrophins in mediating calcium-activated chloride currents will be discussed. (Supported by National Institutes of Health grant GM60448.)

23. Physiological Role of CLC-K Chloride Channels in the Kidney SHINICHI UCHIDA, *Department of Nephrology, Tokyo Medical and Dental University, School of Medicine, Tokyo, Japan*

Chloride channels in the kidney are involved in important physiological functions such as cell-volume regulation, acidification of intracellular vesicles, and transepithelial chloride transport. Among nine mammalian CLC chloride channels, three (CLC-K1, CLC-K2, and CLC-5) were identified to be related to kidney diseases in humans or mice. Of these, the CLC-K channels are localized in plasma membranes of tubular cells and mediate transepithelial chloride transport. CLC-K1 is present in the thin ascending limb of Henle's loop (tAL), the nephron segment with the highest transepithelial chloride permeability. According to our comparison of the functional characteristics of CLC-K1 expressed in *Xenopus* oocytes with the characteristics of chloride permeability elucidated by the in vitro perfusion studies of the tAL (Uchida et al. 1995. *J. Clin. Invest.* 95:104–113), CLC-K1 is very likely to be responsible for the transepithelial chloride transport in the tAL. This was later confirmed by our analysis of CLC-K1 knockout mice (Matsmura et al. 1999. *Nat. Genet.* 21: 95–98) Furthermore, CLC-K1 mice showed nephrogenic diabetes insipidus, clearly demonstrating that chloride transport in the tAL was essential for the urinary-concentrating mechanisms in the kidney. Unlike the case with CLC-K1, intrarenal localization of CLC-K2 could not be clearly presented since its high homology with CLC-K1 made it difficult to generate CLC-K2–specific antibody. Accordingly, precise intrarenal localization of CLC-K2 was initially determined using CLC-K1 knockout mice. CLC-K2 was identified as a basolateral chloride channel in distal nephron segments (Kobayashi et al. 2001. *J. Am. Soc. Nephrol.* 12:1327–1334), and the identification of CLC-KB (a human homologue of rat CLC-K2) as a causative gene for Bartter syndrome proved that CLC-K2 is required for chloride reabsorption. Barttin, a fourth gene responsible for Bartter syndrome, was recently identified as a beta-subunit of CLC-K channels. This paper also describes interaction of barttin and CLC-K2 and clearly demonstrates the molecular pathogenesis of Bartter syndrome caused by barttin mutations.

24. The Physiological Role of ClC Channels in CF-affected Epithelia RAHA MOHAMMAD-PANAH, SONJA DHANI, NAJMA AHMED, KATALIN GYOMOREY, MOHABIR RAMJEESINGH, CANHUI LI, LING-JUN HUAN, YANCHUN WANG, and CHRISTINE E. BEAR, *Program in Structural Biology and Biochemistry, Hospital for Sick Children and University of Toronto, Toronto, Canada* (Sponsor: Criss Hartzell)

CFTR functions as a phosphorylation and nucleotide-regulated chloride channel in the plasma membrane of epithelial cells**.** Mutations that cause the loss of CFTR function on the apical membrane cause cystic fibrosis, a disease characterized by the plugging of tubular organs with thick sticky mucus. ClC-2, ClC-3, ClC-4, and ClC-5 are expressed in intestinal and airway epithelia,

tissues that are normally affected in cystic fibrosis and there has been considerable speculation that the expression of ClC channels may influence the severity of CF disease, possibly by conferring an alternative pathway for chloride ion flux. Confocal and electron micrographs of rodent and human intestinal epithelia revealed that ClC-2 and ClC-4 are expressed in the apical membrane of the intestine. Further, using patch clamp electrophysiology and an antisense strategy, we determined that these channels are functional in the plasma membrane. Therefore, these channels are poised to functionally compensate CFTR and future studies are required to define pharmacological tools for their activation.

ClC channels may also affect CF disease severity by modifying the regulated trafficking of CFTR mutant proteins. The functional expression of CFTR and the major mutant (CFTRΔF508) at the cell surface is regulated by endosomal trafficking, endocytosis into a sorting endosomal pool from which it can be directed for subsequent degradation, or recycled to function again at the cell surface. Our recent work shows that ClC-4 and ClC-5 are expressed in the apical membrane and endosomal vesicles of CF-affected tissues including the respiratory and gastrointestinal epithelia and function to regulate the trafficking of membrane proteins. Therefore, in our future work we plan to assess the role of these and related ClC channels in the regulated trafficking of normal and mutant versions of CFTR.

(This research was supported by operating grants to C.E. Bear awarded by National Institutes of Health [#P50 DK49096] and CIHR.)

25. Pharmacology of Chloride Channels Belonging to the CLC Family DIANA CONTE CAMERINO,[1] ANNAMARIA DE LUCA,[1] SABATA PIERNO,[1] ANTONELLA LIANTONIO,[1] ALESSIO ACCARDI,[2] ALESSANDRA PICOLLO,[2] and MICHAEL PUSCH,[2] [1]*Section of Pharmacology, Department of Pharmacobiology, University of Bari, Italy;* [2]*Institute of Biophysics-CNR, Genova, Italy*

Many unresolved questions about the function of the chloride channels belonging to the CLC family are partially caused by the lack of specific pharmacological agents. The most studied classes of organic substances that affect CLC channels are the 9-anthracenecarboxylic acid (9AC) and the 2-(p-chlorophenoxy)propionic acid (CPP) derivatives. CPP stereoselectively modulates the macroscopic chloride conductance (gCl) sustained by the muscle CLC-1 channel (Conte Camerino et al. 1988. *Pfluegers Arch.* 413:105–107). In particular, S(−)CPP blocks gCl of native skeletal muscle fibers in a concentration-dependent manner whereas the R(+) enantiomer produces a typical biphasic effect, indicating the presence of two different binding sites able to enhance and decrease, respectively, channel activity. Phosporylation-dephosporylation pathways physiologically regulate the activity of CLC-1 (Rosenbohm et al. 1999. *J. Physiol.* 514:677–685) modulating also its sensitivity to the CPP enantiomers (De Luca et al. 1998. *Br. J. Pharmacol.* 125:477–482). The expression of CLC-1 in heterologous system allowed to investigate the mechanism of drug action (Pusch et al. 2000. *Mol. Pharmacol.* 58:498–507). CPP blocks CLC-1 in a voltage-dependent manner and interferes with channel gating from the intracellular side. Furthermore, CPP is a specific CLC-1 blocker being ineffective on CLC-2, CLC-5, and CLC-K. Recently, by screening newly synthesized CPP derivatives on the various members of the CLC family, we identified a set of "bis-phenoxy" derivatives of CPP capable to specifically inhibit from the extracellular side CLC-K chimeras and CLC-K1 coexpressed with barttin (Liantonio et al. 2002. *Mol. Pharmacol.* 62:265–271). These derivatives represent a useful tool to study the structure and the function of CLC channels and a starting point for the identification of drugs therapeutically useful in genetic and acquired diseases in which these channels are involved. (Supported by Italian CNR PS n.00.00147.74 and PRIN Project 2001.)

26. Slick and Slack: Chloride and Sodium-activated Potassium Channels in Neurons and other Excitable Cells ARIN BHATTACHARJEE, WILLIAM J. JOINER, and LEONARD K. KACZMAREK, *Department of Pharmacology, Yale University School of Medicine, New Haven, CT*

The flux of Cl^- across the plasma membrane has dramatic effects on neuronal excitability. In addition to its direct effects on membrane potential, it is possible that Cl^- directly regulates the conductances of other cellular ions. It has been shown recently that the gene *Slack*, which encodes a large-conductance K+ channel, can be gated by both Cl^- and Na^+ ions. We now report the cloning and expression of another large conductance K^+ channel, *Slick*, which shares 74% homology to *Slack*. Expression of Slick in CHO cells shows, however, that Slick channels are even more sensitive to Cl^- than Slack channels, and are also activated by Na^+. The Slick channel subunit also contains a consensus ATP binding site and we have found that wild-type Slick channels are inhibited by intracellular ATP and by nonhydrolysable ATP analogs. In contrast, a Slick mutant lacking the ATP binding site, is not inhibited by ATP. Slick currents are insensitive to iberiotoxin, charybodotoxin, glyblencamide, and diazoxide but are blocked by quinidine, barium, and 20 mM TEA. By Northern blot analysis, Slick is found to be expressed primarily in brain and heart. By immunohistochemistry, Slick immunoreactivity is widely distributed in the nervous system, and is detected in the calyces of Held in the auditory brainstem and in dorsal root ganglia, which are known to have high $[Cl^-]_i$. Having the ability to sense $[Cl^-]_i$ may allow Slack and Slick to modify neuronal and cardiac excitability by linking Cl^- fluxes to the activation of Cl^--sensitive K^+ conductances. (Supported by National Institutes of Health grant P01 NS 42202.)

27. Reconstitution of Function in ClC-1: Coexpression of its Transmembrane Domain and Small Fragments of its Carboxyl Tail WEIPING WU,[1] BRONNI SIMPSON,[1] GRIGORI RYCHKOV,[2] BERNIE HUGHES,[1] and ALLAN BRETAG,[1] [1]*Centre for Advanced Biomedical Studies, University of South Australia, Adelaide, Australia;* [2]*Department of Physiology, University of Adelaide, Adelaide, Australia* (Sponsor: Criss Hartzell)

Truncations and point mutations in the carboxyl tail of the skeletal muscle chloride channel, ClC-1, are associated with myotonic muscle disease, e.g., Q658X, A659V, R669C, F708L, E717X, Q807X, G859D, R894X, and P932L and many of these are abnormal or nonfunctional when expressed in heterologous systems (Pusch. 2002. *Human Mutation.* 19:423–434). Functionally significant domains in the tail are, however, still largely unknown and crystal structures for two bacterial ClC isoforms (Dutzler et al. 2002. *Nature.* 415:287–294) have not helped our understanding, because, although able to catalyse a transmembrane chloride flux, they lack most of the tail. One thing that is clear is that all eukaryotic ClCs normally have two cystathionine β synthase (CBS) domains, one located near the beginning of the tail and the other, in ClC-1, about two thirds of the way along it. Both ClC-0 and ClC-1 can be functionally reconstituted from complementary pairs of coexpressed mRNA, where the split is between the first CBS domain (CBS1) and the second (CBS2), whereas similar attempts to restore function in splits immediately before or within CBS1 are unsuccessful. We have attempted reconstitution from several truncated transmembrane constructs of ClC-1 (ranging between Y601X and N879X—all of which are nonfunctional when expressed alone), by coexpressing them with a variety of carboxyl tail fragments. Of particular interest are G721X coexpressed with its 721 to COOH-terminal complement (G721-C) and G721X coexpressed with L863-C, both of which give currents indistinguishable from wild-type even though the latter is missing 142 amino acids (aa), including most of CBS2. We have

shown previously that truncations of up to 100 aa from the full length ClC-1 have only minor effects. So is all of the remaining tail, L863-C, essential for reconstitution? Even coexpression with the 26 aa peptide, L863-N889X, restores function to G721X.

28. Effect of Mutation C256A in Rat ClC-2 on its Gating Behavior LEANDRO ZÚNIGA, MARÍA ISABEL NIEMEYER, DIEGO VARELA, FRANCISCO V. SEPÚLVEDA, and L. PABLO CID, *Centro de Estudios Científicos (CECS), Valdivia, Chile*

ClC-0 and ClC-1 chloride channels have a double-barrelled structure with a fast single-pore gate and a slow common gate. Mutation of a single conserved cysteine (C212 in ClC-0) in these channels locks the slow gate in the open state without affecting the fast gate (Lin et al. 1999. *J. Gen. Physiol.* 114:1–12; Accardi et al. 2001. *J. Physiol. (London)*. 534:745–752). Although there is some evidence for a double-barrelled structure for ClC-2, it has not been possible to define the presence of these two gating mechanisms. C212 of ClC-0 is also conserved in ClC-2 (C256 in the rat) and we have mutated it to see if information concerning the two types of gating mechanisms can be obtained. Experiments were conducted to compare the behaviour of rClC-2 with that of rClC-2C256A expressed in HEK-293 cells by whole-cell patch-clamp recording. Extracellular Cd^{2+} inhibits rClC-2 by ~95% with a half maximal concentration of 50 μM. rClC-2C256A was also inhibited with similar affinity but only by ~50%. Mutation C256A shifted voltage-activation curve by ~30 mV in the depolarizing direction. There was no change in the slope factor but the minimal apparent P_O increased in the mutant. The activation of the channel by hyperpolarization can be fitted to a two-exponential plus an instantaneous component model, the exponentials becoming faster with hyperpolarization. Mutation C256A did not change these time constants, but increased the contribution of the fastest component at the expense of the two slower ones. The dependence of rClC-2 activity upon extracellular pH was also studied and found to be altered in the C256A mutant. Our results point to a slow gating mechanism in ClC-2 that might be similar to that present in ClC-0 and 1. (Supported by Fondecyt 1020652. CECS is a Millennium Science Institute funded by Fundación Andes.)

29. Biophysical Analysis of Two *C. elegans* ClC Variants Reveals a Role of Amino and Carboxyl Termini in Voltage-dependent Gating and Extracellular Cl^- and H^+ Sensitivity JEROD DENTON,[1] KEITH NEHRKE,[2] and KEVIN STRANGE,[1] [1]*Departments of Anesthesiology, Molecular Physiology and Biophysics, and Pharmacology, Vanderbilt University Medical Center, Nashville, TN;* [2]*Department of Medicine, Digestive Unit, University of Rochester Medical Center, Rochester, NY*

The structural basis of ClC gating has been studied extensively using mutagenesis strategies and by characterizing disease-causing mutations. However, the role of alternative splicing in regulation of normal ClC function has received far less attention. We therefore initiated a biophysical analysis of two ClC variants, CLH-3a and CLH-3b, expressed in *C. elegans*. CLH-3a and CLH-3b exhibit 100% amino acid identity with the exception of their NH_2 and COOH termini; CLH-3b lacks 71 NH_2-terminal amino acids present in CLH-3a and has 261 COOH-terminal residues absent in CLH-3a. We examined several voltage-dependent properties of CLH-3a and CLH-3b expressed in HEK293 cells. Several notable differences were observed. For example, the kinetics of CLH-3a activation at −120 mV were significantly slower than those of CLH-3b. CLH-3a exhibited time- and voltage-dependent inactivation over prolonged test potentials that was not observed in CLH-3b. The peak current amplitude of CLH-3a recorded at −120 mV was potentiated by previous depolarization, whereas no such prepotentiation was observed in CLH-3b. CLH-3a was strongly inhibited by a reduction in extracellular Cl^- concentration, but CLH-3b was virtually insensitive to this maneuver. In addition, CLH-3a was activated to a much greater degree by extracellular acidification than CLH-3b. The voltage dependence of the pH responses suggests that extracellular H^+ interact differently with the two channels. Together, these results indicate that the NH_2 and COOH termini of CLH-3 participate in voltage-dependent channel gating. The unique gating properties of CLH-3a (i.e., inactivation and prepotentiation) suggest the existence of an additional gating mechanism that is absent in CLH-3b. Differences in external H^+ and Cl^- sensitivities of CLH-3a and CLH-3b suggest that the NH_2 and COOH termini modulate channel sensitivity to these ions. We are currently using molecular biological techniques to determine the structural basis of these differences in gating characteristics. (Supported by DK51610, DK61168, DK58212.)

30. Why the Chloride Ion Is Important in Biology: Transient Anion Binding to Structural Proteins in Cornea, Muscle, and other Biological Polyelectrolyte Gels JUSTYN REGINI, STUART HODSON and GERALD ELLIOTT, *Department of Optometry and Vision Sciences, Cardiff University, Cardiff CF10 3NB, Wales, UK*

A group of biological systems called "biological polyelectrolyte gels" consist of arrays of filaments or fibrils,

etc., bearing a net negative charge at physiological pH (Elliott and Hodson. 1998. *Rep. Prog. Physics* 61:1325–1365). The ion-exchange capacity of such gels (the net negative charge on the fibrils, etc.) consists of two components: (a) charged amino-acid side chains at the surfaces of the fibrils and (b) a charge resulting from the transient binding of anions, principally chloride ions. to sites on the same surfaces. These sites are probably extended networks of interacting charged amino-acid sides chains ("Saroff sites"; Loeb and Saroff. 1964. *Biochemistry.* 3:1819–1826).

In corneal stroma, anion binding is responsible for about half of the ion-exchange capacity, with its consequent Donnan-osmotic swelling effects (Elliott and Hodson, *loc cit).* The dynamic balance between this swelling tendency and the continual dehydration provided by the ion pumps and channels in the endothelium provides the two major properties of cornea, resilience and transparency. Here we show by X-ray diffraction how the ordering of the fibrils, and therefore the tissue transparency, depends on the presence of a critical level of chloride ions.

The concept of transient anion (chloride) binding is also important in muscle physiology (Bartels et al. 1993. *Biochim. Biophys. Acta.* 1157:63–73), in the eye lens (Bartels and Elliott. 1993. *J. Physiol.* 467:279P) and probably in red blood cells and many other biological systems. In biological tissues free chloride is the only small anion present in appreciable quantities, though phosphate and bicarbonate levels can also affect some of these systems. We think this is a fundamental mode of biological interaction, operating in many guises, and it is for this reason that the membrane control of chloride levels is important in the function of so many different systems, both in sickness and in health. (Funded by grant Ref. M43057, EPSRC, UK.)

31. Functional and Structural Conservation of CBS Domains from CLC Channels RAÚL ESTÉVEZ,[1] MICHAEL PUSCH,[2] CARLES FERRER-COSTA,[3] MODESTO OROZCO,[3] and THOMAS J. JENTSCH,[1] [1]*Zentrum für Molekulare Neurobiologie Hamburg (ZMNH), Hamburg University, Falkenried 94, D-20246 Hamburg, Germany;* [2]*Istituto di Biofisica, Via de Marini 6, I-16149 Genova, Italy;* [3]*Departament de Bioquímica i Biología Molecular, Facultat de Quimica, Universitat de Barcelona, Marti i Franques 1, Barcelona 08028, and Institut de Recerca Biomédica. Parc Científic de Barcelona, Josep Samitier 1-5. Barcelona 08028, Spain* (Sponsor: Criss Hartzell)

All eukaryotic CLC Cl^- channels posses a long cytoplasmatic COOH terminus that contain two so-called CBS (cystathionine-β-synthase) domains. These domains are found in various unrelated proteins from all phylae. The crystal structure of the CBS domain of inosine monophosphate dehydrogenase (IMPDH) is known, but the function of these domains remains obscure.

Working primarily with ClC-1, we use deletion scanning mutagenesis, coimmunoprecipitation and electrophysiology to demonstrate that these domains interact. Truncation after the first CBS domain abolish the ability of the protein to generate currents, but not its dimerization, suggesting that the interaction between CBS domains is not essential for the dimerization of the channel. Replacing CBS domains of ClC-1 channel with the corresponding CBS domain from other CLC channels and even human IMPDH yielded functional channels, indicating a high degree of structural conservation. Based in a homology model of the pair of CBS domains of CLC channels, we identify some residues that when mutated affect the gate that regulates both monomers (common gate). Thus, we propose that CBS domains play a major role in the regulation of the common gate of CLC channels.

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32. Theoretical Study of the Passage of Chloride Ions through a Bacterial ClC Chloride Channel GENNADY V. MILOSHEVSKY and PETER C. JORDAN, *Department of Chemistry, Brandeis University, Waltham, MA* (Sponsor: Criss Hartzell)

The X-ray structures (Dutzler et al. 2002. *Nature.* 415: 287–294) permit theoretical study of Cl^- permeation along the curvilinear pores of the bacterial ClC Cl^- channel. A priori the coordinates of the conduction pathways are unknown. Metropolis Monte Carlo simulations using the Kinetic Monte Carlo Reaction Path Following technique (Miloshevsky et al. 2003. *Biophys. J.* 84:412a) were used to determine the coordinates of the conduction pathways and the helices and amino acids that line these lowest energy paths and that coordinate the translocating ion. We represent the channel in full atomic detail, including missing residues and explicit hydrogens. We examine the effect that the charge state of positively or negatively charged amino acids lining the curvilinear pores has on ion permeation by calculating electrostatic potential profiles, which clearly demonstrate electrostatic barriers and wells that Cl^- ex-

periences during translocation. The effect of mutation of some important amino acids on the potential energy profiles is also examined. We have found that (a) the crystallographic structure of the bacterial ClC Cl^- channel corresponds to a closed state. E148 and S107 side chains form a steric barrier on both sides of the crystal binding site, respectively. (b) Cl^- conduction from the extracellular region to the cytoplasmic region is governed by the electrostatic potential gradient. An anion's potential energy is much higher on the extracellular side than on the cytoplasmic side. (c) Cl^- ions permeating from outside towards the cytoplasm experience no electrostatic energy barrier near mid-membrane. (d) Mutations and charge states of the amino acids lining the pores substantially effect the potential energy profiles, suggesting the possibility of an electrostatic mechanism for controlling ion flow through these pores. (Supported by National Institutes of Health grant GM28643.)

33. Induction of Gating of Constitutively Open Mutants of CLC-0 by P-chlorophenoxy-acetic Acid (CPA) SONIA TRAVERSO, LAURA ELIA, and MICHAEL PUSCH, *Istituto di Biofisica, Sezione di Genova, CNR, Via De Marini, 6, I-16149 Genova, Italy* (Sponsor: Peying Fong)

The 3-D structure of bacterial CLC homologues (Dutzler et al. 2002. *Nature.* 415:287–294) confirmed the model of a dimeric protein with two identical, independent permeation pathways. Dutzler et al. (2002) indicated a glutamate as the sensor of the extracellular Cl^- and proposed it as being responsible for the voltage dependence of gating.

Here we show that mutating this critical residue (Glut 166) in the *Torpedo* CLC-0 to aspartate slowed down opening while mutating it to alanine, lysine, and serine led to constitutively open channels. Constitutively open mutants were inhibited by p-chlorophenoxy-acetic acid (CPA) at negative voltages with a >200-fold larger affinity than for WT CLC-0. To describe CPA block of E166A we developed a kinetic 3-state model composed of an open state, a blocked non-conducting state, and a closed CPA-bound state ($O \leftrightarrow O_B \leftrightarrow C_B$). Open-channel block is fast and of low affinity as suggested by the observed reduction of the single-channel current in the presence of 5 mM CPA. The closed CPA-bound state had to be included to explain the [CPA] dependence of the relaxation rates. The conformational change between O_B and C_B is analogous to regular protopore gating. This conclusion is based on three lines of evidence. First, the parameters obtained by fitting the three-state model to the experimentally observed relaxation rates have a similar voltage dependence as those that describe regular WT gating. Second, inhibition by CPA is affected by extracellular Cl^- similarly as the gating of WT CLC-0. Third, the relaxations seen in the presence of CPA are similarly affected by mutations (K519E and S123T) that alter the gating kinetics in WT CLC-0. Thus, CPA is able to mimic regular channel gating. (Supported by grants from Telethon Italy [grant 1079] and the Italian Research Ministry [FIRB RBAU01PJMS]. S. Traverso receives a CNR doctoral fellowship].)

34. Basolateral ClC-2 Chloride Channels in Surface Colon Epithelium: Regulation by a Direct Effect of Intracellular Chloride MARCELO CATALAN, MARÍA ISABEL NIEMEYER, L. PABLO CID, and FRANCISCO V. SEPÚLVEDA, *Centro de Estudios Científicos (CECS), Valdivia, Chile*

Important functions of the colon are the absorption of NaCl, short-chain fatty acids, and water. Apical membrane Na^+ channels, Na^+/H^+ and $Cl^-/HCO3^-$ exchangers have all been postulated to mediate NaCl entry into colonocytes. Based on in situ hybridization, immunohistochemical and patch clamp studies we have postulated that basolateral exit pathway for Cl^- could be via ClC-2 channels present in that membrane domain of surface epithelium. This study aims at obtaining functional data for a basolateral localization of ClC-2 and explores a possible direct regulation by intracellular Cl^-. Distal colon epithelium from guinea-pig with the apical membrane perforated with nystatin in Ussing chambers is used to reveal a basolateral Cl^- conductance. Heterologous expression of the recombinant channel and the patch-clamp technique are used to investigate a direct regulation by intracellular Cl^-. A basolateral membrane conductance with the characteristics of ClC-2 channels, including Cd^{2+}-sensitivity, selectivity and inhibition by extracellular alkalinization is present in distal colon epithelium. The effect of intracellular Cl^- on this conductance suggests a modulation of its gating by the permeant anion. Using the recombinant ClC-2 channel a strong dependence of its activity on intracellular Cl^- is demonstrated, with a shift of activation to more positive voltages as $[Cl^-]_i$ is increased. Functional basolateral ClC-2 channels upregulated by intracellular Cl^- are present in distal colon. It is suggested that ClC-2 serves as exit pathway for Cl^- in the basolateral membranes of distal colon, and that its dependence on $[Cl^-]$ might provide a cross talk mechanism to match fluxes at the apical and basolateral domains of these epithelial cells. (Supported by Fondecyt 1030627. CECS is an Millennium Science Institute funded by Fundación Andes.)

35. Amino- and Carboxyl-terminal Determinants of NKCC1 Function: A Study of Deletion Mutants in *Xenopus laevis* Oocytes ERIC DELPIRE, ROGER ENGLAND, and KERSTIN PIECHOTTA, *Department of Anesthesiology, Vanderbilt University Medical Center, Nashville, TN*

The open reading frame of the mouse NKCC1 cDNA was inserted into the vector pBF for expression in *Xenopus laevis* oocytes. NKCC1 function was assessed through ^{86}Rb-uptakes in oocytes exposed to isotonic (200 mOsM) or hypertonic (260 mOsM) solutions. Fluxes were routinely measured 3-d postinjection, which represents 81% of the maximal flux (measured at days 4–6). Injection of 27.5 ng NKCC1 cRNA, which represents a saturated amount, yields fluxes at room temperature of 5,057 ± 281 pmoles K^+/oocyte/h (n = 20) in isotonic solution and 17,055 ± 1,180 (n = 20) in hypertonic solution, compared to 1,270 ± 91 (isotonic) and 2,312 ± 57 (hypertonic) for water-injected oocytes. The entire NKCC1-mediated K^+ flux was inhibited by 50 μM bumetanide. When the uptake was performed at temperatures ranging from 10°C to 34°C, a sharp increase in NKCC1 flux was observed between 10°C and 20°C, followed by a lesser increase thereafter. This temperature effect, leading to a nonlinear Arrhenius plot showing an abrupt change in activation energy around 20°C to 23°C, suggests a significant effect of lipid phase transition. In order to identify key determinants of NKCC1 function, we created deletion mutants and tested their function in isotonic and hypertonic conditions. In the carboxyl terminus, we deleted exon 21 (16 residues located in the middle of the carboxyl-terminal tail of NKCC1) to reproduce an isoform found in abundance in the brain (Randall et al. 1997. *Am. J. Physiol. Cell Physiol.* 272:C1267–C1277). There was no significant difference between the function of wild-type and alternatively spliced variant, as measured in isotonic and hypertonic conditions. In the amino terminus, we mutated each of the two binding domains of the Ste20-related kinase SPAK. Prevention of SPAK binding at one site alone did not affect NKCC1 function. Deletion of a 91 amino acid fragment containing both SPAK binding domains reduced NKCC1 function by 40%, but did not affect the hypertonic stimulation. Using additional deletion mutants, we identified a threonine (T211) and a small upstream fragment containing multiple serine residues, essential for NKCC1 function. The identity of the residues surrounding T211 were also critical for NKCC1 function, suggesting the presence of a kinase recognition site. (Supported by National Institutes of Health grant NS36758.)

36. Determination of Intracellular Chloride Concentration in Dendritic Knobs of Olfactory Sensory Neurons Using Fluorescence Lifetime Imaging HIROSHI KANEKO,[1,2] ILVA PUTZIER,[1] THOMAS GENSCH,[1] U. BENJAMIN KAUPP,[1] and STEPHAN FRINGS,[2] [1]*Institute for Biological Information Processing, Juelich Research Center, Juelich, Germany;* [2]*Department of Molecular Physiology, University of Heidelberg, Heidelberg, Germany* (Sponsor: Paul J. Bauer)

The sensory membrane of rat olfactory sensory neurons (OSNs) contains two types of transduction channels: cAMP-gated Ca-permeable channels and Ca-activated Cl channels. To understand the physiological role of the Cl channels, it is necessary to know the chloride distribution across the sensory membrane. Here we employ a novel method to measure the intracellular Cl concentration in the dendritic knobs of rat OSNs which involves two-photon excitation of the fluorescent Cl indicator MQAE and lifetime analysis of the emitted fluorescence signal. This method is designed to examine Cl distributions in small cellular compartments of neurons in intact tissue.

We have found that the Cl concentration is above equilibrium in all dendritic knobs, indicating that OSNs actively accumulate Cl. Intracellular Cl concentrations range from 40 to 60 mM in the presence of a physiological extracellular Cl concentration (50 mM). The Cl equilibrium potential in vivo is thus near 0 mV. The uptake mechanism that mediates Cl accumulation appeared to be independent of extracellular Na and was not sensitive to bumetanide. No expression of Na-coupled transport proteins was detected in OSNs by immunohistochemistry or PCR. K-coupled Cl extrusion could be detected by its sensitivity to the KCC-specific inhibitor DIOA. KCC1 message was identified in OSNs, but KCC2 message was absent.

Most OSNs lost their ability to accumulate Cl after isolation from the olfactory epithelium; intracellular Cl concentration in many isolated cells dropped to values below 30 mM. Our results show that Ca-activated Cl channels in OSNs conduct a depolarizing Cl efflux as long as the tissue is intact. However, cell isolation impairs the Cl uptake mechanism which is necessary to sustain the Cl fraction of the receptor current. (Supported by the Deutsche Forschungsgemeinschaft, FR 937-4.)

37. pH Activation of a Prokaryotic Chloride Channel ALESSIO ACCARDI and CHRISTOPHER MILLER, *HHMI/Brandeis University, Waltham, MA*

Recently the field of CLC channels has been blessed by a newfound wealth of structural information on prokaryotic channels. This growth in structural knowledge has not been paralleled in the understanding of

the electrophysiology of these channels. Information on channel characteristics can be obtained using a nonelectrophysiological technique, concentrative uptake of radioactive $^{36}Cl^-$.

We show that Eric, one of the *E. coli* CLCs, is a pH-activated channel. The rate of $^{36}Cl^-$ uptake increases upon acidification. We are in the process of investigating the sidedness of this pH activation. Preliminary results indicate asymmetrical pH activation, suggesting the presence of only one pH sensor.

The side chain of E148 occludes the permeation pathway in the original Eric structure (Dutzler et al. 2002. *Nature.* 415:287–294). This led to the proposal that it represents the closed state. More recently, the crystal structure of two mutants, E148A and E148Q, showed that neutralization of the glutamate side chain resulted in its displacement from the permeation pathway (Dutzler et al. 2003. *Science.* 19:285–292). This suggested that the new structure represents a conducting state. The protonation of the side chain of E148 could thus be important in determining the conduction state of the channel, making it a candidate for the pH sensor. We show that both mutations, E148A and E148Q, remove any pH sensitivity in the protein: the rate of $^{36}Cl^-$ uptake becomes pH independent. We find that the rate of $^{36}Cl^-$ uptake in these mutants is faster than in the WT channel at pH 7. This shows that the mutants have a higher activity at a neutral pH. Conversely, at lower pHs, 4.5 and 3, the rate of $^{36}Cl^-$ uptake for the WT channel is faster than that of the mutants. This suggests that the mutants are less active than the native protein at low pH range.

38. Functional Genomic Analysis of Osmoregulation in the Nematode *C. elegans* S.T. LAMITINA, K. BAMAN, R. MORRISON, G. MOECKEL, and K. STRANGE, *Departments of Anesthesiology, Molecular Physiology, and Biophysics, Pharmacology and Pathology, Vanderbilt University Medical Center, Nashville, TN*

The ability to control solute and water balance is essential for cellular life. Osmotic homeostasis is maintained by the regulated accumulation and loss of inorganic ions and organic osmolytes. While cellular osmoregulation has been studied extensively in many cell types, major gaps exist in our molecular understanding of this essential process. Because of its numerous experimental advantages, *C. elegans* provides an ideal model system in which to define the genes and genetic pathways required for cellular osmoregulation in animals. To begin defining the genetic basis of cellular osmoregulation in *C. elegans,* we characterized the ability of worms to survive extreme hypertonic stress by adding NaCl to the growth agar. Exposure to high salt agar causes rapid shrinkage followed by slow recovery of body volume over several hours. Survival is normal on agar containing up to 200 mM NaCl. When grown on 200 mM NaCl for 2 wk, worms are able to survive and reproduce on agar containing 400 mM NaCl. Worms adapted to hypertonic stress swell and then rapidly return to their initial body volume when returned to low salt agar. Using HPLC analysis, we have observed that the organic osmolyte glycerol increases 15-20-fold in nematodes grown on 200 mM NaCl plates. Accumulation of glycerol begins after 6 h of high salt stress and peaks by 24 h. Whole genome microarray analyses demonstrated that ~150 genes are significantly upregulated by hypertonic stress. These included genes that encode proteases, cytoskeletal proteins, extracellular matrix proteins, and components of stress response and signaling pathways. Putative metabolic genes accounted for 41% of the genes that were transcriptionally upregulated. We are currently using forward genetic screens, RNA interference reverse genetic analysis, and molecular, biochemical, and physiological approaches to define the molecular mechanisms utilized by *C. elegans* to adapt to and survive osmotic stress. (Supported by DK51610, DK58212.)

39. Voltage- and pH-gated Chloride Conductance in TRK Proteins ALBERTO RIVETTA,[1] GEFEI ZENG,[1] CLIFFORD SLAYMAN,[1] and TERUO KURODA,[2] [1]*Yale School of Medicine, New Haven, CT;* [2]*Gene Research Center, Okayama University, 700-8530 Okayama, Japan*

Proteins of the so-called "TRK family," which mediate moderate- to high-affinity uptake of potassium by bacteria, fungi, and plants, function as active carriers of K^+ ions, but are folded like K^+ channels: i.e., in a single polypeptide chain they form four MPM motifs which cluster into a K^+-channel like selectivity filter supported by eight transmembrane helices. The yeast *Saccharomyces cerevisiae* contains two of these proteins, Trk1p and Trk2p, of which the first is larger (1235 amino acids, cf. 889 in Trk2p) and is absolutely required for high-affinity K^+ accumulation.

Whole-cell patch-clamp measurements of K^+ transport through the yeast TRK proteins have revealed inward currents no larger than 20 pA/cell at large negative voltages, consistent with the small size of yeast cells (~100 μm^2 surface area; 0.1 pL volume), but have also revealed much larger currents—up to 200 pA/cell—which vary in proportion to pipette chloride concentration, depend on low extracellular pH, and are strongly rectified. The rectification closely resembles that reported for chloride currents through the excitatory

amino acid transporter in mammalian brain (EAAT1) and can be described by a simple, single-barrier gating function superimposed on a "constant-field" conductance, where the critical parameter is the apparent gating voltage, E_g, which shifts positive ~35 mV for each pH unit of acid shift, from −249 mV at pH 7.5 to −145 mV at pH 4.5.

In wild-type yeast cells, the TRK-dependent Cl^- currents are mediated largely by Trk1p, but Trk2p can be up-regulated, in *TRK1*-deleted strains, to produce currents of similar amplitude. Currents for bromide and iodide closely mimic those for chloride. The TRK proteins are also susceptible to more strictly chaotropic ions, such as nitrate and thiocyanate, but the resultant currents are essentially pH independent and nonrectifying. (Supported by National Institutes of Health research grant GM 60696.)

40. The Yeast CLC Chloride Channel Is Proteolytically Processed by the Furin-like Protease Kex2p ANDREA WAECHTER and BLANCHE SCHWAPPACH, *Zentrum für Molekulare Biologie, Universität Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany* (Sponsor: Peying Fong)

Inactivation of the CLC (*GEF1*) gene in yeast has been shown to result in two main phenotypes: loss of high affinity iron uptake and reduced resistance to toxic cations (Greene et al. 1993. *Mol. Gen. Genet.* 241: 542–553; Gaxiola et al. 1998. *PNAS.* 95:4046–4050; Schwappach et al. 1998. *JBC.* 273:15110–15118). High-affinity iron uptake is lost because Fet3p (an oxidase involved in high affinity iron uptake at the cell surface) does not mature normally in *Δgef1* strains. Copper loading to the active center of Fet3p in the lumen of the late secretory pathway requires Cl^- ions that enter the compartment via the CLC channel (Davis-Kaplan et al. 1998. *PNAS.* 95:13641–13645). The sequestration of toxic cations is presumably affected because this process depends on anions that can counterbalance the accumulation of positive charge on the lumenal side. The subcellular localization of Gef1p to the Golgi and prevacuolar compartments (Schwappach et al. 1998. *JBC.* 273:15110–15118; Gaxiola et al. 1999. *PNAS.* 96:1480–1485) is consistent with the functions suggested by the knockout phenotypes.

However, we report here that previous accounts of Gef1p subcellular localization have to be reconsidered since the channel protein undergoes proteolytic processing in the secretory pathway. Cleavage occurs in the first extracellular loop of the protein at residues KR136/137 and is carried out by the Kex2p protease. A mutant form of the channel that cannot be processed is functional and accumulates in the prevacuolar compartment. Fragments mimicking the NH_2- and COOH-terminal products of the cleavage reaction are nonfunctional when expressed alone. However, functional channels can assemble when the two fragments are coexpressed. Cleavage by Kex2p does not seem to represent a simple on or off switch for Gef1p channel activity. Rather, we propose that this mechanism is in place to achieve subtle regulation of the localization or function of the chloride channel.

41. Loss of Chloride Channel ClC-5 Impairs Endocytosis by Defective Trafficking of Megalin and Cubilin in Kidney Proximal Tubules ERIK I. CHRISTENSEN, OLIVIER DEVUYST, GENEVIÈVE DOM, RIKKE NIELSEN, PATRICK VAN DER SMISSEN, PIERRE VERROUST, MICHÈLE LERUTH, WILLIAM B. GUGGINO, and PIERRE J. COURTOY, *University of Aarhus, Department of Cell Biology, DK-8000 Aarhus C, Denmark; Université Catholique de Louvain, Medical School, Division of Nephrology and Cell Unit, B-1200 Brussels, Belgium; INSERM U538, CHU Saint Antoine, Paris, France; Johns Hopkins University School of Medicine, Departments of Physiology and Medicine, Baltimore, MD 21205* (Sponsor: Criss Hartzell)

Loss of the renal endosome–associated chloride channel, ClC-5, in Dent's disease and knock-out (KO) mice is reflected by low-molecular weight proteinuria, indicating impaired endocytic uptake of filtered proteins by kidney proximal tubular cells (PTC). The underlying mechanism remains unknown. We therefore tested whether this endocytic failure could primarily reflect a loss of reabsorption by the multiligand receptors, megalin and cubilin, due to a trafficking defect. Impaired endocytosis in PTC of ClC-5 KO mice was demonstrated by a (a) ~85% decreased uptake of injected ^{125}I-β_2-microglobulin; (b) reduced labelling of endosomes by injected peroxidase and the endogenous megalin/cubilin ligands, vitamin D- and retinol-binding proteins; and (c) urinary excretion of low-molecular weight proteins and the selective cubilin ligand, transferrin. Contrasting with preserved mRNA levels, megalin and cubilin abundance was significantly decreased in kidney extracts of KO mice. Percoll gradients resolving early and late endosomes (Rab5a, Rab7), brush border (villin, aminopeptidase M) and a dense peak comprising lysosomes (acid hydrolases) showed a disappearance of the brush border component for megalin and cubilin in KO mice. Quantitative ultrastructural immunogold labelling confirmed the overall decrease of megalin and cubilin in PTC and their selective loss at the brush border. Percoll gradients also

showed an altered distribution of injected ^{125}I-β_2-microglobulin and a shift of lysosomal enzymes from high-density in controls towards low-densities in KO mice, pointing to defective routing and progression along the endocytic apparatus. In contrast, total contents of the rate-limiting endocytic catalysts, Rab5a and Rab7, were unaffected.

These data suggest that defective protein endocytosis due to invalidation of ClC-5 is due to a major and selective loss of megalin and cubilin at the brush border, reflecting a trafficking defect in PTC.

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42. Adaptor AP-3–dependent Targeting of a Chloride Channel to Synaptic Vesicles G. SALAZAR,[1] R. LOVE,[1] M. STYERS,[1] E. WERNER,[1] A.A. PEDEN,[2] AND V. FAUNDEZ,[1] [1]*Department of Cell Biology, Emory University, Atlanta, GA;* [2]*Genentech, South San Francisco, CA* (Sponsor: C. Hartzell)

Membrane protein transport by vesicle carriers relies on the recognition of peptide signals present in the cargo molecules. Adaptor complexes decode these signals. One of these adaptors, the adaptor complex 3 (AP-3), is involved in the biogenesis of lysosomal-related organelles and synaptic vesicles (SV). Accordingly, an AP-3–deficient mouse model is characterized by mistargeting of lysosomal proteins, behavioral phenotypes and defects in the luminal ionic composition of SV.

In order to identify SV membrane proteins whose targeting was affected by the absence of AP-3, we analyzed the protein profile of SV isolated from wild-type and AP-3 mutant brains. We identified two AP-3–dependent cargo molecules, the zinc transporter 3 (ZnT3), and the chloride channel 3 (ClC-3). ClC-3 was enriched in SV of wild-type brains. In contrast, the channel was retained in endosomal compartments in membranes from AP-3–deficient brains. The subcellular distribution of a GFP-tagged ClC-3 was also AP-3–dependent in fibroblasts isolated from mutant mice. We further examined the SV targeting of ZnT3 and ClC-3 in PC 12 cells. In this cell line AP-3–dependent SV biogenesis from endosomes is abrogated by brefeldin A. Both ZnT3 and ClC-3 were localized in early endosomes and SV. Their targeting to SV was brefeldin A sensitive, indicating that both vesicles antigens were packed on AP-3–derived vesicles. We confirmed that these two proteins converge on the same organelles using biochemical and functional strategies. Vesicular zinc transport assays revealed that the ClC-3 content on membranes was the rate-limiting step on the vesicular zinc transport.

Our data identify new AP-3 cargo molecules and provide insight into the mechanisms that control chloride channel subcellular distribution. (Supported by National Institutes of Health NS42599-01A1 and March of Dimes 5-FY2001.)

43. Loss of the ClC-7 Chloride Channel Leads to Neurodegeneration Resembling Neuronal Ceroid Lipofuscinosis DAGMAR KASPER, ROSA PLANELLS-CASES, JENS C. FUHRMANN, OLAF SCHEEL, JULIANA PARK, MICHAELA SCHWEIZER, UWE KORNAK, and THOMAS J. JENTSCH, *Zentrum fuer Molekulare Neurobiologie, ZMNH, Universitaet Hamburg, Falkenried 94, D-20246 Hamburg, Germany* (Sponsor: Criss Hartzell)

Loss of the intracellular chloride channel ClC-7 leads to osteopetrosis in mice and humans. In osteoclasts, ClC-7 is present in late endosomes/lysosomes and in the ruffled border, where it shunts the current generated by V-type H^+-ATPases. In its absence, efficient proton secretion cannot occur, and bone resorption is severely impaired. Additionally, the retina of ClC-7 knock-out mice degenerates after eye opening. We now show that ClC-7 deficiency in mice leads to a phenotype resembling lysosomal storage disease and neurodegeneration. Progressing from postnatal day 20 (p20) to the time of death at around p40, electron-dense and autofluorescent material accumulated at neuronal perikarya. Neurodegeneration was most pronounced in the CA3 region of the hippocampus and associated with astrogliosis. The amount of several lysosomal enzymes was increased in the brain of ClC-7 knock-out mice. Additionally, we observed accumulation of subunit c of the mitochondrial H^+-ATPase, a major component of the storage material in human neuronal ceroid lipofuscinosis. In contrast, osteopetrotic mice homozygous for a deletion in the gene encoding the α3-subunit of the V-type H^+-ATPase (osteosclerotic, oc/oc) showed no comparable retinal or neuronal degeneration. Thus, the retina and neuronal degeneration caused by ClC-7 deficiency are not secondary to the osteopetrosis phenotype, and loss of the α3 subunit is probably compensated for by other α subunits in brain and retina. Lysosomal pH in neurons and fibroblasts of ClC-7 knock-out mice was significantly increased, offering a mechanistic explanation for the phenotypes described. ClC-7 acts to shunt the current generated by H^+-ATPases on late endosomes and lysosomes. In its absence, impaired acidification results in decreased lysosomal function and storage of undegraded material. (Supported by the Deutsche Forschungsgemeinschaft.)

44. The Chloride Channel ClC-4 Contributes to Endosomal Acidification and Trafficking RAHA MOHAMMAD-PANAH, RENE HARRISON, SONJA DHANI, CAMERON ACKERLEY, LING-JUN HUAN, YAN-CHUN WANG, and CHRISTINE E. BEAR, *Program in Structural Biology and Biochemistry, Hospital for Sick Children and University of Toronto, Canada* (Sponsor: Criss Hartzell)

Mutations in the gene coding for the chloride channel; ClC-5 cause Dent's disease, a disease associated with proteinuria and renal stones. Studies in ClC-5 knockout mice suggest that this phenotype is related to defective endocytosis of low molecular weight proteins and membrane proteins by the renal proximal tubule. In the present studies, confocal micrographs of proximal tubules and cultured epithelial cells revealed that the related protein: ClC-4, is expressed in endosomal membranes, suggesting that this channel may also contribute to the function of this organelle. In order to determine the relative role of ClC-4 in endosomal trafficking, we used an antisense strategy. By immunofluorescence quantification and using the patch clamp technique, we showed that both antisense ClC-4 and antisense ClC-5 are specific and functionally effective in disruption of endogenous ClC-4 and ClC-5 proteins respectively. In cultured epithelial cells (Caco-2 and LLCPK-1), we found that depletion of endogenous ClC-4 expression by transfection of ClC-4 antisense cDNA, acidified endosomal pH, and altered transferrin trafficking to the same extent as the specific disruption of ClC-5. Our pulse-chase experiments suggest that ClC-4 and ClC-5 may contribute to multiple steps in transferrin receptor trafficking with a primary role in a fast component of receptor recycling. In addition, both channels can be coimmunoprecipitated, arguing that they may partially contribute to endosomal function as a channel complex. These studies prompt future investigation of the role of ClC-4 in renal function in health and in Dent's disease. Future studies will assess whether the severity of Dent's disease relates not only to the impact of particular mutations on ClC-5 but also on the consequences of those mutations on the functional expression of ClC-4. (Supported by operating grants to C.E.B. awarded by National Institutes of Health [# P50DK49096] and CIHR. R. Mohammad-Panah is awarded the CIHR fellowship.)

45. Regulation of *C. elegans* ClC Activation Kinetics by an Ste20-related Kinase JEROD DENTON,[1] KEITH NEHRKE,[2] and KEVIN STRANGE,[1] [1]*Departments of Anesthesiology, Pharmacology, and Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, TN;* [2]*Department of Medicine, Digestive Unit, University of Rochester Medical Center, Rochester, NY*

We recently identified an inwardly rectifying ClC channel, CLH-3b, expressed in the *C. elegans* oocyte that is activated by osmotic cell swelling and during oocyte meiotic maturation. Using pharmacological techniques we showed that CLH-3b is inhibited under basal conditions by ser/thr phosphorylation and that dephosphorylation of these residues by PP1-type phosphatases mediates channel activation. Using RNA interference (RNA_i) we identified two PP1-type phosphatases, CeGLC7α and CeGLCβ, that mediate swelling- and maturation-induced activation of CLH-3b. To identify additional CLH-3b regulatory proteins, we conducted yeast two-hybrid studies in which the channel COOH terminus was used as bait. One protein identified in these studies is an Ste20-related kinase (SRK) that exhibits 53% and 55% amino acid identity to human and *Drosophila* SRKs, respectively. SRKs are ser/thr kinases that play critical roles in cell-cycle progression. In yeast, Ste20 activation by hyperosmotic stress is required for survival and cellular osmoregulation. CLH-3b is inhibited by ser/thr phosphorylation and hyperosmotic stress. Given the central role of SRKs in cell-cycle control and hyperosmotic adaptation, we postulated that the SRK identified in our yeast two-hybrid screen regulates CLH-3b activity. To test this hypothesis, we have begun to characterize the functional properties of CLH-3b coexpressed with the SRK in HEK293 cells. Coexpression of the SRK and CLH-3b causes significant slowing of channel activation kinetics. For example, the time required to reach 50% peak current amplitude of CLH-3b expressed alone was 6 ± 1 ms (n = 7). However, coexpression of the SRK and CLH-3b increased the half-activation time to 109 ± 18 ms (n = 8). Interestingly, coexpression of the SRK with CLH-3a, which lacks 261 COOH-terminal amino acids found in CLH-3b, has no effect on CLH-3a activation kinetics. This suggests that the SRK interacts specifically with the COOH terminus of CLH-3b and indicates a role of the COOH terminus in regulation of channel gating. (Supported by DK51610, DK61168, DK58212.)

46. Trafficking of CLC Cl Channels in the Golgi and Endosomal System KATJA HEUSSER and BLANCHE SCHWAPPACH, *Zentrum für Molekulare Biologie, Universität Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany* (Sponsor: Peying Fong)

We have chosen the single CLC (*GEF1*) protein present in the yeast *Saccharomyces cerevisiae* (Greene et al. 1993. *Mol Gen. Genet.* 241:542–553) as a model to investigate the sorting and regulation of CLC channels

present in the Golgi and endosomal system. To this end, we have systematically mutagenized putative binding sites for vesicle coat proteins present in the COOH terminus of Gef1p. We will report how the absence of these protein–protein interaction motifs affects the functionality of the channel as assessed by complementation assays. The localization of mutated channel variants—as followed by microscopy and subcellular fractionation—will be described.

The most related mammalian counterparts of Gef1p are found in the ClC-3,4,5 subbranch of the family. In mammals, alternative splicing has been shown to determine the localization of ClC-3 to the Golgi or endosome (Gentzsch et al. 2003. *J. Biol. Chem.* 278:6440–6449). This mechanism as well as the presence of more than one isoform of the ClC-3,4,5 sub-branch in many cell types may allow for exquisite fine tuning of chloride flux to the lumen of the secretory pathway. Most differences in localization will be due to different protein–protein interaction profiles of the channel isoforms.

To identify interaction partners that affect the subcellular localization of these channel proteins we have embarked on a genetic screen in mammalian cells. Employing CD4- and CD8-reporter fusion proteins as well as extracellularly epitope-tagged variants of the channels we use retroviral gene transduction of cDNA libraries combined with flow cytometry to enrich cDNA inserts that change the localization of the respective channel protein. A characterization of the reporter cell lines and initial results from the screen will be discussed. (Supported by Graduiertenkolleg 230 der Deutschen Forschungsgemeinschaft.)

47. CLC-3 Activation by CaMKII-dependent Phosphorylation in HT29 Cells NICOLE C. ROBINSON and DEBORAH J. NELSON, *Department of Neurobiology, Pharmacology, and Physiology, University of Chicago, Chicago, IL* (Sponsor: Deborah J. Nelson)

CLC-3, a member of the CLC family of chloride channels, mediates function in many cell types in the body. The multifunctional calcium/calmodulin-dependent protein kinase II (CaMKII) has been shown to activate recombinant CLC-3 stably expressed in tsA cells and natively expressed channel protein in T84 cells. A colonic tumor cell line, HT29, also expresses endogenous CLC-3, where it is associated with cytoplasmic vesicles as well as the plasma membrane. We introduced autonomous CaMKII into voltage-clamped cells via the patch pipette in order to examine the CaMKII-dependent Cl^- conductance in regulation of CLC-3 in HT29 cells. We observed the activation of a Cl^- conductance after intracellular introduction of the isolated autonomous CaMKII into HT29 cells. Similar current activation was not observed after exposure of the cells to CaMKII + 1 μM autocamtide inhibitory protein (AIP), a selective inhibitor of CaMKII. Evidence supporting the hypothesis that the kinase-dependent Cl^- conductance in HT29 cells is ClC-3 was established in experiments in which phosphorylation-dependent current activation was prevented in the presence of a COOH-terminal antipeptide antibody, α-hCLC-$3_{730–744}$, or CLC-3 antisense. We then examined whether the channel could be phosphorylated using GST fusion proteins of CLC-3 NH_2 and COOH termini. In vitro phosphorylation studies showed that the NH_2-terminal is phosphorylated by CaMKII. The putative CaMKII consensus site on the NH_2-terminal was mutated (S109A) and electrophysiological studies were performed on transfected tsA cells. Cells transfected with the extracellularly Flag-tagged mutant construct did not demonstrate current activation in the presence of the kinase. Immunofluorescence demonstrated proper trafficking to the plasma membrane. These data indicate that CLC-3 is a CaMKII-regulated Cl^- conductance with phosphorylation-dependent gating. (Supported by National Institutes of Health grant DK57882.)

48. Dominant-negative Effect of Misspliced ClC-1 RNA in Myotonic Dystrophy JIM BERG,[1] CHRISTINA JIANG,[2] CHARLES THORNTON,[2] STEPHEN C. CANNON,[3] [1]*Department of Neurobiology, Harvard Medical School, Boston, MA;* [2]*Department of Neurology, University of Rochester Medical Center, Rochester, NY;* [3]*Department of Neurology, UT Southwestern Medical Center, Dallas, TX*

Muscle degeneration and myotonia (repetitive action potentials and impaired relaxation) are the hallmarks of myotonic dystrophy (DM). Mankodi et al. (2002. *Mol. Cell.* 10:35–44) showed that the expanded CTG and CCTG repeats associated with DM1 and DM2 cause an increased frequency of aberrant ClC-1 pre-mRNA splicing, decreased ClC1 at the sarcolemma, and 10-fold reduced Cl current. Sarcolemmal hyperexcitability and myotonia result from loss of the resting Cl conductance. We tested whether misspliced transcripts with intron retention/premature stop codons produced functional ClC1 protein when expressed in *Xenopus* oocytes. No ClC-1 current was detected in oocytes injected with either of two mutant mRNAs with abnormal introns retained between exons 6 and 7. In oocytes coinjected with either mutant plus wild-type mRNA, the Cl current amplitude was reduced about two-fold beyond the 50% reduction produced by diluting wild-type mRNA with water. Coinjection of mutant and wild-type mRNA re-

sulted in currents with an accelerated rate of deactivation at hyperpolarized potentials. These results imply misspliced mRNA in DM is translated and exerts a dominant-negative effect on net Cl current density. The detection of altered deactivation kinetics suggests WT-mutant heterodimers are able to conduct Cl current. Dominant inheritance in DM may result from both a transdominant effect of abnormal RNA with expanded CTG or CCTG repeats on ClC-1 pre-mRNA splicing and from a dominant-negative effect of miscoded and truncated ClC-1 protein. (Supported by the MDA.)

49. Altered Skeletal Muscle Chloride Channel (ClC-1) Activity in a Mouse Model for Myotonic Dystrophy Type 1 (DM1) JOHN D. LUECK, AMI MANKODI, CHARLES A. THORNTON, and ROBERT T. DIRKSEN, *University of Rochester School of Medicine and Dentistry, Rochester, NY*

Myotonic dystrophy type 1 (DM) is the most common inherited muscle disorder in adults and is caused by a CTG repeat expansion in the 3′-untranslated region of the gene, which encodes a serine/threonine DM protein kinase (DMPK). However, the precise ionic mechanism underlying increased myotonias susceptibility in DM1 is not known. Mice that overexpress an actin mRNA containing expanded CUG repeats in skeletal muscle exhibit aberrant splicing of pre-mRNA for the muscle chloride channel (ClC-1) and robust myotonias, both of which are also observed in skeletal muscle of DM1 patients. In whole-cell voltage clamp experiments, we compared the density, voltage dependence, and kinetics of 9-AC–sensitive macroscopic chloride currents recorded from acutely dissociated *flexor digitorum brevis* (FDB) muscle fibers of young (8–21-d-old) wild-type and HSA^{LR} mice. Interestingly, we found an ~80% decrease in ClC-1 chloride current density in HSA^{LR} FDB fibers as compared to control at 9, 13, and 18 d postnatal. Immunocytochemistry (ICC) experiments correlate well with the electrophysiology results, with a marked decrease in ClC-1 t-tubule expression in FDB fibers of HSA^{LR} mice. These results indicate that altered skeletal muscle excitability in DM1 involves a marked reduction in t-tubule ClC-1 channel expression/activity. In additional experiments, we are comparing the biophysical properties (e.g., voltage dependence of relative open probability and deactivation kinetics, rectification, and ion selectivity) of native ClC-1 activity recorded from young FDB fibers with that observed after homologous expression of the cloned murine ClC-1 cDNA (mClC-1) in primary cultures of mouse skeletal myotubes (which lack ClC-1 activity). (Supported by National Institutes of Health and MDA.)

50. Regulation and Gating of a CFTR Chloride Channel without the Regulatory Domain TOMOHIKO AI, XIAOHUI WANG, JEONG-HAN CHO, MIN LI, XIAOQIN ZOU, and TYZH-CHANG HWANG, *Departments of Physiology and Biochemistry, Dalton Cardiovascular Research Center, University of Missouri-Columbia MO*

The CFTR chloride channel is activated by phosphorylation of serine residues in the regulatory (R) domain and then gated by ATP binding and hydrolysis at the nucleotide binding domains (NBDs). Studies of the ATP-dependent gating process in excised inside-out patches are very often hampered by channel rundown partly caused by membrane-associated phosphatases. Since the severed CFTR-ΔR (Csanady et al., 2000), whose R-domain is completely removed, can bypasses the phosphorylation-dependent regulation, this mutant channel might be a useful tool to explore the gating mechanism of the CFTR. To this end, we investigated the regulation and gating of the CFTR-ΔR expressed in CHO cells. In the cell-attached mode, basal CFTR currents were always obtained in the absence of cAMP agonist. Application of cAMP agonists or PMA, a PKC activator, failed to affect the activity, indicating that the activity of CFTR-ΔR channels indeed is phosphorylation-independent. Consistent with this conclusion, in excised inside-out patches, application of the catalytic subunit of PKA did not affect ATP-induced current. ATP increases the activity of CFTR-ΔR channels by increasing *Po* in a dose-dependent manner with an apparent K_d of 55.7 ± 14.6 μM. ADP inhibits the ATP-dependent currents in a dose-dependent manner. The apparent K_d values for ADP inhibition are inversely related to the ATP concentrations (57.9 ± 17.0 μM with 200 μM ATP, 181.3 ± 27.6 μM with 500 μM ATP, and 232.5 ± 46.7 μM with 1 mM ATP). These results suggest that ADP inhibits CFTR channel opening by competitively binding to the ATP-binding site(s). Similarities of nucleotide-dependent gating between wild-type and CFTR-ΔR suggest that this phosphorylation-independent mutant can be used to explore more extensively the gating mechanism of CFTR. (Supported by National Institutes of Health.)

51. Structure-Activity Studies of Fluorescein Derivatives as Inhibitors of the CFTR Cl^- Channel ZHIWEI CAI and DAVID N. SHEPPARD, *Department of Physiology, University of Bristol, Bristol BS8 1TD, UK*

The fluorescein derivative phloxine B (2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachlorofluorescein) is a potent open-channel blocker of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. Unlike other open-channel blockers of CFTR that penetrate deep into the intracellular vestibule of the CFTR

pore, phloxine B binds at a superficial intracellular site to prevent Cl^- permeation. To understand better channel block by fluorescein derivatives, we studied ethyl eosin (2′,4′,5′,7′-tetrabromoeosin ethyl ester), eosin Y (2′,4′,5′,7′-tetrabromofluorescein), TCF (4,5,6,7-tetrachlorofluorescein) using excised inside-out membrane patches from C127 cells expressing wild-type human CFTR. When added to the intracellular solution, fluorescein derivatives inhibited CFTR Cl^- currents with rank order of potency: ethyl eosin (K_d (0 mV) ~6 μM) > phloxine B (K_d (0 mV) ~50 μM) > eosin Y (K_d (0 mV) ~100 μM) > TCF (K_d (0 mV) ~165 μM). Like phloxine B, channel block by eosin Y was voltage dependent and enhanced when the external Cl^- concentration was reduced. In contrast, channel block by TCF was voltage-dependent, but unaffected by changing the external Cl^- concentration while CFTR inhibition by ethyl eosin was voltage independent. Single-channel studies indicated that fluorescein derivatives have distinct effects on channel gating with eosin Y (100 μM) greatly prolonging the interburst interval, TCF (100 μM) causing a flickery block of open channels and phloxine B (20 μM) exhibiting blocking characteristics similar to both eosin Y and TCF. These data suggest that first, the negatively charged carboxylic group of the fluorescein molecule plays an important role in voltage-dependent block. Second, chlorine atoms at positions 4,5,6,7 are required for flickery block of open channels. Third, bromine atoms at position 2′,4′,5′,7′ are required to prolong the interburst interval separating bursts of channel openings. Thus, modifications to the chemical structure of fluorescein derivatives have marked effects on channel block of the CFTR Cl^- channel. (Supported by the CF Trust.)

52. Regulation of CFTR Channel Gating by Intracellular pH JENG-HAUR CHEN, ZHIWEI CAI and DAVID N. SHEPPARD, *Department of Physiology, University of Bristol, Bristol BS8 1TD, UK*

The cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl^- channel with complex regulation. Using excised inside-out membrane patches from C127 cells expressing wild-type human CFTR, we found that intracellular pH (pH_i) modulates CFTR activity with alkaline pH_i decreasing open-probability (P_o) and acid pH_i increasing P_o. To understand better how pH_i modulates CFTR activity, we investigated gating kinetics at pH 6.3, 7.3, and 8.3. The pipette (external) solution contained 10 mM Cl^- at pH 7.3, whereas the bath (internal) solution contained 147 mM Cl^-, PKA (75 nM) and ATP (0.3 mM); voltage was −50 mV. To ensure that the Cl^- concentration was identical, all bath solutions were first titrated to pH 7.3 with HCl before adding either H_2SO_4 to decrease pH or Tris to increase pH. At pH 6.3, mean burst duration (MBD) was increased (pH 7.3, MBD = 138 ± 10 ms; pH 6.3, MBD = 204 ± 26 ms; $n = 5$; $P < 0.05$), whereas interburst interval (IBI) was decreased (pH 7.3, IBI = 154 ± 13 ms; pH 6.3, IBI = 104 ± 12 ms; $n = 5$; $P < 0.001$). In contrast, at pH 8.3, MBD was decreased (pH 7.3, MBD = 128 ± 5 ms; pH 8.3, MBD = 80 ± 7 ms; $n = 6$; $P < 0.001$), whereas IBI was increased (pH 7.3, IBI = 163 ± 8 ms; pH 6.3, IBI = 225 ± 23 ms; $n = 6$; $P < 0.001$). Analysis of open- and closed-time constants indicated that these alterations in MBD and IBI likely occur through changes in the short and long closed time constants and not the open time constant. We interpret these data to suggest that pH_i modulates CFTR activity by regulating intra- and interburst closed times. (Supported by the CF Trust and University of Bristol.)

53. Inhibition of CFTR Channels by a Peptide Toxin of Scorpion Venom MATTHEW D. FULLER,[1,2] ZHIREN ZHANG,[2] GUIYING CUI,[2] and NAEL A. MCCARTY,[2] [1]*Program in Molecular and Systems Pharmacology, Emory University, Atlanta, GA;* [2]*School of Biology, Georgia Institute of Technology, Atlanta, GA*

Peptide toxins have been used to identify amino acid residues that line the permeation pathway of cation channels. However, no peptide toxins have been identified that interact with known anion channels, such as the cystic fibrosis transmembrane conductance regulator (CFTR). Several organic blockers have been used to investigate the structure of the CFTR chloride permeation pathway; investigations of the wider cytoplasmic vestibule have been hindered due to the absence of a high affinity blocker that interacts with residues in this area. In this study we show that venom of the scorpion *L. quinquestriatus hebraeus* inhibits CFTR, in a voltage-independent manner, by decreasing the single channel mean burst duration (60 ± 10% inhibition at 0.1 mg/mL venom, $n = 5$) and open probability (41 ± 9% inhibition, $n = 5$) when applied to the cytoplasmic surface of the channel. The inhibitory effects were reversed upon washout of the venom. Decreases in burst duration and open probability were also seen when CFTR channels, previously locked open by treatment with either vanadate or 5′-adenosine(β,γ-imino)triphosphate, were exposed to the venom, suggesting inhibition by a pore block mechanism. Interestingly, the inhibitory activity of venom was abolished by protease treatment. We conclude that a peptide toxin contained in scorpion venom inhibits CFTR channels by a pore-block mechanism. (Supported by National Institutes of Health grant DK-56481 and NSF grant MCB-0077575.)

54. Probing the CFTR Channel Pore with Hydrophobic Aromatic Compounds YOSHIRO SOHMA, TOMOHIKO AI, and TZYH-CHANG HWANG, *Department of Physiology, Dalton Cardiovascular Research Center, University of Missouri-Columbia, MO*

To investigate the structural properties of the anion conducting pore of the cystic fibrosis transmembrane conductance regulator (CFTR) channel, we investigated the kinetics of the open pore block induced by aromatic carboxylases, using single channel recording of wild-type CFTR and whole-cell current recording of an locked-open CFTR mutant, K1250A. The 9-anthracene carboxylic acid (9-AC) induced a fast flickery block that can be well explained by a single binding site scheme ($n = 0.8$ and $K_d(0) = 6.9$ mM). The 9-AC block is voltage dependent with an effective valence ($z\delta$) of ~0.43, suggesting that the binding site of 9-AC might be identical to that of glibenclamide ($z\delta = \sim 0.45$), a well characterized CFTR blocker. A positional isomer of 9-AC, 1-anthracene carboxylic acid (1-AC) induced a more potent block than 9-AC with a $K_d(0)$ of 1.0 mM ($n = 0.95$) and a $z\delta$ of ~0.50. Amplitude distribution analysis showed that the off-rate of 1-AC block is 3-fold slower than that of 9-AC, whereas the on rate is ~3-fold faster. The different position of the carboxylic group in 9-AC and 1-AC should lead to a different orientation of the hydrophobic anthracene main body (consisting of three benzene rings) against the channel pore when the negatively charged carboxylic group binds to the presumed positively charged anion binding site. The difference of the binding affinity suggests a hydrophobic interaction between the compounds and the pore. Less hydrophobic compounds, 1-naphthalene carboxylic acid (1-NA, two benzene rings) and benzoic acid (BA, one benzene ring) showed lower binding affinity to the site (1-NA: $K_d(0) = \sim 14$ mM, BA: ~110 mM). Together, these results support the idea of the existence of a hydrophobic region close to the anion binding site. (Supported by National Institutes of Health.)

55. Purification and Functional Reconstitution of the Second Membrane-spanning Domain of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) FRANCISCA UGWU, MOHABIR RAMJEESINGH, CANHUI LI, SONJA DHANI, LING-JUN HUAN, YAN-CHUN WANG, and CHRISTINE BEAR, *Programme in Structural Biology and Biochemistry, Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada* (Sponsor: Criss Hartzell)

CFTR is a phosphorylation- and nucleotide-regulated chloride channel, essential for maintaining proper hydration of the fluid-lining epithelia in multiple hollow organs, including the gastrointestinal tract, airways, and other ductular organs (Sheppard and Welsh. 1999. *Physiological Reviews.* 79:S23–S45). The molecular basis for conduction through the CFTR chloride channel is unknown because we lack structural information. However, there has been remarkable progress in our understanding of the structure of related MsbA (Chang and Roth. 2001. *Science.* 293:1782–1794) and BtuCD (Locher et al. 2002. *Science.* 296:1091–1098). These structures revealed that the intact transporter is comprised of two membrane-spanning domains (MSDs), and two nucleotide-binding domains (NBDs). Further structural information is therefore, required to define the molecular basis for chloride conduction through CFTR. As progress toward this long-term goal, we have expressed the second transmembrane domain of CFTR (MSD2), encompassing residues 857 through 1158 in Sf9 cells using the baculovirus system. Sf9 expression of MSD2 confers the appearance of an anion permeation pathway suggesting that MSD2 can form an anion channel. MSD2 was purified from Sf9 cells by virtue of its polyhistidine tag and nickel affinity and was found to adopt primarily an alpha helical conformation in detergent micelles by circular dichroism. Reconstitution of MSD2 into phospholipid liposomes conferred a DIDS-inhibitable, chloride-selective electrodiffusion pathway. Reaction of the single cysteine residue in MSD2 using Nα-(3-maleimidylpropionyl) biocytin (MPB) inhibited chloride flux, supporting the claim that MSD2 directly mediates this function. Further, it is likely that dimeric MSD2-mediated chloride flux as this structure is specifically labeled by MPB. In summary, we have shown that the second membrane domain of CFTR can be purified and functionally reconstituted as a chloride channel, supporting its possible contribution to the architecture of the CFTR pore.

56. Regulation of CFTR by Extracellular Anions Is Modulated by Cytosolic Calcium and Phosphatase Activity ANGELA M. WRIGHT, BARRY E. ARGENT AND MIKE A. GRAY, *School of Cell and Molecular Bioscience, University of Newcastle Upon Tyne, Newcastle Upon Tyne, UK* (Sponsor: Mike A. Gray)

We have shown that Cl^- efflux through CFTR is inhibited by raising external HCO_3^- concentration (O'Reilly et al. 2000. *Gastroenterology* 118:1187–1196). Here we demonstrate that changes in external Cl^- concentration ($[Cl^-]_{ext}$) alone underly the inhibitory effect and that cytosolic factors modulate the extent of inhibition. Experiments were performed on CHO cells stably expressing human CFTR using both fast and slow whole-cell recordings (WCR). Replacing 100 mM extra-

cellular Cl^- with HCO_3^-, NO_3^-, Br^-, or Aspartate$^-$ produced an equal inhibition of outward and inward Cl^- currents through CFTR, despite marked differences in relative anion permeability. Identical inhibition occurred with iso-osmolar mannitol replacement, indicating that the reduction in $[Cl^-]_{ext}$ alone is sufficient to produce the inhibitory effect. Comparable results were obtained using slow WCR; however, the concentration response curve was significantly different. During fast WCR, when Cl^- was lowered from 155.5 mM to 116.5, 91.5, or 71.5 mM (mannitol replacement), the mean percent inhibition of inward current were 36.6 ± 3.6, 43.6 ± 8.7 and 59.1 ± 7.6, respectively, (n = 4–6) compared to 12.2 ± 4.4, 7.2 ± 1.6, and 24.4 ± 2.3, respectively, during slow WCR (n = 5), implicating a cytosolic factor in the inhibitory response. Increasing cytosolic $[Ca^{2+}]$ from 1 to 100 nM in fast WCR decreased the inhibition caused by $[Cl^-]_{ext}$ to levels comparable with slow WCR. The mean percent inhibition were 9.3 ± 8, 23.5 ± 3.5, and 30.7 ± 5 at 116.5, 91.5, and 71.5 mM external $[Cl^-]$, respectively (n = 4–7). Adding 1 mM bromotetramisole to the pipette solution also alleviated the block to levels comparable with slow WCR (28.7 ± 8.3% at 71.5 mM Cl^-, n = 4). Combining 100 nM Ca^{2+} and bromotetramisole had no additional inhibitory effect. These data implicate a novel Ca^{2+}-dependent phosphatase step in the inhibitory effect of reduced $[Cl^-]_{ext}$ on Cl^- efflux through CFTR, and therefore suggest that the phosphorylation state of the CFTR is involved in this response. (Supported by the Wellcome Trust.)

57. Effects of Genz-97179 and Genz-98826 on Chloride Currents in CFT1 Cells YONG-FU XIAO, QINGEN KE, YU CHEN, JILL GREGORY, BRADFORD HIRTH, JOHN L. KANE JR., LISA CUFF, SHUANG QIAO, and MARKO PREGEL, *Cardiovascular Division, Beth Israel Deaconess Medical Center, Harvard Medical School, Genzyme Corporation, Cambridge, MA* (Sponsor: Alexander Leaf)

The cystic fibrosis transmembrane conductance regulator (CFTR) protein is a Cl^- channel regulated by cAMP-dependent phosphorylation. The genetic disease cystic fibrosis (CF) is due to defective epithelial ion transport caused by mutations in the CFTR. CFTR is predominantly located in the apical membrane of epithelia and plays a crucial role in the transport of salt and water. In this study, the effects of two compounds, Genz-97179 and Genz-98826 (Genzyme Corporation), on whole-cell Cl^- currents were investigated in CFT1 human airway epithelial cells expressing the mutant ΔF508 CFTR. Genz-97179 and Genz-98826 were discovered by screening a small molecule library for compounds that restore chloride channel function in cells expressing ΔF508 CFTR. Small Cl^- currents were observed in the control CFT1 cells treated with 0.1% DMSO (n = 11) and the currents increased by 38 ± 24% ($P > 0.05$) after extracellular application of 1 μM forskolin plus 100 μM 1-isobutyl-3-methylxanthine (IBMX). Forskolin and IBMX stimulation of CFT1 cells which were incubated with 1 or 10 μM Genz-97179 for 24 or 48 h significantly increased Cl^- currents by 200% (n = 8, $P < 0.05$) and 961% (n = 9, $P < 0.01$), respectively. The increased currents returned toward the prestimulated levels after 30-min washout of the cAMP agonists. In contrast, Cl^- currents in the absence or presence of forskolin plus IBMX did not show significant differences in CFT1 cells with or without incubation of Genz-98826, an analog of Genz-97179, for 24 to 48 h. The present data suggest that Genz-97179, but not Genz-98826, was able to restore the function of the mutant CFTR in CFT1 cells.

58. Structural Modeling of the Nucleotide Binding Domains of the CFTR Using the Crystal Structures of Other ABC Transporter Proteins HAO-YANG LIU, MIN LI, TZYH-CHANG HWANG, and XIAOQIN ZOU, *Departments of Biochemistry and Physiology, Dalton Cardiovascular Research Center, University of Missouri-Columbia, Columbia, MO*

The atomic structural information of the nucleotide binding domains (NBDs) of the cystic fibrosis transmembrane conductance regulator (CFTR) is vitally important to understand CFTR gating at a molecular level. In the absence of X-ray or NMR structure of the CFTR, we have constructed a homology model of the NBD1-NBD2 dimeric structure of the CFTR based on the crystal structures of MalK (Diederichs et al. 2001. *EMBO.* 19:5951–5961) and HisP (Hung et al. 1998. *Nature.* 396:703–707) using a secondary structure-driven multiple sequence alignment. CFTR, HisP, and MalK all belong to the ABC transporter superfamily, whose members are likely to have similar structures. We have optimized our homology model by mechanical dynamic simulations with the AMBER software. The modeled dimeric structure is quite asymmetric. The roles of highly conserved residues such as Walker A lysines, Walker B aspartates, and Q-loop glutamines are analyzed from the structure and compared with patch-clamp studies. Both the side chain and main chain nitrogen of the Walker A lysine in NBD2 (K1250) interact with the β-phosphate of the bound ATP via hydrogen bonding, supporting the observations that the K1250A CFTR mutant has a significantly prolonged open time. However, only the main chain nitrogen of the same

lysine in NBD1 (K464) forms a hydrogen bond with the β-phosphate of the bound ATP, reflecting the findings that single-channel gating kinetics of the K464A mutant is almost indistiguishable from that of the wild-type CFTR. The electrostatic properties of the modeled NBD1-NBD2 dimer are also studied. Discussions will be made on the crystal structures of other closely-related proteins in the ABC transporter superfamily (e.g., Rad50 ATPase, MJ0796, MJ1267, and BtuCD) (Supported by National Institutes of Health grants DK61529 and HL53445.)

59. Cysteine String Protein (Csp) Provides a Checkpoint for CFTR Maturation HUI ZHANG, FEI SUN, KATHRYN W. PETERS, and RAYMOND A. FRIZZELL, *Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261*

The common CF mutation, ΔF508, impairs CFTR folding leading to its degradation by ubiquitin-proteasome pathways. Only ~30% of immature wt CFTR and virtually none of the ΔF508 mutant escape the ER. Csp localizes to synaptic vesicles or secretory granules to regulate exocytosis. In epithelial cells, the localization of Csp to the ER and its functional and physical interactions with CFTR implicate Csp in protein maturation (Zhang et al. 2002. *J. Biol. Chem.* 277:28948–28958). Csp overexpression blocks CFTR maturation and causes CFTR to accumulate in perinuclear aggregates. Excess Csp also stabilized immature CFTR (band B), suggesting that Csp may lie at a branch point between CFTR maturation and degradation. Csp knock-down using dsRNA elicited a fivefold increase in the expression of mature wt CFTR and reduced band B. However, decreasing Csp did not promote maturation of ΔF508 CFTR. In vitro binding studies showed that Csp interacts physically with the CFTR NH_2 terminus and R domain. The Csp structures involved in CFTR binding and maturation were examined in HEK 293 cells expressing CFTR and Csp variants. A J-domain mutant, H43Q, disrupts Csp's Hsc70 interaction motif and eliminates the inhibitory effect of Csp on CFTR maturation, but it does not disrupt CFTR binding. The J-domain also showed the strongest interaction with the CFTR NH_2 terminus. Thus, while Csp's interaction with Hsc70 is required for its effect on CFTR maturation, it does not employ Hsc70 as a linker protein in its association with CFTR. Protein binding and coimmunoprecipitation assays indicate that Csp is part of a chaperone complex that brings CFTR into association with Hsc70, HOP, and Hsp90. Our findings suggest that Csp monitors CFTR conformation via its interactions with the NH_2 terminus and R domain to regulate the progression of CFTR to post-ER compartments. (Supported by National Institutes of Health DK56490 and the CF Fdn.)

60. Pharmacology and Regulation of the Acidic pH-activated Voltage-dependent Chloride Current ($rClC_{pH}$) in Rat Sertoli Cells C. AUZANNEAU, V. THOREAU, C. NOREZ, and F. BECQ *Laboratoire des Biomembranes et Signalisation Cellulaire, CNRS, UMR 6558, Université de Poitiers, 86022 Poitiers, France* (Sponsor: Criss Hartzell)

Sertoli cells from mammalian testis are involved in development and maintenance of spermatogenesis, support and nourishment of germ cells and synthesis and release of several proteins and a potassium-rich fluid into the lumen of seminiferous tubules. Sertoli cells express a variety of ionic channels among them voltage-dependent Ca^{2+} and calcium-dependent Cl^- channels. We recently identified a novel voltage-dependent chloride current activated by extracellular acidic pH (proposed named $rClC_{pH}$) in rat Sertoli cells (Auzanneau et al. 2003. *J. Biol. Chem.* In press). Based on a molecular analysis of rClC proteins, this new chloride current does not correspond to rClC-2, rClC-3, rClC-6, or rClC-7 channels. It is activated only in the presence of an extracellular acidic pH with an estimated half maximal activation at pH 5.5. The current is strongly outwardly rectifying. It is inhibited by DIDS, DPC, glibenclamide, $CdCl_2$, and $BaCl_2$, but not by calixarene or 9-AC. The protein kinase inhibitors H89, chelerythrine chloride, and KN62 fully prevented the pH activation of this current, suggesting that phosphorylation by multiple kinases may control its activity. Sertoli cells are known to secrete lactate but its mechanism of transport was unknown. We found by RT-PCR analysis and immunocytochemistry that the monocarboxylate tranporter MCT1 is expressed in Sertoli cells. Moreover, lactic acid stimulated rClCpH with exactly the same properties (current, pharmacology, and kinases regulation). We concluded that in rat Sertoli cells, lactic acid is secreted via MCT1, which regulates $rClC_{pH}$. Both MCT1 and $rClC_{pH}$ may play an important role in the control of spermatogenesis by providing an acid environment that maintain sperm cells in a quiescent state to prevent the premature activation of acrosomal enzymes. (Supported by le Conseil régional du Poitou-Charentes.)

61. Chloride Inward Rectifier Channels in Mammalian Heart May Be Encoded by Alternatively Spliced Variants of ClC-2 F.C. BRITTON, L. YE, B. HOROWITZ, J.R. HUME, and D. DUAN *Center of Biomedical Research Excellence, Departments of Physiology and Cell Biol-*

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A novel volume-regulated hyperpolarization-activated chloride inward rectifier (Cl.ir) channel has been identified in mammalian heart (Duan et al. 2000. *Circ Res.* 86:E63–E71; Komukai et al. 2002. *Am. J. Physiol.* 283:H715–H724; Komukai et al. 2002. *Am. J. Physiol.* 283:H412–H422). To investigate whether ClC-2 is the gene responsible for Cl.ir channels in the heart we cloned a full-length cDNA of ClC-2 from rat heart (rCLC-2). When expressed in mammalian NIH/3T3 cells, rClC-2 yielded a volume regulated inwardly rectifying whole-cell current with properties similar to cardiac Cl.ir channels, and were inhibited by extracellular cadmium and by intracellular dialysis of anti–ClC-2 antibody. The single-channel rClC-2 currents behaved as a "double-barrel"-like channel with a unitary slope conductance of 3.9 ± 0.2 pS (from RP-60 to RP-120 mV). In addition, splice variants of ClC-2 cDNA with a loss of part of exon 15 corresponding to a deletion of amino acids in the transmembrane domains, with no disruption of the protein reading frame were cloned from both rat and guinea pig hearts. Although the exon 15 skipping violates the principle of conserved transmembrane domains, expression of guinea pig ClC-2 (gpClC-$2\Delta_{509–543}$) resulted in functional channels with activation kinetics and volume sensitivity similar to endogenous Cl.ir channels in native guinea pig heart (Duan et al. 2000. *Circ. Res.* 86:E63–E71) but different from expressed rClC-2 channels in mammalian cells. These results suggest that endogenous Cl.ir channels in the heart may be encoded by different cardiac ClC-2 variants. These may contribute to regulation of membrane potential and pacemaker activity, to affect cardiac electrophysiology and induce arrhythmias during ion channel remodeling under pathophysiological conditions. (Supported by AHA GIA 9950153N, NHLBI HL63914, and NCRR P20 RR 15581.)

62. Identification of Novel Murine Bestrophin Transcripts That Are Expressed in Mouse Heart H.R. BURKIN, B. HOROWITZ, J.R. HUME, and F.C. BRITTON *Center of Biomedical Research Excellence, Departments of Physiology and Cell Biology, Pharmacology, University of Nevada School of Medicine, Reno, NV 89557-0046*

Bestrophin is the protein product of the VMD2 gene of which four human isoforms have been identified (Stohr et al. 2002. *Eur. J. Hum. Genet.* 10:281–284). Mutations of bestrophin proteins are associated with vitelliform macular dystrophy (VMD, Best disease) and recently these proteins were shown to elicit a calcium-activated chloride current when expressed heterologously in mammalian cells (Sun et al. 2002. *P.N.A.S.* 99:4008–4013). We performed a search of the mouse genomic database and have identified five novel VMD2-related murine homologs (mVMD2, mVMD2L1, mVMD2L2, mVMD2L3, and mVMD2L3b). We determined that the mVMD2L3b variant is a result of alternative exon processing of mVMD2L3 transcript. The predicted translation products of the murine VMD2 transcripts show 50–89% amino acid identity with the human VMD2 family members. Kyle-Doolittle hydrophathy analysis revealed that these proteins have four putative transmembrane domains (TMDs). The murine VMD2 proteins are most conserved in these putative TMDs, whereas each of the VMD2 proteins has a unique COOH terminus. The expression of VMD2 transcripts in mouse cardiac tissue was investigated by RT-PCR analysis using gene specific primers and sequencing of the amplification products. All five murine VMD2 transcripts were found to be expressed in atrial tissue while only mVMD2 and the mVMD2L3 variants were expressed in ventricular tissue. (Supported by National Institutes of Health grant NCRR P20RR15581.)

63. Renal CLC-K Chloride Channels Heterologously Coexpressed with Barttin Are Specifically Affected by 2-(p-chlorophenoxy)propionic Acid Derivatives ANTONELLA LIANTONIO,[1] MARIA PAOLA DIDONNA,[1] ALESSANDRA PICOLLO,[2] SONIA TRAVERSO,[2] MICHAEL PUSCH,[2] and DIANA CONTE CAMERINO,[1] [1]*Section of Pharmacology, Department of Pharmacobiology, University of Bari, Italy;* [2]*Institute of Biophysics-CNR, Genova, Italy* (Sponsor: Criss Hartzell)

CLC-K channels are selectively expressed in kidney and ear where they are important for transepithelial chloride, sodium, and potassium transport. In a previous study (Liantonio et al. 2002. *Mol. Pharmacol.* 62:265-271), we identified a class of compounds, the "bis-chlorophenoxy" derivatives of the 2-p-chlorophenoxy propionic acid (CPP), capable of blocking currents sustained by CLC-K chimeras. The identification of barttin as an essential β-subunit of CLC-K channels (Estévez et al. 2002. *Nature.* 414:558–561), allowed us to perform a pharmacological characterization of wild-type CLC-K1 channels expressed in *Xenopus* oocytes. We screened the effect of a series of bis-chlorophenoxy derivatives using the two-microelectrode voltage-clamp technique. Several chemical modifications regarding the phenoxy group of the side chain (elimination of the oxygen atom or methylenic groups, substitutions of the chlorine atom) did not alter the drug-blocking activity, with five different derivatives showing a similar potency. Among these, GF-47, a derivative of CPP carrying a benzyl group on the chiral center

in the place of the methyl group, represented the minimal structure for blocking CLC-K1 currents. GF-47 inhibited CLC-K1 from the extracellular side with an affinity in the 150 μM range. The blocking potency of GF-47 is fourfold increased by lowering the extracellular chloride concentration from 112 to 22 mM, suggesting that the drug interacts with the channel pore. Concomitantly, we screened the effect of some of the "classical" Cl^- channel blockers (9-AC, DIDS, SITS, DPC, Niflumic acid, NPPB). DIDS was the only one capable of blocking CLC-K1 with a potency similar to GF-47 although in an irreversible manner. These newly identified substances provide a useful tool to investigate the biophysical and physiological role of these renal channels other than a starting point for the development of possible therapeutic drugs. (Supported by PRIN Project 2001; Telethon Italy [grant 1079]; MIUR [FIRB RBAU01PJMS].)

64. Voltage and Activity Dependence of a Novel Chloride Conductance Dynamically Controls the Resting Status of the Intact Rat Sympathetic Neuron FESCE RICCARDO,[2] MARIA LISA ROSSI,[1] RITA CANELLA,[1] and OSCAR SACCHI,[1] [1]*Department of Biology, University of Ferrara, Ferrara;* [2]*Center of Neuroscience, University of Insubria, Varese, Italy* (Sponsor: David C. Gadsby)

We discovered a novel voltage-dependent, noninactivating chloride conductance in the intact, mature rat-sympathetic neuron, by means of the two-electrode voltage-clamp technique. When the neuron is hyperpolarized, inward transient currents ensue; thereafter they decay over tens of seconds, accompanied by internal chloride ion redistribution (which however remains more concentrated than at equilibrium) and a parallel decrease in cell-input conductance, suggesting that the current is sustained by channels initially open and then slowly closed by hyperpolarization.

Here we report a remarkable activity dependence of this conductance. Both direct and synaptic neuron tetanization (15 Hz, 10-s duration to saturate the response) result in a long-lasting (not <15 min) increase of cell input conductance (+70–150% 10 min after tetanus) and an inward current with the same time course. After synaptic stimulation, both processes display similar properties under current- or voltage-clamp conditions, and upon direct stimulation they are unaffected by external calcium.

The posttetanic effects are sustained by g*Cl* increase, since both conductance and current modifications are blocked by 0.5 mM 9AC (a chloride channel blocker), but unaffected by TEACl or cesium chloride treatments. The chloride channel kinetic properties are modified by stimulation: their voltage sensitivity and rate of closure in response to hyperpolarization strongly increase.

When the voltage dependence of the three major conductances governing the cell subthreshold status (g*Cl*, g*K*, and g*L*) is evaluated over the −40/−110 mV membrane potential range in resting or stimulated neurons, voltage-conductance profiles drastically change, due exclusively to g*Cl* increase.

This previously neglected, active chloride conductance moulds neuronal excitability below threshold, and appears to host an intrinsic mechanism, a memory of previous neuron activity, which makes the chloride current a likely candidate for natural controller of the balance between opposite resting currents, and thus of membrane potential level. (Supported by COFIN 2001.)

65. Regulation of the Endogenous Transient Inward Cl Current in *Xenopus* Oocytes by $G_q\alpha$- and G_q-coupled Receptors SO YEONG LEE and SCOTT M. O'GRADY, *Department of Physiology, University of Minnesota, St. Paul, MN* (Sponsor: Criss Hartzell)

Nucleotide stimulation of G_q-coupled P2Y receptors expressed in *Xenopus* oocytes produces activation of an endogenous voltage-gated ion channel, previously identified as the transient inward (T_{in}) channel. The T_{in} current was activated by agonist stimulation of G_q-coupled receptors or G_q α subunit injection into *Xenopus* oocytes. In this study, the effect of mouse (m) G_q expression in *Xenopus* oocytes on the functional and pharmacological characteristics of T_{in} channels was compared with T_{in} currents elicited by G_q-coupled receptors with appropriate agonists. V_{50} values of G_q-activated currents were similar to the human (h) B_1-bradykinin receptor and the rat (r) M_1-muscarinic receptor, however, significantly more positive compared to the $hP2Y_1$ receptor. Time-to-peak measurements reflecting activation gating were also similar to those of the hB_1-bradykinin receptor and the rM_1-muscarinic, however, significantly increased compared to the $hP2Y_1$ receptor. Zn^{2+} sensitivity of the T_{in} current activated by the mG_q alpha subunit itself was similar to the hB_1-bradykinin receptor and the human $P2Y_{11}$ receptor. In addition, critical amino acid residues of G_q α subunit that are important in activating T_{in} channels were identified. These residues were found to be distinct from known amino acid sequences in G_q that are critical for activation of phospholipase C (PLC) β1. (Supported by AES grant 082, University of Minnesota.)

66. The Role of Cl^- in Contractions of the Opossum Esophageal Circular Muscle MADHU PRASAD,

STEVEN L. FLAMM, JOY K. SAHA, and RAJ K. GOYAL, *Center for Swallowing and Motility, West Roxbury VA Medical Center, Harvard Medical School, Boston, MA* (Sponsor: Criss Hartzell)

Myenteric neurons within the esophagus orchestrate a complex series of electrical events in the smooth muscle (SMC) membrane resulting in mechanical changes that characterize peristalsis. Electrical field stimulation (EFS) of the esophagus results in a small ACh-mediated "on" contraction that occurs at the onset of the stimulus followed by a larger NO-mediated "off" contraction that occurs at the end of the stimulus. Emerging evidence suggests that the effect of NO on gastrointestinal smooth muscle cells results from suppression of Cl^- channels. Recent studies indicate that Cl^- plays a vital role in regulating SMC membrane potential (Chipperfield and Harper. 2000. *Prog. Biophys. Mol. Biol.* 74:175–221). This study examined the role of Cl^- ions in contractions of the opossum esophageal smooth muscle. Methods: Strips of esophageal smooth muscle attached to a tension transducer in an organ bath (37°C) were perfused with oxygenated PSS and stimulated with EFS (pulse strength of 60 V for 1 ms; frequencies of 2, 20, and 40 Hz; 10-s duration). Results: EFS evoked "off" contractions were almost fourfold larger than "on" contractions (7.8 ± 0.7 *g* vs. 2.1 ± 0.2 *g*, respectively, n = 38) at 40 Hz in control tissues. Cl^-depletion (0 Cl^-) resulted in marked (90%) attenuation of the "off" contraction amplitude, with minimal effect on the "on" contraction. Reintroduction of Cl^- slowly restored the "off" contraction to initial magnitude. The Cl^- channel blocker DIDS (1 mM), and the selective Ca^{2+}-activated Cl^- ($I_{Cl,\,Ca}$) channel blockers flufenamic acid and niflumic acid (100 μM each) inhibited the "off" contraction, with minimal effect on the "on" contraction. Conclusions: In contrast to the intrastimulus ACh-mediated "on" contraction, the nitrergic "off" contraction is sensitive to both Cl^- depletion and blockers of $I_{Cl,Ca}$ in opossum esophageal smooth muscle. These results suggest that Cl^- plays an essential role in esophageal peristalsis by its selective effect on nitrergic responses. (Supported by National Institutes of Health RO1 DK 31092.)

67. Chloride Channels in Taste Cells: Multiple Subtypes and Functions SWETA RAO, DANE R. HANSEN, WEI CHEN, and TIMOTHY A. GILBERTSON, *Department of Biology, Utah State University, Logan, UT*

Chloride channels have been implicated in a variety of functions in the mammalian taste system, including being involved in the gustatory response to water, contributing to anionic effects in the salt taste response, and modulating other conductances during taste stimulation. Despite their importance in the peripheral taste system, there have been few attempts to identify Cl^- channels in taste-receptor cells. As a first approach, we have used RT-PCR and immunocytochemistry to try and identify subtypes of the ClC family members in rat taste-receptor cells. To date, we have demonstrated expression of several members of this family, including ClC-2, ClC-3, ClC-5, ClC-7, and ClC-K in taste buds from the fungiform, foliate, and vallate papillae of the tongue. Sequencing of the PCR products has demonstrated that these Cl^- channels in the taste system are identical to those found in the kidney. We are currently using a multiplexed real-time PCR assay in order to quantify expression of ClC family members in the taste system. Due to the importance of epithelial sodium channels (ENaC) in salt taste, we have also looked for expression of another Cl^- channel, the cystic fibrosis transmembrane conductance regulator (CFTR), a known modulator of ENaC function. CFTR was expressed in all taste bud–containing regions in parallel with expression of α, β, and γ ENaC subunits. Functionally, we have shown previously that a swelling-activated Cl^- conductance mediates the ability to respond to hypoosmotic stimuli (e.g., "water taste"; Gilbertson. 2002. *Chemical Senses.* 27:283–294). The properties of this conductance were remarkably similar to the ubiquitous swelling-activated Cl^- conductance ($I_{Cl,swell}$) found in many tissues. Like $I_{Cl,swell}$, however, the molecular identification of the Cl^- channel subtype mediating this conductance in the taste system has remained elusive. (Supported by National Institutes of Health grant DC02507 to T.A. Gilbertson.)

68. Inhibition of ClC-2 by a Component of Scorpion Venom CHRISTOPHER H. THOMPSON,[1] DAVID M. FIELDS,[1] KATE HUBBARD,[2] ZHI-REN ZHANG,[1] NAEL A. MCCARTY,[1] [1]*School of Biology, Georgia Institute of Technology, Atlanta, GA*; [2]*Program in Molecular and Systems Pharmacology, Emory University, Atlanta, GA*

The molecular architecture of voltage-gated ClC channels differ fundamentally from the well-studied voltage-gated cation channels. Despite the recent solution of the crystal structure of two bacterial ClCs, the gating mechanism for these channels remains unclear. The discovery of a high-affinity peptide inhibitor of ClC channels would allow study of the gating mechanism in greater detail. Recently a peptide component of the venom of the scorpion *L. quinquestriatus hebraeus* was found to inhibit CFTR chloride currents (Fuller et al. 2003. *Biophys. J.* 84:82a–83a). To determine if scorpion venom contained a component that inhibits ClC chloride currents, we studied macroscopic currents of ClC-0,

ClC-1, and ClC-2 using two-electrode voltage clamp. Despite the molecular homology between these channels, only ClC-2 was affected by the venom. We found that 0.1 mg/ml venom inhibited ClC-2 quasi-steady state currents at $V_M = -160$ mV (49.0 ± 5.9% inhibition, $n = 6$). This inhibition was reversible and occurred in a voltage-dependent manner. There was no inhibition of currents at potentials that do not strongly activate the channel. Similar inhibition of ClC-2 was seen in macropatches in the outside-out configuration. Fitting the activation pulse (at −160 mV) with a double exponential showed that the venom induced an increase in both τ_1 and τ_2 compared to control records. The observation of a change in activation kinetics coupled with the voltage dependence of inhibition indicates that venom includes a component that serves as a gating modifier. Venom inhibited ClC-2 current in a dose-dependent manner. The apparent on-rate of venom was dose-dependent, while the off-rate was not, but the off-rate was very low, suggesting that the component of the venom responsible for inhibition may be a peptide.

69. Chloride Channel Regulation of Membrane Potential and Steroidogensis Differs between Steroidogenic Cells of Testis and Ovary FRED VON STEIN, YAN LI, SUHASINI GANTA, LISA C. FREEMAN, *Department of Anatomy and Physiology, Kansas State University, Manhattan, KS* 66506-5802 (Sponsor: Philip M. Best)

Chloride ions are known to play a significant role in the regulation of testicular (Leydig cell) steroidogenesis (for review see Cooke et al. 1999. *J. Steroid Biochem. Mol. Biol.* 69:359–365). Leydig cells express two distinct chloride currents: (a) depolarization-activated, Ca^{2+}-dependent and (b) hyperpolarization-activated, cAMP- and volume-sensitive (Noulin et al. 1996. *Am. J. Physiol.* 271:C74–C84). Enhancement of chloride efflux by luteinizing hormone/cAMP is believed to be a key regulatory event in stimulation of steroidogenesis by luteinizing hormone. Here, we show that although chloride is also an important regulator of steriodogenesis in ovarian granulosa cells (Li et al. 2003. *Repro. Biol. Endocrinol.* In press), the role of chloride channels differs signficantly between ovarian and testicular cells. Primary cultures of pig ovarian granulosa cells and MA-10 (Leydig cell line) were grown for 24 h in the presence and absence of the chloride channel antagonist DIDS (100 μM). Progesterone accumulation in cell culture media was measured by radioimmunoassay. Drug-induced changes in resting membrane potential were assessed by $DiBAC_4(3)$ fluorescence as well as patch-clamp methods. DIDS had no significant effects on granulosa cell progesterone production, and depolarized granulosa cell-resting potential. In contrast, DIDS-treated MA-10 exhibited decreased progesterone production and hyperpolarized membrane potential. Western analysis detected protein for the voltage-gated chloride channels CLC-2 and CLC-3 in MA-10 cell membrane fractions, but only CLC-3 in granulosa. The relationships between chloride-channel activity, expression of steroidogenic acute regulatory protein and mitochondrial membrane potential are under investigation. Differences in chloride channel expression between testicular Leydig and ovarian granulosa cells may reflect tissue-specific dynamic responses and local environments. (Supported by National Institutes of Health HD36002.)

70. Role of Chloride in Activation of Human Neutrophil CD11b/CD18 (Mac-1) Integrin PHILIP A. KNAUF and JOANNE B. SCHULTZ, *Department of Biochemistry and Biophysics, University of Rochester, Rochester, NY*

Upon activation by cytokines or bacterial products, neutrophils undergo an activation process that results in increased adhesiveness of surface integrins, enabling neutrophils to firmly attach to endothelial cells lining the interior of venules. Experiments with pharmacological agents and low-chloride media have suggested a possible role for chloride fluxes as part of the signaling process whereby the integrins are converted from a resting to an activated state (Menegazzi et al. 2000. *J. Immunology.* 165:4606–4614). We have used the CBRM1/5 antibody (Diamond and Springer, 1993. *J. Cell Biol.* 120:545–556) to detect the activated conformation of the alpha chain, CD11b, that forms part of the major neutrophil integrin, Mac-1. Suspension of neutrophils in medium with glucuronate replacing chloride caused a rapid, >10-fold increase in the binding of CBRM1/5, as detected by flow cytometry. When chloride was restored, the binding of CBRM1/5 decreased nearly to the level seen before replacement of chloride. Parallel measurements of binding of an anti-CD11b antibody that binds to both the resting and activated showed no significant changes in glucuronate relative to cells in chloride medium. Removal of chloride recruited 40–50% of the CD11b molecules to the activated state, as compared with <10% after treatment with the bacterial product FMLP. The alteration in CD11b conformation caused by decreasing external chloride takes place within 1 min at 37°C, when the decrease in intracellular chloride is <5%, making it unlikely that changes in intracellular chloride concentration are responsible for the observed activation. It seems more likely that the activation mechanism in-

volves changes in membrane potential or structural changes caused directly by extracellular anions. The data not only suggest a new role for chloride in an important signaling mechanism, but also provide an experimental means for converting Mac-1 into the activated state for studies of the functional consequences of such activation. (Supported by National Institutes of Health grant P01 HL18208.)

71. Regulation of Volume-sensitive Chloride Channels by H^+ and Cl^- Ions in HEK 293 Cells CARMEN YUDITH HERNANDEZ-CARBALLO,[2] PATRICIA PEREZ-CORNEJO,[1] and JORGE ARREOLA,[2] [1]*Instituto de Física and* [2]*Facultad de Medicina, Universidad Autónoma de San Luis Potosí, San Luis Potosí, México*

The effects of external pH (pH_e) and Cl^- ions on volume-sensitive Cl^- channels (I_{ClVol}) were studied in HEK 293 cells using the whole cell patch clamp technique and compared to control conditions, $[Cl^-]_e = [Cl^-]_i =$ 140 mM and $pH_e = 7.3$. pH_e was varied between 5.5 and 9 while $[Cl^-]$ remained constant. pH_e 5.5 accelerated channel inactivation from 624 ± 73 ms to 285 ± 24 ms ($n = 13$) at +120 mV and produced a larger fraction of inactivated channels. These effects were observed only at positive voltages (Vm). In contrast, pH_e 9.0 resulted in complete loss of channel inactivation and linear I-V curves with currents larger than control at all Vm. pK values (7.51–7.45) determined in the range of 40–120 mV, were Vm insensitive, suggesting that the effects of H^+ on I_{ClVol} are confined to the external side. Decreasing $[Cl^-]_e = [Cl^-]_i$ from 140 to 70 to 25 mM while pH_e was maintained at 7.3 reduced the fraction of channel inactivation from 41 ± 4% ($n = 12$) to 19 ± 2% ($n =$ 11) to 8 ± 2% ($n = 4$) at +120 mV, respectively. By contrast, a reduction of $[Cl^-]_e$ from 140 to 25 mM with $[Cl^-]_i = 140$ mM accelerated channel inactivation and increased the fraction of channels inactivated from 17 ± 2 to 24 ± 2% ($n = 11$) at +80 mV, all this at $pH_e =$ 7.3. However, when lowering both pH_e to 5.5 and $[Cl^-]_e$ to 25 mM the fraction of channel inactivation further increased to 38 ± 5% ($n = 7$). We conclude that the inactivation of I_{ClVol} is controlled by the simultaneous interaction of H^+ and Cl^- ions acting on two different binding sites on the channel. Furthermore, the effects of H^+ were observed within the normal physiological range suggesting that this regulation could be relevant to channel function. (Supported by Conacyt NC0072 and National Institutes of Health DE09692.)

72. Angiotensin (AT1) Receptors and Sarcolemmal NADPH Oxidase Regulate a Cl^- Current Elicited by β1 Integrin Stretch in Rabbit Ventricular Myocytes DAVID M. BROWE and CLIVE M. BAUMGARTEN, *Department of Physiology, Medical College of Virginia, Richmond, VA*

Specific stretch of β1 integrin activates an outwardly rectifying, tamoxifen-sensitive Cl^- current (Cl^- SAC) via focal adhesion kinase (FAK) and/or Src. Cl^- SAC resembles $I_{Cl,swell}$, the volume-sensitive Cl^- current. Myocyte stretch releases angiotensin II, which binds to myocyte AT1 receptors and activates FAK and Src in an autocrine loop. Therefore, we tested whether AT1 receptors and their downstream signaling cascades are involved in mechanotransduction. Superparamagnetic beads (4.5-μm diameter; Dynal) coated with anti–β1 integrin mAb (Chemicon) were applied to rabbit ventricular myocytes and pulled upward with an electromagnet (35 G, 4–6 min). Losartan (100 μm), a specific AT1 receptor antagonist, blocked 125 ± 20% ($n = 5$) of Cl^- SAC. In the absence of stretch, losartan (100 μm) had no effect ($n = 4$). Angiotensin II–induced signaling is mediated largely by H_2O_2 generated from O_2^- produced by sarcolemmal NADPH oxidase. In the absence of stretch, exogenous H_2O_2 (500 μm) activated an outwardly rectifying Cl^- current (1.0 ± 0.2 pA/pF at +40 mV, $n = 4$, $P < 0.01$) that resembled Cl^- SAC and was completely inhibited by tamoxifen (121 ± 15%, $n = 4$). After β1 integrin stretch, diphenyleneiodonium (DPI, 60 μm), a blocker of NADPH oxidase, rapidly (<1 min) inhibited 151 ± 4% ($n = 3$) of Cl^- SAC. A structurally unrelated NADPH oxidase blocker, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, 0.5 and 2 mM), rapidly inhibited 106 ± 7% ($n = 3$) and 139 ± 28% ($n = 3$) of Cl^- SAC, respectively; block by 2 mM AEBSF was reversible upon washout (91 ± 16%, $n = 3$). These data suggest that integrin stretch activates AT1 receptors and NADPH oxidase and reveal a close coupling between NADPH oxidase and Cl^- SAC. Because NADPH oxidase transports O_2^-, the possibility that NADPH oxidase contributes to Cl^- SAC merits consideration. (Supported by National Institutes of Health HL-46764.)

73. Activation of a Cl^- Current by Specific Stretch of β1 Integrin Is Mediated by FAK and Src in Rabbit Ventricular Myocytes DAVID M. BROWE and CLIVE M. BAUMGARTEN, *Department of Physiology, Medical College of Virginia, Richmond, VA*

A volume-sensitive Cl^- current, $I_{Cl,swell}$, is activated by osmotic swelling and pressure-induced inflation of myocytes, but it remains unclear whether Cl^- currents are activated by direct mechanical stretch. Because force normally is transmitted by integrins that activate multiple signaling cascades including tyrosine kinases, we

tested whether specific stretch of β1 integrin regulates a stretch-activated Cl^- current (Cl^- SAC). Superparamagnetic beads (4.5-μm diameter; Dynal) coated with mAb to β1 integrin (Chemicon) were applied to rabbit ventricular myocytes. Beads were pulled upwards with an electromagnet (35 G, 4–6 min) while Cl^- currents were recorded in Na^{+-}, K^{+-}, and Ca^{2+}-free solutions using the ruptured patch configuration. Specific stretch of β1 integrin activated an outwardly rectifying Cl^- current (1.3 ± 0.3 pA/pF at +40 mV, $n = 12$, $P < 0.001$) that partially inactivated at positive potentials and reversed at E_{Cl}. When stretch was terminated for >10 min, Cl^- SAC decreased by 70 ± 8% ($n = 3$). Tamoxifen (10 μM), a specific blocker of $I_{Cl,swell}$, inhibited Cl^- SAC by 112 ± 4% ($n = 4$). Genistein (100 μM), a broad spectrum tyrosine kinase inhibitor previously shown to suppress activation of $I_{Cl,swell}$, inhibited Cl^- SAC by 62 ± 6% ($n = 4$). Focal adhesion kinase (FAK) and Src are upstream protein tyrosine kinases that are activated by both integrin and nonspecific forms of mechanical stimulation. PP2 (10 μM), a specific and potent blocker of both FAK and Src, inhibited Cl^- SAC by 127 ± 13% ($n = 6$), whereas its inactive analog, PP3 (10 μM), had no significant effect on Cl^- SAC ($n = 4$). These data suggest that specific stretch of β1 integrin activates a Cl^- SAC that resembles $I_{Cl,swell}$. Moreover, the mechanism of mechanotransduction involves protein tyrosine kinases, particularly FAK and/or Src kinase. (Supported by National Institutes of Health HL-46764.)

74. Cholesterol Depletion Results in Stiffening of Aortic Endothelial Cells as Determined by Micropipette Aspiration Analysis FITZROY J. BYFIELD, HELIM ARANDA-ESPINOZA, VICTOR ROMANENKO, GEORGE ROTHBLAT, and IRENA LEVITAN, *Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA*

This study investigates the effect of cellular cholesterol on the mechanical properties of aortic endothelial cells. Cellular cholesterol content was manipulated by exposing the cells to methyl-β-cyclodextrin (MβCD): cholesterol solutions at increasing molar ratios of MβCD to cholesterol while the MβCD concentration remained constant. The mechanical properties of the cells depleted of and enriched with cholesterol were determined by measuring the degree of membrane deformation for each condition using micropipette aspiration. Membrane deformation of substrate-attached cells was induced by applying a negative pressure to a micropipette attached to the cell membrane. These experiments were conducted using a 3-D deconvolution microscopy system. Our observations show that while cholesterol enrichment had no effect on the degree of membrane deformation, cholesterol depletion had a significant stiffening effect on the membrane. Young Elastic modulus calculated using a standard linear viscoelastic half-space model was 292 N/m^2 for cholesterol enriched and control cells but increased to 365 N/m^2 for cholesterol-depleted cells. These results are contrary to previous findings in model membranes where a decrease in membrane cholesterol increased the elasticity of the bilayer. This apparent discrepancy can be explained by the effect of cholesterol on the submembrane cytoskeleton that has a major impact on the mechanical properties of plasma membranes in living cells. Indeed, we show that cholesterol depletion induced a significant increase in F-actin polymerization as estimated with rhodamine-phalloidin. Our results suggest that cholesterol depletion decreases the elasticity of the membrane–cytoskeleton complex of the endothelial cells. (Supported by the American Heart Association Scientist Development grant 0130254N [to I. Levitan], AHA Postdoctoral fellowship 0225412U [to V. Romanenko] and National Institute of Health grants HL22633 and HL63768 [to G. Rothblat] and HL64388-01A1 [to Dr. Peter Davies].)

75. Volume-regulated Cl^- Channels Play an Important Role in the Reduction of Ischemia-induced Cardiomyocyte Swelling by Ischemic Preconditioning ROBERTO J. DIAZ, KUMARESAN YOGESWARAN, ALINA HINEK, PETER H. BACKX, and GREGORY J. WILSON, *Division of Cardiovascular Research, The Hospital for Sick Children, Toronto, Canada.* (Sponsor: Criss Hartzell)

We have shown previously that regulatory volume decrease (RVD) is primarily dependent on Cl^- channel activity in oxygenated cardiomyocytes and that ischemic preconditioning (IPC) triggers a signaling mechanism which enhances RVD and substantially reduces ischemia-induced cardiomyocyte swelling (Diaz et al. 2003. *J. Mol. Cell Cardiol.* 35:45–58). However, it is not known if this enhanced RVD state is directly responsible for the substantial reduction of ischemia-induced cardiomyocyte swelling produced by IPC. To address this issue, we studied 48-h cultured rabbit ventricular myocytes subjected initially to 30 min of stabilization (incubation in culture medium 199). Myocytes were then subjected to 30 min of simulated ischemia (SI, severe hypoxia and metabolic inhibition). Preconditioned myocytes were also subjected to 10 min of SI followed by 10 min of simulated reperfusion (SR, incubation in oxygenated media 199) prior to the long SI. Control myocytes were not preconditioned. Cl^- channels were inhibited by adding 50 μM indanyloxyacetic acid 94 (IAA94) both during 10-min SR and the following 30-min SI. Myocyte volumes were measured just before the

long SI and after 30 min SI with a laser-scanning confocal microscope and the percentage myocyte volume change was determined in each cell. IPC significantly ($P < 0.0001$) reduced myocyte swelling during SI (pooled controls 28 ± 10% (mean ± SEM) vs. IPC −8 ± 5%, $N = 4$ hearts/group, $n = 21$–22 cells/group). This cell volume effect of IPC was significantly attenuated by Cl^- channel inhibition with IAA94 (IPC+IAA94 18 ± 4%, $N = 5$ hearts, $n = 25$ cells, $P < 0.001$ vs. IPC, $P = 0.29$ vs. pooled controls). No difference was found among controls (± vehicle or drug). These data indicate that volume-regulated Cl^- channels play a key role in the reduction of ischemia-induced ventricular myocyte swelling by IPC. (Supported by Ontario Heart and Stroke Foundation Grant #T-4179.)

76. Disruption of *Clcn3* Gene in Mice Facilitates Heart Failure during Pressure Overload L. LIU, L. YE, C. MCGUCKIN, W.J. HATTON, and D. DUAN, *Center of Biomedical Research Excellence, Department of Pharmacology, University of Nevada School of Medicine, Reno, NV 89557-0270* (Sponsor: J.R. Hume)

Myocardial hypertrophy is a critical adaptive response to pressure overload in the early stages of cardiac remodeling. When the adaptive effort fails to compensate the pressure overload, heart failure appears. The volume-sensitive Cl^- channels seem to be involved in the remodeling because these channels are constitutively activated in hypertrophied ventricular myocytes even under isotonic conditions in congestive heart failure animal models (Clemo et al. 1999. *Circ. Res.* 84:157–165), but the exact role of these channels is not known. To investigate whether ClC-3 channels play a role in heart failure we developed a pressure overload model through a minimally invasive transverse aortic banding (MTAB) at the level of suprasternal notch in age-matched wild-type and *ClCn3*$^{-/-}$ mice (Dickerson et al. 2002. *Brain Res.* 958: 227–250). Changes in cardiac function of the animals before and after MTAB were monitored using echocardiography (GE System 5, 12 MHz linear transducer). The anterior and posterior wall thickness, chamber dimensions, and shortening fraction of the left ventricle were measured every week after the surgery. The changes in heart weight and histology and histopathology were also examined in mice of 1st and 10th week after MTAB. In comparison with the wild-type sham-operated mice, *ClCn3*$^{-/-}$ mice developed myocardial hypertrophy significantly faster (<1 wk vs. >4 wk to reach the peak of increase in wall thickness). While the hypertrophied changes in wild-type mice maintained at a similar level for >10 wk after MTAB, dilated changes (decrease in wall thickness and increase in chamber dimensions) and decrease in heart function were observed in *ClCn3*$^{-/-}$ mice after 6 wk of MTAB. We conclude that disruption of *Clcn3* gene in mice may facilitate the development of myocardial hypertrophy and promote the decompensation process and heart failure during pressure overload. (Supported by National Institutes of Health NCRR P20 RR15581.)

77. Cl^- Flux during Cell Volume Regulation Recorded Using Modulation of Ion-selective Electrodes SARAH S. GARBER,[1,2] MARK A. MESSERLI,[1] KATHERINE HAMMER,[1] MARCY D. HUBERT,[2] ELISABETH INDYK,[2] and PETER J.S. SMITH,[1] [1]*BioCurrents Research Center, Marine Biological Laboratory, Woods Hole, MA;* [2]*Department of Physiology and Biophysics, FUHS/The Chicago Medical School, North Chicago, IL*

Self-referencing Cl^--selective electrodes were positioned 1–3 μm from human embryonic kidney cells (tsA201a) and used to record Cl^- flux during regulated volume decrease in response to a sustained hyposmotic challenge. The response of the Cl^--selective electrode was close to Nernstian when comparing DC potentials measured in 100 and 10 mM NaCl (DDC = 57 ± 2 mV) but were slightly greater than ideal when comparing 1 and 10 mM NaCl (DDC = 70 ± 3 mV). Addition of 1 mM glutamate, 1 mM gluconate, or 1 mM acetate, 10 μM tamoxifen or 0.1, 1, or 10 mM HEPES at pH 6 or 7, had little effect on anion selectivity in 100 mM NaCl. A stable Cl^- flux was recorded from four out of five cells in isotonic (313 ± 5 mOsm) Ringer's solution, in the absence of a volume change. Cl^- efflux was recorded in the first 500 s after exposure to hypotonic (250 ± 5 mOsm) Ringer's solution. During this time, cells became swollen but had not yet recovered volume. An initial Cl^- efflux is consistent with regulatory volume decreases observed in separate experiments using a similar osmotic protocol. Addition of 10 μM tamoxifen to hypotonic Ringer's blocked Cl^- efflux. Tamoxifen also blocked regulated volume decrease and a volume-regulated anion current in this cell type. These results establish the feasibility of using modulation of electrochemical electrodes to monitor chloride movement during volume regulatory events. (Supported by Erik B. Fries Endowed Fellowship [MBL, Woods Hole, MA] and National Institutes of Health P41 RR01395.)

78. Inhibition of an Insulin-like Signaling Pathway in *C. elegans* Increases Resistance to Hypertonic Stress S.T. LAMITINA, L. WATERS, and K. STRANGE, *Departments of Anesthesiology, Molecular Physiology and Biophysics,*

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The molecular mechanisms that protect animal cells from osmotic stress are not well understood. In *C. elegans*, mutations in the *daf-2* signaling pathway increase resistance to thermal, oxidative, UV, and hypoxic stress. We therefore analyzed the effect of these mutations on osmotic stress resistance. Nematodes strains with mutations in the insulin-like receptor gene *daf-2* or the PI-3–like kinase gene *age-1* exhibit 80–100% survival after 24 h exposure to 400 mM NaCl growth agar, whereas wild-type worms are all killed. *daf*-2-induced survival is observed at 20 and 25°C, but not at 16°C, consistent with the temperature-sensitive nature of the *daf-2(e1370)* allele. Hypertonic stress resistance in *daf-2;daf-16* or *age-1; daf-16* double mutants is indistinguishable from wild-type worms, suggesting that *daf-2* and *age-1* effects are mediated by the DAF-16 transcription factor. DAF-2 and AGE-1 function to prevent nuclear accumulation of DAF-16. We tested the hypothesis *daf-2* signaling is osmotically sensitive by analyzing subcellular distribution of a DAF-16::GFP fusion protein. Heat shock caused a dramatic nuclear localization of DAF-16::GFP within 1 h. DAF-16::GFP worms showed significantly increased resistance to 400 mM NaCl, but hypertonic stress had little effect on nuclear localization. These results suggest that hypertonicity does not activate the *daf-2* signaling pathway. Consistent with this hypothesis, we observed that *daf-16* loss-of-function mutants survive well on 400 mM NaCl agar if they are first grown for 2 weeks on agar containing 200 mM NaCl. A similar adaptive response is seen in wild-type animals. Our results suggest that the transcriptional targets of DAF-16 protect cells from hypertonic stress. However, other transcription factors and signaling pathways likely mediate the adaptive response to hypertonicity. Current studies are aimed at identifying these transcription factors, their transcriptional targets, and the signaling pathways that lead to their activation. (Supported by DK51610, DK58212.)

79. Bcl-2–dependent Modulation of Swelling-activated Cl^- Current and ClC-3 Expression in Human Prostate Cancer Epithelial Cells LOIC LEMONNIER, YAROSLAV SHUBA, ALEXANDRE CREPIN, MORAD ROUDBARAKI, CHRISTIAN SLOMIANNY, BRIGITTE MAUROY, BERNDT NILIUS, NATALIA PREVARSKAYA, and ROMAN SKRYMA, *Laboratoire de Physiologie Cellulaire, INSERM EMI 0228, USTL Bât. SN3, 59655, Villeneuve d'Ascq, France*

Cell shrinkage is an integral part of apoptosis. However, intimate mechanisms linking apoptotic events to the alterations in cell volume homeostasis remain poorly elucidated. Here we investigated how overexpression of Bcl-2 oncoprotein—a key antiapoptotic regulator—in LNCaP prostate cancer epithelial cells interferes with the volume-regulated anion channel (VRAC), a major determinant of regulatory volume decrease (RVD). Bcl-2 overexpression resulted in the doubling of VRAC-carried swelling-activated Cl^- current ($I_{Cl,swell}$), RVD enhancement, and weakened $I_{Cl,swell}$ inhibition by SOC (store-operated Ca^{2+} channel)-transported Ca^{2+}. This was also accompanied by substantial upregulation of ClC-3 protein—a putative molecular candidate for the role of VRAC. ClC-3–specific antibody suppressed $I_{Cl,swell}$ in the wild-type and Bcl-2 overexpressing LNCaP cells. EGF treatment of wild-type LNCaP cells, promoting their proliferation, resulted in the enhancement of endogenous Bcl-2 expression and associated increases in ClC-3 levels and $I_{Cl,swell}$ magnitude. We conclude that Bcl-2–induced upregulation of $I_{Cl,swell}$, due to enhanced expression of ClC-3 and weaker negative control from SOCs-transported Ca^{2+}, would strengthen the ability of the cells to handle proliferative volume increases, and thereby promote their survival and diminish their proapoptotic potential.

80. Cholesterol Modulates Volume-regulated Anion Current (VRAC) by Changing the Physical Properties of the Lipid Environment VICTOR ROMANENKO,[1] GEORGE H. ROTHBLAT,[2] and IRENA LEVITAN,[1] [1]*Institute for Medicine and Engineering, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA;* [2]*Department of Pediatrics, The Children's Hospital of Philadelphia, Philadelphia, PA 19104*

Volume-regulated anion current (VRAC) plays a major role in the maintenance of cell-volume homeostasis as well as in regulation of cell cycle, cell proliferation, and angiogenesis. Earlier we have shown that VRAC activation in bovine aortic endothelial cells (BAECs) is inversely dependent on the level of cellular cholesterol. The goal of this study is to discriminate between the two general mechanisms proposed for the regulation of membrane proteins by cholesterol: (a) cholesterol-induced changes in the physical properties of the membrane or (b) specific sterol–protein interactions. To discriminate between the two mechanisms we substituted endogenous cholesterol with its structural analogues, which either mimic membrane-ordering effect of cholesterol or do not exert significant effect on membrane physical properties. The substitution was performed by exposing BAECs to methyl-β-cyclodextrin (MβCD) complexed with the appropriate sterol and the cellular sterol composition was determined by gas-liquid chromatography. To avoid the interference of sterols-

induced alteration in cell swelling, we studied activation of VRAC in swelling-independent manners by dialyzing the cells either with low ionic strength solution (Γi) or with GTPγS. We show that VRAC do not discriminate between cholesterol and its chiral analogue, epicholesterol, as well as between the two cholesterols and β-sitosterol. In contrast, substitution of cholesterol by coprostanol resulted in VRAC enhancement, similar to cholesterol depletion. Since it is known that cholesterol, epicholesterol, and β-sitosterol have similar effects on the physical properties of the lipid bilayer, whereas the effect of coprostanol is drastically different, our observations strongly support the hypothesis that physical properties of the membrane bilayer play a significant role in VRAC regulation. (Sponsored by AHA Scientist Development grant 0130254N, AHA Postdoctoral fellowship 0225412U, and National Institutes of Health grants HL22633 and HL63768.)

81. Inhibition of Volume-sensitive Osmolyte and Anion Channels (VSOACs) by Novel anti–ClC-3 Antibodies in Cardiac and Smooth Muscle Cells GE-XIN WANG, WILLIAM J. HATTON, GRACE L. WANG, JUMING ZHONG, ILIA YAMBOLIEV, DAYUE DUAN, and JOSEPH R. HUME, *Center of Biomedical Research Excellence, Department of Pharmacology, University of Nevada School of Medicine, Reno, NV 89557-0270*

Whether ClC-3 encodes VSOACs remains controversial. Native VSOACs in cardiac and vascular myocytes were shown previously to be blocked by a commercial anti–ClC-3 carboxyl terminus antibody ($C_{592\text{-}661}$ Ab), although recent studies have raised questions related to the specificity of $C_{592–661}$ Ab. Therefore, we developed three new anti–ClC-3 Abs and investigated their functional effects on native VSOACs in freshly isolated canine pulmonary artery smooth muscle cells (PASMCs) and guinea-pig cardiac myocytes. These new Abs produced a common prominent immunoreactive band with an apparent molecular mass of 90–92 kD in guinea-pig heart and PASMCs, and a similar molecular weight immunoreactive band was observed in brain from homozgygous *Clcn3*$^{+/+}$ mice, but not from homozygous *Clcn3*$^{-/-}$ mice. VSOACs elicited by hypotonic cell swelling in PASMCs and guinea-pig atrial myocytes were nearly completely abolished by intracellular dialysis with two new anti–ClC-3 Abs specifically targeting the ClC–3 carboxy ($C_{670–687}$ Ab) and amino terminus ($A_{1–14}$ Ab). This inhibition of native VSOACs can be attributed to a specific interaction with endogenous ClC-3, since: (a) preabsorption of the Abs with corresponding antigens prevented the inhibitory effects, (b) extracellular application of a new Ab raised against an extracellular epitope ($Ex_{133–148}$) of ClC-3 failed to inhibit native VSOACs in PASMCs, (c) intracellular dialysis with an Ab targeting Kv1.1 potassium channels failed to inhibit native VSOACs in guinea-pig atrial myocytes, and (d) anti–ClC-3 $C_{670–687}$ Ab had no effects on swelling-induced augmentation of the slow component of the delayed rectifying potassium current (I_{Ks}) in guinea-pig ventricular myocytes, although VSOACs in the same cells were inhibited by the Ab. These results confirm that endogenous ClC-3 is an essential molecular entity responsible for native VSOACs in PASMCs and guinea-pig cardiac myocytes. (National Institutes of Health grants HL49254 and NCRR P20RR15581.)

82. Volume Regulation in the Rat Lens: Roles for Chloride Channels and Transporters KEVIN F. WEBB, KAA-SANDRA CHEE, JOERG KISTLER, and PAUL J. DONALDSON, *Department of Physiology, University of Auckland, Auckland, New Zealand*

Since lens transparency critically depends on an ordered cellular structure, cell volume must be tightly controlled and extracellular spaces minimized to avoid light scattering and development of cataract. Previously we have shown that exposure of lenses to inhibitors of chloride channels and transporters, under isotonic conditions, disrupts cell structure due to localized changes in cell volume, which mimic diabetic cataract pathologies (Young et al. 2000. *Invest. Ophthalmol. Vis. Sci.* 41:3049–3055). Lens volume is thought to be maintained by a novel internal circulation system that facilitates nutrient delivery and waste removal from the internalized fibres cells in the deeper lens (Mathias et al. 1997. *Physiol. Rev.* 77:21–50). Central to the operation of this circulation system are ion channels and transporters whose differential expression promotes a vectorial movement of ions, water, and nutrients. To identify, localize, and characterize the components responsible for the damage phenotypes we have observed, we have used a combination of molecular biology, immunocytochemistry, and functional experiments to develop a molecular model that describes chloride transport in the lens.

An RT-PCR–based screening approach detected the anion channels CLC2, CLC3, and RVDAC-1, as well as the potassium chloride cotransporters KCC1, KCC3, and KCC4 in lens fibre cells. Localization of these proteins by immunocytochemistry highlights a unique distribution of labelling. Differences are observed radially within the whole lens, and labelling appears both within the cytoplasmic and membrane domains within different lens regions. Functional experiments utilizing inhibitors of chloride channels and transporters sug-

gest that the lens possesses the means to regulate its volume under conditions of stress. Examination of single cells isolated from the lens cortex identified an outwardly-rectifying anion channel, whose broad characteristics mimic those of the channel responsible for regulatory volume decrease in a number of cell systems. We believe that elements of this system are constitutively active under in vivo conditions, and further dissection of the underlying mechanisms could identify novel drug targets for anti-cataract therapy by modulating lens hydration. (Supported by Health Research Council of New Zealand, Auckland Medical Research Foundation and Foundation for Research Science and Technology.)

83. Role of SGK in Hypotonic Activation of Volume-sensitive Outwardly Rectifying Chloride Channels (VSOACs) in Cultured PASMCs G.-X. WANG, C. MCCRUDDEN, Y.-P. DAI, J.R. HUME, and I.A. YAMBOLIEV *Center of Biomedical Research Excellence, Department of Pharmacology, University of Nevada School of Medicine, Reno, NV 89557-0270*

The serum and glucocorticoid-inducible kinase (SGK) is a serine/threonine protein kinase transcriptionally regulated by corticoids, serum, and cell volume. SGK regulates cell volume of various cells by effects on Na^+ and K^+ transport through membrane channels. Based on this, we hypothesized a role for SGK in the activation of volume-sensitive osmolyte and anion channels (VSOACs) in canine-cultured pulmonary artery smooth muscle cells (PASMCs). Intracellular dialysis through the patch electrode of active SGK, but not heat-inactivated SGK, partially activated VSOACs under isotonic conditions. Dialysis of active SGK prior to cell exposure to hypotonic medium significantly accelerated the activation time-course and increased the maximal density of VSOAC currents. Hypotonic swelling of PASMCs caused activation of phosphatydilinositol 3-kinases (PI3Ks) and their downstream target SGK. Inhibition of PI3Ks with wortmannin abolished the hypotonic activation of SGK, and reduced the activation rate and maximal amplitude of VSOACs. Native ClC-3 chloride channels in PASMCs were directly phosphorylated by PKCε but not by SGK. These data indicate that the PI3K-SGK cascade is activated upon hypotonic swelling of PASMCs, and is further linked to activation of downstream signaling molecules and to activation of VSOACs. (NCRR P20 RR15581 and HL 49254.)

84. F-actin–dependent Translocation and Activation of PKCε during Osmotic Stress of Cultured Pulmonary Arterial Smooth Muscle Cells G.-X. WANG, K.J. MURRAY, Y.-P. DAI, J.R. HUME, and I.A. YAMBOLIEV, *Center of Biomedical Research Excellence, Department of Pharmacology, University of Nevada School of Medicine, Reno, NV 89557-0270*

Activation of endogenous PKCε inhibits native volume-sensitive osmolyte and anion channels (VSOACs) preactivated by cell swelling, while inhibitors of PKCε activate VSOACs under isotonic conditions in pulmonary arterial smooth muscle cells (PASMCs) (Zhong et al. 2002. *AJP Cell.* 283:C1627). Hypotonic swelling is known to activate several intracellular signal transduction pathways and cause reorganization of the actin cytoskeleton (Pedersen et al. 2001. *Comp. Biochem. Physiol.* 130:385), but the exact mechanism of VSOAC activation remains elusive. In this study we tested the hypothesis that upon hypotonic swelling, PKCε is translocated away from the sarcolemmal membrane, presumably allowing protein phosphatases to dephosphorylate and activate native VSOACs. Also, we examined cell volume–induced activation of PKCε and the possible link between PKCε activity and actin cytoskeleton reorganization. Cultured PASMCs, stained with Alexa Fluor 546 phalloidin, exhibited characteristic filamentous actin structures, and fluorescent labeling of PKCε produced a uniform diffuse pattern throughout the cells under resting conditions. Hypotonic swelling (230 mOsm) was associated with disruption of peripheral actin filaments and translocation of PKCε from the cell membrane to cytoplasmic regions, without changes in catalytic activity. Incubation of cells with cytochalasin D (10 microM) mimicked the effects of cell swelling. Hypertonic (370 mOsm) stress increased the density of actin filaments, induced translocation of PKCε into membrane peripheral regions, and inhibited VSOACs. Translocation of PKCε from cell membrane to cytoplasmic and perinuclear regions appears to be driven by disruption of peripheral actin structures rather than by changes in kinase activity, consistent with dephosphorylation and activation of VSOACs in the sarcolemma. In contrast, hypertonic cell shrinkage is associated with recovery of peripheral actin structures and PKCε content, and robust activation of PKCε. (National Institutes of Health grants NCRR P20 RR15581 and HL 49254.)

85. β-Adrenergic Regulation of the Swelling-induced Cl^- Current in Human Atrial Myocytes JONAS JUREVICIUS and RODOLPHE FISCHMEISTER, *Laboratoire de Cardiologie Cellulaire et Moélculaire, INSERM U-446, Faculty of Pharmacy, University Paris-XI, Châtenay-Malabry, France* (Sponsor: H. Criss Hartzell)

The Cl^- currents $I_{Cl,CFTR}$ and $I_{Cl,swell}$ exhibit almost similar characteristics in heart and are barely distinguishable on the basis of the current records once they are activated. This has created some confusion concerning the functional expression of $I_{Cl,CFTR}$ and/or the regulation of $I_{Cl,swell}$ by cAMP, particularly in human heart. In this study, we reevaluated this question by measuring whole-cell Cl^- currents in isolated human atrial myocytes (HAM) under symmetrical Cl^- concentrations (120 mM), using step changes in membrane potential or voltage ramps (1 V/s). Cell swelling induced by hypotonic (150 mOsm/kg) bath solution activated $I_{Cl,swell}$. $I_{Cl,swell}$ current density averaged 3.2 pA/pF at −100 mV and 7.4 pA/pF at +60 mV ($n = 4$) and outward current was strongly inhibited by DIDS (150 μM) and glibenclamide (600 μM). In normotonic conditions (310 mOsm/kg), isoprenaline (1 μM) failed to elicit any Cl^- current, although isoprenaline strongly stimulated the L-type Ca^{2+} current. However, upon cell swelling, isoprenaline dramatically increased $I_{Cl,swell}$ (+96% at −100 mV, +60% at +60 mV). The isoprenaline-activated $I_{Cl,swell}$ was not affected by 60 μM glibenclamide, a concentration sufficient to inhibit $I_{Cl,CFTR}$ (Tominaga et al. 1995. *Circ. Res.* 77:417–423), but was inhibited by larger concentration of glibenclamide (600 μM) or by 150 μM DIDS. Isoprenaline-activation of $I_{Cl,swell}$ persisted in nominally free bath Ca^{2+} concentration, excluding the participation of a Ca^{2+}-activated Cl^- current. Surprisingly, both basal and isoprenaline-activated $I_{Cl,swell}$ were strongly antagonized (by 75%) by chelerythrine (3 μM), suggesting an obligatory role of PKC. These experiments refute the existence of a functional $I_{Cl,CFTR}$ current in HAM and support the demonstration of a cAMP-activation of $I_{Cl,swell}$ (Oz and Sorota. 1995. *Circ. Res.* 76:1063–1070). A dual stimulation of $I_{Cl,swell}$ by cAMP and PKC in human heart may contribute to electrophysiological changes in the setting of cardiac ischemia, when cell swelling and catecholamines release occur concomitantly.

86. Modulation of Calcium-activated Chloride Channels from Mouse Parotid Acinar Cells by Intracellular cAMP PATRICIA PEREZ-CORNEJO and JAMES E. MELVIN, *University of Rochester, 601 Elmwood Avenue, Box 611, Rochester, NY 14642* (Sponsor: Jorge Arreola)

Several types of chloride channels have been characterized in acinar cells from the parotid gland of mice, among those calcium-activated Cl^- channels (I_{ClCa}). I_{ClCa} is the most important Cl^- exit pathway during fluid secretion of salivary glands. Our laboratory has extensively studied the biophysical properties of these channels; however, the regulatory mechanisms that modulate the channel activity have not been yet explored. Using the whole-cell configuration of the patch-clamp technique we studied the modulation of I_{ClCa} by cAMP. I_{ClCa} was activated by dialyzing the cells with an internal solution containing 250 nM $[Ca^{2+}]_i$. In preliminary experiments ($n = 3$) cells were stimulated with an cocktail mix that contained 20 μM forskolin, 100 μM IBMX, and 500 μM CPT-cAMP (a permeable analog of cAMP). The cocktail mix applied extracellularly decreased the amplitude of the current only at membrane potentials more positive than +40 mV. The same effect was observed when 1 mM of CPT-cAMP ($n = 16$) was used, but not when 1 mM of the impermeable acidic form of cAMP ($n = 10$) was used. Moreover, the effect of 1 mM of CPT-cAMP was prevented by cell dialysis with 20 nM PKI 6-22 ($n = 6$), the PKA inhibitor amide–fragment 6-22. Thus, our results suggest that the I_{ClCa} from mouse parotid acinar cells is modulated by intracellular cAMP, and that this effect is probably mediated by PKA. (Supported by National Institutes of Health DE09692 and DE13539.)

87. Intracellular Ca^{2+} Directly Regulates the Ca^{2+}-activated Cl^- Conductance in Mouse Inner Medullary Collecting Duct Cells S.H. BOESE,[2] O. AZIZ,[3] N.L. SIMMONS,[1] M.A. GRAY,[1] [1]*School of Cell and Molecular Biosciences, University Medical School, Newcastle upon Tyne, NE2 4HH, UK;* [2]*Zoophysiology, Institute for Biochemistry & Biology, University Potsdam, Lennéstr. 7a, D-14471 Potsdam, Germany;* [3]*Laboratory of Signal Transduction, NIEH-NIH, Research Triangle Park, 111 TW Alexander Drive, NC 27709*

Mouse renal inner medullary–collecting duct (mIMCD-K2) cells express a Ca^{2+}-activated Cl^- conductance (CaCC) involved in ATP-stimulated transepithelial Cl^- secretion (Boese et al. 2000. *J. Physiol.* 523:325–338). We used whole-cell patch-clamp recordings, short circuit current (I_{SC}), and Fura-2 Ca^{2+} measurements to investigate the regulation of CaCC in mIMCD-K2 cells.

Extracellular ATP causes a transient mobilization of $[Ca^{2+}]_i$, which shows a very similar time course to the activation of CaCC by ATP. There is no significant difference in the rise time (∼25 s) or decay time (∼140 s) between these two responses. The time course of transepithelial inward secretory I_{sc} in ATP-stimulated epithelial layers is also very similar to the whole-cell CaCC current and the $[Ca^{2+}]_i$ transient.

Removal of extracellular Ca^{2+} had no significant effect on basal $[Ca^{2+}]_i$ or slope conductance. ATP stimulation still resulted in a transient increase in $[Ca^{2+}]_i$ and associated current response. Although peak current/Ca^{2+} values were not different to controls, there was a significant reduction in the decay time of both signals.

This suggests that part of the activation of CaCC may be due to Ca^{2+} influx during the late phase of the ATP response. In contrast, $[Ca^{2+}]_i$ chelation reduced basal $[Ca^{2+}]_i$ significantly but had no effect on basal slope conductance, indicating that the CaCC is not active under resting conditions. Furthermore, the Ca^{2+} transient as well as CaCC activation by ATP were abolished.

Current activation started at $[Ca^{2+}]_i$ levels as low as 50–100 nM and maximal activation was achieved at levels of around 1μM. Activation by $[Ca^{2+}]_i$ was also voltage-dependent with a Hill coefficient of 3.0 at −80 mV and 1.7 at 80 mV.

In conclusion, activation and maintenance of CaCC in mIMCD-K2 cells is tightly coupled to changes in $[Ca^{2+}]_i$, suggesting that calcium directly regulates these channels. (Supported by the NKRF, UK.)

88. Virtual Cloning of Novel *Xenopus laevis* Transcripts Showing Homology to the Calcium-activated Chloride Channel (CLCA) Family F.C. BRITTON, N. LEBLANC, L. STEVENSON, J.R. HUME, and B. HOROWITZ, *Center of Biomedical Research Excellence, Departments of Physiology and Cell Biology, Pharmacology, University of Nevada School of Medicine, Reno, NV 89557-0046*

Calcium-activated chloride currents are the best characterized anion channels in *Xenopus* oocytes (Weber. 1999. *Biochim. Biophys. Acta* 1421:213–233), but the molecular component(s) underlying this current has not been identified. Members of the CLCA family of proteins have been shown to elicit a calcium-sensitive chloride conductance when heterologously expressed in mammalian cells (Fuller et al. 2001. *Pfluegers Arch.* 443:S107–S110). In this study, conserved amino acid regions of CLCA family members were used as query sequences to search the *Xenopus laevis* EST database. Two *Xenopus laevis* EST sequences showed partial homology to the NH_2 terminus of the CLCA family. Sequence analysis of the isolated cDNAs showed two transcripts (xCLCA a and b) of different length (3,596 and 5,088 nucleotides, respectively). Transcript sizes of ~4 and ~5.5 kb were confirmed by Northern analysis of oocyte RNA. Both cDNAs translate to give an identical predicted protein of 911 amino acids that show ~48% homology to the CLCA family of proteins. Sequence analysis predicted a protein with four transmembrane spanning domains, a signal sequence, and a CLCA family cysteine motif (C-$(X)_{12}$-C-$(X)_4$-C-$(X)_4$-C-$(X)_{12}$-C). Consensus phosphorylation sites are present in the predicted xCLCA protein for Ca^{2+}/camodulin-dependent protein kinase (CaMKII, 5 sites), protein kinase A (9 sites), protein kinase C (6 sites), as well as potential sites for N-linked glycosylation (9 sites). RT-PCR identified the expression of xCLCA transcripts in several *Xenopus* tissues, with the highest transcript expression in colon, oocyte and spleen. xCLCA mRNA expression relative to L8 ribosomal RNA (arbitrary units) was calculated to be 0.045 ± 0.02 for colon, 0.006 ± 0.0003 for oocyte, 0.006 ± 0.004 for spleen (mean ± SEM, $n = 3$ each tissue). We propose that xCLCA is a new CLCA orthologue that may contribute to I_{ClCa} in native oocytes. (Supported by National Institutes of Health NCRR P20RR15581.)

89. The Nonhydrolyzable Forms of ATP, AMP-PNP, and ATPgS, Modulate Rundown of the Ca^{2+}-activated Cl^- Current in Rabbit Pulmonary Arterial Myocytes NORMAND LEBLANC[1] and IAIN A. GREENWOOD,[2] [1]*Center of Biomedical Research Excellence, Department of Pharmacology, University of Nevada School of Medicine, Reno, NV 89557-0270;* [2]*Department of Pharmacology and Clinical Pharmacology, St. George's Hospital Medical School, London SW17 0RE, UK* (Sponsor: J.R. Hume)

Activation of Ca^{2+}-activated Cl^- current ($I_{Cl(Ca)}$) is an important mechanism to increase membrane excitability in smooth muscle cells (Large and Wang. 1996. *Am. J. Physiol.* 271:C435–C454). The underlying channels appear to be gated directly by intracellular Ca^{2+}, but recent studies have shown that phosphorylation by calmodulin-dependent protein kinase II regulates channel activity in airway (Wang and Kotlikoff. 1997. *Proc. Natl. Acad. Sci. USA.* 94:14918–14923) and vascular (Greenwood et al. 2001. *J. Physiol.* 534:395–408) myocytes. The aim of the present study was to investigate the impact of phosphorylation status on the activation of $I_{Cl(Ca)}$ in rabbit pulmonary artery myocytes using the nonhydrolyzable forms of ATP, AMP-PNP, and ATPγS. In myocytes dialyzed with a solution containing 500 nM Ca^{2+} and 3 mM ATP, a large increase in holding current (≈300 pA) was observed immediately after rupturing the cell membrane that decreased over the next 2 min to reach a steady-state level (≈40 pA). Rundown was associated with a decrease in the amplitude of the outward current at +70 mV (HP = −50 mV) from 202 ± 17 pA to 60 ± 12 pA (71% decrease, $n = 23$). When phosphorylation was limited by substitution of AMP-PNP for ATP the amplitude of the outward relaxation at +70 mV decreased from 196 ± 25 pA to 115 ± 12 pA (33% decrease, $n = 13$). Similar results were obtained for $I_{Cl(Ca)}$ evoked by 250 nM $[Ca^{2+}]_i$. Current-voltage relationships constructed after 3 min access were markedly larger with AMP-PNP in the pipette compared to ATP. Irreversible thio-phosphorylation with ATPγS substituted for ATP in the pipette solution produced significantly smaller $I_{Cl(Ca)}$. Our data are consistent with

the notion that activation of $I_{Cl(Ca)}$ in rabbit pulmonary myocytes is crucially dependent on the phosphorylation status. (CIHR MOP-10863 [N. Leblanc], National Institutes of Health NCRR P2015581 [N. Leblanc], and Wellcome Trust 053794 [I.A. Greenwood] grants.)

90. Modulation Dynamics of Calcium-activated Chloride Channels by Niflumic Acid in Rabbit Coronary Myocytes JONATHAN LEDOUX,[1] IAIN A. GREENWOOD,[3] and NORMAND LEBLANC,[2] [1]*Department of Physiology, University of Montréal, Montréal, Québec, Canada;* [2]*Center of Biomedical Research Excellence, Department of Pharmacology, University of Nevada School of Medicine, Reno, Nevada 89557-0270;* [3]*Department of Pharmacology and Clinical Pharmacology, St. George's Hospital Medical School, London SW17 0RE, UK* (Sponsor: J.R. Hume)

Ca^{2+}-activated Cl^- channels (Cl_{Ca}) serve as an excitatory depolarizing mechanism in the regulation of vascular smooth muscle contractility. Niflumic acid (NFA) has been widely used as a pharmacological tool to study the properties of currents generated by Cl_{Ca} ($I_{Cl(Ca)}$). A recent study has shown that NFA can both inhibit and stimulate $I_{Cl(Ca)}$ in rabbit pulmonary artery myocytes (Piper et al. 2002. *J. Physiol.* 539:119–131). We investigated the dynamics of such phenomena in coronary artery myocytes dialyzed with 500 nM $[Ca^{2+}]_i$ using the whole-cell patch-clamp technique. Exposure to 100 μM NFA inhibited $I_{Cl(Ca)}$, whereas an increase in current amplitude beyond control level was observed upon washout of the drug. The outward current elicited by 1-s pulses to +90 mV (HP = −50 mV) increased by ≈75% after washout of the drug. We next examined the concentration dependence of enhanced $I_{Cl(Ca)}$ using a fast flow delivery system. Currents were elicited by 20-s depolarizing steps to +60 mV and cells were exposed for 2 s to NFA during such steps. While the relative increase in $I_{Cl(Ca)}$ was dose independent in the range of 10 μM to 1 mM, the time to reach 50% of maximal current amplitude increased 0.20 ± 0.02 s with 10 μM NFA to 1.3 ± 0.3 s with 1 mM NFA. Furthermore, enhanced $I_{Cl(Ca)}$ recovered faster at more negative potentials. A second exposure to NFA 500 ms after washout inhibited the enhanced $I_{Cl(Ca)}$ to a similar extent to the initial exposure, suggesting that both control and enhanced $I_{Cl(Ca)}$ can be blocked by NFA. A kinetic model is proposed whereby NFA stimulates $I_{Cl(Ca)}$ by binding to high and low affinity binding sites which are occluded by the interaction of NFA with the inhibitory site. (CIHR MOP-10863 [N. Leblanc], National Institutes of Health NCRR P2015581 [N. Leblanc], and Wellcome Trust 053794 [I.A. Greenwood] grants.)

91. Endothelin Modulates Calcium-activated Chloride Channels in a Biphasic Manner via a Phosphorylation by PKC in Rabbit Coronary Myocytes JONATHAN LEDOUX[1] and NORMAND LEBLANC,[2] [1]*Department of Physiology, University of Montréal, Montréal, Québec, Canada;* [2]*Center of Biomedical Research Excellence, Department of Pharmacology, University of Nevada School of Medicine, Reno, NV 89557-0270* (Sponsor: J.R. Hume)

Endothelin (ET) is one of the most potent vasoactive hormones. It has been suggested that the vasoconstriction mediated by ET involves a depolarization caused by activation of Ca^{2+}-activated Cl^- channels (Cl_{Ca}) via IP_3-mediated Ca^{2+} release (Klöckner and Isenberg. 1991. *Pflügers Arch.* 418:168–175). In a recent study by our group, we showed that Cl_{Ca} appears to be regulated by calmodulin-dependent protein kinase II in vascular myocytes (Greenwood et al. 2001. *J. Physiol.* 534:395–408). The aim of the present study was to determine whether macroscopic Cl_{Ca} current ($I_{Cl(Ca)}$) is modulated by ET in coronary artery myocytes dialyzed with 500 nM $[Ca^{2+}]_i$ using the whole-cell patch-clamp technique. Such a technique allows for studying the regulation of Cl_{Ca} in the absence of changes in $[Ca^{2+}]_i$. ET (10 nM) applied onto the cells with a fast flow superfusion system induced a transient inhibition of $I_{Cl(Ca)}$ (−25 ± 2%) followed by a delayed increase in current level above control (+64 ± 38%). Similar to ET, PMA (100 nM), a PKC activator, caused a transient inhibition of $I_{Cl(Ca)}$ (−24 ± 3%) followed by a delayed stimulation of the current beyond the control level (+30 ± 7%). Inhibition of PKC by Calphostin C (Calp C; 1 μM) inhibited basal current to 44 ± 5% of the control level but this effect partially reversed as the current reached a new steady-state at −11 ± 12% of the control value. PMA had no effect on $I_{Cl(Ca)}$ in the presence of Calp C. These results suggest that ET modulates $I_{Cl(Ca)}$ in a biphasic manner in the absence of changes in $[Ca^{2+}]_i$. Both basal or direct stimulation of PKC can mimic some of the effects of ET in regulating $I_{Cl(Ca)}$ in coronary smooth muscle cells. (CIHR MOP-10863 and National Institutes of Health NCRR P2015581 grants.)

92. A Role for cAMP and Ca^{2+} in CLCA-dependent Regulation of Chloride Transport MATTHEW E. LOEWEN,[1] LANE K. BEKAR,[2] WOLFGANG WALZ,[2] SHERIF E. GABRIEL,[3] and GEORGE W. FORSYTH,[1] [1]*Veterinary Biomedical Sciences and* [2]*Physiology, University of Saskatchewan, Saskatoon;* [3]*Pediatrics, University of North Carolina, Chapel Hill, NC* (Sponsor: Criss Hartzell)

Proteins in the CLCA family have diverse effects, including cell adhesion, tumor suppression, and chloride

channel activity. Although the CLCA name refers to a Ca^{2+}-dependent chloride channel activity, Ca^{2+}-dependent chloride conductance may not be a general property of expressed isoforms of this protein family. We cloned pCLCA1 from porcine intestinal mucosa using a monoclonal antibody that inhibited a cAMP-dependent chloride conductance in brush border vesicles. Predicted protein structure of pCLCA1 included both C and A kinase consensus phosphorylation sites in the same putative regulatory cytoplasmic loop of the transmembrane protein (Gaspar et al. 2000. *Physiol. Genomics* 3:101–111). Expression of the pCLCA1 gene in NIH3T3 fibroblasts was accompanied by the appearance of a Ca^{2+}-dependent chloride conductance. However, the chloride permeability of the pCLCA1-expressing 3T3 cells was not sensitive to forskolin and IBMX. When the pCLCA1 protein was expressed in differentiated Caco-2 cells with brush border epithelial phenotype, the Ca^{2+} dependence of chloride conductance was lost, and endogenous cAMP-dependent chloride conductance in the Caco-2 cells was enhanced (Loewen et al. 2002. *Biochem. Biophys. Res. Comm.* 298:531–536). We probed this agonist sensitivity by sequential addition of the Ca^{2+} ionophore, ionomycin, followed by forskolin and IBMX to Caco-2 cells expressing pCLCA1. As observed previously, there was no effect of ionomycin alone, on ^{36}Cl efflux from, or on short circuit current measured across differentiated Caco-2 cells. However, the rate of ^{36}Cl efflux from cells, and the short circuit current response to cAMP agonist addition was significantly enhanced by prior exposure of cells to ionomycin. Pretreatment with the C-kinase activator, phorbolmyristoylacetate, had the same effect. The data support a synergistic cross-talk between C and A-kinase consensus phosphorylation sites for the regulatory effects exerted on Caco-2 chloride conductance by pCLCA1. (Supported by the Canadian Cystic Fibrosis Foundation.)

93. A Novel cGMP-dependent Calcium-activated Chloride Current from the Mesenteric Resistance Artery VLADIMIR MATCHKOV, CHRISTIAN AALKJAER, and HOLGER NILSSON, *Department of Physiology, University of Aarhus, Aarhus, Denmark* (Sponsor: Criss Hartzell)

We have previously demonstrated the presence in vascular smooth muscle cells of a cyclic GMP (cGMP)-dependent calcium-activated inward current, and suggested this to be of importance in synchronizing smooth muscle contraction. We have here characterized this current. Using conventional patch-clamp technique, whole-cell currents were evoked in freshly isolated smooth-muscle cells by elevation of intracellular calcium with either 10 mM caffeine, 1 μM BAY K8644, 0.4 μM ionomycin, or by high calcium concentration (900 nM) in the pipette. The current had an absolute requirement for cyclic GMP with an EC_{50} of 6.4 μM, and activation was blocked by inhibition of protein kinase G either by the antagonist Rp-8-Br-PET-cGMP or by the nonhydrolysable ATP analog AMP-PNP. The amplitude of the current closely followed the chloride gradient. The anion permeability sequence of the channel was: $Cl^- > F^- > I^- >$ acetate $> Br^- > SCN^-$, but the conductance sequence was: $I^- > Br^- > Cl^-$ > acetate $> F^- > SCN^-$. The current had no voltage or time dependence. It was inhibited by nickel and zinc ions in the micromolar range, but was unaffected by cobalt and had a low sensitivity to inhibition by the chloride channel blockers niflumic acid, DIDS and IAA-94. The properties of this current in mesenteric artery smooth muscle cells differed from those of the calcium-activated chloride current in pulmonary myocytes, which was cGMP independent, exhibited a high sensitivity to inhibition by niflumic acid, was unaffected by zinc ions, and showed outward current rectification as has been reported previously for this current. Under conditions of high calcium in the patch pipette, a current similar to the latter could be identified also in the mesenteric artery smooth muscle cells. This cGMP-dependent Ca^{2+}-activated current seemed to have all properties required to participate in the reciprocal interaction of membrane potential and intracellular calcium release, and, therefore, it is probably involved in the mechanism of generation of vasomotion. (Supported by the Danish Heart Foundation 02-2-2-15A-22033.)

94. A Candidate Protein for Calcium-activated Chloride Channels in Olfactory Sensory Neurons ILVA PUTZIER,[1] HIROSHI KANEKO,[2] JONATHAN BRADLEY,[1] and STEPHAN FRINGS,[2] [1]*Institute for Biological Information Processing, Juelich Research Center, Juelich, Germany;* [2]*Department of Molecular Physiology, University of Heidelberg, Heidelberg, Germany* (Sponsor: Paul J. Bauer)

The receptor current in olfactory sensory neurons (OSNs) is triggered by cAMP-gated cation (CNG) channels and mainly carried by Ca-activated Cl channels. Although much is known about structure and function of the CNG channels, genes that encode the Cl channel protein have not yet been identified. The CLCA family of proteins forms Ca-activated Cl channels in various epithelia. Here we examine the hypothesis that a member of this family functions as transduction channel in the sensory cilia of rat OSNs.

Using CLCA-specific primers, we cloned a gene from rat olfactory epithelium cDNA, *rclca1*, which is highly

homologous to *mclca4*, expressed in smooth muscle cells. The gene is expressed in OSNs but not in the supporting cells of the epithelium. rCLCA1-specific antibodies recognize a 97-kD protein in a preparation of ciliary membrane proteins. This protein is enriched in cilia compared to whole olfactory epithelium and displays a size of 80 kD after deglycosylation. When expressed in HEK 293 cells, *rclca1* produces a major 120-kD protein and, in addition, two minor proteins of 97 and 35 kD. Using a set of antibodies, we identified the two smaller proteins as NH_2- and COOH-terminal fragments of the 120-kD protein. Both fragments are glycosylated membrane proteins. Using photolysis of caged Ca to activate channels, functional studies were performed with native Ca-activated Cl channels in OSNs and with HEK 293 cells expressing rCLCA1. rCLCA1-transfected cells expressed a Ca-dependent Cl conductance that displayed lower Ca sensitivity compared to native channels in OSNs.

These data suggest that rCLCA1 may contribute to the Ca-dependent Cl conductance in sensory cilia of OSNs but that additional factors are required to generate the properties of the native channel. (Supported by the Deutsche Forschungsgemeinschaft, SPP 1025.)

95. Nucleotide Sensitivity of the ATP-activated Chloride Conductance in Mouse Parotid Acinar Cells JORGE ARREOLA and JAMES E. MELVIN, *Instituto de Fisica, Universidad Autonoma de San Luis Potosi, SLP, Mexico; Center for Oral Biology, University of Rochester, Rochester, NY*

We have shown previously that extracellular ATP (ATP_e) activates a novel Cl conductance (I_{ClATP}) in mouse parotid acinar cells (Arreola and Melvin. 2003. *J. Physiol.* 547:197–208). We have further characterized the time course of ATP response to prolonged exposure to ATP_e and the nucleotide sensitivity of I_{ClATP} using the whole-cell patch clamp. At +80 mV, 5 mM ATP_e produced a sustained I_{ClATP} that rapidly returned to control level upon removal (see Fig. 1, top trace). However, at −80 mV (Fig. 1, bottom trace), I_{ClATP} rapidly activated and decayed to about 22 ± 4% (n = 6) of the peak current after a monoexponential time course; upon removal of ATP_e, I_{ClATP} displayed a robust rebound before returning to control level. These effects of ATP_e were dose dependent and observed in to the presence of 0.5 mM Cibacron Blue, a purinergic antagonist. At 0.1 mM Tris-ATP, I_{ClATP} shows no decay and no rebound (n = 4). Increasing the ATP_e to 0.15 mM induced a slight current decay (24 ± 6% in three out of five cells), without rebound. We also determined the nucleotide sensitivity of I_{ClATP}. Na_2-, Mg- or Tris-ATP, Li_4-ATP-γ-S, and Li_4-AMP-PNP (5 mM) induced activation of I_{ClATP}. In contrast, neither Tris-UTP (5 mM; n = 5) nor Na_2-GTP (2.5 mM; n = 2) activated I_{ClATP}. Caged-ATP (n = 7) and TNP-ATP (n = 3) at 0.25 mM were also ineffective on I_{ClATP}. However, single UV flash to cells (n = 4) bathed in the external solution containing 0.25 mM Caged-ATP was sufficient to increase Cl current at −80 mV. These data suggest that activation of I_{ClATP} is strictly dependent on the presence of ATP_e and that hydrolysis of ATP_e is not required for channel activation. (Supported by National Institutes of Health grants DE09692 and DE13539.)

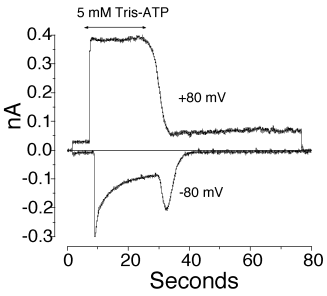

FIGURE 1.

96. Evidences of a Neuropeptidic Control of CL^- Secretion in Airway Epithelia KAREN BERNARD, STÉPHANIE BOGLIOLO, and JORDI EHRENFELD, *Laboratoire de Physiologie des Membranes Cellulaires, CNRS - Université Nice-Sophia Antipolis (UMR 6078), Villefranche-sur-Mer, France*

Arginine vasopressin (AVP) and arginine vasotocin (AVT) are two neuropeptides known to modulate electrolytes and fluid transport across the renal epithelia of mammals or inferior vertebrates. Three isoforms of AVP receptors have been identified. V1a and V1b receptors initiate a calcium-dependent signaling pathway while V2 receptors induce a cAMP-dependent one. In airways, the knowledge of mechanisms underlying the modulation of ionic conductances and fluid transport may help to determine pharmacological targets in the treatment of airway diseases, including cystic fibrosis or asthma. In this context we studied effects of neuropeptides on the electrolyte secretion of the human bronchial cell line 16HBE14o-, using a pharmacological approach combined to the short-circuit current (Isc) technique. When epithelia were bathed with 120 mM Cl^- on both sides, basal application of AVT (1 μM) induced a transient Isc peak (ΔIsc of 16.2 ± 1.2 $\mu A.cm^{-2}$) while AVP did not. This current was inhibited by Cl^- channel blockers (glybenclamide, NFA, or NPPB) applied on the apical side. A dose–response curve gave an EC_{50} of 96 nM. AVT stimulation of Isc was abolished when cells were pretreated for 72 h in presence of AVT (1 μM). In addition, the AVT stimulation was also blocked by the SR49059 compound (a V1 receptor antagonist) and by clotrimazole (a specific SK4 channel inhibitor) when applied on the basolateral side. Clotrimazole-sensitive ^{86}Rb effluxes

through basolateral membranes were also transiently increased by AVT application. These results suggest that AVT binds to a V1-type receptor located on the basolateral membranes of 16HBE14o-cells. The stimulation of calcium-dependent K^+ channels SK4 would create a favorable electrochemical gradient for Cl^- exit.

In conclusion, this study provides for the first time evidences of the control of Cl^- secretion in airway cells by AVT through the activation of a V1-type receptor. (This work is supported by the association "Vaincre la Mucoviscidose".)

97. cAMP-activated Maxi-Cl^- Channels in Native Bovine Pigmented Ciliary Epithelial Cells: Implications on the Net Secretion of Aqueous Humor CHI-WAI DO,[1] KIM PETERSON-YANTORNO,[1] CLAIRE H. MITCHELL,[1] MORTIMER M. CIVAN,[1,2] [1]*Department of Physiology and* [2]*Medicine, University of Pennsylvania, Philadelphia, PA19104-6085*

ATP stimulates whole-cell cAMP-activated Cl^- currents and reduces volume of immortalized bovine-pigmented ciliary epithelial (PE) cells. We investigated the effects of cAMP on excised patches and whole cells derived from freshly dissociated native bovine PE cells.

Cl^- currents were measured by whole cell and inside-out excised-patch patch-clamping. Volume was monitored by measuring cell area with calcein fluorescence.

cAMP stimulated whole-cell current by 22 ± 10% (N = 3); the increase was reversibly inhibited by the Cl^--channel blocker NPPB (100 μM). Maxi-Cl^- channels were detected in excised inside-out patches, displaying a linear conductance of 273 ± 2 pS (N = 77). Open channel probability (P_o) was measured at transmembrane voltages clamped at −80 to +80 mV in 20-mV steps. Maxi-Cl^- channels were infrequently observed under baseline conditions. The addition of cAMP to the cytoplasmic side of the patches produced a concentration-dependent increase in P_o at all potentials without changing unitary conductance. The cAMP-triggered activation was usually completely reversible. The Cl^- channel blockers SITS (1 mM) and NPPB (100 and 500 μM) significantly and reversibly inhibited the cAMP-activated maxi-Cl^- channels. At 500 μM cAMP, decreasing the cytoplasmic Cl^- concentration from 130 to 65 and 30 mM produced stepwise reductions in P_o. The inhibition was more significant when the membrane potential was negative. Consistent with Cl^- channel activation, addition of the membrane-permeable cAMP analogue 8-Br-cAMP triggered shrinkage of intact PE cells under isosmotic conditions. The shrinkage was mediated by enhanced Cl^- release because NPPB significantly prevented the cAMP-induced volume change.

The results suggest that cAMP activates Cl^- channels in native PE cells as in immortalized cells. The cAMP acts directly on its target, the maxi-Cl^- channels, and not through protein kinase A. The cAMP-activated maxi-Cl^- channels provide a potential pathway for reducing the rate of net aqueous humor secretion by facilitating Cl^- reabsorption back into the stroma. (Supported by National Institutes of Health research grant EY08343 and core grant EY01583.)

98. Control of Cl^- secretion by SK4 Channels in Airway Epithelia KAREN BERNARD, STÉPHANIE BOGLIOLO, OLIVIER SORIANI, and JORDI EHRENFELD, *Laboratoire de Physiologie des Membranes Cellulaires, CNRS - Université Nice-Sophia Antipolis (UMR 6078), Villefranche-sur-Mer, France*

cAMP- or calcium-activated Cl^- secretion in secretory epithelia necessitate the presence of luminal Cl^- and basolateral K^+ channels. Ca^{2+}- and cAMP-dependent K^+ conductances have been reported to participate in Cl^- secretion by favoring the driving force for Cl^- exit. The aim of this study was to identify the K^+ channels involved in ion transport in airway cells. The 16HBE14o-cell line, derived from the human bronchial epithelia, and primary cultures of human bronchial cells (NHBE) constitute models of Cl^- secretion. The NCI-H292 cell line, derived from a human pulmonary mucoepidermoid carcinoma, is a model of mucin secretion. Characterization of Cl^- and K^+ conductances was assessed by a pharmacological approach combined to the measurement of the short-circuit current and ^{86}Rb effluxes. The presence of SK and KvLQT1 channels, candidates to Ca^{2+}-and cAMP-dependent K^+ conductances, was investigated by RT-PCR.

In all airway cells, 1-EBIO stimulated while clotrimazole, charybodotoxin, or W-7 inhibited the K^+ permeability, indicating the presence of SK4-like K^+ channels. Furthermore, blockade of SK4 channels by SK4 blockers resulted in a Cl^- secretion inhibition. These channels are essentially expressed in basolateral membranes of 16HBE14o- and NHBE cells while mainly expressed in apical membranes of NCI-H292 cells, a finding linked to the cell phenotype and to its associated function. Conversely, we failed to stimulate the K^+ permeability in 16HBE14o- and NCI-H292 cells by increasing intracellular cAMP, suggesting the absence of functional KvLQT1 channels. RT-PCR experiments confirmed the presence of SK4 mRNA in the three cell types, but KvLQT1 mRNA was also detected. Thus, the lack of cAMP-dependent K^+ conductance in 16HBE14o- and NCI-H292 cells may be due to the absence of a KCNE subunit associated with the KvLQT1 channel.

In conclusion, our results underlie a key role in Cl^- secretion for SK4 channels in human bronchial cells. (This work is supported by the association "Vaincre la Mucoviscidose".)

99. Transport of Urea across Epithelial Membranes of MDCK Cells Expressing UT-A1 ROBERT B. GUNN, OTTO A. FROEHLICH, PAULINE M. SMITH, JANET D. KLEIN, SERENA M. BAGNASCO, and JEFF M. SANDS, *Departments of Physiology, Pathology, and Medicine, Emory University, Atlanta, GA*

The gene for the largest transcript of the rat UT-A isoforms, UT-A1, of the renal urea transporter was cloned into pcDNA5/FRT and transfected into MDCK cells with an integrated Flp recombination target site (Invitrogen). The cells from a single clone were grown to confluence on collagen-coated membranes until the resistance was >1,500 ohm-cm^2. Transepithelial ^{14}C-urea fluxes were measured at 37°C in Hank's balanced salt solution, pH 7.4, with 5 mM urea. The baseline fluxes were not different between unstimulated UT-A1–transfected MDCK cells and nontransfected or sham-transfected MDCK cells. However, only the UT-A1–transfected cells expressed UT-A1 protein (as measured by Western analysis) and were stimulated by 10 μM forskolin (1.8-fold, n = 20) or by 100 pM vasopressin (2.2-fold). Thionicotinamine (5 mM, TN) inhibited the unstimulated and stimulated ^{14}C-urea fluxes in the UT-A1–transfected MDCK cells by 27% and 55%, respectively, indicating that these cells have some constitutive TN-sensitive urea transport and that all of the stimulated flux is TN sensitive. The stimulated flux was also inhibited by 1 mM phloretin. These characteristics mimic those seen in isolated perfused rat terminal inner medullary collecting ducts (Chou and Knepper. 1989. *Am. J. Physiol.* 257: F359). We conclude that we have created a polarized epithelial cell line that stably expresses UT-A1 and reproduces several of the physiologic responses observed in rat terminal inner medullary collecting ducts. (Supported in part by National Institutes of Health grant DK63657.)

100. A Chloride Channel at the Basolateral Membrane of the Connecting Tubule of the Mouse Kidney ANTOINE NISSANT and JACQUES TEULON, *CNRS UMR 7134, Université Pierre et Marie Curie, Paris, France* (Sponsor: Criss Hartzell)

The connecting tubule (CNT) is a part of the renal tubule located between the distal convoluted tubule and the collecting tubule, and comprised of CNT cells and intercalated cells. It is involved in Na^+ absorption and K^+ secretion. No information is available on Cl^- transport at this site. We investigated basolateral Cl^- channels on microdissected CNTs, isolated from collagenase-treated mouse kidneys, using the patch-clamp technique. The bath solution contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 10 HEPES (pH 7.4).

With high-Na^+ solution (145 NaCl) in the pipette, we recorded one channel that had a linear i/v relationship reversing at 0.6 ± 6.5 mV (n = 5, means ± SEM) in cell-attached patches, and a unit conductance of 10.5 ± 1.4 pS. With high-K^+ solution (145 KCl) in the pipette, we recorded an inwardly rectifying K^+ channel (g_{in}: ~35 pS) in 45% of cell-attached patches (total number of patches: 42). Under this condition, the 10-pS channel was recorded in all but one case from cell-attached patches that did not show K^+ channel activity (43% of patches). Upon excision, the anionic selectivity of the channel was assessed by changing the solution on the intracellular side. Relative permeabilities with respect to chloride were 0.08 ± 0.02 (n = 6) for Na^+, 0.44 ± 0.07 (n = 5) for Br^-, 0.56 ± 0.09 (n = 5) for NO_3^-, 0.77 ± 0.08 (n = 3) for I^-, and 0.17 ± 0.04 (n = 6) for F^-.

In summary, Cl^- channels seem to be only present in a sub-class of cells in the CNT, possibly intercalated cells, which are not endowed with K^+ channels. This agrees with the notion that intercalated cells are involved in Cl^- transepithelial transport.

101. The Role of the NH_2 terminus of Kir6 in the Interaction with the NH_2-terminal Transmembrane Domain (TMD0) of SUR MONIKA K. SZENK and KIM W. CHAN, *Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH*

The activities of ATP-sensitive potassium (K_{ATP}) channels are regulated by the ATP and MgADP levels exist within a cell and they are important for linking the metabolic state of a cell to its membrane excitability. K_{ATP} channels are made up of two subunits: the pore-forming Kir6 subunit, which belongs to the inwardly rectifying potassium (Kir) channel family and the regulatory SUR subunit, which is a member of the ATP-binding cassette (ABC) proteins. We have demonstrated previously that a truncated SUR protein that comprises only the NH_2-terminal domain (TMD0) of SUR (i.e., containing only the first 195 amino acids) can associate strongly with Kir6 (Chan et al. 2002. *Biophys J.* 82:590A). Furthermore, TMD0 can greatly enhance the cell surface expression of Kir6 once its ER-retention motif (RKR) is abolished (e.g., Kir6.2Δ26). Deletion of the NH_2 terminus of Kir6.2 has been shown to increase the opening probability (Po) of the heteromeric K_{ATP} channels without affecting the Po of the ΔNKir6.2Δ25 (Koster et al. 1999. *J Physiol.* 515:19–30). Hence, the NH_2 terminus of Kir6.2 interacts with SUR. We found that TMD0 cannot enhance the surface expression of ΔN2-14Kir6.2Δ26. We will investigate whether ΔNKir6.2Δ26 can associate with TMD0 and whether the surface expression of ΔNKir6.2Δ26 can be enhanced by the ER-export motif (FYCENE) (Ma et al. 2001. *Science.* 291:316–319) that is present in Kir2.1. Our preliminary data suggest that the NH_2 terminus of Kir6.2 is an important structural element in the interaction

with TMD0 of SUR. (Supported by grant NIH-R01-DK60104-01A1.)

102. Electrophysiological Characterization of Store-independent and Store-dependent Ca^{2+} Conductances in *C. elegans* Intestinal Epithelial Cells ANA Y. ESTEVEZ and KEVIN STRANGE, *Departments of Anesthesiology, Molecular Physiology and Biophysics, and Pharmacology, Vanderbilt University Medical Center, Nashville, TN*

Inositol-1,4,5-trisphosphate (IP_3)–dependent Ca^{2+} oscillations in *C. elegans* intestinal epithelial cells play an important role in regulating the nematode defecation cycle (Dal Santo et al. 1999. *Cell.* 98:757–767), an ultradian rhythm with a periodicity of 45–50 s. Patch-clamp studies combined with behavioral assays and forward and reverse genetic screening would provide a powerful approach for defining the molecular details of oscillatory Ca^{2+} signaling in eukaryotic cells. However, electrophysiological characterization of the intestinal epithelium has not been possible because of its relative inaccessibility. We developed primary intestinal epithelial cell cultures that circumvent this problem. Using the whole cell patch clamp technique we demonstrate that intestinal cells express two highly Ca^{2+}-selective conductances. One Ca^{2+} conductance, I_{ORCa}, shows strong outward rectification, inhibition by intracellular Mg^{2+}, and insensitivity to intracellular Ca^{2+} store depletion. The biophysical characteristics of I_{ORCa} are similar to those of some members of the transient receptor potential (TRP) cation channel superfamily. Inhibition of I_{ORCa} with high intracellular Mg^{2+} concentrations revealed the presence of a store-operated conductance I_{SOC}. The characteristics of I_{SOC} include strong inward rectification, activation by passive or active intracellular Ca^{2+} store depletion, inactivation during store refilling, and depotentiation upon removal of extracellular Ca^{2+}. These characteristics resemble those of the Ca^{2+} release–activated Ca^{2+} current, I_{CRAC}, studied extensively in mammalian cells. The molecular identity of the I_{CRAC} channel is unknown. Our studies provide the first detailed electrophysiological characterization of voltage-independent Ca^{2+} conductances in *C. elegans* and form the foundation for ongoing genetic and molecular studies aimed at identifying the genes that encode the ORCa and SOC channels, for defining mechanisms of channel regulation, and for defining their roles in oscillatory Ca^{2+} signaling. (Supported by DK60829.)

103. Identification and Functional Analysis of Kir2.X Potassium Channels in Human Aortic Endothelial Cells YUN FANG, CONGZHU SHI, PETER F. DAVIS, and IRENA LEVITAN, *Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA*

Membrane potential of aortic endothelial cells under resting conditions is dominated by inwardly recitifier K^+ (Kir) channel. Vascular endothelial Kir channels also play a pivotal role in regulation of Ca^+ signaling and in shear stress mechanotransduction. Previous molecular cloning and reverse transcriptase-polymerase chain reaction (RT-PCR) studies indicated the rectifier potassium current in vascular endothelial cells most probably belongs to Kir2.1. Here we show that not only Kir2.1 transcript but also those of the other three members of Kir2 subfamily are expressed in human aortic endothelial cells (HAECs). In these experiments, RT-PCR was performed with total RNA extracted from human aortic endothelial cells using primer pairs specific to different Kir2 channel subunits. The integrity and quality of the total RNA were evaluated by Aglient 2100 Bioanalyser. Both intron-spanning and nonintron-spanning primers were designed for each gene, except intronless Kir2.2, to prevent false positive results of genomic DNA contamination. Message RNAs for all four members of Kir2 subfamily were detected in HAECs, including Kir2.4, which was reported previously to be uniquely expressed in brain, nervous system, and neuronal cells. The relative Kir 2.X mRNA abundance in HAECs was defined with fluorescence-based real time RT-PCR using Lightcycler™ technology. Our results demonstrate that Kir2.1 and Kir2.2 transcripts are present at twofold higher level than the transcripts of Kir2.3 and Kir2.4. Furthermore, the concentration and voltage dependence of Ba^+ block and single-channel conductance for the inwardly rectifier channels in HEACs were characterized to explore the functional significance of the four members of Kir2 subfamily. (Supported by the American Heart Association Scientist Development grant 0130254N [to I. Levitan], AHA Postdoctoral fellowship 0225412U [to V.R.] and National Institutes of Health grants HL22633 and HL64388-01A1 [to Dr. Peter Davies].)

104. Functional Characterization of a Novel Gβγ-activated Ion Channel in Mammalian Heart EMIL N. NIKOLOV and TATYANA T. IVANOVA-NIKOLOVA, *Department of Pharmacology and Toxicology, Brody School of Medicine, East Carolina University, Greenville, NC*

Muscarinic K^+ (K_{ACh}) channels underlie the response of the heart to parasympathetic stimulation. These channels are members of a larger family of G protein regulated inward rectifier K^+ (GIRK) channels that play a unique role of detecting and translating changes in the levels of specific G protein βγ subunits into membrane hyperpolarization. The K_{ACh} channels are heterotetrameric proteins with unitary conductance of ~35 pS and are composed of two homologous subunits, GIRK1 and GIRK4. Here we report the functional characteriza-

tion of a small conductance GIRK (scGIRK) channel present in the membrane of rat atrial myocytes. The scGIRK channel has a conductance of approximately 16 pS and brief open durations characterized by two time constants τ_{O1} and τ_{O2} of 0.22 and 1.11 ms, respectively. At electrophysiological level, the atrial scGIRK channels resembled the previously described recombinant GIRK4 homomultimeric channels, indicative of possible structural homology between the scGIRK channels and the GIRK4 homomultimers. Populations of both K_{ACh} and scGIRK channels were found present in the same atrial myocytes. Upon Gβγ activation both channels behaved in qualitatively similar manner. The scGIRK channel, like its K_{ACh} counterpart, adopted multiple functional states bestowed by the apparent binding of a different number of Gβγ subunits to the channel structure. However, the electrogenic capacity of the K_{ACh} and scGIRK channels was substantially different. While the K_{ACh} channels were able to generate robust responses upon G protein activation, the scGIRK channels dissipated the G protein signals, thus modulating the sensitivity of the atrial myocytes to parasympathetic stimulation. Utilization of the same Gβγ-activation mechanism by the K_{ACh} and scGIRK channels offers compelling evidence that Gβγ subunits employ a limited repertoire of signaling strategies to convey extracellular signals to effector molecules with different physiological roles in the membrane. (Supported by Brody SOM Faculty Development Grant.)

105. Interactions of CO_2 and pH on Thermosensitive Neurons in Rat Hypothalamic Tissue Slices CHADWICK L. WRIGHT,[1,2] M. LORRY KAPLE,[1] PENNY W. BURGOON,[3] and JACK A. BOULANT,[1] [1]*Department of Physiology and Cell Biology,* [2]*Medical Scientist Program, Ohio State University, Columbus, Ohio;* [3]*Department of Cell and Structural Biology, University of Illinois, Urbana, IL* (Sponsor: Jack A. Rall)

Body temperature is regulated by a temperature-sensitive region of the brain, the preoptic-anterior hypothalamus (PO/AH). Temperature-sensitive neurons in the PO/AH control all thermoregulatory responses. Carbon dioxide (CO_2) is a metabolic waste product of cellular respiration and is normally kept at low levels in the body. When CO_2 levels become elevated, however, body temperature changes inappropriately, i.e., body temperature decreases in cool environments and increases in warm environments. The present study employed extracellular and intracellular electrophysiological recordings in rat hypothalamic tissue slices to determine neuronal responses to increasing CO_2 concentration from normal 5% to elevated 10%. Elevated CO_2 decreased the firing rate of most (60%) warm-sensitive neurons. By comparison, only 29% of the temperature-insensitive neurons decreased firing rate during elevated CO_2. In addition, a small number of neurons (9%) increased their firing rates in response to elevated CO_2, and this occurred in both temperature-sensitive and -insensitive neurons. The most prevalent effect among temperature-sensitive neurons was a decreased firing rate during elevated CO_2. With elevated CO_2, this decreased firing rate was often associated with both membrane depolarization and a more depolarized action potential threshold. The change in threshold appears to be the primary determinant of the decreased firing rate. The inhibitory effects of elevated CO_2 on warm-sensitive neurons may explain why elevated CO_2 impairs body temperature regulation. To differentiate between elevated CO_2 and CO_2-induced acidosis (i.e., decreased pH due to elevated CO_2), some neurons were also exposed to solutions with either normal CO_2 but decreased pH (isocapnic acidosis) or elevated CO_2 but normal pH (isohydric hypercapnia). The neuronal responses to elevated CO_2 are due primarily to decreases in pH rather than a direct effect of elevated CO_2. (Supported by National Institutes of Health grant NS14644.)