Speaker Abstracts

1. Assembly and Activation of an Erythropoietin Receptor (EpoR) Signaling Complex HARVEY F. LODISH, *Professor of Biology and Professor of Biological Engineering, M.I.T. Member, Whitehead Institute for Biomedical Research, Cambridge, MA*

Erythropoietin (Epo) is the principal hormone regulating production of red blood cells. Epo production by the kidney is enhanced by anoxia, and it functions by inhibiting apoptosis of a class of committed erythroid progenitors. The erythropoietin receptor, like all cytokine receptors, requires a JAK tyrosine kinase protein for signal transduction. In the resting state cell surface EpoR is a dimer and Epo binding alters the conformations of the two receptor extracellular domains.

We showed that JAK2, more specifically just its NH_2-terminal "FERM" domain, binds in the endoplasmic reticulum to the membrane-proximal cytosolic domain of newly made EpoR to promote receptor folding and cell surface expression. The amount of JAK2 is thus one of the factors that limit the amount of the EpoR on the cell surface. Another is five conserved amino acids in the extracellular domain of all cytokine receptors, WSXWS; mutation of the middle residue of this sequence, A in the EpoR, to E (found in the majority of cytokine receptors) enhances receptor folding several fold. A third factor is the rapid endocytosis and degradation of cell surface EpoR whether or not ligand is present. Together these regulate the number of receptors able to bind Epo ligand and enable erythroid progenitors to respond appropriately to changing levels of Epo in the circulation.

Three amino acids in the EpoR membrane proximal segment, highly conserved among all cytokine receptors, are essential for JAK2 activation but not for surface appearance of the EpoR in response to JAK2. These key amino acids appear to be on one face of a helix that is physically continuous with the membrane spanning a helix; we suggest that the conformation of these residues changes upon Epo binding such that they bind to JAK2 and "flip" the kinase to the "on" state.

2. Potassium Channel Trafficking DZWOKAI MA, NOA ZERANGUE, KIMBERLY RAAB-GRAHAM, YUH NUNG JAN, and LILY YEH JAN, *Howard Hughes Medical Institute, Departments of Physiology and Biochemistry, University of California, San Francisco, CA*

Potassium channels serve a wide range of physiological functions; mutations of different potassium channels cause epilepsy, episodic ataxia, cardiac arrhythmia, hearing loss, hypertension, hyperinsulinemic hypoglycemia, and developmental abnormalities of the neural crest-derived tissues (Andersen's syndrome). The function of potassium channels is partly determined by the number of these channels on the cell membrane, which may be controlled at the level of trafficking at various steps in the secretory pathway. Recent studies on the trafficking signals identified in potassium channels and potential regulation of potassium channel trafficking will be discussed. (Supported by NIH grant MH63981.)

3. Differential Targeting of AMPA and NMDA Receptors after Assembly with SAP97 O. JEYIFOUS and W.N. GREEN, *Department of Neurobiology, Pharmacology, and Physiology, University of Chicago, Chicago, IL*

Synapse-associated proteins (SAPs) are a family of membrane-associated guanylate kinases (MAGUKs) implicated in the synaptic localization of both ligand- and voltage-gated ion channels. One member of this family, SAP97, localizes at the lateral membrane of epithelial cells and at postsynaptic densities of neurons where it associates with GluR1, an AMPA receptor subunit. Both AMPA and NMDA receptors possess a COOH-terminal

T/SXV motif capable of interacting with the first two PDZ binding domains of SAP97. In this study, we examine the effect of SAP97 coexpression on the cell-surface targeting of AMPA and NMDA receptors. We find that GluR1 colocalizes with SAP97 both intracellularly, and extensively in patches, at the cell surface of transiently transfected COS7 or HEK cells. Coexpression of SAP97 and NMDA receptors results in a cellular distribution strikingly different from that seen when either protein is expressed alone. There is intracellular clustering and colocalization in the ER, which prevents the membrane targeting of both proteins. This ER retention is mediated through an association between SAP97 and the NR2B subunit. Our findings indicate that SAP97 assembles with both AMPA and NMDA receptors in the ER and targets the two different glutamate receptor subtypes to separate locations in the cells.

4. Assembly, Transport, and Targeting of the Yeast Vacuolar H^+–ATPase Complex LAURIE A. GRAHAM and TOM H. STEVENS, *Institute of Molecular Biology, University of Oregon, Eugene, OR*

The proton-translocating ATPase found on the vacuolar membrane of the yeast *Saccharomyces cerevisiae* is one of the most extensively characterized members of the V-type ATPase family (V-ATPase). The V-ATPase is composed of a catalytic V_1 sector of peripherally associated subunits facing the cytosol, which is assembled onto a V_0 sector of integral membrane proteins responsible for the translocation of protons across the membrane. Various membrane-localized transporters utilize the proton gradient generated by the V-ATPase to drive the accumulation of ions, amino acids, and metabolites into intracellular organelles.

Recent work in our lab has focused on characterizing the assembly, transport, and targeting of the V_0 subcomplex. The V_0 subcomplex is composed of a single 100-kD multispanning membrane protein, a 36-kD peripheral protein (Vma6p), and three extremely hydrophobic proteins (or proteolipids) of molecular mass 16 kD (Vma3p), 17 kD (Vma11p), and 23 kD (Vma16p). The 100-kD V_0 subunit is encoded by two genes in yeast (*VPS1* and *STV1*), and whereas the Vph1p-containing V-ATPase complex is localized to the vacuole, the Stv1p-containing V-ATPase is localized to Golgi and endosomal membranes. Upon synthesis, the V_0 subunits are translocated into the membrane of the endoplasmic reticulum (ER), where they assemble to form the V_0 subcomplex before being transported to the vacuole via the Golgi apparatus. This assembly process is aided by three ER-localized proteins, Vma12p, Vma21p, and Vma22p, which are not components of the V-ATPase complex present on the vacuolar membrane. Cells lacking any one of the V-ATPase "assembly factors" (Vma12p, Vma21p, and Vma22p) lack V-ATPase activity due to failure to properly assemble the V_0 subcomplex.

We have found that the Vma12p and Vma22p form a complex that interacts directly with the 100-kD V-ATPase subunit Vph1p in the ER. We have also found that Vma21p interacts with the V_0 subunit Vph1p; however, this interaction is mediated by the small hydrophobic subunits Vma3p, Vma11p, and Vma16p. Surprisingly, in cells lacking Vma12p or Vma22p, the V_0 subunits associate but the resulting "V_0 subcomplex" is unable to exit the ER. Our current model envisions a separate role for the Vma12p–Vma22p complex from the Vma21p in the assembly of the V-ATPase complex. Since Vma21p interacts directly with the proteolipids in the ER, it may play a role in directing the correct stoichiometry of the assembling V_0 subdomain, in particular the assembling proteolipids.

Besides functioning in assembly of the V-ATPase, Vma21p also functions in escorting the V-ATPase out of the ER. Vma21p possesses a COOH-terminal di-lysine ER retrieval motif required for the ER localization, and we have found that the retrieval defective Vma21 mutant protein exits the ER with the V-ATPase and remains associated with the complex even once the V-ATPase is localized to the vacuole. Recent experiments indicate that wild-type Vma21p cycles between the ER and a post-ER compartment (presumably the cis-Golgi). We propose that Vma21p exits the ER with the V-ATPase, dissociates from the V-ATPase complex in the cis-Golgi, and is then retrieved back to the ER for another round of assembly and transport. (Supported by NIH grant GM38006.)

5. Analysis of Intracellular Trafficking Pathways and Protein Dynamics within Living Cells JENNIFER LIPPINCOTT-SCHWARTZ, *Cell Biology and Metabolism Branch, NICHD, NIH, Bethesda, MD*

With the advent of green fluorescent protein (GFP), the subcellular localization, mobility, transport routes, and binding interactions of proteins can be studied in living cells. We have used live cell imaging of GFP fusion proteins in combination with photobleaching and photoactivation techniques to investigate three areas of membrane trafficking: (a) protein turnover and mobility in the ER, (b) the kinetic properties of secretory protein transport, and (c) protein interactions and dynamics underlying membrane trafficking machinery. Results from this work will be discussed in relation to their significance for understanding fundamental principles of secretory membrane trafficking and sorting of molecules within cells.

6. Checkpoints for Transmembrane Protein Folding in the Secretory Pathway LAURENCE FAYADAT and RON R. KOPITO, *Department of Biological Sciences, Stanford University, Stanford, CA*

To understand the relationship between conformational maturation and quality control–mediated proteolysis in the secretory pathway, we engineered the well-characterized degron from the α-subunit of the T cell antigen receptor (TCRα) into the α-helical transmembrane domain of homotrimeric type I integral membrane protein, influenza hemagglutinin (HA). Although the membrane degron does not appear to interfere with acquisition of native secondary structure, as assessed by the formation of native intrachain disulfide bonds, only ~50% of nascent HA^{2+} chains become membrane-integrated and acquire complex N-linked glycans indicative of transit to a post-ER compartment. The remaining ~50% of nascent HA^{2+} chains fail to integrate into the lipid bilayer and are subject to proteasome-dependent degradation. Site-specific cleavage by extracellular trypsin and reactivity with conformation-specific monoclonal antibodies indicate that membrane-integrated HA^{2+} molecules are able to mature to the plasma membrane with a conformation indistinguishable from that of HA^{wt}. These apparently native HA^{2+} molecules are nevertheless rapidly degraded by a process that is insensitive to proteasome inhibitors but blocked by lysosomotropic amines. These data suggest the existence of at least two sequential quality control checkpoints that operate the secretory pathway to ensure the fidelity of protein deployment to the plasma membrane. (Supported by NIH grant DK43994.)

7. Using the ER Quality Control Pathway for Regulation of the Sterol Synthesis RANDOLPH Y. HAMPTON, OMAR BAZIRGAN, STEPHEN CRONIN, THOMAS CUNNINGHAM, JOYCE DEFRIES, CHRISTINE FEDEROVITCH, ISABELLE FLURY, RENEE GARZA, TUYET LAM, ALEXANDER SHEARER, and ERIN QUAN, *Section of Cell and Developmental Biology, University of California at San Diego Division of Biology, La Jolla, CA*

Protein degradation functions in the regulation of specific cellular processes and in quality control to remove damaged or misfolded proteins. In physiological regulation, specific features of a target protein must be uniquely identified, whereas quality control recognition is based on unknown hallmarks of misfolding that transcend individual protein sequence. The degree to which regulation and quality control are mechanistically distinct is an open question. A long-standing and medically important example of regulated degradation is found in HMG-CoA reductase (HMGR), a key enzyme of cholesterol synthesis. Mammalian HMGR undergoes feedback-regulated ER degradation in response to changing cellular demand for sterol pathway products, and this process is conserved in yeast. Capitalizing on this conservation, we have shown that HMGR undergoes regulated ubiquitination at the ER surface that is under the control of a conserved sterol pathway molecule. ER ubiquitination of Hmg2p is mediated by the HRD complex, a transmembrane ubiquitin ligase (E3) of the growing RING-H2 class. In addition to Hmg2p, the HRD complex is required for the ER-associated degradation (ERAD) of a large number of misfolded proteins with no obvious sequence similarity. Thus, Hmg2p appears to undergo degradation by a quality control pathway for purposes of physiological regulation. The implications of this functional juxtaposition will be discussed in terms of approaches to cholesterol management and general strategies for selective in vivo alteration of proteins.

8. Protein Degradation at the Endoplasmic Reticulum THOMAS SOMMER, *Max-Delbrück-Center for Molecular Medicine, Berlin, Germany*

The secretory pathway of eukaryotic cells harbors an elaborate protein quality control system, which prevents the deployment to the secretory pathway of misfolded or unassembled proteins. This system is localized in the endoplasmic reticulum (ER). ER-associated protein degradation (ERAD) is an important component of this quality assurance system and directs misfolded proteins for destruction by the cytoplasmic ubiquitin–proteasome pathway.

ERAD can be divided mechanistically into four steps: first, misfolded proteins are detected in the ER–lumen. Second, the proteolytic substrates are targeted to and inserted into an aqueous transport channel that includes the multispanning membrane protein Sec61p. Third, the substrates are transported back into the cytosol (dislocation). Fourth, dislocated substrates are marked with the polypeptide ubiquitin by membrane-bound components of the ubiquitin system. These include the ubiquitin-conjugating enzymes Ubc1p, Ubc6p, and Cue1p assembled Ubc7 and the ubiquitin ligase Hrd1p. Fifth, the ubiquitin-conjugated and dislocated molecules, which are still attached to the cytosolic surface of the ER membrane, are mobilized by the Cdc48p–Ufd1p–Npl4p ATPase complex. Finally, the cytosolic 26S–proteasome complex digests the misfolded proteins.

9. The Calnexin/Calreticulin Cycle A. HELENIUS, K. QUIRIN, C. RITTER, E.-M. FRICKEL, R. RIEK, K.

WÜTHRICH, and L. ELLGAARD, *Institutes of Biochemistry and Molecular Biology and Biopphysics, Swiss Federal Institute of Technology (ETHZ), Zurich, Switzerland*

Intracellularly, N-linked oligosaccharides are used as universal "tags" that allow specific lectins and modifying enzymes to promote folding, quality control, and sorting of glycoproteins in different stages of maturation. Substrate binding to calnexin and calreticulin in the ER is, for example, determined by UDP-glucose:glycoprotein glucosyltransferase (GT), a soluble, lumenal enzyme that specifically reglucosylates incompletely folded proteins. We have investigated the molecular basis by which this important folding sensor distinguishes between folded and unfolded glycoprotein conformers by analyzing its interaction with RNase B in vitro. The main findings were: (a) GT prefers partially folded over unfolded proteins as its substrate, (b) no carbohydrate moiety is needed in the specifying interaction, (c) it can distinguish between folded and incompletely folded domains in the same protein, and (d) it recognizes small folding defects within otherwise folded domains, and only reglucosylates nearby glycans. In this way, it confers stringent, local conformational criteria to the export and deployment of glycoproteins. Studies using TROSY NMR and biochemical methods have, moreover, revealed that ERp57, a glycoprotein-specific thiol oxidoreductase, interacts with the outermost tip of the long finger like P-domain of calreticulin. Together with the recently published X-ray structure of calnexin's ectodomain, these result lead to a more detailed model for the mechanism of calnexin and calreticulin function in the ER.

10. Lipid Rafts and Membrane Trafficking KAI SIMONS, *Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany*

We are studying the mechanism of raft clustering and apical sorting in MDCK cells. Lipid rafts are assemblies of sphingolipids and cholesterol in the exoplasmic leaflet of the fluid bilayer probably interacting with the underlying cytosolic leaflet. These assemblies function as platforms in membrane trafficking and signaling. A number of proteins specifically interact with rafts and these can be identified by biochemistry and mass spectrometry. One important property is the size of lipid rafts. Lipid rafts are small, ~50 nanometers in diameter. The key characteristic of these small units is that they can be clustered by different means and this property is essential for their function. We have been analyzing the clustering of rafts in several different cellular processes. We have done this artificially by applying antibodies to raft and nonraft components in living cells. We have analyzed apical sorting, which involves clustering of rafts into apical transport containers. We are studying the role of rafts in amyloid precursor protein (APP) processing. We have found that rafts play a decisive role in the generation of β-amyloid from APP. Moreover, we are also analyzing the role of ergosterol-sphingolipid rafts in exocytosis in yeast and their role in regulating yeast cell surface polarity.

11. Post-Golgi Carrier Exocytosis in Epithelial Cells GERI KREITZER,[1] JAN SCHMORANZER,[3] SENG HUI LOW,[4] XIN LI,[4] YUNBO GAN,[1] THOMAS WEIMBS,[4] SANFORD M. SIMON,[3] and ENRIQUE RODRIGUEZ-BOULAN,[1,2] [1]*Margaret M. Dyson Vision Research Institute, Weill Medical College of Cornell University, New York, NY;* [2]*Department of Cell Biology, Weill Medical College of Cornell University, New York, NY;* [3]*Laboratory of Cellular Biophysics, The Rockefeller University, New York, NY;* [4]*Department of Cell Biology, Lerner Research Institute and Urological Institute, Cleveland Clinic, Cleveland, OH*

We used time-lapse total internal reflection and confocal fluorescence microscopy to study changes in localization and exocytic sites of post-Golgi transport intermediates (PGTIs) carrying GFP-tagged apical or basolateral membrane proteins during epithelial polarization. In nonpolarized MDCK cells, apical and basolateral PGTIs were present throughout the cytoplasm and were seen fusing with the basal domain of the plasma membrane. Upon polarization, apical and basolateral PGTIs were restricted to different regions of the cytoplasm and their fusion with the basal membrane was completely abrogated. Quantitative analysis revealed that basolateral, but not apical, PGTIs fused with the lateral membrane in polarized cells, correlating precisely with the restricted localization of syntaxins 4 and 3 to lateral and apical membrane domains, respectively. Microtubule disruption induced syntaxin 3 depolarization and fusion of apical PGTIs with the basal membrane, but effected neither the lateral localization of syntaxin 4 or sec6 nor promoted fusion of basolateral PGTIs with the basal membrane. Our experiments provide novel insights on the mechanisms responsible for polarized exocytosis in epithelial cells. (Supported by NIH grants to E. Rodriguez-Boulan and S.M. Simon and a Jules and Doris Stein Professorship to E. Rodriguez-Boulan.)

12. Transport and Localization of AMPA-type Glutamate Receptors in the *C. elegans* Nervous System CHRISTOPHER RONGO, *Waksman Institute, Rutgers University, Piscataway, NJ*

Synaptic connections undergo a dynamic process of stabilization or elimination during development, and this process is thought to play a critical role in memory, learning, and in establishing the specificity of synaptic connections. To better understand how central synapses form and are regulated, we are examining the subcellular localization of an AMPA-type ionotropic glutamate receptor, named GLR-1, in the nematode *C. elegans*. Our previous results demonstrated that a GLR-1::GFP fusion protein, which retains activity in vivo, is localized to punctate structures in synaptic regions of the neuropil that correspond to specific synaptic inputs. We have identified two proteins, LIN-10 and UNC-43, that function to localize GLR-1 to synaptic connections. LIN-10 is a PDZ protein that also functions in apical–basolateral protein trafficking in epithelial cells. We find that in *lin-10* mutants, GLR-1 fails to tightly cluster at synapses. UNC-43 encodes a Ca^{2+} and calmodulin-dependent protein kinase II (CaMKII), a protein that has been proposed to play a pivotal role in regulating synaptic strength and in maturation of synapses during development. Mutant nematodes that lack UNC-43 have a low density of GLR-1 synaptic clusters. GLR-1 is found at high levels in vesicular pools within the neuron cell bodies of these mutants, suggesting that Ca^{2+} signaling through CaMKII is required for the transport of GLR-1 from neuron cell bodies to neurites and synaptic connections. UNC-43/CaMKII also shows synaptic localization, and we have identified several key residues involved in regulating the activity-dependent localization of UNC-43. We are also currently characterizing a number of new factors (identified through screens) that genetically interact with UNC-43 and LIN-10, and play a pivotal role in GLR-1 trafficking and synaptic localization. To have a complete understanding of the mechanism of GLR-1 localization, our aim is to identify all of the important molecules involved in the localization process. (Supported by the Pew Scholars for Biomedical Sciences and the Johnson and Johnson Discovery Award.)

13. Subunit Control of AMPA Receptor Dynamics at Neuronal Synapses SANG HYOUNG LEE, JERRY LIN, MARIA PASSAFARO, and MORGAN SHENG, *Center for Learning and Memory, Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA*

The AMPA-type ionotropic glutamate receptor mediates most of the fast synaptic transmission at excitatory synapses. Changing the number of postsynaptic AMPA receptors represents a powerful means of modifying the strength of synaptic transmission. In hippocampus, AMPA receptors are largely composed of heteromers containing GluR1 and GluR2, or GluR2 and GluR3 subunits. Via their cytoplasmic tails, GluR1 and GluR2/3 subunits bind to different intracellular proteins. In recent years, it has emerged that AMPA receptors can be rapidly delivered to, or removed from, synapses to potentiate, or depress, synaptic transmission, respectively. We have studied how individual subunits control the trafficking of AMPA receptors by following the exocytotic and endocytotic behavior of epitope-tagged homomeric receptors transiently expressed in hippocampal neurons. Our results show that the exocytosis of GluR1 is inducible by activity, whereas that of GluR2 is constitutive. In heteromeric GluR1/GluR2 receptors, GluR1 is "dominant" over GluR2 in terms of this exocytotic behavior. On the other hand, the endocytosis of GluR2 is inducible by activity, whereas that of GluR1 is not. In heteromeric receptors, GluR2 is dominant over GluR1in terms of endocytotic behavior. The differential regulation of the surface delivery and internalization of GluR1 and GluR2 is determined by the COOH-terminal cytoplasmic tails of these receptor subunits.

14. Ankyrins: Molecular Keys to the Cellular Code for Directing Ion Channels to Sites of Physiological Function VANN BENNETT, PETER MOHLER, and ANTHONY GRAMOLINI, *Departments of Cell Biology, Biochemistry, and Neuroscience and the Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC*

Ankyrins are a ubiquitously expressed family of membrane-adaptor proteins that interact with structurally diverse membrane proteins via ANK repeats. Currently identified ankyrin partners include ion channels/pumps (Na/K and H/K ATPases, the voltage-dependent sodium channel, and the Na/Ca exchanger), calcium-release channels (IP3R and RyR), and cell-adhesion molecules (L1CAM family members). We have found in studies involving gene knock-outs in mice that ankyrins are required for targeting ion channels/pumps to excitable membranes in the central nervous system as well as to the calcium homeostasis compartment within the endoplasmic reticulum of striated muscle. Knockout of ankyrin-B causes mistargeting of ryanodine (Ry) and IP3 receptors in the sarcoplasmic reticulum of neonatal cardiomyocytes. Mice heterozygous for a null mutation in the gene encoding ankyrin-B are haploinsufficient with reduced levels of 220 kD ankyrin-B in multiple tissues, and exhibit a syndrome we refer to as *couch potato*: cardiac arrhythmia, reduced exercise tolerance, and obesity. Ankyrin-B(+/−) ventricular cardiomyoctes have normal levels and localiza-

tion of RyR, but reduced levels of Na/K ATPase, Na/Ca exchanger, and IP3 R. Ankyrin-B(+/−) mice have a cardiac arrhythmia characterized by sinus bradycardia, delayed cardiac conduction, and a stress-induced ventricular tachycardia resembling patterns observed in humans with long QT syndrome. A candidate human disorder involving ankyrin-B is dominantly inherited type 4 long QT syndrome, which includes features of sinus bradycardia and prolonged QT interval, and maps to the same chromosome site of 4q25 as the gene encoding ankyrin-B. These studies establish a physiological requirement for ankyrins in expression of functionally coupled ion channels, and suggest a new class of functional channelopathies due to abnormal cellular processing.

15. Na^+ Channel Clustering at CNS Nodes of Ranvier: The Role of Contactin KATIE KAZARINOVA-NOYES,[1] JYOTI D. MALHOTRA,[2] DYKE P. McEWEN,[2] LORI L. ISOM,[2] and PETER SHRAGER,[1] [1]*Department of Neurobiology and Anatomy, University of Rochester, Rochester, NY*; [2]*Department of Pharmacology, University of Michigan, Ann Arbor, MI*

As axons develop, Na^+ channels cluster at sites destined to be nodes of Ranvier under the influence of myelinating glial cells. Nodal Na^+ channels exist in a complex with a number of surface and cytoskeletal proteins. Contactin is an immunoglobin-superfamily molecule with homology to the β2 subunit of the Na^+ channel. Contactin and Na^+ channels can be reciprocally coimmunoprecipitated (coIP) from rat brain lysates. In transfected cell lines, contactin increased both Na^+ channel current and saxitoxin binding fourfold, but only in the presence of the β1 subunit. Correspondingly, in doubly transfected cells β1 (but not α or β2) coIPs with contactin. In the CNS, contactin was colocalized with Na^+ channels at nodes. Contactin is also present at lower immunofluorescence intensity in paranodes, where it is associated with caspr/paranodin in the formation of axo–glial junctions. Thus, contactin may play a role in enhancing axonal Na^+ channel expression through association with β1, and also participate in myelin formation. This idea was tested further in contactin null mutant mice provided by Dr. B. Ranscht (Berglund et al. 1999. *Neuron.* 24:739–750). In the optic nerve of P15 contactin −/− mice there were only 45% of the nodal clusters of Na^+ channels seen in wt littermates. Further, whereas in +/+ animals 48% of these clusters were highly focal, in −/− mice only 15% were normal, and the remainder more diffuse and misshapen. In wt axons caspr was clustered at high density in paranodes, but in −/− optic nerves no such sites were seen, suggesting a defect in myelination. The compound action potential recorded from P15 wt optic nerves consisted of two clearly resolved peaks, while −/− nerves had only the slower of these two components. This could reflect the deficiency in myelination, but may also result from the absence of a rapidly conducting class of retinal ganglion cells. Thus, genetic elimination of contactin in the CNS results in (a) a loss of paranodal localization of caspr, a component of the axo-glial junctions important for electrical isolation of the node, (b) a significant reduction in Na^+ channel expression and localization at nodes, and (c) a major functional deficit in optic nerve conduction. (Supported by NIH grants NS17965 and MH59980, and NSF grant IBN 9734462.)

16. The Role of AMPA Receptor Endocytosis in Long-Term Depression ROBERT C. MALENKA, *Department of Psychiatry and Behavioral Sciences, Nancy Pritzker Laboratory, Stanford University School of Medicine, Palo Alto, CA*

The mechanisms of synaptic plasticity in the mammalian brain, in particular NMDA receptor–dependent long-term potentiation (LTP) and long-term depression (LTD), are of considerable interest because of their putative roles in the modification of neural circuits during learning and development. For many years there was controversy about whether the initial changes in synaptic strength occur primarily because of modifications of postsynaptic glutamate receptors or changes in presynaptic transmitter release. There is now, however, general agreement that one important mechanism by which activity can regulate the strength of individual synapses is by modifying the number of AMPA receptors in the synaptic plasma membrane. Consistent with this hypothesis, we found that application of glutamate, AMPA or NMDA to cultured hippocampal cells for periods as short as 5 s causes a significant internalization of surface AMPA receptors. A number of lines of evidence indicate that this endocytosis requires dynamin function and is mediated by clathrin-coated pits. Like the triggering of LTD, this pharmacological stimulation of AMPA receptor endocytosis appears to require influx of calcium and activation of the calcium/calmodulin-dependent protein phosphatase calcineurin.

Does AMPA receptor endocytosis actually contribute to synaptic plasticity, specifically LTD? By stimulation of hippocampal cells in culture, we generated an NMDA receptor–dependent LTD, which was accompanied by a decrease in both the amplitude and frequency of miniature excitatory postsynaptic currents. Immunocytochemical analysis revealed that this LTD was associ-

ated with a decrease in the percentage of synapses that contained detectable levels of AMPA receptors in the synaptic plasma membrane. In hippocampal slices, the generation of LTD was prevented by loading cells with inhibitors of dynamin-dependent endocytosis. These results suggest that endocytosis of synaptic AMPA receptors contributes to NMDA receptor–dependent LTD and that modulation of the synaptic localization of glutamate receptors may be a ubiquitous mechanism by which synaptic strength can be adjusted in response to changes in the pattern of neuronal activity.

17. Surface Membrane Turnover Studied by Electrical Methods DONALD W. HILGEMANN, RUOLIN YE, and SIYI FENG, *Department of Physiology, University of Texas Southwestern Medical Center, Dallas, TX*

The rates of membrane cycling between intracellular compartments and the surface membrane remain controversial in many cell types, particularly for "constitutive" membrane trafficking that may not be coupled to classical calcium-dependent signaling mechanisms. Therefore, we have initiated electrophysiological studies to quantify membrane turnover and analyze its regulation, starting with an electrophysiologically advantageous cell line, namely the M1 mouse distal tubule cell. Primarily, we are using whole-cell voltage clamp of small cells with high resolution capacitance measurements, but it also seems possible to activate a diacylglycerol-dependent exocytosis mechanism in giant excised patches. Golgi-to-surface membrane trafficking is blocked at low temperatures, and we are therefore probing whether membrane turnover from and to intracellular compartments can be quantified by temperature perturbations. Tentatively, cell warming results in substantial membrane insertion within just 3 min, and the insertion process occurs in calcium-free conditions (10 mM EGTA on both membrane sides). Tentatively, diacylglycerol surrogates accelerate membrane turnover in calcium-free conditions. And tentatively, cytoplasmic PIP2 and GTP can activate substantial endocytosis in calcium-free conditions. Our long-term goal is to analyze the roles of diacylglycerols, phosphatidylinositides, and other phospholipids in regulating constitutive membrane turnover.

Poster Abstracts

18. COOH-terminal Tail of the Human Anion Exchanger AE1 Is Important for its Proper Trafficking to the Plasma Membrane EMMANUELLE CORDAT, JING LI, and REINHART A.F. REITHMEIER, *Departments of Biochemistry and Medicine, CIHR Group in Membrane Biology, University of Toronto, Toronto, Ontario, Canada*

AE1 or Band 3 is an abundant glyco-protein of the erythrocyte plasma membrane where it catalyses the electro-neutral exchange of chloride for bicarbonate. To determine the role of the COOH-terminal tail of AE1 in its expression, function, and targeting, we generated five different constructs encoding deleted proteins on the last 5 (Δ5), 11 (Δ11), 15 (Δ15), 20 (Δ20), or 35 (Δ35) amino acids. These constructs were transiently transfected in HEK 293 cells. All the proteins except Δ20 and Δ35 were expressed at the same level than the WT. We confirmed by pulse-chase that this low expression was due to a fast degradation of Δ20 and Δ35. Furthermore, the last 15 amino acids are not required for AE1 normal folding in the membrane since Δ5, Δ11, and Δ15 AE1 were able to bind to an inhibitor affinity matrix. Immunofluorescence, pulse chase, and deglycosylation results showed that Δ11 is retained intracellularly, whereas part of Δ5 traffics to the plasma membrane. These results indicate that COOH-terminal tail of human AE1 is important for proper AE1 trafficking and targeting to the plasma membrane, probably via a potential class-II-PDZ binding domain located at the extreme COOH terminus of the protein. (Supported by CIHR and la Fondation Pour La Recherche Medicale.)

19. Interactions of the NaPi Cotransporter IIa in the Brush Border of Proximal Tubular Cells NADINE DELIOT, SERGE GISLER, SANDRA PRIBANIC, NATI HERNANDO, JURG BIBER, and HEINI MURER, *Institute of Physiology, University Zürich, Zürich, Switzerland*

The NaPi-cotransporter IIa is localized in the brush border membrane of proximal tubular cells and is responsible for reabsorption of most of the filtered phosphate (Pi). In addition, NaPi-IIa represents an important target for physiological and pathophysiological alterations of Pi-reabsorption that are achieved by regulated endocytosis.

To identify proteins interacting with the type-IIa cotransporter, yeast two-hybrid screens against the intracellular COOH-terminal and NH_2-terminal domains were performed. Based on immunohistochemistry, identified candidate proteins that localized to the brush border and/or supapical compartment were considered for further analysis. Proteins interacting with the COOH terminus were identified as the PDZ proteins NaPi-Cap1 (PDZK1), NaPi-Cap2 and NHERF-1. These interactions were established to occur via a COOH-terminal TRL-motif. MAP17 (a cancer-associated protein) and VILIP-3 (a Ca-binding protein) were identified by the screen against the NH_2 terminus; MAP17 was also found to interact with NaPi-Cap1, but not with NHERF-1. Verification of the various interactions were performed by in vitro assays, such as by GST-pull downs and by gel overlays.

To reveal possible functions of the identified NaPi-IIa–interacting proteins, OK cells were transfected with single PDZ domains, PDZ domain 1 of NHERF-1 and PDZ domain 3 of NaPi-Cap1. Both were shown to interact specifically with NaPi-IIa. Transfection of these PDZ domains resulted in a disturbance of the apical appearance of NaPi-IIa, while the microvillar organization (β-actin) remained intact. Thus, this data indicate that interactions of NaPi-IIa with NaPi-Cap1 and NHERF-1 are important for the apical sorting/positioning of NaPi-IIa. Expression of both proteins in OK cells was verified. The roles of the other proteins identified remains to be determined.

In addition, evidence was obtained that both PDZ proteins act as anchoring sites for regulatory components such as PK-A (shown by others for NHERF-1) or AKAP2 (unpublished data). (Supported by the Swiss National Science Foundations, grants 31.46523.96 and 31.65397.01.)

20. Regulation of Type IIa NaPi Cotransporters by Endocytosis NATI HERNANDO, NADINE DELIOT, DESA BACIC, SANDRA PRIBANIC, ZOUBIDA KARIM-JIMENEZ, JÜRG BIBER, and HEINI MURER, *Institutes of Physiology and Anatomy, University of Zürich, Zürich, Switzerland*

Many factors such as parathyroid hormone (PTH), atrial natriuretic peptide (ANP), or nitric oxide (NO) lead to phosphaturia by inhibiting renal proximal reabsorption of phosphate (P_i). This inhibition is achieved by reduction of the number of type IIa Na/Pi-cotransporters (NaPi IIa) expressed in the brush border membrane, due to enhanced endocytosis.

Studies in isolated proximal tubules and cultured cells (OK cells) demonstrated that endocytosis is initiated by elevation of cAMP (PTH) or cGMP (ANP and NO). Although the steps connecting secondary messenger activation with retrieval of NaPi IIa remain unknown, we obtained evidences that phosphorylation of NaPi IIa does not play a regulatory role, at least after PK-A activation. Endocytosis does not seem mediated by known endocytic signals such as tyrosine-dileucine– or diacidic-based motifs, although endocytosed cotransporters partially colocalized with clathrin. Internalized cotransporters do not recycle, but instead they are targeted to lysosomes for degradation.

To elucidate which domains of NaPi IIa are required for endocytosis, chimeras between the type IIa (renal/ PTH-regulated) and type IIb (non renal/ non PTH-regulated) cotransporters were fused to the enhanced green fluorescent protein (EGFP) and expressed in OK cells. This experiments documented that a dibasic motif (R/K-R) located in the third intracellular loop (IL3) is necessary for PTH-induced endocytosis of type IIa cotransporters. To identify proteins interacting specifically with the IL3 of NaPi IIa, this loop was used as bait in a yeast two hybrid screening of a kidney cDNA library. Only one protein, HP33, turned out to specifically interact with the IL3 of NaPi IIa. This intracellular protein, suggested to bind microtubules, is expressed in proximal tubular cells, but its role in the hormonal-induced endocytosis of NaPi IIa remains to be tested. (Supported by the Swiss National Foundations, grants 31.46523.96 and 31.65397.01.)

21. The Role of Phospholipase D2 in Glut4 Glucose Transporter Translocation PING HUANG, GUANG-WEI DU, and MICHAEL A. FROHMAN, *Department of Pharmacology and Center for Developmental Genetics, State University of New York, Stony Brook, NY*

Insulin-stimulated glucose uptake in muscle and adipose cells is mediated by the Glut4 glucose transporter, which translocates to the plasma membrane from a storage compartment upon insulin receptor stimulation and increases intracellular glucose flux. Phospholipase D (PLD), a signal-transducing membrane-associated enzyme, has recently been proposed to promote Glut4 translocation through insulin receptor activation (Emoto et al. 2000. *J. Biol. Chem.* 275:7144–7151). However, the underlying mechanisms and the potential roles of the two individual isoforms, PLD1 and PLD2, are not well understood. Using novel isoform-specific PLD antibodies generated in our lab, we have found that both PLD1 and PLD2 are expressed in 3T3-L1 adipocytes and CHO cells but in different subcellular compartments. In 3T3-L1 adipocytes, both insulin and endothelin-1 (ET-1) activate PLD, and they have an additive effect on PLD activation that is only partially inhibited by wortmannin, a PI3-kinase inhibitor, suggesting that PLD is downstream of both the insulin/PI3-kinase and ET-1 signaling pathways. Using tetracycline-inducible PLD overexpressing CHO cells as a model system, we have found that PLD2 (but not PLD1) activation phenocopies the effect of insulin and/or ET-1, that is, it results in Glut4 translocation to the plasma membrane. In contrast, overexpression of the lipase-inactive PLD2 (but not lipase-inactive PLD1) decreases insulin-stimulated Glut4 translocation. Mechanistically, induction of PLD2 causes F-actin and microtubule polymerization, and the PLD2-mediated Glut4 trafficking is blocked by cytochalasin D (an F-actin disassembler) and Nocodazole (a microtubule-depolymerizing agent). Taken together, we propose that PLD2 may play a role to promote Glut4 translocation upon insulin and/or ET-1 stimulation through F-actin and microtubule reorganization. Other findings suggest that PLD1 in contrast may play a role in fusion of Glut4-containing vesicles into the plasma membrane or their replenishment from the peri-nuclear storage compartment. (Supported by grants from NIH and the American Diabetes Association.)

22. Roles of Amino Acid Trafficking Signals in the Assembly and Trafficking of Ion Channel Proteins STEVE H. KELLER and JASON YUAN, *Department of Medicine, University of California at San Diego, San Diego, CA*

We have been investigating roles of amino acid trafficking signals in the assembly and trafficking of ion channel proteins. The nicotinic acetylcholine receptor has a series of conserved adjacent basic amino acid residues positioned in the large cytoplasmic loop of each subunit. Basic amino acids in the configuration BB or BXB (B; basic amino acid, arginine or lysine: X; any amino acid) are known determinants for retrieval back to the endoplasmic reticulum (ER). Exposure of these signals in the unassembled subunit could prevent its trafficking upstream in the secretory pathway. We asked the question why is subunit assembly required to bring the subunits to the cell surface? Alteration of a basic amino acid signal in the α-subunit at the conserved site promotes its trafficking to the medial Golgi, as evidenced by overlap with a Golgi marker in double-labeling immunofluorescence and confocal microscopy. However, this modification is not sufficient to bring the α-subunit to the cell surface, suggesting a second checkpoint in the trafficking pathway. Subsequently, we found that inhibiting ubiquitination by expressing the modified α-subunit with the altered trafficking signal in ubiquitinating deficient cells results in detection of the subunit at the cell surface. These observations suggest the presence of two checkpoints in the trafficking pathway; one between the ER and Golgi, and the second beyond the Golgi.

Trafficking deficiencies correlated with mutations in ion channel proteins might also be associated with misfolding or lack of assembly and exposure of the trafficking signals. We have also been examining the roles of putative signals in the trafficking deficits associated with ΔF508 CFTR and Long QT syndrome HERG potassium channel mutations. (Supported in part by grants from the Cystic Fibrosis Foundation and the American Heart Association to S.H. Keller.)

23. In Caco-2 Cells, Most of the "Apical" SGLT1 Resides in Intracellular, Microtubuli-associated Vesicles HELMUT KIPP, SAEED KHOURSANDI, DANIEL SCHARLAU, and ROLF K.H. KINNE, *Department of Epithelial Cell Physiology, Max-Planck-Institute of Molecular Physiology, Dortmund, Germany*

We investigated the distribution of the endogenous sodium/D-glucose cotransporter (SGLT1) in polarized Caco-2 cells, a model for enterocytes. A cellular organelle fraction was separated by free flow electrophoresis and subjected to the analysis of endogenous and exogenous marker enzymes for various membrane vesicle components. Furthermore, the presence of SGLT1 was tested by an ELISA assay using newly developed epitope-specific antibodies. Thereby it was found that the major amount of SGLT1 resided in intracellular compartments and only a minor amount in apical plasma membranes. The distribution ratio between intracellular SGLT1 and apical membrane–associated SGLT1 was ~2:1. Further immunohistochemical investigation of SGLT1 distribution in fixed Caco-2 cells by epifluorescence and confocal microscopy revealed that the intracellular compartments containing SGLT1 were associated with microtubuli. Elimination of SGLT1 synthesis by incubation of cells with cycloheximide did not significantly reduce the size of the intracellular SGLT1 pool. Furthermore, the half-life of SGLT1 in Caco-2 cells was determined to be 2.5 d by metabolic labeling followed by immunoprecipitation. Our data suggest that most of the intracellular SGLT1 are not transporters en route from biosynthesis to their cellular destination, but represent an intracellular reserve pool. We therefore propose that intracellular compartments containing SGLT1 are involved in an endo-/exocytosis process, which regulates SGLT1 abundance at the apical cell surface.

24. Folding of the T1 Recognition Domain of Kv1.3 ANDREY KOSOLAPOV and CAROL DEUTSCH, *Department of Physiology, University of Pennsylvania, Philadelphia, PA*

Voltage-gated K^+ channels contain an NH_2-terminal domain that is responsible for subfamily-specific coassembly of four subunits into a functional tetramer (Li et al. 1992. *Science.* 257:1225–1230; Shen et al. 1993. *Neuron.* 11:67–76; Xu et al.1995. *J. Biol. Chem.* 270:24761–24768). This NH_2-terminal region is known as the "T1 domain" (Shen et al. 1993. *Neuron.* 11:67–76). Four T1 domains, one from each Kv subunit, form a folded tetrameric structure (Kreusch et al. 1998. *Nature.* 392:945–948). To directly study the folding of individual T1 domains, we developed a new assay. This assay uses (a) bis-maleimides to cross-link pairs of cysteines engineered into an internally folded interface of a T1 domain, and (b) a gel shift strategy that extends current pegylation techniques (Lu and Deutsch. 2001. *Biochemistry.* 40:13288–13301). This approach allows us to detect intramolecularly cross-linked Kv1.3 monomers. We used this assay to determine T1 folding events during Kv1.3 biogenesis. (Supported by NIH grant GM 52302 and NRSA # HL-07027.)

25. Secretion and Activation of Chitin Synthase III in *Saccharomyces cerevisiae* HÉCTOR A. LUCERO and PHILLIPS W. ROBBINS, *Department of Molecular and Cell Biology, Goldman School of Dental Medicine, Boston University Medical School, Boston, MA*

Chitin synthase III (Chs3p) in *Saccharomyces cerevisiae* produces 90% of the cell wall chitin and is targeted to the bud-neck region during budding. Chs3p is, so far, the only known yeast secretory, membrane protein that undergoes complex recycling involving the trans-Golgi network (TGN), the plasma membrane (PM) and the early endosomes (EE) (Valdivia et al. 2002. *Dev. Cell.* 2:283–294). This recycling includes the accumulation of Chs3p in "chitosomes," a putative cytoplasmic reservoir of Chs3p.

Chs6 is essential for the exit of Chs3p from the chitosome to the PM and deletion of Chs6 gene enhances the accumulation of chitosomes. Chs3p activity in chitosomes is about six times lower than in the PM, suggesting the activation of the enzyme may occur when Chs3p reaches the PM. Chs4 is thought to be an essential activator of Chs3p since its deletion results in a complete loss of Chs3p activity without affecting its trafficking. Chs5 is essential for both trafficking and activity of Chs3p.

We have established a correlation between secretory stations of Chs3p and its enzymatic activity using Chs3p enriched fractions from yeast mutants impaired at different stages of secretion. To facilitate the assay of Chs3p activity in multiple samples derived from our purification schemes we have developed a high-sensitivity, high-throughput, nonradioactive chitin synthase assay (Lucero et al. 2002. *Analytical Biochem.* In press). An antipeptide antibody against the Chs3p NH_2-terminal region allowed us to detect and correlate the presence of Chs3p with its activity. Fractions enriched in Chs3p are now being subjected to final immune-purification using magnetic beads coated with an anti-peptide antibody reactive with a cytoplasmic loop of Chs3p. The protein composition of immune-purified vesicles bearing Chs3p is being determined using gel electrophoresis and protein sequencing by mass spectrometry. The accumulation of Chs3p in a cytoplasmic reservoir, standing-by for budding signals to be targeted in a polarized fashion to the bud-neck region, brings to mind the familiar picture of regulated secretion in highly specialized mammalian cells. (Supported by NIH grants GM31318 and AI44070 to P.W. Robbins.)

26. Cholesterol-induced Protein Sorting: An Analysis of Energetic Feasibility J.A. LUNDBÆK,[1] T. WERGE,[1] O.S. ANDERSEN,[2] and C. NIELSEN,[3] [1]*Department of Biological Psychiatry, St. Hans Hospital, Roskilde, Denmark;* [2]*Cornell University Weill Medical College, New York, NY;* [3]*A. Krogh Institution, University of Copenhagen, Copenhagen, Denmark*

The hydrophobic coupling between a membrane protein and the host bilayer means that the bilayer hydrophobic thickness will tend toward matching the hydrophobic length of the protein. Several lines of evidence show that membrane protein sorting between the Golgi and the plasma membranes is determined, in part, by the length of the protein's transmembrane domains. This led Bretscher and Munro (1993. *Science.* 261:1280–1281) to propose that cholesterol could regulate protein sorting as the cholesterol-enriched plasma membrane is thicker than the cholesterol-poor Golgi membrane. According to this suggestion, proteins, which have shorter hydrophobic stretches than plasma membrane proteins, would be retained in the Golgi. We have evaluated the energetic consequences of cholesterol-induced protein sorting using the theory of elastic bilayer deformations and experimental bilayer material moduli. The mismatch between the bilayer thickness and the protein length will incur an energetic cost that varies as a function of the bilayer thickness and material properties. As cholesterol alters both the bilayer thickness and material properties it should in principle be able to regulate protein sorting, the question is whether these changes are large enough to be important. We find that there is sufficient energy for cholesterol-induced protein sorting. Surprisingly, the major effect of cholesterol is due to changes in membrane material moduli rather than thickness. This will have implications for understanding the uneven distribution of cholesterol in eukaryotic cell membranes e.g., lipid rafts.

27. pH of TGN of H/K-ATPase-expressing HEK293 cells: Implications for pH Regulation in the Secretory Pathway T.E. MACHEN,[1] H.-P. MOORE,[1] T. KIMURA,[3] M.-J. LEIGH,[1] S. ASANO,[3] and C. TAYLOR,[1,2] [1]*Department of Molecular and Cell Biology, and* [2]*Graduate Group in Bioengineering, University of California at Berkeley, Berkeley, CA; and* [3]*Molecular Genetics Research Center, Toyama Medical and Pharmaceutical University, Toyama, Japan*

The H/K-pump in parietal cells of the stomach produces pH 1.0 HCl. Because the trans-Golgi network (TGN) has large permeabilities to K^+ and Cl^- (Demaurex et al. 1998. *J. Biol. Chem.* 273:2044–2051), the only ions required for H/K-ATPase function, we hypothesized that this pump would acidify the TGN during its trafficking to the plasma membrane. HEK cells stably expressing both α and β subunits of the rabbit H/K-ATPase ("H/Kα,β cells") or vector-transfected ("mock") cells were transiently transfected with a chimera plasmid encoding the pH-sensitive GFP derivative pHlu-

orin fused to the lumenal aspect of TGN38 to measure pH of the TGN (pH_{TGN}). Immunofluorescence showed that H/K-ATPase was present in the plasma membrane and the TGN of H/Kα,β cells. When the TGN was alkalinized (after a brief pulse of acetate, 30 mM), reacidification was completely blocked by bafilomycin (inhibitor of the H+ v-ATPase) in mock cells, but full block of reacidification in H/Kα,β cells required both bafilomycin and SCH28080 (inhibitor of H/K-ATPase). SCH28080 had no effect on pH_{TGN} in mock cells but caused pH_{TGN} to alkalinize slowly (2×10^{-4} pH/s) from pH 6.4 by 0.15 pH units in H/Kα,β cells; subsequent addition of bafilomycin caused pH_{TGN} to alkalinize 8–10 times more rapidly than during SCH28080 treatment, up to a new steady-state pH 7.0–7.5, similar to cytosol. Bafilomycin caused similarly rapid rates of alkalinization of pH_{TGN} in mock and H/Kα,β cells, indicating that the TGN (like the Golgi; see Wu et al. 2001. *J. Biol. Chem.* 276:33027–33035) had a large H^+ permeability. Thus, the TGN has large permeabilities to K^+ and Cl^-, which provide the ions required for H/K-ATPase function, and also to H^+, which constantly leak H^+ accumulated by both H^+ v-ATPase and H/K-ATPase. Model calculations indicated that pH_{TGN} was only 0.15 pH lower in H/Kα,β cells than in mock cells because the high H^+ permeability of the Golgi (10^{-3} cm/s) demands a large number (10,000) of H^+ v-ATPases to be permanently present in the TGN, making the effects of only 1,000–1,250 H/K-ATPases in transit to the plasma membrane relatively minor. Our data indicate that the TGN has large numbers of H^+ v-ATPases that constantly pump "against" a large H^+ permeability so that the smaller numbers of other H^+ pumps or leaks trafficking through the TGN on their way to the plasma membrane have minimal effect on pH_{TGN}. This H^+ pump-leak buffering mechanism provides an inefficient, but effective system for assuring that the TGN acidity will be consistent across all cell types. (Supported by NIH DK51799 and NSF MCB9983342.)

28. Intracellular Trafficking of the Human Reduced Folate Carrier Is Mediated by the Microtubular Cytoskeleton JONATHAN S. MARCHANT,[1] VEEDAMALI S. SUBRAMANIAN,[2,3,4] IAN PARKER,[1] and HAMID M. SAID,[2,3,4] [1]*Departments of Neurobiology and Behavior,* [2]*Medicine, and* [3]*Physiology/Biophysics, University of California, Irvine, CA; and* [4]*Veterans Affairs Medical Center, Long Beach, CA* (Sponsor: R. Josephson)

The major cellular pathway for uptake of the vitamin folic acid is via a plasma membrane carrier protein known as the reduced folate carrier. We have investigated the mechanisms that control the intracellular trafficking and plasma membrane targeting of the human reduced folate carrier (hRFC) by using confocal microscopy to monitor the cellular distribution of an hRFC fusion protein tagged with the green fluorescent protein (hRFC-EGFP). Transient expression of hRFC-EGFP in a variety of epithelial cell lines (MDCK, HuTu-80, HEK293, and Caco-2 cells) resulted in strong expression of the fusion protein at the cell membrane, assessed by colocalization with the plasma membrane marker FM4-64 (~65% overlap of fluorescence signals after 40 h). Incubation of cells with low concentrations of nocodazole (500 nM for 10 h) attenuated cell surface expression of hRFC-EGFP, whereas treatment with cytochalasin D (500 nM for 10 h) had little effect. Video-rate confocal imaging of a HuTu-80 cell line stably transfected with hRFC-EGFP permitted resolution of the motion of hRFC-containing vesicles. At 37°C, individual vesicles exhibited rapid linear motions (average velocity ~1.9 μm/s) toward as well as away from the cell membrane, interspersed with periods of low motility. Short term incubation with nocodazole (10 μM, <5 min) or colchicine (10 μM, <10 min) inhibited the rapid linear vesicular movements, whereas γ-lumicolchicine (50 μM) and cytochalasin D (10 μM) were ineffective. These data underscore previous evidence (Subramanian et al. 2001. *Am. J. Physiol.* 281: G1477–G1486) that implicate a crucial role for microtubules, but not microfilaments, in the transport of hRFC to the cell surface. (Supported by the Department of Veterans Affairs and NIH grants DK-56061, DK-58057, and GM-48071.)

29. Molecular Determinants for Plasma Membrane Sorting of the Renal Sulfate Transporter Sat-1 DANIEL MARKOVICH and RALF R. REGEER, *Department of Physiology and Pharmacology, School of Biomedical Sciences, University of Queensland, Brisbane, Queensland, Australia*

Sulfate is an essential anion required for bone/cartilage formation, proteoglycan synthesis, and cellular metabolism. Body sulfate homeostasis is primarily maintained through renal tubular mechanisms. The sulfate anion transporter (sat-1) encodes a 703–amino acid protein (75.4 kD) with 12 putative transmembrane domains, which mediates sulfate/chloride/bicarbonate exchange across the basolateral membrane of the renal tubule. To identify the molecular determinant(s) responsible for this basolateral expression pattern in vivo, deletion mutants were made within the intracellular COOH-terminal tail of the rat sat-1 (rsat-1) protein, which were fused to the enhanced green fluorescence protein. These constructs were transiently transfected

into Mardin-Darby Canine Kidney (MDCK) cells in which the wild-type rsat-1 was sorted exclusively to the basolateral membrane. The removal of the last 30 amino acids of the COOH-terminal tail showed intracellular expression, suggesting the presence of a sorting motif within the last 30 amino acids of this protein. The removal of the PDZ domain (SAL) located at the last three residues on the COOH terminus of rsat-1, had no effect on the plasma membrane sorting of the protein, suggesting that PDZ-binding proteins may not be required for sat-1 basolateral sorting. Sequence analysis of the last 30 amino acid residues of rsat-1 identified a dileucine motif at position $L_{677}L_{678}$ as a putative targeting signal. Site-directed mutagenesis of this dileucine motif (to alanines) led to a loss of basolateral expression, suggesting that this motif was necessary for basolateral trafficking of the rsat-1 protein. The intracellular compartment where this mutant is accumulated is presently being investigated. (Supported by an Australian National Health and Medical Research Council grant to D. Markovich.)

30. Synaptic Targeting of N-type Calcium Channels in Hippocampal Neurons ANTON MAXIMOV and ILYA BEZPROZVANNY, *Department of Physiology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX*

N-type calcium (Ca^{2+}) channels play a critical role in synaptic function, but the mechanisms responsible for their targeting in neurons are poorly understood. N-type channels are formed by α_{1B} pore–forming subunit associated with β and α2δ auxiliary subunits. By expressing epitope-tagged recombinant α_{1B} subunits in rat hippocampal neuronal cultures, here we demonstrate that synaptic targeting of N-type channels depends on neuronal contacts and synapse formation. We further establish that the COOH-terminal 163 amino acids (2177–2339) of $\alpha_{1B\text{-}1}$ ($Ca_V2.2a$) splice-variant contain sequences, which are both necessary and sufficient for synaptic targeting. By site-directed mutagenesis we demonstrate that Mint1-PDZ and CASK-SH3 binding motifs located within this region of α_{1B} subunit (Maximov et al. 1999. *J. Biol. Chem.* 274:24453–24456) act as redundant synaptic targeting signals. We further show that the recombinant modular adaptor proteins Mint1 and CASK colocalize with N-type channels in synapses. We found that $\alpha_{1B\text{-}2}$ ($Ca_V2.2b$) splice variant is restricted to soma and dendrites and postulated that "somatodendritic" and "axonal/synaptic" isoforms of N-type channels are generated via alternative splicing of α_{1B} carboxy termini. These data lead us to propose that during synaptogenesis $\alpha_{1B\text{-}1}$ ($Ca_V2.2a$) splice-variant of N-type Ca^{2+} channels pore-forming subunit recruited to synaptic locations by means of interactions with modular adaptor proteins Mint1 and CASK. Our results provide a novel insight into molecular mechanisms responsible for targeting of Ca^{2+} channels and other synaptic proteins in neurons. (Supported by the Robert A. Welch Foundation and NIH R01 NS39552.)

31. N287Y, a Mutation in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Gives Rise to Clinical Disease by Enhancing Endocytic Retrieval JOHN A. PICCIANO, MARK S. SILVIS, CAROL BERTRAND, ROBERT J. BRIDGES, and NEIL A. BRADBURY, *Cystic Fibrosis Research Centre, Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, PA* (Sponsor: Kirk L. Hamilton)

Mutations in the primary sequence of CFTR give rise to the characteristic phenotype of the genetic disease cystic fibrosis (CF). Such phenotype includes chronic respiratory infections, impaired sweat electrolyte levels and, in severe cases, malfunction of the exocrine pancreas. The most common mutation in CFTR, accounting for ~70% of all mutant alleles is the loss of a phenylalanine residue at position 508 (ΔF508 CFTR). Mechanistically, ΔF508 CFTR results in a protein that folds inappropriately and fails to exit the endoplasmic reticulum. 2,000 mutations have now been described in the CFTR gene, yet the molecular bases for most of these mutations are not known. N287Y, a clinical mutation in the second intracellular loop of CFTR, gives rise to patients with pancreatic sufficiency, mild airway disease, and elevated sweat electrolytes. We, and others, have shown that critical tyrosine-based motifs are important for the correct endocytic trafficking of CFTR. We hypothesized that the N287Y mutations results in the generation of a novel endocytic signal, causing CFTR to be removed prematurely from the plasma membrane. We have stably expressed both wild-type (wt) and N287Y CFTR in Hek293 cells. No difference in total cellular CFTR was observed between the two cell lines. However, N287Y CFTR was present in the plasma membrane at a level ~50% of that observed for wt CFTR. In addition to cell surface labeling, CFTR is also present in early endosomes (EE). Significantly more N287Y CFTR was present in EE compared with wt, suggesting that the reduction in cell surface signal was due to an increase in endocytic retrieval. Indeed, N287Y CFTR was endocytosed at twice the rate of wt CFTR. Whole-cell patch clamp analysis revealed that CFTR activity was also significantly reduced in N287Y CFTR, showing a physiological consequence of increased endocytosis. In summary, the N287Y mutation reveals a novel class of CFTR mutation, whose primary

defect is in increased rates of endocytosis. Such increased endocytosis leads to reduced steady-state levels in the plasma membrane and a reduction in stimulated chloride secretion. (Supported by NIH grant DK57583 and the Cystic Fibrosis Foundation.)

32. Distinct Effects of Insulin and Hyperosmolarity on GLUT4 Glucose Transporter Traffic VARINDER K. RANDHAWA,[1,2] DAILIN LI,[1] JIWON RYU,[3] DAWN LIM,[1] RAMI GARG,[1] LEONARD FOSTER,[1] CHAN JUNG,[3] TIMOTHY McGRAW,[4] and AMIRA KLIP,[1,2] [1]*Program in Cell Biology, Hospital for Sick Children and* [2]*Department of Biochemistry, University of Toronto, Toronto, Ontario;* [3]*Department of Physiology and Biophysics, State University of New York, Buffalo, NY; and* [4]*Department of Biochemistry, Weill Medical College of Cornell University, New York, NY*

The GLUT4 glucose transporter is a recycling membrane protein that resides intracellularly in the basal state, and is recruited to the cell surface by insulin and hyperosmolarity. The purpose of this study was to explore the regulation of the various arms of GLUT4 traffic in response to these stimuli using L6 muscle cells stably expressing exofacially myc-tagged GLUT4. Insulin and hyperosmolarity both decreased the recycling time of GLUT4 from 2 h to 40 and 70 min, respectively. While insulin increased cell surface GLUT4 by enhancing exocytosis and inter-endosomal transit, hyperosmolarity did so through retention. Unlike insulin, hyperosmolarity-induced GLUT4 traffic did not require PI 3-kinase, Akt, or actin remodelling. However, although hyperosmolarity-stimulated GLUT4 traffic was not toxin-sensitive, it was clearly NSF-dependent along with insulin since the cell surface accumulation of GLUT4 was decreased by transient expression of dominant negative NSF. Additionally, insulin redistributed the intracellular GLUT4 in a manner distinct from hyperosmolarity. Finally, GLUT4 was found to reside in at least two internal pools both taxable by insulin using endosomal ablation and hypotonic lysis. Thus, we propose that insulin and hyperosmolarity regulate the exocytic and endocytic arms of GLUT4 traffic, respectively. Moreover, insulin recruits GLUT4 from two pools in an NSF-dependent manner: the toxin-insensitive endosomes (also taxed by hyperosmolarity) and the toxin-sensitive specialized vesicles (requiring also PI 3-kinase, Akt, and actin remodelling). (Supported by CIHR-4072026.)

33. A PDZ-interacting Domain Regulates Plasma Membrane Recycling of CFTR AGNIESZKA SWIATECKA-URBAN,[1] MARC DUHAIME,[1] BONITA COUTERMARSH,[1] KATHERINE H. KARLSON,[1] JAMES COLLAWN,[2] MICHAL MILEWSKI,[3] GARRY R. CUTTING,[3] WILLIAM B. GUGGINO,[4] and BRUCE A. STANTON,[1] [1]*Department of Physiology, Dartmouth Medical School, Hanover, NH;* [2]*Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL;* [3]*Johns Hopkins University, Center for Medical Genetics, Johns Hopkins Hospital/CMSC 1004, Baltimore, MD;* [4]*Department of Physiology, Johns Hopkins University, School of Medicine, Baltimore, MD*

The COOH terminus of CFTR contains a PDZ-interacting domain that is required for the polarized expression of CFTR in the apical plasma membrane of polarized epithelial cells. To elucidate the mechanism whereby the PDZ-interacting domain mediates the polarized expression of CFTR, MDCK cells were stably transfected with full-length wild-type (wt-CFTR) or COOH-terminally truncated human CFTR (CFTR-ΔTRL). We tested the hypotheses that the PDZ-interacting domain regulates either polarized sorting and/or polarized plasma membrane expression of CFTR. Pulse-chase studies in combination with domain-selective cell surface biotinylation revealed that newly synthesized wt-CFTR and CFTR-ΔTRL were targeted equally to the apical and basolateral plasma membrane domains in a nonpolarized fashion. Thus, the PDZ-interacting domain is not an apical-sorting motif. To determine if the PDZ-interacting domain plays a role in polarized plasma membrane expression of CFTR we measured the half-life of wt-CFTR and CFTR-ΔTRL in the apical and basolateral membranes. Deletion of the PDZ-interacting domain reduced the half-life of CFTR in the apical plasma membrane from ~24 h to ~13 h, but had no effect on the half-life of CFTR in the basolateral membrane. Thus, the PDZ-interacting domain is important for CFTR expression in the apical plasma membrane. Next, we examined the hypothesis that the PDZ-interacting domain affects the apical plasma membrane half-life of CFTR by altering its endocytosis and/or recycling. Endocytosis of wt-CFTR and CFTR-ΔTRL did not differ. However, recycling of CFTR-ΔTRL was decreased when compared with wt-CFTR. Thus, deletion of the PDZ-interacting domain reduced the half-life of CFTR in the apical plasma membrane by decreasing CFTR recycling. We conclude that the polarized expression of CFTR in the apical plasma membrane of MDCK cells is regulated by a PDZ-interacting domain that links CFTR with a PDZ protein located in the apical plasma membrane.

34. Novel Role for a Multibasic RKR Motif in the Trafficking of an Ion Channel, hIK1 COLIN A. SYME, GLENN D. PAPWORTH, KIRK L. HAMILTON, HEATHER M. JONES, SIMON C. WATKINS, NEIL A.

BRADBURY, and DANIEL C. DEVOR, *Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, PA*

Jan and colleagues previously demonstrated that the RXR motif of the K_{ATP} channel acts as an ER retention signal (Zerangue et al. 1999. *Neuron.* 22:537–548). As all members of the KCNN gene family contain a conserved multibasic RKR sequence in their cytoplasmic NH_2-terminal domains we determined the role of this motif in the trafficking of hIK1 (^{13}RRRKR^{17}), rSK2 (^{131}KRKR^{134}), and rSK3 (^{280}KRKR^{283}) following stable expression in HEK293 cells. Mutation of ^{15}RKR^{17} to alanines (RKR/A) in hIK1 resulted in the near complete loss of plasma membrane localized channel as assessed by immunofluorescence, cell surface biotinylation, and whole cell patch-clamp recording. We obtained similar results following expression of the double mutants R15A/K16A and R15A/R17A. In contrast, the double mutation R13A/R14A as well as mutation of the individual amino acids R15A, K16A, and R17A did not affect cell surface expression of hIK1. Finally, incubating cells expressing RKR/A at 26°C for 24 h resulted in a partial restoration of cell surface expression. In contrast to these results, mutating ^{132}RKR^{134} in rSK2 or ^{281}RKR^{283} in rSK3 to alanines had no effect on cell surface expression of this channel. Furthermore, mutating other potential RXR motifs in rSK2 (^{559}RSR^{561} or ^{565}RRR^{567}) or rSK3 (^{155}RHR^{157}) to alanines also had no effect on channel expression. These results demonstrate that, in contrast to the K_{ATP} channel, mutation of the multibasic RKR motif in hIK1 abrogates cell surface expression. However, the role of this motif in channel trafficking is not conserved in other members of the gene family. Thus, our results suggest a novel role for this RKR motif in channel function; regulating export to the cell surface rather than ER retention. (Supported by NIH DK54941–02 and AHA 0120544U.)

35. Trafficking of the Ca^{2+}-activated K^+ channel, hIK1 Is Dependent upon a COOH-terminal Leucine Zipper COLIN A. SYME, AARON C. GERLACH, LEE-ANN GILTINAN, KIRK L. HAMILTON, NEIL A. BRADBURY, SIMON C. WATKINS, and DANIEL C. DEVOR, *Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, PA*

We demonstrate that COOH-terminal truncations of hIK1 (427 aa) result in a loss of functional channels. This could be caused by either (a) a failure of the channel to traffic to the plasma membrane or (b) the expression of nonfunctional channels. To delineate amongst these possibilities the HA epitope was inserted into the extracellular loop between transmembrane domains S3 and S4. Surface expression and channel function were measured by immunofluorescence and whole-cell patch-clamp techniques, respectively. Truncation at amino acid K402 resulted in a complete loss of membrane expression, whereas truncation at L414 had no effect. Within this region are two important structural motifs, a di-leucine motif (L409-L410) and a leucine heptad repeat ending at L406. Mutation of the di-leucine motif resulted in a small decrease in surface expression and function, whereas mutation of the two terminal leucines (L399A/L406A, ZIP4.5) within the leucine zipper abrogated membrane localization. Additional mutations within the heptad repeat (L385A/L392A, ZIP2.3; L392A/L406A, ZIP3.5) or of the *a* positions (I396A/L403A) resulted in a near complete loss of membrane localized channel. In contrast, mutating individual leucines compromised neither channel trafficking nor function. Colocalization studies revealed that ZIP4.5 failed to escape the ER, although membrane localization and function could be restored by incubation at 26°C. Co-IP studies using differentially epitope-tagged hIK1 demonstrate this channel exists as a multimer, and that this association is not disrupted by mutations in the leucine zipper. We also Co-IPed V5 and Xpress epitope–tagged leucine zipper regions of hIK1 (C59), demonstrating this region of the channel self-assembles. Furthermore, mutation of ZIP4.5 compromised the ability of C59-hIK1 to self-assemble, whereas mutation of ZIP4 (L399A) or ZIP5 (L406A) individually had no effect. In conclusion, these findings demonstrate that the COOH-terminal leucine zipper is critical in facilitating the correct folding and plasma membrane trafficking of hIK1 (Supported by NIH DK54941–02 and AHA 0120544U.)

36. Cellular and Molecular Biology of Nramp2, a Metal Transporter NICOLAS TOURET,[1] WENDY FURUYA,[1] JOHN FORBES,[2] STEVEN LAM,[2] PHILIPPE GROS,[2] and SERGIO GRINSTEIN,[1] [1]*Program in Cell Biology, The Hospital for Sick Children, Toronto, Ontario, Canada;* [2]*McGill University, Department of Biochemistry, Montreal, Quebec, Canada*

Iron is a vital element which participates in various metabolic processes. Its concentration in tissues is tightly regulated, because iron deficiency or overload can have serious consequences. Nramp2 (DMT1 or DCT1), a newly identified iron transporter which is involved in the intestinal absorption of iron, is a key player in metal homeostasis. The epithelial isoform is thought to be expressed in the apical membrane of epithelial cells, whereas the nonepithelial isoform colocalizes with transferrin receptors.

To analyze intracellular traffic of Nramp2, we generated cell lines expressing the transporter tagged with an extracellular HA epitope. The location and accessibility of the exofacial tag enabled us to define the subcellular localization and traffic of the transporter between compartments.

The nonepithelial isoform of Nramp2 was found mainly in recycling endosomes, where it colocalizes with labeled transferrin and with the transferrin receptor. A small fraction of the protein, sensitive to cycloheximide treatment, is found in the endoplasmic reticulum, where it colocalizes with a GFP-KDEL chimera. We were able to quantify the rate and extent of Nramp2 endocytosis using radiolabeled antibodies. The fraction of the transporter found at the surface membrane corresponded to 34 ± 6% of the total Nramp2. By prelabeling surface transporters with antibodies, we showed that Nramp2 is gradually internalized, reaching a plateau after 2 h. By addition of labeled transferrin, we confirmed that internalized Nramp2 is targeted to recycling endosomes. A dominant-negative form of dynamin-1, which inhibits the formation of clathrin-coated vesicles, drastically inhibited the internalization of Nramp2. Lastly, we studied the effect of the PI3-Kinase inhibitor, wortmannin on Nramp2 traffic. Wortmannin inhibited the recycling of the protein, leading to its depletion from the plasmalemma.

These data show that Nramp2 recycles between the surface membrane and endosomes, where it likely mediates the transfer of iron from transferrin into the cytosol.

37. Recycling Dynamics of Apical Membrane Protein in Polarized WIF-B9 Hepatic Cells YOSHIYUKI WAKABAYASHI and IRWIN M. ARIAS, *Department of Physiology, Tufts University School of Medicine, Boston, MA* (Sponsor: Bill Green)

Previous studies in rat liver and WIF-B9 hepatic cells revealed that newly synthesized canalicular membrane protein traffic directly from Golgi to the bile canalicular membrane, hepatocyte apical plasma membrane, and which cycle between the canalicular membrane and intracellular pools (Gatmaitan et al. 1997. *Am. J. Physiol.* 272:G1041–G1049; Sai et al. 1999. *J. Cell Sci.* 112:4535–4545; Kipp et al. 2000. *J. Biol. Chem.* 275: 15917–15925; Kipp et al. 2001. *J. Biol. Chem.* 276:7218–7224). To understand the apical recycling system in the hepatic cells, we identified the intracellular pool of apical membrane proteins and visualized GFP-tagged apical membrane protein trafficking dynamics in WIF-B9 hepatic cells. Apical membrane proteins were restricted to the canalicular membrane, intracellular punctate organelle and tubular structures. Punctate organelle and tubular structures exactly colocalized with rab11, a marker of recycling endosome. Selective photobleaching shows that the canaliculus pool and punctate organelle pool constantly and rapidly exchanges with the intermediate carrier that located entire cells. Time-lapse fluorescence imaging revealed that large tubular structures were found to be the primary vehicles for punctate recycling endosome organelle to canalicular membrane transport of GFP-tagged apical membrane protein. These structures continuously budded as identical domain from the punctate recycling endosome organelle and underwent dynamic shape changes as they moved along microtubule tracks to the bile canalicular membrane. Movement of these tubular structures occurred bi-directional and oscillatory change the speed at a maximal speed of 0.93 μm/s. Microtubule depolymerization disrupted tubular structures, but not affected punctate structure. Apical membrane protein endocytosis was also visualized using time-lapse fluorescence microscopy. Apical membrane protein budded from the bile canalicular membrane as a small vesicular structure, detached and subsequently moved along microtubules to the punctate organelle. Movement of these small vesicles occurred also oscillatory change the speed at a maximal speed of 1.85 μm/s. These data provide the first indication that apical membrane proteins rapidly cycle between canalicular membrane and punctate organelle use a tubular or vesicular intermediate in hepatic cells.

38. Strial Marginal Cells of the Inner Ear Contain the KCNQ1-KCNE1 K^+ Channel in Their Apical Membrane with KCNE1 Being Required for Translation and/or Trafficking of KCNQ1 PHILINE WANGEMANN, BEATRICE ALBRECHT, ERIN WHITE, and LILI MALEKI, *Anatomy and Physiology Department, Kansas State University, Manhattan, KS*

The KCNQ1-KCNE1 K^+ channel (formerly called I_{sK}, minK or KvLQT1/I_{sK} channel) is responsible for K^+ secretion in strial marginal cells of the inner ear (Wangemann. 1995. *Hearing Res.* 90:149–157) and for action potential repolarization in the heart (Varnum et al. 1993. *Proc. Natl. Acad. Sci. USA.* 90:11528–11532). Observations in expression systems and in mice lacking KCNQ1 or KCNE1 suggest that both subunits are essential for channel function. Recent observations in heart suggest that an additional subunit, YOTIAO, contributes to channel function by conferring to the channel cAMP-mediated sensitivity to β_1-adrenergic stimulation (Marx et al. 2002. *Science.* 295:496–499). We hypothesized that the KCNQ1-KCNE1 K^+ channel in strial mar-

ginal cells contains YOTIAO, since β_1-adrenergic receptors stimulate K^+ secretion via cAMP-mediated stimulation of the channel (Sunose et al. 1997. *Hear Res.* 114: 107–116; Wangemann et al. 2000. *J. Membr. Biol.* 175: 191–202). Further, we hypothesized that KCNE1 may be responsible for transcription, translation, or trafficking of KCNQ1. Transcripts were identified by RT-PCR in total RNA isolated from microdissected tissue samples containing strial marginal cells using gene-specific primers. Further, proteins were localized by confocal immunocytochemistry using subunit-specific antibodies. Stria vascularis of normal 129Sv mice contained transcripts for KCNE1, KCNQ1, and YOTIAO. Transcripts for KCNQ1 and YOTIAO were also present in stria vascularis of mice lacking KCNE1 although functional channels are known to be absent in these mice (Vetter et al. 1996. *Neuron.* 17:1251–1264). KCNQ1 and KCNE1 were colocalized (distance <200 nm, physical limit of light microscopy) in the apical membrane of strial marginal cells. Colocalization was confirmed by immuno-FRET (distance <80 nm). Immunolocalization of KCNQ1 in mice lacking KCNE1 resulted in no apparent stain of the apical membrane of strial marginal cells and in a diffuse cytosolic stain that was often difficult to distinguish from background. These observations confirm that both subunits of the KCNQ1-KCNE1 K^+ channel are present in the apical membrane of strial marginal cells and demonstrate that KCNE1 is not required for transcription but for translation and/or trafficking of KCNQ1 to the apical membrane of strial marginal cells. (Supported by grant NIH-RO1-DC1098.)

39. Mopp, Monovalent PDZ Protein, Is a PDZ Scaffold Complex Antagonist That Affects Polarized Sorting of the Kir 2.3 Channel PAUL A. WELLING, JAMES B. WADE, and OLAV OLSEN, *Department of Physiology, University of Maryland School of Medicine, Baltimore, MD* (Sponsor: M. Blaustein)

We recently reported that the basolateral membrane sorting determinant in the inwardly rectifying potassium channel, Kir 2.3, is comprised of a unique arrangement of trafficking motifs, containing tandem, conceivably overlapping, biosynthetic targeting and PDZ-based signals (Le Maout et al. 2001. *Proc. Natl. Acad. Sci.* 18:10475–10480). PDZ interactions appear to control cell surface stability. Indeed, we found that Kir 2.3 interacts with the mLin-7–CASK PDZ complex to retain the channel on the basolateral membrane (BLM) (Olsen et al. 2002. *Am. J. Physiol.* C183–C195). Here we report that retention may be controlled by a novel competition mechanism. Using the yeast two-hybrid system, we identified an unusual and novel PDZ protein, MOPP, that also interacts with the Kir 2.3 channel. MOPP, (monovalent PDZ protein), has a unique domain structure; nearly its entire open-reading frame encodes a single PDZ domain, suggesting that it may act as a natural dominant negative protein. Antibodies raised against MOPP specifically detected the predicted size protein in the kidney (16 kD), confirming our assignment of the open-reading frame. MOPP was immunolocalized within a diffuse cytoplasmic compartment of the renal collecting duct, colocalizing with internalized Kir 2.3. Bona fide interaction of MOPP with Kir 2.3 was confirmed by coimmunoprecipitation. Consistent with our hypothesis about MOPP function, we found that MOPP can compete with Lin-7 for Kir 2.3 binding. When recombinant MOPP was incubated in vitro with a preformed Kir2.3–Lin 7 complex, Lin-7 interaction with the channel decreased as the amount of MOPP associated with the channel increased. To test the consequence of MOPP interaction, Kir 2.3 localization was examined in MDCK cells stably transfected with MOPP. MOPP caused Kir2.3 to redistribute from the BLM into a perinuclear vesicular compartment, similar to the endosomal localization of Kir 2.3 channels lacking the PDZ ligand. In conclusion, MOPP is a unique PDZ protein that functions as an endogenous negative regulator of a basolateral membrane PDZ-based scaffolding complex, having dominant negative effects on basolateral membrane sorting of Kir 2.3. We speculate that MOPP may play a similar role in regulating the expression of other membrane proteins containing a type I PDZ ligand. (Supported by NIH grants DK54231 [P.A. Welling] and DK 32839 [J.B. Wade], and the American Heart Association [Established Investigator Award, P.A. Welling]).

40. PKA Phosphorylation of Kir1.1 Regulates Surface Expression DANA YOO and PAUL A. WELLING, *Department of Physiology, University of Maryland School of Medicine, Baltimore, MD* (Sponsor: M. Blaustein)

The Kir 1.1 (ROMK) subtypes of inward rectifier K^+ channels mediate potassium secretion and regulate sodium chloride reabsorption in the kidney. The density of Kir 1.1 channels on the renal-collecting duct apical membrane is exquisitely regulated in concert with physiological demands. While the precise mechanisms have remained unclear, PKA-dependent phosphorylation of one of the three phospho-acceptors in Kir1.1, S44, has been suggested to control the number of active channels (MacGregor et al. *Am J. Phyiol.* 275:F415–F422). Here, we test this hypothesis. Removal of the phosphorylation site by point mutation (Kir1.1, S44A) dramati-

cally attenuated the macroscopic current density without a change in open-probability or single channel conductance, implying a decrease in cell surface expression or the formation of an inactive population of channels at the membrane. To distinguish between these possibilities, we developed an external epitope (HA)-tagged form of Kir 1.1 so that relative amounts of surface HA-tagged Kir 1.1 could be quantified in individual *Xenopus* oocytes. Inclusion of the tag did not affect channel properties. As measured by antibody binding and a method that combines enzyme amplification with the sensitivity of analytical luminometry, surface expression of the HA-tagged channel was linear over a wide range of expression levels. As measured by this technique, surface expression of Kir1.1 S44A was <10% of the wild-type channel, closely paralleling the difference in macroscopic current. In contrast, a phosphorylation mimic mutant, Kir1.1 S44D, exhibited comparable surface expression and macroscopic current density as the wild-type channel. In conclusion, these results indicate that a high basal state of phosphorylation at S44 drives surface expression of the Kir 1.1 channel in *Xenopus* oocytes. (Supported by NIH grants DK54231 [P.A. Welling], and the American Heart Association [Established Investigator Award, P.A. Welling]).

41. Domain Interactions between Csp and CFTR HUI ZHANG,[1] KATHRYN W. PETERS,[1] MEIR ARIDOR,[1] JOCHEN LANG,[2] and RAYMOND A. FRIZZELL,[1] [1]*Department of Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, PA;* [2]*Institut Européen de Chimie et Biologie, France*

The biogenesis of CFTR is a complex process, involving multiple chaperone protein interactions that stabilize folding intermediates and facilitate CFTR maturation. However, these interactions can target CFTR for degradation by ubiquitin-proteosome pathways when acquisition of the native structure is not achieved. Cysteine string proteins (Csps) are J-domain–containing, Hsc70-binding proteins that have a documented role in regulated exocytosis. We have identified two isoforms of Csp colocalized with CFTR in the ER and apical membrane domain in mammalian epithelial cells, and interacted with the R domain and NH_2 terminus of CFTR in protein binding studies. Overexpression of Csp inhibited cAMP-dependent CFTR currents and interfered with the synthesis of mature CFTR (submitted to *J. Biol. Chem.*). Here, we examined the domain requirements for this effect. Unlike wt Csp, a J-domain mutant (H43Q) that disrupts the HPD-based Hsc70 interaction motif, did not impair CFTR maturation. CFTR was coprecipitated with myc-tagged H43Q Csp, so that the HPD site is not responsible for the physical interaction of Csp with CFTR. Nevertheless, the J-domain was required for interaction with the R domain of CFTR in vitro, indicating that the physical determinants of Hsc70 and R domain binding differ. Since the HPD motif of Csp is required for stimulation of Hsc70 ATPase activity, Csp may serve as a CFTR cochaperone that facilitates its interactions with other chaperone proteins, like Hsc70. This concept is supported by protein binding and coimmunoprecipitation assays, which show that Csp forms complexes with CFTR and Hsc70, Hsp90, and calnexin. The effect of Csp coexpression was specific for CFTR, since Csp did not alter the VSV-G protein levels. Unlike CFTR, Csp did not interact with ubiquitin-conjugated beads, suggesting that it may not directly involve in degradation machinery. (Supported by NIH DK54814 and CF Fdn.)

42. Quest for a "Standardized" *Nitella* Cell CHARLES E. BARR, *Biology Department, State University of New York College, Brockport, NY*

The isolated internodal cells of *Nitella clavata* and its characean relatives are quite variable in their transport and electrical characteristics. Once excised from the intact strand such a cell has given up its function as an integral part of an organism, and consequently its potassium-accumulating role must change. In general the limited goal of obtaining well-behaved cells as experimental objects must focus on keeping the cells in a quasi-stationary state for as long as possible. Preliminary results indicate that the diurnal pattern of the resting potential is less variable for low light intensities and very dilute solutions. If 0.01 mM ammonia is present, isolated cells retain their green color and survive for about two months. But greater uniformity of behavior among cells and from day to day depends upon how the cells grow and mature. Culture conditions have been developed whereby internodal cells in a growing strand attain a final length of ~20 mm at maturity. These cylindrical cells produce alkalinity and acidity in alternating bands along the length: a central alkaline band, and two adjoining acidic bands extending to the cell ends. Under different conditions longer cells develop, these having several bands. Culture solutions containing various combinations of NTA, EDTA, glucarate, galactarate, and glyoxylate as metal-ion chelators are being tried. The details and some examples of the diurnal course of the resting potential will be presented.

43. Interaction between Retinal Rod and Cone Na/Ca-K Exchanger and CNG Channel Probed by Chemi-

cal Cross-linking after Heterologous Coexpression in Insect Cells PAUL J. BAUER,[1] ROBERT T. SZERENCSEI,[2] ROBERT J. WINKFEIN,[2] and PAUL P.M. SCHNETKAMP,[2] [1]*Institute for Biological Information Processing, Research Center Jülich, Jülich, Germany;* [2]*Department Physiology and Biophysics, University of Calgary, Faculty of Medicine, Calgary, Alberta, Canada*

Recent experimental evidence indicates that in bovine rod photoreceptors the Na/Ca-K exchanger (NCKX) and the cyclic nucleotide–gated channel (CNG channel) are associated (Schwarzer et al. 2000. *J. Biol. Chem.* 275:13448). Moreover, covalent cross-linking of the bovine rod NCKX molecules yields dimers, suggesting that two NCKX molecules are bound to each CNG channel (Schwarzer et al. 1997. *Biochemistry*. 36:13667). Interestingly, there is only little affinity between NCKX molecules, whereas a distinct affinity between NCKX and CNG channel has been observed. We, therefore, reasoned that the interaction of the NCKX and CNG channel is reflected in the efficiency of NCKX dimerization as probed by chemical cross-linking. We examined chemical cross-linking of heterologously expressed chicken rod and cone NCKXs both in the absence and the presence of the corresponding CNG channel. The cDNAs of NCKX alone, or of NCKX and CNG channel of rod and cone were transfected into insect cells (High Five, Invitrogen). For the rod NCKX, thiol-specific reagents yielded a cross-link band at twice the molecular mass of the monomer, indicating a NCKX dimer. While this band was observed for cells expressing the NCKX alone, cross-linking was much more efficient and faster when the cells expressed both NCKX and CNG channel. This finding suggests that two NCKX molecules interacted with the CNG channel and, thus, became more readily cross-linked. The adduct band disappeared when the reagent was chemically cleaved. A similar result was obtained for the chicken cone NCKX: again an adduct band was observed which was significantly stronger in the presence than in the absence of the CNG channel. In this case, however, the apparent mass of the adduct band was higher than the double monomer mass, indicating a higher oligomer. Together, these results suggest that, similar to bovine rod photoreceptors, two NCKX molecules interact with the CNG channel in chicken rod photoreceptors. Moreover, an interaction of the cone NCKX with the cone CNG channel was observed, suggesting that both proteins are also assembled in this photoreceptor. (Supported by grants from the Alberta Heritage Foundation for Medical Research, from the Canadian Institutes of Health Research, and from the Deutsche Forschungsgemeinschaft.)

44. COOH Terminus Splice Isoforms of the N-type $Ca_V2.2\alpha_1$ Subunit ANDREW J. CASTIGLIONI and DIANE LIPSCOMBE, *Department of Neuroscience, Brown University, Providence, RI*

The N-type $Ca_v2.2\alpha_1$ subunit undergoes extensive alternative splicing. The COOH-terminal, cytoplasmic tail constitutes ~25% of the total protein and contains at least 11 exons. The COOH terminus has been implicated in selective G-protein inhibition (Simen et al. 2001. *J. Neurosci.* 21:7587–7597) and synaptic targeting (Maximov et al. 1999. *J. Biol. Chem.* 274:24453–24456). There is evidence for divergence among $Ca_v2.2\alpha_1$ subunits in the COOH terminus, which results from alternative splicing. Three locations of sequence divergence among $Ca_v2.2\alpha_1$ cDNAs correspond to exon–exon junctions in the cDNAs. RT-PCR from different brain regions amplified isoforms of mRNAs at two of these sites: the mutually exclusive e37a and e37b, and e46 (Williams et al. 1992. *Science.* 257:389–395; Pan et al. 2001. *Soc. Neurosci. Abstr.* 27:381.12). We investigated the distribution pattern of e37a and e37b in the rat nervous system and have begun to address functional significance. We also used RT-PCR to analyze the expression of e39 and alternative splicing in e46. We demonstrate interesting differential expression patterns of e37a and e37b described below, but we find no evidence for splice isoforms involving e39 or e46 in any of the regions analyzed. Exons 37a and 37b both contain 97 nucleotides and are located at the proximal end of the $Ca_v2.2\alpha_1$ COOH terminus. RT-PCR with e37b-specific primers indicates that $Ca_v2.2\alpha_1$ mRNAs containing e37b are distributed approximately homogenously in all brain regions analyzed. In contrast, RT-PCR using e37a-specific primers indicates preferential expression of $Ca_v2.2\alpha_1$ mRNAs containing this exon in dorsal root ganglia. Tissue-specific expression of e37a in dorsal root ganglia implies an important functional role for this $Ca_v2.2\alpha_1$ isoform. We expressed $Ca_v2.2\alpha_1$+e37a and $Ca_v2.2\alpha_1$+e37b splice isoforms in *Xenopus* oocytes and compared their properties. $Ca_V2.2\alpha_1$ channels containing e37a activate at voltages ~6 mV more hyperpolarized when compared with $Ca_V2.2\alpha_1$ channels containing e37b. This difference in the voltage dependence of activation between these two splice isoforms is present when either calcium or barium is used as the charge carrier. These results imply that sequence modifications in the COOH terminus can influence the voltage-dependence of N-type Ca channel gating. The COOH terminus of $Ca_V2.2\alpha_1$ also contains a putative EF hand only when e37b is expressed. Replacement of e37b with e37a is predicted to disrupt the EF hand. This is analogous to alternative splicing in the closely related $Ca_V2.1\alpha_1$ gene where mutually exclusive splic-

ing of equivalent 97 nucleotide exons determines the expression of an EF hand (Chaudhuri et al. 2001. *Soc. Neurosci. Abstr.* 27:14.5). Mutually exclusive splicing of e37a and e37b in $Ca_V2.2\alpha_1$ is also predicted to change phosphorylation and N-myristoylation patterns. Future experiments will focus on analysis of these splice isoforms in mammalian cells where the level of second messengers may be controlled more precisely. (Supported by NIH grants NS29967 and GM07601.)

45. The Increase in Conductance of *Xenopus* Cystic Fibrosis Transmembrane Conductance Regulator Produced by both PKA and PKC Agonists Are Not Mediated by Channel Insertion in the Plasma Membrane YONGYUE CHEN, GUILLERMO A. ALTENBERG, and LUIS REUSS, *Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, TX*

The cystic fibrosis transmembrane conductance regulator (CFTR) is a phosphorylation- and ATP-hydrolysis–dependent chloride channel expressed in the apical membrane of secretory epithelial cells. Mutations of CFTR cause cystic fibrosis, an inherited autosomal recessive disease affecting predominantly Caucasian individuals. We have found that *Xenopus* CFTR (*X*CFTR), in contrast to human CFTR (hCFTR), can be activated by phorbol 12-myristate 13-acetate (PMA), a PKC agonist. In addition, PMA induces an approximately fivefold membrane conductance increase when it is applied after 8-Br-cAMP to *Xenopus* oocytes expressing *X*CFTR. We call this phenomenon PKC potentiation. Dissecting the mechanism of PKC potentiation will provide information about the regulation of CFTR by phosphorylation and may help design an improved CFTR for gene therapy. Using the approach of Dawson's group (Smith et al. 2001. *J. Gen. Physiol.* 118:407–432), we replaced R335 with cysteine in *X*CFTR and R337 with cysteine in hCFTR. This site is located in the extracellular mouth of the CFTR chloride channel. The highly-polar, membrane-impermeable thiol reagent 2-(trimethylammonium) ethyl methanethiolsulfonate [MTSET(+)] elicited an ~80% increase of PMA- and cAMP-evoked membrane conductance. This effect was reversed by the reducing agent 2-mercaptoethanol (2-ME). The negatively charged thiol reagent (2-sulfonatoethyl) methanethiolsulfonate [MTSES(−)] induced a 2-ME-reversible conductance decrease of ~50%. Hence, CFTR conductance is modified by the thiol reagents in directions consistent with their electric charges. To test for insertion of new channels, we performed double exposure to thiol reagent. The first exposure (before kinase stimulation) is expected to modify all CFTR channels on the membrane; the second exposure (after kinase stimulation) would modify only newly inserted channels and hence if the thiol reagent changes the conductance, then we would conclude that new (unmodified) channels have been inserted. hCFTR-expressing oocytes were pretreated with thiol reagent, washed (but not exposed to 2-ME), stimulated with 8-Br-cAMP, and then exposed to thiol reagent for a second time. The thiol reagents had no effect on the conductance, as previously shown for hCFTR (Liu et al. 2001. *J. Gen. Physiol.* 118:433–446). *X*CFTR-expressing oocytes were subjected to the same double-exposure protocol. The second exposure to thiol reagent had no effect on the conductance elicited by both cAMP and PMA, although the apparent membrane capacitance did increase under these conditions. These results indicate that insertion of new *X*CFTR channels in the plasma membrane does not occur during the simultaneous stimulation of *X*CFTR by PKA and PKC agonists. Therefore, the potentiation must be the result of changes in single-channel conductance and/or open probability. (Supported by NIH grant DK38734.)

46. Actin-based Endocytosis Contributes to Zebrafish Epiboly JACKIE C. CHENG, ANDREW L. MILLER, and SARAH E. WEBB, *Department of Biology, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong SAR, PRC*

The basic body plan of teleost embryos emerges during the gastrula period when a series of extensive cell movements and rearrangements—epiboly, involution, convergence, and extension—lead to the formation of the three germ layers, the endoderm, mesoderm, and ectoderm as well as the dorsoventral and anterioposterior body axes. Starting toward the end of the blastula period, epiboly consists of the thinning and spreading of both the blastoderm and the yolk syncytial layer (YSL) over the yolk cell toward the vegetal pole until, by the end of the gastrula period, the yolk is completely encompassed. It has been previously proposed that in *Fundulus heteroclitus* endocytosis plays a significant role in these morphogenic movements (Trinkaus. 1984. *American Zoologist.* 24:673–688). Here, we present data to suggest that this may also be the case in zebrafish. We report the appearance of a punctate actin band located in the external-YSL at the enveloping layer margin. We propose that this structure may be responsible for the endocytosis of yolk cell membrane and, as in *Fundulus*, this contributes to the driving force for epiboly. Treatment with cytochalasin B results in the disruption of the punctate band, leading to a slowing down of epiboly compared with controls, a failure of

yolk plug occlusion, an elongation of the embryo along its animal pole-vegetal pole axis, and eventual lysis of the embryo through the yolk plug. Our new data thus suggest a key role for actin-based endocytosis during the process of epiboly in zebrafish. (Supported by RGC-CERG grants HKUST6106/01M and N_HKUST007/01.)

47. Insulin-stimulated Na^+/Mg^{2+} Exchange Is Mediated via an Okadaic Acid–sensitive Pathway in Normal Subjects ANA FERREIRA,[1] ALICIA RIVERA,[2] JOSE R. ROMERO,[1] [1]*Endocrine-Hypertension Division, Department of Medicine, Brigham and Women's Hospital/Harvard Medical School, Boston, MA, and* [2]*Department of Pathology, Harvard Medical School, Children's Hospital, Boston, MA*

Cellular Mg^{2+} levels have been shown to be associated with insulin resistance and hypertension. However, the mechanism(s) for cellular Mg^{2+} regulation are not well defined. We tested the hypothesis that insulin regulates intracellular Mg^{2+} levels via activation of the plasma membrane Na/Mg exchange. We studied the effect of insulin on Na/Mg exchange in freshly isolated human erythrocytes from normal subjects after an overnight fast. We measured Na/Mg exchange activity as net total magnesium efflux driven by an inward Na^+ gradient in Mg^{2+}-clamped red cells in the presence or absence of insulin. All subjects studied showed a Na/Mg exchange activity that was 0.60 ± 0.06 mmol/L cell × h (mean ± SE, $n = 44$) when cells were loaded with magnesium to an average of 13.01 ± 0.32 mM. When 400 μU/ml of insulin were added to the flux media, Na/Mg activity increased to 0.75 ± 0.08 mmol/L cell x h ($P < 0.01$, $n = 44$). A dose response curve for insulin on Na/Mg activity showed an EC_{50} for insulin of 70.5 ± 8.6 μU/ml. We also measured the Na/Mg exchange activity as a function of extracellular sodium and cellular magnesium. We found that 400 μU/ml insulin decreased the K_m for extracellular Na^+ from 72.5 ± 2 to 54.8 ± 3.8 mM ($n = 6$). However, the K_m for intracellular magnesium ($n = 3$) was not significantly affected by preincubation with insulin. It is well established that insulin's effects are mediated in part via protein phosphatases. We now report that insulin-stimulated Na/Mg activity was partially inhibited by 100 nM of okadaic acid, a protein phosphatases 1 and 2A inhibitor (from 1.13 ± 0.3 to 0.62 ± 0.2 mmol/L cell × h, $n = 4$, $P < 0.05$). In summary, insulin stimulates the Na/Mg exchanger via a pathway that includes protein phosphatases, which may be responsible for increasing the affinity for the external sodium regulatory site of the exchanger without affecting its intracellular Mg^{2+} regulatory site. Therefore, our results may in part explain the low cellular magnesium levels observed in vivo under hyperinsulinemic conditions and suggest altered regulation of the exchanger by insulin in diabetes.

48. Alternative Splicing in the II-III Loop of the $Ca_v2\alpha_1$ Family of Voltage-gated Calcium Channels ANNETTE C. GRAY, REBECCA R. LEDERER, and DIANE LIPSCOMBE, *Department of Neuroscience, Brown University, Providence, RI*

Voltage-gated calcium channel (Ca_v) α_1 subunits undergo extensive premRNA alternative splicing. Isoforms generated by this mechanism possess unique biophysical properties and display distinct spatial and temporal expression patterns (e.g., Lin et al. 1999 *J. Neurosci.* 19:5322–5331). Splicing within related $Ca_v\alpha_1$ genes displays a high degree of conservation. For example, splice isoforms of both $Ca_v2.1\alpha_1$ and $Ca_v2.2\alpha_1$ exist that differ by only two amino acids in the linker connecting transmembrane segments S3 and S4 in the fourth conserved domain (Lin et al. 1999. *J. Neurosci.* 19:5322–5331). We decided to investigate whether additional sites of alternative splicing in $Ca_v2.2\alpha_1$ were conserved among other $Ca_v2\alpha_1$ family members. We focused on alternative splicing in the intracellular loop connecting domains II and III ($L_{II\text{-}III}$) because (a) isoforms of $Ca_v2.2\alpha_1$ and $Ca_v2.3\alpha_1$ that contain similar sized insertions within this region have already been reported (Williams et al. 1994. *J. Biol. Chem.* 269: 22347–22357; Pan and Lipscombe, 2000. *J. Neurosci.* 20:4769–4775), (b) genomic analysis of the human $Ca_v2.1\alpha_1$, $Ca_v2.2\alpha_1$, and $Ca_v2.3\alpha_1$ genes reveals homologous putative cassette exons (exon18a) within this region, and (c) the $L_{II\text{-}III}$ regions of $Ca_v2.1\alpha_1$ and $Ca_v2.2\alpha_1$ are implicated in excitation-secretion coupling (e.g., Sheng et al. 1994. *Neuron.* 13:1303–1313). We performed RT-PCR on RNA isolated from a variety of adult rat neuronal tissues and on human adult brain, adult spinal cord, and fetal brain RNA (CLONTECH Laboratories, Inc.). First, our results reveal that exon18a isoforms of $Ca_v2.2\alpha_1$ are expressed within the human central nervous system. Exon18a expression increases along a rostro-caudal gradient, as in rat, and is developmentally up-regulated in the human brain. Second, the putative exon identified within the $Ca_v2.1\alpha_1$ gene does not appear to be expressed in any rat or human tissue analyzed so far. Third, $Ca_v2.3\alpha_1$ splice isoforms are expressed in both rat and human neuronal tissues, consistent with previous reports (Pereverzev et al. 1998. *Eur. J. Neurosci.* 10:916–925; Schramm et al. 1999. *Neuroscience.* 92:565–575). Interestingly, expression of $Ca_v2.3\alpha_1$ exon18a follows an expression pattern that is approxi-

mately opposite, both spatially and temporally, to the homologous exon18a of $Ca_v2.2\alpha_1$. In adult rat, expression of $Ca_v2.3\alpha_1$ exon18a is highest in neocortex and hippocampus, but almost completely absent from $Ca_v2.3\alpha_1$ mRNA in peripheral neurons. Further, during development of human brain, exon18a of $Ca_v2.3\alpha_1$ is down-regulated. The expression patterns of $Ca_v2.2\alpha_1$ and $Ca_v2.3\alpha_1$ splice isoforms suggest that they may play specific roles in regulating synaptic transmission at particular developmental stages and in certain areas of the nervous system. To test this question, we have begun to look more closely at the function of $Ca_v2.2\alpha_1$ N-type and $Ca_v2.3\alpha_1$ R-type Ca channels in superior cervical ganglia. In this tissue, $Ca_v2.2\alpha_1$ exon18a expression is up-regulated from immature levels to adult levels between postnatal days 9 and 13, a critical developmental stage in rat when eyes open and whisking begins. (Supported by NIH grant NS29967. A.C. Gray is a Howard Hughes Medical Institute Predoctoral Fellow.)

49. K-Cl Cotransport Is Increased in Nearly All Sickle Cell Red Cells, Not Just the Reticulocytes or a Small Sub-fraction R.B. GUNN and J.K. HAYNES, *Department of Physiology, Emory University School of Medicine and Department of Biology, Morehouse College, Atlanta, GA*

Normal hemoglobin AA cells have a small portion (2–4%) of cells, rich in reticulocytes, that can activate their K-Cl cotransport upon swelling in hypotonic media and thereby shrink and volume regulate (O'Neill. 1989. *AJP:Cell.* 25:858–864). These volume-regulating cells are then resistant to subsequent hypotonic lysis. Patients with sickle cell disease have hemoglobin SS in their red cells, an elevated percentage of reticulocytes and an elevated rate of red blood cell K-Cl cotransport in isotonic media which is strongly activated in hypotonic media. Whether this increased activity is a property of most of the cells or solely due to more reticulocytes or a small population of volume-regulating cells is unknown. We measured (a) K-Cl cotransport activation by hypotonicity; (b) lysis curves of fresh cells, cells after 20 min of incubation in hypotonic media, and the residual volume-regulating cells after incubation and subsequent washing in 91 mOsm NaCl. We find that AA cells do not shift their lysis curve after incubation, but that the volume-regulating AA cells have a much lower osmolality for half hemolysis. In contrast, SS red blood cells shift their lysis curve after incubation, few are lysed in the 91 mOsm NaCl medium and the volume-regulating cells have nearly the same half-hemolysis point as the whole population after incubation. These results support the idea that nearly all of the SS cells have an activated and activatable K-Cl cotransporter. (Supported in part by NIH grant HL66173.)

50. Expression of Multiple KCl Cotransport Isoforms in Erythroid Cells C.H. JOINER, S. CRABLE, and K.P. ANDERSON, *Comprehensive Sickle Cell Center, Division of Hematology/Oncology, Department of Pediatrics, University of Cincinnati, Cincinnati, OH*

The KCl cotransporter plays an important role in cellular cation and volume regulation. To date, four KCC isoforms have been identified in various tissues. It is not clear which isoform mediates volume- and pH-sensitive, Cl-dependent K fluxes in reticulocytes. KCC1 mRNA is present in erythroid tumor cell lines (K562 and mouse eythroleukemia), and immuno-reactive KCC1protein has been demonstrated in human and mouse reticulocytes. Nevertheless, the anion dependence of KCC in red cells (Lauf. 1988. *Am. J. Physiol.* 255:C331) differs from that of KCC1 expressed in oocytes (Mercado et al. 2000. *J. Biol. Chem.* 275:30326). Recently, Lauf et al. (2001. *Cell Physiol. Biochem.* 11:143) reported immunochemical evidence for the presence of both KCC1 and KCC3 in sheep RBC. The present study examined human erythroid cells for the presence of all four KCC mRNA transcripts. Undifferentiated K 562 cells were cultured under standard conditions. Erythroid precursors were grown in semi-liquid culture from isolated peripheral blood mononuclear cells under the influence of stem cell factor and erythropoitein. Cells harvested at 14 d and isolated magnetically by binding to micro magnetic beads coated with transferrin receptor were 95–98% positive for the erythroid antigen glycophorin A by flow cytometry. RNA was extracted and analyzed by semi-quantitative, reverse transcription PCR (RT-PCR), using primers specific for various KCC isoforms, with an internal actin control. The veracity of each PCR product was confirmed by subcloning and nucleotide sequence analysis. As shown in Table I, transcripts for KCC 1, 3, and 4 were detected in both K562 and erythroid precursors. Two COOH-terminal splicing isoforms for KCC1 were detected: the full-length transcript (KCC1a) and a variant in which IVS22 remains in the processed transcript (KCC1b), theoretically producing a truncated protein due to a stop in the IVS. KCC2 message was not detected. Also present in erythroid cells are two KCC3-splicing isoforms, previously described in mouse kidney, which differ in exon 1 and produce unique NH_2 termini. A KCC4 transcript was also present, which is interesting, because the anion dependence of RBC KCC transport is more similar to KCC4 than to KCC1. (The anion dependence of KCC3 has not been reported.) In summary, transcripts for three

TABLE I

Isoform	K562	Cultured cells
KCC1a	+	+
KCC1b	+	+
KCC2	–	–
KCC3a	+	+
KCC3b	+	+
KCC4	+	+

isotypes of KCC, with two additional splicing isotypes, were detected in RNA from erythoroid cells. Recent structure/function studies of KCC (Casula et al. 2001. *J. Biol. Chem.* 276:41870) raise the possibility that interactions among these KCC species may have functional significance in red cells. (Supported by NIH grant P60 HL58421.)

51. The Volume Set Point of KCl Cotransport in Normal and Sickle Reticulocytes: Effects of Cell Age and Sulfhydryl Reduction C.H. JOINER, R.K. RETTIG, and R.S. FRANCO, *Comprehensive Sickle Cell Center, Divisions of Pediatric and Medical Hematology/Oncology, University of Cincinnati, Cincinnati, OH*

KCl Cotransport (KCC) is excessively active in sickle red blood cells (SSRBC) and contributes to pathological dehydration of sickle reticulocytes. To explore the physiological basis for this pathological behavior, methodologies are needed that: (a) reflect KCC activity in the entire reticulocyte population (as opposed to a subpopulation selected on the basis of cell density or response to osmotic stimuli) and (b) permit comparison of aged-matched populations of reticulocytes (SS vs. normal, AA). To this end, we have examined the changes in RBC density profile (as a surrogate marker for flux measurements) upon activation of KCC, coupled with flow cytometric detection of reticulocytes to track the density changes of this distinct population. Whole blood (SS or AA) was washed in isotonic HEPES-buffered saline (HBS) and treated with nystatin to adjust hemoglobin concentration (MCHC) to 30 g/dl. After incubation at 37°C under specified conditions and times, cells were washed in isotonic HBS pH 7.4 and subjected to density analysis on stepwise gradients prepared from OptiPrep®. The percentage of RBC at each density level was calculated from cell counts to give the RBC density profile. From the percentage of reticulocytes in each density fraction, the reticulocyte density profile was generated. A density score (DS) was calculated for RBC and reticulocytes as: $DS = \Sigma\ (N \times P_N)$ where N = gradient fraction number, P_N = percentage of cells in fraction N. There was a linear correlation between DS and MCHC (r = 0.97) when gradients were calibrated using cells treated with nystatin to yield various MCHC. Thus, the DS of a cell population could be used to calculate its MCHC. When KCC was activated by acidification at 37°C to pH 7.0 in HBS, retic density increased rapidly, and then stabilized between 1 and 2 h. Minimal changes occurred in nitrate media. Thus, the rapid increase in MCHC is a manifestation of KCC activity, and the MCHC obtained at 2 h ($MCHC_{final}$) reflects the "volume set point" (VSP) for KCC activity under those conditions. When adjusted to MCHC 30 g/dl, neither SS nor AA reticulocytes changed volume significantly when incubated at pH 7.4. However, when acidified to pH 7.0, both types of RBC became more dense; SS retics had a more rapid change in MCHC than AA retics, and $MCHC_{final}$ was significantly higher in SS than in AA retics (SS, 35.6 ± 1.0 vs. AA 31.2 ± 1.0, $n = 6$, $P < 0.01$ by unpaired t test). Thus, the VSP of SS cells appears to be different from that of AA retics. When the maturity of SS reticulocytes was assessed by fluorescence intensity of reticulum staining by thiazole orange, older SS retics were found to have higher $MCHC_{final}$ than younger cells (35.0 ± 0.2 vs. 34.0 ± 0.4, $n = 6$, $P < 0.04$ by paired t test), suggesting that VSP changes as reticulocytes mature. Finally, treatment of SS cells with the sulfhydryl reducing agent, dithiothreitol, lowered $MCHC_{final}$ (control, 34.4 ± 1.3 vs. treated, 33.4 ± 1.2, $n = 6$, $P < 0.002$, paired t test), suggesting that sulfhydryl oxidation contributes to the abnormally high $MCHC_{final}$ of SS retics. This is plausible in view of the known activation of KCC by sulfhydryl oxidation, and the increased oxidant stress and membrane oxidation seen in SS RBC. (Supported by NIH grant P60 HL58421.)

52. GABA Transport in Retinal Horizontal Cells of the Skate: Modulation by Glutamate MATTHEW A. KREITZER[1] and ROBERT PAUL MALCHOW,[1,2] [1]*Departments of Biological Sciences and* [2]*Ophthalmology and Visual Sciences, University of Illinois at Chicago, Chicago, IL*

The inhibitory neurotransmitter GABA plays an important role in the processing of visual information within the vertebrate retina. Second order neurons called retinal horizontal cells are thought to use this amino acid in establishing the surround portion of the center-surround receptive field of retinal neurons. The transport of GABA into and out of horizontal cells is believed to be a key regulator of extracellular GABA concentrations. We have examined modulation of the transport of GABA into horizontal cells by glutamate, the likely neurotransmitter released by vertebrate photoreceptors. Enzymatically isolated horizontal cells

from skate (*Raja erinacea/R. ocellata*) were examined using the whole-cell version of the patch clamp technique. Addition of 1 mM GABA induced an inward electrical current that likely reflects the uptake of GABA into the cells. Prior application of 100 uM glutamate significantly reduced the GABA-elicited current. The inhibition occurs through the activation of ionotropic glutamate receptors: the ionotropic agonist kainate mimics the inhibition, and the ionotropic antagonist CNQX abolishes modulation by glutamate. The metabotropic glutamate receptor agonists L-AP4 and ACPD were without effect. This reduction appears to be a calcium-dependent process: glutamate inhibition was markedly reduced when extracellular calcium was removed from the bath, and when intracellular recording solutions contained high concentrations of calcium buffers such as BAPTA. Interestingly, calcium rises of similar magnitude induced by activation of voltage-gated calcium channels (measured using the calcium indicator dye Oregon Green) did not reduce GABA-elicited currents. Our data suggest that glutamate released by vertebrate photoreceptors may play an important role in regulating the transport of GABA into retinal horizontal cells. This modulation has the potential to significantly alter the receptive field properties of retinal neurons. (Supported by NIH EYO9411 and NSF 0091240.)

53. Accessibility Measurements of Kv1.3 Using Pegylation Kinetics JIANLI LU and CAROL DEUTSCH, *Department of Physiology, University of Pennsylvania, Philadelphia, PA*

Each subunit of a voltage-gated potassium channel (Kv) contains six putative transmembrane segments, S1–S6, and a cytosolic NH_2-terminal recognition domain, T1. Although it is well established that Kv channels are tetrameric structures, the protein–protein, protein–lipid, and protein–aqueous interfaces are not precisely mapped. The topological accessibility of specific amino acids may help to identify these boundaries. Toward this end, we have developed a variant of the substituted-cysteine-accessibility method that relies on mass-labeling accessible SH groups with a large SH reagent, methoxy-polyethylene glycol maleimide (MAL-PEG) and a gel shift assay (Lu and Deutsch. 2001. *Biochem.* 40:13288–13301). Pegylation of full-length Kv1.3, integrated into microsomal membranes, has allowed us to topologically characterize native cysteines as well as cysteines engineered into intersubunit interfaces. We have now used pegylation to identify the environment of specific residues, starting with the S6 transmembrane segment of Kv1.3. We measure the pegylation rates in either zero detergent, nondenaturing detergent, or in denaturing sodium dodecylsulfate. As opposed to other accessibility methods that rely on electrophysiological read-outs, this approach allows us to study electrically silent residues. (Supported by NIH grant GM 52302.)

54. The $Ca_V\alpha_2\delta$ Subunit Modifies Splice Isoforms of N-type, $Ca_V2.2\alpha_1$ Calcium Channels STEFAN I. MCDONOUGH,[1] YINGXIN LIN,[2] and DIANE LIPSCOMBE,[2] [1]*Marine Biological Laboratory, Woods Hole, MA; and* [2]*Department of Neuroscience, Brown University, Providence, RI*

Two splice isoforms of the neuronal N-type calcium channel $Ca_v2.2\alpha_1$ subunit have been described previously that involve alternative splicing of short cassette exons in two S3-S4 extracellular linkers (IIIS3-IIIS4 and IVS3-IVS4). Alternative splicing in these domains is tissue-specific and affects the voltage-dependence and kinetics of channel gating (Lin et al. *Neuron.* 1997. 18: 153–166). When coexpressed with the ancillary $Ca_v\beta_3$ subunit in *Xenopus* oocytes, one $Ca_v2.2\alpha_1$ isoform that is predicted to dominate in brain activates more rapidly compared with a second isoform that is more prevalent in peripheral neurons. The difference in activation kinetics between these two $Ca_v2.2\alpha_1$ splice isoforms was localized to the expression of one exon (e31a) in domain IVS3-IVS4 that encoded the dipepetide, ET (Lin et al. *J Neurosci.* 1999. 19:5322–5331). To characterize the differences between these two splice isoforms in more detail, we have analyzed channel deactivation kinetics and gating currents in tsA-201 cell lines stably expressing these N-type Ca channel isoforms. Four cell lines were generated that expressed the two $Ca_v2.2\alpha_1$ splice isoforms together with $Ca_v\beta_3$ in the absence and presence of $Ca_v\alpha_2\delta$.

Coexpression of $Ca_v\alpha_2\delta$ increased Ca channel current density and was necessary to measure gating currents. The $Ca_v\alpha_2\delta$, however, also had major effects on gating kinetics and on the voltage-dependence of ionic currents carried by each splice isoform. Ionic currents of both $Ca_v2.2\alpha_1$ splice isofoms activated and deactivated more rapidly at all voltages in the presence of $Ca_v\alpha_2\delta$ compared with currents in cells not expressing this subunit. The effect of $Ca_v\alpha_2\delta$ on activation and deactivation kinetics was voltage-independent. The differences in activation and inactivation kinetics between $Ca_v2.2\alpha_1$ splice isofoms was nonetheless maintained in cells expressing $Ca_v\alpha_2\delta$. Macroscopic rates of N-type Ca channel activation were $\sim$0.6 ms^{-1} faster and macroscopic rates of deactivation were $\sim$0.8 ms^{-1} faster for $Ca_v2.2\alpha_1$ subunits lacking exon 31a in domain IVS3-

IVS4, consistent with previous findings. Ca channel current densities were increased sufficiently in cells expressing $Ca_v\alpha_2\delta$ to enable the recording of gating currents. "On" gating currents were measured with step depolarizations to the ionic reversal potential where there was no net flow of ionic current. The average time constant of decay of "on" gating currents, reflecting the speed of charge movement, was significantly faster in cells expressing the $Ca_v2.2\alpha_1$ subunits lacking exon 31a (0.14 ms) compared with for $Ca_v2.2\alpha_1$ subunits containing exon 31a in domain IVS3-IVS4 (0.28 ms) ($P = 0.0005$). These data suggest that the faster activation kinetics of the brain-dominant splice isoform that lacks exon 31a results from faster movement of the channel voltage sensor.

Coexpression of $Ca_v\alpha_2\delta$ also changed the equilibrium voltage-dependence of N-type Ca channel opening such that channels opened at more hyperpolarized voltages. Further, the activation curves generated from measuring tail currents from cells expressing both splice isoforms were best described by the sum of two Boltzmann functions. In contrast, activation curves measured in cells expressing $Ca_v2.2\alpha_1$ and $Ca_v\beta_3$ in the absence of $Ca_v\alpha_2\delta$ were fit well by a single Boltzmann function. These data suggest that in the presence of $Ca_v\alpha_2\delta$, N-type Ca channels exist in two states that differ in their sensitivity to voltage. The presence of $Ca_v\alpha_2\delta$ also reduced the difference in the voltage-dependence of activation between $Ca_v2.2\alpha_1$ splice isoforms reported previously.

Finally, to test whether the different properties of $Ca_v2.2\alpha_1$ splice isoforms described here and in previous studies are reflected in native currents, we recorded N-type Ca channel currents from central and peripheral neurons. Calcium channel currents were recorded from acutely dissociated rat superior cervical ganglion and hippocampal pyramidal neurons and the N-type component isolated from other Ca channel currents by ω-conotoxin-GVIA subtraction (1 μM). N-type Ca currents recorded from hippocampal pyramidal neurons activated and deactivated more quickly compared with N-type Ca currents in sympathetic neurons. The difference in activation kinetics between N-type Ca channel currents recorded from these two neurons was voltage-independent. These data are consistent with the hypothesis that alternative splicing in domain IVS3-IVS4 of $Ca_v2.2\alpha_1$ generates functional diversity among N-type Ca channels in different regions of the nervous system. (Supported by NS29967 [D. Lipscombe] and by grant 0160282T from the American Heart Association [S.I. McDonough]).

55. Generation, Expression, and Function of Cysteine-less CFTR MARTIN MENSE, DENNIS M. WHITE, PAOLA VERGANI, ANGUS C. NAIRN, and DAVID C. GADSBY, *The Rockefeller University, New York, NY*

To address biochemically the controversial issues of oligomeric structure, and of inter- and intramolecular domain interactions, in functional CFTR chloride channels, we generated cDNA encoding a cysteine-less mutant (18CS) of human epithelial CFTR in which each of the 18 native cysteines was replaced by a serine, the amino acid most similar in structure. To assay function, 18CS CFTR cRNA was injected into *Xenopus laevis* oocytes in which two-microelectrode voltage-clamp recordings revealed a forskolin-induced transient increase in conductance with time course similar to that observed for oocytes expressing wild-type CFTR. Preliminary unitary current recordings indicated that the 18CS mutant has a single-channel conductance, and gating dependence on PKA and nucleotides, qualitatively comparable to that of wild-type CFTR channels expressed in oocytes. However, expression of this 18CS mutant at the oocyte surface appeared markedly reduced compared with that of oocytes injected with similar amounts of wild-type CFTR cRNA. A pair of native cysteines, C590 and C592, was identified as critical for the abundant surface expression of partially cysteine-substituted CFTR mutants. Indeed, a 10-fold larger whole-oocyte conductance than that obtained with cysteine-free 18CS CFTR was exhibited by a 16CS mutant which retained cysteines C590 and C592. To further investigate the significance of cysteines C590 and C592, they were replaced by alanines or valines. The cysteine-less mutant 16CS(C590V/C592V) resulted in whole-oocyte conductances ≥5-fold larger than obtained with the 18CS mutant channel under comparable conditions. In contrast, cysteine-less 16CS(C590A/C592A) CFTR appeared even less well expressed at the oocyte surface than the 18CS mutant. Western blot analysis confirmed that *Xenopus* oocytes produce measurable amounts of fully glycosylated cysteine-less 16CS(C590V/C592V) CFTR protein. These results demonstrate successful creation of a useful tool that should facilitate investigation of interdomain and intermolecular interactions in the CFTR chloride channel. (Supported by NIH DK51767 and the Revson/Winston Foundation.)

56. Mechanical Properties of Biological Membranes C. NIELSEN,[1] T. EIDE,[1] J.A. LUNDBÆK,[2] R.E. KOEPPE II,[3] and O.S. ANDERSEN,[4] [1]*August Krogh Institute, University of Copenhagen, Copenhagen, Denmark;* [2]*St. Hans Psychiatric Hospital, Roskilde, Denmark;* [3]*University of Arkansas, Fayetteville, AR;* [4]*Weill Medical College of Cornell University, New York, NY*

In this study we characterize the deformation energy associated with membrane protein conformational

change. We use gramicidin (gA) channels as molecular force transducers to quantify the membrane deformation energy. We incorporate different gA analogs (gA channels of different hydrophobic length) in Sf9, EATC, and HEK293 cell plasma membranes, and we assess the mechanical properties of the plasma membrane by monitoring channel activity and mean lifetime. Incubation of cells with gA analogs causes a gradual increase of whole cell currents, and the currents can be potentiated by Triton X-100, β-octyl glucoside, and docosahexanoic acid (DHA). Single channel (gA) recordings in Sf9 plasma membranes show that [val]gA has an average lifetime of ~5,100 ms comparable to that seen in di-palmitoleoylphosphatidylcholine (diC16:1PC) bilayers formed with decane. The mean channel lifetime increases with increased channel hydrophobic length, which allows for quantification of the membrane deformation energy in vivo. The results are interpreted using an elastic membrane model describing the energetics of protein-lipid interactions. (Supported by the Carlsberg Foundation 980106/20–1249.)

57. Structure/Function Analysis of Na,K-ATPase α1 And α2 Central Isoform-specific Regions Reveals Their Involvement in Regulation by Protein Kinase C SANDRINE V. PIERRE, MARIE-JOSEE DURAN, DEBORAH L. CARR, and THOMAS A. PRESSLEY, *Department of Physiology, Texas Tech University Health Sciences Center, Lubbock, TX*

The Na^+-K^+-ATPase is critical for ionic homeostasis in animal cells. The sequences of the four isoforms of its catalytic α-subunit are nearly identical, with the exception of the amino terminus and a 10-residue region near the center of the molecule: the isoform specific region (ISR). Differences in the protein kinase C (PKC) response displayed by the isoforms may originate within these regions of divergent structure. Although previous experiments have shown that the amino terminus is involved, it does not explain all the variability, suggesting that additional regions are involved. We therefore examined the importance of the central ISR in the regulation of enzyme activity by PKC. Complementary DNA of rat ouabain-resistant α1, as well as the α2 isoform modified to display α1-like ouabain-resistance (α2*), were used to obtain chimeras in which the central ISR were exchanged. After transfection into ouabain-sensitive mammalian cells (OK cells), recipient colonies were selected by exposure to ouabain concentrations sufficient to kill nontransfected cells. The response to PKC was assessed by measuring Na,K-ATPase-mediated Rb^+-uptake in transfectants treated with the PKC-activator, phorbol 12-myristate 13-acetate (PMA). PKC activation induced an increase in Na,K-ATPase-mediated Rb^+ transport in α1 and α2* transfected cells. The insertion of α2 ISR in α1α2α1 increased the response to twice that seen with α1. On the other hand, the insertion of α1 ISR in α2α1α2 abolished the response of α2* to PMA. These data suggest that the ISR near the center of the molecule is involved in the regulation of enzyme activity by PKC. In OK cells, PMA-induced activation of transfected rat Na,K-ATPase transport activity is due to an increased membrane expression of the enzyme complex. The ISR of α1 contains a di-Leu motif that could be used by adaptor molecules for membrane recycling. The absence of this motif in the α2 ISR sequence might explain some of the isoform-specific differences observed for PKC regulation. (Supported by a grant from the National Center for Research Resources, RR-19799.)

58. Glucocorticoid Receptors Stimulate Sodium Absorption via Epithelial Sodium Channels (ENaC) in Semicircular Canal Duct Epithelium SATYANARAYANA R. PONDUGULA, JOEL D. SANNEMAN, PIERRE G. MILHAUD, and DANIEL C. MARCUS, *Department of Anatomy and Physiology, Kansas State University, Manhattan, KS*

It was recently found that semicircular canal duct (SCCD) epithelium contributes to the homeostasis of vestibular endolymphatic ion composition by secretion of Cl under adrenergic control (Marcus et al. 2001. *FASEB J.* 15:A842). We hypothesized that SCCD was capable of bi-directional ion transport and investigated whether SCCD is capable of cation absorption in response to stimulation by corticosteroid receptors.

SCCD cells of neonatal and adult rats were cultured to confluence on permeable supports and transepithelial voltage (V_T) and resistance (R_T) were measured in an Ussing chamber at 37°C. Time (15–36 h) -dependent dexamethasone (DXM; 100 nM) -stimulated short circuit current (I_{sc}) was inhibited by the glucocorticoid receptor (GR) antagonist RU-38486 (100 nM) but not by the mineralocorticoid receptor antagonist spironolactone (1μM), indicating stimulation of I_{sc} by activation of GR. DXM-stimulated I_{sc} was also inhibited by the transcription blocker actinomycin D (10–100 nM) and by the translation blocker cycloheximide (10 μM), suggesting genomic regulation of I_{sc} by DXM.

I_{sc} in the presence of DXM was acutely reduced by blockers (amiloride and benzamil) of the epithelial sodium channel (ENaC) but not by a blocker (ethyl isopropyl amiloride) of Na^+/H^+ exchanger. Apical, but not basolateral, amiloride reduced V_T with an IC_{50} of

~1 μM and the more-potent ENaC blocker benzamil with an IC_{50} of ~0.1 μM. Reduction of V_T was accompanied by an increase of R_T. The amiloride-sensitive I_{sc} was nearly abolished when apical Na^+ was replaced with K^+, demonstrating a high Na^+-selectivity of ENaC in this epithelium.

It was found that inhibition of ENaC by amiloride in DXM-treated epithelia did not prevent the usual stimulation of I_{sc} by forskolin, a stimulator of adenylyl cyclase. We conclude that SCCD contribute to the homeostasis of endolymph via bi-directional transport of Na^+ via ENaC under control of GR and of Cl^- under control of β_2-adrenergic receptors coupled to intracellular cAMP. (Supported by NIH NIDCD grant R01-DC212.)

59. The TRK Carriers of Yeast Plasma Membrane Can Behave Like Chloride Channels ALBERTO RIVETTA,[1] ESTHER BASHI,[1] CLIFFORD SLAYMAN,[1] and TERUO KURODA,[1,2] [1]*Yale School of Medicine, New Haven, CT;* [2]*Gene Research Center, Okayama University, Okayama, Japan*

Trk1p and Trk2p, closely related proteins responsible for the major potassium accumulation by *Saccharomyces cerevisiae*, belong to a class of K^+ transporters which is widespread among plants, fungi, and bacteria, but has not yet been found in animal systems. Genetic experiments (Gaber et al. 1988. *Mol. Cell. Biol.* 8: 2848–2859; Ko et al. 1990. *Genetics* 125:305–312) had suggested that Trk1p and Trk2p have complementary functions, supporting high-affinity and moderate-affinity K^+ influx, respectively. But direct physiological experiments have produced a more complicated picture and, in particular, patch-clamp experiments have demonstrated inward currents through both proteins, which can be much larger, at the expected resting membrane voltage (−200 mV), than the total proton pump current through yeast membranes, and which are only weakly dependent on extracellular K^+.

These same currents depend strongly upon intracellular chloride and upon acidic extracellular pH (Bihler et al. 1999. *FEBS Lett.* 447:115–120). Steady-state currents at negative membrane voltages are approximately linearly related to intracellular Cl^- concentration, but are logarithmically related to extracellular proton concentration. The data can be modeled by a chloride channel having a constant-field open-channel conductance due 90% to chloride, a gating charge of 0.5, and a very negative gating voltage: −340 mV at pH_o = 7.5, which shifts 45 mV positive with each unit acid shift of pH. The effect of high intracellular Cl^- is closely mimicked by Br^-, and less closely by I^-, but not by the non-halide chaotrope, $SC0^-$. This behavior is reminiscent of the chloride-channel function already well described for neurotransmitter transporters (Lester et al. 1996. *Neuron.* 17:807–810), and is probably related to the chloride-dependent breakdown of membrane resistance which has previously been observed in a variety of plants and fungi. It may be important agriculturally, by contributing to salt intolerance in higher plants. (Supported by NIH research grant GM60696 and by a sabbatical grant from the Japanese Science Foundation.)

60. Arginine Supplementation of Sickle Transgenic Mice Reduces Red Cell Hemoglobin Concentration and Ca^{2+} Activated K^+ Channel Activity J.R. ROMERO,[1] S.M. SUZUKA,[2] and M.E. FABRY,[2] [1]*Endocrine-Hypertension Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA; and* [2]*Hematology Division, Albert Einstein College of Medicine, Bronx, NY*

Nitric oxide (NO), essential for maintaining vascular tone, is produced from arginine by nitric oxide synthase (NOS). Increased levels of inducible and constitutive NOS have been reported in cremaster muscle, kidney, liver, and brain of sickle transgenic mouse models. Plasma arginine levels are low in sickle cell anemia and may be related to increased NOS activity. We found low plasma arginine in sickle mice when compared with control mice (C57BL). We supplemented sickle and C57BL mice with a fourfold increase in dietary arginine (5% arginine) for several months. This restored the plasma arginine level in sickle mice to that found in C57BL mice and decreased the mean corpuscular hemoglobin concentration (MCHC) in sickle mice only. We measured deoxy-stimulated K^+ efflux that is characteristic of red cells in sickle cell disease and contributes to the disease process by increasing MCHC and rendering red cells more susceptible to polymer formation. We observed a decrease in the clotrimazole-sensitive deoxy K^+ efflux suggesting involvement of the Ca^{2+} activated K^+ channel [K(Ca)]. Therefore, we studied the kinetic properties of K(Ca) in room air by measuring its activity as a function of cellular calcium. V_{max} of K(Ca) was reduced from 4.1Å0.6 FU (nonsupplemented) to 2.6 Å 0.4 FU (supplemented) (n = 7 and 8, $P < 0.04$, t test) in sickle mice and was similarly reduced in C57BL mice. We measured the time course of response to arginine supplementation and observed that maximal inhibition of K(Ca) did not occur until more than 40 d after the onset of supplementation. We conclude that the major mechanism by which arginine supplementation reduces red cell density (MCHC) in sickle mice is by inhibiting the Ca^{2+} activated K^+ channel. Reduc-

tion of MCHC does not occur in C57BL mice because Ca^{2+} influx following deoxygenation is unique to cells with polymerizable hemoglobin. (Supported by P60HL38655.)

61. Acute and Chronic EGF-induced Inhibition of Amiloride-sensitive Sodium Absorption in Renal Collecting Duct Cells Is Mediated by ERK1/2 JIE-PAN SHEN and CALVIN COTTON, *Departments of Pediatrics and Physiology and Biophysics, Case Western Reserve University, Cleveland, OH*

Addition of epidermal growth factor (EGF) to the basolateral bathing solution of isolated, perfused rabbit cortical collecting ducts is known to cause rapid inhibition of sodium absorption (Vehaskari et al. 1989. *Am. J. Physiol.* 256: F803–F809); however, the signal transduction pathways responsible for inhibition of sodium absorption remain undefined. Furthermore, the effects of long-term exposure to EGF on renal collecting duct ion transport are not known. We used a conditionally-immortalized murine collecting duct cell line (mCT1 cells; Takacs-Jarrett et al. 1998. *Am. J. Physiol.* 275:F802–F811) to examine EGF-mediated signaling and its effects on electrogenic sodium absorption. MCT1 cells express all three subunits of the epithelial sodium channel (ENaC), and the basal short-circuit current of mCT1 cells (I_{sc}, 15 Å 1 $\mu A/cm^2$; $n = 10$) is amiloride sensitive (~90% inhibition). Acute exposure (30 min) to EGF (50 ng/ml) reduced I_{sc} by 15 Å 3% ($n = 4$), whereas chronic exposure (24 h) to EGF reduced I_{sc} by >60%. The inhibitory effects of long-term exposure to EGF were sustained as long as EGF was present (up to 5 d); however, upon removal of the EGF, sodium absorption returned to control levels within 24 h. Addition of EGF (20 ng/ml) or PMA (150 nM) to mCT1 cells induced rapid phosphorylation of p42/p44 (ERK1/2). Pretreatment of the epithelial monolayers with PD98059 (30 μM; MEK inhibitor) prevented phosphorylation of p42/p44. Similarly, pretreatment of mCT1 monolayers with PD98059 prevented both the acute and chronic EGF-induced inhibition of amiloride-sensitive sodium absorption. Quantitative RT-PCR analysis of mCT1 cells treated with EGF (20 ng/ml; 24 h) revealed a 60–80% reduction in the mRNA levels for α-, β-, and γ-ENaC subunits. The results of these studies demonstrate that both the acute and long-term effects of EGF on amiloride-sensitive sodium absorption are mediated by activation of the Extracellular signal–regulated protein kinase pathway (ERK1/2). The EGF receptor is abnormally expressed on the apical cell membrane of renal cysts in both autosomal dominant and autosomal recessive polycystic kidney disease (ADPKD and ARPKD). Activation of ERK1/2 signaling by lumenal EGF and EFG-like molecules may contribute to alterations in ion and water transport across renal cystic epithelium and promote cyst formation and/or enlargement. (Supported by grants from the Polycystic Kidney Research Foundation #99013, NIH DK-27651, and DK-57306.)

62. Strain Energy Storage in Contracting Single Muscle Fibers STUART R. TAYLOR, *Mayo Foundation, Rochester, MN*

Torsional rigidity of an isolated frog skeletal muscle fiber increases to its maximum during contraction in advance of tension, and remains at this level after relaxation sets in (Sten-Knudsen. 1953. *Acta Physiol. Scand.* 28:1–240). These results were linked to the theory that torsional stiffness during contraction discloses forces of interaction in the transverse direction. Mechanisms for lateral force transmission are functionally important in integrating the forces from the single fiber to the whole muscle (Monti et al. 1999. *J. Biomechanics.* 32:371–380). To better understand these mechanisms we directly measured torsional strain, cross-sectional area (CSA), and contractile force from isolated frog skeletal muscle fibers (Neering et al. 1991. *Biophys. J.* 59:926–932). CSA rose and fell closely following the rise and fall of tetanic force (10–15 Hz; 1°C; 2.5 μm). CSA fell below its resting value near the end of contraction and recovered during the ~0.5 s after tension relaxation was complete. The Z band lattice stores some strain energy because a tetanus induces forces with lateral components that are absent in muscle that is passively stretched or shortened (Goldstein et al. 1988. *J. Gen. Physiol.* 92:113–119). If the CSA reflects the capacity of the Z bands to store strain energy, it is small compared with the structures strained by torsion. In contrast to CSA, the time course of torsional strain matched that of the torsional rigidity described by Sten-Knudsen. Torsional strain reached maximum in ~15 ms, which was one third of the time to the onset of tension. Torsional strain did not begin to decline until 200 ms after the completed, isometric phase of tension relaxation. Sten-Knudsen found that torsional stiffness at rest increases exponentially with stretch, while it decreases linearly with stretch before an isometric contraction. Both relationships are associated with a decrease in the fraction of the thin filaments overlapped by thick filaments. The possibility that the thin filaments can store strain energy has previously been raised, in view of the discovery that their axial extensibility is substantially greater than expected (Goldman and Huxley. 1994. *Biophys. J.* 67: 2131–2136). The changes in torsional strain are consis-

tent with this idea. (Supported by NSF grants DMB 8503964 and IBN 9213160, and NIH grant HL52760.)

63. Splice Isoforms of the N-type Ca Channel Differ in Their Sensitivity to Cumulative Inactivation Induced by Trains of Action Potential Wave-forms CHRISTOPHER THALER and DIANE LIPSCOMBE, *Department of Neuroscience, Brown University, Providence, RI*

Alternative splicing in the voltage-gated calcium channel Cav α1 subunit family has been shown to produce modifications in kinetics and voltage-dependence of channel gating (Lin et al. 1997. *Neuron.* 18:153–166; Lin et al. 1999. *J. Neurosci.* 19:5322–5331; Pan and Lipscombe 2000. *J. Neurosci.* 20:4769–4775). Little is known about whether modifications in channel properties arising from alternative splicing alters the profile of calcium entry during the relatively short voltage excursions found in action potential (AP) wave-forms. Here we examine two splice isoforms involving cell-specific expression of exon 18a in the II-III loop of N-type Cav2.2 α1. This region of the Cav2.2 α1 subunit has been implicated in coupling presynaptic Cav2.2 α1 channels to the synaptic vesicle release machinery. Cav2.2 α1 splice isoforms that differ in the expression of exon 18a differ in their sensitivity to cumulative inactivation during trains of AP wave-forms. N-type Ca channel Cav2.2 α1 subunits were transiently expressed in tsA201 cells together with Cav β1b and Cav α2δ. Currents were recorded with 1 mM calcium as the charge carrier. Following a train of 15 AP waveforms N-type currents induced by the expression of Cav2.2 α1 subunits containing exon 18a inactivated 24 ± 5% ($n = 6$). In contrast, currents induced by the expression of the Cav2.2 α1 isoform lacking exon 18a inactivated 40 ± 6% ($n = 6$); Student's t test, $P < 0.001$ (1st pulse − 15th pulse/1st pulse × 100 = % inactivation). To begin to explore the mechanism by which alternative splicing of exon 18a affects the degree of cumulative inactivation during AP wave-forms, we compared the time courses of recovery from inactivation of the two splice isoforms. During a 400-ms prepulse to peak current, both Cav2.2 α1 splice isoforms generated calcium currents that inactivated to the same extent (+18a: 90 ± 4%, $n = 6$; Δ18a: 86 ± 3%, $n = 3$; statistically not significant). Test pulses were applied at various intervals to assess the level of recovery. The currents showed no recovery after 10 ms relative to the current remaining at the end of the 400 ms prepulse (+18a, 96 ± 4%, $n = 6$), and in fact the Δ18a splice isoform continued to inactivate (87 ± 5%, $n = 3$) ($P < 0.05$) consistent with closed state inactivation. After 100 ms, the +18a splice isoform increased by almost 50% (43 ± 12%, $n = 6$). In contrast, the splice isoform lacking exon 18a showed little or no additional change relative to the 10-ms time point (87 ± 12%, $n = 3$). One possible conclusion from these data that will require further testing, is that the +18a isoform exhibited less or no closed state inactivation. This could explain differences in the time course of recovery from inactivation observed between exon 18a splice isoforms of Cav2.2 α1 and could, at least in part, underlie differences in cumulative inactivation seen during trains of AP waveforms. Our findings are consistent with the idea that alternative splicing is an important mechanism for optimizing calcium channel activity and calcium entry in specific regions of the nervous system. (Supported by NS 29967 and MH19118.)

64. Isolation of Murine Renal Collecting Duct Cells by Flow Cytometry ELIAS VEIZIS and CALVIN COTTON, *Departments of Pediatrics, Physiology and Biophysics, Case Western Reserve University, Cleveland, OH*

Isolated, perfused tubules represent a versatile and highly relevant biological preparation for the study of renal tubule function; however, they are technically difficult to obtain, long-term experiments are problematic, and it is difficult to obtain large numbers of pure collecting duct cells. Amphibian kidney cells (A6) and immortalized mammalian renal collecting duct cell lines (M1, IMCD, mCT1) are widely used as models for studies of collecting duct function. The goal of this work is to develop a method for the isolation of large numbers of murine collecting duct cells suitable for primary cell culture. We used a transgenic mouse in which enhanced green fluorescent protein (EGFP) expression is driven by the Hoxb7 promoter (Srinivas et al. 1999. *Dev. Genet.* 24:241–251). EGFP expression is specific for the embryonic ureteric bud and its derivatives (collecting ducts). Kidneys were dissected, washed, and digested for 30–40 min in collagenase Type IV at 37°C. Tubule fragments and single cells were seeded in tissue culture dishes with standard collecting tubule culture media. After 5–8 d in culture, the cells were removed from the tissue culture plates, suspended in Hepes-buffered Ringers solution and subjected to fluorescence-activated cell sorting (FACS). The EGFP positive (EGFP+) cells were collected and seeded in collagen-coated permeable filter inserts (Millicell CM) for electrophysiology and immunohistochemistry. The fluorescence intensity of the cell population was bimodal and the EGFP+ cells exhibited ~100-fold greater intensity compared with EGFP− cells (autofluorescence). We routinely isolate 1–1.5 × 10^6 cells per

mouse with a purity of >95%. EGFP+ cells remain positive for more than two weeks in culture and EGFP- cells remain negative. The primary cultures of EGFP+ cells form high resistance monolayers (0.8–1.7 kE-cm^2), express ZO1 tight junction protein, lateral membrane E-cadherin, and are polarized. Small secretory responses are elicited by elevation of intracellular cAMP or calcium. Furthermore, the basal short circuit current (I_{sc} = 23 Å 5 μA/cm^2, R_t = 1.2 Å 0.3 k3 -cm^2; n = 6) is inhibited by ~90% by lumenal amiloride, indicative of electrogenic, ENaC-mediated sodium absorption, a feature of collecting duct principal cells. This approach provides a convenient and reliable method for the isolation of pure populations of collecting duct cells suitable for functional evaluation from a variety of mouse models. Studies are currently underway using CFTR knockout and autosomal recessive polycystic kidney disease mice. (Supported by grants from the Polycystic Kidney Research Foundation # 99013, NIH DK-27651 and DK-57306.)

65. Sidedness Determination Supports the MPM (Channel-analogue) Model for Yeast TRK Proteins

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The so-called MPM structural motif comprises two transmembrane α-helices (M) sandwiching a less hydrophobic (P) segment that in turn loops to the interior of the membrane bilayer from the extracellular ends of the M helices. This motif, including a signature triad glycine-tyrosine-glycine, has been canonized for potassium-selective ion channels by the crystallographic studies of Doyle et al. (1998. *Science.* 280:69–77). But amino acid sequence comparisons, throughout the proteome databases, suggest that this motif also characterizes a class of "active" K^+-transport proteins common among bacteria, fungi, and plants (Durell et al. 1999. *Biophys. J.* 77:775–788; Durell and Guy, *Biophys. J.* 77:789–807). Two such proteins, designated Trk1p and Trk2p, are found in the yeast *Saccharomyces cerevisiae.* Simple hydropathy analysis (Gaber et al. 1988. *Mol. Cell. Biol.* 8:2848–2859) posits a chain of 12 transmembrane α-helices for both proteins, but MPM folding predicts a chain of four such motifs, implying only eight transmembrane helices. Detailed comparison of these models reveals several segments of the protein which should clearly reside on opposite sides of the yeast plasma membrane by the two models, thus affording sidedness tests of the actual folding pattern.

Starting with *TRK2*, which encodes the smaller of the two yeast proteins, we inserted an HA (hemagglutinin) tag into the largest single test loop and also attached the EGFP (enhanced green fluorescent protein) tag at the COOH terminus of the protein, which lies intracellular by both models. The tagged proteins were then expressed in a TRK-negative yeast strain (*trk1Δ trk2Δ*), via a galactose-driven plasmid, and examined by indirect immunofluorescence and immunoelectron microscopy. In permeabilized cells, both TRK2-HA and TRK2-EGFP were found in two locations: on the plasma membrane and within intracellular membrane clumps. However, neither location was accessible to the antibodies in nonpermeabilized cells, thus demonstrating that the tag-site for the HA epitope lies on the intracellular side of the plasma membrane, as predicted by the MPM model. Both tagged proteins were functional, supporting growth of the *trk1Δ trk2Δ* strain on low K^+; and the intracellular clumping artifact could be suppressed by growth of the yeast in 5–10% glycerol, which serves as a chemical chaperone (Figler et al. 2000. *Arch. Bioch. Biophys.* 376:34–46). (Supported by NIH research grants GM60696 and by a James Hudson Brown—Alexander Brown Coxe Fellowship from Yale School of Medicine [to G. Zeng].)