Speaker Abstracts

1. Phosphoinositide Signaling LEWIS CANTLEY,[1,2] KATJA LAMIA,[1,2] SASKIA BRACHMANN,[1,2] JI LUO,[1,2] JONATHAN HUROV,[1,2] YOUNG-BUM KIM,[2] ODILE PERONI,[2] BARBARA KAHN,[2] and LUCIA RAMEH,[3] [1]*Department of Systems Biology, Harvard Medical School,* [2]*Beth Israel Deaconess Medical Center, Boston, MA;* [3]*Boston Biomedical Research Institute, Watertown, MA*

We have generated mice in which genes for subunits of various phosphoinositide kinases have been deleted and are investigating the role of these enzymes in development, in insulin signaling, and in cancers. We have focused on the relative roles of the p85 regulatory subunits and the p110 catalytic subunits of class Ia phosphoinositide 3-kinase (PI 3-kinase) in insulin and PDGF signaling. Surprisingly, we previously found that mice lacking either p85α or p85β have increased insulin-dependent glucose clearance from blood, despite having reduced insulin-dependent activation of PI 3-kinase (Fruman et al. 2000. *Nature Genetics.* 26:379–382; Ueki et al. 2002. *Proc. Natl. Acad. Sci. USA.* 99:419–424.). Possible biochemical mechanisms for this effect will be discussed. We have also generated mouse embryo fibroblasts in which all splice forms of p85α and p85β have been deleted and IGF-1 and PDGF signaling in these cells will be discussed. Finally, we have also generated mice in which Type II phosphatidylinositol-5-phosphate 4-kinase genes have been deleted. Characterization of these mice suggests that the phosphatidylinositol-5-phosphate pathway indirectly regulates the PI 3-kinase pathway. (Supported by NIH grants GM41890 and GM36624.)

2. PI Kinase Signaling in Membrane Trafficking SCOTT EMR, ALEX RUSNAK, JI SUN, TONY CHU, JON AUDHYA, and CHRIS STEFAN, *Department of Cellular and Molecular Medicine, University of California, San Diego School of Medicine & HHMI, La Jolla, CA 92093-0668*

Phosphoinositide lipids (PIs) function as spatially restricted membrane-signaling molecules that regulate diverse cellular processes including cell growth, differentiation, cytoskeletal rearrangements, and membrane trafficking. Activation of the Vps34 PI 3-kinase results in production of the lipid second messenger PI3P, which triggers the recruitment/activation of FYVE domain and PX domain–containing effector proteins on endosomal membranes. One FYVE domain–containing protein, Vps27, and its mammalian homologue, HRS, are required for the formation of late endosomal compartments called multivesicular bodies (MVBs). The MVB sorting pathway is required for the down-regulation of numerous activated cell-surface receptors (e.g., EGFR) that subsequently are degraded in the lysosome. Ubiquitination of both biosynthetic and endocytic cargo by the Rsp5 HECT-domain Ub ligase in yeast serves as a signal for sorting into the MVB pathway. We recently identified three distinct protein complexes referred to as the endosomal sorting complex required for transport (ESCRT) complexes that function in the recognition and sorting of ubiquitinated MVB cargoes. Vps27 appears to serve as a docking site for the ESCRT-I complex, thereby initiating the MVB sorting reaction. Vps27 recruits ESCRT-I to endosomes via a short peptide sequence in the COOH terminus of Vps27 that is related to a sequence in the HIV-1 Gag protein required for both viral budding and interaction with human ESCRT-I. Our observations indicate that PI kinase signaling and monoubiquitination function as critical regulators of endosomal sorting, receptor down-regulation, and HIV viral budding.

3. A New Model for Activation of the Epidermal Growth Factor Receptor (EGFR) STUART MCLAUGHLIN,[1] STEVEN O. SMITH,[2] and DIANA MURRAY,[3] [1]*Department of Physiology and Biophysics, and* [2]*Department of Biochemis-*

try and Cell Biology, SUNY Stony Brook, NY; [3]Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, NY

The cytoplasmic domain of the EGFR comprises a juxtamembrane (JM) region, a protein tyrosine kinase (PTK) core, and a COOH-terminal tail with tyrosine residues that can be trans autophosphosphorylated. The PTK core structure reveals the catalytic elements are "primed and ready for phospho-transfer", suggesting regulation "must be exerted by control of the delivery of the COOH-terminal substrate tyrosines to the active site" (Stamos et al. 2002. *J. Biol. Chem.* 277:46465–46472). Our model explains how this control is exerted and accounts for many established observations: e.g., either the influx of Ca^{2+} into a cell or hyperosmotic shock can trigger autophosphorylation in the absence of physiological ligands. We postulate that residues 645–660 of the JM region (RRRHIVRKRTLRRLLQ) bind to the inner leaflet of the bilayer through electrostatic and hydrophobic interactions. Poisson-Boltzmann calculations show the PTK core has a positively charged face and should also bind to the bilayer, preventing free rotation and full activity. EGF stimulation of cells produces a rapid (3–10 s) increase in free [Ca^{2+}], and we postulate that Ca^{2+}/calmodulin (Ca/CaM) binds to the JM 645–660 region, reversing its charge from +8 to <0. This electrostatically repels the Ca–CaM-JM complex from the negatively charged bilayer, ripping the PTK off the membrane, which in turn allows it to rotate freely and exert full activity. We tested our "electrostatic engine" model for EGFR by showing that an EGFR(645–660) peptide binds with high affinity to PC/PS bilayers (molar partition coefficient K $\sim 10^6$ M^{-1} for 2:1 PC/PS vesicles), and that high salt or Ca/CaM effectively remove it from the membrane (K_d for Ca/CaM-EGFR(645–660) ~10 nM). FRET measurements show that membrane-bound EGFR(645–660) electrostatically sequesters PIP_2, the substrate for EGFR-bound PLC-γ. (Supported by NIH grant R-37 GM24971 to Stuart Mclaughlin.)

4. Elasticity and Strength of Lipid Bilayers EVAN EVANS, *Physics and Pathology, University of British Columbia, Vancouver; Biomedical Engineering and Physics, Boston University, Boston, MA*

Enabled by precision micropipette aspiration and high-resolution membrane-edge tracking, measurements of apparent vesicle area under tension have revealed both the elastic bending modulus k_c (due to smoothing of thermal undulations at very low tensions ~0.001–0.5 mN/m) and the direct-stretch modulus K_a (due to increase in area per lipid at high tensions >1 mN/m). After removal of thermal undulations, stretch moduli of PC bilayers are found to vary little with chain length (from diC13:0 to diC22:1) or unsaturation and have a common value of ~240 mN/m, as predicted by a simple physical theory based on the free energies of chain entropy confinement and a hydrocarbon–water interaction of ~40 mJ/m^2 at each interface. Also expected from the theory, the measurements of bending moduli for saturated and monounsaturated PC bilayers increase as the square of the hydrocarbon thickness h derived from x-ray diffraction with $k_c \sim 0.56 \times 10^{-19}$ J and $h \sim 2.4$ nm for diC13:0 up to $k_c \sim 1.2 \times 10^{-19}$ J and $h \sim 3.4$ nm for diC22:1. However, exposing the capriciousness of chemistry, bending moduli drop precipitously when two or more cis-double bonds are present in one or both chains, which shows that poly-cis unsaturated chains are anomalously flexible. Tested in the same way, but with pipette suction ramped at fixed speeds spanning four orders in magnitude, measurements of the instantaneous tensions at vesicle rupture have provided a spectroscopic method to connect bilayer strength to the kinetics of defect nucleation and rupture pore dynamics. Revealed by the dependence of vesicle rupture tension on the rate of tension application, the key determinants of bilayer strength are the spontaneous frequency ν_δ and size (~1 nm^2) of defects plus the edge energy ε of a nascent hole. Again measured for the same set of PC bilayers, the defect frequency (~10^{-2}–10/s) and hole edge energy (~1–20 pJ/m) are found to correlate with thickness and bending modulus, respectively. What's intriguing is that bilayer strength is lowered dramatically by amphiphilic proteins at concentrations well below lytic levels. Consequently, even modest tensions greatly enhance bilayer permeation by antimicrobial and apoptotic peptides (as well as likely impact other membrane-protein interactions). (Rawicz, W., K. Olbrich, T. McIntosh, D. Needham, and E.J. Evans. 2000. *Biophys. J.* 79:328–339. Evans, E., V. Heinrich, F. Ludwig, and W. Rawicz. 2003. *Biophys. J.* 85: 2342–2350.)

5. Phosphoinositides Control Actin Remodelling during Phagocytosis and Bacterial Invasion SERGIO GRINSTEIN, *Cell Biology Program, Hospital for Sick Children, Toronto, Canada*

The engulfment and elimination of microorganisms by neutrophils and macrophages is an essential component of the innate immune response. This process, known as phagocytosis, involves extensive remodeling of the actin cytoskeleton as well as membrane fusion and fission events. These responses are rapid, transient, and highly localized, which complicates their analysis by conventional biochemical means. We therefore studied the signals involved in phagosome generation by

digital imaging of live cells. In particular, we analyzed the role of phosphoinositides using chimeric constructs of PH, PX, FYVE, or C1 domains fused to fluorescent proteins. PIP_2 was found to undergo a biphasic change at the phagosomal cup: an early accumulation that was rapidly superseded by complete disappearance of the phosphoinositide. The former phase was associated with the accumulation of actin at the cup. Elimination of PIP_2 was accompanied by generation of PIP_3 and also by the appearance of diacylglycerol in the sealed phagosome. Of note, disappearance of PIP_2 coincided with the dissociation of actin from the nascent phagosome. Conditions that precluded the hydrolysis of PIP_2 resulted in thickening of the submembranous F-actin layer, prevented the dissociation of actin from the cup and arrested particle internalization by impairing scission of the phagosomal vacuole.

Some bacteria, including *Salmonella*, gain entry to nonphagocytic cells by an invasion process akin to phagocytosis. Invading *Salmonella* elicit massive ruffling of the membrane of the host cell, followed by pinching off of small vacuoles at the base of the ruffles. We found that while PIP_2 is abundant at the tip of the ruffles, the phosphoinositide is absent from their base, where scission of bacteria-containing vacuoles is occurring. The focal depletion of PIP_2 from the base of the ruffles is induced by a bacterial protein, SigD/SopB, which is injected into the host cell cytosol by the microorganism. When transfected into mammalian cells, SigD/SopB suffices to deplete cellular PIP_2 and induces the spontaneous formation of blebs and of large intracellular vacuoles. Transfection of SigD/SopB also alters the cellular F-actin skeleton. Jointly, these experiments suggest that uptake of bacteria or large particles by animal cells requires two phases of actin remodeling: an early stage of actin recruitment and remodeling at the tip of pseudopodia or ruffles, and a secondary phase of actin dissociation which is necessary for membrane fission and vacuole sealing. The early phase requires PIP_2 and is associated with a local increase in the concentration of the inositide. The second phase is accompanied by, and likely requires removal of PIP_2.

Thus, localized phosphoinositide metabolism appears to play a critical role in both phagocytosis and bacterial invasion.

6. The Lipid Binding Pleckstrin Homology Domain in UNC-104 Kinesin Is Necessary for Synaptic Vesicle Transport in *C. elegans* DIETER R. KLOPFENSTEIN[1,3] and RONALD D. VALE,[1,2] [1]*Department of Cellular and Molecular Pharmacology and* [2]*The Howard Hughes Medical Institute, University of California San Francisco, San Francisco, CA 94143;* [3]*DFG Research Center for Molecular Physiology of the Brain, Georg August University, Humboldtallee 23, 37073 Göttingen, Germany*

Directional vesicle movement in neurons critically depends on molecular motors that facilitate the long-range transport into axon and dendrites. Many such transport processes depend on members of the kinesin motor protein family that specifically bind to cargo membranes through protein–protein interactions. However, the role of lipids in membrane transport is less well understood. We are studying a kinesin motor, UNC-104 (KIF1A), which has been shown to interact with the membrane lipid phosphatidylinositol(4,5)bisphosphate (PIP_2) with its pleckstrin-homology domain (PH). UNC-104 is a neuronally expressed kinesin motor that transports synaptic vesicles from the cell body to the terminal. In the nematode *C. elegans*, synaptic vesicle transport requires a motor with an intact PH domain and point-mutations that reduce binding to PIP_2 show reduced membrane transport activity with the consequences of mislocalized synaptic vesicles and uncoordinated worm movement. In parallel, in vitro transport studies suggest that clustering of PIP_2 in the membrane can stimulate cargo transport in a cooperative manner. These results suggest that PIP_2 is an important lipid for motor-cargo interaction and may be used to regulate axonal transport of synaptic vesicles.

7. Lipid Signaling, Directional Sensing, and Cell Shape Control PETER DEVREOTES, ELAINE HUANG, MIHO IIJIMA, CHRIS JANETOPOULOS, FRANCESCA VAZQUEZ, and LINGFENG CHEN, *Department of Cell Biology, Johns Hopkins University, Baltimore, MD 21205*

The ability to sense and respond to shallow gradients of extracellular signals is remarkably similar in *Dictyostelium discoideum* amoebae and mammalian leukocytes. Consideration of the features of a chemotactic response presents several fascinating and unique challenges. Shallow external gradients must generate sharply localized internal responses at the leading edges of the cells. Moreover, cells at different points in the gradient sense equally well so there is a powerful mechanism for background subtraction or adaptation. We have suggested that a balance between local excitatory and global inhibitory processes controls the response to chemoattractants. An extensive series of studies in the last several years have indicated that the upstream components and reactions in the signaling pathway are uniformly localized in cells exposed to a chemoattractant gradient. However, downstream responses such as PI $(3,4,5)P_3$ accumulation and actin polymerization are sharply localized toward the high side of the gradient, suggesting that these responses are selectively acti-

vated at the cell's leading edge. We have recently found that uniform stimuli transiently recruit and activate PI3Ks while PTEN is released from the plasma membrane. Although chemoattractant receptors and G-proteins are evenly distributed along the cell surface, gradients of chemoattractant cause PI3Ks and PTEN to bind to the membrane at the front and the back of the cell, respectively. This reciprocal regulation provides robust control of $PI(3,4,5)P_3$ and leads to its sharp accumulation at the anterior. Interference with PI3Ks modifies chemotaxis while disruption of PTEN broadens PI localization and actin polymerization in parallel. Thus, counteracting signals from the upstream elements of the pathway converge to regulate the key enzymes of PI metabolism, localize these lipids, and direct pseudopod formation. (Supported by RO1GM28007 and RO1GM34933.)

8. Functional Coupling of Ion Transport, Membrane Recycling and Phosphoinositide Turnover DONALD W. HILGEMANN, PING DONG, and PATRICK FOLEY, *Department of Physiology, University of Texas Southwestern at Dallas, Dallas, TX*

Numerous ion transporters and channels are activated by the ATP-dependent production of phosphatidylinositides (Ptides) in excised membrane patches, and in a few cases Ptides are implicated to be plasmalemma-delimited messengers in channel regulation. A general role, however, may be that Ptides regulate ion channel and transporter activity during their trafficking, which is closely coupled to Ptide metabolism, as a means to maintain specific ion channel and transporter activities in specific membrane compartments. Studying the cardiac Na/Ca exchanger (NCX1) in excised patches, exogenous PIP_2 results in complete transporter activation by relieving multiple inactivation reactions. But in intact BHK cells, with rapid control of the cytoplasmic medium, PIP_2 powerfully inhibits transporter activity in parallel with a decrease of cell capacitance. Endocytosis appears to be involved, and similar effects occur in multiple cell lines with several (but not all) transporters and channels analyzed to date. Generation of PIP_2 by overexpression of lipid kinases also favors internalization of transporters and channels. Not only manipulation of lipids, but fast manipulation of the major cytoplasmic monvalent ions causes ~15% of the cell surface to be added or removed in 1 min. The possibility will be discussed that ion channels, including PIP_2-sensitive K-potassium channels, regulate membrane cycling and Ptide metabolism by mediating changes of vesicular volume. (Supported by NIH HL0679420 and HL051323 to Donald W. Hilgemann.)

9. Gating of K^+ Channels by Phosphatidylinositol Bisphosphate (PIP_2) DIOMEDES E. LEGOTHETIS,[1] TAIHAO JIN,[1] AVIA ROSENHOUSE-DANTSKER,[1] COELI M.B. LOPES,[1] TIBOR ROHACS,[1] HAILIN ZHANG,[2] and JINLIANG SUI,[1] [1]*Mount Sinai School of Medicine, Department of Physiology and Biophysics, New York, NY 10029;* [2]*Hebei Medical University, Department of Pharmacology, Shijiazhuang, China*

Representative members from all structural superfamilies of ionic channels have been shown to be directly regulated by phosphoinositides. Inwardly rectifying K^+ (Kir) channels are important regulators of resting membrane potential and cell excitability. The activity of Kir channels is critically dependent on the integrity of channel interactions with PIP_2. Several basic amino acid residues in the NH_2 and COOH termini of Kir channels are localized in three dimensions into a pocket that is juxtaposed to the inner cell membrane, forming electrostatic interactions with PIP_2 to control channel gating. Interactions of a number of intracellular modulators (e.g., protons, Mg^{2+}, Na^+, the $\beta\gamma$ subunits of G proteins, protein kinase C, phospholipase C) with regions neighboring to the basic PIP_2-interacting residues modulate channel activity by affecting channel PIP_2 interactions. Mutations on specific PIP_2-interacting basic residues or neighboring sites result in disease states, such as Andersen's and Barter's syndromes. Such mutations alter the single channel gating kinetics of Kir channels. We have characterized single-channel kinetics of mutants of IRK1 that displayed weakened channel PIP_2 interactions and mutants of GIRK4* that displayed enhanced channel–PIP_2 interactions. All tested neutralizations of the positively charged residues of IRK1 affect the burst behavior of the channels, while some mutations also affect the open time kinetics of the channels. All tested mutations on GIRK4* increased the burst duration of the channels. Mutations on GIRK4* that increase the channel–PIP_2 interactions and proline mutations on the pore-lining TM2 helix that render the GIRK channels G protein independent show distinct effects on the single-channel kinetics of GIRK4* channel, reflecting the distinct time-scale at which GIRK channels interact with different gating molecules. We propose a mechanical model of gating, where channel–PIP_2 interactions provide a tether to the cell membrane, generating a tangential adjustable force that pulls the gate open.

10. Lipid Signaling in *Drosophila* Phototransduction ROGER C. HARDIE, *Department of Anatomy, University of Cambridge, UK*

In *Drosophila* photoreceptors the depolarising response to light is mediated by two classes of Ca^{2+}-permeable channels, TRP and TRPL, activated downstream of rhodopsin, G-protein, and phospholipase C (PLC). Elements of the transduction cascade, including the TRP channels are organized into signaling complexes within densely packed microvilli, forming a light-guiding rhabdomere. While some members of the TRP family are activated indirectly via inositol 1,4,5 trisphosphate—e.g., as store-operated or Ca^{2+} activated cation channels—it now appears that many TRP channels are regulated by lipid products of phosphatidyl inositol 4,5 bisphosphate (PIP_2) hydrolysis. In *Drosophila* photoreceptors a combination of genetic, electrophysiological, and pharmacological evidence implicates diacylglycerol (DAG) and/or downstream metabolites (polyunsaturated fatty acids) as excitatory messengers. Some of the most compelling evidence for the role for DAG comes from studies of the DAG kinase mutant (*rdgA*), which massively sensitizes responses to light on genetic backgrounds where PLC activity is reduced.

To understand better the role of PIP_2 in phototransduction, PIP_2 levels have been tracked in vivo using genetically targeted electrophysiological biosensors (PIP_2-sensitive Kir2.1 channels). Measurements using these biosensors reveal high in vivo basal levels of PLC activity, equivalent to ~50% depletion of total PIP_2 min^{-1}. After activation by light, PLC depletes PIP_2 at rates in excess of 100% s^{-1}. PIP_2 is resynthesised with a half time of ~1 min, which would be incapable of maintaining PIP_2 levels in the face of such high activity. However, Ca^{2+} influx via the highly Ca^{2+}-permeable TRP channels into the restricted lumen of the microvilli results in extremely high Ca^{2+} levels (>100 μM), which rapidly inhibit PLC activity. When Ca^{2+} influx is prevented by recording in Ca^{2+}-free solutions, or reduced by genetic elimination of the TRP channel, even moderate illumination rapidly results in depletion of all detectable PIP_2. (Supported by Grants from Medical Research Council and Biotechnology and Biological Sciences Research Council.)

11. Protein Insertion into the ER Membrane: Can Hydrophobicity be Quantitated? GUNNAR VON HEIJNE, *Department of Biochemistry and Biophysics, Stockholm University, Sweden*

Even though membrane protein insertion into the ER membrane of eukaryotic cells has been studied for years, a quantitative understanding of the process is still lacking. We are using an in vitro system based on cotranslational integration of model proteins into dog pancreas microsomes to analyze this process. By carefully designing the model protein constructs, we have derived the first true "biological" hydrophobicity scale and have been able to get a first idea of how the position of a given kind of residue within a transmembrane segment affects its ability to promote membrane insertion.

12. Crystal Structure of a Phosphoinositide Phosphatase, MTMR2: Insights into Myotubular Myopathy and Charcot-Marie-Tooth Syndrome BEGLEY MJ, TAYLOR GS, KIM SA, VEINE DM, STUCKEY JA, and DIXON JE, *Department of Pharmacology, University of California, San Diego School of Medicine, School, La Jolla, CA 92093-0602*

Myotubularin-related proteins are a large subfamily of protein tyrosine phosphatases (PTPs) that dephosphorylate D3-phosphorylated inositol lipids. Mutations in members of the myotubularin family cause the human neuromuscular disorders myotubular myopathy and type 4B Charcot-Marie-Tooth syndrome. The crystal structure of a representative member of this family, MTMR2, reveals a phosphatase domain that is structurally unique among PTPs. A series of mutants are described that exhibit altered enzymatic activity and provide insight into the specificity of myotubularin phosphatases toward phosphoinositide substrates. The structure also reveals that the GRAM domain, found in myotubularin family phosphatases and predicted to occur in ~180 proteins, is part of a larger motif with a pleckstrin homology (PH) domain fold. Finally, the MTMR2 structure will serve as a model for other members of the myotubularin family and provide a framework for understanding the mechanism whereby mutations in these proteins lead to disease.

Poster Abstracts

13. Unique Spatial Localization of Phosphatidylinositol 3-Kinase in Response to Insulin-like Growth Factor Signaling JI LUO,[1] SETH J. FIELD,[1,2] JENNIFER Y. LEE,[1] and LEWIS C. CANTLEY,[1] [1]*Department of Systems Biology, Harvard Medical School and Division of Signal Transduction, Beth Israel Deaconess Medical Center, Boston, MA*; [2]*Division of Endocrinology, Massachusetts General Hospital, Boston, MA*

Phosphatidylinositol 3-kinase (PI 3-kinase) is an important signaling molecule downstream of many growth factor receptor tyrosine kinases that regulates cell survival, proliferation, growth, and migration. In this study we demonstrate, using EGFP-tagged p85α regulatory subunit of PI 3-Kinase (EGFP-p85α) as a reporter, the unique spatial recruitment of PI 3-kinase to discrete foci in response to IGF-1 but not to PDGF receptor activation. These foci also contained tyrosine-phosphorylated adaptor molecule IRS1 and their formation was dependent on the Src homology-2 domains of p85α. However, the EGFP-p85α foci were not sites of phosphatidylinositol-3,4,5-triphosphate production as they did not colocalize with Akt-PH domain reporter after receptor activation, and Akt activation preceded the formation of the EGFP-p85α foci. Ultrastructural studies revealed that the EGFP-p85α foci localized to the cytoplasm and were protein complexes rather than vesicles. We therefore propose that these novel foci may serve to down-regulate PI 3-kinase signaling. (Supported by Howard Hughes Medical Institute Predoctoral Fellowship Award to Jennifer Lee and Physician-Postdoctoral Fellowship Award to Seth J. Field, and by NIH Grant GM41890 to Lewis C. Cantley.)

14. Neuronal Activity Triggers Reversible Plasma Membrane Dissociation and Golgi Targeting of K-Ras via a Ca^{2+}/CaM–regulated Prenyl/Electrostatic Switch Mechanism MARC FIVAZ and TOBIAS MEYER, *Stanford University, School of Medecine. Department of Molecular Pharmacology, Clark Center, Stanford, CA 94305-5439*

The Ras/MAPK pathway is a key regulator of synaptic plasticity and cell survival in CNS neurons. Mammalian genomes encode three ubiquituous isoforms of the small GTPase Ras (H-Ras, N-Ras, and K-Ras), which essentially differ in their COOH-terminal lipid-based plasma membrane–targeting motif. Little is known however about isoform-specific functions of Ras in CNS neurons. Here, we found that synaptic activity triggers rapid translocation of K-Ras CFP from the plasma membrane to the perinuclear Golgi complex and peripheral dendritic vesicles ($t_{1/2}$: 6 min). This translocation process is dictated by the COOH-terminal membrane-interacting motif of K-Ras. CFP fused to the hypervariable region (HV) of K-Ras undergoes glutamate-induced translocation to intracellular membranes with kinetics similar to that of full-length K-Ras. Interestingly, neither full-length H-Ras CFP, nor H-Ras–tail CFP undergo glutamate-induced translocation. Glutamate-induced translocation of K-Ras tail CFP is reversible, Ca^{2+} dependent, and can be blocked by a NMDA-R antagonist, indicating that Ca^{2+} entry through the NMDA-R channel initiates the translocation process. We further showed that this isoform-selective redistribution of K-Ras correlates with Ca^{2+}-dependant binding of CaM to the COOH-terminal HV region of K-Ras. We propose a mechanism whereby the membrane-interacting motif of K-Ras acts as a Ca^{2+}/CaM–dependent switch that regulates plasma membrane levels of K-Ras. To determine whether translocation of K-Ras modulates its signaling activity, we made use of the Ras binding domain of Raf, fused to YFP (RBD-YFP), to monitor K-Ras CFP activation in living neurons. Our preliminary results indicate that glutamate leads to a significant but transient translocation of RBD-YFP to the plasma membrane, followed by a brief recruitment of RBD-YFP to perinuclear membranes, which parallels glutamate-induced K-Ras

translocation. Our data thus suggest that glutamate-induced translocation of K-Ras transiently relocalizes its activity to perinuclear membranes.

15. Identification of a Phosphatidylinositol 4-phosphate 5-kinase Mediating Ca^{2+} Signaling YING-JIE WANG,[1] JING WANG,[1] KE XU,[2] WEN-HONG LI,[2] HELEN L. YIN,[1] [1]*Department of Physiology, and* [2]*Department of Cell Biology, University of Texas Southwestern Medical Center at Dallas, TX 75390*

Phosphatidylinositol 4,5-bisphosphate (PIP_2) regulates multiple plasma membrane functions. In addition, it is a critical contributor to agonist-sensitive Ca^{2+} signaling because it is cleaved by phospholipase C to generate $InsP_3$, which releases Ca^{2+} from intracellular stores. A previous study has suggested that the agonist-sensitive PIP_2 pool is distinct from the general PIP_2 pool, but the question of how these PIP_2 pools are generated is not known. One possibility is that the three known isoforms (named α, β and γ) of the type I phosphatidylinositol 4-phosphate 5-kinase (PIP5KI), which all synthesize PIP_2, may have unique functions and/or localization. Evidence for this is beginning to emerge, including a role for PIP5KIβ in the receptor-mediated endocytosis of 5 in focal adhesion formation. However, PIP5KIγ also exists as a shorter 87-kD form (PIP5KIγS), which lacks the COOH-terminal extension required for focal adhesion formation. Quantitative PCR studies showed that HeLa cells have 10-fold more PIP5KIγS than PIP5KIγL. We therefore sought, using RNA interference (RNAi), to identify the roles of PIP5KγS. HeLa cells were transfected with siRNA directed against a sequence common to both PIP5KIγL and S forms (L+S) or that in PIP5KIγL only. We found that HeLa cells that were transfected with siRNA(L+S) had a 15% decrease in PIP_2 mass but a >60% inhibition of histamine or UTP elicited Ca^{2+} transients. RNAi of PIP5KIγL did not decrease Ca^{2+} signaling, establishing that the siRNA(L+S) induced inhibition of Ca^{2+} signaling is due exclusively to PIP5KIγS knockdown. Significantly, PIP5KIα or β knockdown also does not perturb Ca^{2+} transients to a similar extent. These results establish that PIP5KIγS is a major contributor to the agonist-sensitive PIP_2 pool. (Supported by NIH R01 GM51112, NIH Burn Center Grant GM21681, and a Career Development Award from the Leukemia and Lymphoma Society.)

16. Mobilities of Phospholipids in the Inside and Outside Leaflets of Cell Membranes ALP YARADANAKU, MANUEL MARTINEZ, HELEN YIN, and DONALD W. HILGEMANN, *Department of Physiology, University of Texas Southwestern at Dallas, Dallas, TX*

Many hypotheses about lipid signaling rely on assumptions about localized diffusion of phospholipids in cell membranes. To address this issue for PIP_2, we analyzed the lateral diffusion of NBD-PC and NBD-PIP_2 by the FRAP technique in membrane "lawns" prepared from BHK cells. Plasma membrane lawns (20×30 μm) were prepared on coverslips by a sonication technique and were incubated in a KCl solution with lipid phosphatase inhibitors. On the basis of the following results, we suggest that the lipid probes accumulate preferentially in the cytoplasmic membrane leaflet in this model: (1) Multiple membrane probes label giant membrane patches more strongly from the cytoplasmic side than from the extracellular side. (2) Hydrophobic anions that rapidly translocate across the membrane (e.g., dipicrylamine) locate to >80% on the cytoplasmic side of membrane patches at 0 mV. (3) Hydrophobic cations (e.g., tetraphenylphosphonium) generate much larger currents from the cytoplasmic than from the extracellular side. (4) The same NBD-phospholipids label intact cells much less well than the membrane lawns. Diffusion constants for NBD-PC and NBD-PIP_2 were similar in the membrane lawns (∼10–8 cm^2/s), being close to those expected for pure lipid bilayers. Both probes diffused ∼5 times slower on the surface of intact cells. The diffusion constants were unaffected by several interventions that disrupt actin cytoskeleton. Thus, for the "membrane lawn" model our results to date do not support the existence of restricted lipid diffusion, significant "buffering" of PIP_2 on the cytoplasmic side, or cytoskeleton control of lipid diffusion. The extracellular membrane leaflet tends to repel hydrophobic compounds, and the reduced diffusion constant of lipids on the extracellular side may reflect this same principle. (Supported by NIH HL0679420 and HL051323 to Donald W. Hilgemann.)

17. Phosphoinositide Turnover, PIP_2, and KCNQ2/3 Channel Modulation via M_1 Muscarinic Receptors BYUNG-CHANG SUH, LISA HOROWITZ, WIEBKE HIRDES, and BERTIL HILLE, *Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle, WA 98195*

The voltage-sensitive and K^+-selective M-current is suppressed through membrane phosphoinositide (PI) signaling. Our previous studies showed that PLC is required for channel suppression, and wortmannin (>10 μM)-sensitive lipid kinases are needed for recovery from suppression. Here we compare membrane PI turnover with M_1 muscarinic modulation of KCNQ2/3

channels in tsA cells. PI metabolism is monitored by confocal microscopy using two GFP-based indicators: PH $_{PLC\delta1}$ for PIP_2 and IP_3; and $C1_{PKC}$ for diacylglycerols (DAG). (1) KCNQ current is suppressed by the muscarinic agonist oxotremorine-M (oxo-M) in 15–20 s and recovery takes 100–200 s. In parallel, PH $_{PLC\delta1}$ translocated from membrane to cytosol and $C1_{PKC}$ from cytosol to membrane with agonist addition, and they returned after agonist washout. The translocation time courses were like that for current suppression. (2) DAG kinase inhibitors R59022 or R59949 did not affect oxo-M–induced translocation of the two probes but slowed recovery of $C1_{PKC}$ selectively. These inhibitors had little effect on the oxo-M-mediated channel modulation. Application of DAG analogs OAG or DOG translocated cytosolic $C1_{PKC}$ to the membrane without affecting the muscarinic modulation of current. (3) We then overexpressed IP_3-5′ phosphatase, which speeds degradation of IP_3. This blocked the increase of intracellular Ca^{2+} upon receptor activation, blocked the translocation of PH $_{PLC\delta1}$ from membrane to cytosol, slightly slowed KCNQ2/3 channel inhibition, and had little effect on the $C1_{PLC}$ translocation. Evidently signals downstream of IP_3 are not required. (4) KCNQ channel recovery was prevented by removal of intracellular Mg^{2+} or addition of hydrolysis-resistant ATP analogs, AMP-PNP, AMP-PCP, or TNP-ATP. ATPγS could not substitute for ATP in the recovery, but Mn^{2+} could replace intracellular Mg^{2+}. Wortmannin (30 μM) and phenylarsine oxide (30 μM) inhibited recovery of $PH_{PLC\delta1}$ and KCNQ current with little effect on $C1_{PKC}$. Our observations fit with PIP_2 being the key regulator of KCNQ channels. (Supported by NIH grant NS08174.)

18. Studying the Regulation of M-channels by Phosphatidylinositol-4,5-bisphosphate (PIP_2) Using Lipidated Peptides That Mimic the PIP_2 Binding Site of KCNQ2 JON ROBBINS, STEVE J. MARSH, DAVID A. BROWN, *Department of Pharmacology, University College London, London WC1E 6BT, UK*

It is difficult to study the intra-membrane modulation of ion channels in intact cells in a dynamic fashion. Here we have developed a method using membrane-targeted lipidated peptides (Covic et al. 2002. *Nat. Medicine.* 8:1161–1165) that can be applied extracellularly, and in some cases reversibly, to address this problem. An NH_2-terminal palmitoylated decapeptide (H-palmitoyl-HRQKHFEKRR-$CONH_2$) based on the amino acid sequence for the putative PIP_2 binding domain of KCNQ2-5 (Zhang et al. 2003. *Neuron.* 37:963–975) was constructed using solid phase synthesis. M-currents were recorded from cultured rat superior cervical ganglion cells (SCGs) using the perforated patch technique. The PIP_2 pal-peptide inhibited M-current in SCGs (IC_{50} 1.49 ± 0.22 μM (n = 7) and KCNQ2+3 current expressed in Chinese hamster ovary cells (IC_{50} 1.17 μM, n = 3–8). The peptide was reversible at submicromolar concentrations. It was ineffective on the A-current and the delayed rectifier. Furthermore, at submicromolar concentrations, it increased the sensitivity of M-current to muscarinic receptor mediated inhibition without significantly altering sensitivity to that of bradykinin receptors. The nonpalmitoylated version of the PIP_2 peptide was ineffective up to 10 μM (n = 6).

Changing the NH_2-terminal fatty acid from palmitic acid (C16:0) to its unsaturated equivalent, palmitoleic acid (C16:1), significantly ($P < 0.05$) reduced its potency to 4.00 ± 0.82 μM (n = 6), but improved reversibility. Increasing the carbon chain length by using the saturated lignoceric acid (C24:0) removed activity completely (no effect up to 10 μM, n = 6). Decalysine (KKKKKKKKKK-$CONH_2$) had no effect on the M-current up to 10 μM (n = 5); however, palmitoylated decalysine was active, (IC_{50} 0.80 ± 0.24 μM; n = 5), but not reversible.

These results are consistent with the view (Su and Hille. 2002. *Neuron.* 35:507–520) that PIP_2 is a major regulator of the M-current and suggest that lipidated peptides may be useful tools for investigating phospholipid–ion channel interactions. (Supported by the UK Medical Research Council.)

19. Phosphatidylinositol-4′5′-bisphosphate (PIP_2) and the Regulation of the Neuronal M-current STEVE MARSH, SIMON HUGHES, JOANNA WINKS, and DAVID BROWN, *Department of Pharmacology, University College London, Gower Street, London WC1E 6BT, UK*

Evidence is accumulating that the M-current, a G-protein receptor inhibitable potassium conductance, is regulated by membrane PIP_2 (Suh and Hille. 2002. *Neuron.* 35:507–520; Zhang et al. 2003. *Neuron.* 37:963–975). We have examined the relation between membrane PIP_2 and M-current inhibition using cultured rat sympathetic ganglion (SCG) neurons by recording simultaneously the membrane-to-cytosol translocation of the fluorescent GFP-tagged PH-domain of PLCδ (GFP-PLCδ-PH) as an indicator of PIP_2 levels and hydrolysis (Varnai and Balla. 1998. *J. Cell Biol.* 143:501–510) and M-current amplitude (using perforated-patch electrodes) after stimulation of muscarinic acetylcholine receptors.

Oxotremorine-M (a muscarinic receptor agonist) produced a reversible translocation of GFP-PLCδ-PH

and concomitant decrease in the amplitude of the M-current, with statistically indistinguishable IC_{50} values (0.65 and 0.68 μM, respectively).

Using whole-cell recording we estimated membrane PIP_2 levels ($[PIP_2]_{Mem}$) by measuring the ability of intracellular IP_3 to induce GFP-PLCδ-PH translocation by competitive binding. Based on previously published affinities of GFP-PLCδ-PH for IP_3 (0.13 μM) and PIP_2 (2.1 μM) (Lemmon and Ferguson. 2000. *Biochem. J.* 350:1–18), $[PIP_2]_{Mem}$ was calculated at ~300 μM. We then raised $[PIP_2]_{mem}$ to an estimated value of ~800 μM by overexpressing the PIP_2-synthesizing enzyme phosphatidylinositol-4-phosphate-5-kinase (PI5-kinase, PI4P-5K; Ishihara et al. 1996. *J. Biol. Chem.* 272:23611–23614). This reduced the M-current inhibition produced by oxotremorine-M (10 μM) from 65.9 ± 3.2% (n = 12) to 14.5 ± 5.4% (n = 13), and also reduced oxotremorine-induced translocation of GFP-PLCδ-PH. These results appear to confirm that membrane levels of PIP_2 and M-current amplitude are closely linked. (Supported by the MRC and the Physiological Society.)

20. PIP_2 Inhibition of a Prokaryotic Kir Channel I. JELIAZKOVA, D. ENKVETCHAKUL, and C.G. NICHOLS, *Department of Cell Biology and Physiology, Washington University, St. Louis, MO*

A prokaryotic family of inwardly rectifying K^+ channels was identified 3 yr ago (Durell. 2001. *BMC Evol. Biol.* 1:1–14) and one member, KirBac1.1, was recently crystallized (Kuo el al. 2003. *Science.* 300:1922–1926). However, nothing is yet known about the function of this protein, even whether it functions as a channel.

We cloned KirBac1.1 from *B. pseudomallei* genomic DNA, in a vector suitable for protein expression in a prokaryotic system, purified 6-histidine tagged KirBac1.1 and reconstituted it in liposomes.

We used an ^{86}Rb flux assay to characterize channel activity in the liposomes (75% POPE, 25% POPG). In such a system, it is possible to directly examine the role of membrane lipid composition on channel function. Interestingly, and in apparent contrast to all known eukaryotic inwardly rectifying K^+ channels, KirBac1.1 is inhibited by phosphatidylinositol-4,5-bisphosphate (PIP_2) and phosphatidylinositol-4-phosphate (PIP) when incorporated at low levels into the liposomes. At PIP_2 0.1% of total lipid (m/m), PIP_2 inhibits ^{86}Rb uptake by ~60%, PIP inhibits uptake by ~30%, and there is no significant inhibition by phosphatidylinositol (PI). At up to 3% of total lipid, PIP_2 was without effect on KcsA, obviating nonspecific effects. To further investigate the nature of the PIP_2–KirBac1.1 channel interaction we examined the effect of diacylglycerol (DAG) (PLC hydrolysis mediated derivative of PIP_2) and polylysine (known to screen PIP_2 effect on eukaryotic Kir channels) on KirBac1.1. At up to 1% total lipid, DAG was without effect, and exogenous application of 100 μg/ml polylysine reversed the PIP_2 inhibition of uptake. The data suggest that, as with the eukaryotic inwardly rectifying channels, KirBac1.1 interactions with PIP_2 are electrostatic in nature. Elucidating the role of KirBac1.1 interactions with phospholipids in the membrane, in this recombinant system, will help to understand the molecular nature of PIP_2 activation of eukaryotic channels.

21. Functional Characterization of a Prokaryotic Inward Rectifying Potassium Channel DECHA ENKVETCHAKUL,[1,2] JAYA BATTACHARYYA,[1] IANA JELIAZKOVA,[1] DARCY GROESBECK,[1] and COLIN G. NICHOLS,[1] [1]*Renal Department, and* [2]*Department of Cell Biology and Physiology, Washington University, Saint Louis, MO*

Inward rectifying potassium (Kir) channels are widespread and critical regulators of cell excitability in eukaryotic cells. A related gene family of putative inwardly rectifying potassium channels (KirBac) was found in prokaryotes (Durell and Guy. 2001. *BMC Evol Biol.* 1:14). Despite having the crystal structure of KirBac1.1 at atomic level resolution (Kuo et al. 2003. *Science.* 300: 1922), there have been no published functional studies of any of the KirBac gene products. Here we present functional characterization of KirBac1.1 reconstituted in liposomes. KirBac1.1 was cloned from *B. pseudomallei* genomic DNA (Bhattacharyya et al. 2002. *Biophys. J.* 84: 78a), expressed in *E. coli* as a COOH-terminal 6-histidine–tagged protein, and affinity purified on a cobalt column. Using an $^{86}Rb^+$ uptake assay, we demonstrate that purified KirBac1.1 protein reconstituted in liposomes (3:1 ratio of POPE:POPG) generates a cation permeation pathway, strongly selective for K^+, Rb^+, and Cs^+, over Na^+, Li^+, and NMG^+, and blockable by extraliposomal Ba^{2+} at submillimolar concentrations. Sensitivity to block by polyamines in KirBac1.1 is weaker than in KcsA, suggesting that KirBac1.1 is not physiologically inward rectifying. KirBac1.1 is fully active at pH > 7 and is inhibited by acidic conditions, in sharp contrast to the activation of the well-characterized prokaryotic K+ channel KcsA by low pH. Similar acid sensitivity of eukaryotic Kirs (Jiang et al. 2002. *Trends Card Med.* 1:5) suggests conservation of function of the structurally conserved cytoplasmic domain that is unique to Kir channels. In contrast to the full–length KirBac1.1, purified COOH-terminal trun-

cated KirBac1.1Δ12] protein, similar to that used in the putatively closed crystal structure (Kuo et al. 2003. *Science.* 300:1922), was nonfunctional in liposomes. Attempts at crystallizing full-length KirBac1.1 are ongoing in hopes of providing insight into the structural mechanism of channel opening and closing, and to date have yielded poorly diffracting crystals. (Supported by NIH grant DK60086.)

22. Does M_1 Muscarinic Stimulation of Protein Kinase C Minimize Ca^{2+} Current Inhibition by the Membrane Pathway? JOHN F. HENEGHAN and ANN R. RITTENHOUSE, *Department of Physiology & Program in Neuroscience, University of Massachusetts Medical School, Worcester, MA*

During transmitter activation of the membrane-delimited pathway, $G_{\beta\gamma}$ subunits from G_i or G_o G-proteins bind directly to N-type Ca^{2+} channels to inhibit their activity. Phorbol ester activation of protein kinase C (PKC) blocks N-type Ca^{2+} current (N-current) inhibition by the membrane-delimited pathway (Swartz. 1993 *Neuron.* 11:305–320). Comparable effects using neurotransmitters have not been demonstrated. Therefore, we sought to find conditions in which a receptor agonist stimulates PKC, minimizing membrane-delimited inhibition of N-current. Receptors such as M_1 muscarinic receptors (M_1Rs) activate PKC via phospholipase C (PLC); however, PLC activation has multiple downstream effects that may obscure N-current modulation by PKC. Specifically, activation of PLC induces a slow pathway, which inhibits N-current. We reasoned that if we blocked the slow pathway during PLC stimulation by the M_1R agonist oxotremorine-M (Oxo-M), we could reveal PKC modulation of channel activity. To do this, we recorded from superior cervical ganglion neurons whole cell Ba^{2+} currents with low BAPTA (0.1 mM) in the pipette solution and the M_2R antagonist methoctramine in the bath to minimize muscarinic stimulation of the membrane-delimited pathway. Under these conditions, Oxo-M inhibited current by the slow pathway. This inhibition could be antagonized when either the diacylglycerol lipase inhibitor RHC-80267 or the phospholipase A_2 inhibitor oleyloxyethyl phosphocholine was present in the bath. If M_1Rs stimulate PKC, its activity should be apparent under these conditions. To test this possibility we determined whether N-current inhibition by the membrane-delimited pathway was altered while M_1Rs were activated. Norepinephrine stimulation of the membrane-delimited pathway normally elicits a robust current inhibition (52 ± 4.8%, $n = 3$). In contrast, when Oxo-M was also present only minimal norepinephrine-induced current inhibition was observed (7.8 ± 12%, $n = 3$). These preliminary findings suggest that M_1Rs may activate PKC, which then antagonizes N-current inhibition by the membrane-delimited pathway. (Supported by NIH grant RO1-NS34195.)

23. PIP_2 Depletion Contributes to $G_{q/11}$-mediated Muscarinic Modulation of N-type Ca^{2+} Channels NIKITA GAMPER,[1] VITALIY REZNIKOV,[1] YOICHI YAMADA,[2] JIAN YANG,[2] and MARK S. SHAPIRO,[1] [1]*Department of Physiology, University of Texas Health Science Center at San Antonio, San Antonio, TX;* [2]*Department of Biological Sciences, Columbia University, New York, NY*

Muscarinic acetylcholine receptors (mAchR) inhibit N-type ($Ca_v2.2$) Ca^{2+} channels via two mechanisms: one fast (<1 s), involving a direct action of pertussis-toxin (PTX)-sensitive G proteins on the channels, and the other slower (>10 s), involving PTX-insensitive $G_{q/11}$ G proteins and intracellular second messengers. As activation of $G_{q/11}$ hydrolyzes $PI(4,5)P_2$ (PIP_2) via PLC, we investigated whether the slower pathway involves depletion of PIP_2, and if N-type channels are sensitive to PIP_2 levels. PIP_2 applied to the cytoplasmic face of excised inside-out macropatches pulled from *Xenopus* oocytes expressing cloned $Ca_v2.2$ channels slowed channel run-down. We then studied PTX-treated superior cervical ganglion (SCG) neurons. Using perforated-patch recordings, the PI-3 and PI-4 kinase inhibitor wortmannin strongly blunted recovery of I_{Ca} from muscarinic modulation, but the PI3-kinase inhibitor LY294002 had no effect. If depletion of PIP_2 is necessary for the modulation, then intracellular dialysis of PIP_2 might block inhibition. Indeed, diC8-PIP_2 included in whole-cell pipettes blunted and slowed muscarinic modulation of I_{Ca} and slowed run-down. We tested the effects of three phosphoinositide sequestering/depleting constructs exogenously expressed in SCG cells using the "gene gun". The PIP_2 binding/sequestering construct EGFP-PLCδ-PH modestly reduced tonic I_{Ca} amplitudes (16 ± 2 vs. 24 ± 2 pA/pF in control, $P \leq 0.05$), and moderately attenuated muscarinic modulation (25 ± 6% vs. 57 ± 5% in control, $P \leq 0.001$). The PIP_2-depleting construct EGFP-Lyn-PH-PP (containing a PIP_2 5′-phosphatase) strongly reduced tonic I_{Ca} amplitudes (9 ± 1 pA/pF, $P \leq 0.001$), but caused only modest reduction in percent modulation of I_{Ca} (39 ± 4%, $P \leq 0.05$). Finally, EGFP-PLCδ-PH can be used as an optical reporter of PIP_2 hydrolysis since it also binds to IP_3 and exhibits translocation from the membrane to the cytosolic compartment upon PLC activation. In SCG neurons expressing EGFP-PLCδ-PH, both muscarinic and

bradykinin stimulation caused translocation. We conclude that N-type Ca^{2+} channels are sensitive to [PIP_2], and its depletion contributes to slow muscarinic inhibition, but other concurrent signals may be involved. Supported by NIH, AHA-Texas, and the EJLB Foundation.

24. Does enh Facilitate Protein Kinase C Modulation of N-type Calcium Current in Sympathetic Neurons? LEE F. STANISH, JOHN F. HENEGHAN, and ANN R. RITTENHOUSE, *Department of Physiology, University of Massachusetts Medical School, Worcester, MA*

Phorbol esters such as PMA activate protein kinase C (PKC), resulting in the block of N-type calcium (Ca) current inhibition via the membrane-delimited pathway. The site of convergence appears to occur at the I-II linker of the pore-forming subunit of the channel with phosphorylation antagonizing G-protein binding. The particular PKC isoform that modulates Ca current in superior cervical ganglion (SCG) neurons has not yet been identified. Maeno-Hikicki et al. (2003. *Nature Neurosci.* 6:468–475) demonstrated that the protein enigma homologue (enh) allows for specific anchoring of PKCε to the carboxy-terminal tail of recombinant N-channels expressed in oocytes. The resulting channel complex augmented PKC modulation of Ca current, whereas inhibiting complex formation minimized modulation. Thus, PKCε may be the primary PKC that mediates N-channel modulation. To determine whether this mechanism of N-channel modulation occurs in SCG neurons, we established which PKC isoforms are present in acutely dissociated neonatal SCG by Western blot analysis. At least 11 PKC isoforms are known and vary in their sensitivities to Ca and phorbol esters. The classical PKCs α, βI, βII, and γ are both Ca and phorbol ester sensitive, while the novel PKCs δ, ε, θ, and η are only phorbol ester sensitive and the atypical PKC's ζ and ι/λ are insensitive to either Ca or phorbol esters. Our results are consistent with those found in cultured SCG neurons, confirming the presence of PKC α, βI, βII, δ, ε, and ζ (Scholze et al. 2002. *J. Neurosci.* 22:5823–5832). We then established the presence of enh transcripts using RT-PCR. These preliminary findings indicate that both PKCε and enh are present in the SCG. We are currently testing their role in PKC modulation of N-current in SCG neurons. (Supported by NIH grant RO1-NS34195.)

25. Phospholipase A_2 Mediates M_1 Muscarinic Inhibition of L-type Calcium Current in Sympathetic Neurons LIWANG LIU, LEE F. STANISH, RUBING ZHAO, and ANN R. RITTENHOUSE, *Department of Physiology, University of Massachusetts Medical School, Worcester, MA*

Calcium (Ca) currents and the M-type potassium current are modulated by an incompletely characterized diffusible second messenger pathway called the slow pathway. Great enthusiasm has greeted a recent hypothesis that PLC metabolism of phosphatidylinositol 4,5 bis-phosphate (PIP_2), constitutively bound to channels, dissociates causing current inhibition; no further downstream processing of PIP_2 is required (Suh and Hille. 2002. *Neuron* 35:507–520; Wu et al. 2002. *Nature.* 419:947–952). In contrast, our data indicate that additional processing of PIP_2 is required for Ca current modulation. In addition to M_1 muscarinic receptors (M_1Rs), G_q and phospholipase C (PLC), we found phospholipase A_2 (PLA_2) and a free fatty acid, most likely arachidonic acid (AA), appear to participate in L- and N-type Ca current inhibition (SGP abstract Liu, Roberts, and Rittenhouse). Demonstrating that PLA_2 is required for Ca current inhibition by the slow pathway is critical for resolving the differences, if any, between these two models. Here, we use patch-clamp and molecular methods to determine which PLA_2 participates in L-current inhibition by the slow pathway in neonatal rat SCG neurons. Antibodies (Abs) selective for different PLA_2s were dialyzed into neurons treated with ω-conotoxin-GVIA to block N-current. Type IV ($cPLA_2$) or type VI ($iPLA_2$) Abs, but not nonimmunized IgG or type IIa PLA_2 ($sPLA_2$) Abs, minimized current inhibition by the muscarinic agonist oxotremorine-M (Oxo-M). Inhibition of FPL-64176 induced long-lasting L-type tail currents, normally observed with Oxo-M, was also minimized when the $cPLA_2$ Ab was dialyzed into cells. Western blot analysis revealed that the $cPLA_2$ Ab recognized a single band at the approximate molecular weight of $cPLA_2$. PCR confirmed $cPLA_2$'s presence in SCG consistent with Western blot analysis. These data identify $cPLA_2$ as a necessary participant in M_1R inhibition of L-current. We are currently probing the role of $cPLA_2$ further using gene knockout technology. (Supported by NIH RO1-NS 34195.)

26. Nonchiral Effects of a Peptide Inhibitor of Mechanosensitive Channels: Evidence for a Bilayer-Dependent Mechanism SONYA E. TAPE,[1] THOMAS M. SUCHYNA,[2] ROGER E. KOEPPE II,[3] OLAF S. ANDERSEN,[1] FREDERICK SACHS,[2] and PHILIP A. GOTTLIEB,[2] [1]*Department of Physiology and Biophysics, Weill Medical College of Cornell University, New York, NY 10021;* [2]*Department of Physiology and Biophysics, SUNY at Buffalo,*

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Neuroactive peptides derived from venom generally are presumed to act as lock and key ligands for the targeted ion channels. We show that the peptide GsMTx4 (Suchyna, T.M., J.H. Johnson, K. Hamer, J.F. Leykam, D.A. Gage, H.F. Clemo, C.M. Baumgarten, and F. Sachs. 2000. *J. Gen. Physiol.* 115:583–98) modifies the gating of mechanosensitive, stretch-activated channels (SACs) and gramicidin A (gA) channels in a manner that does not conform to the lock and key model. Rather, GsMTx4 alters channel kinetics by perturbing the lipid-packing adjacent to the channel, as shown by two lines of evidence. First, GsMTx4 is equally effective on left- and right-handed gA channels, which have proven useful as probes of bilayer-protein interactions (Andersen, O.S., C. Nielsen, A.M. Maer, J.A. Lundbæk, M. Goulian, and R.E. Koeppe II. 1999. *Meth. Enzymol.* 294:208–224), and the effect increases with increasing channel-bilayer mismatch. Second, the enantiomeric GsMTx4 (*en*GsMTx4, composed of D amino acids) inhibits SACs, and modifies gA channels as effectively as GsMTx4. Specific binding interactions therefore are not necessary for GsMTx4 activity, but GsMTx4 is close to the channels because, as expected for the adsorption of a pentavalent peptide, it decreases inward SAC currents with no effect on outward currents. It likewise decreases gA single-channel currents. The results suggest a new, bilayer-dependent, mechanism for membrane protein modulation by amphipathic peptides mechanopharmacology. The ability to use D peptides moreover has therapeutic potential, because D peptides are not hydrolyzed by endogenous proteases. (Supported by NIH grants to Olaf S. Andersen, Roger E. Koeppe, and Frederick Sachs.)

27. Capsaicin Modulation of Gramicidin and Voltage-dependent Sodium Channel Function: Going Beyond Monolayer Curvature JENS A. LUNDBÆK,[1,2,3] PIA BIRN,[2] SONYA E. TAPE,[1] GIL TOOMBES,[4] RIKKE SØGAARD,[3] CLAUS NIELSEN,[5] SOL M. GRUNER,[4] ANKER J. HANSEN,[2] and OLAF S. ANDERSEN,[1] [1]*Department of Physiology and Biophysics, Weill Medical College, Cornell University, New York, NY;* [2]*Novo Nordisk, A/S, Måløv, Denmark;* [3]*Department of Biological Psychiatry, St. Hans Hospital, Roskilde, Denmark;* [4]*Department of Physics, Cornell University, Ithaca, NY;* [5]*QUP, The Technical University of Denmark, Lyngby, Denmark*

Capsaicin is among the many amphiphiles that modulate membrane protein function at two widely different concentration ranges. At pM to nM concentrations they regulate one (or a few) protein(s), by interacting with a specific receptor. At μM concentrations they regulate a large number of seemingly unrelated proteins in a manner that remains poorly understood, but often is ascribed to changes in lipid bilayer physical properties. We explored this possibility in combined measurements on voltage-dependent sodium channels (VDSCs) and gramicidin (gA) channels.

The hydrophobic coupling between a membrane protein's transmembrane domain and the host bilayer means that protein conformational changes may involve an elastic deformation of the adjacent bilayer. If so, protein function will be regulated by the bilayer elastic properties (BEP) (thickness, monolayer curvature, compression, and bending moduli). Changes in BEP can be evaluated using gA channels because a hydrophobic mismatch between bilayer thickness and channel length means that channel formation involves an elastic bilayer deformation. Changes in "bilayer elasticity" therefore will be reflected in changes in gA channel appearance rate and lifetime. Capsaicin and its antagonist capsazepine (at 10–30 μM) promote VDSC inactivation (known to be promoted by increases in bilayer elasticity) and increase gA appearance rate and lifetime. The quantitative correlation between the shift in inactivation and changes in gA lifetime is similar to that previously observed for other (micelle-forming) amphiphiles. The effects of capsaicin on VDSC or gA channels cannot be explained simply by changes in monolayer curvature. Whereas capsaicin has an intrinsic negative curvature, micelle-forming amphiphile have a positive curvature—but both compounds promote inactivation of VDSC. We conclude that capsaicin can modulate membrane protein function by changing the BEP. The effects on inactivation of VDSC were predicted by the changes in bilayer elasticity, measured using gA channels—but not by other measures of bilayer properties.

28. Modification of Gramicidin Channel Function by Polyunsaturated Fatty Acids MICHAEL J. BRUNO,[1] ROGER E. KOEPPE II,[2] and OLAF S. ANDERSEN,[1] [1]*Department of Physiology, Biophysics and Systems Biology, Weill Medical College of Cornell University;* [2]*Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701*

Polyunsaturated fatty acids (PUFAs) at low micromolar concentrations alter (activate or inactivate) the function of many different membrane proteins—including various types of ion channels. Whereas PUFAs might act by binding directly to the membrane protein in question, they exert their effects on structurally and functionally unrelated proteins at remarkably similar

concentrations, which suggests a common mode of action. Given that the lipid bilayer serves as the common "solvent" for all membrane proteins, PUFAs could alter protein function by adsorbing at the solution/bilayer interface, thereby modifying the host bilayer properties and thus protein function. We examined this question using the dimeric channels formed by the 15–amino acid bacterial peptide gramicidin A (gA) to monitor whether bilayer properties are altered by PUFAs. We quantified the changes in channel properties for increasing concentrations (3–30 μM) of DHA, DPA, EPA, AA, OA, and ETYA with gA analogs of 13, 15, and 17 amino acids in $DC_{18:1}PC$ or $DC_{20:1}PC$/*n*-decane bilayers (1.0 M NaCl, pH 7). PUFAs increase the channel lifetimes, meaning that they alter bilayer properties. The changes in channel lifetimes are greater with greater hydrophobic mismatch between channel length and bilayer thickness. PUFAs promote a negative equilibrium monolayer curvature, a change that, according to the conventional model of lipid-protein interactions, would be expected to decrease channel lifetimes. Our results show that the overall bilayer mechanical properties and deformation energy, not simply curvature, are the important determinants of PUFAs' action. (Supported by NIH grants GM21342 and RR15569.)

29. Lipid- and Mechano-sensitivities of Sodium-Hydrogen Exchangers Analyzed by Electrical Methods DANIEL FUSTER,[1,2] ORSON W. MOE,[2] and DONALD W. HILGEMANN,[1] [1]*Departments of Physiology and* [2]*Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX*

$Na^+/H+$ exchangers (NHEs) are ubiquitous ion transporters that serve multiple cell functions. We have used a new method to characterize two mammalian isoforms, NHE1 (ubiquitous) and NHE3 (epithelial-specific), by measuring extracellular proton (H+) gradients during whole-cell patch clamp with perfusion of the cell interior. Maximal Na^+-dependent H+ fluxes (JH+) are equivalent to currents >20 pA for NHE1 in chinese hamster ovary (CHO) fibroblasts, >200 pA for NHE1 in guinea pig ventricular myocytes, and 5–10 pA for NHE3 in opossum kidney (OK) cells. The fluxes are blocked by the NHE inhibitor, ethylisopropylamiloride (EIPA), and are absent in NHE-deficient AP-1 cells. NHE1 activity is stable with perfusion of nonhydrolyzable ATP (AMP-PNP), is abolished by ATP depletion (2 deoxy-D-glucose with oligomycin or perfusion of apyrase), can be restored with PIP2, and is unaffected by actin cytoskeleton disruption (latrunculin or pipette perfusion of gelsolin). NHE3 (but not NHE1) is reversibly activated by PIP3. Both NHE1 and NHE3 activities are disrupted in giant patches during giga-ohm seal formation. NHE1 (but not NHE3) is reversibly activated by cell shrinkage, even at neutral cytoplasmic pH without ATP, and inhibited by cell swelling. NHE1 (but not NHE3) is strongly inhibited from either membrane side by compounds that expand the membrane surface (lyso-phosphatidylcholine (LPC) and β-octylglucoside (OG)), probably resulting in bilayer thinning, but is strongly activated by cholesterol enrichment, which causes bilayer thickening. Thus, NHE1 (but not NHE3) is probably sensitive to hydrophobic mismatch, and rearrangements of lipids at the membrane–transporter interface may underlie its mechano-sensitivity.

30. Computational Studies of the Membrane-mediated Mechanism of the Subcellular Localization of Proteins DIANA MURRAY, *Department of Microbiology and Immunology and the Institute for Computational Biomedicine, Weill Medical College of Cornell, New York, NY*

The reversible binding of proteins to membranes is crucial to many biological processes, such as signal transduction, vesicle trafficking, and viral assembly. Many of these peripheral proteins contain lipid-interacting domains that recruit the proteins to specific intracellular membranes in response to signals, such as an increase in cellular calcium or the production of a phosphoinositide lipid. Our computational research and complementary experimental studies from other labs suggest that the binding of lipid-interacting domains to ligands, such as calcium ions or phosphoinositide head groups, dramatically alters the biophysical properties of the domains and that these changes are responsible for regulating membrane association. Further, it appears that various combinations of two physical factors—electrostatics and hydrophobicity—are major determinants of membrane binding. The finite difference Poisson-Boltzmann (FDPB) method has proved extremely accurate in its ability to account for many of the experimentally determined electrostatic properties of protein/membrane systems. We are using the FDPB method to model the subcellular targeting of proteins to membrane surfaces. Our calculations of the physical forces between atomic-level models of proteins and phospholipid membranes provide insight, at the molecular level, into how different proteins are recruited to specific membranes and how proteins and lipids may be organized at membrane surfaces to facilitate the formation of macromolecular complexes. Specific applications to phosphoinositide signaling and retroviral assembly will be presented. The overall computational approach we are developing provides a comprehensive framework within which to examine how

proteins are designed to effect the wide range of membrane binding behaviors observed. (Supported by NIH grants GM66147 and AI54167, and NSF grant MCB0212362.)

31. Designing a Membrane-spanning Force Transducer HAIYAN SUN,[1,3] SONYA E. TAPE,[2] OLAF S. ANDERSEN,[2] and ROGER E. KOEPPE II,[1] [1]*Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701;* [2]*Department of Physiology and Biophysics, Weill Medical College of Cornell University, New York, NY 10021;* [3]*Department of Physiology, Johns Hopkins University School of Medicine, Baltimore, MD 21205*

Proteins and other "defects" within a lipid bilayer membrane cause the bilayer thickness to vary locally. These bilayer deformations incur an energetic cost that, in principle, could cause membrane proteins to couple to each other at the functional level. For this reason, we have considered methods to measure the bilayer deformation energy and have designed several sets of analogs of gramicidin channel subunits that can serve as molecular force transducers. At least three fundamentally different approaches to measuring the deformation energy can be used, namely, measurements of (i) channel lifetimes as the bilayer thickness is varied, (ii) channel lifetimes as the channel length itself is varied, or (iii) of the ratio of appearance rates for heterodimer channels relative to the corresponding types of homodimer channels when the subunits are of different lengths. We will discuss the relative merits of each approach and report the properties of several types of gramicidin analogs that are suitable for these measurements. For diphytanoylphosphatidylcholine and dioleoylphosphatidylcholine bilayers, we find that the bilayer spring constants are in the range of 30–40 kJ/mol/nm^2. Future improvements to the molecular design of the force transducer subunits will enable us to narrow this range. (Supported by NIH grants GM21342 and RR15569.)

32. Membrane Cholesterol Content Regulates the Mechanosensitivity of a Nonselective Cation Channel in Renal Proximal Tubule JULIE NICOLE, ANDREW R. WARGO, EVAN BISHOP-RIMMER, and ALAN S. SEGAL, *Department of Medicine, University of Vermont, Burlington, VT*

Mechanosensitive or stretch-activated nonselective cation (SA-NSC) channels are thought to play a role in cell volume regulation and may be involved in the response to cellular injury. We have characterized a Ca^{2+}-permeable SA-NSC channel found in ~80% of basolateral membrane (BLM) patches of renal proximal tubule. The channel is ATP sensitive with conductance ~28 pS for Na^+ (Hurwitz et al. 2002. *Am. J. Physiol.* 283: F93–F104). Although the molecular mechanisms by which membrane stress or deformation produce mechanogating have not been elucidated, they probably involve interactions of the channel protein with the cytoskeleton and/or the lipid environment of the plasma membrane. Disruption of the cytoskeleton does not significantly alter behavior of the SA-NSC channel. In contrast, using methyl-β-cyclodextrin (MβCD) to deplete membrane cholesterol in excised inside-out patches produces a rapid and profound increase in channel activity. This effect is reversible upon repletion of patch-membrane cholesterol. Addition of cholesterol to freshly excised patches usually decreases channel activity, suggesting that membrane cholesterol content of the native plasmalemma is not saturated. Modulation of membrane cholesterol does not affect the pore properties (conductance, ATP sensitivity) of the SA-NSC channel, and has no effect on the ATP-sensitive K^+ channel (or any other channel) in the BLM. Surprisingly, inhibition of the channel—by lanthanides (Gd^{3+}, La^{3+}), ruthenium red, or tarantula venom—cannot be overcome by additional negative pressure and renders the channel insensitive to subsequent MβCD exposure. Taken together, these findings suggest that the changes in the lipid bilayer affect channel gating, e.g., stretch and MβCD act as openers by decreasing membrane deformation energy or stiffness on one side of the bilayer, whereas inhibitors indirectly stabilize channel closure by interactions within the membrane. We conclude that the mechanosensitivity of the SA-NSC channel is regulated by membrane cholesterol content, which may be relevant during acute cellular injury.

33. Synthetic Low-molecular Weight Scramblases Induce Facilitated Phospholipid Flip-Flop Across Vesicles and Cell Membranes KRISTY M. DIVITTORIO, TIMOTHY N. LAMBERT, LAKSHMI CHAKKUMKUMARATH, RAMESHWER SHUKLA, and BRADLEY D. SMITH, *Department of Chemistry and Biochemistry, Walther Cancer Research Center, University of Notre Dame, Notre Dame, IN 46556-5670*

The distribution of phospholipids across biological membranes is asymmetric. Maintaining this asymmetry is important for cellular signaling and health. For example, phosphatidylserine (PS) is normally localized in the inner monolayer where it is vital for intracellular fusion processes, such as exocytosis, and for lipid–protein interactions involved in signal transduction pathways.

However, the appearance of PS in the outer monolayer of membranes correlates with blood coagulation and cell clearance through phagocytosis. Recently, we designed a cationic steroidal compound and showed that it promotes the flip-flop of anionic PS across cell membranes (Boon et al. 2003. *J. Am. Chem. Soc.* 125:8195–8201). To further elucidate the flip-flop mechanism of this compound we have performed a mechanistic analysis using vesicle systems with fluorescent probes and PS flop in red blood cells with annexin V-FITC binding. We have also investigated the translocation and binding properties of a series of synthetic PC scramblases in order to develop an efficient PC scramblase. (Supported by NIH grant GM059078.)

34. Partitioning of Kir2.1 Channels into Triton-insoluble Membrane Domains Is Independent of the Level of Cellular Cholesterol VICTOR ROMANENKO,[1] YUN FANG,[1] ALEXANDER TRAVIS,[2] and IRENA LEVITAN,[1] [1]*Institute for Medicine and Engineering, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104;* [2]*Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853*

Our recent study has shown that endothelial Kir channels are regulated by the level of cellular cholesterol and that this effect is maintained when Kir2.x channels are expressed in Chinese hamster ovary cells, a null cell line that has no endogenous Kir2 channels. In this study, we have tested whether Kir2.1 channels partition into cholesterol-rich membrane domains (lipid rafts) and whether changes in the level of cellular cholesterol alter the distribution of the channels between different membrane fractions. Partitioning of the channels into lipid rafts was tested by isolating triton-insoluble membrane fractions and by detergent-free membrane separation on a sucrose gradient. Our results showed that Kir2.1 channels partitioned virtually exclusively into triton-insoluble membrane fractions (.95%). Caveolin, one of the typical lipid raft markers, partitioned into triton-insoluble membrane fractions under the same experimental conditions by 75%. To exclude the possibility of a detergent artifact, we have also tested whether Kir2.1 partitions into low-density membrane fractions separated on a sucrose gradient without exposure to a detergent. The high buoyancy of these factions is considered to be a hallmark feature of lipid rafts membrane fractions. As expected, Kir2.1 clearly appeared in the low-density fractions, however, not as exclusively as in triton-insoluble fractions, indicating that triton-insoluble membrane fractions are heterogenous in their properties. The most surprising observation, however, was the observation that neither depleting cellular cholesterol by methyl-b-cyclodextrin (MbCD), nor enriching the cells with cholesterol by exposing them to MbCD saturated with cholesterol affected the distribution of the channels to triton-insoluble or low-density membrane fractions. These observations suggest that cholesterol sensitivity of Kir2.1 channels is not due to changes in their distribution between raft and nonraft membrane domains but rather is due to altering the lipid environment within the lipid raft domains.

35. Cholesterol Depletion Results in Stiffening of Aortic Endothelial Cells as Determined by Micropipette Aspiration Analysis and Traction Force Microscopy FITZROY J. BYFIELD,[1] HELIM ARANDA-ESPINOZA,[3] VICTOR ROMANENKO,[1] GEORGE ROTHBLAT,[2] DANIEL A. HAMMER,[3] and IRENA LEVITAN,[1] [1]*Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA;* [2]*Lipid Research Group, The Children's Hospital of Philadelphia, Philadelphia, PA 19104;* [3]*Department of Bioengineering, University of Pennsylvania, Philadelphia, PA 19104*

This study investigates the effect of membrane cholesterol on mechanical properties of aortic endothelial cells. We have focused on measuring the stiffness of membrane-cytoskeleton deformation using micropipette aspiration and the strength of cell adhesion to the substrate using traction force microscopy. Cellular cholesterol was manipulated by exposing cells to methyl-β-cyclodextrin (MβCD): cholesterol solutions at increasing molar ratios of MβCD to cholesterol while the MβCD concentration remained constant. Cholesterol levels for each experimental condition were measured using gas liquid chromatography. Aspiration experiments were performed on substrate-attached endothelial cells. Our observations unexpectedly showed that cholesterol depletion resulted in a decrease in membrane deformability, indicating that cells became stiffer. Cholesterol enrichment, however, had no effect on cellular stiffness. Importantly, while cholesterol depletion had no apparent effect on F-actin structure visualized by rhodamine phalloidin, the stiffening effect of cholesterol depletion was abrogated by F-actin depolymerization. Since these experiments were performed on substrate-attached cells, one mechanism for the stiffening effect may be an increase in cellular adhesion. Therefore, these studies were extended to test whether cholesterol depletion affects the strength of cell adhesion by quantifying the deformation of acrylamide gels upon which cells were attached. Gel deformation was quantified by measuring the displacement of beads em-

bedded in the gels. Our results show that cholesterol depletion increased the force that cells exert on a substrate. This observation is consistent with an increase in cell stiffness as earlier studies show that an increase in adhesion strength is correlated with an increase in cell stiffness. Consistent with our observations for cell stiffness, enriching the cells with cholesterol had no effect on the traction forces. We conclude that cholesterol plays an important role in regulating the mechanical properties of actin cytoskeleton and cellular adhesion.

36. What Anchors PLC-ζ to Membranes? A Unique Cluster of Basic Residues in the Catalytic Linker Region May Interact with Acidic Membrane Lipids in Membranes PAYAL PALLAVI,[1] ANTHONY LAI,[2] JIYAO WANG,[3] DIANA MURRAY,[3] and STUART MCLAUGHLIN,[1] [1]*Department of Physiology and Biophysics, SUNY, Stony Brook, Stony Brook, NY;* [2]*University of Wales College of Medicine, Cardiff, UK, Department of Microbiology and Immunology;* [3]*Weill Medical College of Cornell University, NY*

Lai and coworkers have identified a novel PIP_2-specific phospholipase C, PLC-ζ, in sperm (Saunders et al. 2002. *Development.* 129:3533–3544). PLC-ζ shares considerable sequence homology with PLC-δ, but lacks the PH domain that anchors PLC-δ to the membrane. The unstructured region that links the X and Y portions of the catalytic domain in PLC-ζ, however, contains a cluster of basic residues not present in PLC-δ or other PLCs. We hypothesize this basic cluster may anchor PLC-ζ electrostatically to membranes containing acidic lipids. We used a centrifugation assay to measure the molar partition coefficient, K, of a basic peptide that corresponds to this linker region (12 amino acids with 7 basic residues). For 5:1, 3:1, and 2:1 PC/PS vesicles, $K = 5 \times 10^2$, 3×10^3, and 2×10^4 M^{-1}. This binding is strong enough, in principle, to anchor the enzyme to the inner leaflet of a typical plasma membrane. We also used this assay to measure binding of the intact protein: PLC-ζ binds weakly to 2:1 PC/PS vesicles, $K < 10^2$ M^{-1}, but more strongly to vesicles with 1% PIP_2, $K = 5 \times 10^3$ M^{-1}. Applying the nonlinear Poisson-Boltzmann equation to atomic models of the peptide and membrane produces a satisfactory description of the binding; these calculations also suggest the membrane-adsorbed basic cluster produces a local positive electrostatic potential and thus should enhance the local concentration of PIP_2 adjacent to the catalytic domain. FRET experiments confirm that the membrane-adsorbed basic peptide does indeed induce lateral sequestration of PIP_2: we observed strong energy transfer between a Bodipy-TMR label on PIP_2 and Texas Red label on the peptide, even when the bulk membrane contains 30% PS and only 0.1% PIP_2. (Supported by NIH grant R-37 GM24971.)

37. Do Natively Unfolded MARCKS and GAP43 Proteins Act as Reversible Sources and Sinks for PIP_2? URSZULA GOLEBIEWSKA and STUART MCLAUGHLIN, *Department of Physiology and Biophysics, SUNY at Stony Brook, Stony Brook, NY*

Although phosphatidylinositol 4,5-bisphosphate (PIP_2) comprises only ~1% of plasma membrane lipids, it performs a surprisingly wide variety of functions. We explore the possibility that proteins can control the local free [PIP_2] to help regulate these functions. We hypothesize that certain proteins act as reversible sources and sinks to produce a transient increase in the free [PIP_2] in response to elevated intracellular [Ca^{2+}]. Specifically, MARCKS and GAP43 proteins can act as PIP_2 sources and sinks, respectively. MARCKS is expressed at high levels in neuronal cells, and it can sequester PIP_2 through its basic effector domain (Wang et al. 2002. *J. Biol. Chem.* 277:34401–34412), then release it upon Ca^{2+}/calmodulin (Ca/CaM) binding (Kim et al. 1994. *J. Biol. Chem.* 269:28214–28219). GAP43 is present at high concentrations in neuronal cells, and has a cluster of basic residues that binds apoCaM (Andreasen et al. 1983. *Biochemistry* 22:4615–4618). We postulate that GAP43 can act as a reversible PIP_2 sink: in a quiescent cell, where [Ca^{2+}] is low, GAP43 binds apoCaM; when [Ca^{2+}] rises Ca/CaM dissociates from the basic region of GAP43, which can then bind and sequester PIP_2. We show that a peptide corresponding to the GAP43 basic region (GAP43(30–57)) can sequester PIP_2: for example, there is energy transfer between a Texas red label on GAP43(30–57) and a Bodipy-TMR label on PIP_2. We also show that binding of GAP43 to membranes containing PIP_2 can be reversed by apoCaM. These results support the hypothesis that MARCKS and GAP43 could act in tandem to regulate local free [PIP_2] by producing a "PIP_2 shuttle". (Supported by NIH grant R-37 GM24971.)

38. Peptides Corresponding to Basic/Hydrophobic Regions of Proteins Laterally Sequester PIP_2 ALOK GAMBHIR, *Department of Physiology and Biophysics, SUNY Stony Brook, Stony Brook, NY*

Many important peripheral membrane proteins, e.g., MARCKS and GRK5, contain clusters of basic/hydrophobic residues that interact electrostatically with acidic lipids on the inner leaflet of the plasma membrane. The juxtamembrane cytosolic region of intrinsic membrane proteins such as glycophorin, syntaxin, NMDA receptor, and EGF receptor also contain clus-

ters of basic residues. The role of these basic clusters is unclear. We utilize three techniques to show that a peptide corresponding to the basic cluster in MARCKS, MARCKS(151–175), laterally sequesters PIP_2 (Gambhir et al. 2004. *Biophys. J.* 86:2188-2207). First, we measure FRET from Bodipy-TMR labeled PIP_2 to Texas red–labeled MARCKS(151–175) adsorbed to large unilamellar vesicles (LUVs). Second, we detect self-quenching of Bodipy-TMR labeled PIP_2 in LUVs when unlabeled MARCKS(151–175) binds to vesicles. Third, we show that the PLC-δ_1–catalyzed hydrolysis of PIP_2 decreases markedly as MARCKS(151–175) sequesters most of the PIP_2. For MARCKS(151–175) and several other basic peptides, we show that this sequestration of PIP_2 is primarily due to nonspecific electrostatic interaction by observing the salt dependence on FRET and self-quenching. These results are consistent with theoretical calculations that suggest the local positive electrostatic potential adjacent to the peptide attracts the multivalent (net charge approximately -4) PIP_2. Experiments show that this lateral sequestration occurs even in the presence of 100-fold higher, physiologically relevant mole fractions of monovalent acidic lipids such as phosphatidylserine. The electrostatic sequestration of PIP_2 has biophysical implications in terms of the equilibration and distribution of other lipids in the membrane. (Supported by NIH grant GM24971 to SM.)

39. Characterization of the Membrane Docking Mechanism of the GRP1 Pleckstrin Homology Domain JOHN A. CORBIN and JOSEPH J. FALKE, *Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309*

The Pleckstrin homology (PH) domain is a conserved signaling motif found in a wide array of signaling proteins that dock to membranes. An important class of PH domains are those that dock to membranes in response to phosphatidylinositol phosphate lipid second messengers such as $PI(3,4,5)P_3$ and $PI(3,4)P_2$. Structural evidence indicates that this class of PH domains contains an inositolphosphate binding pocket, but little else is known of the molecular basis of their interaction with membranes. To investigate the mechanism of PH domain membrane docking, the activation and membrane-docking parameters of the $PI(3,4,5)P_3$-specific GRP1-PH domain were measured. A protein-to-membrane fluorescence resonance energy transfer (FRET) assay was employed to quantify both equilibrium dissociation constants (K_D) and kinetic parameters including on-rate constants (k_{on}), and off rate constants (k_{off}) for docking of the GRP1-PH domain to synthetic vesicles containing lipids of a defined composition. The equilibrium and kinetic data is consistent with a three-step mechanism for GRP1-PH membrane docking that involves a 2-D search across the membrane surface for its rare lipid target $PI(3,4,5)P_3$. The data will also serve as the basis for further molecular studies of the PH domain protein–membrane interface. (Funded by NIH Grant GM-63235.)

40. Proteome-wide Screen for Novel Phosphoinositide Binding Proteins MONICA L. KERR,[1,2] SETH J. FIELD,[1,2] and LEWIS C. CANTLEY,[1,2] [1]*Department of Systems Biology, Harvard Medical School;* [2]*Beth Israel Deaconess Medical Center, Boston, MA*

Phosphoinositides (PIPs) are a family of phospholipids involved in many diverse cellular processes. These include the regulation of cell growth, proliferation, and survival, as well as cytoskeletal reorganization and vesicle trafficking events. PIPs can be modified by reversible phosphorylation at three distinct sites, creating eight derivative molecules, each of which may function as unique regulators. PIPs are restricted to either the plasma membrane or intracellular vesicles and organelles, and the formation or degradation of a particular PIP species at a specific membrane compartment impacts the localization and activity of protein targets.

Mediating the binding between proteins and PIPs are highly conserved and common protein domains. While many PIP-binding domains have been characterized to date (PH, PX, FYVE, ENTH, Tubby, PHD, GRAM, PDZ, Sec14, and FERM domains), we hypothesize that many unidentified proteins with novel domain specificities still exist. The identification of these novel PIP-binding proteins will illuminate unresolved PIP-dependent pathways and further define the roles that each PIP plays in cellular processes.

For these reasons, we have devised and performed a proteome-wide screen for PIP binding proteins. We devised a novel assay that permits the rapid and efficient processing of multiple samples for relative binding to all PIP species. Our high-throughput protocol couples a modified lipid blot assay with small pool expression cloning to create a system in which an entire *Drosophila* cDNA library was screened in <10 blots. From these experiments, six known PIP-binders as well as seven novel PIP-binders were identified. We are currently working on the characterization of the most interesting lead, which exhibits a unique specificity toward PI-5-P and PI-3-P.

41. Phospholipid–Protein Interaction on the Membrane Determines a Physiological Mode of Action of RGS Proteins MASARU ISHII and YOSHIHISA KURACHI, *Department of Pharmacology, Osaka University Graduate School of Medicine, Osaka, Japan*

Regulators of G-protein–signaling (RGS) proteins are a family of proteins, which accelerate intrinsic GTP-hydrolysis on α-subunit of trimeric G-proteins and play crucial roles in the physiological regulation of G-protein–mediated cell signaling. If RGS proteins were active unrestrictedly, it would completely suppress various G protein–mediated signalings as has been seen in the overexpression experiments of RGS proteins. Therefore, it is important to understand how the actions of RGS proteins are regulated in various physiological conditions. We have been shown a physiological mode of action of a RGS protein regulated by the interaction with phospholipids. The voltage-dependent formation of Ca^{2+}/calmodulin (CaM) facilitated the GTPase-activity of RGS protein via removing intrinsic inhibition mediated by a kind of phospholipid, phosphatidylinositol-3,4,5,-trisphosphate (PtdIns(3,4,5)P_3). This modulation of RGS-action underlies a characteristic property, named "relaxation", of G-protein–gated K^+ (K_G) channels in native cardiac myocytes. Further examination using protein-lipid cosedimentation assay detected the specific interaction between RGS4 and PtdIns(3,4,5)P_3 (but not other PIPs), which was abolished by Ca^{2+}/CaM. Interestingly, this reciprocal modulation is exclusively performed within RGS domain, which is also responsible for GTPase-accelerating activity. We identified the clusters of positively charged residues in helix 4 of RGS domain as a candidate of the molecular switch of PtdIns(3,4,5)P_3/CaM-modulation. Because the residues are conserved in almost all RGS protein subtypes, the allosteric modulation of RGS proteins should be important in the physiological regulation of G-protein signaling by various RGS proteins in different cell types. Because PtdIns(3,4,5)P_3 is known to be concentrated into the lipid raft fraction, we are now further examining the relation between lipid raft and the physiological control of RGS protein function.

42. The Sac1 Lipid Phosphatase Regulates a Pool of Phosphatidylinositol-4-phosphate Required in Late Endocytic Trafficking SABINA TAHIROVIC[1] and PETER MAYINGER,[2] [1]*Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH), D-69120 Heidelberg, Germany;* [2]*Division of Nephrology and Hypertension, Oregon Health & Science University, Portland, OR 97201* (Sponsor: Don Hilgemann)

Phosphatidylinositol 4-phosphate (PtdIns(4)P) regulates diverse cellular processes, such as actin cytoskeletal organization, Golgi trafficking, and vacuolar biogenesis. Synthesis and turnover of PtdIns(4)P is mediated by a set of specific lipid kinases and phosphatases. Here we show that the polyphosphoinositide phosphatase Sac1p modulates organelle-specific pools of PtdIns(4)P. We have found that *sac1Δ* mutants show pleiotropic synthetically lethal interactions with mutations in genes required for vacuolar protein sorting (Vps). Disruption of the *SAC1* gene caused also a defect in the late endocytic pathway. These trafficking phenotypes correlated with a dramatic accumulation of PtdIns(4)P at vacuolar membranes. In addition, *sac1* mutants displayed elevated endoplasmic reticulum (ER) PtdIns(4)P. The accumulation of PtdIns(4)P at ER and vacuole and the endocytic defect could be compensated by mutations in the PtdIns 4-kinase Stt4p. Our results therefore demonstrate that Sac1p and Stt4p regulate a specific pool of PtdIns(4)P, which is required for late endocytic and vacuolar trafficking. Sac1p also controls an ER-specific pool of PtdIns(4)P and functions as an antagonist of the Pik1p PtdIns 4-kinase in Golgi trafficking. We conclude that Sac1p is a key coordinator of distinct PtdIns(4)P-dependent reactions involved in membrane trafficking and homeostasis. (Grant support from the Deutsche Forschungsgemeinschaft, Ma1363/5-2.)

43. Role of Synaptotagmin–PIP_2 Interactions during Ca^{2+}-triggered Exocytosis JIHONG BAI and EDWIN R. CHAPMAN, *Department of Physiology, University of Wisconsin-Madison, Madison, WI*

Phosphatidylinositol-4,5-bisphosphate (PIP_2) is an important signaling molecule that regulates a number of cellular functions, including cell motility, ion channel function, and membrane traffic. PIP_2 directly interacts with synaptotagmin I (syt) (Schiavo et al. 1996. *Proc. Natl. Acad. Sci. USA.* 93:13327–13332), a putative Ca^{2+}-sensor for exocytosis (Chapman. 2002. *Nat. Rev. Mol. Cell. Biol.* 3:498–508). Syt is anchored to the membrane of secretory vesicles via a single transmembrane domain. The cytoplasmic domain of syt is composed of two Ca^{2+}-sensing modules, C2A and C2B. Here, we report that the C2B domain of syt interacts with PIP_2-harboring membranes via two distinct modes, Ca^{2+}-independent adsorption and Ca^{2+}-dependent membrane penetration. A cluster of lysine residues on the side of C2B binds PIP_2 in the absence of Ca^{2+}. Substitution of Lys residues to either Ala or Arg, disrupts Ca^{2+}-independent syt–PIP_2 interactions, suggesting that both spatial arrangements and net charges of the side chains of Lys residues are critical for binding PIP_2 in the absence of Ca^{2+}. The Ca^{2+}-independent mode of binding predisposes syt to penetrate into PIP_2 harboring target membranes in response to Ca^{2+} with submillisecond kinetics. Reagents that block syt–PIP_2 interactions, such as neomycin, inositol hexaphosphate (IP_6), and phos-

pholipase C δ1 pleckstrin homology (PH) domain inhibit Ca^{2+}-triggered catecholamines release from permeabilized PC12 cells. Furthermore, functional studies using mutant versions of syt indicate that PIP_2 steering of the cytoplasmic domain of syt toward the plasma membrane plays a role in exocytosis, perhaps by driving the close apposition of the vesicle and target membrane in response to Ca^{2+}. (Supported by NIH grants GM 56827 and MH61876, American Heart Association grant 9750326N. Jihong Bai is supported by an AHA postdoctoral Fellowship.)

44. Regulation of Phosphoinositide 3-kinase Signaling by PI-5-P 4-kinases VALERIE CARRICABURU, DEBORAH SARKES, VANESSA JUNG, and LUCIA E. RAMEH, *Boston Biomedical Research Institute, Watertown, MA*

Phosphatidylinositol-5-phosphate (PI-5-P) is a newly identified phosphoinositide with characteristics of a signaling lipid but with no known cellular function. PI-5-P levels are controlled by the type II PI-5-P 4-kinases (PIP4k II), a family of kinases that converts PI-5-P into PI-4,5-P_2. The PI-5-P pathway is an alternative route for PI-4,5-P_2 synthesis as the bulk of this lipid is generated by the canonical pathway in which phosphatidylinositol-4-phosphate (PI-4-P) is the intermediate. PI-5-P levels in cells dramatically increase following *Shigella flexneri* infection, due to the activity of the virulence factor IpgD, a bacterial phosphatase that dephosphorylates PI-4,5-P_2 to generate PI-5-P (Niebuhr et al. 2002. *EMBO J.* 21:5069–5078). Therefore, IpgD reverts the reaction catalyzed by PIP4k II. We have recently shown that overexpression of PIP4k II reduced PI-3,4,5-P_3 levels in cells stimulated with insulin or cells expressing activated phosphoinositide 3-kinase (PI3k). This reduction in PI-3,4,5-P_3 levels resulted in decreased activation of the downstream protein kinase, Akt/PKB. Conversely, expression of IpgD resulted in Akt activation and this effect was partially reversed by PIP4k II. Since PIP4k II expression did not impair insulin-dependent association of PI3k with IRS1 but abbreviated Akt activation, our data support a model in which the PI-5-P pathway controls insulin signaling that leads to Akt activation by regulating a PI-3,4,5-P_3 phosphatase (Carricaburu et al. 2003. *Proc. Natl. Acad. Sci. USA*.100:9867–9872). To further understand the role of PI-5-P and PIP4k II in growth-factor signaling and in PI-3,4,5-P_3 regulation, we suppressed the expression of PIP4k IIα, IIβ, and/or IIγ in various mammalian cell lines using RNA interference. Consistent with our previous results, we find that suppression of PIP4k II can up-regulate insulin and EGF-induced PI3k signaling that leads to Akt phosphorylation and activation. (Supported by NIH grant DK063219 and US Army, DOD, DAMD 017–01-1-0155.)

45. Class II PI3-kinase C2α Is Essential for ATP-dependent Priming of Neurosecretory Granule Exocytosis FRÉDÉRIC A. MEUNIER,[1] FRANK T. COOKE,[2] SHONA L. OSBORNE,[4] GERALD HAMMOND,[1] JAN DOMIN,[3] PETER J. PARKER,[1] and GIAMPIETRO SCHIAVO,[1] [1]*Cancer Research UK, London Research Institute, Lincoln's Inn Fields Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX, UK;* [2]*Biochemistry and Molecular Biology, University of College, London, Darwin Building, Gower Street, W1E 6BT, London, UK;* [3]*Renal Section, Faculty of Medicine, Imperial College, London W12 0NN, UK;* [4]*School of Biomedical Sciences, University of Queensland, St. Lucia, Queensland 4072, Australia*

Phosphoinositide-3 kinase (PI3K) activity is important for several aspects of neuronal differentiation and plasticity. However, its direct involvement in regulated exocytosis is unclear, despite clear evidence for a requirement for phosphoinositides in this process. Neurotransmitter release from synaptosomes and hormonal secretion from chromaffin cells is only sensitive to high concentrations of the PI3K inhibitors wortmannin and LY294002 pointing to a possible role for the less sensitive PI3K-C2α. In neurosecretory cells PI3K-C2α was detected on a subpopulation of mature secretory granules, abutting the plasma membrane. Both PI3K-C2α antibodies and PI3K inhibitors selectively prevented ATP-dependent priming in permeabilized chromaffin cells. Transient expression of PI3K-C2α potentiated secretion, whereas its catalytically inactive mutant abolished exocytosis, suggesting a possible role of the main catalytic product of this enzyme, PtdIns-3-phosphate (PtdIns3*P*), in this process. Treatment of PC12 cells expressing the PtdIns3*P*-binding FYVE domain with a low concentration of wortmannin selectively abolished early endosomal staining and revealed a full colocalisation with PI3K-C2α on PC12 granules. Finally, sequestration of PtdIns3*P* by the overexpression of FYVE domain abolished secretion from PC12 cells. Together these data demonstrate that production of PtdIns3*P* along with PtdIns4,5P_2 is required in the acquisition of fusion competence that secretory vesicles undergo during or following docking to the plasma membrane.

46. Dolichol Phosphate Mannose Synthase Dpm1p Recruits the Lipid Phosphatase Sac1p to the Endoplasmic Reticulum FRANK FAULHAMMER, GERLINDE KONRAD, BEN BRANKATSCHK, ANDREAS KNOEDLER

and PETER MAYINGER, *Department of Medicine, Division of Nephrology and Hypertension, Oregon Health & Science University, Portland, OR*

The phosphoinositide phosphatase Sac1p is an integral membrane protein that regulates local pools of phosphoinositides at endoplasmic reticulum (ER) and Golgi membranes. It is unknown how the localization of Sac1p to distinct organellar membranes is mediated. We could identify a novel mechanism for localizing Sac1p to the ER. We identified a COOH-terminal region in Sac1p crucial for ER targeting. This region contains one of the two transmembrane domains of Sac1p, yet ER localization was independent of Rer1p, the only described candidate sorting receptor for transmembrane sequences. Chemical cross-linking led to the identification of dolichol phosphate mannose synthase Dpm1p as a factor interacting with Sac1p at ER membranes. Our findings indicate that the interaction of Sac1p with Dpm1p represents a device for localizing this enzyme to the ER. In particular, our data suggests that the transmembrane domains of Dpm1p and Sac1p are required for this interaction. At least one of the two transmembrane regions in Sac1p is essential for ER localization, and this region appears to directly interact with the transmembrane region of Dpm1p. According to our data, the ER-resident portion of Sac1p contributes to proper dolichol oligosaccharide biosynthesis, as a *sac1Δ* strain showed significant changes in cellular levels of dolichol-linked glycosyl metabolites compared with a wild-type strain. Our data suggest that the lipid phosphatase Sac1p coordinates oligosaccharide biosynthesis with secretory protein processing in the ER. (Supported by a grant from the Deutsche Forschungsgemeinschaft to Peter Mayinger (Ma1363/5-2) and a PhD scholarship from the Boehringer Ingelheim Fonds to Frank Faulhammer.)

47. $PI(4,5)P_2$ Is Required for Mitotic Cytokinesis SETH J. FIELD,[1,2] NIKKI MADSON,[1] MAMTA TAHILIANI,[1] and LEWIS C. CANTLEY,[1] [1]*Division of Signal Transduction, Beth Israel-Deaconess Medical Center, Department of Systems Biology, Harvard Medical School, Boston, MA;* [2]*Division of Endocrinology, Massachusetts General Hospital, Boston, MA*

Beginning with an unbiased survey of phosphoinositide subcellular localization through the mammalian cell cycle we demonstrate accumulation of $PI(4,5)P_2$ at the cleavage furrow during mitotic cytokinesis. A fusion of PI(4)P-5-kinase-α to EGFP also localizes to the cleavage furrow. Sequestration of $PI(4,5)P_2$ impairs cytokinesis. Potential downstream targets of $PI(4,5)P_2$ at the cleavage furrow are being examined. A model for $PI(4,5)P_2$ function at the cleavage furrow will be presented. (Supported by a Howard Hughes Medical Institute Physician-Postdoctoral Award and NIH grant K08 DK065108-01 to Seth J. Field and NIH grant GM36624 to Lewis C. Cantley.)

48. Modulation of Serotonin Biosynthesis by Sensory Cytoskeleton and TRPV Ion Channel in *C elegans* SZE JI YING, *Department of Anatomy and Neurobiology, University of California, Irvine, CA*

Serotonin (5HT) is a pivotal signaling molecule that controls behavioral and physiological responses to sensory stimuli. We use *C. elegans* as a genetic model to study regulation of 5HT signaling in vivo. The pair of serotonergic chemosensory neurons ADF resides in the bilaterally symmetric amphid sensory organ and has ciliated sensory endings exposed to the external environment. The ADF neurons have been proposed to mediate neuroendocrine response to the environment: worms with the ADF neurons ablated (Bargman et al. 1991. *Science.* 251:1243–1246) or mutated are more susceptible to stressful environment–induced Dauer larvae arrest, and we found this abnormal Dauer formation at least in part due to down-regulation of the DAF-2/insulin receptor signaling pathway (Zhang et al. 2004. *Development.* 131:1629–1638).

By isolation of neuron-specific 5HT-deficient mutants, we discovered that production of 5HT in ADF requires signaling from vanilloid subfamily of the transient receptor potential (TRPV) channels encoded by *ocr-2* and *osm-9* genes (Zhang et al. 2004. *Development.* 131:1629–1638). OCR-2 and OSM-9 are localized to the cytoplasmic membrane and ciliated sensory endings in ADF but not in any other serotonergic neurons. Mutation in either *osm-9* or *ocr-2* specifically down-regulates ADF to express the key 5HT biosynthesis enzyme tryptophan hydroxylase (*tph-1*); *osm-9* and *ocr-2* mutants are more susceptible to form Dauers. Misexpression experiments indicate that *ocr-2/osm-9* acts in ADF to up-regulate the *tph-1* expression. Other signaling pathways may suppress 5HT biosynthesis: *tph-1* expression in *ocr-2* and *osm-9* deletion mutants are restored by mutations in *che-2* and *che-13*, both genes encode intraflagellar transport polypeptides and interact with kinesin II and cytoplasmic dynein (Qin et al. 2001. *Curr. Biol.* 11:457–461). We propose that 5HT production in the ADF chemosensory neurons may be modulated by opposing signals: OSM-9/OCR-2 channel signaling stimulates 5HT production but the pathway associated with *che-2* and *che-13* inhibits it. (Supported by NIH grant MH64747.)

49. Insulin and PDGF Mobilize Different Pools of GLUT4 with Distinct Involvement of the Actin Cytoskeleton

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Insulin promotes the translocation of glucose transporter 4 (GLUT4) from a distinct intracellular pool to the surface of muscle and fat cells via a mechanism dependent on phosphatidylinositol (PI) 3-kinase (PI3-K). The growth factor PDGF also robustly activates PI3-K, raising the question of whether it causes GLUT4 translocation and whether this occurs from the insulin-sensitive pool. The insulin response requires cortical actin remodelling; hence we compared insulin and PDGF actions on the PI3-K signaling axis, actin remodelling, and GLUT4 pool mobilization. In L6 myoblasts stably expressing myc-tagged GLUT4, the insulin-induced gain in surface GLUT4myc peaked at 10 min and remained at 30 min. In contrast, PDGF action was transient, peaking at 5 min and disappearing in 20 min. These GLUT4myc translocation time-courses mirror the phosphorylation of Akt by the two stimuli. Interestingly, insulin and PDGF caused distinct manifestations of actin remodelling. Insulin induced discrete long (>5 μm) dorsal actin structures at the cell periphery, whereas PDGF induced multiple short (<5 μm) dorsal structures throughout the cell and preferentially above the nucleus. The actin dynamics-disrupting drugs latrunculin B and cytochalasin D, while preventing insulin- and PDGF-induced actin remodelling, inhibited vastly the insulin-induced GLUT4myc translocation (by 75–85%) and only slightly the PDGF-induced GLUT4myc translocation (by 20–30%). Moreover, transfection of tetanus toxin light chain, which cleaves the v-SNAREs VAMP2 and VAMP3, reduced insulin-induced GLUT4myc translocation by 70% but did not affect PDGF action. These results suggest that insulin and PDGF mobilize different GLUT4 compartments that require different v-SNAREs for fusion. We conclude that insulin and PDGF rely differentially on the actin cytoskeleton to mobilize distinct GLUT4myc pools, defined by their v-SNAREs. (Supported by a grant from the Canadian Institutes of Health Research MT7307.)

50. Sensing Chemical Signals at Membrane Surfaces JOSEPH J. FALKE, *Molecular Biophysics Program, University of Colorado, Boulder, Boulder, CO*

Chemical sensing of second messengers at membrane surfaces plays a central role in most cellular-signaling pathways. In the chemotaxis pathway of leukocytes, for example, attractant binding to receptors triggers the accumulation of the signaling lipid PIP_3 at the leading edge of the cell, as well as Ca^{2+} signals in the cytoplasm. As a result, information about the extracellular attractant concentration gradient is used to regulate a variety of signaling events occurring at intracellular membrane surfaces. Ultimately these intracellular membrane signals trigger actin filament polymerization at the leading edge that pushes the cell forward, as well as cytoskeletal retraction at the trailing edge of the cell. Current studies in the lab focus on the molecular mechanisms of (a) docking of PH domain proteins to membrane surfaces at the leading edge of the cell, and (b) calcium-triggered docking of C2 domains to specific intracellular membranes. Unpublished results pertaining to target search mechanisms and the structure of the protein-membrane interface will be discussed.

51. A Role for Palmitoylation in Kv1.5 Channel Localization KAREN FOSTER and JEFFREY R. MARTENS, *Department of Pharmacology, University of Michigan, Ann Arbor, MI*

Recent evidence demonstrated that the voltage-dependent K+ channel, Kv1.5, targets to lipid raft microdomains (Martens et al. 2001. *J. Biol. Chem.* 276:8409–8414). However, the molecular mechanisms of targeting these polytopic proteins to lipid rafts are unknown. Protein modifications such as fatty acylation have been shown to increase the affinity of transmembrane proteins, such as hemagglutinin, for the liquid ordered environment of lipid rafts (Melkonian et al. 1999. *J. Biol. Chem.* 274:3910–3917). In asking the question what is the role of fatty acylation in Kv1.5 targeting to lipid rafts and/or caveolae, we discovered a role for palmitoylation in Kv1.5 cell-surface expression. In transfected fibroblasts, 2-bromopalmitate, an inhibitor of palmitoylation, but not 2-hydroxymyristate, and inhibitor of myristoylation, significantly decreased Kv1.5 channel expression. Channel protein degradation was rescued by treatment with the proteosome inhibitiors. Immunostaining of cells expressing Kv1.5 revealed that after 2-bromopalmitate treatment, channel accumulated in intracellular compartments before degradation. Deletion of 57 COOH-terminal amino acids from Kv1.5, which removes three cystine residues, resulted in a loss of the bromopalmiate effect. These results suggest that palmitoylation, directly on the channel or indirectly through a channel binding protein can regulate cell surface expression of Kv1.5. (Supported by NIH grant HL070973.)

52. Phospholipid Matolism Is Required for L-type Calcium Current Inhibition by Muscarinic Receptor Agonist in Sympathetic Neurons LIWANG LIU, MANDY

L. ROBERTS, and ANN R. RITTENHOUSE, *Department of Physiology, University of Massachusetts Medical School, Worcester, MA*

M_1 muscarinic receptor (M_1R) agonists activate a G-protein–mediated signaling pathway, called the slow pathway, that uses an unknown diffusible second messenger to modulate N- and L-type calcium (Ca) currents in rat superior cervical ganglion (SCG) neurons. Our recent studies (Liu and Rittenhouse. 2003. *Proc. Natl. Acad. Sci. USA.* 100:295–300) identified phospholipase C (PLC), phospholipase A_2 (PLA_2), and arachidonic acid (AA) as additional members of the slow pathway modulating N-current. Inhibition of both N- and L-currents by M_1Rs requires a low concentration of intracellular Ca, the pertussis toxin–insensitive G-protein G_q and a diffusible second messenger (Bernheim et al. 1992. *Proc. Natl. Acad. Sci. USA.* 89:9544–9548). Therefore, the same pathway is expected to mediate M_1R inhibition of both currents. To test this hypothesis, whole-cell L-currents were recorded at +10 mV from dissociated neonatal rat SCG neurons using 0.1 mM BAPTA as the internal Ca chelator and 20 mM barium as the charge carrier. We used two methods to isolate L- from N-current. First, cells were treated with ω-conotoxin-GVIA (CgTX; 1 μM) to block N-currents. Second, long-lasting tail currents elicited by FPL-64176 (FPL; 1 μM) and made up entirely of L-current were monitored for inhibition by the slow pathway. Under these conditions, 10 μM oxotremorine-M (Oxo-M) inhibited CgTX-treated current by 45.0 ± 6.6% (n = 12) and FPL-induced tail current by 43.0 ± 4.0% (n = 4). 5 μM AA mimicked inhibition of peak current (50.4 ± 4.8%, n = 15). Pharmacologically inhibiting M_1Rs, G_q, PLC, PLA_2 or AA activity minimized Oxo-M's ability to modulate L current. PLA_2 liberates AA from phospholipids; lysophosphatidic acid (LPA) can be also formed in this process. We tested whether LPA (10 μM) could mimic current inhibition by Oxo-M, but found no significant effect. These data indicate that M_1R agonist modulates L current by the slow pathway via G_q, PLC, and PLA_2, and AA may be the diffusible second messenger. (Supported by NIH RO1-NS34195.)

53. Arachidonic Acid Inhibition of L-type ($Ca_V1.3$) Calcium Currents in ST14A Cells MANDY L. ROBERTS and ANN R. RITTENHOUSE, *Program in Neuroscience, Department of Physiology, University of Massachusetts Medical Center, Worcester, MA*

Arachidonic acid (AA) is released from cell membranes after stimulation with dopamine D_2 receptor (D_2R) agonists in both striatal neurons and D_2R-transfected CHO cells (Schinelli et al. 1994. *J. Neurochem.* 62: 944). Additionally, D_2R agonists decrease L-type calcium (Ca^{2+}) current (L-current) via a diffusible second messenger pathway involving calcineurin (Hernández-López et al. 2000. *J. Neurosci.* 20:8987–8995). Our lab has shown that AA also inhibits L-current (Liu et al. 2001. *Am. J. Physiol. Cell Physiol.* 280:1293–1305). To test whether AA participates in the diffusible second messenger pathway of D_2R-mediated inhibition of L-current, we developed a preparation that uses the striatal cell line ST14A (Ehrlich et al. 2001. *Exp. Neurol.* 167: 215–226). We confirmed the presence of critical signaling molecules, such as D_2Rs, by RT-PCR and Western blot analysis. We found that type IVa phospholipase A_2, which cleaves AA from membrane phospholipids, and calcineurin, a phosphatase which can be activated by AA independently of Ca^{2+}-calmodulin (Kessen et al. 1999. *J. Biol. Chem.* 274:37821–37826), are also endogenously expressed by the cells. L-type Ca^{2+} channel subunits ($Ca_V1.3$, β_{2a}, and $\alpha_2\delta$) and green fluorescent protein were cotransfected into ST14A cells using lipofectamine to test whether AA can modulate $Ca_V1.3$ current. Whole cell L-current was recorded using 20 mM Ba^{2+} as the charge carrier. The current-voltage relationship, inactivation kinetics and tail current of $Ca_V1.3$ in the presence and absence of the L-type Ca^{2+} channel agonist FPL 64176 (1 μM) have similar characteristics of $Ca_V1.3$ currents in oocytes (Xu and Lipscombe. 2001. *J. Neurosci.* 21:5944–5951). In initial experiments, bath applying 10 μM AA inhibited the transfected current by 30% after 3 min (n = 3). Thus, AA may act downstream of D_2Rs inhibiting L-current directly or indirectly through calcineurin. Future studies will address whether inhibition by AA requires phosphatase activity. (Supported by NIH grant 5TS32 NS07366 and AHA 9940225N.)

54. Bidirectional Control of Kir-channel Activity by Phosphoinositides and LC-acyl-CoA esters MARKUS RAPEDIUS, DIRK SCHULZE, MALLE SOOM, HARIOLF FRITZENSCHAFT, and THOMAS BAUKROWITZ, *University of Jena, Department of Physiology II, Am Teichgraben 8, D-07743 Jena*

Recent work introduced two distinct classes of lipids as potent regulators of Kir channels: long-chain acyl-CoA (LC-CoA) esters (e.g., oleoyl-CoA) and phosphatidylinositol phosphates (e.g., PIP2). PIP2 is known to activate all members of the Kir family whereas LC-CoA esters appear to be a specific activators of KATP channels. Here we studied the effect of LC-CoA esters on different Kir channels heterologously expressed in *Xenopus laevis* oocytes. In agreement with previous reports, none of the Kir channels tested (Kir1.1, Kir2.1, Kir3.4,

Kir4.1, and Kir7.1) were activated by oleoyl-CoA. Quite the contrary, we found LC-CoA esters to be potent inhibitors of all Kir channels (except Kir6.2). PIP2 reversed the inhibitory effect of oleoyl-CoA, suggesting a displacement of PIP2 by oleoly-CoA from a common binding site as the mechanism behind the oleoyl-CoA inhibition. This notion is supported by mutant channels with reduced PIP2 affinity and biochemical studies assaying the binding of PIP2 and oleoyl-CoA on NH_2- and COOH-terminal fragments of Kir2.1 and Kir6.2. In addition, we provide evidence that PIP2 and LC-CoA regulate the activity of Kir channels via structural changes of in the selectivity filter of the channel's pore.

55. Inhibition of Inactivation-deficient Cardiac Na^+ Channels by n-3 Polyunsaturated Fatty Acids YONG-FU XIAO,[1,2] LI MA,[1] SHO-YA WANG,[4] GING KUO WANG,[3] JAMES P. MORGAN,[1] and ALEXANDER LEAF,[2] [1]*Department of Medicine, Beth Israel Deaconess Medical Center;* [2]*Massachusetts General Hospital;* [3]*Department of Anesthesia, Brigham and Women's Hospital, Harvard Medical School, Boston, MA*; [4]*Department of Biology, State University of New York at Albany, Albany, NY*

Several studies show that mammalian cardiomyocytes have a small persistent Na^+ current (I_{NaP}) that is potentiated during hypoxia. I_{NaP} found in LQT-3 syndromes can delay repolarization of cardiac action potentials and cause cardiac arrhythmias, including sudden death. In the present study, we investigated the effects of the n-3 polyunsaturated fatty acids (PUFAs) on Na^+ currents in HEK293t cells transfected with an inactivation-deficient mutant (L409C/A410W) of the α-subunit of human cardiac Na^+ channels (hNav1.5) plus β_1 subunits. I_{NaP} was observed in HEK293t cells expressing the mutant. Extracellular application of eicosapentaenoic acid (EPA, C20:5n-3) at 5 μM significantly inhibited both peak and late portions of I_{NaP}, but the late portion was more sensitive and almost completely suppressed. I_{NaP} returned to the pretreated level after washout of EPA. The inhibitory effect of EPA on I_{NaP} was concentration dependent with the IC_{50} values of 4.0 ± 0.4 μM for the peak amplitude and 0.9 ± 0.1 μM for the late portion of I_{NaP}, respectively. EPA shifted the steady-state inactivation of peak I_{NaP} by −19 mV to the hyperpolarizing direction. EPA accelerated the process of resting inactivation of mutant channels and delayed the recovery of I_{NaP} from resting inactivation. Other polyunsaturated fatty acids, docosahexaenoic acid, linolenic acid, arachidonic acid, and linoleic acid, at 5 μM also significantly inhibited I_{NaP}. In contrast, the monounsaturated fatty acid oleic acid or the saturated fatty acids, stearic acid and palmitic acid, at 5 μM had no effects on I_{NaP}. Our data demonstrate that the double mutations at 409 and 410 sites in D1-S6 region of hNav1.5 induce I_{NaP} and the n-3 PUFAs inhibit the current. The ability to inhibit I_{NaP} may have clinical implication for n-3 PUFAs to treat arrhythmias caused by ischemia or by genetic mutations of cardiac Na^+ channels in LQT-3 patients.

56. Studies on Heat- and Lipid-induced Activation of TREK-2 DAWON KANG and DONGHEE KIM, *Department of Physiology, Rosalind Franklin University of Medicine and Science, The Chicago Medical School, North Chicago, IL*

TREK-2 is a member of the two-pore domain K^+ channel family and is activated by various physical and chemical factors, including heat, free fatty acids, oxygen, halothane, and protons. In this poster, we present studies on the effects of temperature and free fatty acids. In COS-7 cells transfected with TREK-2 DNA, TREK-2 was closed at 24°C but easily activated by application of arachidonic acid (AA). The sensitivity of TREK-2 to AA was abolished by substitution of the COOH terminus of TREK-2 with that of TASK-1 or TASK-3 (TREK-2/TASK-3C). A COOH terminus region of 25 amino acids near the fourth transmembrane segment was critical for providing the sensitivity to AA. At 37°C, TREK-2 was active and further activated by AA. The heat sensitivity of TREK-2 was observed in cell-attached patches but not in inside-out patches, indicating the involvement of a cytosolic factor. The gating kinetics of TREK-2 were altered by heat (24°C to 42°C) such that the burst duration was markedly shortened at higher temperatures despite the higher open probability. Therefore, the cytosolic factor was not free fatty acids, as they increased the burst duration of TREK-2. TREK-2 expressed in cultured cerebellar granule neurons exhibited behavior similar to that in COS-7 cells with respect to heat and AA sensitivity. Therefore, TREK-2 at physiological temperatures is likely to contribute to the regulation of resting K^+ conductance and membrane potential, and of cell excitability in response to various modulators. (Supported by AHA and NIH.)

57. Lipid-signaling Pathways Stimulate and Inhibit Cl^- and K^+ Secretion across Guinea Pig Distal Colonic Mucosa SUSAN HALM, JULIAN GOMEZ-CAMBRONERO and DAN HALM, *Department of Anatomy & Physiology, Wright State University, Dayton, OH*

Secretagogue activation of Cl^- secretion across the colonic epithelium also stimulates electrogenic K^+ secretion. Thus, the cellular mechanism of Cl^- secretion in the colon includes apical membrane K^+ channels. The proportion of K^+ secretion to Cl^- secretion varies

with secretagogue type, ranging from primarily K^+ secretion with epinephrine, through equal secretory rates with prostaglandin-E_2, to primarily Cl^- secretion with carbachol + PGE_2. Cyclooxygenase inhibitors indomethacin [2 μM] and NS-398 [2 μM] were used to suppress endogenous production of prostaglandins. Secretory control of ion transporters likely involves other membrane lipid–derived elements, since three major phospholipases were stimulated in guinea pig distal colonic mucosa, as measured by release of arachidonate and DAG or transphosphatidylation. PGE_2 stimulated PLA_2 (110%), PLC (30%), and PLD (25%); CCh stimulated PLA_2 (40%) and PLD (20%); primary K^+ secretion occurred with only increased PLC activity (30%). Inhibition of PLC with ET-18-OCH_3 [100 μM] or D609 [100 μM] reduced epi-stimulated K^+ secretory current in guinea pig distal colonic mucosa, measured as short-circuit current and transepithelial conductance. Similarly, epi-stimulated K^+ secretion was reduced during inhibition of PKC with staurosporine [0.3 μM] or rottlerin [100 μM], but not with Gö6850 [3 μM]. Inhibiting DAG-lipase with RHC-80267 [50 μM] stimulated K^+ secretory current but did not augment the maximal secretory capacity. These results support a signaling pathway for K^+ secretion involving DAG release by PLC followed by PKC-δ activation. PGE_2-stimulated Cl^- secretion was augmented by using the PLA_2-inhibitor aristolochic acid [300 μM], the P450-inhibitor ketoconazole [20 μM] or the lipoxygenase-inhibitor nordihydroguaiaretic acid [30 μM]. These results support the presence of a repressor signaling pathway for Cl^- secretion involving conversion of arachidonate by lipoxygenase and P450. Thus, control of electrogenic Cl^- and K^+ secretion involves release of several membrane lipid components, with PLC/PKC activity leading to K^+ secretion and PLA_2/LOX:P450 activity leading to repression of Cl^- secretory rate. (Supported by NIH grant DK39007.)

58. The Inner Pore Mutation Y652C Reverses the Trafficking-defective Long QT2 Channels G601S and F640V Phenotype BRIAN DELISLE,[1] JESSICA SLIND,[1] JENNIFER KILBY,[1] COREY L. ANDERSON,[1] DAVID J. TESTER,[2] MICHAEL J. ACKERMAN,[2] RAVI C. BALIJEPALLI,[1] TIMOTHY J. KAMP,[1] and CRAIG T. JANUARY,[1] [1]*University of Wisconsin-Madison, Departments of Medicine and Physiology;* [2]*Mayo Clinic, Department of Pediatrics*

Several mutations in the human ether-a-go-go–related gene (HERG or KCNH2)-encoded K^+ channel disrupt intracellular ion channel transport (trafficking) and cause long QT syndrome (LQT2). Compared with WT HERG, trafficking-defective LQT2 channels have a phenotype of minimal or absent complex glycosylation and poor surface membrane expression. We report a novel HERG mutation in the sixth transmembrane segment (F640V) in a family with a history of sudden death, syncope, and long QT syndrome. Heterologous expression of F640V showed a trafficking-defective phenotype, and this phenotype was corrected by a 24-h incubation in the HERG channel blocker E4031(10 mM). We then tested the hypothesis that mutating the putative drug-binding domain at position Y652 would alter the effect of E4031. In a WT background, the Y652C mutation did not alter the WT glycosylation pattern but reduced I_{HERG} (WT = 108 ± 20pA/pF, n = 9; Y652C = 31 ± 8pA/pF, n = 6), presumably by altering channel function. Y652C I_{HERG} also was less sensitive than WT to E4031(100 nM) block; I_{HERG} for WT = 17 ± 1% (n = 5) and Y652C = 78 ± 3% (n = 3). We next tested the double mutation F640V-Y652C. In contrast to F640V, F640V-Y652C was complexly glycosylated; confocal microscopy confirmed cell surface expression; and I_{HERG} was increased (F640V = 8 ± 1 pA/pF, n = 13; F640V-Y652C = 22 ± 2 pA/pF, n = 10) to a level similar to Y652C alone. We also tested the trafficking-defective G601S mutation and obtained similar findings; G601S-Y652C was complexly glycosylated and I_{HERG} density was comparable to that of Y652C alone (G601S = 39 ± 8 pA/pF, n = 6; G601S-Y652C = 25 ± 7 pA/pF, n = 5). E4031 (10 μM) incubation (24 h) did not alter the glycosylation pattern or I_{HERG} in F640V-Y652C or G601S-Y652C. We conclude that mutagenic modification of the inner pore can restore the processing of several trafficking-defective LQT2 channel proteins. (Supported in part by R01 HL60723 to Craig T. January and F32 HL071476-01 to Brian Delisle.)

59. Calcium Influx through L-type Calcium Channels Increases *C-fos* Mrna Levels in Superior Cervical Ganglion Neurons RUBING ZHAO and ANN R. RITTENHOUSE, *Program in Neuroscience, Department of Physiology, University of Massachusetts Medical Center, Worcester, MA 01655*

Calcium (Ca) influx through L-type Ca channels can initiate transcription of several members of a class of genes known as immediate-early genes (IEGs), such as *c-fos* (Finkbeiner and Greenberg. 1998. *J. Neurobiol.* 37: 171–189; Greenberg et al. 1986. *Science.* 234:80–83). To determine whether L-type Ca channels are coupled to activity or depolarization-induced gene expression in superior cervical ganglion (SCG) neurons, we established a semiquantitative RT-PCR protocol for detecting changes in *c-fos* mRNA levels in SCG using cell de-

polarization with KCl (55 mM). Using this method, we examined the role of Ca influx and Ca channel activity on *c-fos* expression. Preincubation of pairs of ganglia were followed by stimulation of one SCG and the contralateral ganglion served as an unstimulated control. Total RNA extracted from each ganglion was reverse transcribed into single-strand cDNA and 15% of the resulting cDNA mix was amplified via PCR. The intensity of amplified products was quantified by densitometric measurement. The ratio of target cDNA to internal standard (GAPDH) was used to calculate relative mRNA levels of *c-fos*. Stimulation for 40–45 min maximally increased *c-fos* mRNA levels by 1.83 ± 0.30 (n = 7). No increase in *c-fos* mRNA levels could be detected when stimulating SCG with high K^+ medium containing low Ca (0.1 mM)/high Mg (10 mM). The L-type Ca channel antagonist nimodipine (1 μM) blocked the increase in *c-fos* mRNA levels due to stimulation, whereas the N-type Ca channel antagonist ω-conotoxin GVIA (100 nM) had no effect. Stimulation in the presence of both nimodipine and ω-conotoxin GVIA had the same results as nimodipine. These results indicate that Ca influx through L-type Ca channels is necessary for depolarization-induced changes in *c-fos* mRNA levels. (Supported by ROINS34195.)

60. TRPC5 Activation Kinetics Are Modulated by the Scaffolding Protein Ezrin/Radixin/Moesin-binding Phosphoprotein-50 (EBP50) ALEXANDER G. OBUKHOV and MARTHA C. NOWYCKY, *Department of Pharmacology and Physiology, UMDNJ, New Jersey Medical School, Newark, NJ*

TRPC1-7 proteins are members of a family of mammalian nonspecific cation channels that mediate receptor-operated, phospholipase Cβ/Cγ–dependent Ca^{2+} influx in various cell types. Uniquely in the TRPC family, TRPC4 and TRPC5 channels possess a COOH-terminal "VTTRL" motif that binds to PDZ-domains of the scaffolding protein, EBP50 (NHERF1; Tang et al. 2000. *J Biol. Chem.* 275:37559–37564). The functional effects of EBP50 on TRPC4/5 activity have not been investigated. We have cloned rat TRPC5 (rTRPC5), functionally expressed it in HEK293 cell, and studied channel regulation with patch-clamp techniques. Both rTRPC5 and its VTTRL deletion mutant (r5dV) were localized to the plasma membrane. rTRPC5 did not display any significant basal activity in unstimulated HEK293 cells. In cells coexpressing rTRPC5 and H1 histamine receptor, rTRPC5 current evoked by GTPγS or histamine developed in two phases: a slowly developing, small inward current at negative potentials ("Phase 1") was followed by a rapidly developing, transient, large inward current ("Phase 2"). Each phase had a characteristic nonlinear current-voltage (I-V) relationship. Phase 2 was followed by a steady-state current whose I-V relationship resembled that of Phase 1. Deletion of the VTTRL motif had no detectable effect on the biophysical or kinetic properties of the channel. Coexpression of EBP50 with rTRPC5 caused a significant delay in the time-to-peak of the histamine evoked, transient large inward current, by prolonging Phase 1. EBP50 did not significantly modify the activation kinetics of the VTTRL-deletion mutant. We conclude that the VTTRL motif is not necessary for activation of TRPC5, but may mediate the modulatory effect of EBP50 on TRPC5 activation kinetics. (Supported by NIH grant NS40167 (Martha C. Nowycky) and AHA grant 0335076N (Alexander G. Obukhov).)

61. Role of Neuronal Calcium Sensor 1 Protein (NCS-1) in Human TRPC5 Calcium Channel Activity DAMIAN. M^{C}HUGH,[1] FANNING ZENG,[1] HUI HUI,[2] JAMIE L.WEISS,[2] and DAVID J.BEECH,[1] [1]*School of Biomedical Sciences, University of Leeds, Leeds LS2 9JT, UK;* [2]*Institute of Molecular Physiology, University of Sheffield, Sheffield S10 2TN, UK*

The Ca^{2+}-dependent nonselective cation channel TRPC5 is a membrane protein with a prominent role in providing a route for Ca^{2+} entry into excitable cells. While the nature of the moieties initiating channel gating remains elusive, it is apparent that TRPC5 gating is complex and subject to regulation by many signals arising close to the plasma membrane (Strübing et al. 2001. *Neuron.* 29:645–655). Here we examine whether NCS-1 plays a role in TRPC5 function in HEK293 cells, stably expressing tetracycline-regulated transcription of human TRPC5 (hTRPC5). $[Ca^{2+}]_i$ was monitored using Fura-PE3. Ca^{2+} signals from other cation channels were inhibited by 10 μM Gd^{3+}. After depletion of intracellular Ca^{2+} stores with 1 μM thapsigargin, store- and receptor-operated hTRPC5 activity were assessed. Muscarinic receptors were activated by 100 μM carbachol. Gd^{3+} (100 μM) was used in separate experiments to activate hTRPC5 directly. All modes of hTRPC5 activity were significantly inhibited by NCS-1 DN (E120Q; $P < 0.001$). By contrast, CaM_{1234} DN resulted in no inhibition of hTRPC5 activity. Mouse TRPC5 is known to be regulated by phospholipase C activity and we suggest a similar link for hTRPC5 because an inhibitor of phospholipase C, U73122 (10 μM), strongly inhibited the activity of hTRPC5. It seems plausible that NCS-1 DN affected hTRPC5 because it displaced endogenous NCS-1 from one of its protein partners, PI-4 kinase

(Zhao et al. 2001. *J. Biol. Chem.* 43:40183–40189), thereby reducing PIP_2 levels. We tested this idea by inhibiting PI-4 kinase with wortmannin (10 μM; 30 min). Unexpectedly, we found that store-operated TRPC5 activity was unaffected after wortmannin treatment. Therefore, it seems unlikely that NCS-1 DN affects TRPC5 via PI-4 kinase. We suggest NCS-1 plays a role in the function of TRPC5, possibly mediating the stimulatory effect of Ca^{2+} on its channel activity. (We thank The Wellcome Trust for support.)

62. Functional Consequences of Alternative Splicing in the II-III Loop of the $Ca_V2.2$ Calcium Channel ANNETTE C. GRAY and DIANE LIPSCOMBE, *Department of Neuroscience, Brown University, Providence, RI*

Calcium entry through $Ca_V2.2$ N-type calcium channels regulates neurotransmitter release from synapses. Alternative splicing within the II-III loop ($L_{II\text{-}III}$) protects this channel from action potential–dependent inactivation (Thaler et al. 2004. *Proc. Natl. Acad. Sci. USA.* 101:5675–5679), and may impact short-term dynamics of synaptic transmission. Specifically, we have shown that a $Ca_V2.2$ isoform that contains 21 additional amino acids encoded by exon 18a ($Ca_V2.2e[+18a]$) is significantly less sensitive to cumulative inactivation induced by trains of action potential waveforms, compared with $Ca_V2.2e[\Delta 18a]$. The $Ca_V2.2e[+18a]$ isoform exhibits reduced closed-state inactivation within a relevant range of membrane potentials and time periods. This likely protects the $Ca_V2.2e[+18a]$ isoform from action potential–induced cumulative inactivation. $L_{II\text{-}III}$ of $Ca_V2.2$ binds to synaptic vesicle proteins that modify channel inactivation. We hypothesized that tissue-specific inclusion of exon 18a modifies protein binding to $L_{II\text{-}III}$, thereby influencing channel inactivation. We performed yeast two-hybrid screens of a human brain cDNA library with baits of both exon 18a isoforms, containing the NH_2-terminal one-third of $L_{II\text{-}III}$. Positively interacting clones were sequenced, identified by BLAST search, and tested directly against both baits to determine isoform selectivity. Potential binding partners were also tested against a COOH-terminal bait of the channel, and many were eliminated from consideration based on cross-reactivity. We have identified a number of novel putative binding partners of $Ca_V2.2$ $L_{II\text{-}III}$, including cytoplasmic linker protein-170 (CLIP-170/Restin), Neimann-Pick disease type C2 (NPC2/HE1), synaptotagmin-like protein 1 (JFC1), and breast cancer–associated gene 3 (BCA3/KyoT). All proteins tested thus far interact with both $Ca_V2.2$ isoforms, although the possibility remains that $Ca_V2.2$ isoforms have different affinities for these novel $L_{II\text{-}III}$ binding partners. Differential protein binding to $L_{II\text{-}III}$ of $Ca_V2.2$ isoforms may influence more than channel inactivation, such as coupling of the channel to downstream effector proteins. (Supported by NIH grant NS29967. Annette C. Gray is a Howard Hughes Medical Institute Predoctoral Fellow.)

63. The Functional Unit for CFTR Cl^- Channels: One Polypeptide = One Pore ZHI-REN ZHANG,[1] XUE-HONG LIU,[2] DAVID C. DAWSON,[2] and NAEL A. MCCARTY,[1] [1]*School of Biology, Georgia Institute of Technology, Atlanta, GA;* [2]*Department of Physiology & Pharmacology, Oregon Health & Science University, Portland, OR*

The CFTR channel forms an anion-permeation pathway that is regulated by ATP binding and hydrolysis at the cytoplasmic nucleotide–binding folds. The domains of the CFTR protein that contribute to forming the pore are still being identified. We used the characteristics of CFTR channels containing engineered cysteine residues to determine the functional unit for the CFTR channel by asking how many channel pores are constructed from each CFTR polypeptide, and how many polypeptides are needed to make a functional channel. We studied the magnitudes and durations of subconductance states in wild-type CFTR and in CFTRs bearing substitutions in transmembrane domain six (TM6); these subconductances could reflect the activity of separate pores, or the different conductance states of a single pore. Within an open burst it was possible to distinguish three distinct conductance levels referred to as f (full), s1, and s2. Mutations in TM6 altered the duration and probability of occurrence of s1 and s2 states, but did not greatly alter the relative amplitudes. Covalent modification of R334C-CFTR by $MTSET^+$, monitored in real-time in inside-out patches, resulted in a simultaneous modification of all three conductance levels in what appeared to be a single step. This behavior suggested that at least a portion of the conduction path is common to all three conducting states. The time-course for modification of R334C-CFTR, measured in outside-out macropatches using a rapid perfusion system, was also consistent with a single modification step, as if each pore contained only a single copy of the cysteine at position 334. These results are consistent with a model for the conduction pathway in which a single pore is formed by a single CFTR polypeptide. (Supported by NIH-DK56481.)

64. Fast and Slow Na^+ Currents in Canine Cardiac Purkinje Cells MARCELLO ROTA and MARIO VASSALLE, *Department of Physiology and Pharmacology, State*

University of New York, Downstate Medical Center, Brooklyn, NY

One fast (I_{Na1}) and two slowly inactivating (I_{Na2} and I_{Na3}) TTX-sensitive Na^+ currents are present in canine cardiac Purkinje cells studied with a patch-clamp method. The aim of the present experiments was to determine by use of different voltage-clamp protocols whether these Na^+ components reflect the behavior of a fraction of the Na^+ channels responsible for the fast Na^+ current (I_{Na1}) or instead are due to different populations of Na^+ channels. Depolarizing steps from a V_h of 80 mV in increments of 2 mV activate I_{Na3} at ~60 mV, I_{Na1} at ~50 mV, and I_{Na2} at ~45 mV. I-V relations of I_{Na3} and I_{Na2} are separated by the activation of I_{Na1}, I_{Na2} being larger than I_{Na3}. The monoexponential decay of I_{Na3} is slower than that of I_{Na2}. Ramp and square pulse protocols to progressively less negative values activate in succession I_{Na3}, I_{Na1}, and I_{Na2}. During a ramp, I_{Na2} activates after I_{Na1} decay and during a step it inactivates slowly after I_{Na1} decay. Membrane conductance decreases gradually during both I_{Na3} and I_{Na2} and abruptly after I_{Na1}. If suitably slow ramps are used, no I_{Na1} is elicited, and I_{Na2} and I_{Na3} are identified by their different negative slopes (the slope for I_{Na2} being steeper and peaking at ~25 mV). During ramps negative to the I_{Na1} threshold only I_{Na3} is activated, whereas similar ramps applied from 50 mV (after I_{Na1} inactivation) elicit only I_{Na2}. Slower ramps negative to I_{Na1} threshold result in smaller I_{Na3}. It is concluded that I_{Na1}, I_{Na2}, and I_{Na3} are three separate Na^+ currents and not components with different kinetics of the same fast Na^+ channels. I_{Na3} appears to be important for the late diastolic depolarization and spontaneous discharge, I_{Na1} for the upstroke and conduction velocity, and I_{Na2} for the plateau duration. (Supported by an NIH grant.)

65. Diastolic Oscillations and the Mechanisms of Sympathetic and Cholinergic Control of the Sino-atrial Node Discharge JOHN N. CATANZARO, MICHAEL P. NETT, MARCELLO ROTA, and MARIO VASSALLE, *Department of Physiology and Pharmacology, State University of New York, Downstate Medical Center, Brooklyn, NY*

The role of the diastolic after-potential V_{os} and prepotentials ThV_{os} in the autonomic control of the sino-atrial node (SAN) discharge was studied with a microelectrode technique in guinea pig SAN perfused in high $[K^+]_o$. In quiescent SAN, norepinephrine (NE, 0.05–1 μM) increases diastolic depolarization (DD) amplitude and induces ThV_{os}, which initiates spontaneous discharge. In slowly active SA node, NE has little effect on the initial diastolic depolarization (DD1), increases V_{os} amplitude and causes larger and earlier ThV_{os} at more negative potentials, thereby increasing the rate. Carbachol (CCh, 0.05 μM) has little effect on DD1, slows discharge by decreasing V_{os} amplitude and causes smaller and later ThV_{os}. Eventually, ThV_{os} fail to reach the threshold and quiescence ensues. Higher concentrations of CCh (0.1–1 μM) hyperpolarize the membrane in diastole with a marked slowing of discharge quickly followed by quiescence. During CCh washout, the hyperpolarization subsides until ThV_{os} appear and reinitiate discharge. Cesium (2 mM) increases the rate through larger V_{os} and ThV_{os}, and does not hinder the actions of either NE or CCh. Barium (0.1 mM) increases the rate. We conclude that NE initiates discharge in quiescent SA node by inducing ThV_{os}, and increases the rate of a spontaneously active SA node mostly by increasing the size of V_{os} and the amplitude and slope of the depolarizing phase of ThV_{os}. CCh decreases the rate by decreasing V_{os} size and ThV_{os} size and amplitude so that the threshold is missed. In higher concentrations, CCh inhibits discharge by shifting the membrane potential negative to the oscillatory zone. The results with cesium suggest that I_f does not play a role either in the basal discharge of SA node dominant pacemakers or in neuromediators' actions. Barium and cesium increases dominant pacemaker discharge apparently by decreasing K conductance. (Supported by an NIH grant.)

66. Relationship between Overdrive Suppression and Overdrive Excitation in the Sino-atrial Node ADAM T. GRAZIANI and MARIO VASSALLE, *Department of Physiology and Pharmacology, State University of New York, Downstate Medical Center, Brooklyn, NY*

Overdrive suppression is much shorter in sino-atrial node (SAN) than in Purkinje fibers, possibly because of simultaneous overdrive excitation of dominant pacemaker cells (slow response due to Ca^{2+} entry) and overdrive suppression of subsidiary pacemaker cells (fast responses due to Na^+ entry). To test this hypothesis, discharge patterns and force changes induced by overdrive were studied in guinea pig SAN superfused in vitro. In Tyrode solution, the short overdrive suppression is little affected by faster or longer drives (in contrast to Purkinje fibers), since both overdrive excitation and suppression would be enhanced. In high $[K^+]_o$ (only slow response action potentials are present), overdrive percent-wise increases force more than in Tyrode solution and often induces overdrive excitation, which tends to be self-sustaining (sustained increase in Ca^{2+} load). In SAN quiescent in high $[K^+]_o$, increasing the driving rate to 60/min or above increases force due to larger Ca^{2+} entry and smaller extrusion of Ca^{2+}

(shorter diastole). Intermittent drives also progressively increases force and tends to cause overdrive excitation through a gradual accumulation of Ca^{2+}. High $[Ca^{2+}]_o$ increases force and rate, and prolongs overdrive excitation in high $[K]_o$. In high $[Ca^{2+}]_o$, a brief drive can initiate persistent discharge. Ni^{2+} decreases force prior and during overdrive and enhances overdrive suppression. TTX and lidocaine affect both overdrive excitation (less Ca^{2+} inside) and overdrive suppression (less Na^+ inside). We conclude that in SAN the short pause after drive is the net result of overdrive excitation of dominant pacemakers (due to a prevalent Ca^{2+} load and a less efficient extrusion of Ca^{2+}) and overdrive suppression of subsidiary pacemakers (due to the frequency-dependent large Na^+ load). The different behavior reflects acceleratory effect of an increased Ca^{2+} load in dominant and the electrogenic extrusion of sodium in subsidiary pacemakers. (Supported by an NIH grant.)

67. Indirect Evidence for Apical Bicarbonate Conductance in Guinea-pig Pancreatic Duct Cells HIROSHI ISHIGURO, SATORU NARUSE, SHIGERU B. H. KO, AKIKO YAMAMOTO, TAKAHARU KONDO, MAYNARD CASE, and MARTIN STEWARD, *Human Nutrition and Internal Medicine, Nagoya University Graduate School of Medicine, Nagoya, Japan, and School of Biological Sciences, University of Manchester, Manchester, UK*

The interlobular duct segments of guinea-pig pancreas secrete HCO_3^- into an HCO_3^--rich (125 mM) luminal fluid after secretin stimulation (Ishiguro et al. 1998. *J. Physiol.* 511:407–422). Under these conditions intracellular HCO_3^- is ~20 mM and intracellular potential is approximately −60 mV, indicating that there is a luminally directed electrochemical gradient for HCO_3^- (Ishiguro et al. 2002. *J. Gen. Physiol.* 120:617–628). To examine whether apical HCO_3^- transport is mediated by an anion conductance, we measured changes in intracellular pH (pH_i) when the cells were de- or hyperpolarized by manipulation of extracellular K^+ ($[K^+]_B$).

The isolated ducts were superfused with HCO_3^-/CO_2-free HEPES-buffered solution and luminally perfused with 125 mM HCO_3^-, 24 mM Cl^-, and 5% CO_2. pH_i was measured with BCECF.

When $[K^+]_B$ was raised from 5 to 70 mM, pH_i in the unstimulated ducts changed only slightly. In the presence of dbcAMP, the depolarization caused a large increase in pH_i from 6.83 ± 0.11 to 7.32 ± 0.09 (mean ± SEM, $n = 4$, $P < 0.01$). When $[K^+]_B$ was reduced from 5 to 1 mM, pH_i decreased by 0.11 ± 0.01 ($P < 0.05$). Under Cl^--free conditions, when $[K^+]_B$ was reduced from 5 to 1 mM and then raised to 70 mM in the presence of dbcAMP, pH_i decreased from 7.15 ± 0.06 ($n = 4$) to 7.06 ± 0.07 and then increased to 7.54 ± 0.16 ($P < 0.05$).

In summary, de- and hyperpolarization caused changes in pH_i that most probably reflected the influx and efflux of HCO_3^- across the apical membrane. These HCO_3^- movements were not dependent on the presence of Cl^- and may be attributed to the presence of a significant HCO_3^- conductance at the apical membrane.

68. Probing for Kv Channel Secondary Structure Inside the Ribosome Tunnel JIANLI LU and CAROL DEUTSCH, *Department of Physiology. University of Pennsylvania. Philadelphia, PA 19104*

Transmembrane segments in the intact voltage-gated K^+ (Kv) channel are helical, whether assayed by tryptophan scanning, alanine scanning, or more recently, by direct crystallographic techniques. But, when is this helicity first manifested? Could it be soon after the peptide segment is synthesized inside the ribosome? The Kv protein is synthesized at the peptidyl transferase center in the cleft between the small and large ribosome subunits and then wends its way along an exit tunnel as the nascent peptide is elongated. The tunnel is ~100-Å long with a minimum diameter of 10 Å near the entrance and 20 Å at the widest point near the exit site. The length of the protein when it first emerges from the ribosome at the exit site should therefore reflect the secondary structure of the nascent peptide. To address the question of when secondary structure is first acquired, we have engineered a cysteine at various positions along the S6 transmembrane segment of Kv1.3 and generated biogenic intermediates still attached to the ribosome (Lu et al. 2001. *Biochem.* 40: 10934–10946). When the cysteine emerges from the tunnel, it can be mass-tagged with PEG-MAL and detected by a gel shift assay (Lu and Deutsch. 2001. *Biochem.* 40: 13288–13301). A new, bulky mass-tag, calmodulin-MAL, confirms the location of these cysteines. A molecular tapemeasure containing only extended loops or β-strands indicates that the functional length of the tunnel, ~109 Å, is longer than the anatomical length and that some regions of S6 form α-helices inside the ribosome tunnel. (Supported by NIH GM 52302.)

69. When Does the T1 Domain of Nascent Kv1.3 Acquire its Secondary and Tertiary Structure? ANDREY KOSOLAPOV, LIWEI TU, JING WANG, and CAROL

DEUTSCH, *Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104*

Voltage-gated K^+ (Kv) channels contain a recognition domain, T1, in the amino terminus that facilitates Kv tetramerization. We have previously shown that this domain begins to fold into its tertiary structure while the nascent peptide is still attached to the ribosome, but before it targets to the endoplasmic reticulum (Kosolapov and Deutsch. 2003. *J. Biol. Chem.* 278:4305–4313). We now ask when after the T1 domain emerges from the ribosome does it fold. Can it fold while still inside the ribosome? Using Kv1.3 biogenic intermediates of different lengths that contain cross-linkable cysteine pairs engineered at the folded intrasubunit interface, we have determined the length of peptide linker at the COOH-terminal end of T1 that permits tertiary structure formation. T1 cannot fold while its COOH terminus is still inside the ribosome, but this same T1 construct can fold when it is released from the ribosome. Thus, it is the constraining ribosome tunnel that prevents folding. Moreover, our data have implications for secondary structure formation (α-helix, β-strand) inside the ribosome, specifically for the T1 domain and for the T1-S1 linker. Regions of the T1 domain acquire a compact secondary structure (likely α-helix) inside the ribosome tunnel. The combined use of pegylation and an intramolecular folding assay (Lu and Deutsch. 2001. *Biochem.* 40:13288–13301; Kosolapov and Deutsch. 2003. *J. Biol. Chem.* 278: 4305–4313) provides a general approach to determining acquisition of secondary and tertiary structure during early biogenesis. (Supported by NIH grant GM 52302, NRSA # HL-07027, and AHA Grant-In-Aid.)

70. Postsynaptic Calcium Stores Modulate Nicotinic Inhibition of Cochlear Hair Cells JEE-HYUN KONG, MARIA LIOUDYNO, ELEONORA KATZ and PAUL FUCHS, *The Center for Hearing and Balance, Department of Otolaryngology, Head and Neck Surgery, the Johns Hopkins University School of Medicine, Baltimore, MD*

Mechanosensory hair cells in vertebrates are subject to feedback inhibition from cholinergic brainstem neurons. Acetylcholine activates nicotinic receptors (nAChRs) that include α9 and α10 subunits (Elgoyhen et al. 1994. *Cell.* 79:705-715; Elgoyhen et al. 2001. *Proc. Natl. Acad. Sci. USA.* 98:3501–3506). Calcium influx through nAChRs triggers the gating of small-conductance, calcium-activated potassium (SK) channels (Fuchs and Murrow. 1992. *J. Neurosci.* 12:800–809). While calcium influx alone can account for many features of the cholinergic response (Fuchs and Martin. 1992. *Proc. Roy. Soc. London B.* 250:71–76), other evidence suggests that intracellular calcium stores might provide additional calcium, or otherwise modulate efferent inhibition (Sridhar et al. 1997. *J. Neurosci.* 17:428–437). In this study we examined the effect of ryanodine and related compounds on the transient synaptic currents produced in outer hair cells by ACh released from efferent endings in an organ of Corti preparation excised from young rats. As shown previously (Glowatzki and Fuchs. 1999. *Science.* 288:2366–2368), "spontaneous" ACh release causes transient activation of SK channels in cochlear hair cells, producing a current of approximately −100 pA at −90 mV (in 25–40 mM K^+ saline) and with a decay time constant of ∼40 ms at 22°C. Extracellular ryanodine at 100 μM reversibly reduced the average synaptic current by ∼30%, with no effect on time course. The ryanodine receptor antagonist, 8-Br-cADPR, caused a smaller (14%) but still significant and reversible reduction in average amplitude. To avoid possibly confounding effects of these drugs on the presynaptic release of ACh, ryanodine or cADPR were added directly to the internal solution of the whole-cell recording pipette. Addition of 100 μM ryanodine produced no significant change in synaptic currents compared with those of control cells. However, 3 μM cADPR, a ryanodine receptor agonist, was associated with a 33% increase in mean amplitude that was highly significant. (Supported by NIH grants DC01508 and TW006247.)

71. Sphingosine-1-Phosphate Controls Early Keratinocyte Differentiation via Intracellular Ca^{2+} Mobilization YOSHIKAZU UCHIDA, WALTER M. HOLLERAN, and THEODORA M. MAURO, *Department of Dermatology, University of California San Francisco and Department of Veterans Affairs Medical Center, San Francisco, CA*

Mammalian epidermis is characterized by the presence of a basal, dividing cell layer, with suprabasal cells undergoing both migration and terminal differentiation to yield the enucleate cells of the outermost skin layer, the stratum corneum. This vectoral process of keratinocyte differentiation is intrinsically linked to both intra- and extracellular calcium levels, with low extracellular calcium promoting proliferation, and elevated calcium initiating and sustaining differentiation. In addition, lipid signaling is central to the control of both epidermal keratinocyte proliferation and differentiation, with the IP-3–mediated response to raised extracellular Ca^{2+} being the most-thoroughly characterized lipid-signaling pathway to date. However, we now find that specific events in early keratinocyte differentiation are mediated through a sphingosine-1-phosphate (S1P)

pathway. In contrast to IP3-mediated events, we found that S1P did not require elevated extracellular Ca^{2+} as a stimulus. Rather, in undifferentiated, proliferative keratinocytes; i.e., maintained in low extracellular Ca^{2+} (0.03 mM) medium, exogenous S1P (5 μM) significantly inhibited cell proliferation. Furthermore, intracellular Ca^{2+}, measured with Fura-2, transiently increased after S1P addition, due to release of Ca^{2+} from intracellular stores. Although S1P itself did not induce capacitive Ca^{2+} entry, it did enhance thapsigargin-induced capacitive Ca^{2+} entry. Unlike IP3-mediated Ca^{2+} signaling, S1P exposure resulted in transient Ca^{2+} signals, suggesting a distinct role for S1P-mediated signaling in the transition from proliferative-to-differentiative keratinocytes (early differentiation) rather than in late (or terminal) differentiation events. Consistent with this hypothesis, exogenous S1P reduced the levels of both the differentiation-specific protein involucrin, as well as procaspase-14 protein, normally expressed only in differentiated keratinocytes. These data suggest that an alternative, S1P-mediated signaling pathway, which signals independently of extracellular Ca^{2+}, enhances early rather than late keratinocyte differentiation processes. (Supported by NIH grant PO1-AR39448 to Walter M. Holleran and Theodora M. Mauro.)

72. Changes in Local Intracellular Ca^{2+} Concentration Triggers Ca^{2+} Current Oscillations in *C. elegans* Intestinal Epithelial Cells ANA Y. ESTEVEZ and KEVIN STRANGE, *Anesthesiology Research Division, Vanderbilt University Medical Center, Nashville, TN*

Inositol-1,4,5-trisphosphate (IP_3)–dependent Ca^{2+} oscillations in *C. elegans* intestinal epithelial cells regulate the nematode defecation cycle (Dal Santo et al. 1999. *Cell* 98:757–767), an ultradian rhythm with a periodicity of 45–50 s. To elucidate molecular mechanisms of oscillatory Ca^{2+} signaling, we are combining patch-clamp electrophysiology with behavioral assays, intracellular Ca^{2+} imaging, and forward and reverse genetic screening. We have shown previously that cultured *C. elegans* intestinal cells express two highly Ca^{2+}-selective conductances. One of these, I_{ORCa} (outwardly rectifying calcium current), shows strong outward rectification, inhibition by intracellular Mg^{2+}, and insensitivity to intracellular Ca^{2+} store depletion. I_{ORCa} bears strong biophysical resemblance to TRPM7 currents. When intestinal cells are patch clamped with buffers containing 10 mM BAPTA and 11 nM Ca^{2+}, I_{ORCa} activates slowly and reaches stable amplitudes of 200–300 pA/pF at +80 mV. However, when cells are dialyzed with 1 mM BAPTA, I_{ORCa} activity oscillates dramatically. Oscillations are blocked by removal of extracellular Ca^{2+} or addition of 100 μM La^{3+}. Readdition of Ca^{2+} to the bath or removal of La^{3+} induces immediate oscillatory current activation. Oscillations are also blocked by clamping cells at hyperpolarized voltages or by elevating intracellular Ca^{2+} concentration. Elevation of intracellular Ca^{2+} in the presence of 10 mM BAPTA has dual effects on channel activity. As Ca^{2+} concentration is increased, both the rate of current activation and peak current amplitude increase. However, at Ca^{2+} concentrations ≥250 nM, current amplitude does not stabilize, but instead declines slowly. We conclude that changes in Ca^{2+} concentration close to the intracellular opening of the channel pore regulate channel activation. Low concentrations of Ca^{2+} activate ORCa. As Ca^{2+} enters and accumulates at the pore mouth, channel activity is inhibited. I_{ORCa} oscillations may play a role in generating intracellular Ca^{2+} oscillations that drive the *C. elegans* defecation rhythm. (Supported by NIH DK51610.)

73. SAPKs and Hyperosmotic Volume Regulation in Mussel Gills LEWIS E. DEATON, *Biology Department, University of Louisiana at Lafayette, Lafayette, LA*

Stress-activated protein kinase (SAPK) signaling pathways have been shown to be activated by hyperosmotic stress in yeast and mammalian cells. Exposure of bivalve gill tissues to hyperosmotic media induces a rapid accumulation of amino acids (primarily alanine) and betaine to increase the cytoplasmic osmotic concentration. The mechanism involved in the initiation and control of this process is unknown. We incubated isolated gills from the euryhaline mussel *Geukensia demissa* in hyperosmotic seawater (SW) or hyperosmotic SW containing the SAPK inhibitor cyclosporin and measured the levels of alanine and betaine in the tissues. Gills transferred from 250 to 1,000 mOsm SW for 4 h contained 107 ± 30.4:mol/g dry wt betaine and 75.1 ± 33.9:mol/g alanine. Gills incubated in 1,000 mOsm SW containing 10:g/ml cyclosporin contained 52 ± 12:mol/g betaine and 21.5 ± 26.5:mol/g alanine. Both of these decreases were statistically significant. Gills were incubated in 250 mOsm SW with the SAPK activators anisomycin (10:mol/g) and ceramide (10:M). Gills maintained in 250 mOsm SW contained 14 ± 4: mol/g betaine and 6.8 ± 7.1:mol/g alanine. In 250 mOsm SW containing anisomycin, the level of betaine in the gills was 17.5 ± 3:mol/g; the alanine content was 20.6 ± 11.6:mol/g. The increases are significant. Gills incubated in 250 mOsm SW containing ceramide contained 15 ± 5:mol/g betaine and 17.8 ± 18.3:mol/g alanine. These levels are not different from the controls. A Western blot showed that the gill tissue contains

two proteins that bind antibody to the yeast kinase Hog1 (high osmotic glycine). The molecular weights of the two proteins are about 80 and 90 kD. These results suggest that SAPKs may play a role in hyperosmotic cellular volume regulation in bivalved molluscs.

74. L-type Ca^{2+} Channels Are Present in Caveolae and Associated with Caveolin-3 and β_2-AR in Ventricular Myocytes RAVI C BALIJEPALLI,[1] JASON D. FOELL,[1] DUANE D. HALL,[2] JOHANNES W. HELL,[2] and TIMOTHY J. KAMP,[1] [1]*Department of Medicine, University of Wisconsin, Madison, WI;* [2]*Department of Pharmacology, University of Iowa, Iowa City, IA*

Caveolae are specialized structures in the plasma membrane composed of cholesterol, sphingolipids, and structural proteins termed caveolins. Given the importance in cell signaling of intracellular Ca^{2+}, we hypothesized that L-type Ca^{2+} channels may be localized to caveolae associated with proteins in the cAMP/PKA-signaling pathway. Canine and neonatal mouse ventricular myocytes were employed for biochemical, confocal line scan and electrophysiology studies. Detergent-free sucrose density gradient fractionation of the ventricular tissue or isolated myocytes yielded 12 membrane fractions (5–42% sucrose). Western blot analysis of the gradient fractions demonstrated that caveolin-3, β_2-adrenergic receptor (β_2AR) and $Ca_v1.2$ subunits of the L-type Ca^{2+} channels were predominantly present in fractions 4–6 (15–21% of sucrose). In contrast, β_1-adrenergic receptors (β_1-AR) were more broadly distributed in the gradient. Indirect immuno-fluorescence confocal microscopy of isolated ventricular myocytes showed colocalization of $Ca_v1.2$ and caveolin-3 in the surface sarcolemma and T-tubules. Furthermore, immunoprecipitation from neonatal and adult ventricular homogenates using an antibody directed against $Ca_v1.2$ followed by Western blot analysis revealed that caveolin-3 and β_2-AR coimmunoprecipitate with $Ca_v1.2$ channels, in contrast the β_1-AR did not. Alternatively, immunoprecipitation using the caveolin-3 antibody confirmed that $Ca_v1.2$ channels and β_2-AR coimmunoprecipitate with caveolin-3. Further investigation of the $Ca_v1.2$ or caveolins-3 immunoprecipitates revealed that adenelyl cyclase (AC), catalytic subunit of protein kinase A (PKA), and protein phosphatase 2A (PP2A) are associated with $Ca_v1.2$ and caveolin-3. Disruption of the caveolar lipid rafts by depleting membrane cholesterol with 1 mM methyl-β-cyclodextrin caused a reduction in Ca^{2+} transients elicited by field stimulation; however, methyl β-cyclodextrin treatment did not affect the Ca^{2+} current density. We conclude that a subpopulation of $Ca_v1.2$ channels and associated proteins of the β_2AR/cAMP/PKA–signaling pathway are compartmentalized to caveolar microdomains and contribute to EC coupling in the heart.

75. Do Adult Skeletal Muscle Traverse Tubules Contain Caveola? G. BARRIENTOS[1] and A. QUEST,[2] [1]*Centro FONDAP de Estudios Moleculares de la Célula,* [2]*Programa de Biología Celular y Molecular, ICBM, Facultad de Medicina, Universidad de Chile*

Transverse tubules (TT) are plasma membrane invaginations that propagate action potentials to the inside of the skeletal muscle fiber. The lipid composition of isolated TT, with 35% cholesterol and 15% sphingomyelin (Rosemblatt, M., C. Hidalgo, C. Vergara, N. Ikemoto. 1981. *J. Biol. Chem.* 256:8140–8148) is equivalent to that of caveolae and lipid rafts. Some reports indicate that caveolin-3 is present only in developing skeletal muscle TT while others describe expression of caveolin-3 in adult skeletal muscle TT. We explored the expression of caveolin-3 in adult skeletal muscle fibers. By means of subcellular fractionation experiments, we found high levels of caveolin-3 in isolated TT and triads. After a classical method to isolate rafts/caveola, we isolated in sucrose density gradients light fractions from TT and triads solubilized with Triton X-100 at 4°C, which were markedly enriched in caveolin-3 and cholesterol, two markers of caveolae. These results indicate that caveolin-3 is present both in free TT and in triad-attached (junctional) TT isolated from adult skeletal muscle fibers, presumably forming part of cholesterol-rich caveola. Light fractions obtained from triads, however, had lower cholesterol contents and appeared at higher sucrose density than light fractions obtained from free TT. Addition of methyl-β-cyclodextrin to triads to remove cholesterol induced the release of calsequestrin, a luminal SR protein, and of DHPR to the supernatant, suggesting that cholesterol removal disrupts SR and TT integrity. We confirmed the distribution of caveolin-3 in junctional TT that were directly isolated from triads. These results show that skeletal muscle TT contain caveolin-3 in a region highly enriched in DHPR and close to RyR and raise the possibility of caveolin regulation of muscle excitation contraction coupling. (Supported by FONDAP 15010006.)

76. A Novel Genetic Approach to IP_3R Inhibition: Design and Characterization of "Dominant-negative" IP_3R2 SCOT J MATKOVICH, SUZHAO LI, and ANDREW R MARKS, *Department of Physiology, Columbia University College of Physicians and Surgeons, New York, NY 10032*

While inositol 1,4,5-trisphosphate receptors (IP_3Rs) are critical mediators of intracellular Ca release in numerous cell types, their precise function in tissues also expressing ryanodine receptors (RyRs), such as the heart, is not clear. To overcome the limited selectivity and pleiotropic effects of pharmacological IP_3R inhibitors, we have designed mutant IP_3R, which can bind IP_3 but cannot conduct Ca, as a basis for studies of IP_3R functional inhibition, taking the approach that overexpression of a given gene is more easily realized than gene knockout. We mutated three key amino acids necessary for Ca channel function in full-length IP_3R2 (V2499Y, D2501A, and D2520A), added a FLAG epitope at the COOH terminus to distinguish mutant IP_3R2 from wild-type IP_3R2, and stably expressed this construct (IP_3R2-DN) in HEK293 cells, which express predominantly IP_3R2 relative to other isoforms. IP_3R2-DN is localized to the endoplasmic reticulum, forms complexes with coexpressed recombinant IP_3R2 and coimmunoprecipitates with endogenous IP_3R2 in HEK293. Expression of IP_3R2-DN significantly reduced carbachol-stimulated Ca release in fura-2 loaded HEK293 in a manner dependent on the level of IP_3R2-DN expression. IP_3R function is critical for both proliferation and induction of apoptosis in Jurkat T lymphocytes (Jayaraman and Marks. 1997. *Mol. Cell. Biol.* 17:3005–3012) and staurosporine (STS)-mediated apoptosis of HEK293 was lower in cells expressing IP_3R2-DN compared with cells expressing wild-type, FLAG-tagged IP_3R2 (wild-type: no STS, 1.70% apoptosis; with STS, 12.0%; IP_3R2-DN no STS, 2.74%; with STS, 4.40%). In addition, proliferation of IP_3R2-DN cells over 7 d (9.4 ± 0.2-fold) was lower than wild-type IP_3R2 cells cultured under identical conditions (20 ± 1.5-fold). These experiments demonstrate that expression of IP_3R2-DN inhibits endogenous IP_3R signaling, thus forming the basis for studies in which IP_3R function will be ablated by transient overexpression in cellular systems or by tissue-specific overexpression of IP_3R2-DN in transgenic animals.

77. Regulation of the Type 1 Inositol 1,4,5-trisphosphate Receptor by Phosphorylation at Tyrosine 353 JIE CUI, SCOT J. MATKOVICH, NIKHIL DESOUZA, SUZHAO LI, NORA ROSEMBLIT, and ANDREW R. MARKS, *Department of Physiology, Columbia University College of Physicians and Surgeons, New York, NY 10032*

The inositol 1,4,5-trisphosphate receptor (IP_3R) plays an essential role in Ca^{2+} signaling during lymphocyte activation. Engagement of the T cell or B cell receptor by antigen initiates a signal transduction cascade that leads to tyrosine phosphorylation of IP_3R by Src family nonreceptor protein tyrosine kinases, including Fyn (Jayaraman et al. 1996. *Science.* 277:1492–1494). However, the effect of tyrosine phosphorylation on the IP_3R and subsequent Ca^{2+} release is poorly understood. We have identified tyrosine 353 (Y353) in the IP_3 binding domain of type 1 IP_3R (IP_3R1) as a phosphorylation site for Fyn both in vitro and in vivo. We developed a phosphoepitope-specific antibody and showed that IP_3R1-Y353 becomes phosphorylated during lymphocyte activation. Furthermore, at low IP_3 concentrations (<10 nM) tyrosine phosphorylation of wild-type IP_3R1 increased IP_3 binding from an EC_{50} of 19.34 ± 1.55 nM (nonphosphorylated) to 8.45 ± 1.36 nM (phosphorylated) ($n = 7$, $P < 0.05$), but an increase in binding affinity was not observed in an IP_3R1-Y353F mutant that cannot be tyrosine phosphorylated at Y353 (EC_{50} 19.34 ± 1.55 nM, $n = 7$, $P > 0.05$ relative to wild-type IP_3R1). Using wild-type IP_3R1 and IP_3R1-Y353F expressed in IP_3R-deficient DT40 B cells, we demonstrate that tyrosine phosphorylation of Y353 permits prolonged intracellular Ca^{2+} release during B cell activation (time constant for normalized maximum decay rate = 0.135 ± 0.011 for wild-type IP_3R1, 0.266 ± 0.020 for IP_3R1-Y353F, $P < 0.01$, $n = 4$). Overexpression of wild-type IP_3R1 in Jurkat increased TCR-stimulated NFAT-luciferase activity above vector-transfected cells but overexpresssion of IP_3R1-Y353F resulted in less enhancement of NFAT activity (vector transfection 16.0 ± 2.00, wild-type IP_3R1 55.3 ± 1.69, IP_3R1-Y353F 40.2 ± 1.75, arbitrary units, $P < 0.05$ between wild-type and –Y353F or vector, $n = 7$). Taken together, these data suggest that one function of tyrosine phosphorylation of IP_3R1-Y353 is to enhance Ca^{2+} signaling in lymphocytes by increasing the sensitivity of IP_3R1 to activation by low levels of IP_3.

78. Cell Surface Organization and Dynamics of the $Serotonin_{1A}$ Receptor SHANTI KALIPATNAPU, THOMAS J. PUCADYIL, and AMITABHA CHATTOPADHYAY, *Centre for Cellular and Molecular Biology, Hyderabad, India*

$Serotonin_{1A}$ receptors are prototypical members of the G-protein–coupled receptor superfamily and represent a prime target for therapeutic actions of several anxiolytic and antidepressant drugs. Organization of lipids and proteins in membranes is believed to be crucial in regulating signal transduction processes. We have investigated the organization and dynamics of the $serotonin_{1A}$ receptor in relation to membrane domains, particularly those that are enriched in cholesterol and sphingolipids, using CHO cells stably expressing the human $serotonin_{1A}$ receptor fused to yellow fluorescent protein ($serotonin_{1A}$-EYFP). This fusion protein mimics

the native receptor in terms of ligand binding properties and cell-signaling functions. Insolubility of membrane constituents in nonionic detergents such as Triton X-100 has been a widely used biochemical criterion to indicate their presence in membrane domains. We observe significant retention of fluorescence of serotonin$_{1A}$-EYFP receptors upon Triton X-100 treatment of intact cells demonstrating their detergent insolubility. Control experiments performed with membrane domain-specific fluorescent lipid probes and protein markers validate this approach. The relationship of the receptor to membrane domains was further explored by analyzing lateral diffusion characteristics of the receptor by fluorescence recovery after photobleaching (FRAP) experiments. We observe considerable heterogeneity in lateral diffusion of the receptor on the cell surface that is reduced upon lowering the cholesterol content of membranes. This heterogeneity could indicate the existence of domains at the plasma membrane which require membrane cholesterol. Our results on the cell surface dynamics of serotonin$_{1A}$ receptors that display significant detergent insolubility provide interesting insights into their organization at the cell surface. (Supported by the Council of Scientific and Industrial Research, Government of India.)

79. Membrane Structures Analyzed by Cryo-focused Ion Beam Imaging JULIE E. M MCGEOCH and GUIDO GUIDOTTI, *Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA*

A stream of gallium ions (1–100 pAmps) were focused on live flash-frozen macrophage and epithelial cells at −120C, 1.4e-6mbars, to sculpt away a few nano meters at a time the cell structure. Images collected in a serial manner as the successive layers of the internal organelles were milled away, provided detailed anatomical detail at a resolution of 30 nm. The cells were sputter-coated with 10 nm of Au to reduce charging and as this coat was removed a pattern of Au particles plugging the pores of ion channels was revealed. Beneath the plasma membrane of epithelial cells the endoplasmic reticulum (ER) was seen to abut to the membrane and then branch repeatedly with reduction inwards toward the nucleus. ER cisternae (100-nm diameter) encountering mitochondria or other vesicles move out and around each organelle. The ER of macrophages (200–500 nm) was much wider than that of epithelia and possessed funnel like openings (40–100-nm diameter) showing 20-nm internal helical membrane structures of helix diameter 60–80 nm. Any of these organelles could be chiseled free from the cell with a tungsten probe and 300–1,000-nm side size cubes could be transported to other substrates (for instance antibody array protein chips) for analysis of the organelle protein profile content. This Cryo-FIB technique provides new insight into cell architecture and points to a way of establishing the identities of membrane protein functional complexes. (Supported by NIH grant HL 08893.)

80. Preferential Regional Contraction in Barnacle Muscle Cells May Be Due to Local Structural Variations JILLIAN THEOBALD,[1] HECTOR RASGADO-FLORES,[1] STUART TAYLOR,[2] JOSEPH P.R. ORGEL,[1] THOMAS C. IRVING,[2] HUGO GONZALEZ-SERRATOS,[3] CECILIA PENA-RASGADO,[1] MIGDALIA GAMBOA,[1] and HECTOR RASGADO-FLORES,[1] [1]*FUMS/Chicago Med. School, North Chicago, IL 60064;* [2]*Illinois Inst. Technology, Chicago, IL 60616;* [3]*University of Maryland, Baltimore, MD 21201*

Skeletal muscle contraction has been shown to produce osmotic and nonosmotic volumes changes that can affect contraction (Bagni, M.A. et al. 1990. *J. Physiol. (Lond.)*. 430:61–75; Neering, I.R. et al. 1991. *Biophys. J.* 59:926–932) Nonosmotic changes are due to lattice spacing changes (Cecchi, G. et al. 1990. *Science.* 250: 1409–1411) while osmotic result from a net loss or gain of osmolytes (Rapp, G. et al. 1999. *Biophys. J.* 75:2984–2995). What role these changes play in barnacle muscle cell (BMC) contraction is currently unknown. BMCs permit simultaneous, direct measurement of intracellular pressure, ionic composition, membrane potential, pH, force generation, and cross-sectional area. Further, they undergo a slow contraction and relaxation cycle, allowing easy detection of volume changes throughout contraction. In BMCs, isometric contractions induced by electrical or KCl stimulation result in a nonhomogeneous contraction. There is a local increase 50–75% in cross-sectional area (bulging) between the midportion and tendon-end of the cell. We propose that bulging results from an intrinsic architectural arrangement of the cell. To test this, we have examined changes in volume, intracellular pressure, sarcomere length, and myofilament lattice spacing during isometric contraction along the length of the cell. The results show that: (a) two populations of thick filaments are observed upon K^+-induced contraction, with d-space spacing values of ∼55.5 and ∼59 nm; (b) two populations exist along the entire length of the cell during isometric contraction and at the cellular ends in the resting cell; (c) sarcomere spacing increases from basal to tendon end; (d) isometric contractions induce similar reductions in intracellular pressure at the basal and tendon end; (e) intracellular pressure changes are reduced on either side of and become null in the bulging region; and (f) mechanical prevention of bulging dampened the cells

ability to generate force by ~75%. We propose that: (a) BMCs are architecturally designed to contract over a small portion of their length, possibly to conserve energy; and (b) the majority of force is transmitted laterally and is prevented by mechanical restriction of bulging. (Support by AHA 0256070ZI.)

81. Mechanisms of Synergistic p70S6 Kinase (p70S6K) Activation by EGF and Thrombin KOK CHOI KONG, CHARLOTTE K. BILLINGTON, and RAYMOND B. PENN, *Department of Internal Medicine and the Center for Human Genomics, Wake Forest University, Winston Salem, NC*

Airway remodeling is a major contributing factor of chronic asthma resulting from airway smooth muscle (ASM) hypertrophy and hyperplasia. We have previously demonstrated that stimulation of human ASM (HASM) with the GPCR agonist thrombin potentiates EGF-mediated proliferation via a p70S6 kinase–dependent, p42/p44 MAPK–independent pathway (Krymskaya et al. 2000. *Am. J. Respir. Cell. Mol. Biol.* 23:546–554). Mechanisms mediating the synergistic activation of p70S6K and HASM growth are unclear. Stimulation of HASM with thrombin increased EGF-induced phosphorylation of p70S6K at residues T389, T229, and T421/S424 at 4 h but not at 30 min. This effect was not attenuated by pretreatment with singular or combined inhibition of PKC, Rho, Rho kinase, or Gi (via pertussis toxin; PTX) signaling. Moreover, proline-directed residues did not appear to be the critical locus through which thrombin potentiated p70S6K activity, as a p70S6K mutant (D4) in which the COOH-terminal proline-directed sites were mutated to acidic residues still exhibited cooperative T389 phosphorylation by EGF+thrombin. However, synergistic p70S6K phosphorylation could also be observed in murine embryonic fibroblasts (MEFs), and this could be reversed only in Gq/11−/− MEFs pretreated with PTX. These data suggest that signals from either Gq/11 or Gi are sufficient to mediate the effects of thrombin on EGF-stimulated p70S6K phosphorylation via a mechanism regulating a T229 or T389 kinase or phosphatase. (Funded by HL65338. Raymond B. Penn is recipient of a Career Investigator Award from the American Lung Association.)

82. Location, Location, Location: Exclusive Granule vs. Membrane Localization of Neutrophil Elastase Causes Two Different Inherited Forms of Neutropenia KATHLEEN F. BENSON,[1] FENG-QIAN LI,[1] RICHARD E. PERSON,[1] DALILA ALBANI,[1] ZHIJUN DUAN,[1] JEREMY WECHSLER,[1] KIMBERLY MEADE-WHITE[1], KAYLEEN WILLIAMS,[1] GREGORY M. ACLAND,[2] GLENN NIEMEYER,[3] CLINTON D. LOTHROP,[3] and MARSHALL HORWITZ,[1] [1]*Department of Medicine, Division of Medical Genetics, University of Washington, Seattle, WA;* [2]*College of Veterinary Medicine, Cornell University, Ithaca, NY;* [3]*College of Veterinary Medicine, Auburn University, Auburn, AL*

Cyclic hematopoiesis is a stem cell disease in which the number of neutrophils and other blood cells oscillates in weekly phases. Autosomal dominant mutations of *ELA2*, encoding the protease neutrophil elastase, found in lysosome-like granules, cause cyclic hematopoiesis and most cases of the preleukemic disorder severe congenitial neutropenia (SCN). Over 20 different mutations of neutrophil elastase have been identified, but their consequences are elusive, because they confer no consistent effects on enzymatic activity. The similar autosomal recessive disease of dogs, canine cyclic hematopoiesis, is not caused by mutations in *ELA2*. Instead it is caused by homozygous mutation of the gene encoding the dog adaptor protein complex 3 (AP3) β-subunit, directing trans-Golgi export of transmembrane cargo proteins to lysosomes. COOH-terminal processing of neutrophil elastase exposes an AP3 interaction signal responsible for redirecting neutrophil elastase trafficking from membranes to granules. Disruption of either neutrophil elastase or AP3 perturbs the intracellular trafficking of neutrophil elastase. Most mutations in *ELA2* that cause cyclic hematopoiesis prevent membrane localization of neutrophil elastase, whereas most mutations in *ELA2* that cause SCN lead to exclusive membrane localization.