ABSTRACTS OF PAPERS AT THE SIXTY-FIRST ANNUAL MEETING OF THE SOCIETY OF GENERAL PHYSIOLOGISTS

MEMBRANE BIOPHYSICS OF FUSION, FISSION, AND RAFTS IN HEALTH AND DISEASE

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Organized by
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Class II Virus Membrane Fusion Proteins: Mechanisms and Inhibition. MARGARET KIELIAN, Department of Cell Biology, Albert Einstein College Of Medicine, Bronx, NY 10461

Alphaviruses and flaviviruses infect cells through low pH-dependent membrane fusion reactions mediated by their structurally similar virus fusion proteins. During fusion these class II fusion proteins trimerize and refold to form hairpin-like structures with the domain III and stem regions folded back toward the target membrane-inserted fusion peptides. Using the alphavirus Semliki Forest virus (SFV) and the flavivirus dengue virus, we have demonstrated that exogenous domain III can function as a specific inhibitor of membrane fusion and infection. Surprisingly, the fusion protein stem region is not specifically required for SFV fusion activity. Our data reveal the existence of a relatively long-lived trimer core intermediate with which domain III stably interacts to initiate membrane fusion. We are using expressed protein domains to define this interaction and to characterize the target membrane insertion, trimerization, and low pH triggering of the fusion protein. We anticipate that comparison of the functional differences and similarities of the alphavirus and flavivirus fusion proteins will define key features of the class II membrane fusion mechanism. (Supported by National Institutes of Health grants GM 057454, AI 075647, and AI 067931.)

How Synaptotagmin Promotes Membrane Fusion. SASCHA MARTENS, MICHAEL M. KOZLOV, and HARVEY T. McMAHON, Laboratory of Molecular Biology, Medical Research Council, Cambridge CB2 2QH, UK

Synaptic vesicles loaded with neurotransmitters exocytose in a SNARE protein–dependent manner after presynaptic depolarization induces calcium influx. The calcium sensor required for fast fusion is synaptotagmin-1. Current models for membrane fusion do not account for how the high activation energy of bilayer–bilayer fusion (40 kBT) can be overcome. We have recently shown that synaptotagmin-1 promotes SNARE-mediated fusion by significantly lowering this activation barrier by inducing high positive curvature in target membranes in response to calcium binding. We propose that synaptotagmin-1 triggers the fusion of docked vesicles by local calcium-dependent buckling/bulging of the plasma membrane in synchrony with the zippering of SNAREs. We further show that the synaptotagmin-related molecules synaptotagmin-3, synaptotagmin-9, and synaptotagmin-like protein 2 are also capable of membrane curvature induction, and thus this mechanism to promote membrane fusion may be widespread.

Recognizing that membrane curvature induction is important also in vesicle recycling, we have characterized various membrane recycling pathways from structures of constituent proteins to functions of these pathways.

Sascha Martens, Michael M. Kozlov, and Harvey T. McMahon. 2007. How Synaptotagmin Promotes Membrane Fusion. Science. 10.1126/science.1142614.

Timing of Protein–Protein Interactions Required for Synaptic Vesicle Exocytosis. GEORGE J. AUGUSTINE, Department of Neurobiology, Duke University Medical Center, Durham, NC 27710 and Marine Biological Laboratory, Woods Hole, MA 02543

Dozens of proteins are involved in neurotransmitter release, including the SNARE proteins that participate in membrane fusion during exocytosis. To discern the role that each protein plays, it is necessary to sort out the temporal order in which they interact. Toward this goal, we have used light-activated binding site peptides to define the precise times at which two SNARE-related proteins act during exocytosis at the squid giant synapse. The time at which NSF works was determined
by a “caged” peptide that inhibits the ATPase activity of NSF only after the cage is photolyzed by UV light. Photolysis of this caged peptide caused an abrupt (0.2 s) slowing of the kinetics of neurotransmitter release and a more gradual (2–3 s) reduction in the amount of release. Based on the rapid rate of these inhibitory effects relative to the speed of synaptic vesicle recycling, we conclude that NSF functions in reactions that immediately precede neurotransmitter release, rather than working after fusion as is widely postulated. Complexin is a protein that participates in neurotransmitter release by binding to SNAREs. A light-activated inhibitory peptide from the syntaxin-binding domain of complexin was used to block the binding of synaphin to SNARE proteins at precise times. We find that this peptide inhibits neurotransmitter release within 180 ms before fusion of synaptic vesicles with the presynaptic plasma membrane. These results indicate that complexin binds to the SNARE complex after synaptic vesicles dock, but well before the millisecond required for calcium ions to trigger synaptic vesicle fusion. Collectively, these observations support models in which NSF dissociates cis-SNARE complexes before fusion and complexin then prepares the resulting trans-SNARE complexes for membrane fusion.

4. The Fusion Pore as a Window into the Molecular Mechanism of Ca\(^{2+}\)-triggered Exocytosis. MEYER B. JACKSON, EDWIN R. CHAPMAN, ZHENJIE ZHANG, ZHEN ZHANG, ENFU HUI, and CAMIN DEAN, Department of Physiology, University of Wisconsin, Madison, WI 53706

Studies of fusion pores in PC12 cells and posterior pituitary nerve terminals have provided insights into the molecular mechanism of Ca\(^{2+}\)-triggered exocytosis, and these insights should help illuminate basic mechanisms by which nerve terminals release neurotransmitter. Analysis of the amplitudes of noradrenaline flux through open fusion pores has implicated the membrane anchors of SNARE proteins as structural components of the fusion pore. Analysis of the lifetime of fusion pore openings has revealed important roles for synaptotagmin and the SNARE complex in functional transitions of fusion pores. One enigmatic isoform of synaptotagmin, synaptotagmin IV (syt IV), has no known Ca\(^{2+}\)-dependent effector interactions, and yet this protein exerts strong effects on the kinetics of exocytosis and on fusion pores. We found that syt IV expression is very high in the peptidergic nerve terminals of the posterior pituitary. Comparisons of whole-terminal capacitance recordings between wild-type and syt IV–null mice indicate that the presence of syt IV depresses Ca\(^{2+}\)-triggered exocytosis in nerve terminals. Furthermore, syt IV also influences synaptic function in the hippocampus. Hippocampal slices from syt IV–null animals show enhanced long-term potentiation of synaptic transmission in the CA1 region and a lower threshold for triggering epileptiform discharges.

5. Cell Fusion: from Early Intermediates to Syncytium Formation. LEONID V. CHERNOMORDIK, Section on Membrane Biology, Laboratory of Cellular and Molecular Biology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

Although cell–cell fusion is an important stage in normal animal development, little is known about the developmental fusogens and mechanisms of this family of membrane fusion events. We are now most interested in later fusion stages that follow hemifusion and opening of an initial fusion pore. To explore the expansion of fusion pores that fully merges the volumes of the fusing cells into syncytium, we study fusion between SF9 cells expressing either developmental fusogens of C. elegans or baculovirus fusion protein gp64 and fusion between NIH 3T3 cells expressing influenza virus hemagglutinin. Confocal microscopy analysis of the morphology of the contact zones between gp64-expressing cells shows that at the micron scale fusion progresses through opening, expansion, and merger of multiple pores. Fusion pores in membrane junction colocalize with openings in the actin cortex. Fusion pores are roughly circular and grow radially until they come close to one another, whence their shapes begin to distort. Fusion is completed with the merging of pores. In contrast to early fusion stages, fusion pore expansion in gp64 and hemagglutinin-mediated fusion reactions requires cell metabolism. Pore growth is driven neither by bending stresses in fusion pore, by membrane tension, nor by microtubule cytoskeleton. Acceleration of fusion pore expansion in the presence of an F-actin–disrupting agent (latrunculin A) and an inhibition of pore growth by an F-actin–stabilizing agent (jasplakinolide) indicate that actin cytoskeleton does not drive syncytium formation but rather slows down fusion pore expansion. We hypothesize that whereas early fusion stages are controlled by protein fusogens that generate relatively small fusion pores, later fusion stages are guided by actin cytoskeleton and driven by protein components of cell vesiculation machinery.

6. Studying Membrane Fusion with Yeast Vacuoles. WILLIAM WICKNER, Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755

Yeast vacuoles fuse and fuse in response to the osmolarity of the cell’s growth medium. We study the fusion of isolated vacuoles with a colorimetric assay that measures the activation of pro-Pho8p (inactive proenzyme form of the major vacuolar phosphatase) by proteases from vacuoles that are deleted for the phosphatase. We find that vacuole fusion uses a Rab GTPase (Ypt7p), a six-subunit Rab effector complex (termed HOPS), which includes the SM protein of this organelle, and
four SNAREs. Purified vacuoles that are incubated together with ATP will fuse. First, the chaperones Sec17p and Sec18p drive disassembly of cis-SNARE complexes that are anchored to one membrane. The Ypt7p and HOPS then catalyze tethering, and the organelles’ membranes continue to be drawn to each other until, in the docked structure, disc-shaped microdomains of each docking partner are closely apposed. Each of the crucial factors for fusion, Ypt7p, HOPS, the SNAREs, and key “regulatory” lipids (ergosterol, phosphoinositides, and diacylglycerol), become highly enriched in a ring microdomain that surrounds these apposed discs. After the SNAREs pair in trans, there is rapid lipid mixing and permeability changes (which may represent hemifusion), and then slow further lipid changes to give complete fusion. This fusion reaction is not accompanied by any measurable lysis, though fusion driven by SNAREs alone is accompanied by major organelle lysis. (Supported by the National Institutes of Health.)

7. Toward a Molecular Understanding of Synaptic Vesicle Fusion. THOMAS C. SÜDHOF, Department of Neuroscience, Howard Hughes Medical Institute, University of Texas Southwestern, Dallas, TX 75390

At a synapse, release of neurotransmitters initiates synaptic transmission. Our laboratory is interested in how a presynaptic terminal executes release in a tightly regulated and topologically restricted manner. Neurotransmitter release occurs by synaptic vesicle exocytosis that is triggered by influx of Ca\(^{2+}\) into the presynaptic terminal. Ca\(^{2+}\)-evoked synaptic vesicle exocytosis is one of the fastest and most tightly controlled reactions in biology and can be modulated during synaptic plasticity. The speed and plasticity of neurotransmitter release are two of the major factors that shape the exquisite precision of synaptic networks.

In a longstanding program, we are investigating the molecular cascades that orchestrate synaptic vesicle exocytosis and neurotransmitter release. Hundreds, maybe thousands, of proteins are involved in release. We envision that these proteins mediate release in a hierarchy of reactions: at the lowest level of this hierarchy, release is effected by exocytosis. Exocytosis in turn is controlled by Ca\(^{2+}\), and the Ca\(^{2+}\)-dependent release machinery is embedded in the active zone of the presynaptic terminal by a protein scaffold that integrates signaling during synaptic plasticity. At the top level of the hierarchy, a transsynaptic cell adhesion apparatus organizes the position and activation of the release machinery with respect to the postsynaptic specializations.

We are trying, in conjunction with other laboratories, to achieve a systematic description of the molecular components that mediate neurotransmitter release and to analyze these components structurally and functionally. Studies in our and other laboratories have identified major players at each level of the presynaptic hierarchy.

Exocytosis is largely mediated by SNAREs (e.g., synaptobrevin, syntaxin, and SNAP-25) and SM proteins (e.g., Munc18-1) and is controlled by synaptotagmins as Ca\(^{2+}\) sensors. Key components of the protein scaffold of the active zone, such as Munc13s, RIMs, and ELKS, integrate synaptic vesicle exocytosis and mediate synaptic plasticity. Unexpected access to transsynaptic cell adhesion was gained in the description of receptors for α-latrotoxin, which triggers neurotransmitter release by binding to a class of presynaptic cell adhesion molecules called neurexins. Neurexins contribute to the transsynaptic organization of nerve terminals as ligands for postsynaptic neuroreceptors and are essential for the activation of presynaptic Ca\(^{2+}\) channels and for the functionality of the presynaptic release machinery. An initial, sketchy description of the molecular machinery that executes neurotransmitter release has thus emerged, a description that hopefully will form the basis for a mechanistic understanding of this central process in synaptic transmission. In my presentation, I will describe key aspects of the machinery as it relates to synaptic vesicle fusion.

8. The Role of Membrane Lipid Composition and Tail Structure in Virus Host Cell Association, Entry, and Infection. H. EWERS, K. BACIA, W. CHAI, G. SCHWARZMANN, T. FEIZI, P. SCHWILLE, A.E. SMITH, and A. HELENIUS; ETH Zurich, Switzerland; Imperial College London, London SW7 2AZ, UK; University of Bonn, D-53115 Bonn, Germany

SV40 is unusual among animal viruses in that it uses a lipid, the GM1 ganglioside, as a cell entry receptor. After binding to GM1 in the outer leaflet of the plasma membrane, the virus moves randomly for 5–10 s, is immobilized by the cortical actin filament network, and induces trans-membrane signaling that leads to endocytic internalization and infection. We have found that the structure of the hydrocarbon chains of GM1 controls the entry process. Unlike native molecules, GM1 isoforms with shorter hydrocarbon chains or cis-double bonds allowed binding but failed to support inclusion of the virus into detergent-resistant lipid rafts, actin-regulated confinement of virus motion along the plasma membrane, caveolar/raft-mediated endocytosis, and infection. They also prevented association of the virus with liquid-ordered (lo) domains in model lipid bilayers. We concluded that once GM1 is clustered by the virus, the hydrocarbon chains control a phase-partitioning step necessary for transmembrane coupling and subsequent receptor-mediated endocytosis and infection. The results provide evidence for one of the central concepts in lipid raft theory, i.e., that the physical and chemical properties of lipid hydrocarbons drive the formation of microdomains in biological membranes and that this can have important functional consequences.
9. Revitalized the Imaging of Membrane Dynamics in Traditional Thin Section Electron Microscopy via a New Fixation/Embedding Protocol. JOHN HEUSER, LYUSSIENA LOULTCHEVA, and TANYA TENKOVA-HEUSER, Department of Cell Biology, Washington University School of Medicine, St. Louis, MO 63110

We will present a wholly revamped and improved protocol for carrying out standard thin-section electron microscopy, one which does not require any of the new and complicated freeze-substitution procedures or complicated tomographic reconstructions, but yields the benefits of both. We will demonstrate how this new protocol can yield amazing insights in the study of endocytosis, exocytosis, and membrane dynamics. We will also show how it totally revitalizes classical HRP-uptake histochemical studies, as well as EM immunocytochemistry using HRP-tagged secondary antibodies or DAB-based photoconversion of fluorescent antibodies and proteins, all because it so dramatically enhances the visibility of oxidized DAB in fixed cells. The two critical aspects of this improved protocol are simply to substitute KMnO₄ for OsO₄ as the secondary fixative after standard glutaraldehyde fixation and to reduce the time devoted to dehydration and plastic infiltration from hours or days, as is done traditionally, to just 3 min! The consequence is far less extraction and distortion of cells and organelles and vastly improved visibility of membranes, lipids, and polyamines like DAB. As two examples of the ease and utility of this new protocol, we will demonstrate, first, that the mechanism of plasmalemmal “healing” after any one of several different forms of cellular injury is in fact the recruitment of endoplasmic reticulum, not the recruitment of lysosomes as is currently believed today. Second, we will revisit synaptic vesicle recycling at the frog neuromuscular junction and illustrate that synaptic vesicles reform from membranous cisternae after intense stimulation by a unique form of membrane tubulation via a mechanism that essentially recapitulates how synaptic vesicles form from axonal endoplasmic reticulum in the first place.

10. Coupling of Exo- and Endocytosis at CNS Synapses. JURGEN KLINGAUF, Department of Membrane Biophysics, Max Planck Institute for Biophysical Chemistry, 37077 Goettingen, Germany

During synaptic transmission, small synaptic vesicles filled with neurotransmitter fuse with the plasma membrane to release their content. For maintaining synaptic transmission, the exocytosed vesicle proteins have to be retrieved thereafter by compensatory endocytosis. Different mechanisms for synaptic vesicle protein retrieval and recycling have been suggested. Although clathrin-mediated resorting, reclustering, and endocytosis of vesicle proteins is thought to be the major pathway, vesicles might connect only briefly to the plasma membrane without full collapse and loss of protein content (“kiss and run”). A third possibility could be that after full vesicle collapse synaptic vesicle proteins and lipids do not disperse in the plasma membrane, but remain clustered in a raft-like structure. What distinguishes these different scenarios fundamentally is the fate of the synaptic vesicle proteins after fusion. For single vesicle fusion events, maintaining molecular identity during exo/endocytic cycling is the hallmark of a kiss-and-run-type mechanism. Both modes of full collapse can be easily distinguished by the dispersion criterion at the single vesicle level. By optically recording single fusion events we show for four different transmembrane vesicle proteins (synaptobrevin 2, synaptotagmin 1, synaptophysin, and VGlut1) fast dispersion after fusion. Proteins diffused within the synaptic bouton membrane with diffusion constants around 0.2 μm²/s, but only 10–20% were lost into the axonal membrane. This suggests a mechanism by which vesicle proteins are rapidly cleared from the release site to allow for the next docking and priming event, but are efficiently recaptured outside the active zone.

11. Systematic Mapping of the Molecular Dynamics of Clathrin-mediated Endocytosis. CHRISTIEN MERRIFIELD, Laboratory of Molecular Biology, Medical Research Council, Cambridge CB2 2QH, UK

Clathrin-mediated endocytosis is one of the principal mechanisms by which eukaryotic cells internalize cell surface receptors. This conserved mechanism is driven by a consortium of different kinds of proteins including kinases, GTPases, proteins involved in lipid modification, and proteins involved in remodeling of the actin cytoskeleton. Although many of these proteins have been well described biochemically and are known to form a network of protein–protein interactions, it is not well understood how they are integrated into the dynamic molecular machine that drives clathrin-coated pit invagination, membrane scission, and the liberation of clathrin-coated vesicles.

In this talk, I will describe experiments designed to systematically unravel the workings of the endocytic machine in living cells by using evanescent field fluorescence microscopy. In this approach, we labeled clathrin-coated pits using transferrin receptor (Tfnr), tagged on the extracellular domain with a pH-sensitive version of green fluorescent protein (super ecliptic phluorin) to form Tfnr-phl. This marker was used to report the accessibility of clathrin-coated pits to imposed changes in extracellular pH. By switching the extracellular pH in conjunction with image acquisition we could detect when scission occurred at individual clathrin-coated pits with a resolution of 4 s. Using dual-color image acquisition we could monitor the recruitment to sites of scission of a second species of protein labeled with monomeric red fluorescent protein.

Using this experimental system we have measured the recruitment/departure profiles of dynamin, amphiphysin,
N-WASP, cortactin, mAbp1, actin, Arp3, Hip1R syndapin, AP-2, and several kinases such as GAK. I will describe how we aim to use these data as the foundation for a more precise molecular model of clathrin-mediated endocytosis.

12. Looking for Lipid Rafts. KEN JACOBSON$^{1,2}$ and YUN CHEN$^{2,3}$. $^1$Department of Cell and Developmental Biology, $^2$Lineberger Comprehensive Cancer Center, and $^3$Department of Biomedical Engineering, University of North Carolina-Chapel Hill, Chapel Hill, NC 27599

Lipid rafts as an organizing principle for biomembrane functionality has gained prominence but the form of the putative microdomains is an area of healthy controversy. One key tenet of the lipid raft hypothesis is that the formation of sphingolipid and cholesterol-rich lipid domains can be driven solely by characteristic lipid–lipid interactions. In fact, using fluorescence microscopy, domains with raft-like properties have been found by many groups to coexist with fluid lipid regions in both planar-supported lipid layers and in giant unilamellar vesicles (GUV) by many groups. However, although many of the tenets of the raft hypothesis can be confirmed in model systems, current membrane models differ measurably from actual biomembranes. In this respect, it is not so surprising that raft existence in unstimulated natural membranes is much more controversial. What is less in doubt is that when glycosyl-phosphatidylinositol–anchored proteins (GPIAPs) are induced to aggregate, distinctive domains form with interesting properties. Using single particle tracking, we found that by robustly cross-linking several GPIAPs under antibody-conjugated 40-nm gold particles, transient anchorage of the gold-labeled clusters occurred for periods ranging from 300 ms to 10 s. Cholesterol depletion and addition of the Src family kinase (SFK) inhibitor PP2 abolished transient anchorage, whereas caveolin-1 knockout cells exhibited reduced transient anchorage. In addition, inhibition of PI3 kinase abolished long duration anchorage and converted $\approx50\%$ of the trajectories to bidirectional movement. Transient anchorage was severely abrogated by expression of siRNA knocking down the transmembrane protein Csk binding protein and by expressing the dominant-negative cytoskeletal adaptor EBP50. In contrast, a transmembrane protein, the cystic fibrosis transmembrane conductance regulator (CFTR), exhibited transient anchorage that occurred without deliberately enhanced cross-linking but was dependent on the interaction of its PDZ binding domain with the adaptor EBP50 linking to the cytoskeleton. We propose that cross-linked GPIAPs become transiently anchored via a cholesterol-dependent, SFK-regulatable linkage between a transmembrane cluster sensor, cbp, and the cytoskeleton that is mediated by the adaptor EBP50. Preliminary results using time series analysis to extract key parameters of the proposed scheme will be discussed. (Supported by National Institutes of Health GM 41402.)
13. Method for Measuring the Effect of Lipid Composition and Fusion-active Peptides on the Gaussian Curvature Elastic Energy of Lipid Membranes. DAVID P. SIEGEL, Givaudan Inc., Cincinnati, OH 45216

Recent measurements show that the Gaussian curvature elastic energy of membranes is a substantial barrier to formation of membrane fusion intermediates and fusion pores (Siegel, D.P, and M.M. Kozlov. 2004. *Biophys. J.* 87:366–374). In some cases this contribution to the total intermediate energy rivals the contribution of the bending elastic energy (Kozlovsky, Y., A. Efrat, D.P. Siegel, and M.M. Kozlov. 2004. *Biophys. J.* 87:2508–2521; Siegel, D.P. 2006. *Biophys. J.* 91:608–618). It was recently found that physiological levels of cholesterol can induce QII phase formation in unsaturated phosphatidylcholines (e.g., DOPC; Tenchov, B.G., R.C. MacDonald, and D.P. Siegel. 2006. *Biophys. J.* 91:2508–2516) and that membrane-spanning WALP peptides (Siegel, D.P., V. Cherezov, D.V. Greathouse, R.E. Koeppe, J.A. Kilian, and M. Caffrey. 2006. *Biophys. J.* 90:200–211) strongly stabilize QII phases. There is preliminary evidence (unpublished data) that influenza hemagglutinin fusion peptides stabilize these phases as well. This raises the question of whether changes in lipid composition or the presence of fusion-active peptides may lower fusion intermediate energies by altering the Gaussian (saddle-splay) elastic modulus of the lipid–peptide membrane. This may be one role of the peptides in catalyzing membrane fusion. Here, an experimental method is presented for measuring the contribution of a small mole fraction of exogenous lipid or peptide to the Gaussian curvature elastic energy of a lipid or lipid/peptide mixture. This permits estimation of the elastic moduli of the pure exogenous lipid or peptide components. Equations are given for data analysis and specific experimental protocols are described. Specific lipid host systems and experimental protocols are described. These are based on measurement of the equilibrium unit cell size of inverted cubic phases as a function of temperature and measurement of the temperature-dependent spontaneous curvature of the mixture.

14. Simulation of Membrane Fusion Mechanisms: Correlation with Experiment. PETER M. KASSON and VIJAY S. PANDE, Department of Chemistry, Stanford University, Stanford, CA 94305

Membrane fusion is critical to biological processes such as viral infection, endocrine hormone secretion, and neurotransmission, yet the precise mechanistic details of the fusion process remain unknown. One challenge in understanding membrane fusion is how changes in physical properties of lipid membranes affect fusion and how they may be used in physiological regulation of fusion events. Molecular dynamics simulations enable detailed mechanistic models for fusion; however, there is a substantial gap in complexity between the systems computationally tractable via molecular dynamics simulation, those used in reduced model systems for fusion, and the complex membrane environment of cellular fusion. We present results of a computational model system for fusion where the ratio of lipid components was systematically varied, using several thousand molecular dynamics simulations up to a microsecond in length to predict the effects of lipid composition on fusion kinetics and mechanism. In our simulations, the ratio of phosphatidylcholine to phosphatidylethanolamine substantially affects both fusion kinetics and the fusion intermediates formed. Examining vesicles with asymmetric inner and outer leaflet composition, we further predict that the kinetics of stalk formation between vesicles depend only on the outer leaflet composition, whereas the kinetics of full fusion show a more complex dependence on both leaflets. Our findings are in good
15. Visualizing Membrane Fusion of Single Viral Particles. DANIEL FLOYD, STEVE HARRISON, and ANTOINE VAN OIJEN, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115

Specific fusion of biological membranes is a central requirement of many cellular processes and is the key event in the entry of enveloped viruses into cells. Though many biochemical and biophysical studies have contributed to an understanding of the mechanisms underlying fusion, important questions remain about the sequence and orchestration of events underlying the process. Conventional fusion assays are generally limited to observation of ensembles of multiple fusion events, making more detailed analysis difficult. We have developed an in vitro two-color fluorescence assay that enables us to monitor the kinetics of individual fusion events. The resulting “molecular movies” allow us to dissect the reaction kinetics at a level of detail previously inaccessible. Analysis of lipid and content mixing trajectories of single particles provides further evidence of a hemifusion intermediate preceding pore formation. Distributions of the delay time before formation of the hemifusion intermediate and the time elapsed between hemifusion and pore formation provide detailed kinetic information on the underlying transitions.

16. Formation of Virus-like Vesicles through Assembly of Proteolipid Domains. ANNA SHNYROVA,1,2 JUAN AYLLON,2 ENRIQUE VILLAR,2 JOSHUA ZIMMERBERG,1 and VADIM FROLOV,1 1Laboratory of Cellular and Molecular Biophysics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20847; 2Departamento de Bioquímica y Biología Molecular, Universidad de Salamanca, 37008 Salamanca, Spain

The membrane of enveloped viruses is lined by a dense protein matrix. The matrix plays a major role in the formation of the virus envelope, yet it remains unclear how matrix proteins guide the shape of the envelope membrane. We reconstituted the budding of Newcastle Disease Virus by purified matrix protein in model membrane systems. Upon adsorption on a phospholipid bilayer, matrix proteins condense into fluid-like domains that cause membrane deformation and budding of spherical vesicles, as revealed by fluorescent and electron microscopy images of the protein condensed on the lipid surface. Measurements of the electrical admittance of the membrane resolved the gradual growth and rapid closure of a bud, followed by its separation to form a free vesicle. The vesicle size distribution, confined by intrinsic curvature of budding domains, but broadened by their merger, matched the virus size distribution. Thus, we revealed a new class of membrane shape deformation with “negative curvature” driven by self-organization of membrane proteins into fluid-like domains.

17. Extensive Membrane Lipid Reorganization Precedes BaxΔC-induced Cytochrome c Release in Isolated Rat Liver Mitochondria. ELAI DAVICIONI,1 RIVKA COHEN-LURIA,1 NATALIA TSESIN,1 ILANA NATHAN,2 and ABRAHAM H. PAROLA,1 1Department of Chemistry, Faculty of Natural Sciences, and 2Department of Clinical Biochemistry, Faculty of Health Sciences and The Hematology Institute, Soroka University Medical Center, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

The excimer/monomer fluorescence emission ratio (E/M) of the excimer-forming lipid 12-(pyrene-1-yl)dodecanoic acid (PDA) and the rotational relaxation of TMA-DPH, incorporated into isolated rat liver mitochondria, were used to study the interaction between the proapoptotic protein BaxΔC and the mitochondria. The addition of BaxΔC caused a rapid decrease in PDA E/M, followed by a slowly saturating linear course indicating that BaxΔC increases mitochondrial membrane microviscosity. The changes in microviscosity were directly proportional to the BaxΔC protein/mitochondrial lipid ratio but were not inhibited by the antiapoptotic protein Bcl-2. Another proapoptotic protein, C-terminal Bid, failed to induce the rapid changes in membrane microviscosity observed with BaxΔC, although it too stimulated cytochrome c release. Hill type analyses of the fluorescence values resulted in slopes of 1, excluding cooperative effects for BaxΔC protein–protein interactions (i.e., oligomerization) as the cause for the increase of membrane microviscosity. Phase transitions were determined by following changes in E/M with temperature, and corresponding Arrhenius plots show that BaxΔC addition lowered the phase transition temperature and increased the activation energy of excimerization. Fluorescence anisotropy studies with TMA-DPH–labeled mitochondria independently show reduced rotational dynamics, which is evidence of increased membrane microviscosity upon interaction with BaxΔC. These findings reflect the initial stages of BaxΔC interactions with mitochondrial membranes, where the binding of BaxΔC lowers the probe mobility in the membrane and induces major reorganization of mitochondrial lipids, possibly through nonlamellar bilayer structures. Thus, Bax-induced lipidic pore model for cytochrome c efflux as a critical process in the commitment to cell death gains support.
18. Conformational Changes that Affect Oligomerization and Initiate Pore Formation Are Triggered throughout Perfringolysin O upon Binding to Cholesterol. ALEJANDRO P. HEUCK,1 CHRISTOS G. SAVVA,2 ANDREAS HOLZENBURG,2,4 and ARTHUR E. JOHNSON,3,4 1Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, MA 01003; 2Department of Biology, 2Department of Chemistry, and 3Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843

Perfringolysin O (PFO) is a pore-forming toxin secreted by Clostridium perfringens. Upon encountering a cholesterol-rich membrane, the toxin oligomerizes and spontaneously inserts into the bilayer to form a large transmembrane pore (~300 Å diameter). We have shown that pore formation involves (a) the initial binding to a cholesterol-containing membrane with a consequent conformational change in a distant domain (>70 Å away from the membrane surface), (b) rearrangement of a β-sheet to allow oligomerization on the membrane surface and formation of a prepore complex, and (c) insertion of two amphipathic β-hairpins per monomer that span the membrane bilayer forming a transmembrane β-barrel created by more than 180 β-strands.

Although significant progress has been made in characterizing PFO structural changes during pore formation, the mechanism by which cholesterol effects PFO binding to cholesterol-rich membranes and pore formation remains unknown. When incubated with cholesterol dispersions, the lytic, lethal, and cardiotoxic properties of PFO and related toxins are inhibited, yet they are able to form structures similar to those observed in natural membranes.

We have found that PFO binds to cholesterol dispersed in aqueous solution in the same manner as to cholesterol-containing liposomal membranes. The topography of domain 4 when bound to cholesterol aggregates is indistinguishable to that observed with membrane bilayers. Moreover, the binding of domain 4 to cholesterol aggregates triggers the same conformational change in a distant domain that occurs when PFO interacts with liposomes. Surprisingly, the transmembrane β-hairpins also interact with cholesterol aggregates, and indeed exhibit the same amphipathic β-hairpin pattern observed after insertion in membranes. Direct PFO–cholesterol interactions are involved in and required for effecting each step of the PFO cytolytic mechanism.

These results reveal that cholesterol alone is sufficient to initiate the irreversible sequence of extensive conformational changes in PFO that accomplish and regulate pore formation.

19. Amperometric Detection of Spontaneous Fusion of Chromaffin Dense Core Granules to a Lipid Bilayer. JAMES M. MCNALLY,1 DIXON J. WOODBURY,2 and JOSÉ R. LEMOS,1 1University of Massachusetts Medical School, Worcester, MA 01655; 2Brigham Young University, Provo, UT 84602

The SNARE fusion complex (composed of syntaxin, SNAP-25, and VAMP) is thought to serve as an ubiquitous mechanism for membrane fusion and exocytosis of both small synaptic vesicles and large dense core granules (LDCG). We have previously demonstrated that LDCG from both bovine neurohypophysial terminals (McNally, J.M., D.J. Woodbury, and J.R. Lemos. 2004. Cell Biochem. and Biophys. 41:11–24) and chromaffin cells (McNally, J.M., D.J. Woodbury, and J.R. Lemos. 2003. Biophys. J 84:A1866) are able to spontaneously fuse with a planar lipid bilayer containing only the t-SNARE protein syntaxin 1A. In these experiments, native LDCG granule membranes were modified to contain nystatin channels, allowing each fusion event to be visualized by monitoring membrane conductance. Now, we have been able to confirm these results with native chromaffin cell LDCGs using amperometry. Amperometric detection was coupled with our planar lipid bilayer model system to successfully detect individual release events. These events displayed the characteristic amplitude and kinetics of catecholamine release events from chromaffin cells. This approach also allowed for analysis of differences between spontaneous and osmotically driven fusion events. Interestingly, small current steps preceded some of the observed amperometric spikes that appear similar in nature to the prespike “foot” observed during native catecholamine release events from chromaffin cells. The prespike foot is attributed to the slow release of transmitter through a fusion pore before pore widening and complete collapse of the vesicle into the plasma membrane. The appearance of prespike feet preceding amperometric events seen in our extremely simple model system implies that all of the components required to form a fully functional fusion pore are present. In conclusion, this method provides an effective model system to study possible components involved in vesicle fusion. (Supported by National Institutes of Health Grants NS048628, MH50003, and NS29470.)

20. Investigating Consequences of Synaptobrevin Binding to SNARE Partners for Calcium-triggered Exocytosis. ALEXANDER M. WALTER and JAROB B. SØRENSEN, Department of Membrane Biophysics, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany

The assembly of SNARE proteins is an essential prerequisite for neurosecretory vesicles undergoing membrane fusion. During exocytosis a four-helical coiled-coil is formed by the three SNARE proteins syntaxin, synaptobrevin, and SNAP-25. Recent findings using mutagenesis of SNAP-25 promoted the notion that sequential N- to C-terminal assembly of the SNARE motif is responsible for vesicular priming and fusion (Sørensen, J.B., K. Wiederhold, E.M. Muller, I. Milosevic, G. Nagy, ...
B.L. de Groot, H. Grubmuller, and D. Fasshauer. 2006. EMBO J. 25:955–966). In the present study we investigate the role of the vesicular SNARE protein synaptobrevin 2 in exocytosis in chromaffin cells. In the clean genetic background of the synaptobrevin 2 deficient mouse, viral reexpression of the wild-type protein was able to rescue the knockout phenotype (Borisovska, M., Y. Zhao, Y. Tsutsuya, N. Glyvuk, S. Takamori, U. Matti, J. Retting, T. Südhof, and D. Bruns. 2005. EMBO J. 24:2114–2126). Expression of mutant proteins bearing destabilizing point mutations in the hydrophobic layers of the SNARE motif was used to test the hypothesis of sequential SNARE complex assembly. N-terminal double point-mutations of synaptobrevin 2 were without effect on fusion and triggering but slowed down priming, suggesting that N-terminal interaction of SNAREs underlies vesicle priming. We are currently investigating mutations in the C-terminal end of the SNARE bundle to test the hypothesis that C-terminal assembly of the SNARE bundle drives exocytosis triggering. (Supported by Deutsche Forschungsgemeinschaft grant SFB523.)

21. Probing Synaptic Vesicle Fusion with an In Vitro System. MATTHEW HOLT, DIETMAR RIEDEL, CHRISTINA SCHUETTE, ALEXANDER STEIN, and REINHARD JAHN, Department of Neurobiology, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany

Fusion of synaptic vesicles with the plasma membrane (exocytosis) is a highly regulated process. Many proteins have been identified and assigned putative roles in exocytosis (Südhof, T.C. 2004. Annu. Rev. Neurosci. 27:509–547). However, interactions between these proteins remain unclear, as a gap exists between results obtained from physiological experiments using gene "knock-outs" and the biochemistry of isolated proteins. It is hoped reconstitution of the fusion apparatus under cell-free conditions, using purified components, can bridge this gap.

SNARE proteins, thought to be the minimal fusion machinery, are the best understood of all exocytic proteins. Although liposomes containing these proteins fuse in a SNARE-dependent manner, other features of exocytosis have not been reproduced (Weber, T., B.V. Zemelman, J.A. McNew, B. Westermann, M. Gmachl, F. Parlati, T.H. Söllner TH, and J.E. Rothman. 1998. Cell. 92: 759–772). Furthermore, the relevance of reconstituted systems for fusion reactions is usually challenged because proteoliposomes become leaky and fusion prone at the high protein/phospholipid ratios typically used, so fusions may not truly reflect exocytosis. Here, we take the in vitro approach further by investigating the fusion of isolated synaptic vesicles with proteoliposomes containing the cognate SNAREs syntaxin 1 and SNAP-25. Using a native membrane as one fusion partner we hope to minimize potential artifacts caused by leaky or fusion-prone liposomes, while allowing the study of the vesicular SNARE synaptobrevin in its native environment. Additionally, our proteoliposomes incorporate recent advances in understanding membrane composition and biochemistry and the fusion apparatus (Pobbatti, A.V., A. Stein, and D. Fasshauer. 2006. Science. 313:673–676).

Using multiple complementary methods, we show that synaptic vesicles fuse constitutively with syntaxin and SNAP-25 proteoliposomes. Fusion possesses some hallmarks of exocytosis, including its completeness and requirement for the full set of neuronal SNAREs. Indeed, synaptic vesicles are constitutively active fusion machines, dependent only on SNAREs. This confirms that SNAREs operate as the "bare-bones" fusion machinery, without needing complicated docking complexes, priming reactions, or activation steps.

22. Multiple Modes of Fusion and Retrieval at the Calyx of Held Synapse. LIMING HE and LING-GANG WU, Synaptic Transmission Unit, National Institute of Neurological Disorders and Stroke, Bethesda, MD 20892

Vesicle fusion opens a pore to release transmitter. The pore may fully dilate to create a full vesicle collapse or close rapidly to generate "kiss-and-run" fusion. The pore size determines the release rate. At synapses, fusion pore size is unclear, kiss-and-run remains controversial, and the ability of kiss-and-run in generating rapid synaptic currents is questionable. Here, by recording fusion pore kinetics, we found full collapse and kiss-and-run fusion at calyx-type synapses. For full collapse, the initial fusion pore conductance (Gp) was usually >375 pS and increased rapidly at ≥299 pS/ms. Kiss-and-run was seen as a brief (~2 s) capacitance flicker with Gp > 288 pS for most flickers, but within 15 to 288 pS for the remaining flickers. Large Gp (>288 pS) may discharge transmitter rapidly and thus cause rapid synaptic currents, whereas small Gp may generate slow and small synaptic currents. These results reveal the existence of kiss-and-run and its ability to generate rapid postsynaptic currents and suggest that various fusion pore sizes contribute to controlling the kinetics and amplitude of synaptic currents.

23. An Unusual SNARE Complex Governs the Regulated Exocytosis of Enlargeosome. EMANUELE COCUCCI and JACOPO MELDOLESI, Department of Neuroscience, Vita-Salute University, Milan 20132, Italy

The aim of our study was to define the SNARE machinery involved in enlargeosome exocytosis. Enlargeosomes are a class of vesicles competent for a regulated exocytosis, which is [Ca\textsuperscript{2+}] dependent and tetanus toxin insensitive; they are identified by the presence of their own marker, the protein Ahnak, retained in their lumen. Enlargeosomes were first described in PC12-27 (Borgonovo, B., E. Cocucci, G. Racchetti, P. Podini,
A. Bachì, and J. Meldolesi. 2002. Nat. Cell Biol. 4: 955–962), a clone of the rat pheochromocytoma cell line PC12, which lacks the neurosecretory organelles (the SNARE proteins involved in their fusion [e.g., VAMP2, SNAP25, and syntaxin1]), but is enlargeosome rich.

A screening by Western blot and immunofluorescence was performed to identify the SNAREs of interest. Among these only VAMP4 exhibited an extensive colocalization with the enlargeosome marker Ahnak.

PC12-27 cells were microinjected with antibodies against SNAREs to impair their functions and were subsequently assessed for enlargeosome exocytosis competence by two approaches. In the first approach, PM capacitance changes were monitored upon [Ca2+] increase triggered by uncaging of caged Ca2+ compounds. In the second approach, exocytosis was triggered by adding ionomycin to the cell media and monitored by the appearance on the cell surface of Ahnak.

Enlargeosome exocytosis was unaffected by anti-VAMP8 and anti-syntaxin4 antibodies, whereas it was strongly inhibited by the microinjection of anti-SNAP23, anti-syntaxin6, and anti-VAMP4 antibodies. In addition each of the last three antibodies was able to coprecipitate the other two.

According to our results, enlargeosome exocytosis therefore appears to be governed by a complex composed by SNAP23 together with VAMP4 and syntaxin6, two SNAREs that so far had been shown to participate only in constitutive membrane fusions in the Golgi/trans-Golgi areas. (Supported by grants from the Telethon Foundation and Fondo per gli Investimenti per la Ricerca di Base, PRIN programmes of the Ministero dell’Università e della Ricerca, Italy; E. Cocucci was supported by a short-term Federation of European Biochemical Societies fellowship.)

24. The COG Complex Interacts with Golgi t-SNARE Syntaxin5 to Direct Trafficking of Recycling Intra-Golgi Vesicles. ANNA SHESTAKOVA, RICHARD D. SMITH, OLEKSANDRA PAVLIV, GALIMAT KHAIDAKOVA, and VLADIMIR LUPASHIN, Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, AR 72205

The conserved oligomeric Golgi (COG) complex is an eight-subunit (COG1-8) peripheral Golgi membrane heterooligomeric protein complex that regulates retrograde intra-Golgi transport, and glycoprotein modifications (Shestakova, A., S. Zolov, and V. Lupashin. 2006. Traffic. 7:191–204). In humans, deficiencies in several COG subunits cause type II Congenital Disorders of Glycosylation (Kranz, C., B.G. Ng, L. Sun, V. Sharma, E.A. Eklund, Y. Miura, D. Ungar, V. Lupashin, R.D. Winkel, J.F. Gipollo, C.E. Costello, E. Loh, W. Hong, and H.H. Freeze. 2007. Hum. Mol. Genet. 16:731–741).

In HeLa cells, both the siRNA-induced knockdown (KD) of COG3 and microinjection of anti-COG3 IgGs caused accumulation of COG complex-dependent (CCD) vesicles and disruption of Golgi ribbon (Zolov, S.N., and V.V. Lupashin. 2005. J. Cell Biol. 168:747–759). In COG3 KD cells a significant fraction (20–50%) of intra-Golgi v-SNAREs and medial-Golgi enzymes was mislocalized into CCD vesicles. Prolonged buildup of CCD vesicles affects stability of both v-SNAREs and Golgi enzymes thus blocking retrograde trafficking of Shiga toxin and distorting glycosylation of plasma membrane and lysosomal glycoproteins. COG4 KD and COG7 KD caused similar defects in respect to both Golgi trafficking and protein glycosylation. Intriguingly, knock-down of both COG6 and COG8 did not distort morphology of the Golgi.

Our studies revealed that COG complex interacts with core components of the vesicle docking/fusion machinery: SNARE and Rab proteins. To further investigate molecular mechanism of the COG complex function we have examined COG–SNARE interaction in detail. Yeast two-hybrid assay identified COG4 and COG6 as binding partners of the Golgi t-SNARE Syntaxin5. FRET assay revealed direct in vivo interaction between Syntaxin5 and the COG complex. siRNA-directed knockdown of the COG complex resulted in decreased Golgi SNARE mobility, accumulation of free uncomplexed form of Syntaxin5, and diminution in a steady-state level of intra-Golgi SNARE complexes. Specific interference with the COG–SNARE interaction caused disruption of Golgi ribbon and decreased formation and/or stability of Syntaxin5-contained intra-Golgi SNARE complex.

We propose that the COG complex interacts with SNAREs, Rabs, and coiled-coil tethering factors to orchestrate recycling of intra-Golgi vesicles to the proper Golgi compartment. (Supported by National Science Foundation grant MCB-0645163.)

25. Is Synaptotagmin IV Involved in Neuropeptide Release in Drosophila melanogaster? SARAH WEBSTER, RUDOLPH BOHM, and THOMAS SCHWARZ, Division of Neuroscience, Children’s Hospital, Boston, MA 02115

Release properties vary between types of synapses and these distinctions are particularly stark when comparing release of small clear vesicles to peptidergic dense-core vesicles. Presumably, some of these physiological distinctions arise from differences in the isoforms of synaptic proteins that are involved. Synaptotagmins are a diverse family of C2 domain containing proteins involved in the calcium regulation of membrane trafficking. Syt I is required for fast neurotransmitter release. However, the physiological roles of the other family members are still poorly understood. We generated a null mutant in Drosophila Syt IV to test its role in neurotransmission. Unlike many mutants essential for neurotransmission, the Syt IV null is homozygous viable and fertile. To further characterize Syt IV’s role in the nervous system, we generated an antibody against Drosophila Syt IV. Syt IV is
localized to both pre- and postsynaptic compartments at the neuromuscular junction (Adolfsen, B., S. Saraswati, M. Yoshihara, and J.T. Littleton. 2004. *J. Cell Biol.* 166:249–260). Syt IV is enriched in terminals within both the PNS and CNS that release dense core vesicles, including the neurohemal organs, the ring gland, and type III boutons on muscle 12. It is not detected in non-neuronal endocrine cells. To test whether Syt IV may be involved in dense core vesicle release, we have adopted an optical assay that makes use of a GFP-tagged atrial natriuretic factor (ANF-GFP) expressed in *Drosophila* under the Gal4/UAS control (Rao, S., C. Lang, E.S. Levi- tan, and D.L. Deitcher. 2001. *J. Neurobiol.* 49:139–172) to monitor dense core vesicle release. Wild-type animals release ≈50% of the ANF-GFP in individual boutons in response to 3 min of high K+ Ca2+ stimulus. In contrast, mutants in Syt IV release only ≈35% of GFP. Collectively, the physiological and anatomical data suggest that Syt IV may have a role in neuropeptide vesicle release.

26. Crystal Structure of the RIM1α C2B Domain. RONG GUAN,1,2 HAN DAI,1,2 DIANA R. TOMCHICK,1 IRINA DULUBOVA,1,2 MISCHA MACHIUS,1 THOMAS C. SÜDHOF,3,5 and JOSEPH RIZO,1,2 1Department of Biochemistry, 2Department of Pharmacology, 3Department of Neuroscience, and 4Department of Molecular Genetics, and 5Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, University of Texas Southwestern Medical Center at Dallas, TX 75390

Neurotransmitter release is essential for normal brain function. Many proteins are involved in neurotransmitter release (Südhof, T.C. 2004. *Annu. Rev. Neurosci.* 27:509–547). Among these proteins, RIMs are particularly interesting because they exhibit multiple interactions to organize the active zone and play multiple roles in regulating neurotransmitter release (Schoch, S. P.E. Castillo, T. Jo, K. Mukherjee, M. Geppert, Y. Wang, F. Schmitz, R.C. Malenka, T.C. Südhof. 2002. *Nature.* 415:321–326). Among the different domains found in the different RIM isoforms, only the C2 domain is present in all of them, suggesting that this domain is a key determinant of RIM function. However, the RIM1 C2B domain is only distantly related to well-studied C2 domains, and its three-dimensional structure and interactions have not been characterized in detail. Using NMR spectroscopy, we found the RIM1 C2B domain requires extended N and C termini for a folded, stable domain. The structure of RIM1 C2B domain was solved by x-ray crystallography at 1.7 Å. The RIM1 C2B domain shows a β-sandwich characteristic of C2 domains and forms a dimmer, which is mediated by the N- and C-terminal extensions. Analytical ultracentrifugation analysis shows that the RIM1 C2B domain forms weak dimers with KD ≈90 μM. Our studies provide a structural basis to understand how the abundance of rate-limiting components of SV exocytosis and endocytosis are regulated and how the dynamics of these molecules at individual nerve terminals in turn dictate the dynamics of the SV pool.

We monitored the distribution of Syntaptobrevin, a vesicle protein required for exocytosis, in *C. elegans* motor neurons using a pH-sensitive Syntaptobrevin GFP fusion protein: SyntaptopHluorin (Dittman, J.S., and J.M. Kaplan. 2006. *Proc. Natl. Acad. Sci. USA.* 103:11399–11404). We estimated that 30% of Syntaptobrevin was present in the plasma membrane. Using a panel of endocytosis and exocytosis mutants, we found that the majority of surface Syntaptobrevin derives from fusion of SVs, and in steady state Syntaptobrevin equilibrates throughout the axon. The size of the reservoir is set by the relative rates of exo- and endocytosis.

We further investigated the spatial localization of Syntaptobrevin using photoactivatable GFP tagged at its N terminus (cytoplasmic domain). We found that Syntaptobrevin was immobile when resident on SVs. In contrast, cell surface Syntaptobrevin exhibited rapid lateral diffusion, and this mobility was regulated by AP180. Loss of AP180 resulted in a significant accumulation of Syntaptobrevin in the axonal membrane, and this surface protein could freely diffuse between presynaptic terminals.

We are currently studying other SV proteins, as well as proteins involved in the regulation of endocytosis, such as endophilin and intersectin. Further investigation is required to understand how changes in synaptic function correlate with altered distribution and mobility of these synaptic proteins. (Supported by National Institutes of Health grant GM54728 [J.M. Kaplan], Damon Runyon Fellowship [J.S. Dittman], and Jane Coffin Childs Fellowship [J. Bai].)

28. Calcium-dependent Exocytosis in Giant Excised Membrane Patches. TZU-MING WANG and DONALD W. HILGEMANN, Department of Physiology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390
The excised patch is a potentially powerful experimental model to study membrane fusion as it allows high resolution capacitance measurements and free access to the cytoplasmic side. However, fusion processes tend to be disrupted by seal formation and patch excision. Here, we describe recent advances in preserving and characterizing Ca-dependent exocytosis in giant excised membrane patches from the rat basophil leukemia (RBL) cells. Using the RBL cells, stable excised patches of 2–4 pF are easily obtained after disrupting the actin cytoskeleton with latrunculin A. Membrane fusion is triggered by switching the patch to a cytoplasmic solution containing 100–200 μM of free Ca. Capacitance and amperometric recordings reveal that large secretory granules (SGs, filled with serotonin) are lost from membrane patches mostly during the excision procedure. Occasionally, when the SGs are preserved in the patch, fusion gives capacitance steps of ~10–30 fF, indicating that the diameter of the granules is in the micrometer range. The major vesicle pool on the giant excised patch consists of much smaller vesicles (non-SGs, presumably similar in size to synaptic-like microvesicles). Fusion of the non-SG population results in capacitance increments of 0.1 to 0.4 pF routinely with time constants of ~1 s at 30°C. Amperometric recording reveals that the non-SG pool does not contain serotonin. Further experiments show that the non-SG fusion is lost in patches incubated without ATP or with the nonhydrolysable ATP analogue AMP-PNP for a period of >2 min. Tetanus toxin light chain blocks non-SG fusion in these cells. However, preincubating the patches with N-ethylmaleimide does not block it, implying the ATP mechanism is not SNARE cycling, and the SNAREs of non-SGs are loosely zipped in the excised patches. High affinity PIP2 binding ligands, neomycin and PIP2 of non-SGs are loosely zipped in the excised patches. ATP mechanism is not SNARE cycling, and the SNAREs with N-ethylmaleimide does not block it, implying the fusion in these cells. However, preincubating the patches of nonhydrolysable ATP analogue AMP-PNP for a period of >2 min. Tetanus toxin light chain blocks non-SG fusion in these cells. However, preincubating the patches with N-ethylmaleimide does not block it, implying the ATP mechanism is not SNARE cycling, and the SNAREs of non-SGs are loosely zipped in the excised patches. High affinity PIP2 binding ligands, neomycin and PIP2 antibody, have no effect on non-SG fusion, whether recorded in excised patches or in intact cells, indicating that PIP2 metabolism plays no evident role in this type of fusion. We also find that latrunculin A promotes the formation of giant excised patches with non-SG fusion in other cell types (BHK and MEF cells).

29. Massive Ca-induced Membrane Fusion (and Related Processes) Triggered by Reverse Na/Ca Exchange in Nonexcitable Cells. ALP YARADANAKUL, VINCENZO LARICCIA, TZU-MING WANG, MEI-JUNG LIN, CHENGCHENG SHEN, and DONALD W. HILGEMANN, Department of Physiology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390

Voltage-clamped fibroblasts with stable overexpression of cardiac Na/Ca exchangers (NCX1) undergo four responses upon activation of reverse exchange current (i.e., Ca influx induced by 2 mM of extracellular Ca with 40 mM Na and 0.5 mM EGTA on the cytoplasmic side): massive membrane fusion, cleavage of PIP2 by phospholipase C’s (PLC’s), phosphatidylinerine (PS) translocation to the outer cell leaflet, and long-term inactivation of NCX1. Cell capacitance (i.e., membrane area) increases 15 to 30% within 2 to 4 s and recovers largely to baseline over 2 to 4 min when cytoplasmic GTP (0.5 mM) is present. This response can be repeated several times. The fluorescent membrane tracer, FM126, is taken up during recovery and can be released at another Ca influx episode. However, the membrane compartment that cycles cannot be prelabeled by incubating “resting” cells with FM126. The membrane that traffics does not bring functional Na/Ca exchangers or Na/K pumps to the cell surface, nor are transporters removed during endocytosis. To follow PLC activities, we expressed PIP2-binding GFP-PH fusion proteins and diacylglycerol (DAG)-binding GFP-C1 domains. As confirmed by biochemical measurements, PIP2 is cleaved during the high Ca response. PIP2 recovers within 2 min, whereas outward NCX1 remains inhibited. A large flux of phosphatidylinerine (PS) to the outer cell monolayer is indicated by binding of fluorescent annexin V from the extracellular side, and faster binding of rhodamine-labeled polylysine at the extracellular side indicates that phospholipid scrambling is very fast. M1 (muscarinic) receptor activation causes PIP2 depletion with only little membrane fusion, and Ca influx by NCX1 still causes membrane fusion after M1 receptor-induced PIP2 depletion. Thus, PLC cleavage of PIP2 is not required for fusion. However, we find biochemically that large amounts diacyl- and monoacylgllycerols are generated in response to high Ca, even when PIP2 is depleted. Furthermore, GFP-C1 domains that have been brought to the cell surface by exogenous DAG’s or phorbol esters translocate rapidly back to the cytoplasm during the “high Ca” response. In summary, high cytoplasmic Ca rapidly activates multiple phospholipase activities and phospholipid scrambling. These processes may facilitate but are evidently not a direct trigger for massive membrane fusion that occurs in parallel.

30. Membrane Tension Promotes Fusion by Activating Rab-mediated Tethering and Docking. CHRISTOPHER L. BRETT and ALEXEY J. MERZ, Department of Biochemistry, University of Washington, Seattle, WA 98195

The topological transformations that underlie membrane traffic are intimately coupled to membrane tension. Vesicle fission events generally increase lateral (in-plane) tension, whereas fusion events generally decrease tension. Moreover, tension is thought to be a key parameter controlling the postulated terminal subreaction of fusion: transition from a hemifusion junction to a dilated fusion pore. Here, we used a cell-free assay of yeast vacuolar lysosome fusion to analyze organelle-autonomous responses to lateral (in-plane) membrane tension. Unexpectedly, we discovered that tension controls early, Rab-dependent subreactions of membrane tethering and docking. Increases in tension stabilize...
the vacuolar Rab Ypt7p in an activated state, stabilize the HOPS Rab effector complex on the membrane, and accelerate tethering, docking, and fusion. Conversely, decreases in tension trigger Rab inactivation, destabilize effector association with the membrane, and prevent tethering, docking, and fusion. Hyperactivation of the Rab, stabilization of the HOPS tethering complex, or addition of the recombinant Qα-SNARE rVam7p promote fusion at low tension. We suggest that modulation of Rab-dependent membrane docking by membrane tension may act as a general feedback mechanism to control organelle surface/volume ratios, to trigger fusion and membrane expansion before tension increases to perilous levels, and to trigger Rab inactivation upon completion of vesicle fusion with target membranes. (Supported by National Institutes of Health grant GM077349 to A.J. Merz.)

31. The Observance Multivesicular Compound Exocytosis of Insulin. MICHAEL B. HOPPA and PATRIK RORSMAN, Oxford Centre for Diabetes, Endocrinology and Metabolism, Oxford University, Oxford OX3 7LJ, UK

Exocytosis is the cellular process that underlies the majority of chemical communication in an organism. We have measured exocytosis in insulin-secreting cells, as ATP-induced currents by expressing purinergic ionotropic P2X2 receptors. The currents generated by P2X2 receptors from secreted ATP had a rise time of 8–12 ms and a decay of 50 ms. The amplitude of these events was an average of 45 pA, which correlated with a capacitance measurement that increased by 1.1 fF. In several cases, the currents generated from calcium infusion were 550 pA or higher and correlated with a capacitance increase of 9.5 fF. In addition, we briefly (>10s) stimulated INS-1 cells with high potassium solution that contained FM1-43FX dye and immediately imaged fixed cells in parafformaldehyde. These images revealed several spots of punctuate fluorescence with an average size of 375 nm. In some instances (15%), we measured chains of punctuate spots found interconnected to one spot engaged at the plasma membrane spreading into the cytoplasm. We propose that dense-core vesicles will fuse with each other before engaging in multivesicular compound exocytosis. (Supported by Euronadia and Welcome Trust, UK.)

32. FRET Reports Dynamic Regulation of Rab27A during Stimulation of Insulin Secretion. RAY WU,1 STEPHEN ERNST,2 ALICE D. LAM,1 MATTHEW D’ANDREA-MERRINS,1 RISHI CHAUDHURI,1 and EDWARD L. STUENKEL,1 1Department of Molecular and Integrative Physiology and 2Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, MI 48109

The GTPase Rab27A potently regulates glucose-stimulated insulin secretion (GSIS) from pancreatic β-cells. Rab27A controls recruitment/tethering of secretory granules to the plasma membrane and also enhances priming. Therefore, defining the spatiotemporal dynamics and the signaling pathways that regulate Rab27A activation is critical to understanding GSIS. We used CFP-Rab27A and cYFP fused to mutants of Slp4a/granulophilina (SG4A) as a FRET pair to report the subcellular localization and activation state of Rab27A in live insulin secreting cells. FRET was monitored by three-cube sensitized emission and acceptor photobleaching. FRET stoichiometry quantitatively determined apparent FRET efficiencies at specific molar ratios of the FRET acceptor (cYFP) to donor (CFP). Both constitutively active (Q78L) and dominant-negative (T23N) forms of CFP-Rab27A demonstrated equivalent FRET efficiency with wild-type cYFP-SG4A when compared at identical molar ratios. These results are consistent with published biochemical data. Mutations within SG4A’s SHD2 domain nearly eliminated FRET with Rab27A(T23N), while retaining FRET with Rab27A(Q78L). Additional mutations within the SHD1 domain eliminated FRET with Rab27A(Q78L). Thus, both SHD1 and SHD2 are required for binding of Rab27A-GDP to SG4A, whereas Rab27A-GTP interacts mainly with SHD1. Also, the FRET can report on a specific bimolecular interaction. Dynamic changes in Rab27A-GTP were then measured by FRET in Min6 cells. Secretory stimulation (16.5 mM glucose and 50 mM K+ for 12 min) induced a Ca2+-dependent biphasic response characterized by an immediate (~30 s) and substantial (~10%) initial hydrolysis of Rab27A-GTP, followed by marked reformation of Rab27A-GTP. FRET signals were localized predominantly to insulin granules, suggesting a necessary dissolution of the Rab27A-GTP tethering complex for insulin granule exocytosis. After hydrolysis, reactivation of Rab27A likely allows recruitment of new secretory granules to docking sites at the plasma membrane. (This work was supported by funding from National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, grant DKO77050.)

33. Single Exocytic Events of Lamellar Bodies in Alveolar Type II Cells are Followed by a Peri-vesicular Ca2+ Elevation and Actin Accumulation. PIKA MIKLAVC,1 OLIVER WITTEKINDT,1 MANFRED FRICK,2 and PAUL DIETL,1 1Institute of General Physiology, University of Ulm, 89069 Ulm, Germany, 2Department of Physiology, University of Innsbruck, A-6020 Innsbruck, Austria

Lung surfactant is a surface active substance synthesized and secreted by alveolar type II cells (ATII cells). We established a new method to visualize fusion of surfactant-containing vesicles (lamellar bodies [LBs]) with the plasma membrane without the need of fluorescent dyes, using the dark field Richardson 2.5 microscope. LBs appeared as bright, spherical structures in isolated rat ATII cells and showed a sudden loss of the measured light intensity after stimulation with secretagogues ATP (100 μM), ionomycin (5 μM), or
phorbol-12-myristate-13-acetate (300 nM), which was followed by uptake of the dye FM 1-43 as an indicator of LB fusion with the plasma membrane. We used this new approach to monitor LB fusions together with (a) changes in cytoplasmic calcium concentration \([\text{Ca}^{2+}]_\text{i}\), using the calcium indicator dyes Fluo-4 and Fura-2 and (b) actin reorganization in ATII cells expressing actin-GFP fusion proteins. Interestingly, we detected an increase in \([\text{Ca}^{2+}]_\text{i}\), after vesicle fusion, which was restricted to the vicinity of the fused LB. This phenomenon was accompanied by accumulation of GFP-labeled actin around the fused vesicles. Our results suggest that LB fusion causes a localized increase of \([\text{Ca}^{2+}]_\text{i}\), which could be important for actin reorganization around fused vesicles and content release. (Supported by Austrian Science Fund (FWF) Projects P15742 and P15743, Deutsche Forschungsgemeinschaft Project D1402, and the 6th framework of the European Union, PulmoNet.)

34. Membrane Tether Forces in Mast Cells of Mouse. BRENDA FARRELL,1 FENG QIAN,2 and BAHMAN ANVAR1,2 1Department of Otolaryngology and Head and Neck Surgery, Baylor College of Medicine, Houston, TX 77030; 2Department of Bioengineering, University of California, Riverside, Riverside, CA 92521

Our objective is to monitor the equilibrium force acting on a membrane tether formed from the plasma membrane of a mast cell with laser tweezers and calculate the material properties of the membrane (e.g., membrane tension and bending modulus of the membrane) with relationships that link the equilibrium force to properties of the membrane (Buckman, J.D., J. Hua Yao, and M. Wortis. 1996. Physical Review E. 54:5463–5468). We report that tethers formed from mast cells obtained from the peritoneum of mouse are affected by actin and appear to adopt the features of filamentous actin as they are noncylindrical and readily bend. In addition, a dynamic force is observed riding on top of the equilibrium force, which increases monotonically up to ~70 pN after long times (>100 s) and rapidly jumps back (50 ms) in a step-wise fashion to the equilibrium value. We suggest that this dynamic force results from activity of actin (e.g., actin polymerization and actin–myosin interactions) because addition of the actin-disrupting drug cytochalasin E caused (a) the tether thickness to become more uniform; (b) the equilibrium force to exhibit a unimodal distribution centered at 6 pN; and (c) the dynamic force to disappear into the noise floor. We report estimates for plasma membrane tension and bending modulus of mast cell membranes in the presence of cytochalasin E. This result highlights the influence of the cortical actin network on the material properties of mast cell membrane and is of interest to fusion given that the network disassembles when mast cells are triggered to exocytose. (Supported by National Institutes of Health grants R90 DK071504-01 and RO1 DC00354.)

35. A Role for the F-Actin Binding Protein mAbp1 in Clathrin-mediated Endocytosis. CORA S. THIEL,1 TEJA W. GROEMER,2 JURGEN WIENANDS,1 and JURGEN KLINGAUFL2 1Department of Cellular and Molecular Immunology, Medical Faculty, Georg-August-University, 37073 Goettingen, Germany; 2Department of Membrane Biophysics, Max Planck Institute for Biophysical Chemistry, 37077 Goettingen, Germany

Clathrin-mediated endocytosis (CME) is a major mechanism for membrane retrieval from the plasma membrane in eukaryotic cells. It can be separated into several distinct steps: recruitment of clathrin and other proteins to the plasma membrane, invagination, budding, and finally fission of the clathrin-coated vesicle from the membrane by the GTPase dynamin. Here GTP-dependent twisting of dynamin results in constriction of the neck. In combination with a mechanism that creates membrane tension this will ultimately result in membrane fission (Roux, A., K. Uyhazi, A. Frost, and P. De Camilli. 2006. Nature. 441:528–531). Actin-based mechanisms may provide the required tension.

Here we investigate the possible role of the mammalian actin and dynamin binding protein mAbp1 in orchestrating dynamin-dependent constriction and actin-based tension. The adaptor protein mAbp1 is able to bind to dynamin via the C-terminal SH3 and to F-actin via the N-terminal ADFH and coiled-coil domains. We previously showed that fibroblasts and neurons from mice lacking mAbp1 display a 20% reduction in receptor mediated endocytosis or dye uptake upon action potentials, respectively (Connert, S., S. Wienand, C. Thiel, M. Krikunova, N. Glyvuk, Y. Tstsyura, D. Hilkfer-Kleiner, J.W. Bartsch, J. Klingauf, and J. Wienands. 2006. EMBO J. 25:1611–1622). Here we visualize the dynamics of clathrin-coated structures of fibroblasts from mAbp1 KO mice using total internal reflection fluorescence (TIRF) microscopy. By simultaneously monitoring pit formation using clathrin light chain–CFP and vesicle fission using a transferrin receptor tagged with the pH-sensitive pHluorin, we could analyze differences in the dynamics of CME in fibroblasts from mAbp1 KO mice. (Supported by the Deutsche Forschungsgemeinschaft through SFB 523.)

36. A Role for the Endosomal Adaptor Protein AP1 in Synaptic Vesicle Cycling. YAROSLAV TSYTSYURA,1 NATALIYA GLYVUK,1 CONSTANZE RIET,2 JENNYFER BALTES,3 PETER SCHU,3 and JURGEN KLINGAUFL1 1Department of Membrane Biophysics and 2Department of Neurobiology, Max Planck Institute for Biophysical Chemistry, 37077 Goettingen, Germany; 3Department of Biochemistry, Georg-August-University, 37075 Goettingen, Germany

Members of the adaptor protein’s (AP) family play an important role in membrane and protein sorting. At least four distinct AP complexes have been identified, designated AP1, AP2, AP3, and AP4. Each member of
this family consists of four subunits: two large subunits of 100 kD or more, one medium subunit of ~20 kD, and one small subunit of ~50 kD. The clathrin adaptor protein complex AP1 has been localized to the perinuclear trans-Golgi network and mediates vesicular protein transport between the trans-Golgi network and early endosomes. Knock-out of the small σ1B subunit lead to severe defects in synaptic vesicle recycling, measured with the optical tracer synaptopHluorin. The rate of endocytosis as well as the rate of recovery of the pool of releasable vesicles after depletion was slowed down significantly; synaptopHluorin signals did not recover fully even 2 min after depleting the pool of releasable vesicles by 600 AP/50Hz. Immunohistochemistry showed specific trans-Golgi network as well as synaptic staining for AP1 revealed by colocalization with the synaptic bouton marker synaptophysin. Preliminary ultrastructural analysis indicates that knock-out σ1B boutons contain fewer vesicles and display large endosome-like intermediates after stimulation in line with the imaging data. According to Heuser and Reese (Heuser, J.E. and T.S. Reese. 1973. *J. Cell Biol.* 57:315–344), endocytosed synaptic vesicles recycle through early endosomes. Our findings are in good agreement with this hypothesis, implicating AP1 in synaptic vesicle recycling at the stage of vesicle reformation from endosome-like intermediates.

37. A New Assay for Investigating Early Endosomal Budding. SINA V. BARYSCH, SILVIO O. RIZZOLI, and REINHARD JAHN, Department of Neurobiology, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany

Early endosomes are an entry point for most internalized molecules, and they sort material toward several other compartments within the cell. For receiving and sending material, early endosomes require both fusion and budding processes. Homotypic fusion of early endosomes has been studied intensively. Budding, on the other hand, is a process that is rather difficult to study, as one needs to analyze separation of material within a highly heterogeneous system. Therefore, this process has not been investigated in great detail and only little is known about the molecular mechanisms underlying early endosomal budding. As a first step toward understanding these mechanisms, we developed a novel microscopy-based cell-free assay to reconstitute budding of recycling vesicles from early endosomes. We cultivated PC12 cells and allowed them to internalize two different fluorescently labeled endocytic tracers simultaneously. After breaking the cells, we collected post-nuclear supernatants (PNS) containing the labeled organelles. We then analyzed the number of double labeled endosomes. After incubating the PNS in vitro in conditions mimicking the cellular environment (i.e., in presence of cytosol, ATP-regenerating system, divalent cations, etc.) we were able to detect a significant decrease in the level of endosomes containing the two labels. This observation suggests that recycling vesicles bud from the donor organelles, resulting in a separation of the two labels. Our microscopy-based assay is more sensitive than biochemical analyses and provides a powerful tool to investigate the molecular mechanisms of budding. Finally, time-lapse imaging confirmed a model where sorting proceeds through formation of multiple small-sized recycling vesicles from relatively large and long-lived early endosomes, as opposed to a model in which the early endosome itself is a transient, short-lived organelle.

38. GLUT4 Internalization in Myocytes Occurs through Two Distinct Pathways Differentiated by Clathrin Dependence and Sensitivity to Mitochondrial Uncoupling. COSTIN N. ANTONESCU1,2 and AMIRA KLIP1,2 1Program in Cell Biology, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8; 2Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8

The muscle- and fat-specific glucose transporter GLUT4 continuously cycles to and from the plasma membrane. Insulin increases the steady-state GLUT4 levels at the cell surface, largely by increasing its exocytosis as demonstrated in muscle and fat cell cultures. Less is known about GLUT4 endocytosis, although participation of clathrin-mediated endocytosis (CME) was shown by mutagenesis of putative adaptor binding motifs (FQIQ1 and GII1). Pharmacological agents also point to CME and non-CME mechanisms of GLUT4 endocytosis in adipocytes, and insulin reduces GLUT4 internalization in adipocytes. The endocytic route(s) used by GLUT4 in muscle cells as well as its regulation remain unscrutinized. Here we show that GLUT4 internalization in L6 myoblasts was only partly inhibited by either hypertonic sucrose or siRNA gene silencing of clathrin heavy chain. Moreover, GLUT4 internalization was partly inhibited by cholesterol depletion, an effect additive to the inhibition caused by abrogating CME, and fully inhibited by dominant-negative (K44A) dynamin2, but unaffected by inhibition of endocytosis via caveolae through siRNA to syntaxin6. Accordingly, cell surface GLUT4 colocalized extensively with clathrin light chain but not with caveolin-1. Insulin or dinitrophenol (DNP) increased the amount of surface GLUT4. Insulin did not alter GLUT4 internalization, nor its sensitivity to CME inhibition or cholesterol depletion. In contrast, DNP reduced GLUT4 internalization, and this inhibition was additive to that of CME elimination, but not to that of cholesterol depletion. These results suggest that, in myocytes, GLUT4 internalizes simultaneously through CME and a CME- and caveolae-independent but cholesterol- and dynamin-dependent route and illustrate how insulin and DNP differentially impact GLUT4 endocytosis. The study highlights differences in the effect of insulin in muscle and adipose cells, and further reveals...
the regulation of GLUT4 endocytosis upon energy compromise, which may shed light on how physiological stimuli such as muscle contraction elevate glucose uptake in response to increased energy demand. (Supported by a Canadian Diabetes Association Doctoral Research Award to C.N. Antonescu and by grant MT7307 from the Canadian Institutes of Health Research to A. Klip.)

39. Role of the Exocyst in Insulin-stimulated Translocation of GLUT4 in Primary Isolated Adipose Cells. VLADIMIR A. LIZUNOV,1 IVONNE LISINSKI,2 SAMUEL W. CUSHMAN,3 and JOSHUA ZIMMERM BERG,1 1Laboratory of Cellular and Molecular Biophysics, National Institute of Child Health and Human Development, and 2Department of Cell Biology, Harvard Medical School/Center for Blood Research Institute for Biomedical Research, Boston, MA 02115

Cellular glucose uptake is regulated by insulin through changes in the amount of glucose transporter-4 (GLUT4) in the plasma membrane (PM). Recent findings indicate that the main insulin-regulated steps are tethering and fusion of specialized GLUT4-containing vesicles. However, the molecular mechanisms are not established. Here we used time-lapse total internal reflection fluorescence microscopy to test the involvement of the exocyst complex as a potential tethering candidate mediating insulin-dependent GLUT4 exocytosis in isolated rat adipose cells.

We compared the mobility of GLUT4 vesicles in cells expressing the wild-type Exo70 subunit of the exocyst complex or a dominant-negative mutant Exo70-N. Cells expressing the Exo70 mutant exhibited a bimodal distribution of vesicle mobility. However, inhibition of GLUT4 vesicle mobility with an Exo70-N mutant did not affect the insulin-stimulated fusion of these immobilized vesicles as indicated by exposure of GLUT4 on the PM: insulin augmented the amount of PM GLUT4 in response to insulin in Exo70-N mutant cells to the same extent as in wild-type cells.

Exo70 was reported to play an essential role in the translocation of GLUT4 in 3T3-L1 adipocytes, which are fibroblasts stimulated to develop into adipocytes. In mature adipose cells, Exo70 is not required for the successful fusion of GLUT4-containing vesicles to PM, although both exocyst and GLUT4 translocate to PM after insulin stimulation. Thus, in mature adipose cells, the exocyst complex is not important in GLUT4 vesicle trafficking and its disruption does not abolish insulin-dependent GLUT4 translocation to PM. We suggest a developmental role of the exocyst complex to set up specialized fusion sites for the GLUT4 trafficking pathway.

40. Reversible Fluctuations of the Endocytic Fission Pore Induced by Dynamin Inhibitor Dynasore. VLADIMIR A. LIZUNOV,1 TOM KIRCHHAUSEN,2 and JOSHUA ZIMMERM BERG,1 1Laboratory of Cellular and Molecular Biophysics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892; 2Department of Cell Biology, Harvard Medical School/Center for Blood Research Institute for Biomedical Research, Boston, MA 02115

The critical stage of endocytic vesicle formation is the pinching-off of a membrane connection (the membrane neck) to the plasma membrane. The GTPase dynamin mediates fission of the membrane neck in both clathrin-dependent and some nonclathrin-dependent endocytic pathways. However, the mechanism of dynamin’s interaction with the membrane neck remains unclear. Here we investigate the millisecond scale evolution of the membrane neck during normal and stunted fission of endocytic vesicles induced by selective inhibition of dynamin. Fission pore conductance and vesicle capacitance are monitored by admittance measurements performed in the cell-attached patch-clamp experiments. The time-resolved course of the fission pore conductance reflects two stages of the membrane neck transformation: a subsecond linear growth of the neck resistance and an abrupt 50-ms jump finalizing fission. The second stage is irreversible and the final fission pore conductance is independent of the size of the forming vesicle. Dynasore selectively inhibits dynamin GTPase activity and induces repetitive fluctuations of fission pore conductance that do not result in membrane neck fission. The invaginated membrane pits fail to detach and accumulate at the plasma membrane, since we observe a 10-fold increase in pit density compared with control. Also, in dynasore-treated cells, we find pits have shorter membrane necks (<10 nm) than pits in non-treated cells (10–40 nm). Thus we conclude that inhibition of GTPase activity of dynamin halts the elongation of the neck and results in repetitive fluctuations of fission pore conductance.

41. Formation of Clathrin-Coated Pits Monitored in Living Cells by Fluorescence Microscopy with 10-nm Axial Resolution. SAVEEZ SAFFARIAN and TOM KIRCHHAUSEN, Department of Cell Biology, Harvard Medical School/Center for Blood Research Institute for Biomedical Research, Boston, MA 02115

We describe a time-dependent differential method based on total internal reflection microscopy to track the relative average vertical position of two different sets of fluorescent proteins contained within a diffraction-limited object with 10-nm resolution. We used this method to follow the recruitment of clathrin and its adaptors AP-2 and Epsin into coated pits as they formed at the cell surface with a temporal resolution of 4 s. We found that Epsin primarily localizes to the growing edge of the coated pit in proximity to the budding membrane. AP-2, in contrast, closely follows clathrin but only during the first 2/3 of the assembly process, after which
AP-2 recruitment slows down. This results in asymmetric disposition of AP-2 within the clathrin coat away from the plasma membrane. These data are consistent with a model of accumulating invagination during clathrin polymerization.

42. Small Angle Neutron Scattering Studies of Clathrin Triskelia in Solution Show Evidence of Molecular Flexibility. MATTHEW L. FERGUSON,1,2 KONDURY PRASAD,3 HACENE BOUKARI,1 DAN L. SACKETT,1 SUSAN KRUEGER,4 EILEEN M. LAFER,3 and RALPH NOSSAL,1 1Laboratory of Integrative and Medical Biophysics, National Institutes of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892; 2Department of Physics, University of Maryland, College Park, MD 20742; 3Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78229; 4Center for Neutron Research, National Institute of Standards and Technology, Gaithersburg, MD 20899

Clathrin is a major component in the protein coats of certain post-golgi and endocytic vesicles. At low pH, or in the presence of assembly proteins, clathrin triskelia will self-assemble to form a complete clathrin lattice or “basket.” Recently, static light scattering (SLS) and dynamic light scattering studies (DLS) of clathrin triskelia in solution showed that triskelia have an intrinsic pucker (Ferguson, M.L., K. Prasad, D.L. Sackett, H. Boukari, E.M. Lafer, and R. Nossal. 2006. Biochemistry. 45:5916–5922), similar to that shown in a recent high-resolution cryoEM structure of a clathrin basket. We extend this study by performing small angle neutron scattering (SANS) experiments on isolated triskelia in solution under conditions where baskets do not assemble. Unlike the static light scattering experiments, SANS probes a q-range beyond the Guinier regime probed by SLS. Results of SANS measurements are consistent with light scattering experiments but show a shoulder in the scattering function at intermediate q-values (~0.016 1/A) just beyond the Guinier regime. This feature cannot be fully accounted for by theoretical calculations based on rigid bead models of the triskelia, previously used to analyze the light scattering measurements, unless conformational fluctuations are taken into account. A flexible bead-spring model of a triskelion, which we simulated by Brownian dynamics, is used to generate a time-averaged scattering function. This model adequately describes the experimental data for flexibilities close to previous estimates from the analysis of electron micrographs (Jin, A.J., and R. Nossal. 2000. Biophys. J. 78:1183–1194). (Supported by National Institute of Child Health and Human Development predoctoral Intramural Research Training Award.)

43. A Possible Role of Curvature Scaffolding in Dynamin-induced Fission of Membrane Nanotube. SERGEY A. AKIMOV,1,2 VLADIMIR A. J. FROLOV,1,2 JOSHUA ZIMMERBERG,2 and VADIM A. FROLOV,2 1Laboratory of Bioelectrochemistry, Frumkin Institute of Physical Chemistry and Electrochemistry, Russian Academy of Sciences, Moscow, Russia, 119991; 2Laboratory of Cellular and Molecular Biophysics, National Institutes of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892; 3Department of Molecular Biophysics and Physiology, Rush University Medical Center, Chicago, IL 60612

We consider the formation of domains, such as rafts, in cell membranes a process of wetting of proteins by lipids. The membrane is modeled as a continuous elastic medium and thermodynamic functions of wetting lipid films are calculated using the formulation of biomembrane mechanics. This approach leads to a new theory
to describe wetting. The equations of the theory allowed us to rigorously consider the conditions necessary for a macroscopic wetting film to form and to calculate film thickness. We found that macroscopic films exist in the case of large (tens of nanometers in diameter) lipid/protein aggregates. But only thin adsorption films form around small proteins. If wetting films merge, multiple proteins will associate within the same domain. We have therefore considered, in a semi-quantitative fashion, the manner by which wetting films can regulate merger. Importantly, we also found that a wetting form stabilizes a protein within an aggregate and thus proximity is maintained between proteins whose interactions are vital for cellular functioning. Radii of aggregates were numerically calculated as a function of spontaneous membrane curvatures and height mismatches between aggregates and bulk membrane, using experimentally measured elastic moduli. These radii can be compared with the experimentally measured radii. (Supported by grants from the Program for Molecular and Cellular Biology of the Russian Academy of Sciences, the Russian Foundation for Basic Research [05-04-49024 and 07-04-01145], State Contract with Russian Federal Agency of Science and Innovations [02.512.11.2059], the Civilian Research and Development Foundation Grant Assistance Program [RUB1-1297(5)-MO-05], Russian Science Support Foundation, National Institutes of Health [R01 GM066837], and the intramural program of the National Institute of Child Health and Human Development.)

45. Isolating Membrane Rafts at Physiological Temperature Through the use of Membrane Tension. ARTEM G. AYUYAN and FREDRIC S. COHEN, Department of Molecular Biophysics and Physiology, Rush University Medical Center, Chicago, IL 60612

We have developed a method to separate domains of plasma membranes, such as rafts, from the remainder of the membrane at physiological temperature and without use of detergents or alkaline pH. We show that producing membrane tension, by osmotic swelling of cells, causes small rafts that exist under physiological conditions to merge and become large enough to observe microscopically. Non-raft domains also merge to become large, and these domains are microscopically distinct from rafts. Centrifugation experiments showed that these large rafts are the buoyant fraction. This fraction could be subfractionated into several portions, each with somewhat different lipid compositions. Not all subfractions were enriched in cholesterol. In fact, when normalized to the total content of membrane protein, some fractions were depleted of cholesterol. The content of a GPI-linked protein also varied between subfractions. Temperature was an important variable—the distribution of lipid and protein content varied with temperature. We osmotically swelled artificial lipid vesicles composed of sphingomyelin, cholesterol, and an unsaturated lipid to determine whether raft formation and properties were largely determined by lipids. After isolating the buoyant fraction by centrifugation, we subfractionated it and determined the lipid composition. The cholesterol distributions were found to be similar to those observed for cell membranes. This similarity supports the idea that domain formation in cellular membrane is largely a consequence of lipid–lipid interactions rather than protein–lipid and protein–protein interactions.

46. Specific Lipids Provide Critical Negative Curvature to Enable Ca\(^{2+}\)-triggered Native Membrane Fusion. MATTHEW A. CHURCHWARD, TATIANA ROGASEVSKAIA, REGAN C. TAYLOR, and JENS R. COORSSSEN, Hotchkiss Brain Institute, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada T2N 4N1

Fast, Ca\(^{2+}\)-triggered fusion of apposed lipid bilayers is the defining step of regulated exocytosis. In native secretory vesicles, cholesterol and sphingomyelin-enriched microdomains define the efficiency of fusion by organizing the protein and lipid components of the fusion machine before triggering (Churchward, M.A., T. Rogasevskaia, J. Hofgen, J. Bau, and J.R. Coorssen. 2005. J. Cell Sci. 118:4833–4848; Rogasevskaia, T., and J.R. Coorssen. 2006. J. Cell Sci. 119:2688–2694), while cholesterol also contributes directly to the fusion step, apparently by virtue of its intrinsic negative curvature. Structurally dissimilar lipids of comparable negative curvature mimic the ability of cholesterol to support triggered fusion. After cholesterol depletion using methyl-\(\beta\)-cyclodextrin or sequestration with filipin, \(\alpha\)-tocopherol, dioleoylglycerol, and dioleoylphosphatidylethanolamine are able to selectively recover the fundamental ability to fuse, but not the efficiency of fusion. In a dose-dependent manner, this recovery correlates with the amount of negative curvature each molecule imparts to the membrane. Lipids of intrinsic negative curvature less than that of cholesterol (including phosphatidic acid, N-methylphosphatidylethanolamine, N,N-dimethylphosphatidylethanolamine, and phosphatidylcholine) are unable to substitute for cholesterol and do not rescue the ability to fuse. Collectively, these data indicate that membrane negative curvature is required in a specific total amount, provided by select membrane components having a critical minimal intrinsic negative curvature. Meeting both of these requirements is essential to effectively support the formation of high curvature lipidic fusion intermediates, consistent with the stalk-pore model (Koslovsky, Y., L.V. Chernomordik, M.M. Kozlov. 2002. Biophys. J. 83:2634–2651). Thus the negative curvature contributed by cholesterol acts directly in the process of Ca\(^{2+}\)-triggered membrane fusion. Different fusion sites, vesicles, or secretory cell types could use other lipidic components, in addition to cholesterol or similar endogenous sterols, to provide optimal local negative curvature and even to modulate the fusion process. (Supported by the Canadian Institutes of Health Research.)
47. Function of the Reggie/Flotillin Scaffolding Proteins of Membrane Microdomains in Signal Transduction and Axon Regeneration. CLAUDIA A.O. STUERMER, MATTHIAS LANGHORST, CHRISTINA MUNDERLOH, and HELMUT PLATTNER, Department of Biology, University of Konstanz, 78 475 Konstanz, Germany

Reggie-1 and -2 (alias Flotillin-2 and -1) were identified in our lab as two proteins being up-regulated during axon regeneration in the fish and mammalian visual system (Schulte, T., K.A. Paschke, U. Laessing, F. Lottspeich, and C.A. Stuermer. 1997. Development. 124:577–587; Lang, D.M., S. Lommel, M. Jung, R. Ankerhold, B. Petrausch, U. Laessing, M.F. Wiechers, H. Plattner, and C.A. Stuermer. 1998. J. Neurobiol. 37:502–523). Further characterization of reggie proteins has demonstrated that they form multimers (Solis, G.P., M. Hoegg, C. Munderloh, Y. Schrock, E. Malaga-Trillo, E. Rivera-Milla, and C.A. Stuermer. 2007. Biochem J. 403:313–322) and thus scaffolds of plasma membrane microdomains (of ~100 nm; Stuermer, C.A., D.M. Lang, F. Kirsch, M. Wiechers, S.O. Deininger, and H. Plattner. 2001. Mol. Biol. Cell. 12:3031–3045) in a wide variety of cells.

We developed a transnegative reggie construct (R1EA), which was transfected into hippocampal neurons and N2A cells and blocked reggie association with the plasma membrane and microdomain formation. R1EA transfected cells failed to produce cylindrical neurites/axons. Instead they exhibited club-shaped abnormal protrusions. Biochemical analyses showed an imbalance in the activation of the small GTPases Rac, Rho, and cdc42. Moreover, R1EA interfered with FAK activation and the formation of focal adhesions defined by CAP (Langhorst et al., submitted). To analyze if reggie protein down-regulation would also impair axon regeneration in vivo, we applied reggie morpholinos to the transected zebrafish optic nerve. 3 d later the retina was excised, miniexplants were prepared and axon outgrowth from these explants was quantified. To this end we found a 30% reduction of axon regeneration in the reggie morpholino-treated retinae in comparison to controls. Thus, reggie proteins participate in the control of axon/neurite formation and axon regeneration in vivo.

Together with our findings that reggies form preformed platforms in lymphocytes (Rajendran, L., M. Masilamani, S. Solomon, R. Tikkanen, C.A. Stuermer, H. Plattner, and H. Ilges. 2003. Proc. Natl. Acad. Sci. USA. 100:8241–8246), which are needed for the clustering of the signal transduction machinery and for signal transduction of, e.g., the cellular prion protein (Stuermer, C.A., M.F. Langhorst, M.F. Wiechers, D.F. Legler, S.H. von Hanwehr, A.H. Guse, and H.Plattner. 2004. FASEB J. 10.1096/04-2150fje), we suggest that reggies associate to form scaffolds of plasma membrane microdomains and function in the formation of multiprotein signaling complexes. (Supported by TRSB11 of the Deutsche Forschungsgemeinschaft and Ministerium für Wissenschaft und Kultur.)

48. Dynamic Aspects of Plasma Membrane Fluidity and Organization. TIONE BURANDA, YANG WU, YELENA SMAGLEY, ALEXANDRE CHIGAEV, ERIC PROSSNITZ, and LARRY SKLAR, Department of Pathology and Cancer Center, and Department of Cell Biology and Physiology, University of New Mexico School of Medicine, Albuquerque, NM 87131

Membrane rafts are generally viewed as ordered clusters of dynamic, cholesterol and sphingolipid-enriched nanoscopic domains that float in a sea of liquid-disordered lipids. These domains are believed to serve as platforms for various cellular events, such as signaling, membrane trafficking, and viral infection. Because of their submicroscopic size, the existence of membrane rafts continues to be the subject of much debate. Here we report the use of lipophilic fluorescent lipid derivatives of ganglioside GM1 (Alexa488/555 GM1) and octadecyl rhodamine B (R18) to map out the plasma membrane distribution of ligand-activated Nformyl peptide receptor (FPR) and α1β integrins. We also use some recently developed quantum dot based, fluorescent calibration beads for flow cytometry to quantify the degree of cell membrane staining (Wu, Y., S.K. Campos, G.P. Lopez, M.A. Ozbun, L.A. Sklar, and T. Buranda. 2007. Anal. Biochem. 364:180–192). Using U937 cells transfected with a nondesensitizing form of the FPR, we show that the FPR forms enduring signaling clusters on the membrane surface in response to ligand stimulation. The activated FPR clusters are shown to be in ordered membrane microdomains based on: (a) colocalization with Alexa555 GM1 and (b) FRET measurements and evidence of a reduced rate of R18 flip-flop in the liquid-ordered regions surrounding the clustered receptors relative to the more fluid, liquid disordered domains away from the receptor clusters. Ligand activation of U937 cells results in polarization and cocapping of Alexa488/555GM1 and α1β1, which is visualized by microscopy. We have used real-time FRET measurements on a flow cytometer to show that in resting cells α1β1 integrins are localized in disordered lipid domains and undergo conformational changes, which involve unbending of the integrin upon FPR stimulation. The magnitude of conformational change is analyzed by FRET in terms of distance of closest approach between the fluorescently labeled small molecule ligand bound to the integrin headgroup and R18 acceptors on the membrane surface. These data highlight the utility of real-time flow cytometry measurements in the elucidation of membrane structure. (Supported by National Institutes of Health grants K25AI 60036, AI36357, and U54MH074425 and National Science Foundation grant CTS0332315.)

49. Distinct Vesicular Transport Properties Of Human Kv11.1 (Herg1a) K+ Channels. BRIAN P. DELISLE,
The human KCNH2 or Ether-a-go-go Related Gene1 encodes the voltage-gated K⁺ channel 11.1 α-subunit (Kv11.1). Dozens of missense mutations in Kv11.1 linked to long QT syndrome decrease the intracellular transport (trafficking) of Kv11.1 to Golgi and cell surface membrane. The purpose of this study was to elucidate the mechanisms that underlie trafficking of wild-type Kv11.1 (WT-Kv11.1). Incubation of HEK293 cells stably expressing WT-Kv11.1 in brefeldin A (24 h), a fungal metabolite that disrupts the Golgi, inhibited the complex glycosylation (Golgi processing) of WT-Kv11.1 and reduced WT-Kv11.1 current (I_{Kv11.1}) by 78%. The small GTPases Sar1 and ARF1 regulate the formation of coat-associated protein complex II (COPII) and coat-associated protein complex I (COPI) vesicles, respectively. COPII and COPI mediate the transport of many proteins between the endoplasmic reticulum and the Golgi. We cloned Sar1 and ARF1 from human heart cDNA, generated dominant-negative mutations for each, and transiently expressed these GTPases with WT-Kv11.1 in HEK293 cells. Expression of WT-Kv11.1 and H79G-Sar1 inhibited the Golgi processing of WT-Kv11.1, decreased I_{Kv11.1}, and altered the cellular localization of WT-Kv11.1; however, the coexpression of Q71L-ARF1 did not alter the Golgi processing of WT-Kv11.1, I_{Kv11.1} or the cellular localization of WT-Kv11.1. We also found that the Golgi processing of Kv11.1 and I_{Kv11.1} were not affected by the coexpression of several dominant-negative Rab-GTPases (Rab1a, Rab6a, or Rab6aβ), which can also regulate the trafficking of proteins to the Golgi. These data suggest that the trafficking of WT-Kv11.1 is unique because it depends on COPI but not COPII vesicular transport. (Supported by Scientist Development grant 0535068N, American Heart Association grant R01 HL60723 [to B.P. Delisle], and a National Heart, Lung, and Blood Institute grant [to C.T. January].)

50. Kv11.1 (ERG1) K⁺ Channels Localize in Cholesterol- and Sphingolipid-enriched Membranes and Are Modulated by Membrane Cholesterol. RAVI C. BALIJEPALLI, BRIAN P. DELISLE, JASON D. FOELL, TIMOTHY J. KAMP, and CRAIG T. JANUARY, Department of Medicine and Department of Physiology, University of Wisconsin, Madison, WI 53706

The cell surface membrane is a heterogeneous mixture of proteins, cholesterol, glycerol-, phospho-, and sphingolipids. Cholesterol- and sphingolipid-enriched membranes are believed to coordinate multiple cellular processes, including second messenger signaling, recycling of proteins to the membrane, and biophysical properties of ion channels. The localization of ion channels to specific membrane microdomains can strongly impact the functional properties of channels and their role in cellular physiology. We determined the membrane localization of human Kv11.1 (hERG1) α-subunit protein, which underlies the rapidly activating, delayed rectifier K⁺ current (I_{Kr}) in the heart. Immunocytochemistry and membrane fractionation using discontinuous sucrose density gradients of adult canine ventricular tissue showed that Kv11.1 channel protein localized to both the cell surface and T-tubular sarclemma. Furthermore, continuous sucrose density gradient fractionation of Triton X-100 insoluble membranes from canine ventricular myocytes demonstrated that Kv11.1 protein, along with MiRP1 and Kv7.1 (KCNQ1) proteins, localize in cholesterol- and sphingolipid-enriched membrane fractions. In HEK293 cells, Kv11.1 channels, but not long QT–associated mutant G601S-Kv11.1 channels, also localized to cholesterol- and sphingolipid-enriched membrane fractions. Depletion of membrane cholesterol from HEK293 cells expressing Kv11.1 channels using methyl-β-cyclodextrin (MβCD) resulted in a shift of WT-Kv11.1 channels to denser sucrose gradient membrane fractions, caused a positive shift in the voltage dependence of activation, accelerated the deactivation rate, and slightly reduced the inactivation rate. Cholesterol loading of HEK293 cells reduced the steep voltage dependence of I_{Kv11.1} activation and accelerated the inactivation kinetics of I_{Kv11.1}. MβCD treatment likewise accelerated the deactivation rate of I_{Kv11.1} in neonatal mouse myocytes. We conclude that Kv11.1 protein localizes in cholesterol- and sphingolipid-enriched membranes and that membrane cholesterol can modulate I_{Kv11.1} and I_{Kr}. In turn, alterations in membrane cholesterol could modulate cardiac repolarization and potentially impact the risk for cardiac arrhythmias.

51. Drosophila Bestrophins Are Dually Regulated by Calcium and Cell Volume. LI-TING CHIEN AND H. CRISS HARTZELL, Department of Cell Biology, Emory University, Atlanta, GA 30322

Mutations in the human bestrophin-1 (hBest1) gene are responsible for Best vitelliform macular dystrophy. However, the mechanisms linking mutant bestrophin to retinal degeneration have not yet been elucidated because the function of the bestrophin protein is not fully understood. Bestrophins have been proposed to comprise a new family of Cl channels that are activated by Ca²⁺. While the regulation of bestrophin currents has largely been focused on intracellular Ca²⁺, little is known about other pathways/mechanisms that may also regulate bestrophin currents. Here we show that Cl currents in Drosophila S2 cells, that we have previously shown are mediated by dBest1 and dBest2, are dually
regulated by Ca\textsuperscript{2+} and cell volume. The bestrophin Cl currents were activated in a dose-dependent manner by osmotic pressure differences between the internal (zero-Ca\textsuperscript{2+}) and external solutions. The increase in the current was preceded by cell swelling. The volume-regulated Cl current was abolished in dBest1 or dBest2 RNAi-treated cells. Furthermore, cells not expressing dBest1 or dBest2 after RNAi treatment were severely depressed in their ability to regulate cell volume. Vol-

tume and Ca\textsuperscript{2+} regulation can occur independently of one another: the volume-regulated bestrophin current was activated in the complete absence of Ca\textsuperscript{2+} and the Ca-activated bestrophin current was independent of alterations in cell volume. However, these two pathways of bestrophin channel activation can interact: the presence of Ca\textsuperscript{2+} potentiates the magnitude of the current activated by changes in cell volume. We conclude that in addition to being regulated by an elevated amount of cytoplasmic Ca\textsuperscript{2+}, Drosophila bestrophins are also novel members of the volume-regulated anion channel family that are necessary for cell volume homeostasis.

52. Calcium-dependent Gating of MthK, a Prokaryotic Potassium Channel. BRITTANY ZADEK\textsuperscript{1} and CRINA NIMIGEAN,\textsuperscript{2} \textsuperscript{1}Laboratory of Physiology, Oxford University, Oxford OX1 3PT, UK; \textsuperscript{2}Department of Physiology and Membrane Biology, University of California, Davis, Davis, CA 95616

MthK is a calcium-gated, inwardly rectifying, prokaryotic potassium channel. Although little functional information is available for MthK, its known high-resolution structure is used as a model for eukaryotic Ca\textsuperscript{2+}-dependent potassium channels, such as BK.

We characterized the mechanism of Ca\textsuperscript{2+} activation for MthK by measuring currents through single channels incorporated into planar lipid bilayers. When Ca\textsuperscript{2+} is increased from 2 to 5 mM, MthK open probability increases over 100-fold. This sharp increase is approximated well by a Hill plot with a Hill coefficient of 8, the number of Ca\textsuperscript{2+} binding sites in the crystal structure. We found that MthK has two distinct gating modes: a fast process that operates on a millisecond timescale and a slow process operating in the 100-ms timescale. The fast and slow gating processes are modulated separately, with slow gating affected mainly by Ca\textsuperscript{2+} and fast gating affected by voltage. The increase in Po with Ca\textsuperscript{2+} was found to be due mainly to an increase in opening frequency rather than an increase in mean open time; unlike the mechanism of Ca\textsuperscript{2+} activation in BK channels where the open durations also increase dramatically with Ca\textsuperscript{2+}. Depolarization affects both the fast gate by dramatically reducing the fast channel closures and, to a lesser extent, the slow gate by increasing MthK open probability. We were able to capture the mechanistic features of MthK with a modified MWC model (Zadek, B., and C.M. Nimigean. 2006. \textit{J. Gen. Physiol.} 127:673–685).