

heterogeneous ACh distribution showed an increased interval for block of electrical propagation with tissue stretch.

Symposium 20: Membrane Trafficking

2899-Symp

The Interplay between lipid and protein trafficking

Frederick Maxfield.

Weill Cornell Medical College, New York, NY, USA.

2900-Symp

Watching t-SNAREs And Their Interaction With Secretory Granules In Live Cells

Wolfgang Almers¹, Michelle Knowles², Sebastian Barg³, Lei Wan¹.

¹The Vollum Institute, Oregon Health and Sciences U., Portland, OR, USA,

²Denver University, Denver, CO, USA, ³University of Umea, Umea, Sweden.

The SNARE proteins Syntaxin and SNAP25 inhabit the plasma membrane, and VAMP/syntaxin in the membrane of secretory vesicles. When all three combine in a 1:1:1 complex they are thought to fuse the secretory vesicle to the plasma membrane. To explore the interaction of Syntaxin and SNAP25 with secretory vesicles, we have imaged live cells using TIRF microscopy in two colors. Cells co-expressed a fluorescent granule marker as well as EGFP-tagged t-SNAREs at low copy number. Fluorescence was calibrated by single molecule measurements. Granules formed nanodomains beneath them, each with room for 100 syntaxin molecules. The nanodomains repeatedly and spontaneously emptied of syntaxin and then re-filled. They exchanged their syntaxin with plasma membrane with a half time of a few seconds, and when a granule performed exocytosis its nanodomain disassembled. SNAP25 was concentrated beneath granules but with 10 fold lower affinity than Syntaxin. Most Syntaxin and nearly all SNAP25 molecules were seen to move freely in the plasma membrane, but a minor proportion of each t-SNARE was almost immobile. Single Syntaxin molecules could be observed as they were captured and released from granule sites. We have tracked the recruitment and release of SNAREs at exocytic sites in a time-resolved manner and with single molecule sensitivity.

2901-Symp

Molding The Plasma Membrane At Sites Of Endocytosis

Pietro De Camilli.

Yale University School of Medicine, New Haven, CT, USA.

An important goal of our laboratory is to elucidate mechanisms in the biogenesis and traffic of synaptic vesicles at neuronal synapses, with emphasis on the processes that mediate their reformation by endocytic recycling after each cycle of secretion. We use a variety of complementary approaches that include reconstitution experiments with purified endocytic proteins and lipid membranes, broken cell preparations, intact cells, model synapses and genetically modified mice. With these studies we hope not only to improve knowledge of synaptic transmission but also to advance the understanding of fundamental mechanisms in endocytosis. In my talk I will discuss the role of the GTPase dynamin, a protein implicated in the fission reaction of endocytosis, and the impact of the lack of dynamin on cell structure and physiology. We have generated KO mice for each of the three dynamin isoforms. These mice, as well as cells derived from them, allow us to study the fundamental function of dynamin as well as isoforms specific functions. Surprisingly, cells without any dynamin live, although they fail to proliferate and they display major alterations in the structure of the cell surface. I will also discuss the function of dynamin binding partners with curvature generating and curvature sensing properties (proteins with BAR and F-BAR domains), and the mechanisms through which these proteins deform membranes (Roux et al. Nature 441: 528-531; Ferguson et al. Science 316: 570-574; Frost et al. Cell 132:807-817).

2902-Symp

Phosphoinositides in Ca²⁺ Signaling and Plasma Membrane Biogenesis: Roles for Electrostatic Interactions

Barbara Baird, Lavanya Vasudevan, Nathaniel Calloway, Alice Wagenknecht-Wiesner, Kirsten Elzer, David Holowka. Cornell Univ, Ithaca, NY, USA.

Phosphoinositides are implicated in a wide range of cellular pathways, both at the plasma membrane and at other organelles. During IgE receptor activation in mast cells, phosphatidylinositol 4,5-bisphosphate (PIP₂) synthesized by PIP5-kinase Igα at the plasma membrane is hydrolyzed by phospholipase Cγ to produce inositol 1,4,5-trisphosphate, which initiates store-operated Ca²⁺ influx (SOCE). In contrast, PIP₂ synthesized by another isoform, PIP5-kinase Iα, regulates SOCE in these cells in an apparently bimodal manner: It promotes the interaction between the endoplasmic reticulum (ER) Ca²⁺ sensor STIM1 and the Ca²⁺ channel protein Orai1/CRACM1, yet it plays a net negative role in SOCE, possibly by inhibiting Orai1/CRACM1 gating. Functional

coupling between STIM1 and Orai1/CRACM1 involves electrostatic interactions: Coupling is blocked by positively charged sphingosine derivatives at the inner leaflet of the plasma membrane, and also by mutation of six acidic amino acid residues in the coiled-coil C-terminus of Orai1/CRACM1. We hypothesize that PIP₂ participates in this electrostatic coupling.

Phosphoinositol 4-phosphate participates in ER-to-plasma membrane biogenic trafficking, and it is synthesized from phosphatidylinositol (PI) at the cytoplasmic face of the ER by PI4-kinase IIIα. We find that expression of the polybasic MARCKS effector domain in the lumen of the ER reduces PI4P content in the Golgi complex and inhibits ER-to-plasma membrane protein trafficking in parallel with this inhibition. We hypothesize that ER-targeted MARCKS effector domain traps PI at the luminal face by an electrostatic interaction to inhibit PI4P synthesis and thereby ER-to-plasma membrane trafficking. These results highlight the importance of negatively charged phosphoinositides in multiple cellular pathways and point to the roles of electrostatic interactions in regulating these processes.

Symposium 21: Receptor-mediated Channel Activation

2903-Symp

Conformational Changes Before Opening And The Activation Mechanism In Glycine And Nicotinic Receptors

Lucia Sivilotti.

UCL, London, United Kingdom.

Channels in the nicotinic superfamily are pentameric membrane proteins that respond to the binding of transmitter molecules to their extracellular domain by opening their integral membrane pore. One of the best ways to obtain information on the chain of events that follows transmitter binding is by single channel analysis. Mechanisms of receptor activation can be fitted to sets of experimental recordings for the purpose of validating a particular model and quantifying the rate at which each step occurs. We use HJCfit, a program developed by David Colquhoun (available from <http://www.ucl.ac.uk/pharmacology/dc.html>) to obtain maximum-likelihood, global mechanism fits with full missed event correction to steady-state recordings obtained at different agonist concentrations and idealised by time-course fitting. By the use of this technique on wild-type glycine receptors, we were able for the first time to detect an intermediate conformational change that follows agonist binding but precedes channel opening. The short-lived, partially-activated intermediate shut state (which we termed "flip") has a higher affinity for the agonist than the resting state, which suggests that this conformational change involves some degree of domain closure in the extracellular domain. Activation models that include this flipped state can also accurately describe the properties of ACh nicotinic receptors and of startle disease mutants of the glycine channel. Analysis of the activation of nicotinic channels and glycine channels by partial agonists showed that the difference between partial and full agonists resides in the first conformational change (flipping) rather than in the open-shut reaction as has always been supposed previously. Partial agonists are poor at eliciting the change from resting to flipped, but once in the flipped state the opening and shutting of the channel is much the same for all agonists.

2904-Symp

Probing Structure on Well-defined Functional States of the Nicotinic Receptor Using Systematically-engineered Ionizable Residues and Proton-transfer Events

Gisela D. Cymes, Claudio Grosman.

University of Illinois at Urbana-Champaign, Urbana, IL, USA.

The conformational changes that underlie the closed-open transition in members of the nicotinic-receptor superfamily remain elusive and controversial. To gain insight into the structural properties of the pore-domain of the muscle-nicotinic acetylcholine-receptor channel (AChR) in the open state, while retaining the advantages of studies on intact cells and in real time, we engineered basic residues along the M1, M2, and M3 transmembrane segments of all four types of subunit and recorded the individual proton-transfer events using single-channel patch-clamp electrophysiology. Proton binding-unbinding reactions to and from individual side chains were manifest as blocking-unblocking events of the passing cation current. Two observables, namely, the extent to which the current is attenuated upon side-chain protonation, and the pK_a-shifts of the engineered ionizable groups relative to bulk water, were analyzed to reveal the electrostatic properties of the local microenvironment around the transmembrane segments in the open-channel conformation. In turn, these data were interpreted in terms of secondary and tertiary structure, and compared with existing structural models of the closed state in order to elucidate the change in conformation that opens the AChR. Our open-channel data suggests that the