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
Wolfhard Almers[1], Michelle Knowles[2], Sebastian Barg[3], Lei Wan[1].
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The SNARE proteins Syntaxin and SNAP25 inhabit the plasma membrane, and VAMP/synaptobrevin 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 (PIP_2) synthesized by PIP5-kinase Igamma at the plasma membrane is hydrolyzed by phospholipase Cgamma to produce inositol 1,4,5- trisphosphate, which initiates store-operated Ca^{2+} influx (SOCE). In contrast, PIP_2 synthesized by another isoform, PIP5-kinase Ialpha, regulates SOCE in these cells in an apparently bimodal manner: It promotes the interaction between the endoplasmic reticulum (ER) Ca^{2+} sensor STIM1 and the Ca^{2+} 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 PIP_2 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 IIIalpha. 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
Conformation 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 pKa-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

orientation of the M1, M2, and M3 transmembrane segments of the AChR with respect to the pore and each other is very similar to that in the closed-channel structural model developed on the basis of the cryo-EM images or Torpedo's receptor at 4-Å resolution. To the extent that this structural model corresponds to the actual closed-channel conformation, our results indicate that the expansion of the pore that underlies channel opening involves only a limited rearrangement of these three helices. Such a modest change seems optimal to ensure rapid closed-open interconversion rates, and hence, a fast postsynaptic response upon neurotransmitter-binding.

2905-Symp
Modulation of AMPA and kainate receptors by accessory subunits
James Howe.
Yale University, New Haven, CT, USA.

2906-Symp
Mechanisms For Information Processing By NMDA Receptors: Insights From Single-channels
Gabriela K. Popescu.
University at Buffalo (SUNY), Buffalo, NY, USA.
NMDA receptors are glutamate-activated ion channels with key roles in synaptic transmission and information processing in the brain. Their calcium permeability and voltage-dependent magnesium block have been long identified as critical to these roles. In addition, NMDA receptors have exceptionally slow kinetics and are sensitive to a broad array of neuromodulators. We hypothesized that these two latter features allow NMDA receptors to recognize and integrate a variety of extracellular signals.
After rapidly binding glutamate and glycine, NMDA receptors activate slowly by navigating a complex gating pathway marked by peaks and valleys in the energy landscape of their activation reaction. At equilibrium, fully liganded receptors distribute among multiple pre-open, open and desensitized states which can be resolved in single-channel records and characterized kinetically with statistical analyses. With this approach, we measured changes in the gating energy landscape induced by individual allosteric modulators to learn how NMDA receptors process and integrate chemical information presented in their extracellular environment,
We characterized the effects of protons, zinc, partial agonists, and the neurosteroids pregnanolone sulfate and pregnenolone sulfate on the gating reaction of NR1-1a/NR2A receptors. We found that each of these modulators exert specific, signature effects on the gating reaction. These changes can be expressed as unique sets of gating rate constants. The resulting quantitative kinetic models represent valuable tools for further structure-function investigations, as well as for determining how neuromodulators affect the NMDA receptor response to a variety of physiologic and pathologic stimulation patterns.

2907-Symp
Problems In Determining A Mechanisms Of Receptor Activation And Relating It To Structure
David Colquhoun.
UCL, London, United Kingdom.
Our aim is to understand receptors well enough to be able to make predictions about the activity of a new ligand or about the effect of mutations. This has yet to be achieved but it will entail knowing about both structure and function, and about the relation between them.
Receptors on ion channels that mediate synaptic transmission are essentially never at equilibrium. To understand how they function in real life requires a physical mechanism to be postulated and, if verified, the rate constants in that mechanism to be estimated. The mechanism must describe states that have real physical reality (to a sufficient approximation) if the aim of achieving predictive ability is to be attained. Recent advances in single channel analysis have allowed measurement of up to 18 rate constants, far more than can be obtained from any macroscopic analysis, and it has allowed more stringent tests of proposed mechanisms too. At a limited level some predictive ability has been achieved. For example, the synaptic current produced by glycine can be predicted from steady state single channel analysis (Burzomato et al. 2004, J. Neurosci. 24, 10924-10940), but the ability to predict the effects on function of changes in structure of either the ligand or the receptor is still very limited (though this fact is often disguised by exaggerated claims). It is impossible to say what the next major step forward will be. My guess, for what it's worth, is that single molecule fluorescence methods, perhaps combined with improved NMR and molecular dynamics, may improve enough to allow a firm structural interpretation of the postulated intermediate states in channel opening, despite their brief lifetime of 10 microsec or less (Lape et al. 2008, Nature, 454, 722 - 728)

Minisymposium 4: Electron Transfer and Energy Coupling Reactions in Organelles

2908-MiniSymp
The Reaction Of Cytochrome aa_3-600 With Radical Trapping Agents
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Cytochrome aa_3-600 or menaquinol oxidase from *Bacillus subtilis* is a member of the heme-copper oxidase family that includes mitochondrial cytochrome *c* oxidase. A distinguishing feature of cytochrome aa_3-600 is that it does not oxidize cytochrome *c* and does not contain a Cu_A center, but instead uses menaquinol as reducing substrate to convert O_2 to water. A radical signal is observed when cytochrome aa_3-600 is frozen during the course of steady-state catalysis. The nature of this radical is not fully characterized and we aim to understand it further by using the radical traps 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), 2-methyl-2-nitrosopropane (MNP) and N-*tert*-butyl-phenylnitrone (BPN). TEMPO appears to inhibit menaquinol oxidase's steady state activity, whereas MNP and BPN are without effect. Heme-copper oxidases form a series of intermediates when exposed to H_2O_2 that are related to the intermediates formed in the much faster reaction with oxygen. Addition of H_2O_2 to oxidized cytochrome aa_3-600 leads to formation of the ''P-state'' (606 nm), which is followed by progression to the ''F-state'' (580 nm). The progress of this reaction is halted at the P-state when performed in the presence of TEMPO (50 μM- 5mM). In addition if TEMPO is added at the end of the H_2O_2 reaction the F-state is converted back to the P-state. We propose that the inhibition of cytochrome aa_3-600 by TEMPO is mediated by its ability to trap the P-state of the enzyme and slow its progress through the catalytic cycle.

2909-MiniSymp
Two Conformations Of The Cytochrome C Oxidase Discriminated By Spectro-electrochemistry Using Seiras
Renate L.C. Naumann.
MPI for Polymer Research, Mainz, Germany.
Electronic wiring of cytochrome c oxidase (CcO) from *R. sphaeroides* to gold surfaces was employed to monitor redox changes through redox centers, Cu_A, heme a, heme a_3 and Cu_B. Electrochemical investigations revealed that under aerobic and reducing conditions the enzyme undergoes a gradual transition into an activated state. It is only in this state that proton pumping and catalytic currents can be observed. The potential of the catalytic current, however, is shifted by 450 mV negative from the standard redox potential of Cu_A. In contrast, ''correct'' standard redox potentials of all the centers in the positive potential range can be observed if the enzyme kept under anaerobic and oxidizing conditions. Then no proton pumping does take place. This state is therefore considered as a non-activated state. The transition between the two states is fully reversible. This was also verified by electrochemically-controlled surface-enhanced infrared absorption spectroscopy (SEIRAS) and surface-enhanced resonance Raman spectroscopy (SERRS).
Activated Non-activted
Ch. Nowak, Ch. Luening, W. Knoll, R. L. C. Naumann, A two-layer gold surface with improved surface-enhancement for spectro-electrochemistry using SEIRAS, JPC(C) under review

2910-MiniSymp
Rapid Freeze-quench Trapping Of Intermediates In The Reaction Of Cytochrome *c* Oxidase With Hydrogen Peroxide
Michelle A. Yu, Gary J. Gerfen, Syun-Ru Yeh, Denis L. Rousseau.
Albert Einstein College of Medicine, Bronx, NY, USA.
Hydrogen peroxide treatment of Cytochrome *c* Oxidase (C*c*O) at low and high pH is commonly used to form the ''P'' and ''F'' oxygen-intermediates. The structure and function of intermediates formed in the reaction of bovine C*c*O with H_2O_2 are studied using a custom rapid freeze-quench device designed to trap fast biochemical intermediates on the 50 to 500 μsec time scale. Oxidized bovine C*c*O was mixed with H_2O_2 at pH 6 and pH 8 at room temperature. Reaction intermediates were freeze-quenched at 77 K and examined by optical absorption and EPR spectroscopy. A new carbon-based radical signature arises, which is distinct from the ''broad'' and ''narrow'' species trapped via manual mixing of H_2O_2 and bCcO at low and high pH. A comparison between the new radical, the narrow, and the broad species was conducted at X-band (9 GHz) and D-band (130 GHz) EPR. Assignment to oxygen-intermediates was made by low-temperature optical absorption. The reaction mechanism is reassessed based on these results.