the plasma membrane of neuroendocrine cells. Previous studies have shown that syntaxin-1A is found in clusters that are different from lipid rafts in neuroendocrine plasma membranes. However, the interactions that promote these clusters have been largely unexplored. Here, we have reconstituted syntaxin-1A into lipid model membranes and show that cluster formation of syntaxin depends on cholesterol in a lipid system that lacks sphingomyelin and therefore does not form typical liquid-ordered phases that are commonly believed to represent lipid rafts in cell membranes. The cholesterol-induced clustering of syntaxin is found to be reversed by as little as 1 mol % of the regulatory lipid phosphatidylinositol-4,5-bisphosphate (PIP2), and PIP2 is shown to bind specifically and electrostatically to syntaxin, presumably mediated by the positively charged juxtamembrane domain of syntaxin. Possible consequences of these results to the regulation of SNARE mediated membrane fusion are discussed.

1837-Pos Board B681

Lipid Mixing and Content Release in Single-Vesicle, SNARE-driven Fusion Assay with 5 ms Time Resolution

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A single-vesicle, fluorescence based SNARE-driven fusion assay enables simultaneous measurement of lipid mixing and content release with 5 ms/frame, or even 1 ms/frame, time resolution. The v-SNARE vesicles, labeled with lipid and content markers of different color, dock and fuse with a planar t-SNARE bilayer supported on glass. A narrow (< 5 ms duration), intense spike of calcein fluorescence due to content release and dequenching coincides with inner-leaflet lipid mixing within 10 ms. The spike provides much more sensitive detection of productive hemifusion events than do lipid labels alone. Consequently, many fast events that were previously thought to be prompt, full fusion events are now re-classified as productive hemifusion events. Both full fusion and hemifusion now occur with a time constant of 5-10 ms. At 60% DOPE lipid composition, productive and dead-end hemifusion account for 65% of all fusion events. However, quantitative analysis shows that calcein is released into three-dimensional space above the bilayer (vesicle bursting), rather than the thin aqueous space between bilayer and glass. Evidently at the instant of inner-leaflet mixing, flattening of the vesicle increases the internal pressure beyond the bursting point. In the future, additional height of the aqueous space may enable proper content release. To achieve this goal, single vesicle-vesicle fusion will be tested by tethering v-SNARE vesicles onto supported lipid bilayers. Also GUVs with t-SNARE proteins will be ruptured onto polymer cushion to create a floating target membrane for v-SNARE vesicles to fuse with.

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Visualizing Viral Fusion At The Single-particle Level

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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 viral particles provides further evidence of a hemifusion intermediate preceding pore formation. Distributions of the lag times of events reveal multiple long-lived kinetic intermediates leading to hemifusion followed by a single rate-limiting step to pore-formation. We interpret the series of intermediates preceding hemifusion as the result of multiple copies of the trimeric hemagglutinin fusion protein participating in a single fusion event.

1839-Pos Board B683

Functional and Structural Measurements of HIV gp41 Fusion Protein Constructs

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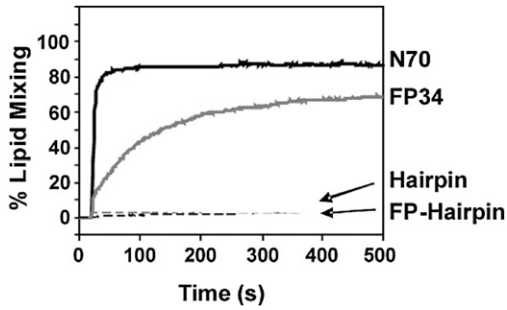
The initial step of HIV infection is fusion between the viral and target cell membranes. Fusion is mediated by the HIV gp41 protein and its N-terminal "fusion peptide" (FP) which binds to target cell membranes. Shorter constructs of gp41 that contain the FP usually catalyze vesicle fusion and such fusion at physiological pH was measured for three different gp41 constructs which differed in their numbers of N-terminal gp41 residues. "FP34" and "N70" were respectively models of the FP and "pre-hairpin intermediate" gp41 conformation while "FP-hairpin" was a model of the final "six-helix-bundle" gp41 structure. N70 induced rapid fusion, FP34 induced moderate fusion, and FP-hairpin induced no fusion and even arrested fusion induced by FP34. The data therefore suggest that the six-helix bundle conformation stops membrane fusion. In related work, solid-state nuclear magnetic resonance measurements probed the membrane locations of three different FP constructs with very different fusion rates. There was a positive correlation between fusion rate and depth of membrane insertion for the FP in either helical or β strand conformation. The key determinant of fusion rate may therefore be FP membrane location rather than conformation.



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SNARE-Mediated Adhesion Kinetics in Giant Membrane Systems

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Giant membrane *in vitro* systems are powerful and relatively unexplored model systems to probe mechanisms of SNARE-mediated adhesion and fusion. We present results of a joint experimental-modeling study of SNARE-mediated adhesion kinetics of giant unilamellar vesicles (GUVs) with supported bilayers (SBLs). The large size of GUVs allows control of physical parameters such as tension and direct observation of adhesion kinetics unavailable in small unilamellar vesicle (SUV) systems. In the gravity-imposed contact zone between v-SNARE GUVs and t-SNARE SBLs, reflection interference contrast microscopy (RICM) revealed initial membrane separation ~50 nm, consistent with Helfrich theory predicting intermembrane repulsion from thermal undulations. Tight adhesion in several discrete patches within the contact zone then develops over ~10 min, with total patch area growing linearly in time. We mathematically modeled the adhesion kinetics, which comprises two stages. (a) Patch nucleation kinetics. Nucleation is limited by the mean membrane separation which exceeds the reach of cognate SNAREs in apposing membranes. Complexation requires fluctuations to bring cognate SNAREs into proximity and the nucleation rate depends on tension and SNARE density. (b) Patch growth. Once nucleated a patch encourages further complexation because the membrane separation is within SNARE reach. We find two classes of patch growth kinetics. (i) Mobile SNARE complexes. Complex osmotic pressure then drives patch growth and patch area grows quadratically in time. (ii) Immobile SNARE complexes. Patches of tightly clustered SNARE complexes are predicted: patch growth, limited by SNARE diffusion, is linear in time. The experimental data are consistent with model (ii). The SNARE complex density in the patch inferred from the areal growth data suggests that complexes were shoulder-to-shoulder as expected in clusters generated by SNARE-SNARE interactions.

1841-Pos Board B685

Relative Rearrangements Of Synaptotagmin3 C2A And C2B Domains Are Influenced ay Calcium, Lipids And SNARE Proteins

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Synaptotagmins are able to bind negatively charged lipids in Ca2+ dependent manner and act as Ca2+ sensors. Together with SNARE and additional auxiliary proteins synaptotagmins coordinate Ca2+ triggered vesicle exocytosis, although the exact mechanism remains unclear. Synaptotagmins are transmembrane proteins comprised of a short intralumenal/extracellular sequence,