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Massive Endocytosis (MEND) Activated By Ca and Polyamines in Fibroblasts and Cardiac Myocytes: Optical Studies of Membrane and Na/Ca Exchanger (NCX1) Internalization, The Possible Role of Ca-activated Transglutaminase in MEND, and The Possible Function of MEND In Cardiac Myocytes

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When cytoplasmic solutions contain physiological concentrations of polyamines (1 mM spermine or spermidine), large Ca transients can cause massive endocytosis in fibroblasts and cardiac myoyctes within 2 to 6 seconds. The magnitudes of responses (30 to 70% of cell surface) are confirmed by both capacitance and FM dye measurements. Using cardiac Na/Ca exchanger (NCX1) fusions with an extracellular Halo tag or an extracellular Phluorin tag, we confirm that NCX1 can be internalized during MEND. We next describe experiments to test candidates for the Ca sensor in MEND. Calmodulin-dependent processes have been largely eliminated by inhibitor studies. Ca-activated transglutaminase activity appears to be an interesting candidate with the potential that critical signaling proteins are activated by polyamination. MEND is inhibited by the transglutaminase blocker, cystamine, and by glutamine-containing peptide substrates of transglutaminase. We also describe that MEND-like processes can be activated in cardiac myoytes during cell isolation protocols. In myocytes from young rodents, NCX1 can be lost 'biochemically' and 'functionally' from the cell surface within 30 min to 1 h, and this loss can be largely blocked by cystamine. Furthermore, HRP labeling progresses rapidly through vesicle, multivesicular, and lysosome stages during this same time period after isolation.

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A New Method for Studying Apical Membrane Trafficking

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Sorting and trafficking of proteins to their target membranes is crucial for the function of epithelia as fluid transporting entities. A powerful tool to study membrane trafficking is total internal reflection fluorescence (TIRF) microscopy at which only a ~100 nm thin layer at the glass buffer interface is illuminated. In particular it allows for monitoring motion and membrane fusion of vesicles carrying membrane proteins with high temporal resolution in living cells. However, TIRF microscopy was limited so far to study membrane trafficking at the basolateral membrane at the sites where cells are attached to the glass cover slip.

To overcome this limitation we developed a microfluidic biochip which allows for approaching the apical membrane of polarized cells towards a glass cover slip in a controlled way. The chip was applied to visualize fluorescently tagged aquaporins at the apical membrane of Madin-Darby canine kidney (MDCK) cells by TIRF microscopy.

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Constitutive and Ca^{2+}-stimulated Turnover of the Plasma Membrane Vacuolar H^+-ATPase (V-ATPase) in Murine Osteoclasts

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Vacuolar-type H^+-ATPases (V-ATPases) are widely distributed at intracellular membranes from yeast to mammalians and could transport H^+ across membranes against negative pH gradients. In osteoclasts, V-ATPases are recruited to the plasma membrane by exocytotic fusion of lysozomal membranes to form the ruffled membrane facing to the bone surface and play an important role in acid secretion. Ca^{2+} released from bone tissue is accumulated in the closed extracellular compartment, which in turn inhibits osteoclast functions. V-ATPases at the ruffled membrane would be exposed to this high level of extracellular Ca^{2+}. We recently succeeded to record proton currents of the plasma membrane V-ATPase in murine osteoclasts using the conventional whole-cell clamp technique, and found that the pump current was decreased by increased extracellular Ca^{2+}. In this study, we investigated whether endocytotic/exocytotic processes were involved in the Ca^{2+}-induced inhibition of the V-ATPase by measuring the H^+ current and the cell capacitance (C_m) simultaneously. C_m is a reliable monitor for surface area. Extracellular Ca^{2+} (5–40 mM) decreased the H^+ current and the C_m simultaneously. The Ca^{2+}-induced inhibitions were dose-dependent and were mimicked by Mg^{2+}. The decrease in the C_m by 40 mM Ca^{2+} was only fractional, corresponding to ~10% of the control (~150 pF), but the decrease in the V-ATPase current was by 70–80%. Bafilomycin A_1, inhibited the Ca^{2+}-induced decrease in the C_m, indicating that V-ATPase-rich membrane may be targeted for endocytosis. These data suggest that extracellular Ca^{2+} facilitates internalization of the V-ATPase through endocytosis, which may be a mechanism for Ca^{2+}-induced inhibition of osteoclastic bone resorption.

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Characterizing the Conformation of the Yeast Endocytic Scaffold Protein Pan1

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Endocytosis is a sequential process that involves the coordination of numerous proteins to internalize membrane bound cargo and extracellular material. The steps of endocytosis include cargo selection, assembly of the endocytic machinery, and the activation of factors that enable vesicle internalization. Transitions between these stages are carefully regulated to ensure successful endocytosis. The essential Saccharomyces cerevisiae protein Pan1 acts as a ''scaffold'', mediating endocytosis by binding factors that act in both early and late stages of endocytosis. The Pan1 N-terminus interacts with adaptor proteins involved in the early process of cargo selection. At its C-terminus, Pan1 binds late-acting factors such as the type I myosins and the Arp2/3 complex, which stimulate actin polymerization to promote vesicle scission. In vitro data suggest that phosphorylation of Pan1 plays a role in its regulation. The Pan1 N-terminus is phosphorylated by the kinase Prk1, which inhibits Pan1 stimulation of Arp2/3 dependent actin polymerization in vitro. These and other data suggest a model whereby Pan1 adopts a conformation that prevents its interaction with late-acting factors in endocytosis. The inhibited state mediated by self-association may be an intra-molecular interaction, where Pan1 folds upon itself, or an inter-molecular interaction, through Pan1 oligomers formed by the central coiled-coil domain. Consistent with this model, initial electron microscopy and biophysical data indicate that Pan1 undergoes Prk1-dependent conformation changes. To characterize the conformations Pan1 can assume, I am defining the domains involved in Pan1 self-interactions and quantifying their affinities and dependence on phosphorylation. I am also analyzing the tryptophan fluorescence and anisotropy values of Pan1 mutants containing only one of four tryptophans. A greater understanding of Pan1 structural changes and how these conformations regulate endocytosis is necessary to develop a complete model of the endocytic process.

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Single Fluorophore Detection And Tracking Of Fluorescent Weibel-Palade Body Membrane Proteins During Exocytosis

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The membranes of mature Weibel-Palade bodies (WPBs), an endothelial cell-specific secretory organelle, contain the leukocyte adhesion molecule P-selectin, the tetraspanin CD63 and the small GTPase Rab27a. Exocytosis delivers these proteins to the cell surface, however, little is known about their mobility in the WPB membrane and how this changes after insertion into the plasma membrane. P-selectin-EGFP, EGFP-CD63 and EGFP-hRab27a were transiently expressed in HUVECs by Nucleofection to label the membrane of WPBs, and WPB exocytosis was evoked by ionomycin (1μM) at 37°C. Using TIRFM and single fluorophore (SF) detection and tracking the diffusion of these proteins in the plasma membrane following exocytotic release was investigated. These data were compared to mobilities for each protein, determined previously by confocal FRAP, in the membranes of individual mature WPBs (Kiskin et al 2007).

We have shown, by FRAP analysis, that P-selectin-EGFP is immobile, EGFP-CD63 mobile ($0.12\mu m^2/s$, (n=31 WPBs) and EGFP-hRab27a highly mobile ($0.68\mu m^2/s$, (n=43 WPBs) in the limiting membrane of individual WPBs (Kiskin et al 2007). During exocytosis P-selectin-EGFP, EGFP-CD63 or EGFP-hRab27a was released and EGFP-SFs detected and automatically tracked. In each case the SFs were found to diffuse freely in the vicinity of the fusion site with diffusion coefficients of $0.14\mu m^2/s$, (P-selectin-EGFP; n=2890 SF), $0.2\mu m^2/s$, (EGFP-CD63; n=3134 SF) and $\sim 0.50\mu m^2/s$, (EGFP-hRab27a; n=1023 SF). These data reveal changes in membrane mobility of some but not other WPB membrane proteins following exocytosis.

Kiskin et al (2007). Eur. Biophys. J. 36, S141.

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A Role For Protein Phosphorylation In Fusion Pore Opening And Transmitter Release

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Activity-dependent regulation of protein kinase A (PKA) and protein kinase C (PKC), is essential for modulation of neurosecretion. To study the role of protein phosphorylation in the dynamics of the fusion pore opening and transmitter