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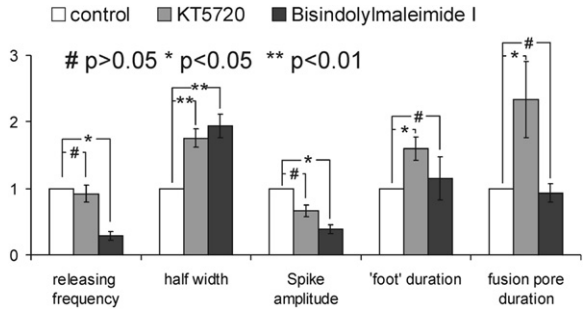
Activity-dependent regulation of protein kinase A (PKA) and protein kinase C (PKC), is essential for modulation of neurosecretion. To study the role of protein phosphorylation in the dynamics of the fusion pore opening and transmitter

release, carbon fiber amperometry and cell-attached capacitance measurements were performed in bovine chromaffin cells. In cells treated with the PKC inhibitor, bisindolylmaleimide I (100nM, 10Ki), release frequency was significantly reduced to 5.0 ± 1.2events/min (from control 17.1 ± 2.0events/min). Amperometric spikes also showed increased half width of 22.3 ± 2.0ms (from 11.5 ± 0.9ms control) and smaller amperometric spike amplitude of 21.9 ± 3.7pA (from 56.2 ± 9.4pA control). In the cells treated with PKA inhibitor, KT 5720 (500nM, 10Ki), the duration of foot signals of amperometric events was prolonged to 14.4 ± 1.6ms (from 9.0 ± 1.0 ms control), consistent with prolonged fusion pore duration in cell-attached capacitance measurements of 27.7 ± 6.8ms (from 11.8 ± 1.4ms control). In contrast to PKC inhibitor, KT 5720 did not affect the release frequency.These results indicate that PKC affects the rate of fusion pore formation and release after full fusion but not early fusion pore expansion, while PKA specifically affects the expansion of the early fusion pore as well as release after full fusion.



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Effects of Calcium and PIP2 on the Membrane Binding of Synaptotagmin I

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Synaptotagmin I (Syt I) appears to act as the Ca^{2+} sensor in neuronal exocytosis and it is known to interact both with membranes and with SNAREs, which form the conserved core protein machinery for the fusion process. The interactions of Syt I with membranes were examined here with a combination of vesicle sedimentation and site-directed spin labeling (SDSL). Several interesting features of the interaction are revealed. First, Syt I binds to PC/PS bilayers in a Ca^{2+}-independent manner though one of its cytosolic C2 domains, C2B. The interaction is mediated by the polybasic region of C2B domain, which associates in the electrostatic double-layer, but does not penetrate into the bilayer interior. Second, the affinity of C2B is increased approximately 20 fold in the presence of Ca^{2+} and now interacts through its Ca^{2+}-binding loops. Remarkably, in the presence of Ca^{2+}, C2A, C2B and a tandem fragment containing both C2A and C2B have approximately the same affinity, indicating the free energy of C2 domain interactions in Syt 1 are not additive. This may be due to demixing of the PS in the bilayer or the effects of curvature strain that are induced by the C2 domains. Finally, $PI(4,5)P_2$ is a lipid that is critical to membrane fusion. Our preliminary data indicate that the addition of 1 mol% $PI(4,5)P_2$ has little effect on the Ca^{2+}-dependent binding of C2A; however, the membrane binding of both C2B and the tandem C2A-C2B domains is enhanced by $PI(4,5)P_2$. As seen for other polybasic segments, the C2 domains appear to sequester or alter the lateral distribution of $PI(4,5)P_2$ in the bilayer.

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A Novel Approach For Wireless Communication Of *In Vivo* Data From Freely Moving Research Animals

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In vivo electrochemistry has become a fascinating research tool allowing neuroscientists to study the release of oxidizable neurotransmitters, such as dopamine and norepinephrine in the brain of freely moving animals (Garris et al., 1997, J. Neurochem., 68(1): 152–161). The main limitation of this technique is the wired connection from the working electrode at the animal's head to the data acquisition apparatus, thus restricting the animal's freedom of motion. To overcome this limitation, we are designing an electronic device with the capability of performing fast-scan cyclic voltammetry measurements and wirelessly transmitting the recorded data. The device consists of two parts: the base station, which is connected to a PC, and the remote unit, which the rat carries on its back. The base station can wirelessly transmit the potential waveform applied to the working electrode, using the Advanced Audio Distribution Profile (A2DP) protocol. At the remote unit, a capacitance compensation circuit partially removes the capacitive background current present in voltammetric measurements due to charging of the Debye double layer. This increases the device's dynamic range, allowing for the detection of lower neurotransmitter levels. Although the forward telemetry (PC to remote unit) is functional, we have not yet characterized the reverse telemetry (remote unit to PC) in A2DP format. After finalizing the design, the device will be tested *in vivo* and subsequently employed in behavioral experiments, allowing researchers to obtain data from freely behaving rodents.

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Intracellular Ca^{2+} In Physiological Range Affects The Forward Rate Of Priming Of Large Dense Core Vesicles, But Not The Backward Rate

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Secretory vesicles which undergo Ca^{2+}-dependent exocytosis pass several consecutive molecular states before release. While docking describes the anchoring of the vesicles to the plasma membrane, priming is necessary to render the vesicles release-competent. Many regulatory proteins and second messengers mediate the transition between these different molecular states.

By combining total internal reflection fluorescence microscopy (TIRFM) and analysis of the caging diameter (CD) we show that different molecular prefusion states of large dense core vesicles (LDCVs) can be distinguished by their different mobility (Nofal et al., J. Neurosci. 2007, 27:1386–95). Furthermore, we established simultaneous TIRFM measurements with whole-cell patch-clamp recordings which enables us to set a stable composition of the intracellular conditions, e.g. intracellular Ca^{2+} (Becherer et al., PLoS ONE 2007, 6:e505).

We investigated the Ca^{2+} dependence of both priming and unpriming reactions by varying the intracellular Ca^{2+} concentration within the physiological range from 50–800 nM.

CD analysis reveals that both lateral and axial mobility of LDCVs under resting conditions (100 nM $[Ca^{2+}]_i$) are elevated, whereas mobilities are reduced with raising $[Ca^{2+}]_i$ from 200 nM to 800 nM. Further increases of Ca^{2+} levels above 800 nM again lead to an increase in mobility. Interestingly, the dwell time of LDCVs appear to be independent of $[Ca^{2+}]_i$ in this range, arguing against the Ca^{2+}-dependence of docking. Quantitative analysis of individual parameters, such as dwelltime in a specific molecular state and frequency of interstate changes, demonstrate that the forward rate of priming is increased with raising $[Ca^{2+}]_i$ while the backward rate remains unaffected.

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Role Of SPIN90 (SH3 Protein Interacting With Nck, 90kda) In The Formation Of Endocytic Vesicle And Its Movement In Receptor-mediated Endocytosis

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Endocytosis is a key mechanism for mediating diverse cellular functions, like uptake of nutrients, recycling of synaptic vesicles and intracellular signaling. The formation and targeting of vesicles to their acceptor compartment from the plasma membrane are tightly controlled for regulating tissue homeostasis. To gain insight into the effect of SPIN90 in the formation of vesicles, we measured the interaction between syndapin and dynamin in SPIN90 overexpressed and deficient fibroblasts. It is reported that syndapin is the phophorylation-regulated dynamin I partner in vivo and its interaction is crucial for SVE. SPIN90-SH3 domain binds with dynamin I-PRD in synapses and PRD domain of SPIN90 interacts to syndapin-SH3 in fibroblasts are already reported. Here, we show that the syndapin-dynamin interaction is maintained in SPIN90-N terminal (SH3 and PRD domain containing part) overexpressed cells comparing to that in mock overexpressed cells. In addition, SPIN90 C terminus (642–722aa) interacts with Rab5a small GTPase which has a role for early endosome movement and fusion were found. For verifying this, immuno-fluorescence and live cell imaging technique were used. We examined that SPIN90 is co-localized with Rab5 in fibroblast, and the movement of gfp-Rab5 positive endosome is delayed when the SPIN90-CC (Rab5 binding) part is overexpressed. From these results, we proposed that SPIN90 has a role in the formation and movement of early endosome.

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TIRF-FRET As An Approach To Quantitative Analysis Of Dynamic Molecular Interactions On Secretory Granules In Live Cells

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