um ERES formation on a mesoscopic scale. The model predicts that ERES are arranged in a quasi-crystalline pattern while their size strongly depends on the cargo-modulated COPII turnover kinetics, i. e. a lack of cargo leads to smaller and more mobile ERES. These predictions are in favorable agreement with experimental data obtained by fluorescence microscopy. The model further suggests that a cooperative binding of COPII components, e.g. mediated by regulatory proteins, is a key factor for the experimentally observed organism-specific ERES pattern. Moreover, the anterograde secretory flux is predicted to grow when the average size of ERES is increased while an increase in the number of (small) ERES only slightly alters the flux.

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Exocytosis & Endocytosis - I

1259-Pos Imaging of Antibody-Antigen Complexes Using Conical Electron Tomography

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Conical electron tomography allows the imaging of macromolecular assemblies in their cellular environments, with isotropic resolution (~3 nm) approximately two orders of magnitude higher than that achieved by optical methods. Furthermore, there is no need to impose symmetry or use averaging methods to increase signal-to-noise ratios. Here, we explore the possibility that proteins in these assemblies can be identified with monoclonal antibodies: in the tomographic reconstructions, antibodies are located on the basis of their size and shape, revealing the identity of their target protein. To test this possibility, we reconstructed two specimens:

(a) meshes of filamentous actin, decorated with monoclonal antibodies; and,
(b) synaptosomal preparations from mouse brain, decorated with antibodies against clathrin, the principal component of coated vesicles.

Conventional thin section microscopy methods were used to prepare both specimens. The maps of actin decorated with IgGs show a dense network of filaments, ~6 nm in diameter, with denser particles of 6–10 nm, jutting out at variable intervals along their length. These particles are absent in pure actin maps. While the shape of the particles varies as expected for flexible molecules oriented at random, many of them exhibit the classical tri-lobar (Y) structure characteristic of IgGs. The synaptosomal maps show the normal synaptic components, including coated vesicles characterized by their polyhedral cages comprised of clathrin triskelions. The antibody molecules appear as particles of 6–10 nm, decorating the clathrin coats. Unexpectedly, we also observe larger particles (15–20 nm), that we interpret as multiple IgGs (2–3) bound to epitopes located in close proximity within the clathrin triskelions. We thus conclude that conical electron tomography represents a novel experimental avenue for identifying proteins comprising key macromolecular assemblies in their cellular environments.

1260-Pos Probing the Endocytosis Pathway of Dendritic Cells Involved in Class I Antigen Presentation

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Board B236

Dendritic cells (DCs), also known as professional antigen presenting cells, are crucial mediators of the immune response and have emerged as prime targets for vaccination. Antigen, foreign or benign, are captured through endocytosis, routed, and processed in a tightly regulated network of compartments that ultimately determines the type of immune response elicited. The luminal pH of endocytic compartments is closely correlated to its trafficking. The kinetics of pH change, which may be regulated by redox reactions, has been implicated as a key mechanism by which DCs selectively process antigen for a particular immune response. In this work, we have developed a microprobe aimed at monitoring pH and redox reactions of the endocytic compartments of DCs in situ. By using this probe, we have examined the mechanisms by which size and pH-sensitivity of carriers affect class I antigen presentation.

1261-Pos Ca2+ Syntillas Decrease Spontaneous Exocytosis In Mouse Chromaffin Cells

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Board B237

Ca^{2+} syntillas are spontaneous transient, focal increases in cytosolic [Ca^{2+}] in excitable secretory cells, resulting from a release from intracellular stores via ryanodine receptors (RyRs). Originally found in nerve terminals of hypothalamic magnocellular neurons (*scintilla*, L.spark, from a synaptic structure, a terminal), syntillas are also found in mouse chromaffin cells. There they do *not* cause exocytosis because they appear to arise in a microdomain different from the one where the final exocytotic steps occur (ZhuGe et al., 2006). What is the function of spontaneous Ca^{2+} syntillas? To find out we studied syntillas and amperometrically recorded spontaneous exocytosis in chromaffin cells in whole-cell patch configuration held at a membrane potential of –80 mV. We found that blocking the stores with ryanodine resulted in significant increases in the mean charge (0.088±0.006 vs 0.233±0.008 pC) and frequency

(0.101±0.017 vs 0.576±0.125 s^{-1}) of the exocytotic events recorded amperometrically. Similar results were obtained by emptying the stores with thapsigargin and caffeine. Ryanodine blockade experiments were also carried out in 0 extracellular [Ca^{2+}] and 200 uM external EGTA, with the same results. In addition when the amperometric events were grouped into those coming from large dense core vesicles (DCVs) and small DCVs, which constitute distinct populations (Grabner et al., 2005), we found that the syntilla-induced decrease in frequency was mainly due to a decrease in the exocytosis of large DCVs. This and not a shift in the amount of transmitter stored in the DCVs accounted for the decrease in amount of transmitter released in the presence of syntillas. We conclude that Ca^{2+} syntillas act to prevent spontaneous exocytosis of DCVs and that this action is primarily the result of a *decrease* in the frequency of the population of larger DCVs.

1262-Pos Turning Cholinergic Nerve Terminals Into Functional Dopaminergic Nerve Terminals

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The nerve terminals of *Torpedo* electric organ contain acetylcholine as the main neurotransmitter and their synaptic vesicles concentrate acetylcholine. These clear synaptic vesicles are homologous to those found in the neuromuscular junction. After incubating the electric organ with dopamine and using an electron microscope technique, we observed that clear cholinergic synaptic vesicles became small dense core synaptic vesicles (SDCSV). We recorded, using carbon fiber amperometry, that isolated nerve terminals previously incubated with dopamine released dopamine, in a $[Ca^{2+}]_0$ dependent manner during K^+ depolarization. When nerve terminals were not treated with dopamine, we could not detect any release. On the contrary, nerve terminals incubated with dopamine secreted dopamine in a quantal manner, recorded as individual transient spikes. These individual exocytic events were symmetrically shaped with an extremely fast ($T_{1/2}$,150 μs). This time course was coincident with that calculated using a Montecarlo simulation, modeled assuming the diffusion of dopamine from synaptic vesicles with the diameter observed in our preparations. Moreover, this calculation indicates that the fusion pore was placed in a distance from 100 to 300 nm of the surface of the carbon fiber. On the other hand, not all the dopamine released is a consequence of synaptic vesicle recycling; the number of SDCSV is larger than the number of recycled synaptic vesicles labeled with horseradish peroxidase, Indeed, we also found that isolated synaptic vesicles transport dopamine and the internal vesicular matrix adsorb and concentrate it as its does with acetylcholine. In summary, these results let us to measure with high precision the real time course of release from a single synaptic vesicle of a fast synapse and suggest that synaptic vesicles can store any kind of neurotransmitters.

Acknowledgement

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1263-Pos A Potentistat Array For Parallel Detection Of Exocytosis

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The quantal transmitter release events are modulated by drugs as well as molecular manipulations of the cells. Quantal release from chromaffin cells can be monitored by cabon fiber amperometry, but there is a need for development of devices that allow parallel detection from several cells for high-throughput screening. Traditional methods of studying quantal release such as amperometry require individual low-noise recording amplifiers. We designed a new shared amplifier structure in which all potentiostats in a given row of the array share a common half circuit permitting the use of fewer transistors per amplifier. The potentiostat circuit that is capable of measuring currents with submillisecond time and picoampere current resolution and can be fabricated as a scalable array of rows and columns on a CMOS chip (Ayers et al., 2007). The rms current noise of the fabricated electrode array at 2 kHz bandwidth was 110fA which is comparable to noise typically achieved with commercially available patch clamp amplifiers. We demonstrate simulataneous on chip electrochemical detection of dopamine at various concentrations. Dopamine was released on a 4×4 on chip electrode array using a pico spritzer. We also demonstrate off-chip detection of exocytotic events from single chromaffin granules through carbon fiber amperometry where the carbon fiber was connected to a single potentiostat circuit. In this case, the noise was higher, up to 8pA at 2kHz, presumably due to antenna effect of the wiring to the carbon fiber. We will present on chip measurements of cellular exocytosis and compare the noise performance.

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1264-Pos Structural and Functional Analysis of Dynamin's GTPase Activity

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Board B240

Dynamin is a 100 kDa GTPase that acts as a key regulator of clathrin-mediated endocytosis. In the unassembled state, dynamin exhibits a high basal rate of GTP hydrolysis that can be stimulated 100-fold by self-assembly on a lipid template and through the activity of an intra-molecular GAP - the GTPase effector domain (GED). While it has been shown that these properties are critical for dynamin's function, the underlying mechanism by which it hydrolyzes GTP remains unknown and the essential catalytic machinery has yet to be identified. Recent structural work has suggested that the GED may physically dock with the GTPase domain to exert its stimulatory effects in the assembled structure, and it is unclear how this may influence basal turnover. To examine how these structural interactions translate into efficient catalysis, we have engineered a minimal GTPase-GED (GG) fusion construct that reconstitutes a robust GTPase activity comparable to the full-length protein. We have produced crystals of this GG construct that diffract to high-resolution and are currently collecting data for structure determination. These findings combined with mutagenesis studies will produce mechanistic insight into the key residues required for dynamin's GTP hydrolysis and the associated conformational changes in the GED and GTPase domains.

1265-Pos Modelling Rapid Diffusive Loss Of Secretory Granule Content In Pancreatic Acinar Cells

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Granule content loss during exocytosis is dependent on multiple factors including the dimensions of the fusion pore. We have now developed two mathematical diffusion partial differential equation models for estimating the emptying times of secretory granules based on recent measurements of pore dimensions in epithelial cells (Larina *et al.* 2007).

In the first model we represent the granule as a sphere with a pore through which content is lost. In the second model we represent the granule as a sphere attached to the plasma membrane by a thin cylindrical neck. We solve the partial differential diffusion equation with a time varying initial condition c(0,t)=v(t), where v(t) is derived from the first model. Using the calculated diffusion constant of amylase, the major content protein=66.2 $\mu m^2.s^{-1}$ (Young and Carroad 1980), a granule radius=0.25 m, a pore radius=15nm and neck length=100nm, the granule contents are calculated to empty in seconds. This time-course of content loss is consistent with previous indirect measures (Thorn and Parker 2005). This approach is universally applicable and could, for example, be used to model insulin release (Marsh *et al.* 2001).

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1266-Pos Spontaneous Fusion of Chromaffin Dense Core Granules to a Lipid Bilayer Detected by Amperometry

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The SNARE hypothesis proposes that a general apparatus (composed of syntaxin, SNAP-25 & synaptobrevin) drives fusion and exocytosis of both small synaptic vesicles and large dense core granules (LDCG). We have previously demonstrated that LDCG from both bovine neurohypophysial terminals (McNally et al. 2004, Cell Biochem. & Biophys. *41*(1): 11–23) and chromaffin cells (McNally et al., 2003, Biophys. J. *84*:A1886) are able to spontaneously fuse with a planar lipid bilayer containing only the t-SNARE syntaxin 1A. In these experiments, native LDCG were modified to contain nystatin channels, allowing each fusion event to be visualized by monitoring bilayer conductance. Here, we confirm these results with native chromaffin cell LDCGs using amperometry. Amperometric detection was coupled with our planar lipid bilayer model system to successfully detect individual release events. These events displayed the characteristic kinetics of catecholamine release events from chromaffin cells. This approach also allowed for analysis of differences between spontaneous and osmotically-driven fusion events. Interestingly, small current steps preceded some of the observed amperometric spikes which appear similar in nature to the pre-spike "foot" observed during native release events from chromaffin cells. The pre-spike foot is attributed to the slow release of transmitter through a fusion pore prior to pore widening and complete collapse of the vesicle into the plasma membrane. The appearance of pre-spike feet preceding amperometric events seen in our extremely simple model system implies that all of the components required to form a fully functional fusion pore are present. In conclusion, this method provides an effective model system to study possible components involved in vesicular exocytosis, as well as shedding some light on the composition of the fusion pore itself.

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1267-Pos Synaptic Transmission Is Modulated By Action Potential Pattern In A Central Synapse In Mouse Brain Slices

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Board B243

The calyx of held is a large glutamatergic synapse in mammalian auditory brainstem. Stimulus-induced secretion on Calyx of Held presynaptic terminal can be recorded by dual patch-clamping recordings on presynapse measuring membrane capacitance (Cm) and on postsynapse measuring EPSCs in mouse brainstem slice. In a previous study, we showed that action potential (AP) pattern modulates secretion in isolated adrenal chromaffin cells (Duan et al, JNS, 2003). The stimulus pattern was defined by a AP "code function" F (N,m,f,d) with "N" is number of total APs, "m" is number of bursts, "f" is frequency, "d" is interval time between two bursts. To investigate whether AP pattern affect synaptic transmission at a CNS synapse, here we study modulation of evoked secretion by AP codes in mice calyx synapses. We found, AP codes strongly modulate secretion signals of both presynaptic Cm and postsynaptic EPSCs. F(100,m,100Hz, 1s)-induced secretion is 59% larger at m = 4 vs. 1, indicating glutamate secretion from calyx synapse is modulated by AP codes. This "burst effect" is relative insensitive to coding parameters "N", "f" and "d", which is in contrast to that in chromaffin cells. The "burst effect" is statistically age-dependent. P15 (after auditoriums function) has less "burst effect" than P8 (before auditoriums function) mice. In summary, CNS fast glutamate synaptic transmission is modulated by spike-timing pattern in mice Calyx of Held.

Supported by grants from 973 program and NSFC.

1268-Pos Facilitation of Calcium-independent Exocytosis at Synaptotagmin-deficient Synapses

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Board B244

Members of the synaptotagmin family of vesicular membrane proteins are likely to function as Ca2+-sensing molecular triggers for rapid, regulated exoctosis at synapses. The precise switching mechanism, however, is under debate. According to the currently favoured hypothesis, synaptotagmins promote membrane fusion by Ca2+-dependent interaction with phopholipids. On the other hand, they might, in the Ca2+-free state, stabilize a pre-fusion or hemi-fusion state and prevent spontaneous progression to full fusion. This "fusion clamp" would be removed upon Ca2+ binding. This hypothesis would predict that vesicles in synaptotagmin-deficient (syt-/-) synapses show higher spontaneous fusion rates and would be more susceptible to Ca2+-independent fusion stimuli, such as osmotic gradients. We have investigated this hypothesis at GABAergic synapses in P0 synaptotagmin-I (syt-I) knockout mouse corpus striatum cultures using whole cell voltage clamp recordings of inhibitory postsynaptic currents (IPSCs). In paired pre- and postsynaptic recordings, the majority (18 of 23) postsynaptic responses evoked by single presynaptic action potentials at syt -/- connections lacked the early synchronous component of control (syt-I+/− and syt-I+/+) IPSCs but consisted entirely of a delayed (latency >30 ms) and long lasting (>1 s) ansychronous sequence of miniature IPSCs. After trains of 10 presynaptic APs, the asynchronous component of release decayed with a time constant of 188, 2 +/− 112.03 ms (control) and 585, 78 +/− 262, 143 ms (syt-I-/-), respectively. The frequency of spontaneous vesicle release as assessed by recording minature IPSC was 0.31+/−0.18 Hz (n=16) in control and 2.50+/−0.84 Hz (n= 11) in syt-I-/- cultures. Finally, the frequency of release induced by a 100 mOsm increase in the tonicity of the extracellular medium was 1.43 +/− 0.5 (control) and 6.49+/− 1.88 Hz, respectively. These findings can be explained by a lowered energy barrier for fusion in synaptic vesicles due to the absence of syt-I.

1269-Pos Molecular Control of Ca^{2+}-evoked Exocytosis in Atrial Myocytes

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Board B245

Ouabain, an endogenous cardiac glycoside, has been shown to evoke the exocytosis of the vasoactive hormone atrial natriuretic peptide (ANP) from atrial cardiac myocytes. However, the Ca^{2+}-dependence and molecular mechanisms that mediate ANP exocytosis currently remain unclear. To judge whether secretory activity could be efficiently evoked by direct elevation of intracellular Ca^{2+} and the role of ouabain in this process, we used time-resolved membrane capacitance measurements (C_m) in combination with the flash photolysis of caged Ca^{2+} to follow the exocytotic activity in neonatal rat atrial myocytes. Two sequential flashes evoked nearly identical Ca^{2+} changes that induced exponential C_m rises (78 and 66 granules, respectively) at room temperature. The application of 100 nM ouabain (to selectively target the α_3-subunit of the Na^+/K^+-ATPase) significantly enhanced the average C_m change to the first stimulus but diminished the response to the second stimulus (138 and 58 granules, respectively) with no considerable effect on Ca^{2+} levels. This suggested that ouabain enhanced the Ca^{+2}-sensitivity of exocytosis. Others have shown that binding of ouabain to the Na^+/K^+-ATPase directly activates Src kinase and subsequent downstream tyrosine phosphoregulation. Using co-immunoprecipitation methods, we demonstrated that the Ca^{2+}-sensing protein, synaptotagmin-1 (previously identified in atrial myocytes), associates with an immune complex consisting of Na^+/K^+-ATPase α3, Src and the SNARE proteins syntaxin 4 and SNAP-23. Treatment with pervanadate (an inhibitor of protein tyrosine phosphatases) or ouabain induced tyrosine phosphorylation of synaptotagmin-1. In contrast, treatment with the Src kinase inhibitor, PP2, reduced ouabain-evoked tyrosine phosphorylation of synaptotagmin-1. Based on these findings, we propose that SNARE proteins are assembled in a signaling complex consisting of Na^+/K^+-ATPase α3, Src, syntaxin 4 and SNAP-23. Moreover, we propose that ouabain induced tyrosine phosphorylation of synaptotagmin-1 enhances the Ca^{2+}-sensitivity of exocytosis and natriuretic peptide release in atrial myocytes.

1270-Pos How BAR Domains And Amphipathic Helixes Recognize Membrane Curvature: A Single Vesicle Study

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Board B246

The BAR domain is a banana shaped a-helical homodimer found in several proteins families that play a major role in endocytosis, actin regulation and signaling. (1) It is shown to sense and/or induce lipid membrane curvature by peripheral binding.

We present a high-throughput single lipid vesicle assay (2) to test the curvature dependant binding properties of several BAR proteins and amphipathic helixes. Fluorescence intensities of immobilized vesicles allowed us to measure accurately their size/curvature (± 5 nm) and the respective densities of BAR proteins. Combining equilibrium concentration dependant studies as well as kinetic information we where able to calculate for the first time the BAR-membrane binding constant and association on/off-rates, further more so in a curvature dependant manner. Our results prompt a thorough reevaluation of the binding mechanism of BAR proteins.

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1271-Pos Real Time Ph-monitoring Of Endosomal Acidification By Ratiometric Live Imaging

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Board B247

Transport of macromolecules from extracellular milieu to the cell interior is mediated by multiple mechanisms of endocytosis involving carrier vesicles, which mature as distinct compartments.

Organelle acidification is an essential element of the endosomal-lysosomal pathway, but the understanding of the mechanisms underlying the progression through this pathway has been hindered by the absence of adequate methods for quantifying intraorganelle pH. To address this issue, we developed a direct quantitative method for accurately determining the pH of endocytic organelles in live cells.

In this report, we demonstrate that the GFP mutant (E1GFP) is a ratiometric fluorescent pH sensor which shows marked changes in emission fluorescence spectra upon pH variations. In order to investigate the intracellular fate of HIV-1 Tat viral toxin, we constructed the chimeric protein Tat-E1GFP. After incubation of the cells with Tat-E1GFP we were able to map pH variations in endosomes with respect to the neutral pH measured in Tat-loaded membrane vesicles.

Furthermore, inhibiting endosomal acidification with chloroquine we restored vesicles -pH to 6.85 with respect to 5.5 measured in absence of lysotromophic agent.

The theoretical framework and conclusions described here, are potentially applicable to other membrane-enclosed compartments that are acidified, such as elements of the Golgi apparatus.

1272-Pos Myosin II And Cortical Actin Are Involved In Fusion Pore Expansion

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Board B248

Chromaffin cells are a widely used model system to study exocytosis. Underneath their plasma membrane, lies a mesh of cortical actin filaments (F-actin), whose dynamic reorganization has been postulated to play a role in the dynamics of exocytosis [1]. It has been speculated that this reorganization is required for molecular motor proteins like myosins to perform their functions [2]. However, the precise roles of myosins and actin in exocytosis are still poorly understood.

Confocal microscopy, carbon fiber amperometry and cell-attached capacitance measurements were performed on pharmacologically treated bovine chromaffin cells. Cytochalasin-D, an inhibitor of actin polymerization, resulted in slower catecholamine release from single vesicles and lower fusion pore expansion rates of chromaffin granules. Similar results were obtained in cells treated with Blebbistatin, which specifically inhibits myosin II ATPase activity [3]. Confocal microscopy showed that Blebbistatin treatment slowed down vesicular motion as previously shown for a myosin regulatory light chain mutation that prevents its phosphorylation [2] and consistent with previous observations in cells treated with Cytochalasin-D [4]. The fusion pore lifetime obtained from amperometric foot-signals or patch capacitance measurements was prolonged in cells treated with Cytochalasin-D but not after Blebbistatin treatment, suggesting that the role of actin in fusion pore dynamics is partly but not exclusively mediated by myosin II. Other myosin subtype(s), presumably myosin Va, which has previously been associated with chromaffin vesicles [5] may be involved in determining fusion pore lifetime. These findings suggest a role for myosin II-F-actin-interactions in fusion pore dynamics in chromaffin cells.

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1273-Pos A New Pathway For Vesicle Fusion And Retrieval At Synapses: Compound Exocytosis Is Followed By Bulk Endocytosis

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Board B249

Vesicle fusion and retrieval is essential for mediating and maintaining synaptic transmission. Our current understanding of this process is that vesicles fuse at the plasma membrane, followed by retrieval of each fused vesicle membrane or a piece of membrane larger than a single vesicle, called bulk endocytosis. Here we report a novel mechanism of vesicle fusion and retrieval. High potassium application induced calcium-dependent capacitance up-steps that reflected single fusion events at cell-attached patches of a synaptic nerve terminal, the calyx of Held. The mean up-step size was much larger than the mean vesicle capacitance, and ~20% of up-steps (called giant up-steps) were larger than the largest membrane capacitance of a synaptic vesicle. Giant up-steps were accompanied with giant miniature EPSCs (mEPSCs), suggesting that giant up-steps were caused by fusion of 'giant' vesicles containing neurotransmitter, called here as compound exocytosis. Our results further suggest that giant vesicles are due to compound fusion between regular vesicles. Giant up-steps were followed by giant down-steps with a similar size range, which reflected bulk endocytosis. High potassium application increased the frequency and the size of giant up-steps and giant down-steps in parallel, suggesting that bulk endocytosis is activated to retrieve the fused giant vesicle membrane. These results establishes a new model of vesicle fusion and retrieval, in which

1. calcium triggers compound fusion between vesicles and the formation of giant vesicles,
2. compound exocytosis of giant vesicles generates giant mEPSCs, and
3. retrieval of the fused giant vesicles is via bulk endocytosis.

The model may be of wide application because giant miniature currents and bulk endocytosis have been observed in a variety of physiological conditions at many synapses.

1274-Pos Morphogen gradient formation: Where does the Dpp go?

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Positional information is essential for the cell's fate in tissue. In the wing imaginal disk of Drosophila melanogaster positional information is provided by a concentration gradient of the morphogens Decapentaplegic (Dpp) and Wingless (Wg). We use a 3D-epifluorescence setup to unravel the spatio-temporal distribution of YFP-labelled Dpp after secretion by specialized producing cells. With our approach we are able to characterize the Dpp distribution in the wing disk in all three dimensions. Dpp forms a gradient in the wing disk. Most Dpp is located apically in a layer of ~5µm. To elucidate how the gradient is maintained individual endosomes containing Dpp are followed. Because endosomes contain large amounts (~50) of Dpp we are able to follow an endosome for hundreds of frames. On timescales up to one hour, endosome mobility is characterized in terms of their diffusion constant and their type of motion, i.e. directed motion and confinement, while intensity fluctuations are used as a readout for changes in Dpp concentration. Detection of 'catastrophes' and 'births', characterized by sudden changes in intensity, combined with positional information allows us to calculate degradation and recycling rates. Simultaneous visualization of the cell membrane enables us to identify endocytosis and exocytosis events. Our study will lead to a mechanistic model for gradient formation.

1275-Pos Electrochemical Characterization Of Serotonin Exocytosis From Human Platelets

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Board B251

Human platelets play a critical role in hemostasis, thrombosis and healing by releasing important molecules, including serotonin, through exocytosis. To date, molecular biology has contributed significantly to the present knowledge of human platelet exocytosis. However, deeper understanding of exocytotic machinery of human platelets has been largely hindered by the lack of suitable tools for direct interrogation of this release process. Recently carbon-fiber microelectrodes have emerged as important tools to directly probe exocytotic processes, offering both exceptional spatial (micron-scale) and temporal (sub-millisecond) resolutions.

Exocytosis from individual activated human platelets was characterized using carbon-fiber microelectrode fast-scan cyclic voltammetry and amperometry. Fast-scan cyclic voltammetry can be used to confirm the identity of the released molecules by revealing the signature locations of the oxidation and reduction current peaks. Amperometry can be used to record serotonin release events. Each exocytosed granule is manifested as a sharp current spike that can be analyzed with sub-millisecond time resolution. Spike features such as area, half-width, and frequency provide insights about the exocytotic machinery of serotonin release. For example, the spike area can be translated to the number of secreted serotonin molecules in individual granules. Accordingly, the homeostasis of serotonin loading into granules can be readily investigated by comparing

averaged spike areas under various conditions such as high extracellular concentration of serotonin.

Experimental results show that, under physiological conditions, an average exocytosed granule secretes ~106 serotonin molecules within a typical ~5 ms duration. This quantitative information suggests highly concentrated serotonin content in each individual granule, typically ~100 mM. Furthermore, fast-scan cyclic voltammetry confirms the secreted molecules as serotonin. These electrochemical methods not only offer a unique opportunity to explore the exocytotic machinery but also to interrogate the relationships between the signaling pathways of platelet activation and exocytotic machinery in human platelets.

1276-Pos Synaptotagmin I Binds to PC: PS Membrane in a Ca2+ -Independent Fashion: Membrane Binding Affinity Measurements

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Board B252

Synaptotagmin I (Syt), which functions as the calcium sensor in neuronal exocytosis, is a membrane protein that is associated with the synaptic vesicle. Syt has a single transmembrane helix linked to two cytoplasmic domains, C2A and C2B, which bind to three and two Ca^{2+} ions, respectively. It is well-accepted that both domains bind to negatively charged phospholipids in a Ca^{2+}-dependent manner, and a number of studies have examined the Ca^{2+} binding affinity of C2A and C2B. However, the Ca^{2+}-dependent and Ca^{2+}-independent membrane binding affinity of Syt to PS containing vesicles is not well-characterized. In the present work, we measured the membrane binding affinity of C2A and C2B to PC:PS bilayers in the presence and absence of Ca^{2+}. The data indicate that C2B and a tandem construct containing both C2A and C2B bind bilayers in the absence of Ca^{2+}, with mM membrane affinity. C2A does not bind PC:PS bilayers in the absence of Ca^{2+}. In the presence of Ca^{2+}, the affinity of C2B increases approximately 20 fold. Power saturation experiments on spin-labeled derivatives of C2B indicate that it interacts electrostatically though its polybasic face, but does not penetrate into the bilayer interior. Ca^{2+}-binding to C2B rotates the domain relative to the membrane interface and alters its membrane interacting face.

1277-Pos Diminished Secretion in CAPS1/2 Double Knockout Mouse Chromaffin Cells

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Board B253

The regulated exocytosis of neurotransmitters and hormones occurs at specialized membrane areas via Ca^{2+} - triggered fusion of secretory vesicles with the plasma membrane. CAPS1 is an essential factor in the exocytosis of large dense core vesicles in PC12 cells. A second CAPS isoform, CAPS2 is also present in neuroendocrine cells. CAPS2 has a high degree of homology to CAPS1 and thus may have a similar function. We have used a knockout approach to determine whether CAPS2 has a role in the secretion of catecholamines from chromaffin cells. In addition we generated a CAPS1/2 double knockout in order to determine if the two CAPS isoforms play a redundant role.

Exocytosis was measured via capacitance measurements in the whole cell mode, and by determination of released catecholamines using carbon-fibre amperometry. The calcium caging compound, NP-EGTA, along with calcium indicators, were included in the intracellular solution. Secretion was induced by stepwise increases in intracellular calcium following flash photolysis of calcium-loaded NP-EGTA.

Chromaffin cells in which CAPS2 was deleted exhibited secretion similar to that in wild type cells. In contrast, secretion from CAPS1/2 double knockout chromaffin cells was diminished by 54%. There was a strong decrease in the exocytotic burst of secretion as well as in the sustained rate. Similar results were observed in the amperometric responses in CAPS1/2 double knockouts. These results indicate that CAPS isoforms can function as priming factors in chromaffin cells.

1278-Pos Synaptotagmin 7 regulates the dynamics of the exocytotic fusion pore

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Board B254

Synaptotamins (Syt) are a family of transmembrane proteins acting as calcium sensors in membrane fusion. All Syts have two C2 (A & B) calcium-binding domains. The most abundant isoforms of this protein are Syt 1 and Syt 7, which have different calcium binding affinities. Syt 1 is the calcium sensor in the stimulus-secretion coupling in neurons and the fast component of exocytosis in chromaffin cells. However, the role of other Syts is controversial. We investigated single vesicle exocytosis in chromaffin cells from KO and KI (C2B) Syt 7 mice using patch amperometry, which allows simultaneously measurement of vesicle fusion and release of catecholamines from single vesicles.

We show that the absence of Syt 7 retards fusion pore expansion. This effect was rescued by increasing external calcium from 5 to 10 mM, suggesting that Ca^{2+} modulation of fusion pore expansion also involves other isoforms of Syt. When removing the calcium-binding

site of the C2B domain in KI mice, we observed the same phenotype, indicating that the C2B domain is required for the more rapid fusion pore expansion in the presence of Syt 7. Fusion pore conductance analysis showed that wild type cells the pore expands in less than 40 ms, increasing up to 100 ms in the C2B KI mice. However, the incidence of flicker events increased in C2B KI compared to wt cells and KO cells, suggesting that Syt 7 without functional C2B domain does participate in closure of the fusion pore. We propose that the Syt 7 lacking a functional C2B domain retains involvement in the fusion machinery and that Syt 7 C2B domain contributes to fusion pore dynamics.

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1279-Pos Postfusional Control Of Quantal Catecholamine Release

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Board B255

During exocytosis, the fusion pore expands to allow release of neurotransmitters and hormones to the extracellular space. The combination of capacitance measurement, by using the whole-cell configuration, simultaneously with amperometry has showed that on fusion pore opening there is a small release of serotonin preceding the amperometric spike which is directly proportional to the pore conductance (1). However, it is still no clear how the fusion pore dynamics affects the release during the bulk release of the spike.

We study single-vesicle exocytosis in mouse chromaffin cells stimulated with caffeine by the technique of patch-amperometry that allows resolve single fusion events by simultaneously measuring the patch membrane capacitance and the release of catecholamines by placing a carbon fiber detector inside the patch pipette. We could measure the time course of the fusion pore during the complete discharge of catecholamines from the vesicle. The amperometric signal closely followed the conductance changes, indicating that the size of the fusion pore always is limiting for release. Interestingly, when the fusion pore opened until conductance values that can no be measured experimentally the vesicle was empty and no more catecholamine release was observed. Caffeine caused a slower expansion of the fusion pore as compared with the fusion events induced by barium and consequently induced a slower release as showed the longer measured kinetic parameters of spikes (halfwidth $=150.67 \pm 11.01$ ms vs 78.59 ± 3.39 ms; RT $= 38.38 \pm 2.54$ ms vs 17.30 ± 0.91 ms). Since the fusion pore expansion rate is sensitive to the concentration of intracellular free calcium (2) it is possible that caffeine and barium showed different intracellular calcium profiles and magnitudes at the release sites.

References

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1280-Pos Co-operative Binding of Syntaxin Molecules to Microdomains in Live PC12 Cells

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Board B256

SNARE proteins mediate membrane fusion and allow for exocytosis to occur. In this work, we used TIR fluorescence microscopy to simultaneously image green Syntaxin, Syx-EGFP, and a red granule marker, NPY-mCherry. On the cell membrane of live PC12 cells Syx was clustered in microdomains that co-localized with secretory granules. We aimed to determine the number of Syx molecules in a granule-associated cluster by measuring the intensity of single Syx-EGFP molecules in the plasma membrane. We took movies of cells expressing low levels of Syx-EGFP and averaged the first 5 seconds. The resulting image contained spots that were located by an automatic algorithm. The spots were transferred into the movie and their fluorescence was found to bleach in a single step. The step magnitude varied more than 10-fold within a single cell. Its size was inversely correlated with the time to bleaching, suggesting that fluorescence varied due to a variation in absorbance. The average time to bleaching was 1.9s, and the average step size corresponded to 66000 photons/s. By fluorimetry and quantitative Western blotting, 38% of all Syx-EGFP molecules in PC12 cells were fluorescent. We conclude that microdomains beneath granules contain 160 molecules at saturating levels of Syx-EGFP expression. At low expression levels, the fluorescence of granule-associated microdomains fluctuated strongly, with microdomains losing all their 10–30 Syx-EGFP molecules in seconds, and re-gaining as many when they refilled. Such a large number are unlikely to bind or un-bind independently, and suggests that Syx-EGFP microdomains empty and refill cooperatively.

1281-Pos Characterization Of Hepatic Endosomes Fractions By Mass Spectrometry

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Board B257

The endosomal apparatus is the intracellular site where signals initiated at the cell surface are terminated, amplified or selected. In order to characterize endosomal membrane in term of protein content we submitted mouse liver endosomes (G/E fraction) to a luminal proteins depletion procedure (Bilodeau et al., FEBS J 2006) before peptides fractionation and proteomic analysis. The quality and reproducibility of the cargo-depletion procedure were charac-

terized in terms of electron microscopy and by N-terminal labeling of tryptic peptides (iTRAQ). On average, 8438 mass spectra sample have been collected leading to the identification of 1169 non-redundant peptides and 244 unique proteins with an average ratio of 1.0 (n=3). In theses conditions, major cargos (albumin, hemopexin, fibronectin) were depleted. Tryptic peptides were separated by several methods (1D electrophoresis, 2D-LC or IEF Strip) and subjected to MS/MS analysis. Proteins belonging to the metabolism, vesicular transport and signaling categories were identified. An affinity-purified subfraction (anti-PY42-column chromatography targeting c-Src phosphorylation substrates) allowed further identification of several phosphorylated proteins including LRP-1, DPP IV, Annexin A1 and the insulin receptor (IR). Therefore, cargo-depletion enabled us to obtain a comprehensive view of the hepatic G/E membrane proteome. Further subfractionation confirmed here the presence of signaling complexes including LRP-1/IR complexes.

1282-Pos Analysis of the Fusion Process Of Glucose Transporter 4 (GLUT4) in Adipocytes By Live-cell Imaging

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Board B258

Because diabetes mellitus is one of the major risk factor for cerebrovascular accident or cardiovascular disease, elucidation of the molecular mechanism of insulin resistance underlying it is important clinically. One of the main insulin action is glucose uptake in skeletal muscles and adipocytes through intracellular translocation of glucose transporter 4 (GLUT4) from perinuclear region to plasma membrane. However, the details of the mechanism regulating this GLUT4 translocation remain unsolved. So we studied the insulin-induced intracellular redistribution of GLUT4 in 3T3L1 adipocytes using live cell imaging that based on green fluorescent protein (GFP) tagging and laser confocal microscopy. Especially, we focused on the dynamics of GLUT4 vesicles at plasma membrane. The behavior of GLUT4 vesicles with plasma membrane was dissected into multiple steps; trapping, docking, and fusion with membrane. The effect of insulin on each step was evaluated. After insulin stimulation, GFP-GLUT4 vesicles translocated from perinuclear region (N) to PM, and the ratio of GFP luminance (PM/N) increased. The proportion of GLUT4 vesicles either at trapping or fusion step with PM also increased by insulin treatment. In addition, after insulin treatment in the perimembranal region, where cortical actin filament proliferated and the anchoring of GLUT4 vesicles were implicated. Besides, siRNA-mediated knockdown of dynamin2, one of cortical actin binding protein diminished insulin effects on GLUT4 vesicles behavior at PM, and insulin-induced glucose uptake. These results suggest that insulin induces GLUT4 vesicles translocation and glucose uptake through activating vesicle trapping and fusion processes at PM, and that the cortical actin plays the key role in these processes. Thus, regulation of the remodeling of cortical actin may provide a new clue to ameliorate the insulin resistance in diabetes.

1283-Pos A Closer Look On Docking And Priming With A Combination Of Tirfm And Patch-clamp Technique

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Board B259

Exocytotic release of neurotransmitters and hormones is a highly regulated process which proceeds in several steps. Messenger-containing large dense core vesicles (LDCVs), after filling, approach and dock with the plasma membrane (PM), then mature via a poorly understood priming process before they are release-ready. The release step is triggered by calcium. Those steps depend on the cooperation of multiple proteins, including the SNARE complex, which modulate and execute the fusion of the vesicle with the PM. Using electrophysiological methods, much has been learned about this final fusion step. Of particular importance has been the whole cell patch clamp method, which allows high time resolution of release via capacitance measurements, with the additional benefit of controlling components in the intracellular milieu.

The absence of methods to study prefusion steps in real time hinders the elucidation of the roles of the various proteins involved in prefusion steps. Total internal reflection flourescence microscopy (TIRFM) allows, with the appropriate use of fluorescent markers, the observation of vesicles in real time prior to fusion. Using this technique we have begun to identify the states of docking and priming based on the behaviour of vesicles. We are combining this technique with whole-cell patch clamping. This allows us to measure simultaneously secretion, by observing capacitance changes, and mobility, while controlling the intracellular conditions.

We have observed that low concentrations of intracellular Calcium lead to an increased mobility of LDCVs, probably meaning lowered priming.

1284-Pos Inferring synaptotagmin function from C2 domain stability: Analysis of structurally defined mutations in synaptotagmin 1 C2A

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Board B260

Synaptotagmin is composed of two water-soluble Ca+2 binding domains (C2A and C2B) attached to the vesicular membrane via a single transmembrane span and an ~130 residue unstructured tether. Interaction of the C2 domains with Ca+2, phospholipid and effector proteins mediates this Ca+2 sensitivity. While synaptotagmin has

been established as the Ca+2 sensor for exocytosis, little is understood about the biophysics that makes this fine-tuned response possible. Using the recent high-resolution crystal structure of human synaptotagmin 1 C2AB as a template, we tested various structurally defined mutations for stability, and cation binding activity. We conclude that the inherent flexibility of loop 3 in C2A is essential to the overall activity of synaptotagmin.

1285-Pos Stimulated Endocytosis in Uptake of Penetratin Peptides: Influence of Arginines and Lysines

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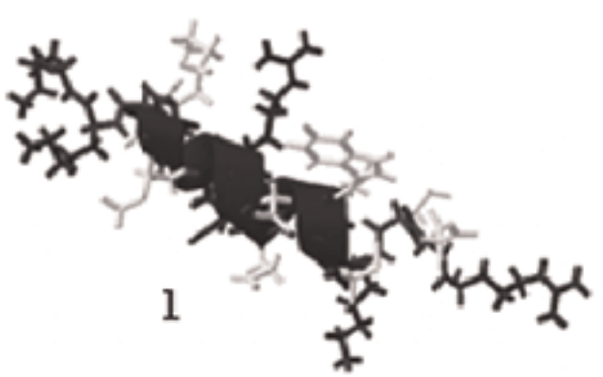
Board B261

The ability to use DNA as a drug target has through advances in genetics and molecular biology become an intriguing possibility. Transport over the hydrophobic plasma membrane constitutes the major obstacle for these gene-targeted drugs and creates the need for intracellular delivery vectors. Cell-penetrating peptides (CPPs) have been shown to efficiently deliver macromolecular cargo into cells and are thus regarded as promising candidates. The uptake mechanisms of these peptides are despite extensive studies not well-described, but occur predominantly through endocytotic routes.

We have studied the ability of penetratin (Ac-R-Q-I-K-I-W-F-Q-N-R-R-M-K-W-K-K-NH2, **1**), a 16-residue cationic and amphipathic CPP, to stimulate endocytosis and thereby trigger its own uptake. To explore the importance of basic amino acids, penetratin is compared with arginine- and lysine- enriched analogs (PenArg and PenLys). We show, using a quantitative uptake assay and confocal microscopy, that penetratin stimulates uptake of large molecular weight markers (70 kDa neutral dextran), without increasing the overall endocytotic activity in CHO-K1 cells. Further, the extent of CPP and dextran uptake follows similar patterns with respect to peptide concentration and arginine/lysine content (PenArg>penetratin>PenLys), suggesting that arginines are beneficial, but not required for stimulated endocytosis.



1286-Pos Stable RNA Interference Results in Variable Expression of Synaptotagmin I that Contributes to Differential Transmitter Release

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Board B262

RNAi has become a powerful technique for specifically suppressing or knocking down the expression of a desired gene. Although different small inhibitory RNAs vary greatly in terms of their effectiveness, it has not been widely appreciated that a single short hairpin RNA-expressing plasmid can produce varying levels of knockdown in different stably transfected cell lines derived from the same transfection. We have targeted three proteins, synaptotagmin I (syt I), SNAP-25 and EGFP, and find that expression varies from minimal to almost complete in independent, stably transfected PC12 cell lines (J Neurosci Methods, 2007, in press). This variability emphasizes the importance of characterizing a number of stable lines, and offers the advantages of studying the effects of graded levels of protein expression. We have used the stable cell lines that express a range of syt I knockdown (minimal to complete) to measure Ca^{2+}-stimulated release of two transmitters, norepinephrine (NE) and neuropeptide Y (NPY). We recently reported that complete knockdown of syt I in a single stable cell line resulted in abolished release of NPY, but maintained 50% release of catecholamine (Am J Physiol, 2006, *291*:C270–81). Stable cell lines that express 50–60% of control levels of syt I have NPY release similar to control cells with normal levels of syt I expression. Release of NPY is reduced to about 18% when expression of syt I is reduced to about 20% of normal, and is abolished when syt I expression is abolished. The range of stimulated catecholamine release (50%–100%) correlates with the range of syt I knockdown as compared to control cells. These results indicate that while syt I is essential for NPY release, there remains a separate calcium sensor that regulates the release of catecholamines.

1287-Pos The Stability And Binding Properties Of Synaptotagmin I C2AB Are Not Simply Due To The Averaged Contributions Of C2A And C2B

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Board B263

Synchronous release from the pre-synaptic terminus requires neurotransmitter-containing vesicles to fuse with the plasma membrane

in response to a Ca+2 flux. Synaptotagmin 1 is the vesicle-localized, Ca+2-dependent phospholipid binding protein that mediates this Ca+2 sensitivity. Synaptotagmin is composed of a single transmembrane span with an ~130 amino acid unstructured tether to a Ca+2 binding domain (C2A). A second Ca2+ binding domain (C2B) is attached to C2A by a 9 amino acid connector. We have suggested that the high local concentration of vesicle membrane sensed by C2A due to this tethering leads to robust binding to lipid (Kertz. 2007. BJ. *92*:1408). This same concept of tethering may also be applied to C2A with regard to C2B. We hypothesized that C2A is connected to C2B to enhance interactions between the domains. Such an interaction is predicted to alter the ability of C2A and C2B to interact with ligands and transduce binding information. To be reversible, the interaction is predicted to be weak due to the high local concentration of one C2 domain to another. We compared the protein stabilities and affinities in the presence and absence of Ca2+ and/or phospholipid of the isolated C2 domains with the full-length soluble construct. If there is not an interaction between the domains, the stability and binding data of C2AB is predicted to be the average of the stabilities and affinities of the individual domains. Deviation from the mean is predicted if interactions occur between the domains. We do not find the mean to describe the stability and binding properties of C2AB. The data will be discussed in context of a high-resolution crystal structure of human synaptotagmin I C2AB indicating an interaction between the domains.

1288-Pos Identification Of SNARE And SNARE-associated Proteins In Cardiac Myocytes

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Board B264

In addition to its role as a pump, the heart also has endocrine functions. Cardiac myocytes contribute to blood volume control by secreting natriuretic peptide hormones, ANP and BNP by both constitutive and regulated exocytotic fusion. Detailed information, however, regarding the identity and function of specific membrane fusion proteins (SNARE proteins) involved in exocytosis in the endocrine heart is deficient. We identified SNARE and SNARE-associated proteins and determined their association with natriuretic-containing secretory granules using primary cultures of neonatal and adult rat cardiac myocytes. Cardiac myocytes were screened for mRNA transcripts by RT-PCR to determine which SNARE proteins were present and SNARE proteins known to be involved in regulated exocytosis in other cell types were further characterized in cardiac myocytes by western blot and co-immunoprecipitation methods. Localization of SNARE and SNARE-associated proteins was determined using cell fractionation and immunofluorescence methods. This study demonstrated that multiple SNARE proteins are present in neonatal and adult cardiac myocytes suggested the importance of SNARE proteins in exocytosis of natriuretic peptides from the endocrine heart.

Exocytosis & Endocytosis - II

1289-Pos Efficiency of Amperometric Catecholamine Detection with Transparent Microelectrodes: Comparison of ITO with Ultrathin Gold

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Board B265

Chromaffin cells of the adrenal gland constitute a model system for neuronal exocytosis where quantal release of catecholamines can be measured amperometrically, usually employing a carbon fiber electrode. Release from single vesicles is indicated by an amperometric current spike from oxidation of the released molecules (Wightman, et al 1991 PNAS *88*: 10754), often preceded by a foot signal (Chow, et al 1992 Nature *356*: 60) indicating opening and expansion of the fusion pore (Albillos, et al 1997 Nature *389*: 509). The charge obtained from the integrated amperometric spike is proportional to the number of molecules released from this vesicle with two electronic charges transferred per molecule.

To gain information about the mechanisms of transmitter release it is desirable to perform simultaneous fluorescence imaging and electrochemical detection of individual events. Additionally, to minimize diffusional broadening, the electrode needs to be in close contact with the cell membrane. For simultaneous fluorescence imaging through the electrode we fabricated transparent electrochemical detector arrays on glass using photolithography. Transparent planar microelectrode arrays were fabricated from either indium tin oxide (ITO), or ultrathin (~14 nm) gold. Amperometric spikes from bovine chromaffin cells could be detected with both materials at low noise, and fluorescence changes monitored through the electrodes with TIRF microscopy. Foot signals were detected with both types of electrodes. For arrays with similar geometry, the mean amperometric charge was 1.11±0.24pC (SEM, n=9 cells) for ultrathin gold, and 0.48±0.10pC (SEM, n=13 cells) for ITO electrodes, indicating that the gold electrodes are more sensitive to catecholamines then ITO. This result suggests that about half of the electrons from catecholamine oxidation are lost at ITO electrodes, presumably in reduction of some components of the ITO surface.

This research is supported by NBTC, NIH.

1290-Pos SV2B Regulates Intraterminal Ca^{2+} and Synaptic Vesicle Dynamics in Retinal Bipolar Neurons

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Board B266

Despite its relationship to human disease, the role that synaptic vesicle protein 2 (SV2) plays in the neuronal secretory pathway is