by employing fast-scan cyclic voltammetry and amperometry at carbon-fiber microelectrodes. While cyclic voltammograms confirm that the released molecules are serotonin, amperometry provides not only the first evidence of quantal secretion of serotonin from platelets, but also a real-time record of the secretion events. Individual platelets secrete an average of ~15 serotonin-containing granules, each with a serotonin concentration of ~0.6M and a secretion time course of ~7 ms. By combining experimental data with simulation results, we conclude that serotonin, along with other small molecules, is stored in the dense-body granules as a protein-free macromolecular complex rather than in a free solution state. This finding is in stark contrast to storage mechanisms proposed for other cell types where a proteinacious matrix is usually present. This work also examines the effects of variations in physiologically relevant extracellular conditions such as osmolarity, pH, temperature and cholesterol concentrationl, on serotonin secretion.

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Ca^{2+} Syntillas Inhibit Spontaneous Exocytosis In Mouse Adrenal Chromaffin Cells

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A central concept in the physiology of neurosecretion is that a rise in cytosolic [Ca^{2+}], resulting from Ca^{2+} influx, triggers exocytosis. But how does Ca^{2+} efflux from intracellular stores affect exocytosis? Here we examine the effect on exocytosis of a focal rise in cytosolic [Ca^{2+}] due to release from internal stores in the form of Ca^{2+} syntillas.

Ca^{2+} syntillas are spontaneous, focal cytosolic transients mediated by ryanodine receptors (RyRs), first found in hypothalamic magnocellular neuronal terminals. (*Scintilla*, Latin for spark, found in a nerve terminal, normally a synaptic structure.) Ca^{2+} syntillas are also found in mouse adrenal chromaffin cells, where they do *not* cause exocytosis because they appear to arise in a microdomain different from the one where the final exocytotic steps occur [1].

We report here that suppressing syntillas in mouse chromaffin cells leads to an increase in spontaneous exocytosis measured amperometrically. Two independent lines of experimentation each lead to this conclusion. In one case release from internal stores was blocked by ryanodine; in another, stores were emptied using thapsigargin plus caffeine. Additionally, the effects of ryanodine were not altered in the presence of reserpine and therefore could not be explained by an inhibitory effect on the vesicular monoamine transporter (VMAT). The effect of syntillas on spontaneous exocytosis can be accounted for by a simple two state model.

We conclude that Ca^{2+} syntillas act to *inhibit* spontaneous exocytosis.

1. ZhuGe, R. et al. Syntillas release Ca2+ at a site different from the microdomain where exocytosis occurs in mouse chromaffin cells. Biophys J 90, 2027–37 (2006).

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F-actin Re-organization Through MARCKS and Myosin II Activity Regulates Quantal Exocytosis

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Chromaffin cells of the adrenal medulla are innervated by the sympathetic nervous system and play a crucial role in determining the appropriate physiological response to stressors. Under diverse physiological conditions, differential release of catecholamines and vasoactive peptide transmitters helps determine the appropriate metabolic status and physiological response. As both classes of transmitters are contained within the same dense core secretory granule, their differential release must be regulated after granule fusion with the cell membrane. At basal firing rates, catecholamines are selectively released though 'kiss-and-run' fusion events characterized by a restricted exocytic fusion pore. Under acute stress, increased sympathetic input elevates cytosolic calcium, driving dilation of the fusion pore and expulsion of both catecholamines and the proteinacious core through the 'full-collapse' exocytic mode. Thus, activity-dependent differential transmitter release is regulated by fusion pore dilation. Previously, we showed that F-actin and myosin II play an important role in regulating the transition from 'kiss-and-run' exocytosis to 'full-collapse' exocytosis. Here, we employ electrochemical, electrophysiological and fluorescence based approaches to further investigate the molecular mechanisms responsible for the transition in secretion mode. We show that under light stimulation, myristoylated-alanine-rich¬-c-kinase-substrate (MARCKS) and myosin II remained inactive and cortical F-actin stabilizes 'kiss-and-run' fusion events. Increased cell stimulation resulted in activation of myosin II and MARCKS. The result was disruption of the actin cortex and collapse of the 'kiss-and-run' fusion event. These data demonstrate a role for activity-evoked cytoskeletal re-arrangement through the action of myosin II and MARCKS and define their roles as regulators of the sympathetic stress response.

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Fusion Pore Regulation of Peptidergic Vesicles

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Neuroendocrine secretory vesicles discharge their cargo in response to a stimulus. This process begins with the formation of a fusion pore, an aqueous channel between a spherical vesicle and the nearly ''flat'' plasma membrane, through which cargo molecules diffuse into the cell exterior. After formation the fusion pore can expand, leading to the complete merger of the vesicle membrane and releasing all of the vesicle cargo (full fusion exocytosis). On the other hand, fusion pore can relatively swiftly close, allowing only partial release of vesicle cargo and retaining vesicle physical integrity (transient exocytosis).

We studied the release of the pituitary hormone prolactin by hypotonicity, because this hormone also contributes to osmoregulation. Perifused cells spontaneously released prolactin at room temperature, and hypotonicity evoked a transient increase in prolactin release, followed by a sustained depression, as monitored by radioimmunoassay. In single cells imaged by confocal microscopy, hypotonicity elicited discharge of the fluorescently-labelled atrial natriuretic peptide cargo from ~2% of vesicles/cell, while KCl-induced depolarization resulted in a response of ~10% of vesicles/cell, with different unloading/loading time-course of the two fluorescent probes. High resolution changes of membrane capacitance were recorded in both, unstimulated and stimulated conditions, reflecting single vesicle fusion/fissions with the plasma membrane. In stimulated cells, the probability of occurrence of full fusion events was low and unchanged, since over 95% of fusion events were transient. However, stimulation prolonged the average pore dwell-time (hypotonicity and KCl depolarization for 25%, respectively), increased the frequency of occurrence (hypotonicity for 35%, KCl depolarization tenfold) and the fusion pore conductance (hypotonicity and KCl depolarization for 50%, respectively). Hypotonicity only rarely elicited new fusion events in silent membrane patches.

The results indicate that transient exocytosis appears to be the dominant mode of exocytosis at spontaneous as well as at stimulated conditions.

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Synchronous Versus Asynchronous Contributions to Frequency-induced Synaptic Depletion in Zebrafish

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Paired spinal motorneuron and target muscle recordings were used to examine transmitter depletion and subsequent recovery during high frequency stimulation. The skeletal muscle is sufficiently compact that the unitary synaptic events (~400pA average) were fully resolved through whole cell voltage clamp. Also, the evoked endplate current reflected the sum of <10 unitary events at physiological calcium concentrations. Stimulating at frequencies >20Hz led to drops in quantal content and eventual failure, with no associated change in unitary quantal size. The time required to deplete 80% of the transmitter corresponded to 25 sec at 20Hz, 10 sec at 50Hz and 5 sec at 100 Hz. Recovery occurred abruptly after a 40 sec rest and the rate of recovery was calcium-dependent. Analysis of the depletion profile during 100 Hz stimulation revealed two partially overlapping processes. Initially, all release was synchronous upon repolarization of the motorneuron action potential. With continued stimulation the release was delayed and asynchronous with respect to the action potential. The quantal size underlying both synchronous and asynchronous modes was unchanged during the depletion and following recovery. At 100 Hz the synchronous endplate current converted to asynchronous unitary openings in a manner that was qualitatively reciprocal. Moreover, traces composed principally of synchronous events had few asynchronous events and conversely traces with the largest numbers of asynchronous events lacked synchronous release. This reciprocity suggested that the two modes share common release sites. However, measurements of total synchronous and asynchronous events during the time of maximal overlap revealed a period of facilitation, suggesting that the synchronous and asynchronous modes were capable of additivity and might represent different release sites. Experiments are ongoing to distinguish between these alternative possibilities.

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Norepinephrine Inhibits Endocytosis In Insulin-secreting Cells

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Norepinephrine (NE) is a well-known physiological inhibitor of insulin secretion in pancreatic β cells. We investigated modulation of exocytosis and endocytosis by NE in INS 832/13 cells using whole-cell capacitance measurements. Exocytosis was stimulated by depolarizing pulses from −70mV to +10mV of variable duration and was followed by compensatory endocytosis. Inhibition of Ca^{2+}-evoked exocytosis by NE was overcome by increasing Ca^{2+} influx, either by increasing the depolarizing pulse duration (up to 500ms) or by increasing the extracellular Ca^{2+} concentration up to 10mM. When stimulated by a short train of 500ms pulses in the presence of NE (5μM), robust exocytosis was observed but endocytosis was markedly inhibited. The NE inhibition of endocytosis was abolished by the α_2-adrenergic receptor antagonist yohimbine (10μM) and was not affected by PTX-treatment (150ng/ml), demonstrating that NE inhibition of endocytosis is mediated via the α_2-adrenergic receptor and not via G_i and/or G_o proteins. When a synthetic peptide that mimicked the last 13 c-terminal amino acids of the $G_{z\alpha}$ subunit was dialyzed into the cells via the whole-cell patch pipette, NE inhibition of endocytosis was fully blocked, suggesting that G_z may be mediating the inhibition. Single vesicle recordings by cell attached capacitance measurements indicate that inhibition of endocytosis by NE is due to a decreased number of endocytic events without a significant change in endocytic vesicle size. Further analysis of fission pore kinetics revealed that NE selectively inhibited the rapid fission events. Our findings establish a novel action for NE and suggest the possibility that NE may modulate endocytosis in the central nervous system and elsewhere.

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Secretory Phospholipase A2 Type III Enhances α-secretase-dependent Amyloid Precursor Protein Processing by its Effect on Membrane Fluidity and Endocytosis

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Owing to the non-amyloidogenic pathway of amyloid precursor protein (APP) processing to produce neuroprotective and neurotrophic α-secretase-cleaved soluble APP (sAPPα), and preclude the amyloidogenic pathway which produces neurotoxic amyloid-β peptide (Aβ), increasing α-secretase activity and sAPPα levels have been suggested as pharmacological approaches for treatment of Alzheimer's disease (AD). In this study, we demonstrated that cytokines including TNFα, IL-1β and IFNγ stimulated immortalized astrocytes (DITNC cells) to secrete secretory phospholipase A2 type III (sPLA2-III) into culture medium. When this conditioned medium was applied to differentiated human neuroblastoma (SH-SY5Y cells), it enhanced sAPPα secretion from cells. To further demonstrate the effect of sPLA2-III on sAPPα secretion, SH-SY5Y cells were exposed to sPLA2-III from bee venom, which is homologous to mammalian sPLA2-IIIs, and hydrolyzed products of sPLA2 including arachidonic acid (AA), lysophosphatidylcholine (LPC) and palmitic acid (PA). We found that either sPLA2-III or AA, but not LPC and PA, increased membrane fluidity, increased the localization of APP at the cell surface without altering the total APP expression in cells, and enhanced sAPPα secretion in SH-SY5Y cells. In addition, neither sPLA2-III nor AA altered the expressions of α-secretases including ADAM 9, 10, and 17. APP has the motif which can target to clathrin-coated pits. We demonstrated that monodansylcadaverine (MDC), clathrin-mediated endocytosis inhibitor, can increase sAPPα secretion from SH-SY5Y cells by reducing APP internalization. Based on these results, sPLA2-III-enhanced sAPPα secretion is suggested to be a consequence in part, due to increased membrane fluidity, and reduced APP internalization through clathrin-mediated endocytosis. Further study will focus on the effect of sPLA2-III on clathrin-mediated endocytosis.

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Quantification of Noise Sources for Amperometric Measurement of Quantal Exocytosis Using Ultramicroelectrodes

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We are developing transparent multi-electrochemical electrode arrays on microchips in order to automate measurement of quantal transmitter exocytosis from individual neuroendocrine cells. Features of interest in amperometric recordings of quantal exocytosis can be <1 pA in amplitude, therefore low current noise is essential. Consequently we are seeking to understand the relationship between current noise and working electrode area, series resistance, bandwidth, and choice of fabrication materials. We have measured the current power spectral density (PSD) from electrode arrays with working areas that vary in size using a low-noise amplifier. Arrays are shielded from interfering signals. The capacitance of the working Indium-Tin-Oxide electrode varies linearly with area with a specific capacitance of 36 fF/μm^2. In the absence of an analyte, current noise is thermal in origin because the PSD is well described by the Nyquist relationship: PSD = 4kT times the Real part of the electrode Admittance. We find the PSD amplitude scales ~linearly with working electrode area and with frequency from ~30 Hz to at least 3 kHz. The dependence of the PSD on electrode area is similar for carbon-fiber electrodes and our patterned chip electrodes, therefore the electrode material and fabrication method are not key determinants of electrode noise. The choice of material and thickness (>2 μm) for insulating the non-working areas of the electrodes also does not affect the PSD. We conclude that the standard deviation of current noise increases ~ linearly with recording bandwidth, and microchip electrodes can achieve the same noise performance as carbon-fiber microelectrodes.

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Calcium/synaptotagmin-mediated Compound Fusion Increases Quantal Size And Causes Post-tetanic Potentiation At Synapses

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Exocytosis at synapses generally refers to fusion between vesicles and the plasma membrane. Although fusion between vesicles, known as compound fusion, occurs in non-neuronal secretory cells and has recently been proposed at ribbon-type synapses, it remains unclear whether it exists, how it is mediated, and what role it plays at the vast majority of synapses, where release occurs at conventional active zones. Here we addressed this issue in rats and mice at a large nerve terminal containing conventional active zones. High potassium application induced giant capacitance up-steps at the release face of nerve terminals, which were larger than the membrane capacitance of regular vesicles. These giant up-steps were not comprised of several smaller steps, nor were they bulk endocytic vesicles that had re-fused. High potassium application also induced giant vesicle-like structures in nerve terminals and giant miniature EPSCs (mEPSCs) that reflected release of a large amount of transmitter. The giant up-steps, giant vesicle-like structures, and giant mEPSCs were abolished by removing the extracellular calcium or by knocking out synaptotagmin II, the calcium sensor mediating fusion at calyces. These results suggest that calcium binding with synaptotagmin II mediates compound fusion and increases quantal size. Compound fusion significantly contributed to the generation of a widely observed synaptic plasticity, post-tetanic potentiation (PTP) of the EPSC, because 1) action potential trains that generated PTP also evoked giant up-steps and increased the mEPSC amplitude, 2) the time course and the degree of the mEPSC amplitude increase paralleled those of PTP, and 3) both the mEPSC amplitude increase and PTP were abolished by the calcium buffer EGTA or synaptotagmin II knockout. Our finding may be of wide application because intense nerve activity, PTP, and giant miniature currents occur in physiological conditions at many synapses.

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Evidence Of A Role For SNAP-25 As A v-SNARE In Vitro

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In neurons, SNARE proteins form a complex that drives membrane fusion leading to neurosecretion. SNAP-25 is an integral part of the neuronal SNARE complex and is clipped by the extremely toxic protease Botulinum Neurotoxin type E (BoNT/E). SNAP-25 is thought to act in the plasma membrane by forming a 1:1 complex with Syntaxin 1A which forms the binding site for the vesicular SNARE, synaptobrevin. SNAP-25 is also found in the vesicle membrane where its physiological role, if any, has yet to be defined. We show that BoNT/E cuts SNAP-25 in rat brain synaptic vesicles (SVs) decreasing their fusion to model membranes (BLM) containing reconstituted syntaxin 1A. We hypothesize that SNAP-25's role *in vivo* may depend on the lipid composition of the plasma and vesicular membranes. SNAP-25 is the only SNARE protein with no membrane spanning domain however, it is anchored in the membrane through the palmitoylation of one or more of its cysteines residues. In order to examine how SNAP-25 functions under different lipid conditions we used our planar lipid bilayer-base fusion system. Fusion rates are determined for target membranes composed of PE:PC (7:3) and with DPPC above and below its Tm. In order to simulate a more native neuronal environment, cholesterol (up to 50mol%) is added to the membrane By understanding the dynamics of the SNARE protein complex in different lipid environment, we hope to understand more about how neurons utilize SNARE proteins to release neurotransmitter.