Instituto Juan March
de Estudios e Investigaciones

110 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

# Regulated Exocytosis and the Vesicle Cycle

Organized by

R. D. Burgoyne and G. Álvarez de Toledo

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# Introduction

G. Álvarez de Toledo

Neurons, neuroendocrine cells, exocrine cells, mast cells and many other cell types communicate by the regulated release of molecules from a stored vesicle pool. Fusion of the vesicles with the plasma membrane to release the vesicle contents by exocytosis is activated by an intracellular signal, in many cases a rise in calcium concentration. This is followed by rapid endocytosis and vesicle recycling. Over the past few years, intensive studies of the different cellular processes and of the proteins involved in regulated exocytosis and other intracellular steps in vesicular traffic, by several laboratories, has resulted in significant advances and led to the identification of a number of physiological steps and some of the proteins which are key components of a core machinery leading to vesicle fusion. These proteins are conserved in eukaryotic cells throughout evolution and they and their related family members are likely to function in all cell types.

The functional importance of many identified proteins in regulated exocytosis has been investigated and confirmed using a wide range of experimental approaches including the study of mutants in yeast, *Drosophila*, *C.elegans* and mice as well as manipulations of proteins within isolated cells. In addition, a variety of proteins that function in the acute regulation of exocytosis such as protein kinases and their substrates have been characterised. Currently the most exciting challenge is the task of understanding how these proteins interact in a sequential and ordered manner in order to lead to secretory vesicle docking, membrane fusion and vesicle membrane retrieval. The identification and *in vitro* analysis of proteins of the exocytotic machinery has coincided with significant advances in the application of high resolution techniques for the analysis of single fusion events and vesicle recycling in secretory cells by electrophysiological and single cell imaging techniques. These new approaches have not only allowed the demonstration and definition of multiple steps in the exocytotic pathway and characterisation of the endocytotic pathway but are now allowing the investigation of the defined role of identified proteins.

The field of exocytosis and the vesicle cycle is currently very active with many hundreds of papers being published each year. In this workshop many of the top scientists in the world, addressing the different approaches to better understand synaptic transmission at the cellular and molecular level, gathered in Madrid to communicate to the scientific community their new advances in this highly competitive and rapidly changing field of neuroscience.

Guillermo Álvarez de Toledo

# Session 1: The protein machinery for vesicle docking and calcium dependent fusion
## Chair: Wieland B. Huttner

# Molecular mechanisms of membrane trafficking and exocytosis

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**The organization of membrane compartments:**

If SNARE complex formation is indeed the driving force behind membrane fusion, perhaps the specificity of vesicle trafficking is, at least in part, determined by the selective pairing of SNAREs localized to particular membranes (Söllner et al., 1993; Bennett and Scheller, 1993). If this hypothesis is true, several criteria should be met. First, there should be a large number of SNARE proteins expressed in cells and second, they should be specifically localized to the various membrane compartments within cells. Third, the various SNARE proteins should form specific sets of complexes consistent with the known membrane trafficking pathways within cells. We have initiated a series of studies to rigorously test this hypothesis.

The first step is to determine the number and organization of SNAREs in mammalian cells. Since the number of SNARE proteins has turned out to be quite large, this has been a labor-intensive, although interesting, project. We have used several approaches to characterize SNARE proteins. The first approach makes use of an observation from the nervous system that reveals immunoprecipitation of a particular SNARE results in the coprecipitation of interacting proteins. This approach was used to characterize many of the SNAREs important in vesicle trafficking between the ER and the Golgi (Hay et al., 1997). Another approach has been to define potentially new SNARE proteins through the expressed sequence tag database. In this approach, we use EST sequence information to define predicted proteins and then, through epitope tagging and generation of specific antibodies, characterize the protein (Advani et al., 1998; Steegmaier et al., 1998). We have now defined the localization of numerous SNARE proteins within the secretory pathway of mammalian cells at the light and electron microscopic levels. We certainly conclude at this stage that many, if not all, membrane compartments have a distinct composition of SNARE proteins consistent with their having important roles in determining the specificity of membrane compartment organization. We are also actively engaged in studying the function of these proteins using permeabilized cell systems. Recently we have been successful in devising systems to study the recycling of transferrin receptors and the degradation of EGF as probes of the endocytic membrane trafficking pathways within cells (Prekeris et al., 1998).

Now that we have characterized many SNARE proteins, we are in a position to ask if their binding to each other is consistent with the known trafficking pathways within cells. We demonstrated that 21 pairs of complexes formed *in vitro* and that they were all of high, although somewhat variable, thermal stability. From these studies, we conclude that the information for the specificity of membrane fusion is not defined by the ability of the proteins to form specific complexes (Yang et al., 1999). This does not rule out a role for the SNAREs in determining the specificity of membrane fusion events, nor do we conclude that SNARE pairing is not specific *in vivo*. We simply conclude that the information for the specificity is likely not encoded by the ability of the proteins to form complexes.

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# The SNARE complex – structural studies

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Neurons release neurotransmitters by calcium-dependent exocytosis of synaptic vesicles. In recent years, many of the proteins involved in mediating exocytosis have been identified but their mechanism of action and their regulation is only incompletely understood. Central to exocytotic membrane fusion are a group of small and evolutionary conserved membrane proteins, commonly referred to as SNAREs. In neurons, the SNAREs include the synaptic vesicle protein synaptobrevin (VAMP), and the synaptic membrane proteins SNAP-25 and syntaxin 1. In vitro, these proteins spontaneously assemble into ternary core complexes involving homologous regions referred to as SNARE motif. Core complexes are disassembled by the chaperone-like ATPase NSF in conjunction with cofactors.

Structural studies involving high-resolution electron microscopy, circular dichroism spectroscopy, and thermal denaturation yielded a refined picture of SNARE assembly. In vitro, synaptobrevin and SNAP-25 are largely unstructured whereas the SNARE motif of syntaxin is partially helical. Upon assembly, the proteins undergo dramatic conformational changes resulting in the formation of a tight bundle of four intertwined α-helices that form a coiled-coil. Since the transmembrane domains of syntaxin and synaptobrevin are aligned at one end of the elongated complex, it was proposed that the assembly reaction pulls the fusing membranes closely together, thus initiating the fusion reaction.

The solution of the X-ray structure of the neuronal core complex revealed unique and conserved features. Among those are layers of interacting and highly conserved amino acids that may define both the alignment between the helices and the specificity of the various SNARE motifs. In the middle, there is an asymmetric layer consisting of three glutamines (contributed by syntaxin and SNAP-25) and one arginine (contributed by synaptobrevin) that is highly conserved throughout the SNARE superfamily. Using the yeast complex as model, we have recently „rotated" the arginine to a different position which was tolerated without a loss of function. In contrast, introduction of two arginines in this position resulted in a severe disruption of exocytotic function.

Recent results will be discussed that involve interactions of the core complex with other proteins, flexibility of side chains during different stages of assembly, and features of SNAREs in an intact membrane environment.

## Molecular genetics studies in *Drosophila*

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We have investigate the mechanisms of synaptic transmission with a combination of *Drosophila* genetics with biochemistry, electrophysiology and microscopy. Via "reverse genetics" hypotheses concerning the *in vivo* physiological function of a known protein can be tested by the isolation and characterization of a mutation in that protein. An example of this approach is represented by our studies of synaptotagmin, a protein that is hypothesized to serve as a Ca-dependent trigger for release and whose binding properties suggest involvement in many nerve terminal processes. Null mutations in the fly *synaptotagmin 1* gene largely disrupt synaptic transmission, though a fraction of nerve-evoked release remains. Into this background, synaptotagmin transgenes were introduced that had undergone site-directed mutagenesis. Mutations in the Ca++-binding site of the C2A domain potently disrupt the Ca++-dependent interactions of C2A with syntaxin and phospholipids but have remarkably little effect on the ability of the gene to support synaptic transmission.

The completion of the sequence of the fly genome provides an excellent opportunity for classical "forward" genetics to uncover novel proteins involved in exocytosis. To this end we have undertaken a mutant screen in which flies are engineered to be homozygous for mutations in their photoreceptors but not in other cell types. In this screen a series of tests for phototactic defects and ERG defects led to the isolation of a gene that appears important for transmission at photoreceptor terminals. This gene (*milton*) encodes a novel cytosolic protein of the nerve terminal.

## Mechanics and regulation of synaptic vesicle fusion

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Our laboratory is interested in understanding how a presynaptic terminal secretes neurotransmitters in a tightly regulated, highly targeted manner. Studies performed in a number of membrane trafficking systems have identified three protein families that are universally involved in intracellular membrane fusion: the SNARE proteins, SM-proteins (sec1/munc18-like proteins), and rab proteins. Most of these proteins were first discovered at the synapse (the SNAREs syntaxin/HPC-1, synaptobrevin/VAMP, and SNAP-25, and the SM-protein munc18/nsec1/rbsec1), but later shown to be universally involved in membrane fusion. However, in spite of a large effort, the functions of these proteins have not yet been definitively identified in any system. Confusingly, different membrane trafficking reactions point to distinct functions. For example, at the synapse SNARE proteins are likely involved in the fusion reaction itself as evidenced by the selective defect in membrane fusion observed when nerve terminals are poisoned with toxins that degrade SNARE proteins (tetanus and botulinum toxins) whereas in the yeast vacuole, they appear to function at a step prior to membrane fusion. Similarly, at the synapse binding of munc18 to syntaxin involves the closed conformation of syntaxin that is incompatible with core complex formation by SNAREs while at the yeast plasma membrane, sec1 binds to the open conformation of the syntaxin homolog Sso1 when it is in the SNARE complex. To contribute to a clarification of these issues, we have embarked on a project in which we are aiming to correlate the binding activities of various proteins in vitro with their in vivo functions as defined in mouse knockouts or yeast mutants. In these studies we have focused on the SM proteins because they form the most homologous protein family in membrane fusion that is not immediately identified with a functional activity. In my present contribution, I will try to compare our results in analysing the functions of SM proteins with data obtained by other laboratories in other systems, and attempt to delineate what general conclusions about membrane fusion are currently possible in light of the apparent contradictions between different systems.

# Ring-like SNARE oligomers suggest a mechanism for fast synaptic exocytosis.

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Neurons transmit information across the synapse by the fast exocytotic release of neurotransmitters. Three evolutionary conserved proteins, known as SNARE proteins, are thought to be directly involved in exocytosis from single cell eukaryotes to neurons. Among neuronal SNARE proteins, syntaxin and SNAP-25 reside in the plasma membrane, whereas synaptobrevin (also known as VAMP) is present on the synaptic vesicle membrane. Recombinant parts of the SNARE proteins form a parallel four helix bundle which has been proposed to promote membrane fusion. However, the molecular nature of the native SNARE complex, specifically its oligomeric structure, is not fully understood. Using electron microscopy we show that the SNARE complex is a ~27 nm particle with a ring of variable size at its centre. Ninety-percent of SNARE particles possess between three and six spokes (four-helix SNARE bundles) emanating from their centre. Oligomers of SNARE bundles exhibited an extraordinary stability which may be explained by a domain swapping mechanism where the hinge loop of SNAP-25 links SNARE bundles in a ring-like structure. We present a model for synaptic vesicle exocytosis in which the movement of SNARE bundles in a ring-like oligomer results in the opening of a fusion pore.

## Session 2: Molecular modulation of vesicle docking and fusion
## Chair: Wolfhard Almers

# GTP-binding proteins in the signal transduction pathway for exocytosis in cells of haematopoietic origin

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The stimulus to secretion in mast cells (and other cells of haematopoietic origin) is best viewed as a signal transduction pathway, not an abrupt response to elevation of $Ca^{2+}$, as in neuronal cells. For sure, stimulation through the IgE receptor, by receptor-mimetics (mastoparan, compound 48/80 etc) and $Ca^{2+}$ ionophores requires the presence of $Ca^{2+}$ and the concentration of intracellular $Ca^{2+}$ is elevated as a consequence of stimulation. It also requires intracellular ATP and GTP. On the other hand, substantial secretion can be elicited from permeabilized cells by provision of GTPγS, in the absence of ATP and $Ca^{2+}$. To unravel the sequence of reactions involved, we used SLO-permeabilisation to introduce proteins and related reagents into the cytosol, aiming to modify (enhance or inhibit) the secretory process. It takes about 5 mins for exogenously applied proteins to diffuse and find their targets within the SLO permeabilized cells. During this time the soluble proteins of the cytosol leak out and the system becomes progressively resistant to stimulation. We therefore refer to run-down cells. The progress of run-down can be slowed, but not prevented by provision of ATP. For reasons that we do not understand, $Ca^{2+}$ becomes an essential component.

Run-down can be modulated by proteins isolated from bovine brain cytosol. In particular, RhoGDI inhibits GTPγS-induced secretion. It follows that one or more Rho-related GTPases act as regulators. Conversely, the complex of RhoGDI with the GTPase Rac enhances secretion, though Rac alone is without effect. However, recombinant Rac and Cdc42, preactivated by coupling with GTPγS both induce secretion. Applied in the presence of an optimal concentration of preactivated Rac, Cdc42 induces further secretion. Free GTPγS also enhances the extent of secretion, increasing the sensitivity to Cdc42 (but not Rac), and giving an indication of another GTP-binding protein upstream.

The PT sensitive Gi3 had been identified as the G-protein mediating signals due to receptor-mimetic agents. While αi-subunits are without effect, βγ subunits retard run-down and enhance the secretion induced by GTPγS. Unlike Rac and Cdc42, βγ subunits are unable to induce secretion when applied in the absence of GTPγS. They therefore collaborate with another GTPase, most likely Cdc42.

Communication downfield of βγ subunits is unlikely to be mediated solely through PLCβ/PKC since the enhancement is additive with phorbol ester. Soluble PH domains, (mutants of βARK having preference for polyphosphoinositides), inhibit secretion. We conclude that βγ subunits communicate to down-field GTPases through PH domains and that proteins having PH-domains again mediate the communication onwards from Rac/Cdc42. We have therefore focussed on reagents that interfere with polyphosphoinositides.

Although without significant effect on secretion induced by GTPγS from abruptly stimulated cells, neomycin, which binds to inositide head-groups and blocks PLC, inhibits secretion more potently as run-down proceeds. Conversely, phosphatidylinositol transfer

proteins, Sec14p (yeast) and PITP (α and β, mammalian) enhance secretion. Indeed, Sec14p protects against neomycin. We get the idea that not only is the presence of the polyphosphoinositide essential, but that the PITP protein may also be an essential component of the secretory machinery. PITP leaks from permeabilized cells, exposing the inositide headgroups, rendereing the system vulnerable to neomycin, yet it acts as an enabling component for exocytosis.

PITPs support synthesis of PI 4,5-$P_2$ by PI-4P 5-kinase. In a cell-free, minimal reconstitution system, consisting only of phospholipids, ATP and recombinant forms of PITP and PI(4)P 5-kinase, both mammalian PITP isoforms α and β enhance (approaching 20x) the activity of mammalian PI(4)P 5-kinase (α, β and γ isoforms). We are currently testing if this process occurs in the permeabilized cell system and if this could be the means by which exocytosis is supported. Preliminary data indicate that the α- (but not the β-) isoform of PI(4)P 5-kinase enhances secretion from run-down cells.

While the details of the membrane fusion event are likely to be conserved in all systems, it is apparent that here we are confronted with a set of complex regulatory processes. This could be the means of controlling the strength of the stimulus to exocytosis in a class of cells in which the total granule content remains poized and at-the-ready, over extended periods of time (months, in the case of mast cells), so providing a fail-safe mechanisms and ensuring graded release. It could also arise from the need to trigger other processes besides exocytosis such as the activation of eicosanoid production and protein synthesis.

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## Cysteine string protein controls late steps in exocytosis

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We have used amperometric measurement of exocytosis in adrenal chromaffin cells in order to assess the involvement of identified proteins in late stages of exocytosis including in fusion pore formation and expansion. In these experiments, chromaffin cells were co-transfected with plasmids either encoding GFP to mark transfected cells for recording or encoding the protein of interest [1]. Individual cells were permeabilised and stimulated by application of digitonin/10μM $Ca^{2+}$ to allow direct assessment of effects of treatments on exocytosis.

α-SNAP and NSF are implicated in early priming steps leading to exocytosis but not in fusion itself [2,3]. We examined the extent of the requirement for α-SNAP/NSF in priming the exocytotic machinery by using a dominant inhibitory mutant, α-SNAP(L294A) [4]. Overexpression of α-SNAP(L294A) was found to substantially inhibit amperometric events due to exocytosis of the stable preformed granule pool indicating an essential requirement for α-SNAP/NSF-mediating priming for their exocytosis. Despite the fact that α-SNAP(L294A) can irreversibly bind to syntaxin 1, the kinetics of individual release events in the residual amperometric spikes were no different from those in control cells [5]. Similarly, transfection with BoNT/C1 neurotoxin, which cleaves syntaxin 1 thus reducing the amount available to participate in exocytosis, inhibited the extent of exocytosis but did not modify the release kinetics of the residual amperometric spikes.

Cysteine string protein (Csp) is a synaptic vesicle and secretory granule protein that has the biochemical properties of a molecular chaperone able to activate Hsc70 [6]. Recent work has demonstrated its ability to bind to syntaxin 1 both in *Drosophila* and mammalian cells. Csp has been suggested to act as a regulator of $Ca^{2+}$ channel function, but our data have instead supported a more direct role in $Ca^{2+}$-activated exocytosis [7,8]. Over-expression of Csp was found to reduce the number of amperometric spikes evoked by permeabilisation in the presence of $Ca^{2+}$ where $Ca^{2+}$ channels are bypassed. In addition, the half-width of the residual spikes and the rise times were increased compared to those from control cells. These data suggest that Csp over-expression has direct effects on the exocytotic machinery leading to a slowing of fusion pore expansion. The lack of effect of α-SNAP(L294A) and BoNT/C1 on release kinetics suggest that this effect of Csp over-expression was not simply due to sequestration of syntaxin 1.

Changes in the kinetics of single release events were also observed following treatment with the phorbol ester PMA which resulted in a significant decrease in half-width and rise-times of release events. These changes were abolished by the PKC inhibitor bisindolymaleimide. These data are consistent with regulation of fusion pore expansion (acceleration) by PKC-mediated phosphorylation.

In conclusion, we have used amperometric measurement to demonstrate a potential late role of Csp in exocytosis and the possibility that Csp and PKC can modulate the kinetics of fusion pore expansion.

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## Molecular analysis of active zone function

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In contrast to most other secretory processes, neurotransmitter release from presynaptic axon terminals is restricted to designated release sites. These so called active zones are electron dense regions of the presynaptic plasma membrane at which the final steps of synaptic vesicle exocytosis, *docking*, *priming*, and *$Ca^{++}$-triggered fusion*, take place with extreme spatial and temporal accuracy.

Based on data from deletion mutant mice, we have recently identified the presynaptic active zone component Munc13-1 as an essential synaptic vesicle priming protein in glutamatergic neurons (Augustin et al., 1999). In order to identify molecular mechanisms by which Munc13-1-mediated vesicle priming is functionally integrated into the docking and fusion machinery of the active zone, we began to systematically characterize proteins that either interact physically with Munc13-1 or are affected in their expression levels by the Munc13-1 deletion.

In a first approach using yeast-two-hybrid technology, we discovered that the conserved N-terminal region of certain Munc13 isoforms (Munc13-1 and ubMunc13-2) serves as a novel binding domain for RIM, which is a putative Rab3 effector in presynaptic active zones (Wang et al., 1997) that may be involved in vesicle docking. In a detailed biochemical analysis we found that the Munc13-1 N-terminus competes with Rab3A for the same zink finger-like binding site in RIM. Overexpression of the RIM-binding N-terminus of Munc13-1 in hippocampal primary neurons leads to a reduction in the pool of readily releasable vesicles. This in turn causes a strong reduction in synaptic transmitter release, essentially creating a phenotype that is similar to that of Munc13-1-deficient neurons. Our data suggest that the interaction between RIM and Munc13-1 functionally couples vesicle docking through a Rab3A/RIM interaction to Munc13-1-mediated vesicle priming in the active zone. Like complete deletion of Munc13-1, disruption of the RIM/Munc13-1 interaction arrests the synaptic vesicle cycle between the docking and priming steps.

The second approach was based on the observation that the expression levels of Complexins are significantly reduced in Munc13-1 deletion mutant mice (Augustin et al., 1999). Complexins interact stoichiometrically with the assembled exocytotic 'core complex' (McMahon et al., 1995), but their exact function in the synaptic vesicle cycle is unknown. In order to determine the role of Complexins in synaptic vesicle fusion, we generated double mutant mice lacking both known Complexin isoforms (Complexin 1 and 2). Double mutants die immediately after birth. Electrophysiological analyses showed that double mutant neurons have normal readily releasable vesicle pools but exhibit drastically reduced release

efficiencies. A detailed analysis suggested that Complexin 1/2 double mutants are compromised at the $Ca^{++}$-triggering step of transmitter release.

Our data identify two protein families that interact physically or genetically with Munc13-1. While the physical RIM/Munc13-1 interaction may occur upstream of Munc13-1-mediated vesicle priming and create a molecular link between vesicle docking and priming, Complexins appear to function downstream of Munc13-1 by regulating the $Ca^{++}$-sensitivity of the exocytotic fusion machinery.

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## Functional genetics of cysteine string protein (CSP) in mice

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Synaptic vesicle proteins are implicated in multiple aspects of the synaptic vesicle cycle and the regulation of neurotransmitter release. We have generated mutant mice for several synaptic vesicle proteins in order to study their function *in vivo* (1, 2). Cysteine string protein (CSP) is a peripheral synaptic vesicle protein that contains a Dna-J domain and interacts with other proteins containing Dna-K domains presumably to function as a chaperone. CSP has been only characterized genetically in *Drosophila* (3). Now, we have generated null-mutant mice for CSP. The mutants are born alive and are undistinguishable from their littermates during the first 2 weeks. Interestingly, at around 2 weeks of age their growth slows and they stop gaining weight. They have difficulties to walk and to maintain an upright position. At 4-5 weeks of age their weight is ~30% of wild type littermates, their movement is severely impaired, and they die. Morphological and inmunocytochemical analysis did not show any obvious abnormality on the development or architecture of the brain. CSP is also expressed in other tissues outside the brain. We performed histological examination of multiple tissues like liver, muscle, intestine, kidney or adrenal gland, but they failed to show major changes versus wild type. Our preliminary data indicate that the lethal phenotype of CSP mice is not due to any major developmental or structural defect. In current experiments we are testing if a functional deficit either in synaptic transmission at the central nervous system or in hormone release, or both, are responsible for the strong phenotype found on CSP knock-out mice. These mice constitute a very useful tool to clarify the physiological role of CSP in the vesicle cycle and to understand *in vivo* the cascade of protein-protein interactions responsible for the chaperone activities of CSP.

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## The role of HRS in vesicular trafficking

Hugo J. Bellen

HRS (Hepatocyte growth factor Regulated tyrosine kinase Substrate) has been implicated in signal transduction, endosomal trafficking, and neurotransmitter exocytosis. To determine the *in vitro* role of HRS, we have cloned and sequenced the sole *Drosophila* homolog, and have determined the structure of the aminoterminal domain which contains the VHS and FYVE zinc finger domains. *Drosophila* HRS protein is expressed ubiquitously in the cytoplasm, and is localized to early (recycling) and late (perinuclear) endosomes; however, it is not present at the synapse. We have identified a mutation in *hrs*. This mutation is a null mutation based on both biochemical and genetic criteria. Consistent with the inability to detect HRS at the synapse, no defects in neurotransmitter release or endocytosis were observed in *hrs* mutant larvae using electrophysiology of third-instar larval neuromuscular junctions. Uptake of various dyes into garland cells demonstrates that endocytosis occurs normally in *hrs* null mutant cells, but endosome formation is impaired. Electron microscopic analysis shows the presence of large, prelysosomal structures in *hrs* mutants which contain the endosomal protein Hook. Overexpression of HRS leads to even larger endosomes than in mutants. These data strongly suggest that HRS is an essential regulator of endosomal trafficking. Interestingly, the t-SNARE SNAP-25 is also present on perinuclear endosomes, binds tightly to HRS, and is mislocalized in *hrs* mutant larvae.

Both loss and gain of function studies suggest that HRS functions in normal *Drosophila* development. Because the phenotype of HRS overexpression can be suppressed by overexpression of a dominant active form of the EGF receptor and is enhanced by a dominant negative form of the EGF receptor, our data suggest that HRS may play a critical role in development by regulating endosomal trafficking of tyrosine kinase growth factor receptors. This regulation may occur by regulation of SNARE complex formation through the interaction of HRS with SNAP-25.

## Session 3: Functional studies of vesicle docking and fusion at synapses and neuroendocrine cells and molecular modulation of vesicle docking and fusion
## Chair: Thomas Schwarz

## Exocytosis in adrenal chromaffin cells studied at high time resolution

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It is widely accepted that the SNARE proteins (Syntaxin, synaptobrevin, and SNAP-25) form a tight complex, which plays an essential role in membrane-to-membrane fusion during intracellular trafficking, neurotransmitter release, and hormone secretion. In regulated secretion the final step of vesicle fusion and release of contents is very rapid, when triggered by an abrupt increase in intracellular free calcium concentration ($[Ca^{++}]_i$). We made use of carbon fiber amperometry and membrane capacitance measurement, as rapid assays of secretion, and combined these techniques with flash-photolysis of caged-$Ca^{++}$ to rapidly trigger exocytosis. Three distinct kinetic components could be observed, which were tentatively associated with SNARE complex formation and with two interconvertible forms of such complexes. Manipulations on specific SNARE proteins, such as complexation with a specific antibody, cleavage by clostridial neurotoxins, or expression of mutated forms of the proteins led to distinct changes in the kinetic components.

## Transport, capture and exocytosis of single synaptic vesicles at active zones

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Among many thousands of secretory vesicles in an endocrine cell or a synaptic terminal, only a few percent are capable of rapid exocytosis. The size of this "release-ready pool" determines the response of an endocrine cell to an action potential, and sets the strength of a synapse. To understand how vesicles become release-ready, electrophysiologic studies have documented the kinetics whereby this pool, once depleted, is replenished[1]. This approach is indirect insofar as it relies on a single event, exocytosis, to study multiple precursor states. To observe directly both exocytosis and some of the preceding events, evanescent field fluorescence microscopy was used to image single vesicles near the plasma membrane. This technique was combined with electrical recording[2]. Ribbon synaptic terminals from goldfish[3,4] were stained with the lipophilic dye FM1-43[5].

Resting terminals were abuzz with moving vesicles, and the plasma membrane was constantly buffeted by vesicles colliding with it. Some vesicles were captured reversibly and then became *resident* at small, discrete "active zones" on the terminal surface. An electric stimulus caused nearly all resident vesicles to undergo rapid exocytosis, seen as the release of fluorescent lipid from the vesicle into the plasma membrane. It also triggered the arrival of reserve vesicles at the plasma membrane. These became release ready after a 0.25 s priming period. While most vesicle capture and exocytosis occurred at active zones, vesicles could fuse also elsewhere on the membrane, and approached active zones and other regions at about equal speeds. But active zones were favored in two ways. (1) The probability of exocytosis was 90% at active zones and 40% elsewhere. (2) Active zones appeared to hold vesicles in reserve, with reserve vesicles visible some 20 nm from the plasma membrane, and close enough for v- and t-SNAREs to touch[6].

Previous work has suggested that synaptic ribbons act as a "conveyor belt" facilitating the transport of vesicles to the plasma membrane. If the active zones observed here represent ribbons, then ribbons are absolutely necessary neither for vesicle transport nor for exocytosis. Hence the role of ribbons is not only transport but presentation: to present vesicles to the plasma membrane at the right distance for fusion, and at locations close to postsynaptic receptors.

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# A functional "Triad" to control cytosolic calcium and exocytosis in chromaffin cells

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Adrenal medullary chromaffin cells are prepared to synthetize and store the catecholamines noradrenaline and adrenaline, in large dense-cored vesicles (20.000 per cell). Fast release of these catecholamines into the circulation occurs during stressful conflicts, to adapt the cardiovascular system to an emergence situation, the "flight or fight" response. Because of their ready availability, their ease of maintenance in primary cultures, and their homogeneity these cells, particularly those of the bovine adrenal gland, have been widely used as models to study basic neurosecretory mechanisms.

Chromaffin cells express various subtypes of high threshold voltage-dependent $Ca^{2+}$ channel, L, N, and P/Q; their relative densities vary drastically among several mammalian species. The channels are subject to modulation by the concentration of cytosolic $Ca^{2+}$ ($[Ca^{2+}]_c$), the membrane potential and by various neurotransmitters. Particularly relevant is the autocrine/paracrine modulation by endogenous ATP and opioid peptides, that are co-stored and co-released with the catecholamines. This modulation is membrane-delimited via G-proteins, and likely constitutes the basis for the so called voltage-dependent facilitation of N or P/Q $Ca^{2+}$ channels.

Using aequoring selectively targeted to the endoplasmic reticulum (ER), we have recently discovered that $Ca^{2+}$ entry through those channels can refill the ER to near millimolar concentrations; in addition, $Ca^{2+}$ entering through $Ca^{2+}$ channels during cell depolarization causes the release of ER $Ca^{2+}$ (CICR). By using mitochondrially-targeted aequorins of different $Ca^{2+}$ affinities we have just shown that mitochondria undergo surprisingly rapid millimolar $Ca^{2+}$ transients ($[Ca^{2+}]_m$), upon stimulation of chromaffin cells with acetylcholine (ACh), high $K^+$ or caffeine. These transients are 2-3 orders of magnitude higher than those measured up to now using fluorescent probes. We believe that these microdomains can be formed because of the existence of a tight functional coupling between $Ca^{2+}$ channels, ER ryanodine receptors, and mitochondria. Physiological stimuli generate $[Ca^{2+}]_c$ microdomains at these functional complexes in which the $[Ca^{2+}]_c$ rises abruptly from 0.1 μM to 20-40 μM. This triggers CICR, mitochondrial $Ca^{2+}$ uptake and exocytosis in nearby secretory active sites. That this is true is shown by the observation that protonophores abolish mitochondrial $Ca^{2+}$ uptake, and drastically increase catecholamine release 3-5 fold. This increase is likely due to acceleration of vesicle transport from a reserve pool to a ready-release vesicle pool; such transport might be controlled by $Ca^{2+}$ delivery to the transport machinery (i.e. the cytoskeleton), through CICR and/or mitochondrial $Ca^{2+}$ release.

P/Q $Ca^{2+}$ channels seem to be located closer to exocytotic active sites than L- or N-types of $Ca^{2+}$ channels. In addition, mitochondria take up more readily the $Ca^{2+}$ entering through P/Q channels. Hence, a functional unit to control $[Ca^{2+}]_c$ transients and exocytosis seem to be more coupled to P/Q channels, indicating that depending of the stimulus, chromaffin cells might undergo different modes of exocytosis, faster and transient (P/Q

channels) or slower and sustained (L channels). This cell specialization keeps pace with our recent finding that chromaffin cells in culture secrete preferentially through their pole of attachment to the culture dish. To discover this, my coworkers and I have combined exocytotic fluorescent probes (i.e. FM1-43 and dopamine beta-hydroxylase antibodies) with confocal microscopy.

We are exploring now the hypothesis that in the intact adrenal medullary tissue, chromaffin cells become polarized to secrete towards their pole close to the endothelium, because of the influence of endothelial factors, i.e. nitric oxide. Chromaffin cells are innervated by cholinergic nerve terminals in a cell pole. Upon stressful conflicts, endogenously released ACh activates $\alpha_7$ and $\alpha_3\beta_4$ of the neuronal type of nicotinic receptors, triggers action potentials, $Ca^{2+}$ entry, and the release of catecholamines into the circulation at the opposite, specialized secretory pole.

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## The exocytotic fusion pore

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Fusion of secretory vesicles with the plasma membrane during exocytosis is thought to be mediated by the SNARE complex [1] as indicated by the inhibitory effects of tetanus toxin and a number of botulinum neurotoxins which specifically cleave SNARE proteins [2]. The molecular mechanism of fusion pore formation, however, is still not understood. Properties of the fusion pore that forms the connection between the intravesicular compartment and the extracellular space have been investigated using electrophysiological techniques [3]. The initial fusion pore conductance is variable but usually in the range of 50-500 pS, a range similar to that of ion channels. In horse eosinophils the initial fusion pore conductance is independent of the intracellular calcium concentration ($[Ca^{2+}]_i$) but the subsequent expansion of the fusion pore is accelerated when $[Ca^{2+}]_i$ is elevated [4]. Fusion pore expansion is also accelerated in PMA treated cells and this effect is abolished by staurosporine suggesting that fusion pore expansion is modulated by protein kinase C (PKC) [5]. It has been shown that the SNARE protein SNAP-25 is phosphorylated by PKC [6]. However, in eosinophils a role for SNAP-25 in specific granule exocytosis is not established.

Chromaffin cells are a widely used model to study neurosecretion and it is well established that SNARE proteins are essential in exocytosis of chromaffin granules [7]. However, in chromaffin cells only exocytosis of unusually large granules can be resolved as single events in the whole-cell configuration [8,9]. Based on the pioneering work of Erwin Neher and Alain Marty [10] we developed an improved method to study exocytosis of small granules by cell-attached capacitance measurements [11]. The low noise in patch recordings made it possible to investigate single channel currents with superb resolution [12] and correspondingly extremely low noise can be obtained in patch capacitance measurements [10,13]. Transmitter release from single chromaffin granules can be studied with exquisite resolution by amperometry using carbon fiber electrodes (CFEs) [14] and it was found that rapid release was frequently preceded by a phase of slow leakage of catecholamines indicated by a so-called 'foot signal' [15].

The relation between fusion pore opening and transmitter release from single chromaffin granules could be recorded simultaneously using the method of patch amperometry where a CFE is inserted into the patch pipette allowing detection of catecholamine released from the patch [16]. These experiments revealed that the foot signal is due to catecholamine release through fusion pores with typical conductance around 400 pS.

The amperometric signals reflect convolution of the release kinetics with the time course of diffusion. In the patch amperometry configuration only release events in the patch under the pipette are measured and the geometry of diffusion is well defined. The distance between CFE and membrane patch can be estimated from the microscope image and the time course of release can thus be determined by numerical deconvolution of amperometric signals thus correcting for diffusion. This analysis revealed two distinct release phases in individual events: a brief spike followed by a slow release phase with a time constant around 100 ms.

Exocytotic events in the cell-attached configuration occurred spontaneously and were strictly dependent on the presence of $Ca^{2+}$ in the pipette solution. Single capacitance steps in the fF range indicating exocytosis and endocytosis as well as amperometric signals can also be recorded in cell-free patches. Step size distributions were very similar in cell-attached and excised patches. With $Ca^{2+}$ present in the pipette solution $\geq$0.5 mM many patches showed spontaneous events as in the cell-attached configuration. When $Ca^{2+}$ was buffered at 150 nM on both sides of the patch membrane no spontaneous events were observed. However, in about half of these patches (4 out of 7) exocytotic events were stimulated by direct application of $Ca^{2+}$ to the cytoplasmic face using a puffer pipette.

The average initial fusion pore conductance was indistinguishable in cell-attached and excised inside-out patches (330 ± 30 pS (sem)). However, the average initial fusion pore expansion rate was 14.6 ± 2.3 nS/s in cell-attached and 7.4 ± 2.3 nS/s in excised patches. The slower fusion pore expansion rate in excised patches indicates that expansion of the fusion pore in chromaffin cells depends on the experimental conditions, as previously observed for the giant granules of horse eosinophils. These results suggest that the fusion pore expansion rate may provide a means to investigate the molecular machinery mediating fusion pore formation and expansion in exocytosis.

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## INTRACELLULAR $CA^{2+}$-DEPENDENCE OF TRANSMITTER RELEASE RATES AT A LARGE CENTRAL SYNAPSE

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Despite of the fundamental importance of $Ca^{2+}$ as the final trigger for synaptic vesicle fusion, the intracellular concentration of $Ca^{2+}$ ($[Ca^{2+}]_i$) attained close to the sites of vesicle fusion in conventional active zones is not known. We have used spatially homogenous $[Ca^{2+}]_i$ elevations by flash-photolysis of *'caged-$Ca^{2+}$'*, and postsynaptic measurements of transmitter release rates at a large central synapse, the calyx of Held, to quantify the intracellular $Ca^{2+}$-dependence of transmitter release. Simultaneous pre- and postsynaptic whole-cell recordings were made in brainstem slices of 8-10 days old rats. Presynaptic $[Ca^{2+}]_i$ was elevated by flash-photolysis of $Ca^{2+}$-loaded DM-nitrophene (1 or 2 mM), or NP-EGTA (5 mM), and was quantified by ratiometric imaging with fura-2FF, a low affinity $Ca^{2+}$-indicator. As a measure of transmitter release, flash-evoked excitatory postsynaptic currents (EPSCs) mediated by AMPA-type glutamate receptors were deconvolved with an average quantal response waveform. Transmitter release was observed at $[Ca^{2+}]_i$ as low as 2 µM, and up to ≈ 10 µM, release rates increased as a power function of $[Ca^{2+}]_i$, with an exponent in the range of 3.5 - 4. At 10-20 µM $[Ca^{2+}]_i$, release rates of 500-1000 quanta/ms were reached. Release rates during action potential-evoked EPSCs under conditions of normal release probability peaked at 200-500 quanta/ms, implying that the local $[Ca^{2+}]_i$ triggering transmitter release is in the range of 10 - 20 µM. This experimental estimate of local $[Ca^{2+}]_i$, which is about 5-10 times smaller than current estimates based on theoretical considerations, might have important implications for the interpretation of $Ca^{2+}$-dependent mechanisms in short-term synaptic plasticity.

## Session 4: Functional studies of transmitter release
## Chair: Manfred Lindau

## Genetic control of secretory vesicle biogenesis

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Acquisition of secretory vesicles characterized by neurotransmitter specificity is usually considered as a step in the differentiation of neurosecretory cells and neurons. Studies on multiple cell populations, isolated from the line PC12 which is known to release both acetylcholine and catecholamines, have however demonstrated that the vesicles (including all their membrane and lumenal proteins), together with the proteins known to be necessary for their discharge (i.e. the plasmalemma tSNAREs: SNAP27 and syntaxin1; various cytosolic proteins, such as rab3 and munc18) can be completely missing from clones that maintain numerous molecular (e.g. synapsins; nkinesin; tyrosine hydroxylase) and structural (neurofilaments; NGF-induced differentiation) properties of nerve cells. Reappearance of the secretory vesicle complement can be induced by fusion of the defective PC12 with many (possibly all) types of cells and by transfection of a cDNA library prepared from wild type PC12 cells. The correlation of the secretory cell defect with the expression of genes involved in the differentiation of nerve cells will be discussed.

## Rapid reuse of readily releasable pool vesicles at hippocampal synapses

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In central nerve terminals, the set of secretory vesicles immediately capable of transmitter release upon stimulation by depolarization-dependent $Ca^{2+}$ entry or by a hypertonic challenge has been operationally defined as the readily releasable pool (RRP). This pool probably correspond to the coterie of morphologically docked vesicles. There is compelling evidence that the size of the RRP governs the probability of transmitter release during presynaptic activation, and that the rapidity of its refilling after previous release events determines how well transmitter release can be maintained in the face of repeated $Ca^{2+}$ entry.

According to the prevailing picture of vesicular cycling, transmitter release is mediated by fusion and collapse of RRP synaptic vesicles into the presynaptic plasma membrane. After a vesicle releases its contents, its place at the active zone is taken up by another transmitter-containing vesicle. Thus, "refilling" of the RRP has been equated with repopulation, the replacement of spent vesicles with fresh ones. The source of the fresh vesicles is the reserve pool (RP), defined as another population of vesicles that are not morphologically docked and thus not primed for fusion. As it resupplies the RRP, the contents of the reserve pool are restored by various processes of vesicle recycling.

There are at least two reasons to question this picture. The first concerns the limitations that it imposes on the functional capabilities of small central nerve terminals. During continual presynaptic spiking, the rate of repopulation will limit neurotransmission, once the RRP has been depleted by the first few action potentials. If the number of recycling vesicles were <40, and repopulation of the RRP took ~40 s, the average steady-state frequency of quantal transmission would limited to <1 Hz. The second issue concerns the empirical underpinnings of the notion of repopulation. Selective depletion of the RRP is followed by a rapid exponential recovery ($\tau$~10 s), attributed to "refilling". However, upon exocytosis of a majority of the releasable vesicles, recovery is significantly slower, and follows a bi-exponential time course, with time constants of ~10 s and >30 s. As pointed out by Stevens and Wesseling, these and other kinetic features are inconsistent with a simple two-pool model whereby RRP and RP are connected by kinetically well-defined rate constants.

One source of difficulty is that the RRP has been operationally defined by electrical recordings and not by imaging of fluorescent dyes. The electrophysiological data is inherently difficult to interpret on its own because quantal EPSCs could in principle arise from either first-time exocytosis or repeated release events. Thus, repopulation by fresh vesicles could not be distinguished from repeated deployment of the same vesicles that have recently undergone fusion. Vesicular reuse would be of potential importance for sustaining secretory activity, given the limited number of vesicles within a presynaptic bouton and the long time that may be required to retrieve fully collapsed vesicles or to construct vesicles de novo. However, reuse has not attracted much attention due to the lack of experimental approaches to establish its existence.

The styryl dye FM1-43 has been extremely valuable for studies of exocytosis and endocytosis, but is not useful for studying the RRP; stimuli that completely exhaust the RRP (for example, hypertonic challenges) fail to cause enough loss of FM1-43 fluorescence to measure easily. To circumvent this problem, we have taken advantage of another FM dye, FM2-10, that dissociates from neuronal membranes much more quickly than FM1-43. We studied recycling properties of RRP vesicles in two ways, through differential retention of FM1-43 and FM2-10 (see also Klingauf et al., 1998), and by varying the time window for FM dye uptake. Both approaches indicated that vesicles which had been held in the RRP (~25% of the total recycling population) underwent rapid endocytosis ($\tau$~1 s); vesicles newly recruited from the reserve pool were recycled by conventional slow endocytosis ($\tau$~20-40 s). Neurotransmission was monitored in conjunction with destaining of the rapidly dissociating FM2-10 during repeated hypertonic challenges. The ability to release neurotransmitter showed a rapid recovery ($\tau$~10 s), as previously reported, whereas restoration of the FM2-10 fluorescence decrease took >10-fold longer. At 15 s following exocytosis of the RRP, neurotransmission had recovered but FM2-10 destaining had not. Finding neurotransmission in the absence of destaining implied that RRP vesicles that had rapidly endocytosed were then reused. Reuse of RRP vesicles is distinct from repopulation of the RRP by recruitment from the reserve pool, the hitherto recognized mechanism. As a means for expanding the functional capabilities of the limited number of presynaptic vesicles, reuse would be particularly effective during intermittent bursts of activity.

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# Regulated exocytosis and fast endocytosis in chromaffin cells: a reflection of the state of the fusion pore ?

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Release of neurotransmitters and hormones by neurons and endocrine cells is a key event in cellular communication and whole body homeostasis. The release process occurs by exocytosis, a phenomenon characterized by the addition of vesicular membrane to the plasma membrane through the formation of a fusion pore between both membranes. Exocytosis allows the release of vesicle contents, meanwhile endocytosis is responsible for the retrieval of plasma membrane to maintain the cell surface constant. For many years, exocytosis has been considered an all-or-none and irreversible event, triggered by a cellular signal, which in most cases is an increase in cytosolic calcium through the opening of voltage dependent $Ca^{2+}$ channels. Conversely, endocytosis was demonstrated to be an independent phenomenon from exocytosis.

We are studying the dynamics of exocytosis and endocytosis with time-resolved (milliseconds) electrophysiological techniques. Using a combination of patch-clamp capacitance measurements and electrochemistry we can monitor exocytosis of single secretory vesicles and to record the dynamics of neurotransmitters as they exit secretory cells. In addition, these techniques offer a unique opportunity to follow dynamic changes in cell surface area due to exo and endocytosis. We show that during exocytosis the cell surface area increases and transmitter release occurs at least in two stages: 1) leak of transmitter through the nascent fusion pore, 2) bulk release when the pore has fully dilated. These phases might be under cellular control, and can be aborted at any time, reversing the course of exocytosis (flicker or transient fusion). This mechanism represents the fastest and most selective way of endocytosis, helping to maintain cell integrity and rapid reuse of secretory vesicles. We try to show that fast endocytosis, the fastest known mechanism of coupling exocytosis and endocytosis is just the reflection of fusion pore closure.

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## Exocytosis of catecholamine-free granules in chromaffin cells

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In chromaffin cells, the combination of cell-attached patch-clamp capacitance measurements and the simultaneous electrochemical recording of catecholamines (CA) by amperometry (patch amperometry), have demonstrated that a granule can reversibly fuse with the plasma membrane and release its CA content with a time course indistinguishable from that of irreversible fusion events. The occurrence of such events is directly proportional to the extracellular $Ca^{2+}$ concentration. Release of CA and closure of the fusion pore may lead to a granule located near the plasma membrane that could be refilled of transmitter without undergoing a complete cycle of synthesis through the endosome. However no proof has been reported showing the ability of such endocytosed secretory granules to undergo a new round of exocytosis.

We now show by means of patch amperometry in chromaffin cells that two types of granules can fuse with the plasma membrane, one containing CA and other CA-free (blanks). Frequency histograms of capacitance step sizes showed that CA-containing granules average size (±SD) was 1.25 ± 0.7 fF ($n$=142, 8 cells) and that of CA-free granules 0.86 ± 0.46 fF ($n$=53, 17 cells). Transformation of granule capacitance to granule radius predicts an average (±SD) granule radius of 96 ± 25 nm for CA-containing granules and of 80 ± 21 nm for CA-free granules. Most granules had a homogenous CA concentration, with the exception of granules that did not release CA at all or very little, indicating that granules that contain CA were normally full. No preferred time distribution of blank events along the recordings were apparent, indicating that these granules, like CA-containing granules, were ready for fusion.

The average time (± S.E.M.) for fusion pore expansion was almost five times shorter in CA-containing (12 ± 1.8 ms, n=50) than in CA-free chromaffin cell granules (58 ± 21 ms, n=20). CA-free granules spent more than 80% of its expansion time with a conductance below 200 pS and the average pore conductance was 195 pS (n= 234). By contrast, CA-containing granules had an average conductance of 560 pS (n=86) , spending less than 20% of its expansion time with a conductance below 200 pS. These results indicate that, although chromaffin cell granules fuse with the membrane regardless of the presence or not of CA inside the granule, CA-free granules tend to enlarge their fusion pores slowly, staying for longer periods at smaller conductance values.

In summary, our results demonstrate the existence of CA-free granules in chromaffin cells and the ability of such granules to fuse with the plasma membrane.

## Leucine 203 plays a critical role in the function of SNAP-25 on exocytotic membrane fusion in chromaffin cells

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Green fluorescent protein (GFP) fused to SNAP-25 (synaptosomal protein of 25 kDa) can be overexpressed in chromaffin cells and used to investigate essential amino acids supporting t-SNARE exocytotic function, overcoming some of the limitations of neurotoxin based studies. Constructs containing either the whole SNAP-25 polypeptide or several deleted forms lacking its C-terminal domain were heavily overexpressed in transfected cells and present in both the cytoplasm and the plasma membrane. By using single cell amperometry we have observed that a construct containing complete SNAP-25 sequence sustained normal secretion, while removal of only four amino acids of its C-terminus greatly altered the rate and extent of exocytosis. Further mutational analysis proved that, in fact, Leu203, the fourth residue from the C-terminus, is critical for secretion. Kinetics of single granule fusions from cells expressing truncated forms showed slow onset and decay times when compared to control cells expressing endogenous SNAP-25 or the construct containing the full SNAP-25, while both displayed similar population of rapidly releasable vesicle pools secreted in response to hyperosmotic solutions. Single vesicle analysis suggest that as a matter of fact the final events of vesicle fusion and catecholamine release may be altered by alterations in SNAP-25 above mentioned. Further studies on the "in vitro" formation of the SNARE complex with the altered forms of SNAP-25 indicated that both the formation and dissasembling of this structure is changed with respect to the normal proteins. Taking together, these data provide direct evidence for the involvement of a specific residue of SNAP-25 in the exocytotic membrane fusion event happening in chromaffin cells, and show that overexpression of GFP-SNARE constructs combined with single vesicle fusion measurements constitutes a powerful approach to dissect the precise structural elements supporting a role in individual steps of the exocytotic cascade.

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## Session 5: Vesicle biogenesis and recycling
## Chair: Richard H. Scheller

## Molecular mechanism of clathrin-mediated endocytosis

Harvey T. McMahon

Clathrin-mediated endocytosis is essential for many processes in the cell from internalisation of receptors, the maintenance of membrane integrity, to its specialised function in nerve terminals the pathway is used to recycle synaptic vesicle components. To understand the molecular mechanism of clathrin-mediated endocytosis our laboratory has taken a structural and biochemical approach.

We have provided evidence that in nerve terminals, endocytosis has a distinct calcium trigger from exocytosis, the calcium dependent phosphatase calcineurin. This catalyses the dephosphorylation of endocytic proteins (including amphiphysin and dynamin) on nerve stimulation. Amphiphysin recruits dynamin to sites of endocytosis by binding to the adaptor protein complex AP-2, which in turn binds to membrane proteins to be endocytosed. We have solved the structures of domains from the adaptins, AP180 and amphiphysin to understand the specificity and adaptations of these proteins in endocytosis. Furthermore we have investigated the function of dynamin in vesicle fission using electron microscopy. We find that dynamin only associates with PIP2 containing lipids and have identified critical residues for lipid binding in its PH domain. On multimerisation around the neck of a vesicle, dynamin's GTPase activity is activated. We observe that on GTP hydrolysis dynamin spirals undergo a lengthwise extension- which we believe drives the vesicle away from the membrane causing lipid fission.

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## The role of lipids in the biogenesis of synaptic-like microvesicles

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We have studied the biogenesis of synaptic-like microvesicles (SLMVs), the neuroendocrine counterpart of synaptic vesicles of neurons, in the neuroendocrine cell line PC12 (1). SLMVs originate from a specialization of the plasma membrane (2). Using a cell-free system (3), the cytoplasmic machinery mediating SLMV formation has been studied and found to include dynamin and the dynamin-interacting protein endophilin I, a lysophosphatidic acid acyl transferase (4). Synaptophysin, a major oligomeric membrane protein of synaptic vesicles and SLMVs (5), specifically interacts with cholesterol, and cholesterol is required for SLMV biogenesis (6).

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# Vesicle cycling at the synapse of a retinal neuron

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We are investigating exocytosis and endocytosis by the fluorescent labelling of vesicles with the dye FM1-43 (Lagnado et al.,1996), and by the measurement of changes in membrane capacitance (Neves and Lagnado, 1999; Burrone and Lagnado, 2000). We apply these methods to a neuron with an unusually large synaptic terminal – the depolarizing bipolar cell from the retina of the goldfish. Like hair cells in the ear, these neurons possess synaptic structures ("ribbons") that tether vesicles close to the active zone.

Both capacitance and fluorescence measurements indicate that there are three kinetically distinct pools of vesicles in this pre-synaptic terminal (Neves & Lagnado, 1999). The two most rapidly released pools probably correspond to vesicles docked at the active zone and attached to the ribbon. The slowest component of exocytosis occurs continuously during maintained depolarization, and involves a pool of about 700,000 vesicles that is rapidly replenished by (Lagnado et al, 1996). Calcium stimulates the transfer of new vesicles to the most rapidly released pool of vesicles on the plasma membrane (Gomis et al., 1999).

Capacitance measurements indicate that endocytosis does not occur at a fixed rate. After a brief stimulus, all the membrane is retrieved rapidly (time-constant ~1 s). But after a longer stimulus that triggers more exocytosis, a proportion of the membrane is retrieved more slowly (time-constant ~10 s). This modulation of endocytosis may be dependent on the calcium signal, since the rapid mechanism of retrieval can be blocked by introducing 2-10 mM EGTA into the terminal.

Fluorescence imaging of FM1-43 uptake indicates that membrane is recycled into two compartments, both localized to a region within 2-3 μm of the plasma membrane. The first compartment is diffuse and can release the dye again, so we believe that it represents synaptic vesicles. The second compartment appears as large "hot-spots" from which the dye cannot be released, and may represent endosomes.

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## Keeping up with glutamate release

Robert Edwards

Synaptic transmission involves the exocytotic release of neurotransmitter stored at high concentrations inside synaptic vesicles. Since many nerve terminals sustain high rates of vesicular release, the amounts of transmitter released can be enormous and may greatly exceed the relatively small amounts present in the cytoplasm, where most classical transmitters are made and from which they must be transported into synaptic vesicles for exocytotic release. In the case of several classical transmitters such as dopamine, the plasma membrane reuptake systems that are generally considered to terminate synaptic signalling also serve to recycle the released transmitter. Indeed, recent work has shown that this recycling, rather than de novo synthesis, provides the bulk of stored neurotransmitter. Many plasma membrane neurotransmitter transporters occur presynaptically, supporting this role. However, the glutamate transporters that have been identified thus far occur mostly on astrocytes or postsynaptically rather than on the nerve terminal, suggesting different mechanisms for recycling of the principal excitatory neurotransmitter.

Work over the last 30 years has suggested that glutamate recycles through the related amino acid glutamine and through astrocytes rather than directly back into the nerve terminal. Specifically, it has been proposed that the glutamate taken up by known glial excitatory amino acid transporters undergoes conversion to glutamine by glutamine synthetase. The glutamine produced by this mechanism is then released from the astrocyte, taken up the neuron and converted back to glutamate by glutaminase prior to packaging into synaptic vesicles. However, the mechanisms responsible for glutamine transfer from astrocyte to neuron have remained unknown. In the course of studying a family of proteins that transport amino acid transmitters into synaptic vesicles, we have identified several novel members that contribute to this glutamine-glutamate cycle.

The vesicular GABA transporter (VGAT) uses a proton electrochemical gradient to pump GABA into synaptic vesicles. In particular, it exchanges lumenal protons for cytoplasmic transmitter (McIntire et al., 1997). We have now identified a subfamily of proteins related to VGAT that occur at the plasma membrane rather than secretory vesicles, and on astrocytes as well as neurons. These proteins recognize a variety of amino acids in addition to GABA. One cotransports $Na^+$ with primarily glutamine in exchange for a proton. As a result of the ionic coupling and stoichiometry, this protein mediates flux reversal under physiological conditions (Chaudhry et al., 1999). Localization to astrocytic processes surrounding synapses further suggests that it catalyzes the efflux of glutamine required for the glutamine-glutamate cycle. The function also corresponds to a classical amino acid transport activity described years ago as System N. Closely related to the System N transporter (SN1), we have now found that two other proteins that correspond to classical amino acid transport System A and catalyze the $Na^+$-dependent uptake of a wide range of neutral amino acids, including glutamine. However, unlike SN1, the System A transporters (SA1 and SA2) do not appear to translocate protons (Reimer et al., ), making them entirely dependent on cotransport with $Na^+$. As a result, these proteins do not mediate flux reversal under physiological conditions. SA1 and SA2 occur on neurons and may thus mediate the uptake of glutamine required for the glutamine-glutamate cycle. In summary, a family of closely related transport proteins mediates multiple aspects of the neurotransmitter cycle, from vesicle packaging to

recycling through astrocytes. Differences in ionic coupling to $H^+$ and $Na^+$ contribute to specific roles in efflux and uptake.

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## INWARD CURRENT THROGUH CALCIUM CHANNELS IS REGULATED BY THE TRANSMEMBRANE DOMAIN OF SYNTAXIN 1A

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Syntaxin 1A plays a critical role in exocytosis by participating in several protein-protein interactions that have been implicated in neurotransmitter release. To probe the functional interaction of syntaxin 1A with the N- and the Lc-type $Ca^{2+}$ channels we prepared a Sx1-2 chimera by replacing the syntaxin 1A transmembrane (TM) domain with the TM domain of syntaxin 2. Sx1-2 expressed in *Xenopus* oocytes along with the N-type or Lc-type $Ca^{2+}$ channels unlike syntaxin 1A, failed to inhibit inward current but retained the syntaxin 1A induced slowdown of channel-activation. The exchange of TM domains of syntaxin 1A with that of syntaxin 2 showed a loss of function in terms of current amplitude but no difference in the effect on the rate of activation. To explore whether the two sites influence the channel independently we used cleavage by Botulinum toxin C1 (Bot-C1). After syntaxin 1A cleavage with Bot-C1, the cytosolic cleaved fragment decreased the activation rate of the channel similar to intact syntaxin 1A, while inhibition of ion flow was lost. These results confirm two interacting sites of syntaxin 1A with N- and L-type $Ca^{2+}$ channels: a TM domain that inhibits maximal current, most likely by interacting with TM domain(s) of the channel, and a syntaxin cytosolic domain, responsible for lowering the rate of channel activation. Furthermore, these results demonstrate that the cytosolic domain interacts with the channel independently of the TM domain. Loss of ion flow regulation by Bot-C1 cleavage demonstrates the pivotal role played by TM-domain of syntaxin in controlling the gating properties of voltage-sensitive $Ca^{2+}$ channels.

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# POSTERS

## R-TYPE $Ca^{2+}$ CHANNELS ARE PRESENT IN MOUSE ADRENAL CHROMAFFIN CELLS AND CONTROL THE RAPID COMPONENT OF SECRETION

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The characterization of $Ca^{2+}$ channels subtypes in chromaffin cells had been performed up to now under conditions far from the physiological situation, due to the use of: (i) isolated cells maintained in culture, which might exhibit substantial differencies with slice cells; (ii) the whole-cell configuration of the patch-clamp technique, which might allow dialysis of relevant factors for $Ca^{2+}$ channels and secretion; (iii) $Ba^{2+}$ instead of $Ca^{2+}$ as a charge carrier. In the present study, we characterized for the first time $Ca^{2+}$ channel subtypes in chromaffin cells of mouse adrenal slices, using the perforated-patch configuration of the patch-clamp technique. Under these conditions, we revealed the presence of a subtype of $Ca^{2+}$ channel never described in chromaffin cells, a toxin and dihydropyridine resistant channel, with similar properties to the neuronal R-type channel. The resistant $Ca^{2+}$ channel in mouse adrenal slice chromaffin cells contributes 22% to the total $Ca^{2+}$ current, and 55% to the rapid secretory response evoked by short depolarizing pulses, measured with capacitance techniques. These data imply that R-type channels are colocalized with the exocytotic machinery to rapidly control the secretory process.

Concerning the other high-threshold voltage activated $Ca^{2+}$ channel subtypes (L-, N-, P-, and Q-type channels), our data demonstrate substantial quantitative differences with $Ca^{2+}$ channel subytpes described in mouse chromaffin cells maintained in culture (Hernández-Guijo et al., 1998). While in mouse adrenal slice chromaffin cells the contribution of P-, Q-, L-, and N-type channels was 22.4±4%, 22.6±7%, 27±4% and 35±5%, in mouse cultured chromaffin cells these values corresponded to 0%, 39±2.5%, 41±2% and 27.4±2%, respectively. Besides that, $Ca^{2+}$ channel subtypes in adrenal slice cells exhibit important kinetic differencies with neuronal $Ca^{2+}$ channels.

Discrepancies with previous studies in chromaffin cells emerged not only from the use of adrenal slice cells, but also from the patch-clamp configuration used. Thus, our data possess relevant physiological and methodological importance.

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# Fusion between *Saccharomyces cerevisiae* secretory vesicles and cytoplasmic-side-out plasma membrane vesicles *in vitro*

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The fusion event between secretory vesicles and the plasma membrane is the final step in the secretory pathway, which also plays an important role in the control of cell morphogenesis. The process has been reconstructed *in vitro* using purified secretory vesicles from a *sec 6-4* temperature sensitive mutant and cytoplasmic-side-out plasma membrane vesicles. Fusion was monitored through the dilution of the lipophilic self-quenching fluorescent probe octadecylrhodamine B-chloride (R18) incorporated into secretory vesicle membranes. Immunoelectron microscopy, using gold-labeled antibodies directed against the probe, reconfirmed the evidence obtained by fluorimetric measurements. The fusion process was strongly dependent on temperature and the cation composition of the medium, showing a half maximal fluorescence increase of 10 % after 17 minutes at 30°C. The fusion process was observed irrespective of ATP and cytosolic extract addition or N-ethylmaleimide (NEM) treatment, but is strongly inhibited by mild proteolytic treatment, and separate preincubation with polyclonal antibodies against Sso1p (t-SNARE) or Snc1p (v-SNARE) proteins. Taken together, the results show that secretory vesicle priming precedes transport to the fusion site and no further steps are needed on contact with the target membrane.

## ANTIBODIES PRODUCTION FOR A NOVEL CALCIUM DEPENDENT EXOCYTOSIS

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During our investigation on the mechanisms involved in the regulated secretion pathway, we have isolated a mutant clone, derived from the neurosecretory cell line PC12, that, still retaining the neuronal phenotype, has lost the vesicles and almost all the machinery for the regulated secretion.
In this particular clone Haruo Kasai (*PNAS 96:945-949, 1999)* was able to identify, by capacitance measurements, an exocytotic event triggered by photolysis of a caged calcium compound, characterized by slow kinetics and, probably, driven by different proteins, since classical SNAREs (i.e.VAMP2, synaptotagmin I, SNAP25) are not present and since this phenomenon is Tetanus toxin insensitive. In parallel, the discovery of calcium induced exocytotic events in non specialized cells such as fibroblasts, adipocytes and epithelial cells, and the cloning of new non neuronal isoforms of neurosecretory proteins, suggested new questions on membrane trafficking.
In order to investigate the subcellular compartments involved in this novel calcium dependent cell surface increase, we have generated a panel of antibodies able to recognize the proteins exposed on plasma membrane during calcium influx in the PC12 mutant clone.
Hybridoma cells were obtained by fusing immunized mice lymphnodes with myeloma cell line and the antibodies therefrom derived were tested in ELISA, Western blot, and immunofluorescence analysis for the reactivity against proteins located on the plasmamembrane of ionomycin treated PC12 mutant clone. The aim of our study is the identification of these antigens, their tissue distribution and the physiological meanning.

## Depolimerization of actin cytoskeleton enhances the frequency of unitary exocytotic events in rat melanotrophs

Helena H. Chowdhury and Robert Zorec

Actin cytoskeleton is involved in exocytosis and endocytosis. Both processes affect the area of the plasma membrane. An elegant way to monitor these processes is to measure membrane area continuously by measuring membrane capacitance (Cm), wich is lineary related to the membrane area. Previously, we measured Cm in cultured rat melanotrophs pretreated with Clostridium spiroforme toxin (CST), which specifically depolymerises F-actin. We have observed a transient and a sustained effect of CST-treatment on changes in Cm at high or low [Ca2+]i respectively, which suggest a distinct role of cytoskeleton in Ca2+-dependent and Ca2+-independent changes in Cm. Transient enhancement of the Cm rate by CST is consistent with a barrier role of cytoskeleton in regulated exocytosis. The sustained effect of CST on Ca2+-independent changes in Cm suggests cytoskeletal involvement in endocytosis (Chowdhury et al., 1999). Here we used cell-attached mode of patch-clamp technique to measure attofarad changes of patch-membrane capacitance. Each positive step of Cm was interpreted as an exocytotic event and each negative step as an endocytotic event. The frequency of exocytotic events was significantly higher in toxin treated cells than in control cells. Frequency of exocytotic events in control cells was lower than the frequency of endocytotic events but in toxin treated cells the predominant process was exocytosis. Results are consistent with the view, that actin cytoskeleton acts as a barrier for secretory activity in rat melanotrophs and that endocytosis requires an intact cytoskeleton.

**Properties of *Drosophila* synaptic boutons under patch-clamp: Neurotransmitter release in *ariadne* mutants.**

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Aiming to a comprehensive analysis of release, we developed a *Drosophila* preparation in which electrophysiological recordings from peptidergic terminals are feasible. Using patch-clamp techniques, we analyzed voltage-dependent potassium currents at the macroscopic and single-channel level. We found that *Shaker* $I_A$ currents are not present in this terminal. Instead, *Shal*, *Shab* and *Shaw* currents are found based on the biophysical properties of the corresponding channels identified. The $Ca^{2+}$ current in this type of terminal have been studied with $Ba^{2+}$ as charge carrier. The presynaptic membrane expresses a current type with high-activation threshold and little inactivation. This current is blocked by verapamil and diltiazem at micromolar concentrations, it is relatively insensitive to nifedipine and completely resistant to non L-type $Ca^{2+}$-channel antagonists.

On the other hand, we have identified a novel protein family, Ariadne, characterized by the presence of two RING-finger motifs. Mutants in this gene exhibit severe abnormalities in axonal projections. We have studied the possible involvement of this protein in synaptic secretion as indicative of growth cone projection defects. *ari* mutants present abnormal synaptic release at the larval stage. The frequency of spontaneous release is significantly reduced in all *ari* alleles tested. Also, a point mutation within the RING finger that interacts with UbcD10 results in potentiation of evoked release likely due to an increased probability of vesicle fusion in response to calcium.

## The heterotrimeric Gi3 protein is required in slow exocytosis of rat melanotrophs

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Besides playing a role in the signal transduction cascade operating at the plasma membrane, trimeric G-proteins may also be involved in the regulation of calcium-evoked secretion. The precise role of G proteins at specific stages of the exocytotic and endocytotic machinery remains to be determined. Our aim was to study the role of Gi3 and Gi1/2 in regulated secretion in rat melanotrophs. By immunocytochemistry, we provide the evidence that in rat melanotrophs Gi3 resides mainly in the plasma membrane whereas Gi1/2 is preferentially associated with the membrane of secretory granules. We used patch-clamp membrane capacitance measurements to monitor exocytotic activity in single rat melanotrophs. Mastoparan, a specific activator of trimeric G-proteins was found to enhance calcium-dependent secretory activity in rat melanotrophs. We introduced the synthetic peptides corresponding to the C-terminal domain of the alpha-subunit of Gi3- and Gi1/2-proteins. Peptide Gi3 but not Gi1/2 specifically blocked the mastoparan-stimulated secretory activity, which indicates an involvement of a trimeric Gi3-protein in secretory activity. In a series of experiments, cells were stimulated by increasing cytosolic calcium quickly and uniformly using flash photolysis of caged Ca2+. Photolysis of caged Ca2+ activated a biphasic membrane capacitance response, revealing an early and a slower phase of exocytosis, followed by endocytosis. Injection of specific antibodies against the alpha-subunits of Gi3-protein selectively affected the slow but not the fast component of the membrane capacitance response. We propose that the plasma membrane-bound Gi3-protein may be involved in regulated secretion by specifically controlling the slow kinetic component of exocytosis.

## Effect of protein kinase C activation on catecholamine secretion from PC12 cells

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Exocytosis from excitable cells has long been known to occur following rapid, local rises of $[Ca^{2+}]_i$ due to $Ca^{2+}$ influx via voltage gated $Ca^{2+}$ channels. However, recent studies have shown that even in some excitable cells, exocytosis can be triggered without a rise of $[Ca^{2+}]_i$, when protein kinase C (PKC) is activated (reviewed by Hille *et al.*, 1999). With this in mind, we investigated the effects of PKC activation on catecholamine release from individual PC12 cells, using amperometric techniques (Taylor *et al.*, 1999a,b).

Bath application of TPA (3-300nM) caused a concentration-dependent increase in the appearance of exocytotic events (1.44 ± 0.14Hz, at 100nM, n=12 cells), which are normally absent under these conditions (24°C, $[K^+]_o$ 5mM). This action of TPA was not mimicked by 4α-PDD (a phorbol ester inactive with respect to PKC activation, n=10), and was fully inhibited by bisindolylmaleimide (BIM; 3μM), a PKC inhibitor (n=8). Thus, PKC activation alone was sufficient to induce secretion from PC12 cells. During TPA-induced secretion, removal of $Ca^{2+}$ or addition of $Cd^{2+}$ (200μM) to the perfusate completely abolished secretion, indicating a complete dependence of PKC-mediated exocytosis on $Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels. TPA (100nM), but not 4a-PDD or TPA in the presence of BIM, also caused a marked, reversible rise of $[Ca^{2+}]_i$ as measured in cells pre-loaded with Fura-2.

Nifedipine (2μM), an inhibitor of L-type $Ca^{2+}$ channels, almost completely abolished PKC-mediated exocytosis and rises of $[Ca^{2+}]_i$, suggesting that PKC selectively activated $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels. Our previous studies have shown that in PC12 cells depolarization-evoked rises of $[Ca^{2+}]_i$ and exocytosis (using 50mM $K^+$ as the stimulus) are almost completely inhibited by ω-conotoxin GVIA-sensitive N-type $Ca^{2+}$ channels, with only a small contribution from L-type and P-type channels (Taylor & Peers, 1999). Thus, our results suggest that PKC activation somehow selectively promotes $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels to trigger exocytosis. Preliminary patch-clamp recordings indicate that TPA shifts the activation of L-type $Ca^{2+}$ channels to more negative potentials, allowing $Ca^{2+}$ influx at potentials close to resting membrane potential, without affecting other $Ca^{2+}$ channels present in these cells.

Our results indicate that PKC can evoke secretion of catecholamines from PC12 cells without any depolarizing stimulus being applied. In contrast to the effects of depolarizing stimuli, this secretion is largely mediated via L-type $Ca^{2+}$ channels, possibly via a PKC-mediated hyperpolarizing shift in their activation.

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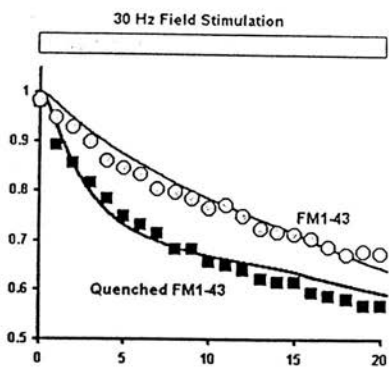
# Discrete, Stochastic Modeling of Vesicle Traffic in the Presynaptic Bouton

Jason L. Pyle and Richard W. Tsien

Presynaptic boutons of the central nervous system are capable of continuous neurotransmitter secretion over extended periods and wide ranges of stimulus frequency. In order to understand better the influence of vesicular turnover on synaptic transmission, we have developed a simulation of presynaptic vesicular trafficking based on experimentally derived parameters from recordings of electrical and styryl dye signals. We describe vesicular turnover with a discrete, stochastic model that treats vesicles as individual entities, tracking their movement along pathways of vesicular transport. Following published experimental evidence, vesicles are functionally and morphologically divided up between a pool that is immediately available for release and another larger pool that stands in reserve. Membrane fusion proceeds with a release probability related to the size of the pool of immediately releasable vesicles (Dobruntz and Stevens, 1997) and is followed by either rapid vesicular retrieval or complete collapse and slow endocytosis (Ryan and Smith, 1995; Klingauf, et al. 1998). We incorporate the recent finding that readily releasable vesicles preferentially undergo rapid endocytosis and become available for additional release events, a process that maintains secretory competence without drawing from the reserve pool of vesicles (Pyle, et al. 1999). The rapid retrieval of readily releasable vesicles can be independently tested by use of bromophenyl blue (BPB) as a quenching agent. Unlike quenchers used in our previous work, which fail to undergo uptake by synaptic vesicles (Pyle, et al. Neuron 1999), BPB rapidly enters vesicles, and when administered in parallel with FM1-43, largely eliminates the increase in fluorescence in dye loading experiments. In the presence of BPB, loss of fluorescence from FM1-43 loaded vesicles upon field stimulation is much faster than in the absence of the quencher (Figure). Both sets of data can be closely fitted with curves generated by the model though the parameters of the simulation were determined without information from styryl dye quenching experiments (Figure, solid lines). We extend the usefulness of our simulation by evaluating the time and frequency response of a presynaptic terminal. By varying input stimulus duration and frequency, we empirically derive a transfer function that describes how information crossing a synapse of the central nervous system is influenced by vesicle trafficking.



# Study on the mechanism of action of presynaptic PLA2 neurotoxins on nerve terminals

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Several animal venoms contain toxins with phospholipase A2 (PLA2) activity. These enzymes hydrolyse the sn-2 ester bond of 1,2-diacyl-3-sn-phosphoglycerides producing fatty acids (FA) and lysophospholipids (LysoPL)1. Some snake venoms contains presynaptic PLA2 neurotoxins that cause a persistent blockade of neurotransmitter release from nerve terminals1. Intra-peritoneal or intravenous injection of these neurotoxins leads to animal death by respiratory failure due to paralysis of respiratory muscles. Three subsequent phases can be distinguished at the NMJ poisoned by PLA2 neurotoxins: a short initial phase with either decreased or unchanged ACh release, is followed by a longer phase (10-30 minutes) of facilitation of ACh release, which then fades into the third phase of complete and irreversible inhibition of neurotransmission. Electron microscopy studies of poisoned NMJ2 revealed appearance of many clathrin-coated W-shaped plasma membrane invaginations, located also in areas facing the Schwann cells indicating a blockage of endocytosis at a stage following formation of the clathrin scaffold, but preceding the closure of the vesicle neck, which requires rearrangement of both proteins (dynamin, amphyphysin, adaptor proteins etc.) and phospholipids3. Therefore PLA2 neurotoxins both promote fusion of SSVs with the presynaptic membrane and inhibit their retrieval, thus causing release of ACh, depletion of vesicles and enlargement of nerve terminals. On the basis of these data we are investigating whether PLA2 neurotoxins block nerve terminals entering the lumen of synaptic vesicles and hydrolyse phopholipids of the inner leaflet of the membrane. We hypothesize that the trans-membrane pH gradient drives the translocation of fatty acids to the cytosolic monolayer, leaving lysophospholipids on the lumenal layer. Such vesicles are highly fusogenic and would release neurotransmitter upon fusion with the presynaptic membrane, but cannot be retrieved because of the high local concentration of fatty acids and lysophospholi

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## Vesicle aggregation and fusion induced by phospholipase C. Effect of cerebroside and sulfatide

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PC-preferring phospholipase C from B. cereus induces aggregation and fusion of large unilamellar phospholipid vesicles through in situ generation of diacylglycerol in the lipid bilayer. The process is highly sensitive to minor changes in vesicle lipid composition. Very low (less than 1 mole%) concentrations of gangliosides markedly inhibit vesicle fusion. In this poster we present our results of phospholipase C-induced liposome fusion with lipid bilayers containing, in addition to PC, PE and cholesterol, 1-20 mole% of cerebroside (galactosylceramide) or sulfatide (sulphogalactosylceramide). Cerebroside inhibits phospholipase activity, and consequently liposomal aggregation and fusion. The negatively charged sulfatide, however, displays a biphasic behaviour, enhancing the above processes at low concentrations (1.2 mole%) and inhibiting them when present at higher ratios. Sulfatide effects are also sensitive to ionic strenght. The marked dependence of membrane fusion events on the presence of glycolipids may have important consequences for intramolecular membrane traffic and biogenesis.

**Fusion of Constitutive Membrane Traffic with the Cell Surface Observed by Evanescent Wave Microscopy.** Derek Toomre*, Jürgen A. Steyer^, Patrick Keller*, Wolfhard Almers^, and Kai Simons*

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Monitoring fusion of constitutive traffic with the plasma membrane has remained largely elusive. Ideally, fusion would be monitored with high spatial and temporal resolution. Recently, total internal reflection (TIR) microscopy was used to study regulated exocytosis of fluorescently labeled chromaffin granules. In this technique only the bottom cellular surface is illuminated by an exponentially decaying evanescent "wave" of light. We have used a prism-type TIR setup with a penetration depth of ~50 nm to monitor constitutive fusion of vesicular stomatitis virus glycoprotein tagged with the yellow fluorescent protein. Fusion of single transport containers (TCs) was clearly observed and gave a distinct analytical signature. TCs approached the membrane, appeared to "dock" and later rapidly fuse, releasing a bright fluorescent cloud into the membrane. Observation and analysis provided insight about their dynamics, kinetics and position prior to and during fusion. Combining TIR and wide-field microscopy allowed us to follow constitutive cargo from the Golgi complex to cell surface. Our observations include: (i) local restrained movement of TCs near the membrane prior to fusion, (ii) apparent anchoring near the cell surface, (iii) heterogeneously sized TCs fused either completely or (iv) occasionally larger tubular-vesicular TCs partially fused at their tips.

## Fusion of secretory vesicles with plasma membrane vesicles 3

Unai Ugalde

Exocytosis is an essential cellular function, which requires the precise control of vesicular trafficking in multiple steps, from the ER to the final fusion reaction of post-Golgi vesicles with the plasma membrane. Despite the versatility with which this process is exploited in all eukaryotic cells, ranging from yeast enzyme secretion and cell morphogenesis to neurotransmitter release, it is understood that the basic process is controlled by a highly conserved group of proteins called SNAREs 1 , with the assistance of chaperone-like molecules called NSF and SNAP (1-5). Other protein groups have been identified, which participate in the specific regulatory features of each cell type, as well as the recycling of the cell components. However, details of the molecular interactions taking place between membrane components remain unclear, and cell-free systems have been proposed as a useful tool to clarify this issue, in combination with structural, biochemical and whole cell methodologies. These have been mostly developed for the study of intravesicular trafficking, but the final step of the secretory pathway has seldom been the subject of cell-free studies, mostly due to technical reasons (6). The study of fusion of pancreatic zymogen granules with isolated plasma membranes (7), or the exocytosis in sea urchin eggs, where the role of proteins, phosphoinositides, ATP and Ca 2+ have been extensively studied (6), are the most notable examples. S. cerevisiae is a well known lower eukaryote, which offers great versatility for genetic manipulation and an extensive background of knowledge on the secretory pathway (8). In yeasts, the last step of this process is constitutive (9). This situation contrasts with the higher eukaryote counterparts mentioned earlier, where the last step is regulated, responding to an intracellular signal, such as an increase in cytosolic Ca 2+ concentration. In yeasts, cell-free membrane fusion studies between ER and Golgi vesicles have been conducted (10, 11), as well as homotypic vacuolar fusion (12), but the last step of the secretory pathway remained unexplored, largely due to the unavailability of unequivocally competent target membrane preparations. A method for the preparation of cytoplasmic-side-out plasma membrane vesicles (13) has provided the missing element for the study of the last exocytotic fusion event. In this paper, we report on the fusion event between two different vesicle populations: secretory vesicles (SVs) and cytoplasmic-side-out plasma membrane vesicles (PMVs), purified from S. cerevisiae. The assay monitors the increase in fluorescence of the lipid-soluble probe octadecylrhodamine B-chloride (R18) loaded to one vesicle population, when it is diluted into the target membrane as the result of fusion (14). The obtained experimental results reveal the existence of a specific heterotypic fusion event, which is dependent on temperature and the cationic composition of the medium. The involvement of proteins in the process has been demonstrated by mild proteolytic treatment, and the participation of SNAREs as central elements of the fusion.

## Docking and fusion of eosinophil granules using optical tweezers

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In eosinophils intracellular application of GTP-gamma-S stimulates heterotypic fusion of granules with the plasma membrane as well as homotypic fusion among granules inside the cell as measured by whole cell capacitance measurements [Scepek & Lindau (1993) EMBO J. 12:1811]. Homotypic fusion increases strongly with increasing GTP-gamma-S concentrations. Horse eosinophil granules are typically 1-3 μm in diameter and are thus well suited to study single fusion events. Isolated granules were obtained by passing the cells through a 0.5 mm syringe needle for 5-10 times. Granules were manipulated using an optical tweezer and monitored by video imaging. When 2 granules are brought into contact using a double trap they adhere to each other. Due to the large granule size the two traps holding the two granules do not overlap when contact is made. When a granule is brought into contact with the extracellular side of the plasma membrane of a cell, the granule also sticks to the membrane. While tethered to the membrane, the granule may still move significantly. Upon addition of GTP-gamma-S a morphological change suggesting a fusion event was observed. The method is presently applied to measure docking forces between granules and in conjunction with fluorescent markers to study the fusion event in-vitro. The present experiments suggest that horse eosinophil granules may be able to fuse without the requirement of specific t-SNAREs in the target membrane.

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