The Eighth Datta Lecture

Molecular mechanisms in synaptic vesicle recycling

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Abstract Synaptic vesicles are specialized secretory organelles which are involved in the fast, point-to-point signaling typical of synapses. They store and secrete non-peptide neurotransmitters and are continuously regenerated in nerve terminals by exo–endocytotic recycling. This recycling represents a highly specialized form of the recycling pathway which occurs at the surface of all cells. Several unique properties make synaptic vesicles a powerful experimental model for studies of vesicular traffic. These unique properties include their abundance in brain, the high specialization of nerve terminals for synaptic vesicle recycling, the possibility of studying their exocytosis at the level of single events by electrophysiology and the availability of toxins which block their recycling. This lecture will summarize current information of molecular mechanisms in synaptic vesicle recycling with emphasis on recent studies carried out in my laboratory on mechanisms of vesicle reformation after exocytosis.

Key words: Synapse; Exocytosis; Endocytosis; Dynamin

1. Introduction

All cells of multicellular organisms have the property to secrete a variety of substances via exocytosis of intracellular vesicles and some cells have developed specialized regulated secretory pathways. Neurons, in addition to constitutive secretory pathways and a regulated pathway for the secretion of neuropeptides, have developed a unique regulated pathway for the secretion of non-peptide neurotransmitters. This secretory system involves synaptic vesicles and it functions with high topological precision and speed. Together, with a specialized transduction machinery localized on postsynaptic membranes, this mechanisms of secretion is responsible for the fast-point-to-point signaling typical of synapses [1].

In an average central nervous system synapse, 10–20 synaptic vesicles are docked at active zones of the presynaptic plasmalemma in a fusion-ready state, and are surrounded by a pool of 100–300 reserve vesicles located deeper in the cytoplasm. Action potential-induced nerve terminal depolarization produces a local rise in cytosolic Ca^{2+} and triggers exocytosis within a narrow temporal window (with a range of hundreds of microseconds) from the pool of docked vesicles (typically, 1 vesicle or less for each action potential at each synapse). The fused vesicle is rapidly replaced by a vesicle from the reserve pool. Its membrane is recaptured by endocytosis and recycled locally for the generation of a new vesicle which is subsequently filled with neurotransmitters [2–5]. Recent estimates indicate that a whole cycle may occur in less than 1 minute [6,7]. During intense stimulation all synaptic vesicles present in a given synapse can be recruited for fusion within short time scales [7] and this rapid recycling ensures the availability of vesicles for release even during prolonged periods of activity.

Over the last several years, our laboratory has been interested in elucidating the properties and the mechanisms of synaptic vesicle exo–endocytosis. This process represents a special case of what takes place at all stations of the secretory and endocytic pathways where anatomically distinct compartments are functionally interconnected via vesicular traffic. More specifically, synaptic vesicle recycling is closely related to the membrane recycling which takes place at the surface of all cells (Fig. 1). Thus, the significance of studies on the properties and life cycle of synaptic vesicles go much beyond the field of neurotransmission. First, these studies are helping to unravel the fundamental mechanisms that are involved in docking, fusion and budding of vesicular carriers [8–11]. Second, they are providing new information on general mechanisms of recycling at the cell surface [12]. Third, studies on the mechanisms which recruit synaptic vesicles and their associated proteins at the synapse may provide new clues on the dynamic interactions between secretory vesicles and the peripheral cytoskeleton.

Synaptic vesicles offer several advantages as a model system for the study of general mechanisms in vesicular traffic. Their exocytosis can be monitored by a variety of assays and single exocytotic events can be studied by electrophysiology. Given the extremely high concentration of these organelles in brain tissue, they can be easily obtained with greater yield and purity than any other secretory vesicle [13,14]. Additionally, since they do not store peptides, their protein composition closely reflects the protein composition of their membrane. These advantages have made possible the identification and cloning of the major protein components of synaptic vesicle membranes using biochemical methods [1,8,15]. Many of these proteins are members of the same protein families that genetic studies in yeast and cell-free assays of other vesicular transport steps have shown to play key roles in membrane traffic [8,10,11,16]. In some cases, neuronal proteins can even function in yeast cells and can be used to complement or suppress yeast mutations [17]. More recently, the use of genetics and of cell-free systems have become possible also for the direct study of synaptic vesicles [18,19].

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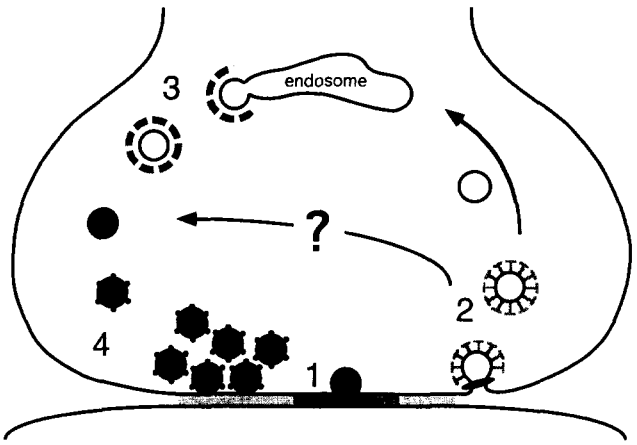

Fig. 1. Schematic drawing illustrating synaptic vesicle recycling in nerve terminals. Step 1 to 4 are discussed in the text. 1, docking and fusion; 2, endocytosis; 3, budding from endosomes; 4, clustering at the synapse. A question mark illustrates a putative recycling pathway which bypasses endosomal intermediates.

2. Docking and fusion

The convergence of information obtained from the biochemical analysis of synaptic vesicles, yeast genetics and cell-free vesicular transport assays have led to the formulation of what is now a widely accepted hypothesis concerning the mechanisms of membrane docking and fusion, the SNARE hypothesis. This hypothesis predicts that a key step in the sequence of events that lead to the fusion of a vesicle with a target membrane is the interaction of small proteins localized on the cytosolic surface of the vesicle (the so-called v-SNAREs) with small proteins localized on the cytosolic surface of the plasmalemma (the so-called t-SNAREs). In the case of synaptic vesicles exocytosis, the v-SNAREs are the synaptobrevins and the t-SNAREs are syntaxin I and SNAP-25. Formation of the SNARE complex is then followed by the recruitment of cytosolic factors, including SNAP proteins and NSF, which in some yet unclear way allow progression to fusion [20–22]. The key role of SNARE molecules in synaptic vesicle exocytosis had been anticipated, and then strongly corroborated, by the demonstration that clostridial neurotoxins block neurotransmitter release by selective proteolysis of SNARE proteins [23–25]. The precise sequence of events which lead to SNARE complex formation and to vesicle fusion is currently the focus of intense research and has been the object of recent reviews [11,20–22].

Formation of the synaptic SNARE complex must be highly regulated given, not only the tight Ca^{2+}-dependent regulation of synaptic vesicle exocytosis, but also the selective occurrence of this process at the synapse. Synaptic v- and t-SNAREs undergo post-translation membrane insertion in the endoplasmic reticulum [26,27] and could, potentially, interact before reaching their final destination. In addition, t-SNAREs which participate in synaptic vesicle exocytosis are localized all along the axon and are not restricted to the presynaptic plasmalemma [28]. Thus, the synaptic v- and t-SNAREs must be regulated by interacting proteins.

Several proteins which may participate in the regulation of SNARE complex formation have already been identified. Synaptotagmin is an integral synaptic vesicle membrane protein which contains in its cytoplasmic portion two domains homologous to the regulatory C2 domains of protein kinase C [29]. It is thought to be one of the Ca^{2+} sensors which mediate the regulatory actions of this ion on exocytosis [18,30,31] and it interacts in a Ca^{2+}-dependent way with components of the SNARE complex [21,32]. Synaptophysin, a synaptic vesicle protein with four transmembrane regions, forms a complex with synaptobrevin on the vesicle membrane and this binding prevents synaptobrevin from interacting with the t-SNAREs syntaxin and SNAP-25 [33,34]. Some regulatory protein in nerve terminals must promote the dissociation of this complex to allow synaptobrevin interaction with the t-SNAREs.

Two cytosolic neuronal proteins which control the formation of the synaptic SNARE complex are rbSec1 [35] (also referred to as Munc18 [36] and N-Sec1 [37]) and Rab3. Both are members of protein families which were first identified via genetic studies in yeast. A direct interaction between Sec1 and syntaxin was first suggested by yeast genetics [38] and then demonstrated by biochemical studies in neuronal tissue [35,37,39]. RbSec1, like syntaxin, is localized along the entire axon where it is partially associated with the plasmalemma [28]. Since the binding of rbSec1 to syntaxin is competitive with the binding of syntaxin to the other SNAREs [28,35,37,39], it had been suggested that Sec1 may act as a negative regulator of SNARE complex formation. It was found, however, that only a small pool of rbSec1 is bound to syntaxin, which is instead primarily associated with SNAP25 [28]. Thus, the role of rbSec1 remains unclear. Since syntaxin overexpression in yeast can suppress Sec1 mutations, but not Sec1 deletions [38], a plausible scenario is that a transient interaction between Sec1 and syntaxin may be required to make syntaxin competent for SNARE complex formation.

Rab3 is a member of the large family of Rab GTPases which appear to regulate all membrane fusion reactions along the secretory and endocytic pathway [40,41]. Current models, supported by a large body of experimental evidence, predict that Rab proteins in their GTP state are bound to the vesicular carrier and act as facilitators (catalysts) in SNARE complex formation [42–44]. Following fusion, which is somehow linked to GTP hydrolysis, Rab proteins are recycled through the cytosol and reused for another cycle of docking and fusion [41,45]. Several accessory proteins were shown to control this putative cycle [46]. GDI (guanylnucleotide dissociation inhibitor) is a cytosolic Rab binding protein which dissociates Rab-GDP from membranes by binding its lipid modified tail. It delivers Rab-GDP to the vesicle membrane where GDP is replaced by GTP [47]. This exchange appears to occur in two steps: first a putative Rab receptor (GDI displacement factor, GDF) disassociates GDI from the GDP-Rab protein and mediates incorporation of Rab-GDP in the membrane. Then, a guanylnucleotide exchange factor (GEF), which acts on the membrane-bound Rab, catalyzes the exchange [48,49].

No GDFs have been identified so far, but the first member of what is predicted to be a family of mammalian GEFs for Rabs was identified using a functional rescue approach in yeast [50]. Briefly, a rat brain cDNA library in a yeast expression vector was used to search for high copy suppressers of the yeast *sec4–8* mutant strain. This strain harbors a temperature sensitive mutation of the Rab protein Sec4 which is essential for yeast exocytosis and therefore for growth [51]. One such suppressor was identified and called Mss4 (mammalian suppressor

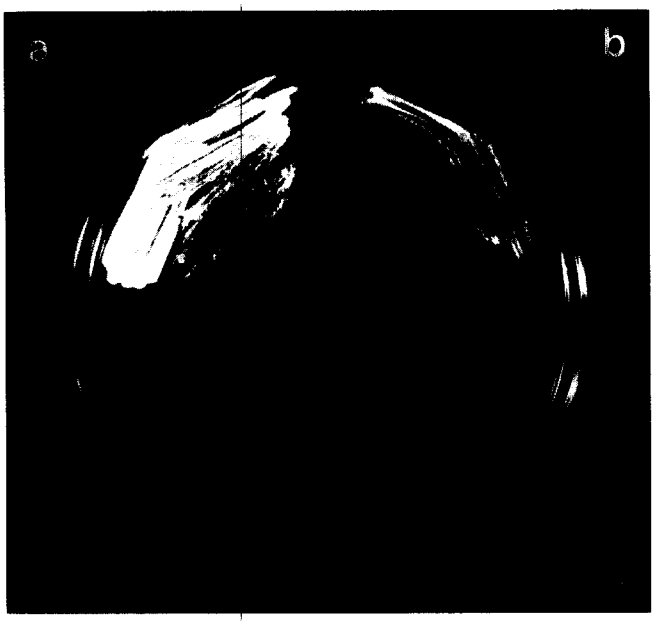

Fig. 2. Restoration of the growth defect of the temperature-sensitive *sec4–8* yeast secretory mutant by expression of the mammalian protein Mss4. Plates were incubated at the restrictive temperature (37°C): a, *SEC4* wild type yeast strain; b, *sec4–8* strain expressing Mss4; c, *sec4–8* parent strain; d, *sec4–8* strain containing expression vector alone (from [17], reprinted with permission).

of Sec4) (Fig. 2). This protein very selectively binds a subset of Rabs including Rab3 and catalyzes GDP-GTP exchange on these proteins [17,50] (Fig. 5). When injected in the squid giant axon, Mss4 enhances neurotransmitter release [50] validating the hypothesis that functional rescue of secretory mutants in yeast can be used to identify proteins which participate in synaptic vesicle exocytosis.

The precise mechanism of action of GTP-Rab3 in controlling docking and fusion reactions remains unclear, and, so far, no unifying model of Rab action has emerged. While Sec4 function is essential for exocytosis in yeast [41,51], disruption of the gene encoding the predominant Rab3 isoform (Rab3a) in mice did not produce major behavioral or neurological defects, nor any change in the size of a secretory response from hippocampal synapses after a single, or a few, depolarizing stimuli. These synapses, however, displayed an increased synaptic depression (an accelerated rundown of the secretory response, after high frequency stimulation), suggesting an inefficient replacement of the fusion-ready pool of vesicles [52]. These data are consistent with a role of Rabs in facilitating one of the steps which directly precede fusion. At least some of the action of Rab3 are likely to be mediated by rabphilin, a protein which specifically binds Rab-GTP but not Rab-GDP. Rabphilin is a hydrophilic protein whose C-terminal half contains two C2 domains and, therefore, resembles the C-terminal half of synaptotagmin [53]. Rabphilin, like Rab3, is selectively localized on synaptic vesicles [54]. In mice which lack the Rab3a gene, it is virtually absent from those nerve terminals which normally primarily express the Rab3a isoform of Rab3, suggesting that Rab3 mediates the recruitment of rabphilin to the vesicles [52,54]. This observation is consistent with the absence of rabphilin from purified brain clathrin-coated vesicles (M. Arribas and P. De Camilli, unpublished observations), because Rab3a was previously shown to dissociate from the vesicle membrane prior to endocytosis (40). An attractive possibility is that rabphilin may cooperate with synaptotagmin in controlling the assembly of the SNARE complex.

3. Endocytosis

Strong evidence implicates the participation of clathrin mediated endocytosis in the recycling of synaptic vesicle membranes after neurotransmitter release (Fig. 1). Clathrin is present at very high concentrations in the central nervous system where it is primarily concentrated in nerve terminals [55,56]. Likewise, the plasmalemma clathrin adaptor, AP2, is concentrated primarily in nerve terminals (M. Robinson and P. De Camilli, unpublished observations). Clathrin-coated pits and vesicles are extremely abundant in nerve endings and their number is increased during the recovery phase after nerve terminal stimulation [57]. The membrane protein cargo of nerve terminal clathrin-coated vesicles is very similar to the membrane protein composition of synaptic vesicles [55,58].

It is widely accepted that clathrin-coated vesicles and early endosomal intermediates play an essential role in the recycling of synaptic vesicle membranes after massive stimulation of neurotransmitter release when the normal balance between exocytosis and endocytosis is impaired and a large fraction of the synaptic vesicle membrane becomes integrated into the plasmalemma. However, it is still debated whether another, more direct, vesicle recycling pathway may coexist and predominate under moderate conditions of stimulation [5,59]. For example, synaptic vesicles may reform by an immediate reclosure of the

Fig. 3. Electron micrograph demonstrating dynamin rings around the tubular stalk of a clathrin coated vesicle. The figure shows a detail from a lysed nerve terminal incubated with brain cytosol, ATP and GTPγS. The origin of the tubule is not visible in the section. Note a second clathrin bud originating from the same tubule (micrograph from Takei and De Camilli [56] reprinted with permission from *Nature*).

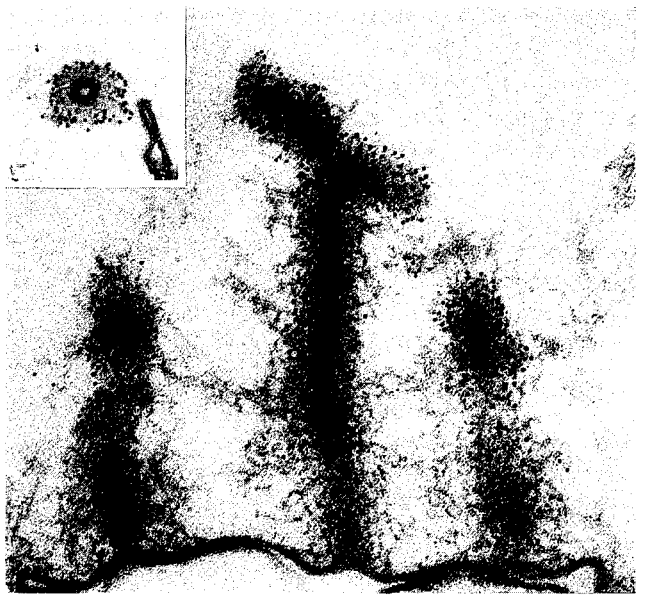

Fig. 4. Immunogold localization of dynamin on fragments of hypotonically-lysed nerve terminals. Three tubular invagination of the plasmalemma are heavily decorated by dynamin immunogold. The inset shows a cross-section of a similar invagination (from [56], reprinted with permission).

exocytotic opening ('kiss-and-run' hypothesis [59]), or directly from clathrin-coated vesicles by a simple uncoating reaction [5].

3.1. Dynamin

Irrespective of whether multiple recycling pathways coexist, a protein which plays a key role in endocytosis of synaptic vesicle membranes is the GTPase dynamin. This GTPase was first identified as a protein which binds microtubules in a nucleotide-dependent way. When mixed with purified microtubules, it polymerizes to form rings and stacks of rings around the tubules [60,61]. Subsequently, Drosophila dynamin was found to be identical to the protein encoded by the *shibire* gene, whose temperature sensitive mutations lead to a block in synaptic transmission [62–64]. At the restrictive temperature, synaptic vesicles still fuse with the plasmalemma, but their endocytosis is blocked at the stage of invaginated pits, indicating a selective impairment of the fission reaction. The neck of these pits is surrounded by an electron-dense ring similar to the rings formed by purified dynamin around microtubules [62]. Transfection of fibroblastic mammalian cells with dynamin mutants defective in guanylnucleotide binding produced a block of clathrin-mediated endocytosis, suggesting a general role of dynamin in the fission of clathrin-coated vesicles [65–67].

Additional information on the function and the mechanism of action of dynamin was provided by cell-free studies on brain membranes [56]. Hypotonically-lysed synaptosomes were incubated with brain cytosol, ATP, and GTPγS to reconstitute vesicular transport, but to arrest it at stages which require GTP hydrolysis. Electron microscopy of these preparations demonstrated the presence of unique membrane intermediates which had never been described previously (Fig. 3). They were tubular membrane invaginations (25–30 nm in diameter), surrounded by regularly spaced, slightly oblique, rings stacked on top of each other to suggest a spiral. Generally (see below), these structures represented invaginations of the plasmalemma and terminated in a clathrin-coated pit. These rings were very similar to the rings formed by purified dynamin around purified microtubules and to the rings observed in the nerve terminals of *shibire* flies at the neck of invaginated pits. Accordingly, they were found to be intensely positive for dynamin immunoreactivity (Fig. 4), while only scattered immunoreactivity was present on the clathrin coat [56].

In a separate study, it was found that purified dynamin alone can self-assemble into rings and spiral-like stacks of rings with an internal diameter of about 25–30 nm [68]. These findings lead to the following model. The clathrin coat is responsible for generating and stabilizing a membrane bud and for recruiting and concentrating selected membrane components to the bud. However, at least one additional factor, dynamin, is required to drive vesicle fission once a narrow stalk is formed [56,68]. It is proposed that elementary dynamin units (most likely tetramers [68]) oligomerize into an open ring (or lock–washer structure) at the neck of invaginated pits, and that GTP hydrolysis correlates with a conformational change of the ring which leads to the fission reaction. Most likely only one, or very few, ring(s) are formed in situ. In contrast, stabilization of the ring by GTPγS allows progressive addition of dynamin units to its open ends and, as a result, to a dynamin spiral and to the elongation of the vesicle neck (Fig. 5) [56].

In principle, the function of dynamin should not be linked to the function of clathrin. For example, one could hypothesize that rapid formation of a dynamin ring at the exocytotic opening of a synaptic vesicle could generate a new vesicle without the participation of clathrin. Such a scenario would fit very well with the 'kiss-and-run' hypothesis. However, in GTPγS treated nerve terminals the great majority of dynamin-coated tubules were capped by a clathrin-coated bud [56], making this possibility unlikely. In addition, as discussed below, formation of a dynamin ring may be closely coupled to the presence of a clathrin coat.

If dynamin is needed for the fission of clathrin-coated vesicles, one may anticipate that dynamin or dynamin-like molecules may function at other sites where generation of vesicular carriers is mediated by clathrin. The function of clathrin is well documented in the vesicle transport pathway from the TGN to lysosomal precursors [69,70]. In fact, a dynamin homologue, VPS1, has been shown to be required in yeast for transport from the TGN to the vacuole (the yeast equivalent of the lysosome) [71,72]. There is no evidence, so far, that dynamin homologues may be involved at vesicle budding reactions which use coats different from clathrin such as COPI [11] and COPII [73]. Accordingly, budding reactions mediated by these coats are not blocked by GTPγS [11,73]. COPII is involved in transport from the ER to the Golgi complex [73] and COPI in transport steps within the Golgi complex and/or between the ER with the Golgi complex [11,74]. Fission of COPI- and COPII-coated vesicles may simply be driven by 'closure' of the coat at the vesicle stalk.

3.2. Dynamin interacting proteins

An interesting open question is what mediates the selective assembly of dynamin at the stalks of clathrin-coated vesicles. The property to form rings or spiral-like stalks of rings is an intrinsic property of dynamin [68]. Formation of these rings around microtubules, the observation which led to the discov-

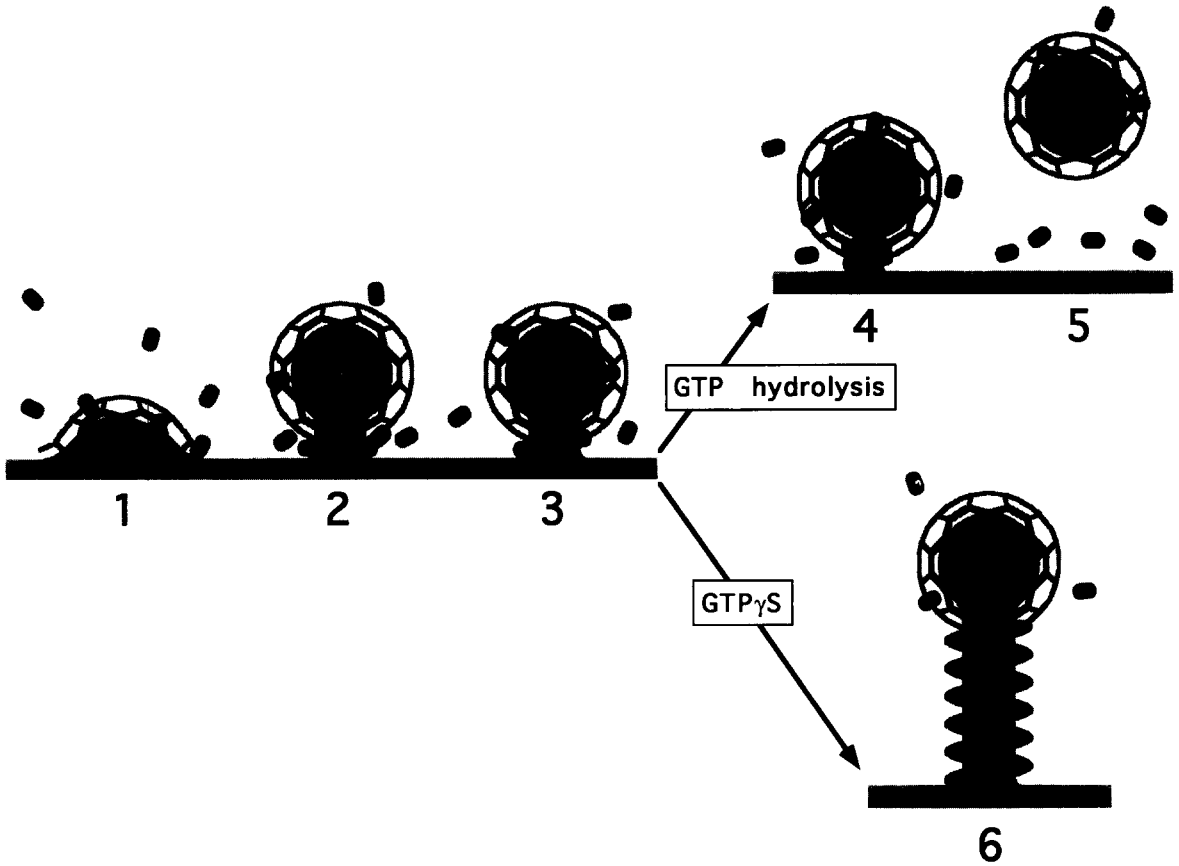

Fig. 5. Diagram illustrating the proposed mechanism of action of dynamin in endocytosis. Dynamin (most likely dynamin tetramers [68]) is concentrated at the cell periphery via interactions with proteins of the peripheral cytomatrix (1). Dynamin is further concentrated in proximity of clathrin coats via reversible interactions with components of the coat (1 and 2). Clathrin coat rearrangement drives vesicle invagination until a narrow stalk is generated. The high local dynamin concentration triggers formation of a ring around the stalk (3). A conformational change of the ring, which correlates with GTP hydrolysis (4) leads to vesicle fission and ring disassembly (5). In the presence of GTPγS the ring is stabilized and multiple ring pile up to generate a narrow tubule surrounded by a dynamin coat (6) (from [89], reprinted with permission).

ery of dynamin, may be explained both by the diameter (approximately the same as the diameter of the stalk of invaginated clathrin-coated vesicles) and by the repetitive unit structure of microtubules which may represent optimal templates for dynamin polymerization. Genetic [62–64] and electron microscopic studies [56], however, indicate that the site of action of dynamin is the stalk of an invaginated clathrin-coated vesicle, and, therefore, that the site at which dynamin polymerizes in situ is tightly controlled. One can draw a parallel with clathrin which can form empty baskets by itself, although, in situ, it forms coats only at very precise sites. Clathrin adaptor proteins (adaptins) have a key role in controlling this assembly [75,76]. Identifying the molecules which interact with dynamin and which control the site of its assembly represents a priority in the field.

Dynamin, the collective name for three similar mammalian isoforms which include the neuron specific isoform dynamin I, contains several domains which have been recognized as important in protein–protein interactions. A domain comprised between the first and the second GTP-binding element may be involved in dynamin self-assembly [78]. In addition, dynamin contains a pleckstrin homology (PH) domain, a proline-rich tail and a region between these two domain with the potential of forming coil-coiled structures [77,78]. The PH domain and the proline-rich tail are only present in the membranes of the dynamin family which participate in budding from the cell surface, suggesting that these domains contain targeting information, but are not involved in the mechanism of action of dynamin.

Several recent studies have focused on the interactions established by dynamin's proline-rich tail. This region is phosphorylated in vivo and in vitro by protein kinase C and is dephosphorylated by the Ca^{2+}-dependent phosphatase calcineurin [79–81]. Stimulation of neurotransmitter release by nerve terminal depolarization leads to a rapid dephosphorylation of dynamin which correlates with a decrease in its GTPase activity and with an increased pool of dynamin recovered in particulate fractions [79,80]. Dephosphorylation may, therefore, facilitate assembly of the dynamin ring.

The proline-rich domain of dynamin contains binding sites for microtubules and for acidic phospholipids [77,80]. Most important, dynamin was found to interact, via this domain, with a variety of SH3 containing proteins including Grb2, the p85 subunit of PI-3 kinase and phospholipase Cg [82–84]. The significance of these interactions is unclear because none of these proteins was shown to be concentrated in nerve terminals. It was found, however, that a major SH3 domain containing protein concentrated in nerve terminals, amphiphysin [85,86],

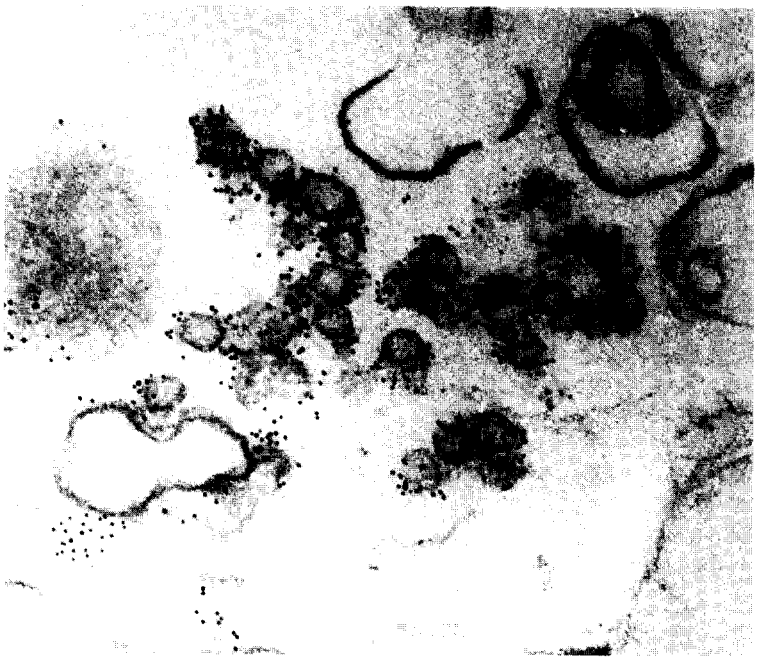

Fig. 6. Immunogold localization of synapsin I on fragments of hypotonically lysed nerve terminals. A cluster of synaptic vesicle heavily decorated by immunogold is visible. Synapsin I immunoreactivity is selectively associated with synaptic vesicles (micrograph from Navone and De Camilli).

binds dynamin with high selectivity and may interact in situ with dynamin [87]. Further support for a physiological significance of the interaction of dynamin with amphiphysin is the primary sequence homology of amphiphysin to two yeast proteins, RVS161 (allelic to END6) and RVS167 [86], whose disruptions produce endocytic defects (H. Riezman, personal communication). Amphiphysin and dynamin were also shown to interact with the plasmalemmal clathrin adaptin AP2 [88]. Based on these observations the following model for the action of dynamin has been proposed (Fig. 5) [89].

Dynamin is concentrated in nerve terminals via interactions with components of the nerve terminal cytomatrix. Binding of the proline-rich C-terminus of dynamin to SH3 domain-containing proteins of the submembranous cytoskeleton may participate in these interactions. Dynamin is then further concentrated at clathrin coats via its binding to AP2. It may also bind SH3 containing adaptor proteins which are recruited by membrane proteins at coated pits [90]. The resulting high concentration of dynamin in proximity to clathrin coats facilitates ring formation once an appropriate template, the narrow stalk of the coated pit, has been generated. Since PH domains were found to bind PIP2 [91], it is tempting to speculate that the interaction of the PH domain of dynamin with inositol-phospholipids may play some role in the assembly of dynamin at the vesicle neck. Next, a GTP hydrolysis-dependent conformational change of the dynamin ring leads to vesicle fission. Dynamin oligomerization was shown to enhance its GTPase activity [92]. It is, therefore, possible that ring formation may correlate with rapid hydrolysis and a catastrophic disassembly of the ring.

A protein of 145 kDa (p145) may function in close relationship with dynamin, although the two proteins do not directly interact. p145 was identified as a major Grb2 binding protein in brain [83]. Overlay experiments, as well as affinity chromatography studies, demonstrated that the three major brain proteins which bind Grb2 via its SH3 domains are dynamin, synapsin I and p145. Since dynamin and synapsin I participate in synaptic vesicle recycling, it was speculated that p145 as well may have a role in synaptic function [83,93]. Characterization of the protein demonstrated that p145, like synapsin I and dynamin, is concentrated in nerve terminals where its subcellular localization resembles the localization of dynamin. Furthermore, p145 is dephosphorylated in parallel with dynamin after nerve terminal stimulation [93]. It is expected that the further characterization of this protein may provide some fresh insights into mechanisms of exo–endocytosis.

4. Budding from endosomes

Endocytosis from the plasmalemma is only one of the two budding steps involved in the recycling of synaptic vesicle membranes. A second budding step (Fig. 1) is synaptic vesicle formation from endosomes. This budding reaction may also be the process through which newly synthesized synaptic vesicle proteins are first assembled into synaptic vesicles [94]. This reaction must ensure the generation of a vesicular carrier of well-defined size and with a precise cargo of membrane proteins. Identification of the mechanisms involved in this process is crucial to elucidate the biogenesis of a synaptic vesicles. Preliminary experiments suggest that the coat involved in budding from endosomes is, again, a clathrin coat and that, accordingly, fission of these vesicle requires dynamin (K. Takei and P. De Camilli, unpublished observations). If these results are confirmed, it will be important to define whether different isoforms of dynamin and of clathrin adaptors are used for vesicle budding from the plasmalemma and from endosomes.

5. Clustering at the synapse

Newly formed vesicles must be made competent for docking

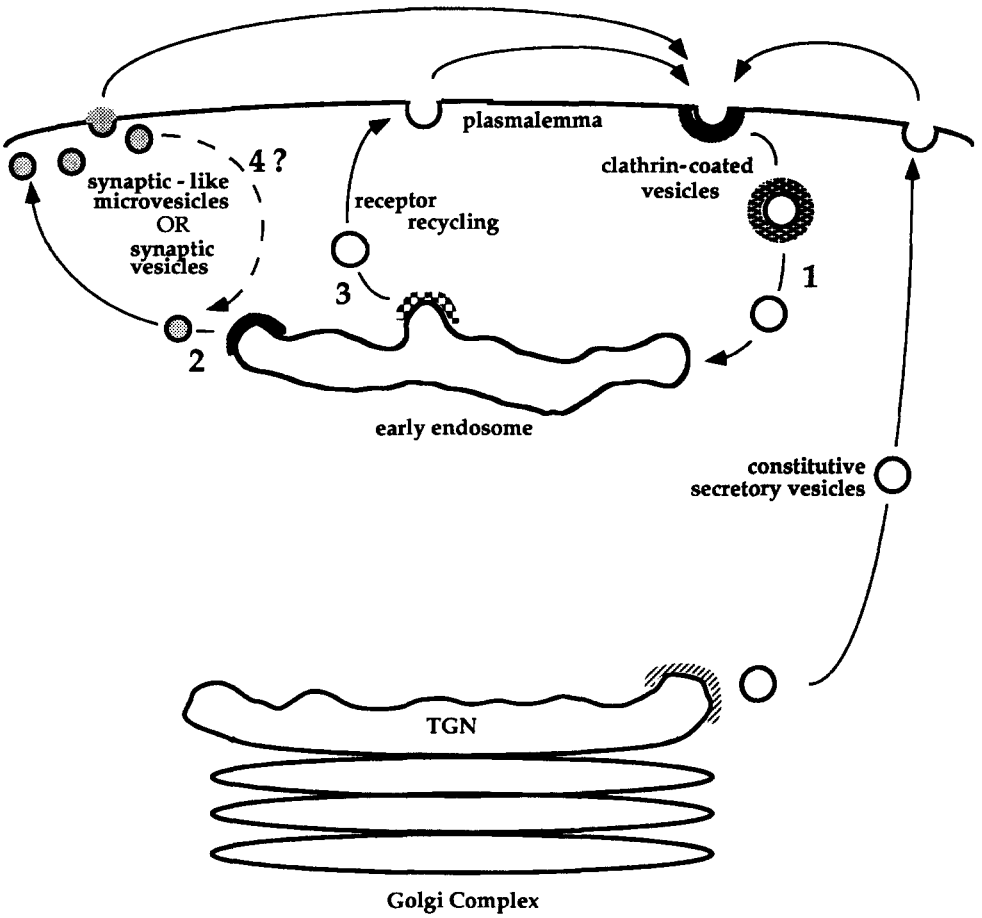

Fig. 7. Schematic diagram illustrating the putative life-cycle of synaptic-like microvesicles which is thought to closely reflect the life-cycle of neuronal synaptic vesicles. The model is primarily based on studies of synaptophysin. After exocytosis, membranes of synaptic-like microvesicles are thought to be internalized via the same clathrin-coated vesicular carriers which recycle plasmalemmal receptors (transferrin receptors, LDL receptors) to early endosomes (1). Synaptic-like microvesicles are re-generated by budding from endosomes when synaptic vesicle proteins are sorted away from recycling receptors (2) which shuttle back to the plasma membrane in distinct vesicular carriers (3). The possible co-existence of a direct recycling pathway (4) which by-pass early endosomes cannot be excluded. Newly synthesized synaptic vesicle proteins are delivered to the cell surface via the constitutive pathway (from [9], reprinted with permission).

and fusion via the recruitment of peripheral proteins (for example Rab3 and rabphilin, see above) and must be incorporated into the reserve pool of synaptic vesicles at synapses. Studies of synaptogenesis in primary cultures of hippocampal neurons have suggested that the ontogenesis of presynaptic clusters is the results of at least two distinct processes [95]. The first process is the spontaneous aggregation of synaptic vesicles to form small clusters which can be observed before synaptogenesis in axons of isolated neurons. These clusters move in bulk along the axonal shaft and in and out of small filopodia [95]. Already at this stage synaptic vesicles undergo exocytosis (and recycling), but exocytosis is not confined to any specialized site [95,96]. The second process, which is dependent upon the contact of the axon with target neurons, is the recruitment at synapses of small clusters to form large vesicle aggregates [95].

A protein which is thought to participate in synaptic vesicle clustering is synapsin, a nerve terminal-specific phosphoprotein. Synapsin (the collective name for synapsin I and II) is selectively associated with the cytosolic surface of synaptic vesicles (Fig. 6) [97] and is not found on brain clathrin-coated vesicles [55], suggesting that it dissociates from the vesicle membrane prior to endocytosis. Synapsin also binds actin and may cluster vesicles by linking them to an actin-based cytomatrix (Fig. 6) [97]. Recent studies have demonstrated the essential role of synapsin in the recruitment of a large vesicle reserve pool at the synapse, but not in the basic process of neurotransmitter release nor in the formation of a synaptic junction. Mice in which both the synapsin I and synapsin II genes have been deleted by homologous recombination can form synaptic contacts which are functionally very similar to normal synaptic contacts under conditions of low frequency stimulation. However, synaptic transmission rapidly fails during sustained stimulation [98]. Similar results were obtained after acute disruption of synapsin function by antibody injection in the giant axons of the lamprey [99]. It would appear that the main function of synapsin is to help build up a sufficient reserve pool of synaptic vesicles to cope with burst of intense synaptic activity.

6. Relationship of synaptic vesicle recycling to membrane recycling in all cells

All the major steps of synaptic vesicle recycling represent a

special case of vesicle transport reactions which occur at various stations of the secretory and endocytic pathways. In addition, as anticipated by the pioneer studies on nerve terminal membrane recycling carried out in the early Seventies [57,100], the synaptic vesicle recycling pathway is closely related to membrane recycling which takes place at the surface of all cells. The molecular information which has been acquired in recent years on synaptic vesicle recycling has further emphasized this similarity. For example, homologues of synaptic vesicle proteins have been identified in membranes of the receptor mediated recycling pathway in non-neuronal cells [101–103], and at least some synaptic vesicle proteins are targeted to this pathway when expressed in non-neuronal cells by cDNA transfection [104,105]. As in the case of synaptic vesicles, the exocytosis of endosome-derived vesicular carriers in non- neuronal cells can be inhibited by the light chain of tetanus toxin, which cleaves proteins of the synaptobrevin family [102]. Although exocytosis of endosome-derived vesicles is primarily a constitutive event, there is evidence that even this process can undergo some regulation by intracellular Ca^{2+} [106]. Dynamin, first identified as a neuron-specific protein, has now been shown to play a general role in assisting endocytosis mediated by clathrin [65–67]. The presence of Rab5 on synaptic vesicle membranes suggests close relationships between early endosomes of nerve endings and all early endosomes [107,108].

On the other hand, special features of synaptic vesicle recycling have also emerged, in addition to those related to the unique topological and temporal regulation of their exocytosis. For example, nerve terminal endosomes exclude a variety of proteins which normally cycle through early endosomes and which in neurons are selectively retained in dendrites [104,105]. In contrast to classical early endosomes, including dendritic endosomes, nerve terminal endosomes do not undergo tubulation in response to brefeldin A treatment [109]. An interesting avenue for future research is the further elucidation of the mechanisms of recycling which are unique to synaptic vesicles and of the evolution of these mechanisms from mechanisms with housekeeping function in all cells.

Endocrine cells are helping to trace this evolution [9]. A subpopulation of endocrine cells, which include pituitary cells, cells of pancreatic islets, chromaffin cells, C cells of the thyroid, parathyroid cells, and cells of the diffuse neuroendocrine system of the gut, have many functional and biochemical similarities to neurons. As a rule, most neuronal proteins which have a very restricted distribution outside the brain, are also expressed by at least some members of this family of endocrine cells. Among such proteins are all major synaptic vesicle proteins, which in endocrine cells are concentrated on 'synaptic-like microvesicles' virtually identical to bona fide neuronal synaptic vesicles: they have similar membrane composition, same size and same sedimentation characteristics [9,104,105,110]. Like neuronal synaptic vesicles they exclude recycling receptors (LDL receptors and transferrin receptors) and accumulate non-peptide neurotransmitter molecules. In pancreatic β-cells they store and secrete GABA, while in chromaffin cells they store and secrete acetylcholine [9]. Their precise function is unclear, but one of their functions may be to participate in paracrine signaling [9].

In some way, these endocrine cells can be seen as a neuron without an axon. Under certain experimental conditions, however (nerve growth factor in the case of chromaffin cells) these cells can grow neurite-like extensions which have many characteristics of axons and which accumulate synaptic-like microvesicles [9].

Studies of synaptic-like microvesicles have already been useful in elucidating the relationship between endosome-derived vesicular carriers of all cells and synaptic vesicles. As depicted in Fig. 7, studies of pancreatic β-cells and chromaffin cells have suggested that synaptic vesicles originate from, and recycle through, early endosomes where they are formed by a specialized budding mechanism [9,94,104,105,110]. Based on our preliminary observations (see above), we suggest that synaptic vesicle budding from endosomes may be mediated by clathrin coats.

7. Concluding remarks

The convergence of studies on synaptic vesicle traffic with studies on vesicular traffic in a variety of other systems, including unicellular organisms, has already produced a remarkable advancement in our understanding of fundamental mechanisms involved in membrane recognition, docking, fusion. Progress is currently being made in the elucidation of the mechanisms of vesicle endocytosis and budding. Due to the unique advantages offered by synaptic vesicles for the analysis of vesicular transport, studies on these organelles will continue to provide major insights into this rapidly developing field. As the proteins which play key functions in each step of the vesicle cycle are identified, the next challenges in the field of neurosecretion will be to obtain a thorough understanding of how each of the steps is regulated by extracellular and intracellular signals and of the mechanisms which lead to the ontogenesis of the presynaptic compartment.

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References

[1] De Camilli, P. and Jahn, R. (1990) Annu. Rev. Physiol. 52, 625–685.
[2] Katz, B. and Miledi, R. (1965) Proc. R. Soc. Lond. B 161, 483–495.
[3] Stevens, C.F. and Tsujimoto, T. (1995) Proc. Natl. Acad. Sci. USA 92, 846–849.
[4] Malgaroli, A. (1994) Sem. Cell Biol. 5, 231–241.
[5] McPherson, P. and De Camilli, P. (1994) Sem. Neurosci. 6, 137–147.
[6] Betz, W.J. and Bewick, G.S. (1992) Science 255, 200–203.
[7] Ryan, T.A. and Smith, S.J. (1995) Neuron 14, 983–989.
[8] Bennett, M.K. and Scheller, R.H. (1994) Annu. Rev. Biochem. 63, 63–100.
[9] Thomas, Reetz, A. and De Camilli, P. (1994) FASEB J. 8, 209–216.
[10] Südhof, T.C., De Camilli, P., Niemann, H. and Jahn, R. (1993) Cell 75, 1–4.
[11] Rothman, J.E. (1994) Nature 372, 55–63.
[12] Mundigl, O. and De Camilli, P. (1994) Curr. Opin. Cell Biol. 6, 561–567.
[13] Huttner, W.B., Schiebler, W., Greengard, P. and De Camilli, P. (1983) J. Cell Biol. 96, 1374–1388.
[14] De Camilli, P., Harris, S.M., Huttner, W.B. and Greengard, P. (1983) J. Cell Biol. 96, 1355–1373.
[15] Jahn, R. and Südhof, T.C. (1994) Annu. Rev. Neurosci. 17, 219–246.
[16] Ferro-Novick, S and Jahn, R. (1995) Nature 370, 191–193.

[17] Burton, J., Roberts, D., Montaldi, M., Novick, P. and De Camilli, P. (1993) Nature 361,464–467.
[18] Schwarz, T.L. (1994) Curr. Opin. Neurobiol. 4, 633–639.
[19] Schulze, K.L., Broadie, K., Perin, M.S. and Bellen, H.J. (1995) Cell 80, 311–320.
[20] Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J.E. (1993) Nature 362, 318–324.
[21] Söllner, T., Bennett, M.K., Whiteheart, S.W., Scheller, R.H. and Rothman, J.E. (1993) Cell 75, 409–418.
[22] Scheller, R.H. (1995) Neuron 14, 893–897.
[23] Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B.R. and Montecucco, C. (1992) Nature 359, 832–835.
[24] Blasi, J., Chapman, E.R., Link, E., Binz, T., Yamasaki, S., De Camilli, P., Südhof, T.C., Niemann, H. and Jahn, R. (1993) Nature 365, 160–163.
[25] Blasi, J., Chapman, E.R., Yamasaki, S., Binz, T., Niemann, H. and Jahn, R. (1993) EMBO J. 12, 4821–4828.
[26] Kutay, U., Ahnert-Hilger, G., Hartmann, E., Wiedenmann, B. and Rappoport, T.A. (1995) EMBO J. 14, 217–223.
[27] Jantti, J., Keränen, S., Toikkanen, J., Kuismanen, E., Ehnholm, C., Soderlund, T. and Olkkönen, V.M. (1994) J. Cell Sci. 107, 3623–3633.
[28] Garcia, E.P., McPherson, P.S., Takei, K., Chilcote, T.J. and De Camilli, P. (1995) J. Cell Biol. 129, 105–120.
[29] Brose, N., Petrenko, A.G., Südhof, T.C. and Jahn, R. (1992) Science 256, 1021–1025.
[30] Geppert, M., Goda, Y., Hammer, R.E., Li, C., Rosahl, T.W., Stevens, C.F. and Südhof, T.C. (1994) Cell 79, 717–727.
[31] Littleton, J.T. and Bellen, H.J. (1995) Trends Neurosci. 18, 177–183.
[32] Li, C., Ullrich, B., Zhang, J.Z., Anderson, R.G.W., Brose, N. and Sudhof, T.C. (1995) Nature 375, 594–599.
[33] Edelmann, L., Hanson, P.I., Chapman, E.R. and Jahn, R. (1995) EMBO J. 14, 224–231.
[34] Calakos, N. and Scheller, R.H. (1994) J. Biol. Chem. 269, 24534–24537.
[35] Garcia, E.P., Gatti, E., Butler, M. Burton, J. and De Camilli, P. (1994) Proc. Natl. Acad. Sci. USA 91, 2003–2007.
[36] Hata, Y., Slaughter, C.A. and Südhof, T.C. (1993) Nature 366, 347–351.
[37] Pevsner, J., Hsu, S.C. and Scheller, R.H. (1994) Proc. Natl. Acad. Sci., USA 91, 1445–1449.
[38] Aalto, M.K., Ronne, H. and Keranen, S. (1993) EMBO J. 12, 4095–4104.
[39] Pevsner, J., Hsu, S.C., Braun, J.E., Calakos, N., Ting, A.E., Bennett, M.K. and Scheller, R.H. (1994) Neuron 13, 353–361.
[40] Fischer v. Mollard, G., Südhof, T.C. and Jahn, R. (1991) Nature 349, 79–81.
[41] Ferro-Novick, S. and Novick, P. (1993) Annu. Rev. Cell Biol. 9, 575–599.
[42] Brennwald, P., Kearns, B., Champion, K., Keränen, S., Bankaitis, V. and Novick, P. (1994) Cell 79, 245–258.
[43] Lian, J.P., Stone, S., Jiang, Y., Lyons, P. and Ferro-Novick, S. (1994) Nature 372, 698–701.
[44] Sogaard, M., Tani, K., Ye, R.R., Geromanos, S., Tempst, P., Kirchausen, T., Rothman, J.E. and Söllner, T. (1994) Cell 78, 937–948.
[45] Simons, K. and Zerial, M. (1993) Neuron 11, 789–799.
[46] Novick, P. and Garrett, M.D. (1994) Nature 369, 18–19.
[47] Nishimura, N., Nakamura, H., Takai, Y. and Sano, K. (1994) J. Biol. Chem. 269, 14191–14198.
[48] Soldati, T., Shapiro, A.D., Svejstrub, A.B. and Pfeffer, S.R. (1994) Nature 369, 76–78.
[49] Ullrich, O., Horiuchi, H., Bucci, C. and Zerial, M. (1994) Nature 368, 157–160.
[50] Burton, J. Burns, M.E., Augustine, G.J. and De Camilli, P. (1994) EMBO J. 13, 5547–5558.
[51] Salminen, A. and Novick, P.J. (1987) Cell 49, 527–538.
[52] Geppert, M., Bolshakov, V.Y., Siegelbaum, S.A., Takei, K., De Camilli, P., Hammer, R.E. and Südhof, T.C. (1994) Nature 369, 493–497.
[53] Yamaguchi, T., Shirataki, H., Kishida, S., Miyazaki, M., Nishikawa, J., Wada, K., Numata, S., Kaibuchi, K. and Takai, Y. (1993) J. Biol. Chem. 268, 27164–27170.
[54] Li, C., Takei, K., Geppert, M., Daniell, L., Chapman, E., De Camilli, P. and Südhof, T. (1994) Neuron 13, 1–20.
[55] Maycox, P.R., Link E., Reetz A., Morris S.A. and Jahn R.J. (1992) J. Cell Biol. 118, 1379–1388.
[56] Takei, K., McPherson, P., Schmid, S.L. and De Camilli, P. (1995) Nature 374, 186–190.
[57] Heuser, J.E. and Reese T.S. (1973) J. Cell Biol. 57. 315–344.
[58] Pfeffer, S.R. and Kelly, R.B. (1985) Cell 40, 949–957.
[59] Fesce, R., Grohovaz, F., Valtorta, F. and Meldolesi, J. (1994) Trends Cell Biol. 4, 1–4.
[60] Shpetner, H.S. and Vallee, R.B. (1989) Cell 59, 421–432.
[61] Obar, R.A., Collins, C.A., Hammarback, J.A., Shpetner. H.S. and Vallee, R.B. (1990) Nature 347, 256–261.
[62] Koenig, J.H. and Ikeda, K.J. (1989) J. Neurosci. 9, 3844–3860.
[63] Chen, M.S., Obar, R.A., Schroeder, C.C., Austin, T.W., Poodry, C.A., Wadsworth, S.C. and Vallee, R.B. (1991) Nature 351, 583–586.
[64] van der Bliek, A.M. and Meyerowitz, E.M. (1991) Nature 351, 411–414.
[65] Herskovits, J.S., Burgess, C.C., Obar, R.A. and Vallee, R.B. (1993) J. Cell Biol. 22, 565–578.
[66] van der Bliek, A.M., Redelmeier, T.E., Damke, H., Tisdale, E.J., Meyerowitz, E.M. and Schmid, S.L. (1993) J. Cell Biol. 122, 553–563.
[67] Damke, H., Baba, T., Warnock, D.E. and Schmid, S.L. (1994) J. Cell Biol. 127, 915–934.
[68] Hinshaw, J.E. and Schmid, S.L. (1995) Nature 374, 190–192.
[69] Farquhar, M.G. (1983) Fed. Proc. 42, 2407–2413.
[70] Seeger, M. and Payne, G.S. (1992) EMBO J. 11, 2811–2818.
[71] Wilsbach, K. and Payne, G.S. (1993) EMBO J. 12, 3049–3059.
[72] Ekena, K. and Stevens, T.H. (1995) Mol. Cell. Biol. 5, 1671–1678.
[73] Barlowe, C., Orci, L., Yeung, T., Hosobuchi. M., Hamamoto, S., Salama, N., Rexach, M.F., Ravazzola, M., Amherdt, M. and Schekman, R. (1994) Cell 77, 895–907.
[74] Letourneur, F., Gaynor, E.C., Hennecke, S., Demolliere, C., Duden, R. and Emr, S.D., Riezman, H. and Cosson, P. (1994) Cell 79, 1199–207.
[75] Pearse, B.M. and Robinson, M.S. (1990) Annu. Rev. Cell Biol. 6, 151–171.
[76] Kirchhausen, T. (1993) Curr. Opin. Struct. Biol. 3, 182–188.
[77] Vallee, R.B. and Okamoto, P.M. (1995) Trends Cell Biol. 5, 43–47.
[78] Robinson, P.J., Liu, J.P., Powell, K.A., Fykse, E.M. and Südhof, T.C. (1994) Trends Neurosci. 17, 348–353.
[79] Robinson, P.J., Sontag, J.M., Liu, J.P. and Fykse, E.M. Slaughter, C., McMahon, H. and Südhof, T.C. (1993) Nature 365, 163–166.
[80] Liu, J.P., Powell, K.A., Südhof, T.C. and Robinson, P.J. (1994) J Biol. Chem. 269, 21043–21050.
[81] Liu, J.P., Sim, A.T. and Robinson, P.J. (1994) Science 265, 970–973.
[82] Gout, I., Dhand, R., Hiles, I.D., Fry, M.J., Panayotou, G., Das, P., Truong, O., Totty, N.F., Hsuan, J., Booker, G.W., Campbell, I.D. and Waterfield, M.D. (1993) Cell 75, 25–36.
[83] McPherson, P.S., Czernik, A.J., Chilcote, T.J., Onofri, F., Benfenati, F., Greengard, P., Schlessinger, J. and De Camilli, P. (1994) Proc. Natl. Acad. Sci. USA 91, 6486–6490.
[84] Herskovits, J.S., Shpetner, H.S., Burgess, C.C. and Vallee, R.B. (1993) Proc. Natl. Acad. Sci. USA 90, 11468–472.
[85] Lichte, B., Veh, R.W., Meyer, H.E. and Kilimann, M.W. (1992) EMBO J. 11, 2521–2524.
[86] David, C., Solimena, M. and De Camilli, P. (1994) FEBS Lett. 351, 73–79.
[87] David, C., McPherson, P.S., Cho, Y., Solimena, M. and De Camilli, P. (1994) Mol. Biol. Cell 5, 194a.
[88] Wang, L.H., Südhof, T.C. and Anderson, R.G.W. (1995) J. Biol. Chem. 270, 10079–10083.
[89] De Camilli, P., Takei, K. and McPherson, P. (1995) Curr. Opin. Neurobiol., in press.
[90] Pawson, T. (1995) Nature 373, 573–580.
[91] Harlan, J.E., Hajduk, P.J., Yoon, H.S. and Fesik, S.W. (1994) Nature 371, 168–171.

[92] Tuma, P.L. and Collin, C.A. (1994) J. Biol. Chem. 269, 30842–30847.
[93] McPherson, P.S., Takei, K., Schmid, S.L. and De Camilli, P. (1994) J. Biol. Chem. 269, 30132–30139.
[94] Regnier-Virouroux, A., Tooze, S.A. and Huttner, W.B. (1991) EMBO J. 10, 3589–35601.
[95] Kraszewski, K., Mundigl, O., Daniell, L., Verderio, C., Matteoli, M. and De Camilli, P. (1995) J. Neurosci. 15,4328–4342.
[96] Matteoli, M., Takei, K., Perin, M.S., Südhof, T.C. and De Camilli, P. (1992) J. Cell Biol. 117, 849–861.
[97] De Camilli, P, Benfenati, F., Valtorta, F. and Greengard, P. (1990) Annu. Rev. Cell Biol. 6, 433–460.
[98] Rosahl, T.W., Spillane, D., Missler, M., Herz, J., Selig, D.K., Wolff, J.R., Hammer, R.E., Malenka, R.C. and Südhof, T.C. (1995) Nature 375, 488–493.
[99] Pierobone, V.A., Shupliakov, O., Brodin, L., Hilfiker-Rothenfluh, S., Czernik, A.J. and Greengard, P. (1995) Nature 375, 493–497.
[100] Ceccarelli, B., Hurlbur, W.P. and Mauro, A. (1973) J. Cell Biol. 57, 499–524.
[101] McMahon, H., Ushkaryov, Y.A., Edelmann, L., Link, E., Binz, T., Niemann, H., Jahn, R. and Südhof, T.C. (1993) Nature 364, 346–349.
[102] Galli, T., Chilcote, T.J., Mundigl, O., Binz, T., Niemann, H. and De Camilli, P. (1994) J. Cell Biol. 125, 1015–1024.
[103] Chilcote, T., Galli, T., Mundigl, O., Takei, K., McPherson, P. and De Camilli, P. (1995) J. Cell Biol. 129, 219–231.
[104] Cameron P.L., Südhof T.C., Jahn R. and De Camilli P. (1991) J.Cell Biol. 115, 151–164.
[105] Linstedt, A.D. and Kelly, R.B. (1991) Neuron 7, 309–317.
[106] Girod, R., Popov, S., Alder, J., Zheng, J.Q., Lohof, A. and Poo, M.M. (1995) J. Neurosci. 15, 2826–2838.
[107] de Hoop, M.J., Huber, L.A., Stenmark, H., Williamson, E., Zerial, M., Parton, R.G. and Dotti, C.G. (1994) Neuron 13, 11–22.
[108] Fischer von Mollard, G., Stahl, B., Walch-Solimena, C., Takei, K., Daniels, L., Khoklatchev, A., De Camilli, P., Südhof, T.C. and Jahn, R. (1994) Eur. J. Cell Biol. 65, 319–326.
[109] Mundigl, O., Matteoli, M., Daniell, L., Thomas-Reetz, A., Metcalf, A., Jahn, R. and De Camilli, P. (1993) J. Cell Biol. 122, 1207–1221.
[110] Bauerfeind, R., Regnier-Vigouroux, A., Flatmark, T. and Huttner, W.B. (1993) Neuron 11, 105–121.