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Molecular Mechanisms in Synaptic Vesicle Endocytosis and Recycling

Review

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Neurons exchange information at synapses with topological precision and speed by capitalizing on the general property of all cells to recycle vesicles at the cell periphery. "Fast" neurotransmitters are stored in synaptic vesicles that release their content into the synaptic cleft by exocytosis. The selective clustering and docking of these vesicles at the presynaptic plasmalemma ensures spatial specificity, and the short delay between nerve terminal depolarization and their exocytosis ensures speed. After fusion, synaptic vesicle membranes are rapidly recycled by endocytosis and reused for the generation of new synaptic vesicles. Given the relationship of this vesicle recycling pathway to the housekeeping recycling pathway used by all cells, elucidation of the mechanisms of synaptic vesicle reformation broadens our knowledge of the general mechanisms of exocytosis–endocytosis.

Time Course of Recycling

The recent development of endocytic probes to label synaptic vesicles membranes in living neurons (Kraszewski et al., 1995; Betz and Wu, 1995) has helped greatly in defining the dynamics of synaptic vesicle recycling in adult and developing axons. The styryl dye FM1-43, which binds with relatively fast "on" and "off" rates (seconds) to lipid bilayers, has permitted the determination of several parameters of synaptic vesicle recycling at motor end plates and at synapses of cultured CNS neurons (Betz and Wu, 1995; Ryan and Smith, 1995). The endocytic reuptake of synaptic vesicle membranes after a short exocytic burst was estimated to occur with a $t_{1/2}$ of about 20 s (Ryan and Smith, 1995), although much shorter half-times (around 1 to a few seconds) have been estimated by capacitance studies in retinal ribbon synapses (von Gersdorff and Matthews, 1994). After endocytosis, the minimum time required for a newly internalized vesicle to become available again for exocytosis is between 15 s and 45 s. Thus, the whole cycle of a synaptic vesicle can be completed in around 1 min or less (Ryan and Smith, 1995; Betz and Wu, 1995). During periods of intense stimulation, the bulk of synaptic vesicles present in nerve terminals of a synapse in the central nervous system can be released in less than 1 min ($t_{1/2}$ of synaptic vesicle depletion at 10 Hz stimulation is about 20 s) (Ryan and Smith, 1995). Considering that synaptic vesicle proteins have half-lives on the order of days, each synaptic vesicle may recycle hundreds and probably thousands of times.

Clathrin Coats Versus "Kiss and Run"

The mechanisms by which synaptic vesicle membranes are internalized and recycled remain a matter of debate. Several lines of evidence implicate clathrin coats in this process. The number of clathrin-coated vesicles and endosome-like structures generally increase in the period of recovery after stimulation (Heuser and Reese, 1973). Both clathrin and clathrin accessory proteins are highly concentrated in nerve terminals, and synaptic vesicle proteins represent the main cargo of brain clathrin-coated vesicles (Maycox et al., 1992). Dynamin plays an essential role in synaptic vesicle reformation (Koenig and Ikeda, 1989), and the evidence discussed below demonstrates that clathrin and dynamin play complementary functions in endocytosis. In fact, as with the proteins involved in exocytosis, clathrin coats were first characterized in brain tissue because neurons are highly specialized for the recycling of synaptic vesicles.

Despite this evidence, a precise correlation between rate of exocytosis and number of either clathrin-coated vesicles or endosomal structures is not always observed. An alternative mechanism of synaptic vesicle reformation involving rapid closure of a transient exocytotic fusion pore—the "kiss and run" hypothesis—was proposed to occur in parallel with the clathrin-mediated pathway and to predominate under moderate conditions of stimulation (Fesce et al., 1994). This model has recently gained support from studies on the effects of the protein kinase inhibitor staurosporine on synaptic vesicle recycling. Stimulation of nerve terminals in the presence of staurosporine produces release of neurotransmitters but not of preinternalized FM1-43, consistent with the formation of a transient exocytotic opening that is insufficient to allow significant equilibration of the dye with the extracellular medium (Henkel and Betz, 1996). However, since FM1-43 dissociates from the neuronal surface with a $t_{1/2}$ of seconds (Ryan and Smith, 1995), this finding does not completely rule out a fast clathrin-mediated endocytic event. The following discussion will focus on the molecular mechanisms implicated in the clathrin dependent reformation of synaptic vesicles.

Generation of a Clathrin-Coated Pit

The basic constituents of clathrin coats, which function both in budding from the plasmalemma and from the *trans*-Golgi network (TGN), are the heavy and light chains of clathrin and the clathrin adaptor proteins. The same clathrin molecules that are present in the cytosol as three-legged oligomers, called triskelia, are used for both budding reactions. However, two distinct, yet similar adaptor complexes, called AP2 and AP1, are used at the cell surface and at the TGN, respectively. Nerve terminal clathrin-coated vesicles, which are cell surface–derived vesicles, contain AP2 adaptors. These are heterotetramers composed of two 100–115 kDa subunits (α and β) and two smaller subunits of 50 and 17 kDa, respectively. The complex has a brick-like structure, containing the main portions of the α and β subunits together with the two small subunits, and two appendages (ears) that correspond to the COOH-termini of the α and β subunits (Figure 1). Assembly of clathrin coats begins when AP2 adaptors, which are not present on

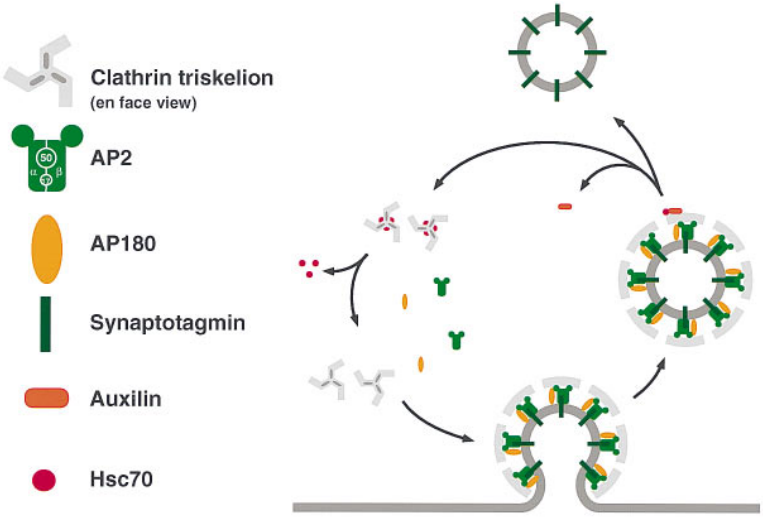

Figure 1. Diagram Illustrating Key Protein Components of Nerve Terminal Clathrin Coats and Their Putative Reciprocal Interactions

Dynamin is not indicated (see Figure 2).

synaptic vesicles, are recruited to the plasmalemma where they assemble into a lattice. The AP2 lattice then acts as a template for clathrin assembly in a hexagonal structure where the vertices are represented by the center of clathrin triskelia (Pley and Parham, 1993; Robinson, 1994).

Current models predict that AP2 recruitment to the plasmalemma is primarily mediated by its high affinity interaction with the cytoplasmic domains of membrane proteins. One major binding site on synaptic vesicle membranes, which may act as the "master" binding site for coat assembly, has been identified as the C2b domain of synaptotagmin I (Li et al., 1995a) (Figure 1). A key role of this protein in endocytosis is supported by genetic studies in Caenorhabditis elegans (Jorgensen et al., 1995) and antibody injection studies in the squid giant nerve terminal (Fukuda et al., 1995). C2b domains that bind AP2 are also present in synaptotagmin isoforms expressed in nonneuronal tissues, raising the possibility that synaptotagmin may play a general role in clathrin-mediated endocytosis (Li et al., 1995a). Additional AP2-binding sites have been identified in short amino acid stretches (endocytosis motifs) present in the cytoplasmic domains of several proteins that are internalized by clathrin coats (Ohno et al., 1995). These additional interactions are thought to trap proteins into clathrin-coated membrane patches, but may also play a role in coat formation or stabilization. Synaptotagmins, and other proteins that bind AP2 directly, may in turn recruit other proteins into the coated membrane patch via direct or indirect interactions. This possibility is consistent with the existence of detergent-resistant protein oligomers in synaptic vesicle membranes.

In addition to AP2, a brain-specific protein called AP180 (also referred to as AP3, F1-20, and NP185) coenriches with clathrin in nerve terminal clathrin-coated vesicle fractions. Like AP2, AP180 alone promotes assembly of clathrin cages in vitro. These cages have the same uniform small size that is characteristic of the majority of nerve terminal clathrin-coated vesicles. AP180 may therefore have a role in defining the unique and uniform size of these organelles (Ye et al., 1995). Another brain-specific protein that is enriched in clathrin-coated vesicles is auxilin (Lindner and Ungewickell, 1992). This protein, however, was recently shown to participate in the uncoating reaction after vesicle budding (see below and Figure 1) (Ungewickell et al., 1995).

Although clathrin triskelia can self-assemble into cages in vitro, de novo formation of clathrin coats on the plasmalemma of semiintact cells requires ATP hydrolysis and GTP. The subsequent bending of the coated membrane patch to form a dome-like structure, and then an invaginated pit with a narrow neck, occurs in ATP-depleted preparations (Schmid, 1993). This process is mediated by the progressive removal of clathrin triskelia that transforms the hexagonal clathrin lattice into a lattice composed of hexagons and pentagons.

A GTPase of the Arf family (Arf1) has been implicated in clathrin-coated versicle budding at the TGN. GTPases of the same protein family are likely to be involved in clathrin-mediated budding from the cell surface, including synaptic vesicle endocytosis, but remain to be identified. The putative Arf protein that acts at the plasmalemma must partially differ in properties from Arf1 because brefeldin A, which blocks GDP/GTP exchange on Arf1, inhibits clathrin-coated vesicle budding from the TGN but not from the cell surface (Robinson and Kreis, 1992).

Vesicle Fission

The fission of a coated invaginated pit to form a free clathrin-coated vesicle requires GTP and ATP hydrolysis (Schmid, 1993). The first indication that budding and fission are mediated by distinct mechanisms came from the ultrastructural analysis of *shibire* mutants of Drosophila. At the restrictive temperature, these mutants become rapidly paralyzed due to a selective block of synaptic vesicle endocytosis at the stage of invaginated pits. These pits have a narrow neck surrounded by an electron-dense collar structure (Koenig and Ikeda, 1989). The *shibire* gene encodes dynamin, a GTPase first identified in mammalian brain owing to its ability to bind and form rings around purified microtubules (Vallee and Okamoto, 1995). Neuronal dynamin (dynamin I), however, is concentrated in nerve terminals, consistent with a primary role for this protein in endocytosis, rather than in microtubule function (Takei et al., 1995a). Furthermore, transfection of nonneuronal cells with dynamin mutants defective in GDP/GTP cycling produces

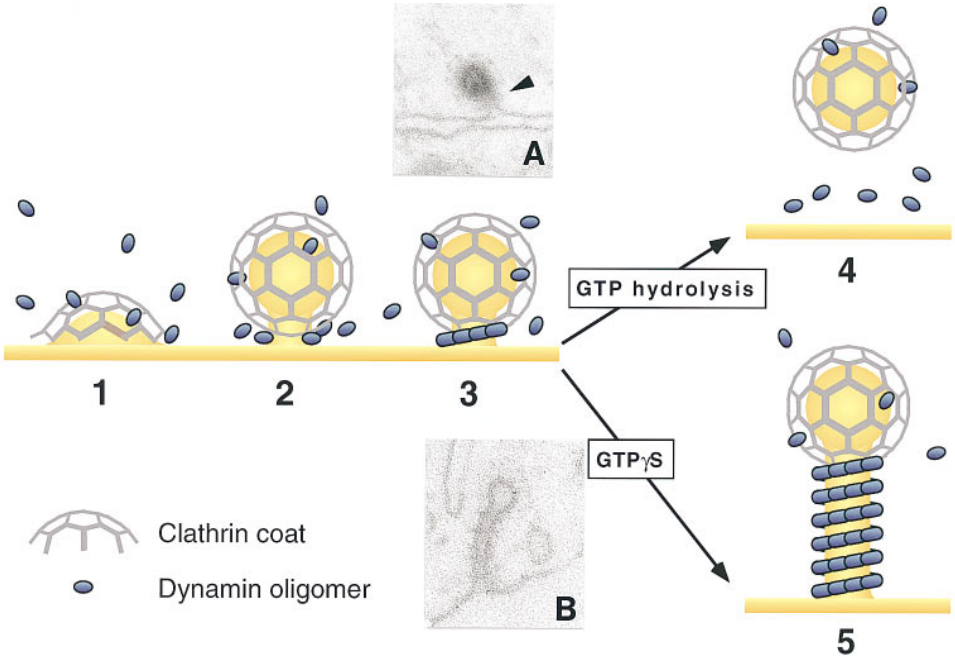

Figure 2. Putative Role of Dynamin in Synaptic Vesicles Endocytosis

Dynamin is recruited at clathrin coats (steps 1 and 2) via interactions with the adaptor AP2. Dynamin subsequently assembles into a ring as soon an appropriate template forms: the neck of the invaginated bud (step 3). Ring formation is immediatly followed by GTP hydrolysis and fission (step 4). In the presence of GTPγS, the ring does not disassemble and several rings may pile up onto each other thus generating a narrow membrane tubule (step 5) (modified from De Camilli et al., 1995). Inset (A) shows an electron micrograph from the nerve ending of a cultured hippocampal neurons demonstrating presence of a dynamin ring (arrowhead) around the neck of a clathrin-coated bud. Prior to fixation, the neuron had been incubated with HRP-conjugated antibodies directed against the lumenal domain of synaptotagmin. Presence of HRP reaction product in the vesicle lumen demonstrates that the bud is involved in synaptic vesicles recycling. Inset (B) shows an electron micrograph from a lysed nerve terminal incubated with GTPγS as described by Takei et al. (1995a). Other micrographs by K. Takei, O. Mundigl, P. L. Daniell, and P. De Camilli.

a potent block of clathrin-mediated endocytosis, suggesting a general role for dynamin in this process (Vallee and Okamoto, 1995).

Recent ultrastructural studies have allowed visualization of dynamin by immunogold electron microscopy. Dynamin forms rings at the neck of clathrin-coated invaginated pits (Figure 2). These rings are very transient structures in physiological conditions and difficult to capture in electron micrographs, although in some rare cases they can be seen (inset A of Figure 2). In the presence of GTPγS, which blocks vesicle fission, rings do not disassemble and often pile up onto each other, thus elongating the vesicle neck into a plasmalemmal tubule approximately 25 nm in diameter (inset B of Figure 2) (Takei et al., 1995a). The collar structures visible at the neck of endocytic pits in *shibire* flies at the restrictive temperature are likely to be dynamin rings locked in the assembled state by the mutation. Dynamin alone, irrespective of its GDP/GTP bound state, can oligomerizes into open rings in vitro (Hinshaw and Schmid, 1995). The formation of dynamin rings around microtubules may reflect the property of microtubules to act as optimal templates for this oligomerization.

In principle, given the open structure of the dynamin ring, a twist of the ring that correlates with GTP hydrolysis could drive vesicle fission by narrowing, and ultimately severing, the vesicle neck. However, since ATP hydrolysis is also required for the fission reaction, other functions for the dynamin rings in fission should also be considered. The rings may serve as templates to recruit effector proteins that act on the vesicle stalk in a GTP-dependent way. Another possibility is that GTP-bound dynamin may interact with proteins already present at the vesicle neck, or with proteins at the free edge of the clathrin coat to activate them and enable them to perform the fission reaction. Although dynamin will form rings in the absence of GTP, GDPβS was reported to inhibit the deep invagination of clathrin-coated pits that appear to precede fission (Schmid, 1993), thus suggesting that GTP-dependent interactions of dynamin may be involved in a late prefission step. GTP hydrolysis may be then needed for the dissociation of a transient intermediate in the series of events leading to fission. Dynamin I is not present at the TGN. Yet in yeast, a dynamin-like protein (VPS1) was shown to be involved in vesicular transport from the TGN to the vacuole (the analogous organelle to the lysosome). This pathway also involves clathrin. Thus, a protein of the dynamin family may be required for all clathrin-mediated budding events (Conibear and Stevens, 1995).

No partners have been identified yet for the GTPase domain of dynamin, the domain that may account for putative GTP-dependent interactions. However, the proline-rich COOH-terminus of the protein, whose deletion prevents targeting to the cell periphery and abolishes the dominant negative effect of dynamin I mutants, interacts in vitro with a variety of Src homology 3 (SH3) domain containing proteins (Gout et al., 1993). One of

these proteins, the neuronal protein amphiphysin, appears to be a major physiological partner for dynamin I (David et al., 1996; McPherson et al., 1996). Amphiphysin is colocalized with dynamin I in nerve terminals and also interacts with AP2 via a domain distinct from the SH3 domain (Wang et al., 1995; David et al., 1996). Thus, it may play a role in recruiting dynamin at clathrin coats. Amphiphysin contains regions of similarity to two yeast proteins, Rvs161 and Rvs167, mutations in which produce endocytosis defects, supporting a putative function for amphiphysin in endocytosis (David et al., 1996; Munn et al., 1995). Dynamin may also interact with AP2 either directly or via Grb2 (Wang et al., 1995, David et al., 1996).

Other SH3 domain–containing proteins that bind dynamin and regulate its GTPase activity include Grb2, the p85 subunit of p85/p110 phosphoinositide (PI) 3-kinase, and phospholipase Cγ. All of the proteins interact via SH2 domains with phosphotyrosine residues of membrane protein internalized via clathrin-coated vesicles (Gout et al., 1993), but the physiological significance of these other interactions for synaptic vesicle endocytosis is still unclear. Reversible interactions between dynamin and proteins of clathrin-coated pits may generate a high local concentration of dynamin, thus facilitating formation of the dynamin ring around their stalks (Figure 2). Dynamin I assembly and function may be further regulated by the Ca^{2+}-dependent dephosphorylation of its proline-rich COOH-terminus that occurs immediately upon nerve terminal stimulation. This dephosphorylation, which was reported to be mediated by calcineurin, reduces the GTPase activity of dynamin I, thereby increasing the GTP-bound pool of the protein (Robinson et al., 1994).

Besides dynamin I, the only other major brain protein that binds the SH3 domain of amphiphysin is synaptojanin. Synaptojanin was first identified as a protein that binds Grb2 in vitro. It is a neuronal protein closely colocalized with dynamin I in nerve terminals, where both proteins undergo stimulation-dependent dephosphorylation (McPherson et al., 1994). Synaptojanin is a member of the family of type II inositol-5-phosphatases that act both on inositolpolyphosphates and phosphoinositides. It was found to dephosphorylate Ins(1,4,5)P_3 (and less effectively Ins(1,3,4,5)P_4) as well as PtdIns(4,5)P_2. Its NH_2-terminal region is similar to the cytosolic domain of the yeast protein Sac1, mutations in which confer inositol auxotrophy and suppress mutations in actin and in the PtdIns transfer protein Sec14 (McPherson et al., 1996). These properties of synaptojanin are consistent with the increasing evidence for a role of the actin-based cytoskeleton and of PtdIns metabolites in endocytosis. An involvement of the actin-based cytoskeleton is indicated by yeast genetics (Munn et al., 1995), while putative links between PtdIns metabolism and endocytosis have emerged primarily from biochemical studies. For example, the AP2 domain–binding C2b domain of synaptotagmin (Fukuda et al., 1995), AP2 itself, and AP180 were all found to bind specifically and with significant affinity to inositolpolyphosphates. Binding of these compunds to AP2 and AP180 was shown to inhibit their clathrin assembly properties (Beck and Keen, 1991; Ye et al., 1995). A pleckstrin homology domain with PtdIns(4,5)P_2 binding potential is present in dynamin. A PI3-kinase is thought to be involved in membrane recycling at the cell surface (Liscovitch and Cantley, 1995; Li et al., 1995b). The precise targets for the physiological function of synaptojanin in endocytosis, however, remain to be elucidated. Synaptojanin is not present at the TGN. Interestingly, the OCRL protein, a type II inositol-5-phosphatase containing a long stretch of similarity to synaptojanin, is concentrated in the Golgi complex region (Olivos-Glander et al., 1995).

The Role of Ca^{2+} in Synaptic Vesicle Endocytosis

Synaptic vesicle endocytosis is tightly coupled to synaptic vesicle exocytosis. Given the strict requirement for cytosolic Ca^{2+} in triggered exocytosis, several studies have investigated the role of Ca^{2+} in synaptic vesicle endocytosis. The recovery from the temperature-sensitive block in *shibire* mutants (Ramaswami, et al., 1994), as well as endocytosis of FM1-43 following a burst of exocytosis (Ryan and Smith, 1995), and the endocytosis that balance spontaneous exocytosis (Kraszewski et al., 1995) were all reported to proceed in the absence of extracellular Ca^{2+}. Other experiments, however, have shown a requirement for extracellular Ca^{2+}. α-Latrotoxin induces synaptic vesicle exocytosis irrespective of the presence of Ca^{2+} in the medium, but exocytosis is followed by endocytosis only if Ca^{2+} is present (Ceccarelli and Hurlbut, 1980). At the lamprey reticulospinal synapse, depletion of synaptic vesicles by intense electrical stimulation followed by exposure to zero Ca^{2+} in the continued presence of stimulation led to a persistent block of synaptic vesicle endocytosis. Endocytosis promptly resumed when extracellular Ca^{2+} was introduced (Gad et al., 1995, Soc. Neurosci., abstract). A possible interpretation of these conflicting findings is that only prolonged incubation in zero Ca^{2+}, which leads to a depletion of cytosolic Ca^{2+}, impairs endocytosis. Finally, in a capacitance study of retinal ribbon nerve terminals, cytosolic Ca^{2+} was found to inhibit synaptic vesicle membrane retrieval (von Gersdorff and Matthews, 1994). Thus, Ca^{2+} seems to have some important effect in endocytosis. Putative sites of action of cytosolic Ca^{2+} in synaptic vesicle endocytosis include the neuronal clathrin light chain (which binds Ca^{2+} and calmodulin) (Pley and Parham, 1993), the cytoplasmic domain of synaptotagmin (Li et al., 1995a), calcineurin (Robinson et al., 1994), and calmodulin (Artalejo and Palfrey, 1995).

The occurrence of Ca^{2+}-dependent, clathrin-independent, rapid endocytosis triggered by membrane depolarization has been detected in several cell types by capacitance measurements (Neher and Zucker, 1993; Thomas et al., 1994; Artalejo et al., 1995). It is not known whether this process occurs in nerve terminals and whether it is involved in synaptic vesicle reformation. A further understanding of this process, which may be mediated by bulk internalization of plasmalemmal fragments, awaits characterization by morphological techniques.

Two Models of Clathrin-Mediated Recycling

Clathrin-coated vesicles shed their clathrin coat rapidly after fission in an ATP-dependent reaction that involves

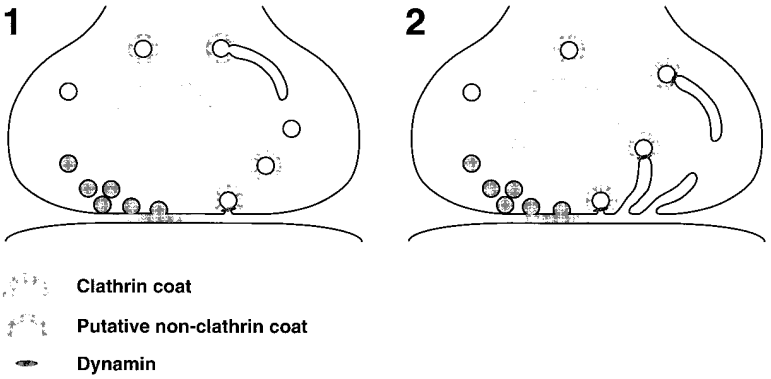

Figure 3. Alternative Models of Synaptic Vesicles Recycling

Model 1 proposes that after clathrin loss the endocytic vesicle fuses with an early endosomes from which a new synaptic vesicle is formed via a specialized budding reaction. Model 2 proposes that a new synaptic vesicle is generated directly from an uncoated clathrin-coated vesicle.

both the heat shock cognate protein Hsc70 (uncoating ATPase) and auxilin (Ungewickell et al., 1995). Auxilin contains a DnaJ motif also found in other proteins implicated in protein folding. It binds to the assembled clathrin lattice and recruits Hsc70 eventually leading to disruption of clathrin–clathrin interactions and to release of soluble triskelia (Ungewickell et al., 1995). The fate of the stripped vesicles remains unclear. One widely accepted model (model 1 of Figure 3) postulates their fusion with early (sorting) endosomes from which new synaptic vesicles originate via a budding reaction that selectively includes synaptic vesicle membrane proteins. Until now, however, no "coat" structures with a putative role in this budding reaction have been identified either biochemically or morphologically in nerve terminals.

An alternative model is that new synaptic vesicles are generated directly from uncoated clathrin-coated vesicles (model 2 of Figure 3). Such a possibility is consistent with the similar size of synaptic vesicles and the majority of nerve terminal clathrin-coated vesicles. This model predicts that at least some of the endosome-like vacuoles visible in nerve terminals after strong stimulation represent deep invaginations of the plasmalemma, or fragments of plasmalemma internalized by bulk endocytosis. The model also predicts that clathrin-mediated budding occurs in parallel from these structures and from the presynaptic membrane. In agreement with model 2, clathrin coats were observed on internal vacuoles in nerve terminals (Heuser and Reese, 1973; Takei et al., 1995b). These images were originally interpreted as clathrin-coated vesicles fusing with endosomes (Heuser and Reese, 1973), but more recent vesicular transport studies have shown that the coats are shedded prior to fusion. Model 2 reduces the steps required for synaptic vesicle reformation and helps to explain the very rapid time course of synaptic vesicle recycling.

A direct pathway of synaptic vesicles reformation (model 2) may coexist with an indirect pathway (model 1) that is primarily used for the recycling of cell surface components, but may represent a salvage recycling pathway for synaptic vesicle proteins. This possibility is supported by the presence in nerve terminals of scattered clathrin-coated vesicles larger than synaptic vesicles. An intriguing finding that remains to be understood is the presence of Rab5, a protein with a putative role in endosome–endosome fusion, on synaptic vesicle membranes and its increased levels in synapsin-deficient mice (Rosahl et al., 1995).

The final steps in synaptic vesicle reformation are neurotransmitter loading (a process that may initiate in large intracellular vacuoles) and recruitment to their membranes of cytosolic factors that are required for the journey toward exocytosis but are shedded after fusion. These factors include the Rab3–rabphilin complex, which has a putative role in docking and fusion (Li et al., 1994), and the synapsins, which have been hypothesized to play a role in recruiting synaptic vesicles into clusters (Pieribone et al., 1995). Formation of a new docking/fusion-competent synaptic vesicles from a newly budded vesicle may also imply conformational changes, and changes in protein–protein interactions, of intrinsic membrane proteins. Very little is known about these processes. An important direction for future studies will be the elucidation of the reversible cyclic modification of the synaptic vesicle membrane that allows it to proceed vectorially through each step of the exocytic–endocytic cycle.

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