The Synaptic Vesicle Cycle: A Single Vesicle Budding Step Involving Clathrin and Dynamin

Kohji Takei, Olaf Mundigl, Laurie Daniell, and Pietro De Camilli

Department of Cell Biology and Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract. Strong evidence implicates clathrin-coated vesicles and endosome-like vacuoles in the reformation of synaptic vesicles after exocytosis, and it is generally assumed that these vacuoles represent a traffic station downstream from clathrin-coated vesicles. To gain insight into the mechanisms of synaptic vesicle budding from endosome-like intermediates, lysed nerve terminals and nerve terminal membrane subfractions were examined by EM after incubations with GTPγS. Numerous clathrin-coated budding intermediates that were positive for AP2 and AP180 immunoreactivity and often collared by a dynamin ring were seen. These were present not only on the plasma membrane (Takei, K., P.S. McPherson, S.L. Schmid, and P. De Camilli. 1995. *Nature (Lond.).* 374:186–190), but also on internal vacuoles. The lumen of these vacuoles retained extracellular tracers and was therefore functionally segregated from the extracellular medium, although narrow connections between their membranes and the plasmalemma were sometimes visible by serial sectioning. Similar observations were made in intact cultured hippocampal neurons exposed to high K^+ stimulation. Coated vesicle buds were generally in the same size range of synaptic vesicles and positive for the synaptic vesicle protein synaptotagmin. Based on these results, we suggest that endosome-like intermediates of nerve terminals originate by bulk uptake of the plasma membrane and that clathrin- and dynamin-mediated budding takes place in parallel from the plasmalemma and from these internal membranes. We propose a synaptic vesicle recycling model that involves a single vesicle budding step mediated by clathrin and dynamin.

SYNAPTIC vesicles are highly specialized secretory organelles that contain nonpeptide neurotransmitters. After exocytosis, synaptic vesicle membranes are rapidly internalized and reused in the formation of new neurotransmitter-filled synaptic vesicles (Heuser and Reese, 1973; Ceccarelli et al., 1973; De Camilli and Takei, 1996). The precise recycling pathway remains to be elucidated, although strong evidence indicates that it is closely related to the housekeeping receptor-mediated membrane recycling of all cells (Thomas-Reetz and De Camilli, 1994). This pathway involves clathrin-coated vesicles and endosomal intermediates.

Clathrin and clathrin accessory proteins are very abundant in nerve terminals, and proteins of synaptic vesicle membranes represent the main membrane cargo of synaptic clathrin-coated vesicles (Pfeffer and Kelly, 1985; Maycox et al., 1992; De Camilli and Takei, 1996). Furthermore, the GTPase dynamin, which is essential for the reformation of synaptic vesicles (Koenig and Ikeda, 1989) and is concentrated in nerve terminals (McPherson et al., 1994; Takei et al., 1995), has been generally implicated in the fission of clathrin-coated vesicles from the plasmalemma (van der Bliek et al., 1993; Herskovits et al., 1993; Damke et al., 1994; De Camilli et al., 1995). Dynamin forms rings (lock washer-like structures) at the neck of clathrin-coated buds. A conformational change in dynamin correlating with GTP hydrolyis is thought to be required for vesicle fission (Takei et al., 1995; Hinshaw and Schmid, 1995).

Stimulation of nerve terminals leads to a transient increase in the number and size of early endosome-like organelles, which can be labeled by endocytic tracers. This change is paralleled by a decrease in the number of synaptic vesicles. The opposite change is observed when the stimulus is terminated, suggesting a precursor–product relationship between these structures and synaptic vesicles (Heuser and Reese, 1973). Based on the widely accepted model of endocytosis in other cells, endosome-like vacuoles of nerve terminals have been typically regarded as stations to which plasma membrane–derived clathrin-coated vesicles are targeted, and from which new synaptic vesicles are generated (Heuser and Reese, 1973; Miller and Heuser, 1984). However, the molecular mechanisms of synaptic vesicle budding from these endosome-like intermediates remain elusive. More generally, mechanisms

Address all correspondence to Pietro De Camilli, Department of Cell Biology, Howard Hughes Medical Institute, 295 Congress Avenue, New Haven, CT 06510. Tel.: (203) 737-4465. Fax: (203) 737-1762.

of vesicle budding from endosomes remain poorly understood.

Studies carried out on other vesicle-budding reactions indicated that a general principle in the generation of vesicular carriers is the recruitment of cytosolic proteins on the donor membrane, which provide a scaffold for the formation of high curvature membrane buds. These proteins are collectively defined as "coats" (Rothman, 1994; Schekman and Orci, 1996). A COPI-like coat has been implicated in endosomal budding (Whitney et al., 1995), but its precise composition and the destination of the vesicles that it generates remain unclear. Sporadic reports (Heuser and Reese, 1973; Matlin et al., 1982; Geuze et al., 1984; Killisch et al., 1992), as well as a very recent study (Stoorvogel et al., 1996), have also demonstrated the occurrence of clathrin on endosomal buds, but even in this case, little is known about the fate of the vesicle. Most likely, budding from endosomes of vesicles destined to the plasmalemma involves more than one mechanism. In neuroendocrine cells, for example, recycling transferrin receptors are excluded from recycling synaptic vesicles, indicating the existence of distinct recycling vesicular carriers (Thomas-Reetz and De Camilli, 1994).

The goal of this study was to investigate coat structures that participate in budding from nerve terminal endosomes. To achieve this, we have applied to nerve terminals an approach similar to that pioneered by Orci et al. (1986) to visualize vesicle coats in ER and Golgi complex traffic. This approach capitalizes on the dependence of coat assembly/disassembly on GTPases, as well as on the properties of GTPγS to block uncoating and to inhibit or delay the fission reaction (Orci et al., 1989; Carter et al., 1993; Rothman, 1994; Tooze et al., 1990; Takei et al., 1995; Schekman and Orci, 1996). The application of this method to nerve terminals has already yielded useful information about the function of dynamin in budding from the plasmalemma (Takei et al., 1995).

We report now that numerous coated buds are found on nerve terminal endosome-like intermediates in the presence of GTPγS, and that the only coat found on such buds is identical to the clathrin/dynamin coat found at the plasmalemma. Prompted by these findings, we have further examined the nature of these endosome-like intermediates, using broken and intact nerve terminal preparations, and found evidence suggesting that they are directly derived from the plasmalemma. Our results point to a model of synaptic vesicle recycling in which new synaptic vesicles are regenerated in parallel both from the cell surface and from internal membranes via a clathrin/dynamin–mediated event.

Materials and Methods

Antibodies

mAbs directed against dynamin (Hudi 1) were a gift from Dr. S. Schmid (The Scripps Research Institute, La Jolla, CA) or from UBI (Lake Placid, NY). mAbs directed against neuronal clathrin light chain (Cl 57.1) were a kind gift from Dr. Reinhard Jahn (Yale University, New Haven, CT). Polyclonal antibodies and mAbs directed against γ-adaptin and α-adaptin (AP.6) were gifts from Dr. Margaret Robinson (University of Cambridge, Cambridge, UK) and Dr. Francis Brodsky (University of California, San Francisco, CA), respectively. mAbs directed against AP180/AP3 and HRP (type VI-A) were purchased from Sigma Immunochemicals (St. Louis, MO). 6-nm protein A–gold conjugates were prepared as described (Takei et al., 1992). Affinity-purified antibodies directed against the luminal domain of synaptotagmin I (Syt_{lum}-Abs)[1] (Mundigl et al., 1993) were conjugated to HRP using the periodate-coupling procedure (Tijssen and Kurstak, 1984). Briefly, 2 mg HRP was dissolved in 600 μl of 20 mM Na periodate/10 mM Na phosphate buffer (pH 7), incubated for 20 min at room temperature, and dialyzed against 1 mM Na acetate, pH 4, at 4°C. 2 mg of affinity-purified antibodies or control rabbit IgGs in 200 μl of 20 mM carbonate buffer (pH 9.5) was combined with the HRP solution and incubated for 2 h at room temperature. 40 μl of $NaBH_4$ (4 mg/ml in H_2O) was added and dialyzed against PBS. The resulting conjugate was separated from unconjugated HRP on a protein A–Sepharose column. The conjugate was eluted with 100 mM glycine (pH 3), and then neutralized with 1 M Tris. The elution buffer was exchanged by gel filtration on a Sephadex PD10 column with PBS. Finally, the conjugate was adjusted to a concentration of 0.5 mg/ml corresponding to a specific peroxidase activity equivalent to 5.5 μg/ml HRP. This material was diluted 1:50 in the neuron incubation media.

Preparation of Membranous Fractions

Synaptosomal fractions (P_2) were prepared from rat brain homogenates as described (Huttner et al., 1983). These fractions were then diluted 1:10 in ice-cold H_2O and gently homogenized by hand in a glass Teflon homogenizer to obtain lysed synaptosomes that were then pelleted at 9,200 *g* for 15 min (lysed P_2) and used without further subfractionation. Alternatively, the diluted P_2 fraction was homogenized vigorously and further processed as described to obtain an LP_2 fraction (Huttner et al., 1983).

Preparation of "Cytosol"

A fraction containing diluted cytosol (S_3) was prepared from rat brain by high speed centrifugation as described (Huttner et al., 1983; McPherson et al., 1994). The S_3 fraction was filtered through a Sephadex G-25M PD-10 column (Pharmacia Biotech Inc., Piscataway, NJ) equilibrated with 5 mM Hepes-KOH buffer, pH 7.4. The final protein concentration of this material, defined "cytosol," was adjusted to 2 mg/ml.

Cell-free Incubations

Gently lysed P_2 fractions or LP_2 fractions were resuspended in "cytosolic buffer" (25 mM Hepes-KOH, pH 7.4, 25 mM KCl, 2.5 mM magnesium acetate, 5 mM EGTA, 150 mM K-glutamate) at a concentration of ~5 mg/ml. 100-μl aliquots of these fractions were added to 730 μl of "cytosol" or "cytosolic buffer," and these mixtures were further taken to 1 ml with various additions to achieve a final concentration of 1× cytosolic buffer and the following final concentrations of nucleotides: 2 mM ATP, 200 μM GTP, or 200 μM GTPγS. Samples containing ATP were also supplemented with an ATP regenerating system at the final concentration of 16.7 mM creatine phosphate, 16.7 IU/ml creatine phosphokinase. These mixtures were incubated for 15 min at 37°C, and incubations were stopped by adding 1 ml of 2× concentrated fixative.

HRP Labeling of Synaptosomes

Intact synaptosomes (P_2 fraction) were washed with Krebs-Ringer-Hepes buffer (KRH: 128 mM NaCl, 25 mM Hepes, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2/K_2HPO_4, and 5.6% glucose; Kraszewski et al., 1995), then incubated at 37°C for 10 min in high K^+-KRH (57 mM K^+ with a corresponding reduction in Na^+) containing 10 mg/ml HRP, and then thoroughly washed in ice-cold KRH. This material was then either fixed or further fractionated to obtain an LP_2 fraction that was used for cell-free incubations as described above.

Labeling with HRP-Syt_{lum}-Abs of Cultured Hippocampal Neurons

Hippocampal neurons from 18-d fetal rats were cultured at a high density (30,000–40,000 cells/cm^2) in defined medium (B27/Neurobasal; GIBCO BRL, Gaithersburg, MD) as described (Mundigl et al., 1995). 2–3-wk-old

1. *Abbreviations used in this paper*: KRH, Krebs-Ringer-Hepes (buffer); Syt_{lum}-Abs, affinity-purified antibodies directed against the luminal domain of synaptotagmin I.

neurons were incubated in high K^+-KRH at 37°C for 10 min with HRP-conjugated Syt_{lum}-Abs and then washed with ice-cold KRH before fixation.

Electron Microscopy

Standard EM. Lysed P_2 and LP_2 fractions that had been exposed to various incubation conditions were fixed with 3% formaldehyde, 2% glutaraldehyde in 0.32 M sucrose, 5 mM Hepes-KOH buffer (pH 7.4), pelleted in an Eppendorf centrifuge, and postfixed in OsO_4. In some samples, OsO_4 postfixation was followed by impregnation with 1% tannic acid to enhance visualization of membrane coats (Orci et al., 1986). Cultured neurons were fixed as described (Mundigl et al., 1993).

Immuno-EM. LP_2 fractions exposed to various incubation conditions were fixed with 3% paraformaldehyde, 0.2% glutaraldehyde in 0.32 M sucrose, 5 mM Hepes buffer. Fixed samples were processed for agarose embedding, immunogold labeling, and EM essentially as described (De Camilli et al., 1983; Takei et al., 1992). For immunogold labeling, samples were reacted sequentially with mouse mAbs, rabbit anti–mouse antibodies, and protein A–gold conjugates, or with rabbit polyclonal antibodies and protein A–gold conjugates.

Morphometry. This analysis was performed on HRP-loaded LP_2 fractions incubated in various conditions and embedded for standard EM. For each condition, 12 EM micrographs (6 from each of 2 epon blocks) were taken at a magnification of 15,500 and printed at the final magnification of 40,300. Regions of the epon blocks containing approximately a similar density of organelles per unit area were photographed. In each micrograph, the total number of coated profiles on HRP-positive and HRP-negative vacuolar structures were counted.

Other Procedures. The HRP reaction product was developed in fixed specimens as described (Mundigl et al., 1993). All EM observations were made on a CM-10 microscope (Philips Technologies, Cheshire, CT).

Results

Coated Intermediates on Endosome-like Vacuoles of Nerve Terminals

Lysed synaptosomal fractions (P_2 fractions) were incubated with rat brain cytosol, ATP, and GTPγS for 15 min at 37°C in a buffer mimicking the cytosolic ionic environment, and then processed for EM. As previously described (Takei et al., 1995), these samples contained numerous clathrin-coated vesicle profiles, as well as clathrin-coated buds on the plasmalemma. In addition, coated buds were present on intraterminal membrane profiles with the pleiomorphic characteristics of early endosomes (Fig. 1, *a–g*). Surprisingly, these coated buds were morphologically indistinguishable from buds present on the plasmalemma and had the bristle-like appearance of a clathrin coat. Furthermore, as observed with the clathrin-coated pit on the plasmamembrane (Takei et al., 1995), their neck was in some cases surrounded by one or several dynamin-like rings (Fig. 1, *c–e* and *g*).

The occurrence of clathrin- and dynamin-mediated budding events from nerve terminal endosomes was confirmed by the analysis of LP_2 subfractions of synaptosomes incubated with GTPγS under the same conditions that were used for lysed synaptosomes (Fig. 1, *h* and *i*). The LP_2 fraction contains the slowly sedimenting membranes of synaptosomal lysates and is thus enriched in synaptic vesicles and endosomes (Huttner et al., 1983). In these preparations, numerous vesicular and tubular membranes decorated by clathrin- and dynamin-like coats, respectively, were present. Coated tubules were often quite long, possibly because in this organelle suspension growth of the tubules is not hindered by surrounding structures and a large portion of the donor vacuole membrane can be recruited. These coated tubules frequently formed bundles (see Fig. 3 *e*) that were reminiscent of previously described bundles of dynamin-coated microtubules and probably result from the association, side-by-side, of dynamin rings (Shpetner and Vallee, 1989).

In both lysed synaptosomes and LP_2 fractions, the large majority of the coated vesicle profiles, irrespective of their localization (free vesicles, plasmalemmal buds, buds on internal membranes), had a small homogeneous size that was in the same range as the size of the synaptic vesicles (Fig. 1).

Clathrin and Dynamin Are Components of the Coats

Since the presence of clathrin on nerve terminal endosome-like intermediates was unexpected, immunocytochemical experiments were performed on lysed nerve terminals and LP_2 fractions to confirm the identity of these coats. At the end of the incubations with cytosol and nucleotides, membranes were fixed and then labeled by immunogold for either dynamin, clathrin light chain, α-adaptin subunit of the plasmalemmal clathrin adaptor AP2 (Pearse and Robinson, 1990), and AP3/AP180, a nerve terminal–specific component of the clathrin coat (Ye and Lafer, 1995). All coated buds, irrespective of whether they originated from the plasmalemma or from a vacuolar structure, were strongly positive for clathrin (Fig. 2, *a–d*). In addition, most coated buds were also positive for AP180 (Fig. 2, *e–g*) and α-adaptin (Fig. 2, *h–j*), although labeling of the coat for these proteins was less homogeneous than labeling for clathrin, possibly because of an uneven accessibility of the relevant epitopes under the clathrin coat. All tubular membranes decorated with rings were positive for dynamin immunoreactivity (Fig. 2, *k–m*). Consistent with earlier work (Damke et al., 1994; Takei et al., 1995), scattered dynamin immunoreactivity was also observed on some clathrin-coated profiles (Fig. 2 *k*). γ-adaptin, a subunit of the TGN clathrin adaptor AP1 (Pearse and Robinson, 1990), was found by immunofluorescence to be absent from nerve terminals (our unpublished observation), speaking against a possible role of AP1 in budding from nerve terminal membranes.

Endosome-like Vacuoles Retain Fluid-phase HRP

To confirm that the vacuoles from which clathrin-coated buds originate are endocytic compartments, we investigated whether they can retain the fluid-phase endocytic tracer HRP (Fig. 3 *a*). Intact brain synaptosomes were incubated for 10 min at 37°C in a depolarizing medium (high K^+-KRH) containing HRP and then rinsed on ice for 5 min to remove all extracellular HRP. Effective removal of the free extracellular tracer was confirmed by EM of samples that were fixed immediately after the rinses (data not shown). The depolarizing conditions were used to stimulate exo-endocytosis and to enhance HRP labeling of endocytic structures. Washed synaptosomes were fractionated to prepare the endosome-enriched fraction LP_2, which was then incubated with cytosol, ATP, and GTPγS, and finally processed for EM and HRP cytochemistry (Fig. 3 *a*). This preparation allowed us to distinguish endocytic compartments that have segregated HRP from small plasma membrane fragments that may have resealed inside out. It also allowed the distinction between endosomes and con-

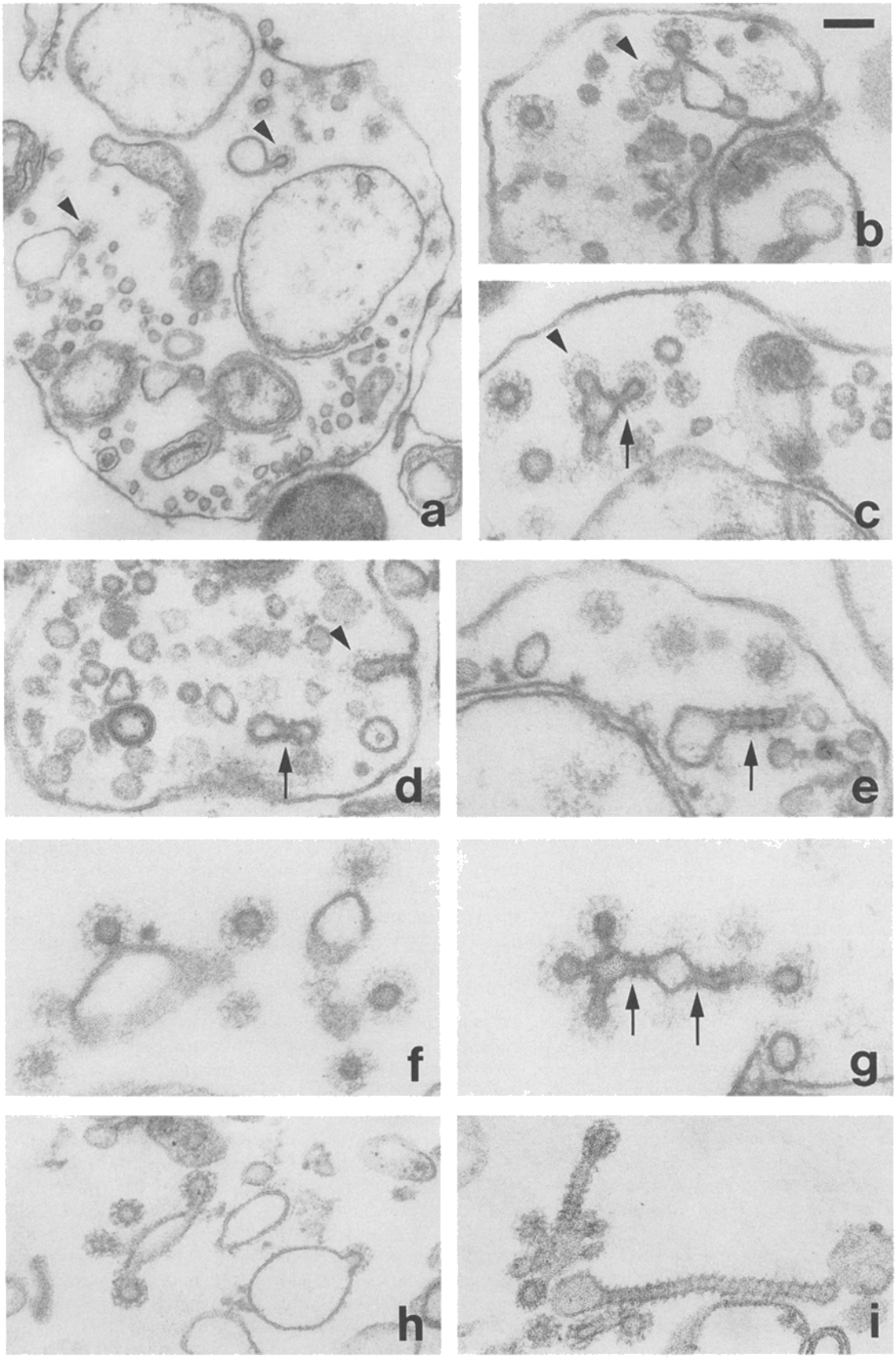


Figure 1. Electron micrographs demonstrating the presence of coated buds and tubules on endosome-like vacuolar profiles of nerve terminal membranes incubated with cytosol, ATP, and GTPγS. (*a–g*) Lysed synaptosomes. (*h* and *i*) LP_2 fractions. (*a*) Low power view of a nerve terminal showing vesicular buds decorated by a clathrin-like coat (*arrowheads*) on two endosome-like vacuoles. (*b–g*) Gallery of coated buds (*arrowheads*) that originate either from the plasmalemma or from internal membranes shown at a higher magnification. Note the presence of dynamin-like rings (*arrows*) at the neck of a coated bud (*d*) and around tubular portions of the vacuoles (*d*, *e*, and *g*). (*h* and *i*) Clathrin-like and dynamin-like coats on vacuolar profiles present in the LP_2 fraction. *a–g* are from tannic acid–impregnated samples. This explains the slightly different appearance of the clathrin coat. Bars: (*a*) 160 nm; (*b–d* and *i*) 100 nm; (*e–g*) 85 nm; (*h*) 110 nm.

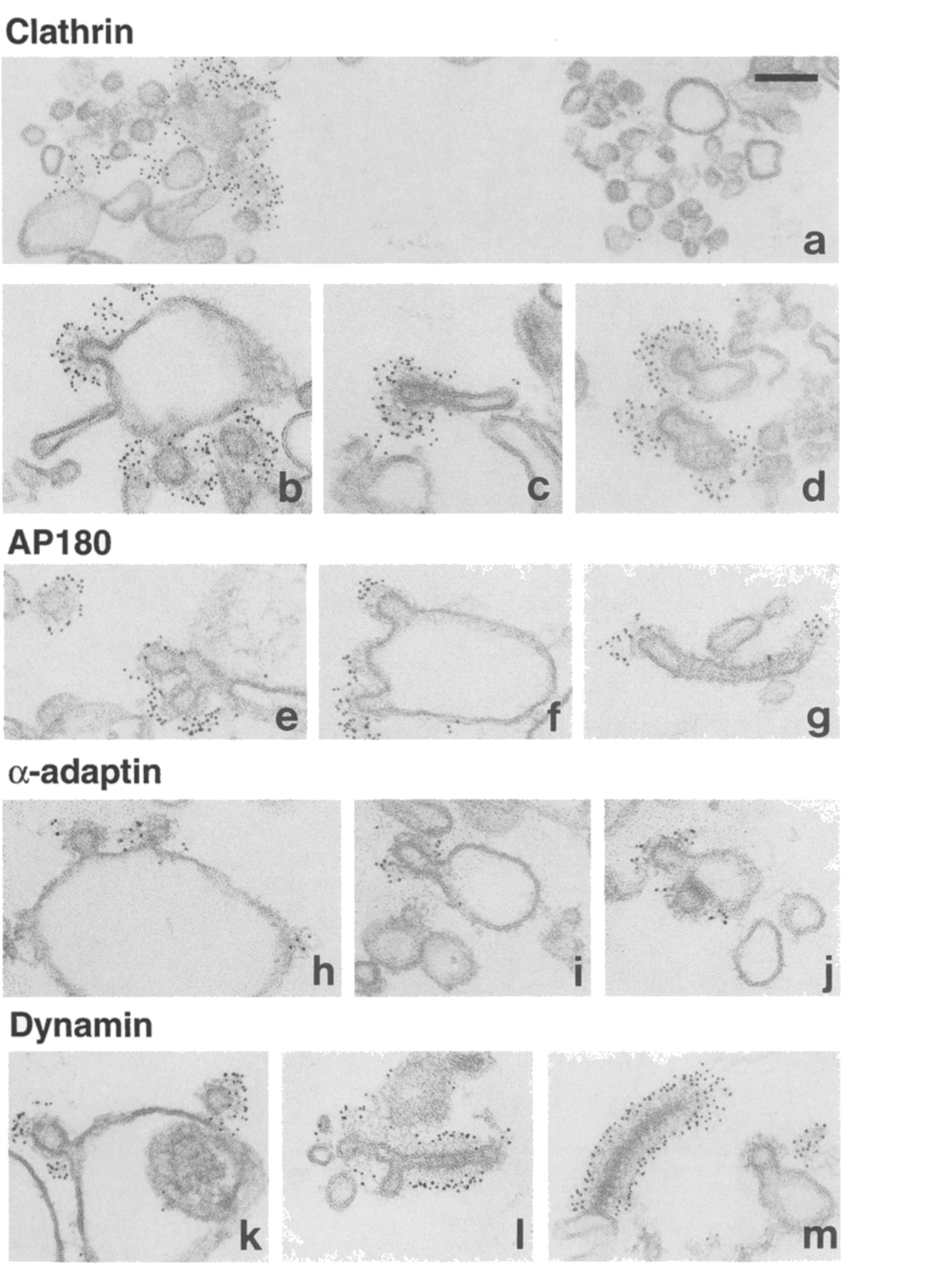


Figure 2. Immunocytochemical characterization of coats present on endosome-like vacuoles of nerve terminals. Immunogold labeling of LP_2 fractions incubated with cytosol, ATP, and GTPγS for the proteins indicated in the figure. (*a–d*) Anti-clathrin immunogold homogeneously surrounds coated vesicular buds. Immunogold is completely absent from synaptic vesicles (e.g., right portion of the field shown in *a*). (*e–g*) Anti-AP180 colocalizes with clathrin on vesicular buds, but is absent from dynamin-coated membrane tubules. (*h–j*) Immunogold for α-adaptin, a subunit of AP2, is present on endosomal buds. (*k–m*) Anti-antidynamin immunogold heavily decorates membrane tubules. It also unevenly decorates clathrin coats. Bars: (*a*) 110 nm; (*b–m*) 100 nm.

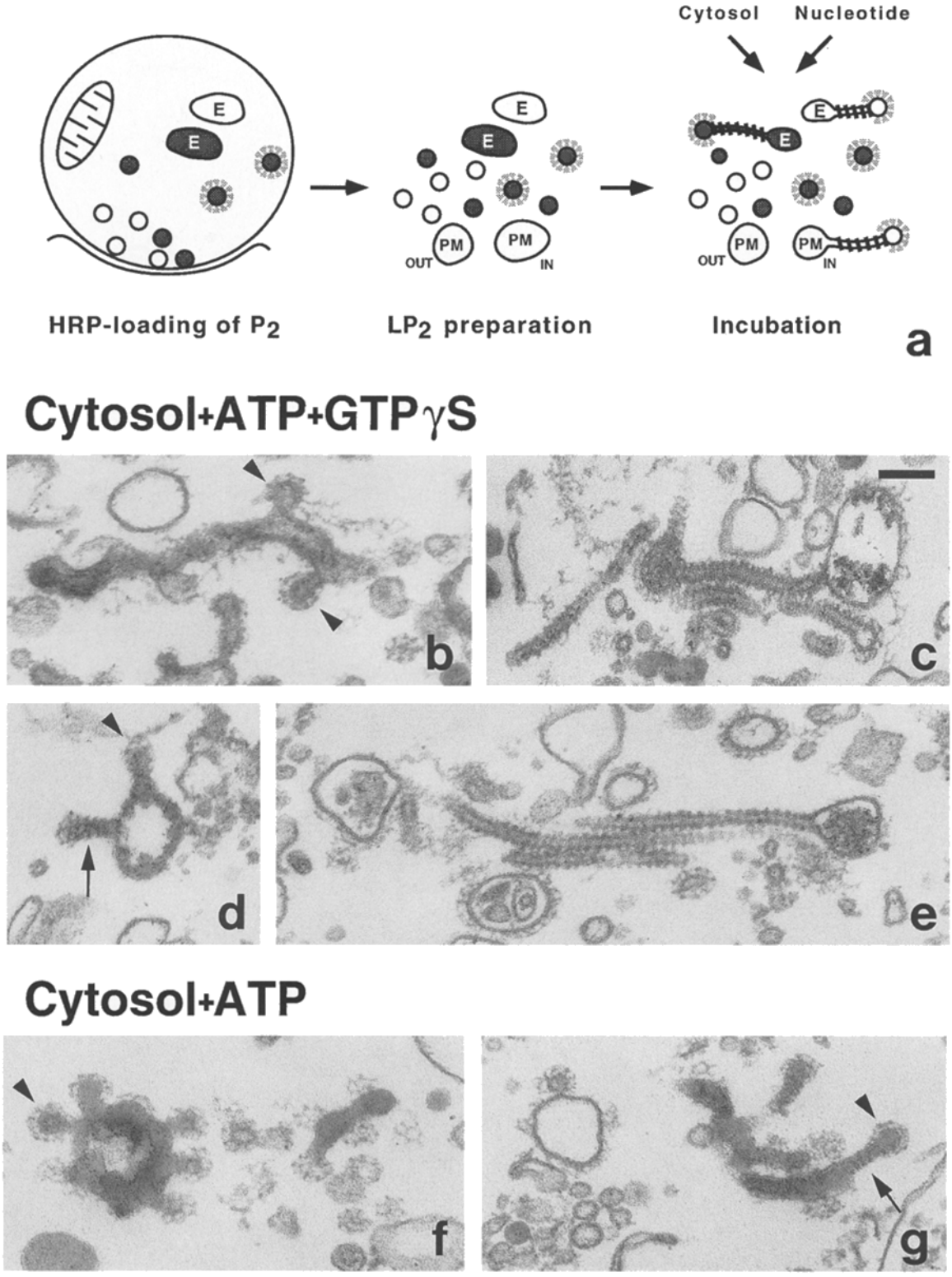


Figure 3. Endosome-like vacuoles from which clathrin-coated buds originate retain fluid-phase HRP. (*a*) Cartoon depicting the experimental protocol. Synaptosomes preloaded with fluid-phase HRP were washed (*left*) and lysed to prepare an HRP-loaded LP2 fraction. The HRP-LP2 fraction was then incubated with cytosol and nucleotides. *E*, endocytic vacuole; *PM*, plasmalemma resealed inside out (*IN*) or outside out (*OUT*). (*b–e*) HRP-LP_2 fractions incubated with cytosol, ATP, and GTPγS. Numerous clathrin-coated buds (*arrowheads* in *b* and *d*) and dynamin-coated tubules (*c*, *e*, and *arrow* in *d*) are present on vacuolar structures, which are labeled by the HRP reaction product. (*f* and *g*) HRP-LP_2 fractions incubated with cytosol and ATP, but no exogenous guanylnucleotides. Note the presence of both clathrin-coated buds (*arrowheads*) and dynamin-coated tubules (*arrow*) on HRP-labeled vacuoles. (*f* represents the high magnification of a portion of Fig. 4 *b*). Bar, 100 nm in *b–g*.

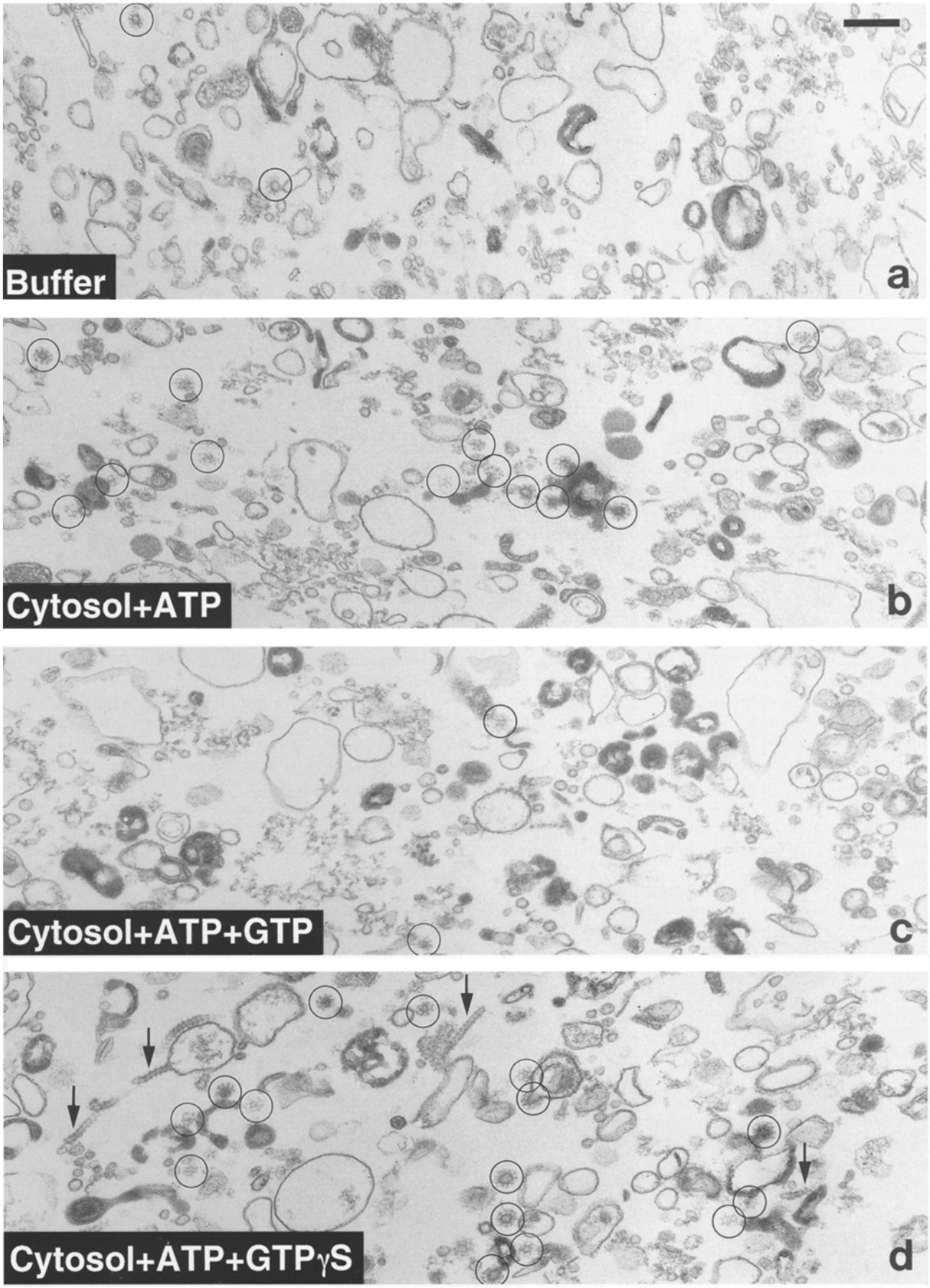


Figure 4. Low power micrographs of LP_2 fractions (from HRP-preloaded synaptosomes) incubated in different nucleotide conditions as indicated. Examples of clathrin-coated buds are indicated by circles. Arrows point to dynamin-coated tubules. Bar, 200 nm.

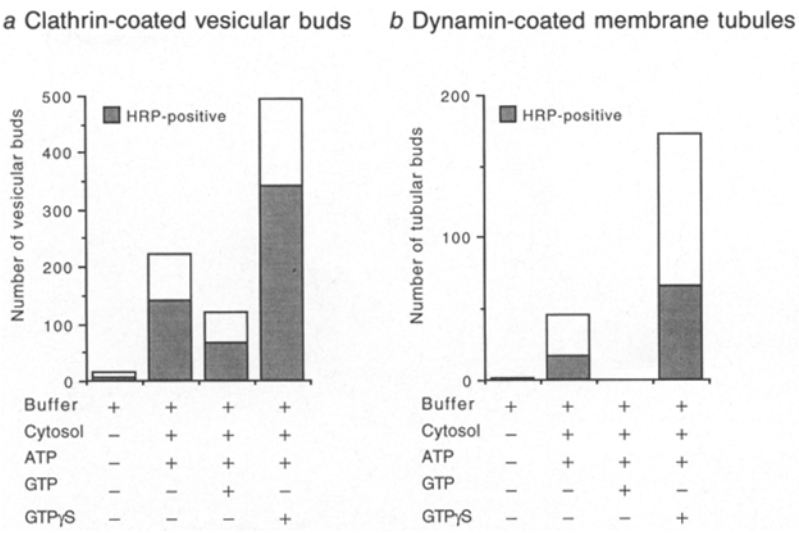


Figure 5. Morphometric analysis of coated membrane evaginations present in LP_2 fractions (from HRP-preloaded synaptosomes) incubated in various conditions as indicated. Bars represent total number of clathrin-coated buds (*a*) or dynamin-coated tubules (*b*) present in the same number of photographic fields taken for the various preparations and containing approximately the same density of organelles per unit area. Shaded portions of the bars represent evaginations originating from HRP-positive vacuoles.

taminating elements of the TGN, from which AP1-positive, clathrin-coated vesicles bud (Pearse and Robinson, 1990). As shown in Fig. 3, *b–e*, many of the vacuolar structures that were positive for clathrin-coated buds and dynamin-coated tubules did contain peroxidase reaction product, validating their identity as endocytic compartments.

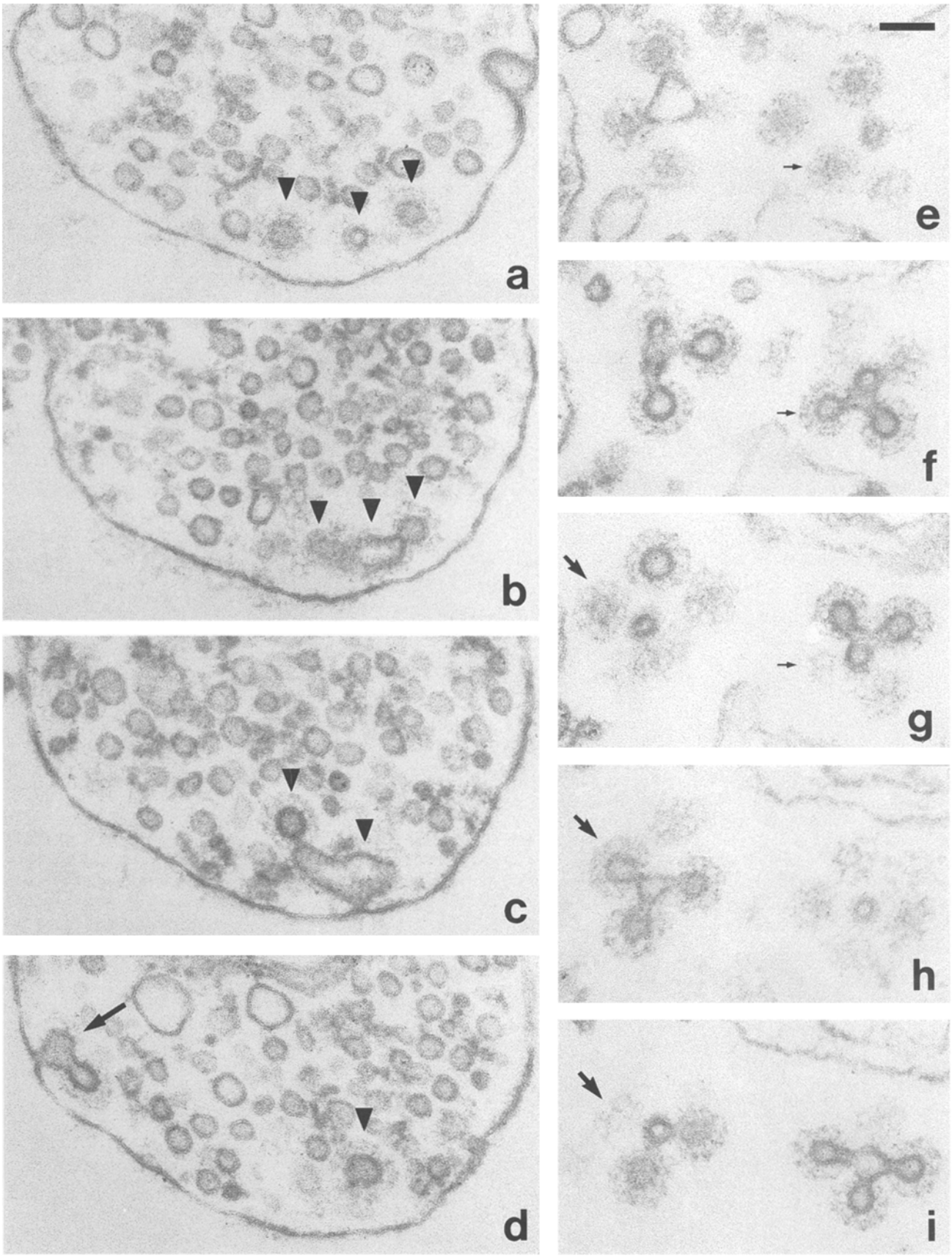
Nucleotide Requirements for the Formation of Coated Buds

To rule out the possibility that clathrin-coated buds on internal membranes may represent a GTPγS-induced artifact, we examined the nucleotide dependence of their formation. LP_2 fractions prepared from HRP-preloaded synaptosomes as described above were incubated under various conditions. As shown by the micrographs of Figs. 3, *f* and *g*, and 4 *b*, and by a morphometric analysis of a large number of micrographs (Fig. 5 *a*), the addition of cytosol and ATP to LP_2 fractions was sufficient to induce the presence of some clathrin-coated buds on endocytic vacuoles. When both GTP and ATP were present in the incubation, a lower number of buds was observed (Figs. 4 *c* and 5 *a*). In contrast, a large increase in clathrin-coated buds was obtained when GTP was replaced by GTPγS (Figs. 4 *d* and 5 *a*). In ATP + GTPγS, clathrin-coated buds were approximately twofold more abundant than in ATP and approximately fourfold more abundant than in ATP + GTP. The low number of buds visible in ATP + GTP may represent steady-state budding. The greater number of buds visible in ATP alone than in ATP + GTP may be explained by a rate-limiting concentration of GTP. Although no GTP was added, some GTP may be generated from a small pool of GDP present in the LP_2 fraction. The stimulatory effect of GTPγS may be explained both by inhibition of fission and uncoating, as well as by a stimulation of coat formation.

The formation of dynamin-coated tubules was greatly stimulated by GTPγS (Fig. 4 *d*), but also occurred in ATP alone (Fig. 5 *b;* see also Fig. 3, *f* and *g*). The increase in the number of tubules produced by the addition of GTPγS to ATP was nearly 400% (Fig. 5 *b*). This stimulatory effect was even stronger, considering that the overall length of the coated tubules was much greater under this condition. No tubules were observed in the presence of ATP + GTP (Figs. 4 *c* and 5 *b*). We hypothesize that in the presence of GTP dynamin rings are extremely transient structures, therefore explaining why they are generally not seen in normal tissue specimens. The stabilization of the GTP-bound conformation of dynamin may greatly favor rings and tubule formation or inhibit their disassembly. On the other hand, the presence of dynamin-coated tubules, even after incubations without exogenous guanylnucleotides, indicates that trapping dynamin in a GTP-bound form is not an essential requirement for ring formation. This is in agreement with the previous demonstration that the polymerization of purified dynamin into rings and spiral-like structures is independent of GTP (Hinshaw and Schmid, 1995). The relative effect of the various nucleotide treatments on the formation of clathrin-and dynamin-coated structures was roughly the same for HRP-positive and HRP-negative vacuoles. Since at least a fraction of the HRP-negative vacuoles are likely to represent inside-out resealed plasmalemma fragments, this observation further establishes the similar mechanisms underlying budding from the plasmalemma and from internal vacuoles.

Both in ATP alone and in ATP + GTPγS, very long dynamin-coated tubules coexisted with clathrin-coated buds that were devoid of even a single dynamin ring. This finding may be explained by a high positive cooperativity in dynamin self-assembly, as previously suggested for the polymerization of dynamin around microtubules (Shpet-

Figure 6. Three-dimensional analysis of lysed synaptosomes incubated in the presence of cytosol, ATP, and GTPγS. (*a–d*) Serial sections demonstrating the morphology of a vacuole with several clathrin-coated buds (*arrowheads*). In *c*, the vacuole appears to be connected to the plasmalemma via a narrow opening. An arrow in *d* points to another vacuole connected to the plasmalemma via a similar opening. (*e–i*) Serial sections demonstrating that most clathrin-coated vesicle profiles that appear as free vesicles are in fact interconnected. Identical coated buds are indicated by identical arrrows. Bar, 100 nm.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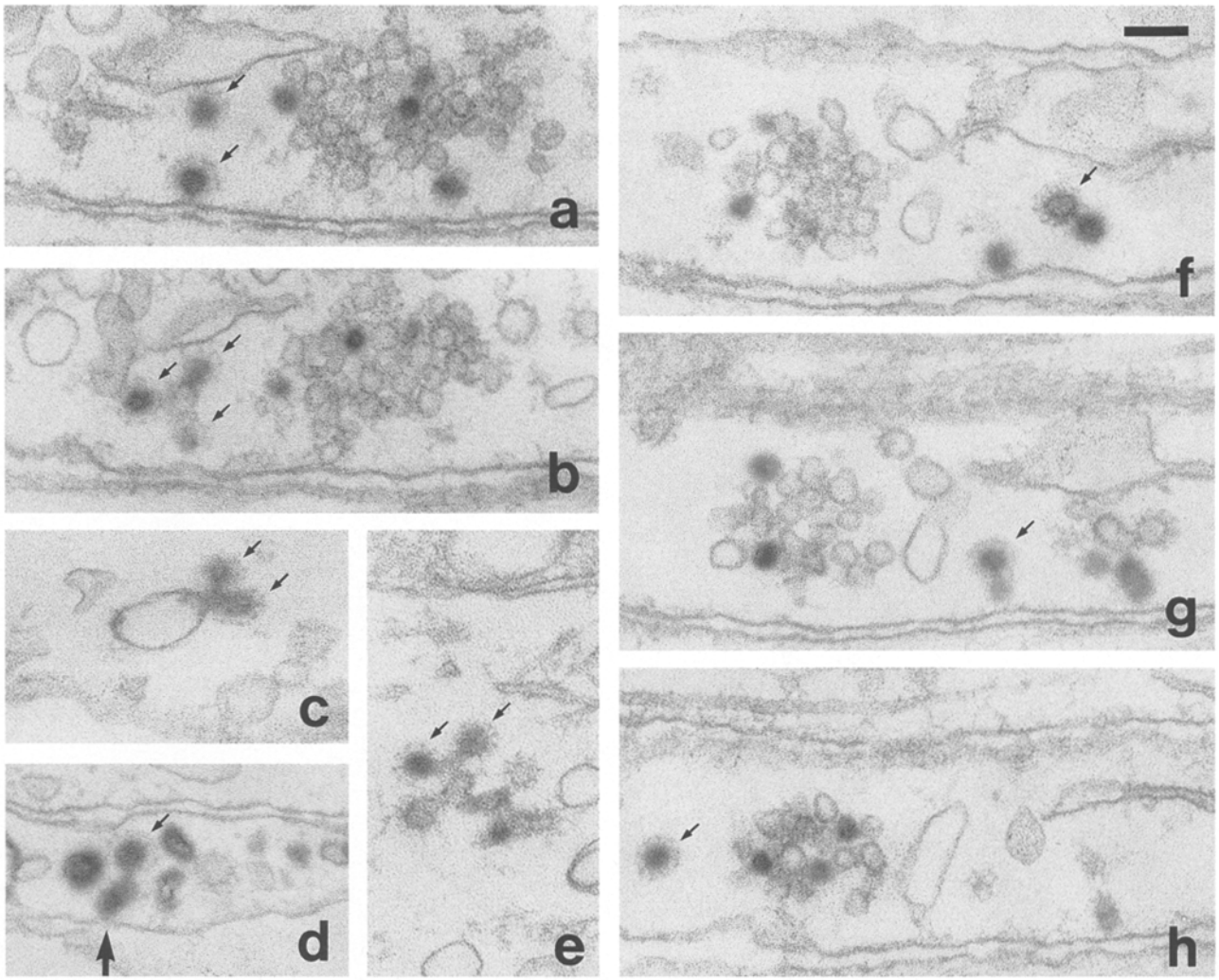

Figure 7. Synaptic vesicle membrane recycling in cultured hippocampal neurons visualized by the uptake of HRP-Syt_{lum}-Ab. Neurons were stimulated for 10 min in depolarizing medium and in the presence of HRP-Syt_{lum}-Ab. (*a* and *b* and *f–h*) Serial sections from two distinct axon terminals. Small arrows point to clathrin-coated vesicular buds or vesicle profiles. Generally, these structures are in close proximity of but not intermixed with synaptic vesicle clusters that include a few HRP-labeled synaptic vesicles. Active zones are out of the plane of sections. Several clathrin-coated buds appear to be interconnected to each other via tubules and cisternae. Interconnections are clearly visible in *c* and *e*. An HRP-labeled vacuole, from which an HRP-labeled clathrin-coated vesicle originates, appears to be continuous with the plasma membrane in *d* (*large arrow*). Bar, 100 nm.

ner and Vallee, 1989). In broken cell preparations, where exogenous dynamin in the added cytosol can freely diffuse, the protein may preferentially assemble at sites where other rings have already formed. In situ, the preferential binding of dynamin to preexisting rings may be counteracted by a limited diffusion and by the short-lived nature of the ring, thereby allowing formation of single rings or very small stacks of rings at each clathrin coated bud.

Narrow Connections between Endosome-like Vacuoles and the Cell Surface

These results are consistent with two possible interpretations of synaptic vesicle recycling. One interpretation is that a vesicle-budding reaction involving clathrin/AP2/AP180/dynamin occurs at two distinct stations of the recycling pathway: budding from the plasmalemma of vesicles targeted to endosomes and budding from the endosome. The other interpretation is that the internal vacuoles from which this budding occurs represent fragments of plasmalemma internalized by bulk endocytosis. To further investigate the relationship of these compartments to the plasmalemma, we analyzed serial sections of lysed synaptosomes incubated with GTPγS. We found that several of these vacuoles were connected to the plasma membrane via narrow necks (Fig. 6, *a–d*), supporting the idea that the membranes of internal vacuoles are directly derived from the plasmalemma. Analysis of serial sections also revealed that most of the profiles that appeared as free clathrin-coated vesicles in these samples were in fact interconnected to each other via highly convoluted tubulovesicular structures (Fig. 6, *e–i*). This observation further emphasizes the abundance of clathrin-mediated budding from intraterminal membranes and confirms the inhibitory role of GTPγS on the fission of clathrin-coated vesicles (Carter et al., 1993; Takei et al., 1995).

Clathrin-mediated Budding from Internal Membranes in Cultured Hippocampal Neurons

The budding reactions described above were observed in lysed nerve terminal preparations under cell-free incubation conditions. It was important to establish whether these events reflect physiological mechanisms that occur in living neurons, and, more specifically, in synaptic vesicle recycling. To address these questions, we examined synaptic vesicle endocytic intermediates in living cultured hippocampal neurons using Syt_{lum}-Abs as a cytochemical marker for synaptic vesicle membranes. These antibodies were previously shown to label specifically synaptic vesicles in hippocampal neurons and to recycle in parallel with the vesicles (Matteoli et al., 1992; Kraszewski et al., 1995). To facilitate the visualization of immunoreactivity, Syt_{lum}-Abs were directly conjugated to HRP. Neurons were exposed to these antibodies for 10 min at 37°C in a depolarizing medium, rinsed, and immediately fixed. EM of these preparations (Fig. 7) revealed typical clathrin-coated buds both on the plasmalemma (e.g., Fig. 7 *a*) and on endosome-like vacuoles (e.g., Fig. 7 *c*). Clathrin-coated buds were in some cases connected to each other to form large tubulovesicular structures (e.g., Fig. 7 *e*). Most of these coated intermediates, as well as many synaptic vesicles and clathrin-coated vesicles, were positive for the HRP reaction product, indicating their direct involvement in synaptic vesicle recycling. When these neurons were analyzed by serial sectioning, some endosome-like membrane profiles were found to be connected to the cell surface (Fig. 7) by narrow openings, as shown above for lysed nerve terminals incubated with GTPγS.

Discussion

We have investigated the mechanisms by which new synaptic vesicles bud from endosome-like intermediates of nerve terminals. Using synaptosomal membranes in a cell-free budding assay and intact cultured hippocampal neurons, we have found that the budding reaction involves both the clathrin coat and dynamin. The similarity of the coats observed on intraterminal vacuoles to the clathrin/dynamin coats present on the plasmalemma was demonstrated by several criteria, including standard EM analysis, sensitivity to different cell-free incubation conditions, and immunogold labeling for clathrin, dynamin, the plasmalemmal clathrin adaptor AP2, and the neuron-specific protein AP180.

These results were complemented by evidence indicating that endosome-like vacuoles of nerve terminals from which clathrin-coated buds originate are closely related to the plasmalemma. Serial section analysis revealed examples of narrow connections between the lumen of some of these structures and the extracellular space. These observations indicate that endosome-like vacuoles originate from deep invaginations of the plasmalemma and are not preexisting internal structures that act as acceptor membranes for plasma membrane–derived endocytic vesicles. On the other hand, these vacuoles become labeled by the fluid-phase marker HRP and retain it when extracellular HRP is removed (see also Fried and Blaustein, 1978). Thus, it is quite likely that they eventually pinch off, although the possibility that they may remain connected to the plasmalemma via narrow openings virtually impermeable to macromolecules cannot be completely ruled out. Even if this was the case, these vacuoles could be considered functionally as internal organelles. Since connections with the plasmalemma were more easily seen in GTPγS-treated preparations than in intact nerve terminals, even the pinching off of these vacuoles, such as the fission of clathrin-coated vesicles, may be controlled by GTPases. For example, the GTPase Rho, which is present in axons (Mackay et al., 1995), was shown to be involved in clathrin-independent endocytosis (Schmalzing et al., 1995). Other GTPases may also be implicated.

If nerve terminal endosome-like intermediates are derived from bulk endocytosis, their membranes are likely to be very similar to the plasmalemma. Thus, clathrin- and dynamin-mediated budding from these internal membranes may be seen as a process homologous to budding of clathrin-coated vesicles from the plasmalemma. This may explain the similar molecular composition of clathrin coats observed on these membranes and at the nerve terminal surface. Sorting of synaptic vesicle membrane proteins from these internalized membrane fragments will leave behind a vacuole that may subsequently fuse again with the plasmalemma. The latter event would represent a sort of homotypic fusion, a type of fusion that is well established for many other cellular membranes (Mellman, 1995).

The possibility that large plasmalemmal fragments may be internalized by bulk endocytosis is supported by several recent findings concerning endocytic mechanisms. Clathrin-independent endocytic pathways, including pathways mediated by large vacuoles, are well documented (Hansen et al., 1993; Cupers et al., 1994; Lamaze and Schmid, 1995). Even in the case of nerve terminals, it had been previously proposed that endocytosis mediated by large cisternae participates in synaptic vesicle recycling after massive incorporation of synaptic vesicle membranes into the plasmalemma caused by either stimulation of exocytosis (Fried and Blaustein 1978; Miller and Heuser, 1984) or a transient block in endocytosis (Koenig and Ikeda, 1989). However, the mechanism of synaptic vesicle formation from these vacuoles remained unclear. A putative role of bulk internalization of plasmalemmal fragments in synaptic vesicle recycling is strikingly suggested by an EM analysis of the recovery from the endocytosis block in nerve terminals of *shibire* mutant flies. These flies harbor a temperature-sensitive mutation of the dynamin (*shibire*) gene. In these flies, synaptic vesicle membranes accumulate in the plasmalemma at the restrictive temperature. After a shift to the permissive temperature, synaptic vesicle reformation is preceded by a transient accumulation of vacuolar intermediates, as if these structures were a precursor of synaptic vesicles (Koenig and Ikeda, 1989). Since vacuoles do not form at the restrictive temperature, this finding raises the question of whether dynamin function is strictly confined to the fission of clathrin-coated vesicles.

A previous study carried out in nonneuronal cells raised the possibility that GTPγS may induce an artifactual mistargeting of clathrin coat components to a perinuclear endosomal compartment (Seaman et al., 1993). Nerve terminal endosomes, however, are organelles well distinct from the perinuclear compartment described by Seaman et al.

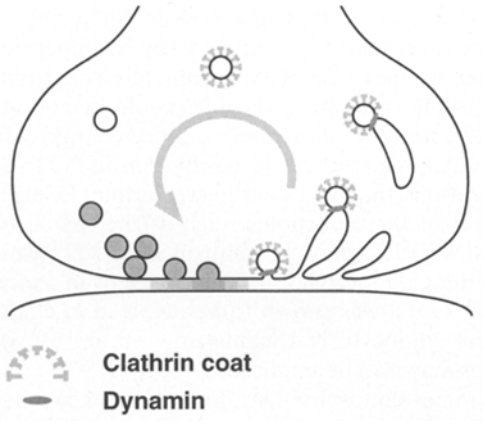

Figure 8. Model of synaptic vesicle recycling suggested by the results reported in this paper. Synaptic vesicles are generated by a single, clathrin coat–mediated, budding step. This budding reaction occurs in parallel from the surface, from deep invaginations of the plasma membrane, or from internal vacuoles that derive from the pinching off of these invaginations. The gray vesicle core represents neurotransmitter content.

(1993). Furthermore, in our experiments, the presence of clathrin-coated buds on membranes of endosome-like structures was clearly not dependent on GTPγS and was observed even in intact neurons. Dynamin rings are generally not seen in intact cells, and we speculate that these negative results may be explained by their transient nature. In fact, we have observed rare examples of electron-dense, ring-like material at the stalk of clathrin-coated vesicles in intact cells (see Fig. 2, inset *A*, in De Camilli and Takei, 1996). Thus, as previously proposed for other types of vesicle transport reactions (Orci et al., 1986; Schekman and Orci, 1996), we speculate that GTPγS is a powerful tool to visualize transient but physiologically important vesicular transport intermediates.

A New Model of Synaptic Vesicle Recycling

Based on our results, we propose a modification of the "classical model" of synaptic vesicle recycling (Heuser and Reese, 1973; Südhof et al., 1993; Schweizer et al., 1995). The "classical model," which received strong support from the characterization of recycling traffic in nonneuronal cells, postulates two vesicle transport steps: one step from the plasmalemma to the endosome mediated by clathrin-coated vesicles, and a second step from the endosome to the plasmalemma mediated by synaptic vesicles. We suggest a model in which the vesicle cycle involves a single coat-mediated budding reaction mediated by clathrin and dynamin. Furthermore, we propose that this budding/fission reaction may occur in parallel from the presynaptic membrane, from deep infolding of this membrane or from plasmalemmal fragments internalized by bulk endocytosis (Fig. 8). The predominance of one of these pathways over the other may depend on the physiological state of the terminal. After massive nerve terminal stimulation, formation of deep infoldings and then vacuoles may be enhanced to compensate for the large increase in the surface area of the nerve ending.

The synaptic vesicle recycling pathway depicted in Fig. 8 helps to explain the rapid kinetics of synaptic vesicle recycling and the lack of morphological and biochemical evidence for the presence of abundant nonclathrin coats in nerve terminals. A recycling pathway mediated by a single vesicle-budding step brings closer together the "classical model" of synaptic vesicle recycling and the "kiss and run" model (Fesce et al., 1994). These two models are generally seen as mutually exclusive. Although the new model eliminates an intermediate sorting station into a bona fide endosome, it preserves a central role of clathrin and the participation of vacuolar structures during intense stimulation. Finally, it reconciles data indicating that both bulk, nonselective, endocytosis by large vacuoles (Fried and Blaustein, 1978; Miller and Heuser, 1984; Koenig and Ikeda, 1989) and selective clathrin-mediated endocytosis (Heuser and Reese, 1973; Miller and Heuser, 1984) participate in synaptic vesicle recycling.

Although images of clathrin-coated buds with a size in the same range of synaptic vesicles were previously observed on vacuolar membranes of nerve terminals (Heuser and Reese, 1973), they were interpreted as incompletely stripped clathrin-coated vesicles in the process of fusion. Several findings obtained after this hypothesis was formulated, including the results of our present study, rule out this interpretation. First, vesicle coats, including clathrin coats, are now known to be shed before docking and fusion (Rothman and Schmid, 1986; Orci et al., 1989; Rothman, 1994). Second, clathrin-coated buds visible in the presence of GTPγS are typically frozen at the deeply invaginated stage, a morphology more consistent with a block at a late stage in budding rather than at an early stage after fusion. Third, the dynamin-coated tubular necks connecting clathrin-coated buds to the donor membranes clearly indicate a block in the budding reaction.

Relationship to Endosomal Budding in Other Cells

The mechanisms of vesicle budding from neuronal endosomes outside nerve terminals and from endosomes of nonneuronal cells remain poorly understood. Brefeldin A, which induces tubulation of the Golgi complex and its collapse into the ER, also induces a tubulation of early endosomes and their mixing with the TGN (Lippincott-Schwartz et al., 1991; Wood et al., 1991). Brefeldin A was reported to produce its effects by inhibiting GDP/GTP exchange on ADP ribosylation factor (ARF) (Donaldson et al., 1992; Helms and Rothman, 1992) and, as a consequence, the ARF-dependent recruitment to membranes of the COPI coat and of the AP1-containing clathrin coat (Orci et al., 1991; Robinson and Kreis, 1992). These findings are therefore consistent with a possible involvment of coats closely related to COPI and AP1 in vesicle budding from endosomes. Evidence for a COPI-like coat on endosomes has recently been reported (Whitney et al., 1995), and sporadic examples of clathrin or clathrin-like coats on endosomal buds have been published over the last several years (Heuser and Reese, 1973; Matlin et al., 1982; Geuze et al., 1984; Killisch et al., 1992; Allen et al., 1992; Rabinowitz et al., 1992). A recent EM study of whole mounts of fibroblastic cells revealed unexpected abundance of brefeldin A–sensitive clathrin-coated buds on endosomal membranes (Stoorvogel et al., 1996). Strikingly, these buds

have a small homogeneous size, similar to the size of synaptic vesicles, which is in contrast to the heterogeneous size of clathrin-coated vesicles that mediate transport from the plasmalemma to early endosomes in nonneuronal cells.

The buds described by Stoorvogel et al. (1996), however, do not appear to be equivalent to the clathrin-coated buds described in our study: they were reported to be negative for the AP2 subunit α-adaptin and, in contrast to synaptic vesicles, they contain transferrin receptors. Furthermore, we have previously shown that nerve terminal endosomes are unaffected by brefeldin A (Mundigl et al., 1993). This is in agreement with the lack of effect of brefeldin A on clathrin coat recruitment mediated by AP2 (Wong and Brodsky, 1992; Robinson and Kreis, 1992). Thus, the clathrin-mediated budding from internal membranes reported here represents a type of budding not reported previously.

Nerve terminal endosome-like intermediates involved in synaptic vesicle recycling may represent a specialized intracellular compartment that is different from classical early endosomes. A unique property of the synaptic vesicle cycle is that it does not require fusion of the recycling membrane with sorting compartments where cargo with different destinations must be sorted away from each other (e.g., segregation of receptors destined for recycling from ligands destined for lysosomes). Thus, an intermediate intracellular station is not necessary. One cannot exclude that a pathway similar to the synaptic vesicle cycle may exist in rudimentary form in all cells. Furthermore, a minor population of more conventional endosomes may be present in nerve terminals and be the target for a subpopulation of clathrin-coated vesicles. A small number of clathrin-coated vesicles larger than synaptic vesicles are present in nerve terminals, and these vesicles were generally unlabeled by HRP/syt_{lum}-Abs (our unpublished observation).

It is now important to further elucidate the relationship between the clathrin/dynamin–mediated budding reaction that occur from nerve terminal endosome-like intermediates and budding mechanisms that occur on other endosomes. It is becoming increasingly clear that "endosome" is the collective name for multiple subcompartments (Robinson et al., 1996). Multiple budding mechanisms can be anticipated. The demonstration that in neuroendocrine cells recycling synaptic vesicle proteins and recycling transferrin and LDL receptors are at least partially segregated into distinct vesicular carriers strongly support this idea (Cameron et al., 1991; Linstedt and Kelly, 1991; Thomas-Reetz and De Camilli, 1994). A further study of neuroendocrine cells that are able to generate bona fide synaptic vesicles, yet do not have the extreme specialization of nerve terminals for synaptic vesicle recycling (Thomas-Reetz and De Camilli, 1994), may help to address these questions by biochemical and morphological techniques.

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