Evidence for a Primary Endocytic Vesicle Involved in Synaptic Vesicle Biogenesis*

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The regulated release of neurotransmitters at synapses is mediated by the fusion of neurotransmitter-filled synaptic vesicles with the plasma membrane. Continuous synaptic activity relies on the constant recycling of synaptic vesicle proteins into newly formed synaptic vesicles. At least two different mechanisms are presumed to mediate synaptic vesicle biogenesis at the synapse as follows: direct retrieval of synaptic vesicle proteins and lipids from the plasma membrane, and indirect passage of synaptic vesicle proteins through an endosomal intermediate. We have identified a vesicle population with the characteristics of a primary endocytic vesicle responsible for the recycling of synaptic vesicle proteins through the indirect pathway. We find that synaptic vesicle proteins colocalize in this vesicle with a variety of proteins known to recycle from the plasma membrane through the endocytic pathway, including three different glucose transporters, GLUT1, GLUT3, and GLUT4, and the transferrin receptor. These vesicles differ from “classical” synaptic vesicles in their size and their generic protein content, indicating that they do not discriminate between synaptic vesicle-specific proteins and other recycling proteins. We propose that these vesicles deliver synaptic vesicle proteins that have escaped internalization by the direct pathway to endosomes, where they are sorted from other recycling proteins and packaged into synaptic vesicles.

The biogenesis of synaptic vesicles is a complex orchestration of events culminating in a vesicle population responsible for the uptake, storage, and regulated secretion of neurotransmitters. Current models of synaptic vesicle biogenesis suggest that at least two pathways exist for the formation of new synaptic vesicles after exocytosis: directly from the plasma membrane and indirectly via an intermediate endosomal compartment (1, 2). In neurons, both a direct pathway, which is at the active zone, and a more distal indirect pathway have been demonstrated at synapses in the Drosophila shibire1ts1 mutant (3). Studies of membrane recycling in hippocampal synapses using the fluorescent dye FM1-43 also suggest that synaptic vesicles formed by endocytosis can fuse with the plasma membrane directly, without passing through an endosomal compartment (4). In the PC12 neuroendocrine cell line, synaptic vesicles are also derived from two different pathways as follows: directly from the plasma membrane (5, 6) as well as from an endosomal intermediate (7–10). Together these data form an emerging model of multiple pathways to recycle both synaptic vesicle proteins and synaptic vesicle membrane.

Although presumably all synaptic vesicle proteins undergo endocytic trafficking, relatively little is known about the intermediate steps in the pathway to mature synaptic vesicles. In order to understand fully the mechanisms underlying synaptic vesicle biogenesis, it is critical that the synaptic vesicle protein trafficking pathways are fully elucidated. In neurons, synaptic vesicle proteins are present in heterogeneous vesicle populations, but the relationship of these compartments to the process of synaptic vesicle formation is almost impossible to determine, given the difficulty of performing a kinetic analysis of synaptic vesicle protein trafficking in brain (11–13). In contrast, PC12 cells have proved to be a useful system for analyzing the trafficking of synaptic vesicle proteins and the biogenesis of synaptic vesicles (5, 6, 8–10, 12, 14–18). We have characterized a vesicular compartment in PC12 cells containing the synaptic vesicle proteins synaptophysin, SV2, and synaptotagmin. These vesicles are distinct in size and protein composition from synaptic vesicles; they contain two different endogenous glucose transporters, GLUT1 and GLUT3, as well as the transferrin receptor (TIR). Moreover, we show that exogenously expressed proteins such as GLUT4 and the sodium-dependent dopamine transporter (DAT) are also present in these vesicles. Our data provide evidence that this compartment is not derived by vesiculation of biosynthetic organelles or the plasma membrane and that vesicles with similar properties are present in rat brain. We suggest that this vesicle is part of the endocytic recycling system in neurons and PC12 cells, potentially operating as a primary endocytic vesicle involved in trafficking synaptic vesicle proteins that have escaped the direct targeting pathway to synaptic vesicles.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—PC12 cells were grown in high glucose Dulbecco’s modified Eagle’s media (Life Technologies, Inc.) supplemented with 2 mM glutamine, 10 units/ml penicillin/streptomycin, 5% horse serum, and 5% defined/supplemented bovine calf serum (HyClone, Logan, UT) in a humidified 37 °C incubator at 10% CO2. For transfection, cells were grown in 15-cm dishes to ~75% confluence (~3 × 105 cells). The transfection method was adapted from Grote et al. (16), and the electroporation conditions were optimized for our cells and

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1 The abbreviations used are: GLUT1, GLUT3, GLUT4, plasma membrane glucose transporter isofoms type 1, 3, and 4, respectively; TIR, transferrin receptor; DAT, dopamine transporter; HA, hemagglutinin; ER, endoplasmic reticulum; Endo H, endoglycosidase H; PNGase F, peptide:N-glycosidase F; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PMSP, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; RT, room temperature; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis.
expression vector. Briefly, cells from one 15-cm dish were harvested by trituration in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS and pelleted for 5 min at 1000 × g. Cells were resuspended in 750 μl per dish electrophoresis buffer (157 mM NaCl, 5 mM KCl, 0.7 mM Na<sub>2</sub>HPO<sub>4</sub>, 6 mM dextrose, 20 mM HEPES, pH 7.2, equilibrated to room temperature (RT) in the presence of 50 μg of proteinase K). Buffer was transferred to a 4.5-ml polypropylene tube (Bio-Rad). After 3–5 min the cells were pulsed at 300 V (rather than 250 V (16)) and 500 microfarads and then transferred immediately to 10 ml of growth media supplemented with 3 mM EGTA prewarmed to 37 °C. After incubating for 30 min at 37 °C with gentle mixing, cells were pelleted for 5 min and re-plated onto two 15-cm polylysine-coated dishes. Sodium butyrate was added to a final concentration of 5 mM 24–30 h after electroporation, and the cells were harvested for experiments 16–18 h after the addition of sodium butyrate.

cDNA Constructs—The rat GLUT1 cDNA in the retroviral expression vector pDOJ was obtained from Dr. Morris J. Birnbaum (19). An epitope specific for the human GLUT1 amino acid sequence was engineered by changing amino acid 239 from Arg to His (19). The GLUT1 cDNA was ligated into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA). The myc epitope (AAAEQKLISEEDLL) was inserted 3′ of the coding region by first engineering by polymerase chain reaction an in-frame NotI restriction site between the last coding nucleotide and the stop codon. The DNA between this NotI site and the XbaI site in the polymerlinker was excised with a synthetic double-stranded DNA fragment containing the myc epitope. The HA epitope (IDYPDYVPDYA) was inserted into this cDNA by polymerase chain reaction between amino acids 53 and 54 in the exofacial loop between transmembrane domains 1 and 2. This chimera has previously been shown to be correctly processed in COS-7 and Chinese hamster ovary cells (20). The sequences of all constructs were confirmed by sequencing with Sequenase (Stratagene, La Jolla, CA). The rat GLUT4 cDNA was also obtained from Dr. Morris J. Birnbaum (University of Pennsylvania) and subcloned into the expression vector pCMV1.2 (21).

The human dopamine transporter was expressed by transfection of pcDNA3.1-DAT (21).

Subcellular Fractionation—PC12 cells were homogenized and analyzed in glycerol velocity gradients as in Clift-O’Grady et al. (12) and Schmidt et al. (8), with modifications. Cells were harvested in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS, pelleted for 5 min, and resuspended at 4 °C in 250 μl of homogenization buffer consisting of either 10 mM HEPES or 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA (HB) with 2 mM PMSF, 2 μg/ml aprotinin/leupeptin/pepstatin. All subsequent steps were carried out at 4 °C unless otherwise noted. Cells were homogenized with 10 passes through a stainless steel ball-bearing homogenizer (Berti-Tech Engineering, Lebanon, PA, CA) with a 12-gauge needle. The homogenate was spun for 10 min at 1,400 × g in a microcentrifuge, and the supernatant was transferred to a Beckman thin-walled polycarbonate tube. The pellet was resuspended in 250 μl of HB plus protease inhibitors and passed through the homogenizer 4 times. This homogenate was then spun as before (P1) and the supernatants combined (S1). The S1 was spun at 40,000 rpm (66,000 × g) for 15 min in a Beckman J2-21 rotor (P2) at 4 °C. The supernatant (P2) was then used for further analysis.

As a control for artifacts of our homogenization method, cells were also homogenized with a Teflon/glass homogenizer with 15 strokes at 1000 × g at 4 °C. The homogenate was spun for 10 min at 1,400 × g, and the pellets were resuspended in 100 μl electroporation buffer (137 mM NaCl, 2.4 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM HEPES, 10 mM glucose, pH 7.4) and centrifuged at 10,000 rpm in an SS34 rotor for 10 min at 4 °C. The pellet was resuspended in 1 μl of ice-cold distilled H<sub>2</sub>O to lyse the synaptosomes, then transferred to a small homogenizer (Thomas number A with type 5 ridged pestle), and resuspended with 5 strokes at 200 rpm. 0.1 ml of intact vesicles was loaded onto 1.5 ml 5–25% glycerol velocity gradients as in Clift-O’Grady et al. (12) and Calabrese et al. (18), centrifuged at 40,000 rpm (66,000 × g) for 1.5 hr in a Beckman TJ 150.4 rotor (Dynal), and were washed twice with 1 ml of HB, 0.1% BSA, once with 1 ml of HB at 4 °C, 10 min each. The supernatants were precipitated with trichloroacetic acid. Beads were resuspended in 20 μl of 1 volume SDS PAGE sample buffer for analysis.

In some cases, organelle immunosialation was performed on individual gradient fractions. Briefly, 0.6 mg of goat anti-mouse IgG-coated M-500 Dynabeads (Dynal, Great Neck, NY) were coated with 0.5 μg of mAb SY38. This amount of beads plus primary antibody was incubated with one gradient fraction overnight at 4 °C. Beads were then washed and resuspended in bound organelles pelleted for 1.5 h at 200,000 × g in a Beckman TLA 100.4 rotor.

Rat Brain Homogenates—Approximately 2 g of frozen rat cerebral cortex (Zivic-Miller, Porterville, PA) was homogenized in 40 ml of ice-cold buffer (0.32 M sucrose, 5 mM HEPES, 0.1 mM EGTA, pH 7.4) with 14 strokes in a glass-Teflon homogenizer. The homogenate was spun in an SS34 rotor at 3500 rpm for 10 min at 4 °C. The supernatant (S1) was then transferred into fresh tubes and spun in the same rotor at 8500 rpm for 10 min at 4 °C. The resulting pellet (P2) was resuspended in 5 ml of ice-cold homogenization buffer, layered on top of 20 ml of 0.8 M sucrose in 5 mM HEPES, 0.1 mM EGTA, pH 7.4, and spun in an SS34 rotor at 8500 rpm for 25 min at 4 °C. The clear middle layer was removed, gradually mixed with an equal volume of 1× synaptosome buffer (137 mM NaCl, 2.4 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM HEPES, 10 mM glucose, pH 7.4), and centrifuged at 10,000 rpm in an SS34 rotor for 10 min at 4 °C. The pellet was resuspended in 1 ml of ice-cold distilled H<sub>2</sub>O to lyse the synaptosomes, then transferred to a small homogenizer (Thomas number A with type 5 ridged pestle), and resuspended with 5 strokes at 200 rpm. 0.1 ml of 1× synaptosome buffer was then added, and a protein assay was performed (BCA protein assay). Protein loading was adjusted to 0.1% of total protein loaded. Beads were excised and replaced with a synthetic double-stranded DNA encoding a myc epitope (AAAEQKLISEEDLL) in-frame with a synthetically created restriction site between the last coding nucleotide and the STOP codon. The cDNA was excised and replaced with a synthetic double-stranded DNA encoding a myc epitope (AAAEQKLISEEDLL) in-frame with a synthetically created restriction site between the last coding nucleotide and the STOP codon. The cDNA was excised and replaced with a synthetic double-stranded DNA encoding a myc epitope (AAAEQKLISEEDLL) in-frame with a synthetically created restriction site between the last coding nucleotide and the STOP codon. The DNA between this NotI site and the XbaI site in the polymer linker was excised with a synthetic double-stranded DNA fragment containing the myc epitope. The HA epitope (IDYPDYVPDYA) was inserted into this cDNA by polymerase chain reaction between amino acids 53 and 54 in the exofacial loop between transmembrane domains 1 and 2. This chimera has previously been shown to be correctly processed in COS-7 and Chinese hamster ovary cells (20). The sequences of all constructs were confirmed by sequencing with Sequenase (Stratagene, La Jolla, CA). The rat GLUT4 cDNA was also obtained from Dr. Morris J. Birnbaum (University of Pennsylvania) and subcloned into the expression vector pCMV1.2 (21).

The human dopamine transporter was expressed by transfection of pcDNA3.1-DAT (21).
Biotinylation of Cell-surface Proteins—PC12 cells were transfected as described above, except that after transfection the cells were plated onto 15-cm dishes coated with 5 μg/cm² rat tail collagen (Collaborative Research, Bedford, MA). Forty-eight hours after transfection the cells were equilibrated to 4 °C for 1 h, after which the cells were washed 2–3 times with cold phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) at 4 °C. All subsequent steps were done at 4 °C. Cells used for the 4 °C labeling control experiment were equilibrated to 4 °C for 20 min prior to the addition of antibody. After incubation with antibody the cells were washed twice with 4 °C PBS, after which the 37 °C cells were allowed an additional 10 min to equilibrate to 4 °C. All subsequent steps were carried out at 4 °C. The 37 °C cells were then incubated with 30 μg/ml Pronase E (Sigma) in PBS, 10 mM EDTA for 5 min to strip antibody remaining at the cell surface, after which all Pronase buffer was removed (22). Pronase treatment was immediately stopped by the addition of complete media containing 2 μM PMFS plus 5 μg/ml aprotonin/leupeptin/pepsatin/benzamidine. Cells were resuspended, pelleted, and washed 2 more times with PBS, 10 mM EDTA plus protease inhibitors. Final cell pellets were resuspended in HB plus protease inhibitors for homogenization and glycerol velocity gradient analysis (see above).

Antibodies—The anti-synaptotagmin monoclonal antibody Cl 41.1 was the generous gift of Dr. Reinhard Jahn (Max Planck Institute, Göttingen, Germany). 12CA5 hybridoma cells were purchased from ATCC (Manassas, VA). For cell-surface labeling mAb 12CA5 was purified from hybridoma supernatant using the Pierce ImmunoPure (G) IgG purification kit. 3F10 anti-HA high affinity rat monoclonal antibody, SY38 anti-synaptophysin, anti-rat IgG, anti-mouse IgG, and anti-rabbit IgG were all purchased from Amersham Pharmacia Biotech. Rabbit anti-GLUT3, rabbit anti-GLUT1, and rabbit anti-GLUT4 polyclonal antibodies were purchased from Chemicon (Temecula, CA). Anti-Thy-1 mouse monoclonal antibody was purchased from StressGen (Victoria, British Columbia, Canada). Streptavidin-horseradish peroxidase was purchased from Amersham Pharmacia Biotech.

RESULTS

GLUT1 and Synaptophysin Co-localize in a Distinct Vesicle Compartment in Glycerol Velocity Gradients—Previous experiments have examined the distribution of exogenously expressed GLUT1 and GLUT4 in PC12 cells. GLUT1 is predominantly expressed in the plasma membrane (23), whereas GLUT4 is found in both large dense core vesicles (23) and a vesicle population that sediments more rapidly than synaptic vesicles in glycerol velocity gradients (24). Initially, we were interested in using GLUT1 as a control protein for experiments on synaptic vesicle protein targeting. When we transfected HA-tagged GLUT1 into PC12 cells and examined its distribution relative to synaptic vesicle proteins in glycerol velocity gradients, we found that although the majority of the protein was found in heavier membranes, including the plasma membrane and endosomes as described previously (see P1 and P2 in Fig. 9) (23), a peak of GLUT1HA immunoreactivity was reproducibly localized to a region of the gradient that overlapped with but was clearly distinct from the peak of immunoreactivity for the synaptic vesicle protein synaptophysin (Fig. 1A). We occasionally observed a shoulder (Fig. 1B, arrow), and infrequently a distinct second peak (Fig. 2), of synaptic vesicle protein immunoreactivity, including synaptophysin, SV2, and synaptotagmin, at the same position as the GLUT1 peak. This distribution was also seen when we expressed a chimeric protein in which the amino-terminal cytoplasmic domain of GLUT1 was replaced with the amino-terminal cytoplasmic domain of SV2A (data not shown). To determine if this colocalization in the second vesicle peak in the gradient represented comigration of two different vesicle populations or colocalization of GLUT1 and synaptophysin in the same organelles, we immunoisolated vesicles containing synaptic vesicle proteins from this region of the glycerol gradient with an antibody to a cytoplasmic epitope of synaptophysin. Vesicles immunoprecipitated with synaptophysin antibodies were then analyzed by SDS-PAGE and Western blotting with antibodies to the HA epitope tag in GLUT1. Fig. 3A shows that antibodies to synaptophysin specifically and quantitatively immunoprecipitate the GLUT1HA present in these fractions, providing strong evidence for the presence of both proteins in the same vesicle population. Furthermore, no detectable GLUT1HA is immuno-
precipitated by synaptophysin antibodies from fractions containing the major peak of synaptophysin immunoreactivity, suggesting that GLUT1 is excluded from the classical synaptic vesicles in PC12 cells. Fig. 3A shows that antibodies to SV2, another synaptic vesicle-specific protein, are also capable of precipitating GLUT1HA, confirming that the presence of other synaptic vesicle proteins in this vesicle population are consistent with the bimodal distribution occasionally seen in Western blots (Fig. 2).

To determine if the presence of GLUT1HA in these vesicles was due to high levels of expression resulting from the transient transfection, we examined the distribution of endogenous GLUT1 by Western blotting and by organelle immunoisolation (Fig. 4). These experiments revealed that endogenous GLUT1 is also present in these vesicles, suggesting that localization to this compartment is not an artifact of the transfection procedure or simply the result of high levels of protein expression.

**Comigration of GLUT3, GLUT4, and GLUT1**—Previous work has demonstrated the presence of a vesicle population in rat brain and PC12 cells that is distinct from synaptic vesicles in size and density and further characterized by the copurification of aminopeptidase B activity, GLUT3, and synaptic vesicle proteins in both sucrose equilibrium and velocity gradients (13, 25). In adipose cells, aminopeptidase activity is enriched in an insulin-responsive compartment that also contains GLUT4 (26, 27). To determine whether the vesicle compartment containing GLUT1 also contains other glucose transporter isoforms, we analyzed the distribution of endogenous GLUT3 in adipose cells using immunoblotting and organelle immunoisolation.

**Fig. 2.** Bimodal distributions of SV2, synaptophysin, and synaptotagmin. SV2, synaptophysin and synaptotagmin occasionally exhibit marked bimodal distributions consistent with their presence in two distinct vesicle populations. Homogenates of PC12 cells were analyzed by separation in glycerol velocity gradients followed by Western blotting as described in Fig. 1, except that the membranes in each gradient fraction were pelleted for 2 h at 200,000 × g prior to solubilization in SDS sample buffer, rather than precipitated with trichloroacetic acid. A, a Western blot illustrating the bimodal distribution of endogenous SV2 observed in two independent gradients. B, quantitation of SV2 protein analyzed in A. Protein bands were digitally scanned and quantitated by densitometry. C, Western blot from a separate experiment, in which gradient fractions were probed for the distributions of both synaptotagmin (Stagmin) and synaptophysin (Synphysin). D, quantitation of the proteins by densitometry: synaptotagmin (□) and synaptophysin (●). Data are expressed as arbitrary units (AU). Both synaptophysin and synaptotagmin are distributed between a peak in fraction 6, corresponding to classical synaptic vesicles, and a peak in fraction 9, coincident with the GLUT1-containing vesicles.
glycerol velocity gradients. As shown in Fig. 5, GLUT3 migrates at the same position as GLUT1 relative to the peak of synaptophysin in these gradients, suggesting that these vesicles are involved in trafficking of this glucose transporter as well in PC12 cells. Furthermore, we found that synaptophysin and GLUT3 in membranes prepared from rat brain showed similar relative distributions in glycerol velocity gradients, as described previously (13, 25). We next examined the distribution of exogenously expressed GLUT4 in the gradient. Previous experiments have demonstrated that exogenously expressed GLUT4 in PC12 cells localizes both to a small vesicle distinct from synaptic vesicles (24) and to large dense core vesicles (23). These studies did not look for the presence of synaptophysin in the small vesicles by organelle immunolocalization, presumably because the peak immunoreactivities of the two proteins are so distinct. When we examined the distribution of GLUT4 in fractions from the glycerol velocity gradient, we found that GLUT4 migrates at approximately 15% glycerol (data not shown), the same concentration as GLUT1 and GLUT3 (see Fig. 5A), and consistent with the distribution described by Herman et al. (24). These results suggest that multiple glucose transporters can be targeted to this vesicle.

Distribution of Other Proteins in the GLUT1 Vesicle—The presence of three different glucose transporter isoforms along with synaptic vesicle proteins suggested that this compartment might be involved in general trafficking of membrane proteins in PC12 cells, without specificity for the glucose transporters or proteins of the regulated secretory pathway. To determine if other proteins are targeted to this vesicle population, we examined the distribution of endogenous transferrin receptor, a protein that constitutively recycles between the plasma membrane and the endosomal recycling system. Transferrin receptor immunoreactivity in the glycerol gradient was concentrated in the same fractions as the second peak of synaptophysin, consistent with the localization of the glucose transporters (Fig. 6). We also looked for the presence of the plasma membrane dopamine transporter, which mediates the high affinity, sodium-dependent uptake of monoamines across the plasma membrane. The dopamine transporter is excluded from synaptic vesicles in rat brain and from synaptic vesicles in PC12 cells following dopamine overexpression (21, 28). The dopamine transporter was enriched at approximately 15% glycerol, the same concentration of glycerol as GLUT1, GLUT3, and GLUT4 (data not shown). The presence of GLUT1, DAT, and the TfR in these vesicles suggests that this vesicle is likely to represent a general component of the endocytic recycling pathway as opposed to a specifically regulated, postendosomal secretory vesicle analogous to synaptic vesicles or insulin-responsive vesicles in adipocytes and skeletal muscle.

Are the Vesicles Derived from ER, Golgi, or Plasma Membrane?—We next sought to exclude the possibility that the vesicle represents fragments of biosynthetic compartments that have vesiculated during homogenization. Both the ER and Golgi are complex tubulovesicular networks involved in the processing of secretory proteins and membrane-bound proteins en route to post-Golgi compartments, including the plasma membrane, lysosomes, and synaptic vesicles. Since synaptophysin and GLUT1 are integral membrane glycoproteins that follow this pathway, the biosynthetic compartment in which they reside can be assessed by the extent of each protein’s glycosylation. Newly synthesized glycoproteins are cotranslationally modified in the ER by the addition of a core high mannose N-linked oligosaccharide that is subsequently modified and elaborated within the ER and the Golgi, resulting in the formation of either hybrid or complex oligosaccharide chains. Glycoproteins within the ER and cis-Golgi still contain N-linked high mannose oligosaccharides that are susceptible to removal by the enzyme Endo H, whereas glycoproteins beyond the cis-Golgi have had these oligosaccharides fully modified to yield hybrid or complex structures that are resistant to Endo H. All three types of oligosaccharides are susceptible to digestion with PNGase F.

We took advantage of these biochemical properties to determine whether the GLUT1 compartment is vesiculated ER or cis-Golgi. Homogenates of PC12 cells transfected with GLUT1HA were resolved in a glycerol gradient and the frac-
tions of the gradient in which GLUT1HA and synaptophysin overlap were pooled and immunosolated with antibodies to synaptophysin. Both synaptophysin and GLUT1HA within this vesicle population were found to be completely resistant to Endo H digestion (Fig. 7A), thus ruling out ER and cis-Golgi as sources of this material. GLUT1HA in the P2 fraction contains a mixture of Endo H-sensitive and Endo H-resistant GLUT1HA protein, suggesting that the majority of ER remains in the P2 (data not shown). In addition, we used calnexin, a resident ER protein, to determine the presence of ER vesicles in our GLUT1HA-containing fractions (Fig. 7B). Although we were able to detect a significant amount of calnexin in the P2 fraction, none was seen in the immunoisolated organelles (data not shown).

Further along the biosynthetic pathway, the enzymes in distal portions of the Golgi complete the modification of oligosaccharides. One of these enzymes, a-mannosidase II, is localized to the medial-Golgi, making it a biochemical marker of this compartment. In our organelle immunoisolation experiments a-mannosidase II was undetectable in the GLUT1 vesicles (Fig. 7C). Although a-mannosidase II was present in the same fractions as GLUT1 in the velocity gradient (as seen in the organelle IP supernatants, Fig. 2), the amount that copurified with synaptophysin after organelle immunoprecipitation was not above background (control mouse IgG beads). Taken together, these experiments suggest that these vesicles are not derived from the ER or Golgi.

The GLUT1 Vesicle Is Not Vesiculated Plasma Membrane—Since approximately 80% of GLUT1 is reportedly in the plasma membrane in PC12 cells (23), while less than 2% of synaptophysin is found in the plasma membrane at steady state (5), we used several approaches to rule out the possibility that this vesicle arises by vesiculation of the plasma membrane. We surface-labeled proteins by biotinylation or antibody binding at 4 °C and looked for the presence of labeled protein in the appropriate fractions in glycerol velocity gradients.

When intact cells were biotinylated at 4 °C, no labeled synaptophysin or GLUT1HA was detected in either peak synaptic vesicle fractions or in the overlapping fractions that contain GLUT1 and synaptophysin. PC12 cells transfected with GLUT1HA were biotinylated with membrane-impermeant sulfo-NHS-SS-biotin at 4 °C. Fractions of a glycerol gradient containing all detectable synaptophysin were individually sol-
FIG. 8. The GLUT1-containing vesicles in the glycerol gradient are not fragments of the plasma membrane. PC12 cells expressing GLUT1HA were labeled at 4 °C with the membrane-impermeant sulfo-NHS-SS-biotin, and the homogenate was resolved in a 5–25% glycerol velocity gradient. Fractions 4–12 were solubilized in detergent and incubated overnight with streptavidin (SA) beads. The beads were pelleted, and the supernatants were precipitated with trichloroacetic acid. A, biotinylated proteins in the beads (left) and non-biotinylated proteins in the supernatants (right) were analyzed by 10% SDS-PAGE and Western blotting. No biotinylated GLUT1HA or synaptophysin (Sphysin) is detectable in the glycerol gradient fractions. Both of these biotinylated proteins were present in the P1 and P2 fractions (data not shown), indicating the presence of plasma membrane in these fractions and providing an internal positive control for biotinylation. The blots were first probed for synaptophysin with SY38 and then for HA with 3F10. B, fractions 7–9 from a second glycerol gradient were pooled, and intact vesicles were analyzed for the presence of biotinylated proteins. Two equal aliquots (100 μg of protein each) of the 7–9 pool were incubated with SY38 α-synaptophysin beads to immunoprecipitate intact vesicles. One sample was solubilized and incubated overnight with streptavidin beads (SY38/SA). Shown here are the streptavidin pellet (P) and trichloroacetic acid-precipitated supernatant (S). The other SY38 immunoprecipitate and its trichloroacetic acid-precipitated supernatant were analyzed directly for comparison. The top half of the blot was probed with 3F10 α-HA monoclonal antibody (GLUT1HA), and the bottom half was probed separately for synaptophysin with SY38 (Sphysin). Taken together, these data suggest that the GLUT1-containing membranes in the glycerol gradient are not fragments of the plasma membrane.

We have identified a vesicular compartment in PC12 cells with a counterpart in rat brain that is characterized by the colocalization of synaptic vesicle proteins with a variety of other proteins, including multiple different transporters and the transferrin receptor. In addition to providing evidence for cosedimentation of membranes containing these proteins with synaptic vesicle proteins, we show by organelle immunosolation that synaptic vesicle proteins and GLUT1 are in the same vesicle and that these vesicles are not derived from a shared component of the biosynthetic pathway or by vesiculation of the plasma membrane.

**DISCUSSION**

We have characterized in this paper have been described in PC12 cells and rat brain by two different laboratories. The earliest work, by Herman et al. (24), demonstrated that GLUT4, exogenously expressed in PC12 cells by DNA transfection, colocalizes with a distinct vesicle population that overlaps in 5–25% glycerol gradients and Western blotting. The mouse 12CA5 antibody internalized by binding to the exofacial HA epitope tag was detected in gradient fractions with anti-mouse biotin and streptavidin-HRP. Specificity of the 12CA5 antibody uptake for the presence of the HA epitope in GLUT1 was confirmed by the absence of antibody uptake by cells expressing transfected GLUT1 without the HA epitope (data not shown). GLUT1HA protein was detected with a rat anti-HA monoclonal antibody 3F10. Comparison of the peak immunoreactivities for internalized antibodies and the GLUT1HA protein is shown in Fig. 10. The major immunoreactivity for the internalized antibody and the GLUT1HA protein overlap in the same region of the glycerol gradient, suggesting that GLUT1 colocalizes with synaptic vesicles in a vesicle derived by endocytosis from the plasma membrane.
Endocytic Trafficking of Synaptic Vesicle Proteins

Precisely with the peak of synaptophysin immunoreactivity (Sphysin) and synaptophysin, the peak of Thy-1 immunoreactivity colocalizes with GLUT1HA cDNA were homogenized and analyzed in glycerol phosphatidylinositol-linked protein that is found in both the plasma membrane and synaptic vesicles. The peak of Thy-1 immunoreactivity colocalizes with synaptic vesicle proteins around fraction 6, whereas membranes containing GLUT1HA are enriched in fractions 8–10. P1 and P2, equal aliquots (0.5% v/v) of the membrane pellets P1 and P2 (66,000 × g for 15 min) were loaded on the gel for comparison to the gradient fractions. B, quantitation of the Western blot in A. A, GLUT1HA; ○, Thy-1; ●, synaptophysin. The absence of Thy-1 in fractions containing GLUT1 provides additional evidence that GLUT1-containing vesicles are not fragments of the plasma membrane. Furthermore, the enrichment of Thy-1 in fractions 6 demonstrates that “classical” synaptic vesicles have a unique protein composition compared with the GLUT1-containing vesicles.

In our glycerol gradient. Because the GLUT4 peak is quite distinct from the synaptophysin peak, these authors assumed that synaptic vesicle proteins and GLUT4 were not in the same vesicles. However, due to substantial overlap between the two peaks, a second minor peak of synaptic vesicle proteins is likely to be obscured by the major overlapping immunoreactivity of synaptic vesicle proteins in the classic position of the gradient. By organelle immunoisolation we have demonstrated the colocalization of synaptic vesicle proteins and the glucose transporters in the same vesicles. Whereas these authors suggested that GLUT4 is targeted to a specialized vesicle corresponding to the insulin-regulated secretory vesicle present in adipocytes, our data suggest little specificity in the targeting of GLUT4 to this compartment.

While this manuscript was in preparation, a second description of these vesicles in PC12 cells appeared in Thoidis et al. (25). Using different gradient systems, these authors demonstrated two populations of vesicles in PC12 cells, one enriched for synaptic vesicle proteins only and a second population characterized by copurification of synaptic vesicle proteins with endogenous GLUT3 and aminopeptidase B. These authors also demonstrated the copurification of GLUT3 and aminopeptidase B with synaptic vesicle proteins in a similar vesicle population in rat brain. Since release of aminopeptidase B is reportedly calcium-dependent in PC12 cells, and aminopeptidase B is found in the insulin-responsive population of GLUT4 vesicles in adipocytes (26, 27), these authors hypothesized that the vesicle population is a distinct type of regulated secretory vesicle, perhaps a second population of synaptic vesicles. The types and diversity of proteins present in these vesicles suggest that these vesicles are unlikely to represent a regulated secretory pathway analogous to synaptic vesicles in neurons and endocrine cells or the insulin-responsive secretory vesicles in adipocytes and skeletal muscle. The proteins we have identified in this vesicle population are all proteins that traffic through the endosomal system, and several of them are constitutively abundant in the plasma membrane of neurons and PC12 cells, including the glucose transporters GLUT1 and GLUT3, the monoamine transporter DAT, and the transferrin receptor. Furthermore, unlike classical synaptic vesicles, this second vesicle population does not appear to exclude any membrane proteins that we have examined. Further evidence for the specificity of synaptic vesicle protein targeting as compared with the vesicle population we have described is the inclusion of the plasma membrane protein Thy-1, but not other plasma membrane proteins, in classical synaptic vesicles in PC12 cells and neurons.

If these vesicles are not likely to represent an alternative population of synaptic vesicles, then what are they? Our data show they are not derived from biosynthetic compartments, including the ER and the Golgi, nor do they appear to arise by vesiculation of the plasma membrane. The characteristics of these vesicles suggest two possibilities, either a generic postendosomal secretory vesicle or a primary endocytic vesicle. In support of a postendosomal identity, we have found that the steady state level of this vesicle population is decreased after

![Fig. 10. The vesicles containing GLUT1 are derived by endocytosis from the plasma membrane. To demonstrate the endocytic origin of the GLUT1 vesicles, we measure the uptake of anti-HA monoclonal antibody 12CA5 into PC12 cells expressing GLUT1HA. PC12 cells expressing GLUT1 with an exofacial HA epitope tag were incubated for 1 h in the presence of 5 μg/ml 12CA5 antibody at either 37 °C (●) or 4 °C (○) to block endocytosis. The cells were then equilibrated to 4 °C and incubated with 30 μg/ml Pronase E for 5 min at 4 °C to strip the cell surface of antibody. The cell homogenates were then resolved in a 5–25% glycerol velocity gradient as in Fig. 1. 12CA5 antibody was detected by Western blotting of the gradient fractions using an anti-mouse IgG-biotin and streptavidin-HRP with enhanced chemiluminescence detection. The distribution of GLUT1HA (○) is shown for comparison. GLUT1HA was detected using the rat anti-HA monoclonal antibody 3F10, followed by anti-rat IgG-biotin and streptavidin-HRP. Levels of immunoreactive proteins were assessed by densitometric scanning of the exposed film and expressed as arbitrary units (AU) for the purpose of comparison. As a control for nonspecific uptake of antibody, cells expressing GLUT1 without an exofacial HA tag were incubated with 12CA5; antibody uptake under these conditions was equivalent to the 4 °C control (data not shown). ●, 12CA5 antibody at 37 °C; ○, 12CA5 antibody at 4 °C; □, GLUT1HA protein.

![Fig. 9. Thy-1, a plasma membrane protein that is also targeted to synaptic vesicles, is not enriched in the GLUT1 vesicle population. A, Western blot illustrating the distribution of Thy-1, a glycoprophatidylinositol-linked protein that is found in both the plasma membrane and synaptic vesicles (30). PC12 cells that were transfected with GLUT1HA cDNA were homogenized and analyzed in glycerol velocity gradients for the relative distributions of GLUT1HA, Thy-1, and synaptophysin. The peak of Thy-1 immunoreactivity colocalizes precisely with the peak of synaptophysin immunoreactivity (Sphysin), around fraction 6, whereas membranes containing GLUT1HA are enriched in fractions 8–10. P1 and P2, equal aliquots (0.5% v/v) of the membrane pellets P1 and P2 (66,000 × g for 15 min) were loaded on the gel for comparison to the gradient fractions. B, quantitation of the Western blot in A. △, GLUT1HA; ○, Thy-1; ●, synaptophysin. The absence of Thy-1 in fractions containing GLUT1 provides additional evidence that GLUT1-containing vesicles are not fragments of the plasma membrane. Furthermore, the enrichment of Thy-1 in fractions 6 demonstrates that "classical" synaptic vesicles have a unique protein composition compared with the GLUT1-containing vesicles.]
prolonged incubation of PC12 cells at 15 °C, consistent with the decrease in a similar vesicle population containing exogenously expressed GLUT4 in Chinese hamster ovary cells (31). However, although endocytosis is not blocked at this temperature, it is likely to be much reduced (32), so sensitivity to 15 °C alone is not sufficient evidence to exclude the possibility that these vesicles are primary endocytic vesicles. In strong support of a primary endocytic identity, two distinct vesicle populations with the characteristics of this vesicle and of synaptic vesicles have been observed in coated vesicle preparations from rat brain after the coats have been removed (13, 25). The presence of two types of clathrin-coated vesicles in brain homogenates has previously been reported, one enriched in Na+,K+-ATPase, consistent with a more generic endocytic pathway (33). The latter pathway was enriched in coated vesicle preparations from cultured glial cells, again consistent with a more generic endocytic function. In agreement with these results, Thoitsid et al. (13, 25) also found that the GLUT3/aminopeptidase vesicles have reduced amounts of the V-type ATPase compared with synaptic vesicles.

Only a small percent of the total protein for each of the proteins we have examined in PC12 cells is expressed in these vesicles. Our ability to detect these proteins in this vesicle compartment is likely to be related to the level of a protein’s flux through the endocytic pathway. For example, proteins with efficient internalization signals such as GLUT4 and the Na+/K+-ATPase are primary endocytic vesicles, and the other enriched in Na+,K+-ATPase, consistent with a more generic endocytic pathway (33). The latter pathway was enriched in coated vesicle preparations from cultured glial cells, again consistent with a more generic endocytic function. In agreement with these results, Thoitsid et al. (13, 25) also found that the GLUT3/aminopeptidase vesicles have reduced amounts of the V-type ATPase compared with synaptic vesicles.

REFERENCES
1. Cremona, O., and De Camilli, P. (1997) Curr. Opin. Neurobiol. 7, 323–330
2. Hannah, M. J., Schmidt, A. A., and Huttner W. B. (1999) Annu. Rev. Cell Dev. Biol. 15, 783–798
3. Koenig, J. H., and Ikeda, K. (1996) J. Cell Biol. 135, 797–808
4. Murthy, V. N., and Stevens, C. F. (1988) Nature 333, 497–501
5. Schmidt, A., Hannah, M. J., and Huttner, W. B. (1997) J. Cell Biol. 137, 445–458
6. Shi, G., Faucon, V., Ross, J., Dell'Angelica, E. C., and Kelly, R. B. (1998) J. Cell Biol. 143, 947–956
7. Grote, E., and Kelly, R. B. (1996) J. Cell Biol. 132, 537–547
8. Desnos, C., Clift-O'Grady, L., and Kelly, R. B. (1995) J. Cell Biol. 130, 1041–1049
9. Lichtenstein, Y., Desnos, C., Faucon, V., Kelly, R. B., and Clift-O'Grady, L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11223–11228
10. Blagoveshchenskaya, A. D., Hewitt, E. W., and Cutler, D. F. (1999) J. Cell Biol. 145, 1419–1433
11. Huttner, W. B., Schiebeler, W., Greengard, P., and De Camilli, P. (1983) J. Cell Biol. 96, 1374–1388
12. Clift-O'Grady, L., Linstedt, A. D., Lowe, A. W., Grote, E., and Kelly, R. B. (1990) J. Cell Biol. 110, 1693–1703
13. Thoitsid, G., Chen, P., Pushkin, A. V., Vallega, G., Leeman, S. E., Fine, R. E., and Kandror, K. V. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 183–188
14. Cameron, P., Mundigl, O., and De Camilli, P. (1995) J. Cell Sci. 17, (suppl.) 95–100
15. Clift-O'Grady, L., Desnos, C., Lichtenstein, Y., Faucon, V., Horng, J. T., and Kelly, R. B. (1998) Methods Companion Methods Enzymol. 16, 150–159
16. Grote, E., Hao, J. C., Bennett, M. K., and Kelly, R. B. (1995) Cell 81, 581–589
17. Norect, J. P., Solaro, R., and Cutler, D. F. (1996) J. Cell Biol. 134, 1229–1240
18. Schmidt, A., and Huttner, W. B. (1998) Methods Companion Methods Enzymol. 16, 160–169
19. Verhey, K. J., Hausdorf, S. F., and Birnbaum, M. J. (1995) J. Cell Biol. 123, 137–147
20. Chalk, M. P., Chawl, A., Woom, C. W., Buxton, J., Armoni, M., Tang, W., Joly, M., and Corvera, S. (1993) J. Cell Biol. 123, 127–135
21. Melikian, H., and Buckley, K. M. (1999) J. Neurosci. 19, 7699–7710
22. Warren, R. A., Green, F. A., Stenberg, P. E., and Enns, C. A. (1998) J. Biol. Chem. 273, 17066–17073
23. Hudson, A. W., Fingar, D. C., Seidner, G. A., Griffiths, G., Burke, B., and Birnbaum, M. J. (1993) J. Cell Biol. 122, 579–588
24. Herman, G. A., Bonzelius, P. Cieciart, A. M., and Kelly, R. B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12750–12754
25. Thoitsid, G., Kupryianova, T., Cunningham, J. M., Chen, P., Cadel, S., Foulen, T., Cohen, P., Fine, R. E., and Kandror, K. V. (1999) J. Biol. Chem. 274, 14092–14096
26. Keller, S. R., Scott, H. M., Mastick, C. C., Aebischer, P., and Lienhard, G. E. (1995) J. Biol. Chem. 270, 23612–23618
27. Kandror, K. V., Yu, L., and Pich, P. F. (1994) J. Biol. Chem. 269, 30777–30780
28. Renick, S. E., Kleven, D. T., Chan, J., Steinitz, K., Milner, T. A., Pickel, V. M., and Fremeau, R. T., Jr. (1999) J. Neurosci. 19, 21–33
29. Green, S. A., and Kelly, R. B. (1992) J. Cell Biol. 117, 47–55
30. Jeng, C. J., McCarroll, S. A., Martin, T. F., Fluorescent, J., Adams, J., Krantz, D., Butz, S., Edwards, R., and Schweitzer, E. S. (1998) J. Cell Biol. 140, 683–698
31. Wei, M. L., Bonzelius, P. Scully, R. M., Kelly, R. B., and Herman, G. A. (1998) J. Cell Biol. 140, 565–575
32. Schmid, S. L., and Smythe, E. (1991) J. Cell Biol. 114, 869–880
33. Uzun, T., Ozomi, K., Yamamoto, A., Inoue, M., and Inagaki, C. (1991) J. Neurochem. 56, 1544–1556
34. Mundigl, O., and De Camilli, P. (1994) Curr. Opin. Cell Biol. 6, 561–567
35. Haecke, V., and De Camilli, P. (1999) Science 285, 1268–1271