

Synaptic-like Microvesicles of Neuroendocrine Cells Originate from a Novel Compartment That Is Continuous with the Plasma Membrane and Devoid of Transferrin Receptor

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Abstract. We have characterized the compartment from which synaptic-like microvesicles (SLMV), the neuroendocrine counterpart of neuronal synaptic vesicles, originate. For this purpose we have exploited the previous observation that newly synthesized synaptophysin, a membrane marker of synaptic vesicles and SLMVs, is delivered to the latter organelles via the plasma membrane and an internal compartment. Specifically, synaptophysin was labeled by cell surface biotinylation of unstimulated PC12 cells at 18°C, a condition which blocked the appearance of biotinylated synaptophysin in SLMVs and in which there appeared to be no significant exocytosis of SLMVs. The majority of synaptophysin labeled at 18°C with the membrane-impermeant, cleavable sulfo-NHS-SS-biotin was still accessible to extracellularly added MesNa, a 150-D membrane-impermeant thiol-reducing agent, but not to the 68,000-D protein avidin. The SLMVs generated upon reversal of the temperature to 37°C originated exclusively from the membranes containing the MesNa-accessible rather than the MesNa-protected population

of synaptophysin molecules. Biogenesis of SLMVs from MesNa-accessible membranes was also observed after a short (2 min) biotinylation of synaptophysin at 37°C followed by chase. In contrast to synaptophysin, transferrin receptor biotinylated at 18° or 37°C became rapidly inaccessible to MesNa. Immunofluorescence and immunogold electron microscopy of PC12 cells revealed, in addition to the previously described perinuclear endosome in which synaptophysin and transferrin receptor are colocalized, a sub-plasmalemmal tubulocisternal membrane system distinct from caveolin-positive caveolae that contained synaptophysin but little, if any, transferrin receptor. The latter synaptophysin was selectively visualized upon digitonin permeabilization and quantitatively extracted, despite paraformaldehyde fixation, by Triton X-100. Synaptophysin biotinylated at 18°C was present in these subplasmalemmal membranes. We conclude that SLMVs originate from a novel compartment that is connected to the plasma membrane via a narrow membrane continuity and lacks transferrin receptor.

THE synaptic vesicle cycle has served as one of the paradigms for understanding the molecular basis of vesicular traffic in the eukaryotic cell (for reviews see Kelly, 1993b; Bennett and Scheller, 1994; Jahn and Südhof, 1994; Südhof, 1995; De Camilli and Takei, 1996). Three principal aspects of the membrane traffic of synaptic vesicle proteins can be distinguished: (a) the de novo biogenesis of synaptic vesicles, (b) their fusion with the presynaptic plasma membrane, and (c) the recycling of the synaptic vesicle membrane after exocytosis for the re-formation of synaptic vesicles. The latter two processes, in particular, have been intensively studied and are relatively well understood (Heuser and Reese, 1973; Bennett and Scheller, 1994; Fesce et al., 1994; Jahn and Südhof, 1994;

O'Connor et al., 1994; Rothman, 1994; Südhof, 1995; De Camilli and Takei, 1996). By contrast, comparatively little is known about the molecular events underlying the de novo biogenesis of synaptic vesicles. Reasons for this scarcity of information include the need for studies involving biosynthetic labeling of synaptic vesicle proteins. Such experiments, however, are not possible with the isolated nerve terminal preparations (synaptosomes) that have been so useful for exocytosis and recycling studies and are technically demanding in the case of primary neuronal cultures because of the numbers of cells required.

It is for these reasons that studies addressing the biogenesis of synaptic vesicles have mostly been carried out with neuroendocrine cell lines, in particular PC12 cells. These cells contain synaptic-like microvesicles (SLMVs),¹ the neu-

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1. *Abbreviation used in this paper:* SLMV, synaptic-like microvesicle.

roendocrine counterpart of synaptic vesicles (Navone et al., 1986; Wiedenmann et al., 1988; Clift-O'Grady et al., 1990; De Camilli and Jahn, 1990). The current view of SLMV biogenesis, based on work from several laboratories, is that SLMVs of neuroendocrine cells originate from the transferrin receptor-containing early endosome. The evidence that has led to this view can be summarized as follows. First, synaptophysin, a major membrane protein of synaptic vesicles (Jahn et al., 1985; Wiedenmann and Franke, 1985) and SLMVs (Navone et al., 1986), has the intrinsic tendency to travel to transferrin receptor-containing endosomes, as revealed by its expression in fibroblasts (Johnston et al., 1989; Cameron et al., 1991; Linstedt and Kelly, 1991). Second, synaptophysin endogenous to neuroendocrine cells is present not only in SLMVs but is also found in membranes containing transferrin receptor (Cameron et al., 1991) and HRP after a 7-min internalization (Bauerfeind et al., 1993). Third, newly synthesized synaptophysin is delivered from the TGN to the cell surface via the constitutive secretory pathway, cycles several times between the plasma membrane and an internal compartment, and then appears in SLMVs (Régnier-Vigouroux et al., 1991). Fourth, HRP is detected in early endosomes but not SLMVs after a pulse internalization (5 min) and short chase (7 min) but appears in the latter organelles after a longer chase (3 h; Bauerfeind et al., 1993). Fifth, VAMP/synaptobrevin, another membrane protein found in synaptic vesicles and SLMVs (Trimble et al., 1988; Baumert et al., 1989; Trimble, 1993), is internalized at 15°C into a compartment that is distinct from the plasma membrane and that generates SLMVs in vivo and in vitro (Desnos et al., 1995).

To dissect the molecular machinery involved in SLMV biogenesis, we have reconstituted this process in a cell-free system derived from PC12 cells that will be described in a separate report (Schmidt, A., and W.B. Huttner, manuscript in preparation). In the course of establishing this cell-free system, we performed a detailed characterization of the compartment into which cell surface-labeled synaptophysin is internalized before its appearance in SLMVs. Surprisingly, we find that SLMVs originate from a novel compartment that is distinct from the transferrin receptor-containing endosome and connected to the plasma membrane via a narrow membrane continuity.

Materials and Methods

Biotinylation and Chase of PC12 Cells

PC12 cells were grown in DME supplemented with 10% horse serum and 5% fetal calf serum as described (Tooze and Huttner, 1990) and used until passage 16. Subconfluent 15-cm dishes were rinsed three times at 37°C with 10 ml PBS²⁺ (136 mM NaCl, 2.5 mM KCl, 1.5 mM KH₂PO₄, 6.5 mM Na₂HPO₄, 0.5 mM CaCl₂, 2 mM MgCl₂), incubated for 20 min in 10 ml PBS²⁺ at 37°C, and rinsed again with 10 ml PBS²⁺ at 37°C.

For biotinylation at 37°C, the rinse was removed, and 3.5 ml PBS²⁺ at 37°C was added per dish. The biotinylation was started by the addition to each dish of 500 μ l PBS²⁺ containing 4 mg of either sulfo-NHS-LC-biotin (Pierce, Rockford, IL) or sulfo-NHS-SS-biotin (Pierce; final concentration 1 mg/ml), followed by incubation at 37°C for 2–30 min as indicated. Biotinylation was terminated by removal of the medium, and dishes were either placed on ice for further treatment at 4°C and analysis, as described in the following sections, or subjected to chase. Dishes were rinsed with 10 ml 37°C PBS²⁺ followed by 10 ml of 37°C growth medium and chased at 37°C in 10 ml of PC12 cell-conditioned and centrifuged growth medium

for various times as indicated in the figure legends. At the end of chase, the medium was removed, and dishes were placed on ice.

For biotinylation at 18°C, the 37°C rinse was removed, the dishes were rinsed with 5 ml of PBS²⁺ preequilibrated at 18°C, and 3.5 ml of 18°C PBS²⁺ added per dish. The dishes were placed in a waterbath set at 18°C in a cold room, and the biotinylation was started as above by the addition to each dish of 500 μ l PBS²⁺ containing 4 mg of either sulfo-NHS-LC-biotin or sulfo-NHS-SS-biotin. Biotinylation was carried out at 18°C for the times indicated in the figure legends (usually 30 min) and terminated by removal of the medium. When indicated (see figure legends), dishes were rinsed after biotinylation with 10 ml of PBS²⁺ at 18°C and chased for 5 min at 18°C with 5 ml PBS²⁺ containing 20 mM glycine. Dishes were then subjected to chase at 37°C as described above, or placed on ice. In some experiments, dishes were incubated for various times at 18°C as above, except that the biotinylation reagent was omitted, followed by biotinylation with sulfo-NHS-LC-biotin for 30 min at 4°C.

Dishes were then subjected to further treatment at 4°C (avidin quench, MesNa [2-mercaptoethanesulfonic acid sodium salt] treatment) and analysis. In some experiments, the MesNa treatment at 4°C was followed by a chase at 37°C, and dishes were then placed on ice.

Avidin Quench

All steps were performed at 4°C, and 10 ml per dish of each solution was used. After biotinylation with sulfo-NHS-LC-biotin, dishes were rinsed with and incubated for 15 min in PBS²⁺ containing 20 mM glycine. Dishes were then incubated for 60 min with PBS²⁺ containing 0.2% BSA and 50 μ g/ml avidin (Boehringer-Mannheim GmbH, Mannheim, Germany), rinsed with PBS²⁺/0.2% BSA, incubated for another 30 min in PBS²⁺/0.2% BSA containing 100 μ g/ml biocytin (Sigma Chemical Co., St. Louis, MO) to quench the free biotin binding sites of avidin, and rinsed extensively with PBS²⁺/0.2% BSA. Control dishes were treated identically except that avidin was omitted. In some experiments, incubation with avidin was performed for 15 min at either 18° or 4°C. Dishes were then either subjected to chase at 37°C as described in the above section, or the cells were harvested and homogenized as described below.

MesNa Treatment

Treatment with MesNa (Sigma Chemical Co.) was performed by modifying the procedure of Carter et al. (1993) as follows. All steps were performed at 4°C, and 10 ml per dish of each solution was used. After biotinylation with sulfo-NHS-SS-biotin, dishes were rinsed with PBS²⁺ followed by NT buffer (150 mM NaCl, 1 mM EDTA, 0.2% BSA, 20 mM N-tris(hydroxymethyl)methyl-3-aminopropane-sulfonic acid, pH 8.6). Dishes were incubated for 10 min in NT buffer, for 30 min in NT buffer containing freshly dissolved 10 mM MesNa, and two times for 60 min each in fresh NT/MesNa buffer. Dishes were rinsed extensively with NT buffer to remove MesNa. In some experiments, residual MesNa possibly left on the dish was inactivated by the addition of 20 mM iodoacetic acid in NT buffer for 15 min; this treatment gave the same results as extensive rinsing in NT buffer alone. Control dishes were treated identically except that MesNa was omitted. Dishes were then subjected to chase at 37°C, or the cells were harvested and homogenized.

For the kinetics of the acquisition of MesNa inaccessibility, 6-cm dishes of PC12 cells were used. Biotinylation, chase, and MesNa treatment were performed as described above, except that the volumes per dish reduced to 0.75 ml for the biotinylation, 2 ml for the chase, and 1 ml for the MesNa treatment. After MesNa treatment and washing in NT buffer, the steps (at 4°C) were as follows. Cells were rinsed twice in homogenization buffer (150 mM NaCl, 0.2 mM MgCl₂, 1 mM EDTA, 10 mM Hepes, pH 7.2) and scraped from the dish in 1 ml of this buffer using a rubber policeman. The cell suspension was centrifuged for 5 min at 850 g, and each pellet was resuspended in 500 μ l of solubilization buffer (1% Triton X-100, 100 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, pH 7.4). The cell lysates were kept on ice for 30 min and centrifuged at maximal speed in an Eppendorf bench-top centrifuge for 15 min. Aliquots (100 μ l) of the cleared cell lysates were diluted fivefold with solubilization buffer and subjected to streptavidin-agarose adsorption.

Subcellular Fractionation

All steps were performed at 4°C. Dishes were rinsed with homogenization buffer containing 0.5 mM PMSF and scraped from the dish in 5 ml of this buffer using a rubber policeman. The cell suspension was centrifuged for 5

min at 850 g, and the pellet from one dish of cells was resuspended in 1 ml of homogenization buffer with PMSF. This suspension was passed six times back and forth through a 0.70 × 30 mm needle attached to 1-ml syringe followed by eight passages through a ball-bearing homogenizer (cell-cracker, 12 μm clearance, European Molecular Biology Laboratory). The homogenate was centrifuged for 10 min at 1,350 g, and the resulting postnuclear supernatant was centrifuged for 15 min at either 12,000 g (15,000 rpm) or 66,000 g (35,000 rpm) in an Optima TL centrifuge (Beckman Instrs., Inc., Fullerton, CA) using a TLA-45 rotor, as indicated in the figure legends. The 12,000 and 66,000 g pellets were resuspended in 200 μl of homogenization buffer containing 1 μg/ml leupeptin and 2 μg/ml aprotinin. The 12,000 and 66,000 g supernatants were loaded onto a 5-ml, 5–25% glycerol gradient (Clift-O'Grady et al., 1990) in homogenization buffer poured on top of a 5-ml, 1.8 M sucrose cushion in homogenization buffer. The gradients were then centrifuged for 55 min at 40,000 rpm (285,000 g_{max}) in a SW40Ti rotor (Beckman Instrs., Inc.). Fractions (500 μl) were collected from the bottom of the gradient (sucrose/glycerol interface, fraction n 1) to its top (fraction n 10, without load) by introducing a needle through the wall of the centrifuge tube just below the sucrose/glycerol interface. Qualitatively similar results were obtained with the 12,000 and 66,000 g supernatants analyzed by glycerol gradient centrifugation; however, after the 66,000 g centrifugation, the immunoblot signals in the endosome-containing fractions of the glycerol gradient relative to those in the SLMV-containing fractions were decreased as compared to the 12,000 g centrifugation.

Streptavidin–Agarose Adsorption

Unless indicated otherwise, all steps were performed at 4°C. Aliquots of the postnuclear supernatants (30–60 μl), the resuspended 12,000 and 66,000 g pellets (5 μl), and the 12,000 and 66,000 g supernatants (50 μl) were mixed with half a volume of 3X concentrated solubilization buffer (see above) and brought to 200 μl with solubilization buffer. Glycerol gradient fractions (500 μl) were mixed with 250 μl of 3X solubilization buffer. The samples were incubated for 30 min on ice followed by addition of 20 μl of a 1:1 slurry of streptavidin–agarose (Sigma Chemical Co.) in solubilization buffer. In initial experiments, this amount of streptavidin–agarose was shown to be sufficient for maximal recovery of biotinylated proteins from the above aliquots of the various subcellular fractions. Samples were incubated for 60 min on a rocking platform and briefly centrifuged in bench-top centrifuge to pellet the agarose beads. The supernatants were collected for the determination of streptavidin-unbound proteins. The agarose pellets were washed three times with 500 μl of solubilization buffer. After addition of 15 μl of 3X Laemmli sample buffer to the streptavidin–agarose pellets (10 μl) and the residual wash (~20 μl), the beads were gently resuspended, the samples were incubated for 4 min at 95°C, and the fluid phase above and around the beads was collected with a 100-μl Hamilton syringe while the samples were kept at 95°C on a heating block. The eluates from the streptavidin–agarose beads were analyzed by SDS-PAGE followed by immunoblotting.

Immunoblotting

SDS-PAGE (9% acrylamide) was performed using the BioRad Laboratories (Richmond, CA) mini-gel system. Proteins were transferred in 20% methanol, 0.1% SDS, 20 mM Tris base, 150 mM glycine, pH 8.3, onto nitrocellulose (BA85; Schleicher & Schuell, Inc., Keene, NH, 0.45 μm) using a Genie electrophoretic blotter (Idea Sci., Minneapolis, MN). The nitrocellulose was incubated for 30 min in blocking buffer (PBS containing 5% low fat milk powder and 0.2% Tween-20). The nitrocellulose was cut between the 69- and 45-kD Rainbow markers (Amersham Intl., Buckinghamshire, UK) to separate the synaptophysin-containing portion of the membrane from that containing transferrin receptor and SV2; the membranes were then incubated for 60 min at room temperature in blocking buffer containing 25 ng/ml of the anti-synaptophysin monoclonal antibody Sy38 (Boehringer Mannheim GmbH), 1 μg/ml of the anti-transferrin receptor monoclonal antibody HTR 68.4 (White et al., 1992; a kind gift from Trowbridge, I. [Salk Institute, San Diego, CA] and P. DeCamilli [Yale University, New Haven, CT]), or a 1:3,000 dilution of an ascites containing the anti-SV2 monoclonal antibody (Buckley and Kelly, 1985; a kind gift from De Camilli, P.). Bound monoclonal antibodies were revealed with HRP-conjugated goat anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA; 0.25 μg/ml in blocking buffer) followed by the ECL detection system (Amersham Intl.).

Quantification of Data

Multiple exposures of the immunoblots were made on Hyperfilm-MP (Amersham Intl.). Exposures whose signals were in the linear range of the procedure as determined from standard curves were quantitated by densitometric scanning using an LKB Ultrascan XL. The values obtained were expressed as arbitrary units. “Biotinylated synaptophysin” and “biotinylated transferrin receptor” are defined as synaptophysin and transferrin receptor, respectively, recovered bound to streptavidin–agarose (except for experiments with avidin quenching, in which the term “streptavidin-bound” is used instead of “biotinylated”). “Total synaptophysin” and “total transferrin receptor” are defined as the sum of streptavidin–agarose-bound plus streptavidin–agarose-unbound synaptophysin and transferrin receptor, respectively, present in either the postnuclear supernatant, the sum of the 12,000 g pellet plus supernatant, or the sum of the 66,000 g pellet plus supernatant; only a minor proportion of synaptophysin and transferrin receptor was recovered in the nuclear pellet (data not shown). “Total biotinylated synaptophysin” is defined as the streptavidin–agarose-bound synaptophysin present in either the postnuclear supernatant, the sum of the 12,000 g pellet plus supernatant, or the sum of the 66,000 g pellet plus supernatant.

Transferrin Uptake and Fluorescence Microscopy

Transferrin Uptake. PC12 cells were grown on polylysine-coated, 9-mm glass coverslips. The growth medium was replaced with DME containing 0.2% BSA, and cells were incubated for 30 min at 37°C. The medium was replaced by DME/0.2% BSA containing 50 μg/ml of Texas red-labeled transferrin (Molecular Probes, Inc., Eugene, OR), and cells were incubated for 30 min at 37°C followed by fixation and fluorescence analysis.

Synaptophysin Immunofluorescence. Unless specified otherwise, all procedures were performed at room temperature. After removal of the transferrin-containing medium, cells were rinsed once with PBS and fixed for 15 min with 3% (wt/vol) paraformaldehyde in PBS. Coverslips were rinsed with and incubated for 10 min in PBS containing 50 mM NH₄Cl. Coverslips were then incubated for 10 min with 100 μl of PBS containing 2 mM EDTA, 0.5 mM PMSF, 10 μg/ml aprotinin, 40 μg/ml leupeptin, and either 0.3% (vol/vol) Triton X-100 (Sigma Chemical Co.) or 50 μg/ml digitonin (Fisher Scientific Co., Pittsburgh, PA; added from a 50 mg/ml stock in DMSO) as indicated. Coverslips were then incubated for 10–20 min in PGA buffer (0.2% [wt/vol] gelatin, 0.02% [wt/vol] azide in PBS) followed by 30 min in PGA buffer containing 0.4 μg/ml of the monoclonal anti-synaptophysin antibody Sy38 (Boehringer-Mannheim GmbH). The coverslips were washed three times in PGA buffer and incubated for 30 min with 3.3 μg/ml Cy2-conjugated goat anti-mouse IgG secondary antibody (Biotrend, Köln, Germany) in PGA buffer. The coverslips were then sequentially washed three times in PGA buffer, three times in PBS, dipped twice in distilled water, and finally mounted in Mowiol.

Confocal Microscopy. Fluorescent images were obtained using a Leica TCS^{SP} confocal laser scanning microscope with a 63X 1.4 numerical aperture objective. The confocal microscope settings were such that the photomultipliers were within their linear range. The images shown were prepared from the confocal data files using Adobe Photoshop, without any alteration of the original fluorescence data.

Synaptophysin Extraction after Fixation

PC12 cells grown on coverslips in 10-cm dishes were biotinylated for 30 min at 18°C using 1 mg/ml sulfo-NHS-LC-biotin. Controls were treated identically except that sulfo-NHS-LC-biotin was omitted. After the chase in PBS²⁺/glycine, dishes were rinsed once with 18°C PBS²⁺, and cells were subjected to one of the following three protocols. (a) Cells were fixed by the addition of 3% (wt/vol) paraformaldehyde in PBS at 18°C and transferred to room temperature. After 15 min fixation, coverslips were incubated with NH₄Cl and treated with Triton X-100, as described under Synaptophysin Immunofluorescence, except that 10 mM *N*-ethyl maleimide was included in the Triton X-100 buffer. All subsequent steps were performed at 4°C unless indicated otherwise. The Triton X-100 extract (100 μl) was cleared by centrifugation in a bench-top centrifuge, incubated for 2 h with 50 μl of a 1:1 slurry of streptavidin–agarose in PBS containing 0.3% Triton X-100 and 2 mM EDTA. The beads were washed in PBS/Triton/EDTA, incubated for 2 h with 100 μl of PBS/Triton/EDTA containing 0.2 μg/ml of the monoclonal anti-synaptophysin antibody Sy38, washed in PBS/Triton/EDTA, incubated for 2 h with 100 μl of PBS/Triton/EDTA containing 1.6 μg/ml of goat anti-mouse IgG antibody conjugated to

HRP, and washed again. Peroxidase activity associated with the beads was revealed by incubation for 5 min at room temperature in 750 μ l of 50 mM sodium phosphate, pH 5, 0.1% Triton X-100, 0.003% H_2O_2 , 100 μ g/ml *O*-dianisidine. Reactions were stopped with 0.7% azide. Absorbances were determined at 455 nm and converted to values of ng HRP-IgG by comparison to a standard curve. (b) Unfixed cells were treated with Triton X-100 as described above except that 10 mM *N*-ethyl maleimide was included, and the extraction was performed for 1 h on ice. The Triton X-100 extract was then processed as in a. (c) Cells were fixed by the addition of 3% (wt/vol) paraformaldehyde in PBS at 18°C and transferred to room temperature. After 15 min fixation, coverslips were sequentially incubated with NH_4Cl , digitonin, and the monoclonal anti-synaptophysin antibody Sy38 and washed with PGA buffer, as described under Synaptophysin Immunofluorescence. Controls were treated identically except that anti-synaptophysin was omitted. Coverslips were then incubated in the Triton X-100 buffer and the resulting extract processed as in a, except that the 2 h incubation with the anti-synaptophysin monoclonal antibody was omitted.

Immunoisolation

For the immunoisolation of SLMVs, the procedure of Burger et al. (1989) was adopted. Affinity purified goat-anti mouse IgG (5 mg; Cappel Laboratories, Cochranville, PA) was coupled to Eupergit C1Z beads (1 g; Röhm-Pharma GmbH, Rodleben, Germany) by incubation for 8 h at room temperature followed by quenching with 1 M glycine overnight. The beads were washed sequentially with three cycles of 0.5 M NaCl, 0.1 M acetate, pH 4.5, followed by 0.5 M NaCl, 0.1 M Tris, pH 8.0, and then rinsed with 150 mM NaCl, 10 mM Tris/HCl, pH 7.3, and stored at 4°C until use. On the day of SLMV isolation, 400 μ l of a 1:1 suspension of beads was washed in homogenization buffer/0.2% BSA and resuspended in 1 ml of this buffer; 500 μ l of the bead suspension was incubated for 2 h at room temperature with 500 ng of the monoclonal anti-synaptophysin antibody Sy38 followed by washing in homogenization buffer/0.2% BSA (immunobeads), while the other 500 μ l of the bead suspension was kept as control beads. Two pools of fractions from the glycerol gradient were prepared, one containing the five fractions corresponding to the SLMV peak (2.5 ml) and the other the two bottom fractions (1 ml), and half of each pool was incubated for 3 h at 4°C on a rocking platform with 250 μ l of either immunobeads or control beads. After immunoadsorption, the beads were washed with ice-cold homogenization buffer/0.2% BSA and examined by electron microscopy.

Electron Microscopy

Immunoisolated Membranes. Immunobeads and control beads were washed with 100 mM cacodylate buffer, pH 7.4, fixed with 1% glutaraldehyde in cacodylate buffer, postfixed in 1% (wt/vol) OsO_4 plus 1.5% (wt/vol) magnesium ferricyanide, and contrasted with 1.5% (wt/vol) magnesium uranyl acetate. Samples were then dehydrated in ethanol and embedded in Epon. Ultrathin sections were contrasted with uranyl acetate and lead citrate and observed in a Zeiss EM 10 CR electron microscope.

Immunogold Labeling of Ultrathin Cryosections. PC12 cells were incubated for 30 min at 18° or 37°C with sulfo-NHS-LC-biotin as described above, followed by a 5 min chase in $PBS^{2+}/20$ mM glycine at the respective temperature. The dishes (10 ml per dish, all steps at 4°C) were rinsed twice with $PBS^{2+}/20$ mM glycine, once with PBS^{2+} , and once with 0.25 M HEPES-NaOH, pH 7.4, and cells were fixed on the dish by addition of 8% paraformaldehyde in the latter buffer at 4°C. After 1 h at 4°C, the fixed cells were scraped off the dish and centrifuged for 5 min at 10,000 *g*. The cell pellets were incubated in fixative for another 3 h at 4°C, infiltrated with 2.1 M sucrose in PBS, and frozen in liquid nitrogen. Fixation at 4°C was chosen to allow a comparison of synaptophysin immunoreactivity between the cells incubated at 18° and 37°C; the epitope recognized by the monoclonal antibody Sy38 is known to be sensitive to the extent of aldehyde fixation (Hoog et al., 1988). Ultrathin cryosections (Griffiths, 1993) were prepared using a Reichert FCS cryoultramicrotome and collected on Formvar carbon-coated grids. Sections were blocked for 10 min with 20 mM glycine in PBS supplemented with 10% fetal calf serum and subsequently incubated with the primary anti-synaptophysin monoclonal antibody (1 μ g/ml; Boehringer-Mannheim GmbH), the secondary rabbit anti-mouse IgG antibody (65 μ g/ml; Cappel Laboratories), and protein A coupled to 9-nm gold, each for 30 min in blocking solution containing only 5% fetal calf serum. Sections were treated with 0.3% uranyl acetate and 1.8% methyl cellulose, dried, and observed in the electron microscope.

Results

Synaptophysin Biotinylated at the Surface of PC12 Cells Is Rapidly Transported to SLMVs

To study the donor membrane from which SLMVs originate, we chose to label synaptophysin of PC12 cells by surface biotinylation, exploiting the fact that the newly synthesized form of this protein travels via the plasma membrane to SLMVs (Régnier-Vigouroux et al., 1991). We first ascertained that biotinylation of synaptophysin, which modifies lysine residues, does not interfere with its ability to be sorted to SLMVs. The appearance in SLMVs of newly synthesized synaptophysin, sulfate labeled in the *trans*-Golgi network, approaches a plateau after 3 h of chase (Régnier-Vigouroux et al., 1991). We therefore labeled PC12 cells for 5 min at 37°C by addition of the membrane-impermeant sulfo-NHS-LC-biotin to the medium, followed by a 3-h chase. After glycerol gradient centrifugation which separates SLMVs from larger membranes (Clift-O'Grady et al., 1990), two differentially sedimenting populations of biotinylated synaptophysin (determined from its binding to streptavidin-agarose) were recovered in the bottom and middle fractions of the gradient, respectively (Fig. 1, A

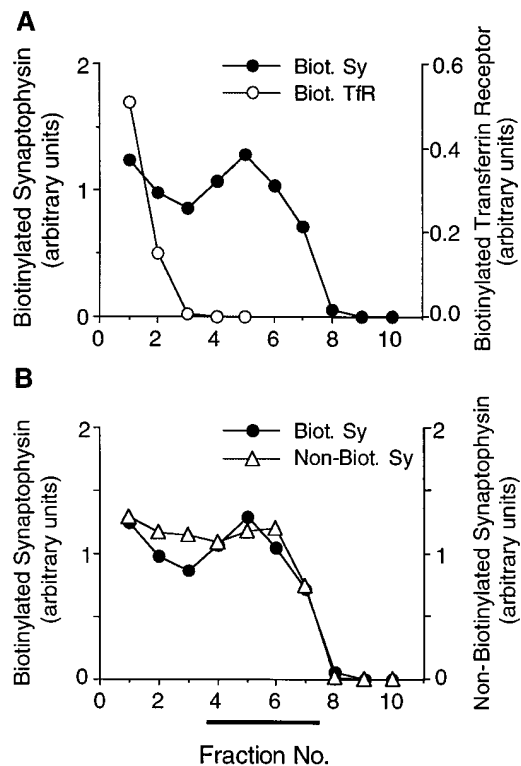


Figure 1. Biotinylated synaptophysin is sorted to SLMVs. PC12 cells were incubated with sulfo-NHS-LC-biotin for 5 min at 37°C and chased for 180 min at 37°C. The 12,000-g supernatant prepared from the cells was subjected to glycerol gradient centrifugation, and the fractions (*n* 1, bottom) were analyzed for biotinylated (A and B, closed circles, same data points for both panels) and nonbiotinylated (B, open triangles) synaptophysin and biotinylated transferrin receptor (A, open circles) by streptavidin-agarose adsorption followed by immunoblotting of bound and unbound material with the respective antibodies. Bar, SLMVs.

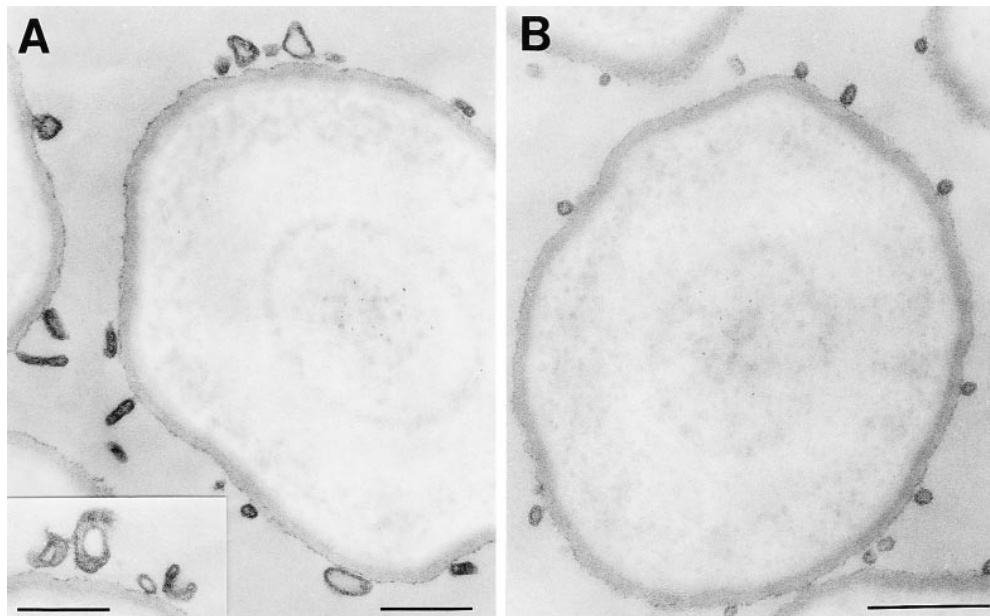


Figure 2. Electron microscopy of glycerol gradient fractions. The 12,000 g supernatant prepared from PC12 cells was subjected to glycerol gradient centrifugation, and the pools of fractions 1 and 2 (**A**) and fractions 5–9 (**B**) were subjected to immunisolation using anti-synaptophysin beads followed by fixation and processing for electron microscopy. Note the heterogeneous membrane structures in **A** and the homogenous population of 50-nm vesicles in **B**. No membranes were adsorbed to the beads if the anti-synaptophysin antibody was omitted (not shown). Bars, 300 nm.

and **B**), both of which also contained nonbiotinylated synaptophysin (Fig. 1 **B**). The bottom, but not the middle, fractions also contained biotinylated transferrin receptor (Fig. 1 **A**), a membrane protein constitutively cycling between the plasma membrane and early endosomes (Trowbridge et al., 1993). Electron microscopy of the membranes immunoadsorbed to beads coated with an antibody against the cytoplasmic tail of synaptophysin showed that the bottom fractions contained synaptophysin in membrane structures of various sizes (Fig. 2 **A**). Given the presence of transferrin receptor in these fractions, and in line with previous reports (Clift-O'Grady et al., 1990; Cameron et al., 1991), the biotinylated synaptophysin in the bottom fractions was presumably present in membranes of early endosomes. Electron microscopy of the synaptophysin-containing membranes in the middle fractions of the glycerol gradient showed that these had the expected morphology of SLMVs (Fig. 2 **B**), corroborating previous conclusions based on biochemical data (Clift-O'Grady et al., 1990; Cameron et al., 1991; Linstedt and Kelly, 1991). We conclude that synaptophysin biotinylated at the cell surface, like nonbiotinylated synaptophysin, is sorted to SLMVs.

We next determined the kinetics of appearance of cell surface-biotinylated synaptophysin in SLMVs. PC12 cells were biotinylated for 5 min, chased for various times, and analyzed by glycerol gradient centrifugation to separate SLMVs from other membranes. This showed that cell surface-biotinylated synaptophysin appeared very rapidly in SLMVs. Already without chase, i.e., at the end of the 5 min biotinylation, a small proportion (~2%) of the biotinylated synaptophysin was detectable in the SLMV-containing fractions of the glycerol gradient (Fig. 3 **A**). A similar observation was made after 3 min of chase (Fig. 3 **B**; 2.5% of total biotinylated synaptophysin recovered in SLMV fractions). Within 10 min of chase, the appearance of biotinylated synaptophysin in SLMVs approached a value corresponding to ~13% of the total biotinylated synaptophysin, which remained constant for at least 3 h (Fig. 3, **A** and **C**).

Surface-biotinylated Synaptophysin Does Not Appear in SLMVs at 18°C

The kinetics of appearance in SLMVs of cell surface-biotinylated synaptophysin was much more rapid than that of newly synthesized synaptophysin sulfate labeled in the TGN (Régnier-Vigouroux et al., 1991). This raised the question whether in PC12 cells, SLMVs originate indeed from early endosomes as previously assumed (Johnston et al., 1989; Clift-O'Grady et al., 1990; Cameron et al., 1991; Linstedt and Kelly, 1991; Régnier-Vigouroux et al., 1991; Bauer-

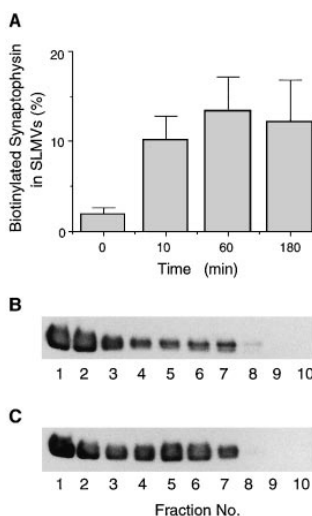


Figure 3. Time course of appearance of biotinylated synaptophysin in SLMVs. PC12 cells were incubated with sulfo-NHS-LC-biotin for 5 min at 37°C and chased at 37°C for various times as indicated (**A**) or for 3 min (**B**) and 180 min (**C**). The 66,000 g (**A**) or 12,000 g (**B** and **C**) supernatants prepared from the cells were subjected to glycerol gradient centrifugation, and the fractions were analyzed for biotinylated synaptophysin by streptavidin-agarose adsorption followed by immunoblotting of bound material with anti-synaptophysin. (**A**) Synaptophysin immunoreactivity in the SLMV-containing fractions is expressed as percent of total biotinylated synaptophysin. Data are the mean of two (0, 10, 180 min) or three (60 min) independent experiments; bars indicate the variation of individual values from the mean or the standard error, respectively. (**B** and **C**) Immunoblots; 2.5% (**B**) and 11% (**C**) of the total biotinylated synaptophysin was recovered in the SLMV-containing fractions (**B**, *n* 5–8; **C**, *n* 4–8).

feind et al., 1993) or perhaps directly from the plasma membrane. In nonneuroendocrine cells, reduced temperature (16–20°C) has been shown to differentially affect certain membrane traffic steps in the endocytic system (Dunn et al., 1980; Wolkoff et al., 1984; Mueller and Hubbard, 1986). We therefore explored whether a reduction in temperature to 18°C could be used to accumulate cell surface–biotinylated synaptophysin in, and hence to facilitate characterization of, the membrane compartment from which SLMVs originate.

Glycerol gradient analysis of PC12 cells incubated for 30 min at 18°C with sulfo-NHS-LC–biotin showed that the reduced temperature indeed blocked the appearance of biotinylated synaptophysin in SLMVs. In contrast to an incubation at 37°C after which biotinylated synaptophysin was found in both bottom and middle fractions of the gradient (Fig. 4 A), synaptophysin biotinylated at 18°C was found in the bottom fractions of the gradient (Fig. 4 B) containing early endosomes, as indicated by the presence of biotinylated transferrin receptor (Fig. 4 C), but not in the middle fractions (Fig. 4 B) containing SLMVs formed before biotinylation, as indicated by the presence of nonbiotinylated synaptophysin (Fig. 4, D and E; two distinct procedures of differential centrifugation before the glycerol gradient; see figure legend). A chase for 30 min at 37°C, performed after the 30 min biotinylation at 18°C, resulted in the appearance of biotinylated synaptophysin in SLMVs (~15% of the total biotinylated synaptophysin; Fig. 4 F), showing that the temperature block was reversible. Our

observations are consistent with the results of Desnos et al. (1995) reported while the present work was in progress, which show that an epitope-tagged VAMP/synaptobrevin mutant labeled by an antibody bound to its extracellular domain does not appear in SLMVs at 15°C but does so at 37°C.

Characterization of the 18°C Compartment

Given that synaptophysin biotinylated at the cell surface did not appear in SLMVs at 18°C, we next investigated whether it was internalized at this temperature. For this purpose, we studied the accessibility of biotinylated synaptophysin to extracellularly added avidin. As a positive control, PC12 cells were rapidly cooled from 37° to 4°C (a temperature preventing further membrane traffic), biotinylated for 60 min at 4°C, and then exposed to avidin at 4°C. In this condition one would expect to biotinylate only those synaptophysin molecules that are exposed at the cell surface; these should be accessible to avidin and, hence, not be able to bind to streptavidin–agarose subsequently. Only ~2% of the total synaptophysin was biotinylated in this condition, in line with previous observations showing that only very little of the total synaptophysin is present at the surface of PC12 cells (Johnston et al., 1989), and most of the biotinylated synaptophysin was accessible to avidin (Fig. 5 A). In contrast, when PC12 cells biotinylated for 30 min at 18°C and chased for 5 min at 18°C were exposed to avidin at 4°C, the amount of biotinylated synaptophysin that subsequently could be adsorbed to streptavidin–agarose was not reduced compared to control (Fig. 5 B). Furthermore, the same amount of synaptophysin bound to streptavidin–agarose when PC12 cells biotinylated for 15 min at 18°C were exposed to avidin at either 18° or 4°C (data not shown). These results indicated that the synaptophysin biotinylated at 18°C was no longer exposed at the cell surface.

Studies on the endocytosis of clathrin-coated vesicles in nonneuroendocrine cells (Carter et al., 1993) have revealed the existence of an intermediate, referred to as a deeply invaginated coated pit, which is thought to still be connected to the plasma membrane via a narrow neck, because the lumen of the pit is not accessible to the 68,000-D protein avidin but is sensitive to the small (150 D) membrane-impermeant, thiol-reducing agent MesNa. Given this finding and the inaccessibility of the synaptophysin biotinylated at 18°C to avidin, we investigated whether or not this synaptophysin was sensitive to MesNa added to the medium. For this purpose, we used sulfo-NHS-SS–biotin in which the biotin moiety after crosslinking to protein can be cleaved off by thiol reduction. As was the case for sulfo-NHS-LC–biotin, labeling of synaptophysin with sulfo-NHS-SS–biotin did not interfere with its ability to be sorted to SLMVs (data not shown; see Fig. 7). When PC12 cells biotinylated for 30 min at 18°C with sulfo-NHS-SS–biotin and chased for 5 min at 18°C were treated with MesNa at 4°C, ~70% of the biotinylated synaptophysin was found to be MesNa sensitive, as indicated by the decrease in binding to streptavidin–agarose compared to the control (Fig. 5 C). In contrast, transferrin receptor biotinylated in the same condition was completely protected from MesNa (Fig. 5 D). These results show that the majority of synap-

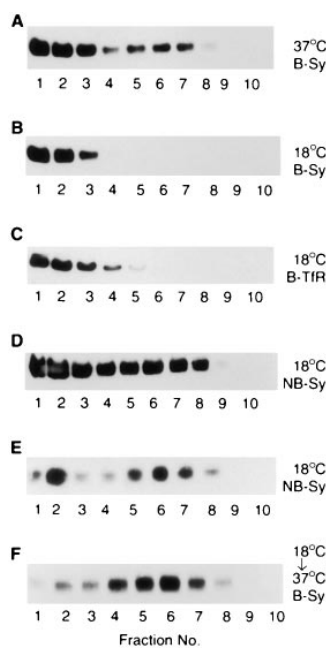
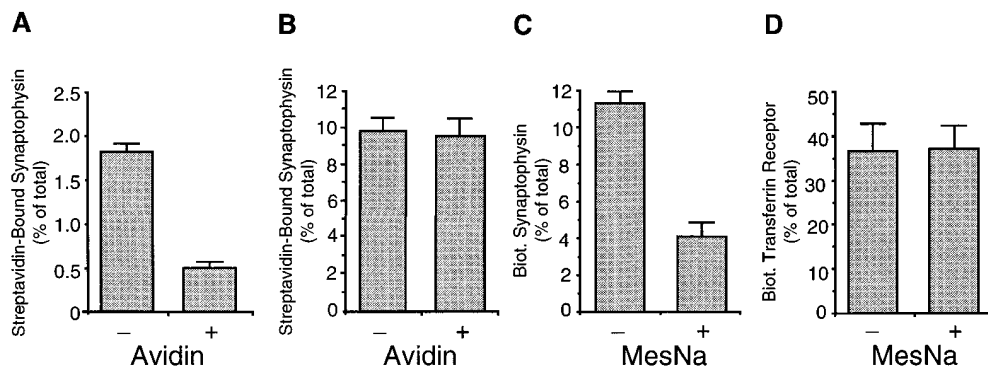


Figure 4. Biotinylated synaptophysin does not appear in SLMVs at 18°C. PC12 cells were incubated with sulfo-NHS-LC–biotin either for 30 min at 37°C (A), for 30 min at 18°C (B–E), or for 30 min at 18°C followed by a 30-min chase at 37°C (F). The 12,000 g (A–D) or 66,000 g (E and F) supernatants prepared from the cells were subjected to glycerol gradient centrifugation, and the fractions were analyzed for biotinylated (B-Sy; A, B, and F) and nonbiotinylated (NB-Sy; D and E) synaptophysin and biotinylated transferrin receptor (B-TfR; C) by streptavidin–agarose adsorption followed by immunoblotting of bound (A–C and F) and unbound (D and E) material with the respective antibodies. The

immunoblots shown in (B) and (C) were obtained from the same filter. 16% (A) and 15% (F) of the total biotinylated synaptophysin was recovered in the SLMV-containing fractions (A, *n* 5–8; F, *n* 4–8); the immunoblot shown in F is a longer exposure relative to that shown in A. Note that the ratio of SLMVs to the larger membranes recovered in the bottom fractions of the gradient is greater in E than D, because a 66,000 g and a 12,000 g supernatant, respectively, was subjected to glycerol gradient centrifugation.



added avidin (A and B) or MesNa (C and D). Synaptophysin and transferrin receptor in the postnuclear supernatants were analyzed for binding to streptavidin-agarose by immunoblotting of bound and unbound material with the respective antibodies. Streptavidin-bound biotinylated synaptophysin and transferrin receptor present in the postnuclear supernatant is expressed as percentage of total (sum of streptavidin-bound and streptavidin-unbound synaptophysin and transferrin receptor, respectively). (A) Data are the mean of two independent experiments; bars indicate the variation of the individual values from the mean. (B–D) Data are the mean of three independent experiments; bars indicate SD.

tophysin biotinylated at 18°C is internalized into a compartment that has a narrow connection to the plasma membrane allowing the passage of MesNa but not avidin and in which there is no detectable transferrin receptor.

The proportion of the total synaptophysin biotinylated after 30 min at 18°C (~10% in Fig. 5 B) was at least five times that biotinylated for 60 min at 4°C (Fig. 5 A). Biotinylation at the latter temperature for 30 min yielded the same value (~2% of the total synaptophysin) as that carried out for 60 min (data not shown), showing that within 30 min at 4°C, every synaptophysin molecule accessible to sulfo-NHS-LC-biotin was biotinylated to an extent sufficient to allow binding to streptavidin. This in turn suggested that the greater proportion of biotinylated synaptophysin obtained at 18° as opposed to 4°C reflected the delivery, at 18°C, either of unbiotinylated synaptophysin molecules from an intracellular pool to the site where biotinylation occurred, or of sulfo-NHS-LC-biotin to an intracellular site where synaptophysin resided. This conclusion was supported by studying the time course of biotinylation of synaptophysin at 18°C, which revealed a continuous increase in the amount of biotinylated synaptophysin over time (Fig. 6, closed circles). Together with the accessibility of the majority of this synaptophysin to MesNa (Fig. 5 C), these results indicate that upon incubation at 18°C, biotinylated synaptophysin accumulated in a membrane compartment connected to the plasma membrane. Interestingly, when PC12 cells incubated for various times at 18°C were subsequently biotinylated for 30 min at 4°C, only ~2% of the total synaptophysin was biotinylated, irrespective of the incubation time at 18°C (Fig. 6, closed triangles). This suggested that upon cooling the cells to 4°C, the lumen of this membrane compartment became inaccessible to sulfo-NHS-LC-biotin, as was the case for avidin (Fig. 5 B) but not MesNa (Fig. 5 C). Biotinylation of the transferrin receptor at 18°C also increased over time, reaching a value of ~35% of total by the end of the 30-min incubation (Fig. 6). Given the inaccessibility of the transferrin receptor to MesNa (Fig. 5 D), this implied its continuous delivery to the plasma membrane and endocytosis at 18°C.

Figure 5. Differential sensitivity to extracellular probes of synaptophysin and transferrin receptor biotinylated at 18°C. PC12 cells were incubated with sulfo-NHS-LC-biotin (A and B) or sulfo-NHS-SS-biotin (C and D) for 60 min at 4°C (A) or for 30 min at 18°C (B–D), chased for 5 min at 18°C in the presence of glycine (B–D) or not chased (A), and incubated at 4°C in the absence (–) or presence (+) of extracellularly

We next investigated whether SLMV biogenesis involved the MesNa-sensitive or -protected population of biotinylated synaptophysin. PC12 cells biotinylated at 18°C were treated with MesNa and then chased for 10 min at 37°C. In contrast to the control, virtually no biotinylated synaptophysin appeared in SLMVs after MesNa treatment (Fig. 7), although ~30% of the biotinylated synaptophysin was protected from MesNa (Fig. 5 C). Hence, the MesNa-sensitive population of biotinylated synaptophysin molecules rather than the protected one participated in SLMV biogenesis.

SV2, Another Membrane Protein of SLMVs, Is Present in the MesNa-accessible Compartment

If the MesNa-accessible compartment gives rise to SLMVs,

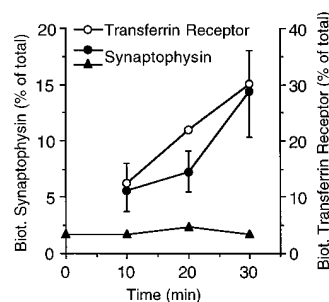


Figure 6. Time course of accumulation of biotinylated synaptophysin and transferrin receptor at 18°C. PC12 cells were incubated with (open and closed circles) or without (closed triangles) sulfo-NHS-LC-biotin at 18°C for the indicated times. The cells incubated without sulfo-NHS-LC-biotin at 18°C were subsequently biotinylated for

30 min at 4°C (closed triangles). Postnuclear supernatants prepared from the cells were analyzed for biotinylated and nonbiotinylated synaptophysin and transferrin receptor by streptavidin-agarose adsorption, followed by immunoblotting of bound and unbound material with the respective antibodies. Biotinylated synaptophysin and transferrin receptor is expressed as percent of total (sum of biotinylated plus nonbiotinylated synaptophysin and transferrin receptor, respectively). Data points without error bars represent single determinations. Data points with error bars represent the mean of three (transferrin receptor), four (synaptophysin at 10 and 30 min), or two (synaptophysin at 20 min) independent determinations; bars indicate SD or the variation of the individual values from the mean.

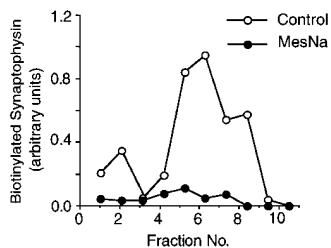


Figure 7. SLMV biogenesis involves the MesNa-sensitive population of synaptophysin molecules biotinylated at 18°C. PC12 cells were incubated with sulfo-NHS-SS-biotin for 30 min at 18°C, chased for 5 min at 18°C in the presence of glycine, incubated at 4°C in the absence (*Control*)

or presence of extracellularly added MesNa, and chased for 10 min at 37°C. The 66,000-g supernatants prepared from the cells were subjected to glycerol gradient centrifugation, and fractions were analyzed for biotinylated synaptophysin by streptavidin-agarose adsorption followed by immunoblotting of bound material with anti-synaptophysin antibodies. 11% and 4% of the total synaptophysin present in the sum of the 66,000 g pellet plus supernatant bound to streptavidin-agarose without and with MesNa addition before the 37°C chase, respectively.

one would expect it to contain SLMV-specific membrane proteins other than synaptophysin. We investigated this issue by analyzing whether one such protein, SV2, showed a similar accessibility to MesNa after cell surface biotinylation for 30 min at 18°C. As shown in Fig. 8, MesNa accessibility of biotinylated SV2 was indeed comparable to that of synaptophysin (the latter confirming the data shown in Fig. 5 C) and in marked contrast to the almost complete MesNa inaccessibility of the transferrin receptor (confirming the data shown in Fig. 5 D).

Synaptophysin Passes through a Compartment Continuous with the Plasma Membrane at Physiological Temperature

Together, the data presented so far suggested that the SLMVs formed upon reversal of the 18°C block originated from a compartment that (a) was continuous with the plasma membrane and (b) lacked transferrin receptor. It was important to determine whether such a compartment

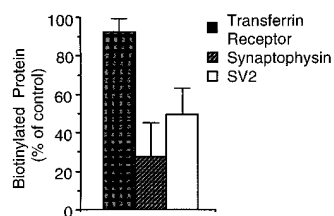


Figure 8. SV2 is accessible to MesNa after biotinylation at 18°C. PC12 cells were incubated with sulfo-NHS-SS-biotin for 30 min at 18°C, chased for 5 min at 18°C in the presence of glycine, and incubated at 4°C in the absence (*control*) or presence

of extracellularly added MesNa. Transferrin receptor, synaptophysin, and SV2 in the postnuclear supernatants were analyzed for binding to streptavidin-agarose by immunoblotting of bound and unbound material with the respective antibodies. Streptavidin-bound biotinylated transferrin receptor, synaptophysin, and SV2 present in the postnuclear supernatant were calculated as percentage of total (sum of streptavidin-bound plus streptavidin-unbound transferrin receptor, synaptophysin, and SV2, respectively), and the individual values obtained after MesNa treatment were expressed as percentage of control. Data are the mean of three independent experiments; bars indicate SD. The mean values of biotinylated protein for the control condition were 25.3% (*transferrin receptor*), 7.5% (*synaptophysin*), and 3.1% (*SV2*).

also exists in PC12 cells maintained at 37°C. To investigate this issue, PC12 cells were biotinylated at 37°C for a very brief period of time (2 min) and chased for 10 min. Analysis of the biotinylated synaptophysin chased to SLMVs for sensitivity to MesNa treatment showed that it was not accessible to MesNa, as expected (Fig. 9 A). However, ~90% of the biotinylated synaptophysin recovered in the 66,000 g pellet, which contained the bulk of the non-SLMV synaptophysin, was still MesNa sensitive at the end of the 10 min chase (Fig. 9 B), although inaccessible to avidin (Fig. 9 C). In contrast, the biotinylated transferrin receptor in the 66,000 g pellet was inaccessible to MesNa (Fig. 9 D), consistent with its rapid endocytosis. These observations show that not only at 18° but also at 37°C, synaptophysin is internalized into a compartment that is characterized by a narrow continuity with the plasma membrane and is devoid of transferrin receptor.

To obtain information about the kinetics with which synaptophysin passes through this compartment, PC12

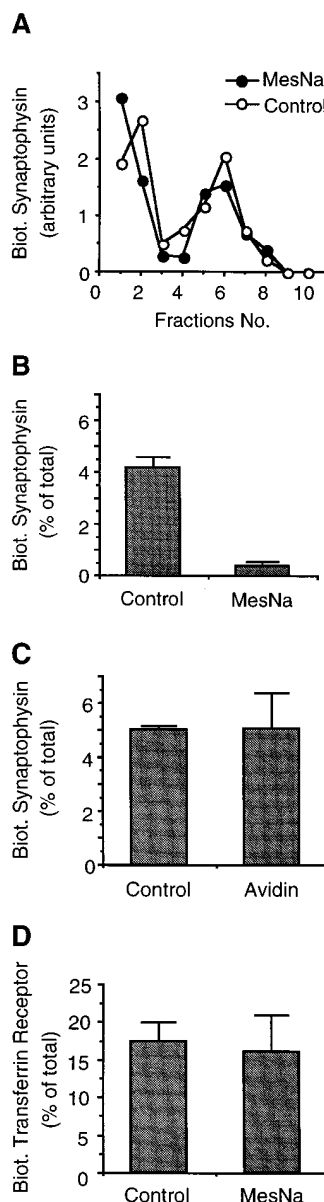


Figure 9. SLMVs originate from an avidin-protected, MesNa-accessible compartment at 37°C. PC12 cells were incubated with sulfo-NHS-SS-biotin for 2 min at 37°C, chased for 10 min at 37°C, and incubated at 4°C in the absence (*Control*) or presence of extracellularly added MesNa (A, B, and D) or avidin (C). The 66,000 g pellets and supernatants were prepared from the cells and the supernatants subjected to glycerol gradient centrifugation. Synaptophysin in the glycerol gradient fractions (A) and transferrin receptor and synaptophysin in the 66,000 g pellets (B–D) were analyzed for binding to streptavidin-agarose by immunoblotting of bound and unbound material with the respective antibodies. (A) A representative experiment showing biotinylated synaptophysin in the glycerol gradient fractions. (B–D) Biotinylated synaptophysin (B and C) and transferrin receptor (D) in the 66,000 g pellet is expressed as percent of total (sum of streptavidin-bound plus streptavidin-unbound synaptophysin and transferrin receptor, respectively, present in the sum of 66,000 g pellet plus supernatant). Data are the mean of four (*MesNa*) or two (*Avidin*) independent experiments; bars indicate SD or the variation of the individual values from the mean.

cells were biotinylated for 2 min at 37°C, chased for increasing periods of time, and subjected to MesNa treatment (Fig. 10). Biotinylated synaptophysin became inaccessible to MesNa with a $t_{1/2}$ of 15–20 min. Acquisition of MesNa inaccessibility by the transferrin receptor occurred much faster, with half of the transferrin receptor being protected from MesNa already at the end of the 2-min biotinylation and maximal protection being reached after 7.5 min of chase (Fig. 10).

A Sub-plasmalemmal, Tubulo-cisternal Membrane System Containing Synaptophysin but Not Transferrin Receptor: Relationship to the 18°C Compartment

By immunocytochemistry, synaptophysin has been previously shown to be colocalized with transferrin receptor in PC12 cells (Cameron et al., 1991). Given the above biochemical data, we reinvestigated the subcellular localization of synaptophysin. Confirming previous observations (Cameron et al., 1991), synaptophysin was indeed found to be colocalized with internalized transferrin when fixed PC12 cells were permeabilized with Triton X-100 and analyzed by double fluorescence using confocal microscopy (Fig. 11, C, D, c, and d). Synaptophysin immunoreactivity and fluorescent transferrin were predominantly observed in a perinuclear location. Surprisingly, a strikingly different subcellular localization of synaptophysin was observed when fixed PC12 cells were permeabilized with digitonin (Fig. 11, A, B, a, and b). In this condition, synaptophysin immunoreactivity was confined to the cell periphery (Fig. 11, A and a) and was remarkably distinct from the largely perinuclear localization of transferrin fluorescence (Fig. 11, B and b) or immunofluorescence for the transferrin receptor using an antibody against its cytoplasmic domain (data not shown). This difference in the intracellular pattern of synaptophysin immunoreactivity depending on the detergent used reflects a phenomenon that will be described in detail elsewhere (Hannah, M.J., and W.B. Hutt-

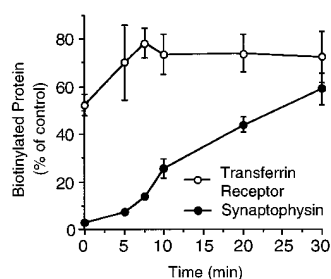


Figure 10. Kinetics of the acquisition of MesNa inaccessibility. PC12 cells were incubated with sulfo-NHS-SS-biotin for 2 min at 37°C, chased for the indicated times at 37°C, and incubated at 4°C in the absence (controls, 0 and 30 min chase only) or presence of MesNa. Synaptophysin and transferrin receptor

in the cell lysates were analyzed for binding to streptavidin–agarose by immunoblotting of bound and unbound material with the respective antibodies. Synaptophysin and transferrin receptor bound to streptavidin–agarose were calculated as percentage of total (sum of streptavidin-bound plus streptavidin-unbound synaptophysin and transferrin receptor, respectively) and are expressed as percentage of control (mean of the control values at 0 and 30 min of chase, which were very similar to each other). Data are the mean of two independent experiments; bars indicate the variation of the individual values from the mean and, for some time points, are within the size of the symbol. In the control, $4.2 \pm 0.3\%$ and $11.0 \pm 1.3\%$ of the total synaptophysin and transferrin receptor were biotinylated, respectively.

ner, manuscript in preparation) and is just briefly summarized here. Upon digitonin permeabilization of fixed PC12 cells, the anti-synaptophysin antibody has access only to synaptophysin localized at the cell periphery but not to that localized in the perinuclear area. By contrast, Triton X-100 permeabilization results in the virtually complete extraction, despite paraformaldehyde fixation, of synaptophysin from the cell periphery and allows access of the anti-synaptophysin antibody to synaptophysin in the perinuclear area. (This extraction of synaptophysin from fixed cells can be demonstrated biochemically [Fig. 12 A]; Hannah, M.J., and W.B. Huttner, manuscript in preparation.)

We exploited (a) the selective access of the anti-synaptophysin antibody to the peripheral synaptophysin upon digitonin permeabilization of paraformaldehyde-fixed PC12 cells and (b) the ability to extract synaptophysin from fixed cells, to demonstrate that the peripheral synaptophysin immunoreactivity included the biotinylated synaptophysin accumulated in the pre-SLMV compartment at 18°C. When Triton X-100 extracts of fixed and nonfixed PC12 cells that had been biotinylated at 18°C were adsorbed to streptavidin–agarose, virtually the same amount of synaptophysin immunoreactivity was detected on the beads (Fig. 12 A). This showed that Triton X-100 quantitatively extracted from paraformaldehyde-fixed PC12 cells, and the synaptophysin biotinylated at 18°C. Next, PC12 cells biotinylated for 30 min at 18°C were fixed, permeabilized with digitonin, and exposed to anti-synaptophysin antibody. The biotinylated synaptophysin was then extracted by Triton X-100, adsorbed to streptavidin–agarose, and analyzed for bound anti-synaptophysin antibody. If the peripheral synaptophysin that is selectively tagged by anti-synaptophysin addition to digitonin-permeabilized fixed cells (see Fig. 11, A and a) included the synaptophysin biotinylated at 18°C, one would expect to detect anti-synaptophysin immunoreactivity associated with the streptavidin–agarose beads in this condition. This was indeed the case (Fig. 12 B).

Electron microscopy of immunogold-labeled ultrathin cryosections revealed synaptophysin immunoreactivity associated with a pleiomorphic subplasmalemmal membrane system in both PC12 cells biotinylated for 30 min at 18°C (Fig. 13, A–D) and cells kept at 37°C (Fig. 13, E–H). Most membranes immunoreactive for synaptophysin had a tubular or cisternal morphology and often were localized in very close proximity to the plasma membrane. Connections between these membranes and the plasmalemma could not be convincingly demonstrated, presumably because the fixation had to be carried out at 4°C to allow a comparison of synaptophysin immunoreactivity between the cells incubated at 18° and 37°C (see Materials and Methods); at 4°C, these connections become very narrow (Fig. 6 and Discussion). Little synaptophysin immunoreactivity was detected on the plasma membrane itself, consistent with the results of biotinylation at 4°C (Fig. 5 A).

Discussion

We have characterized the compartment from which SLMVs originate, using cell surface-biotinylated synaptophysin and SV2 as markers. Our results lead to a modification of the concept about the biogenesis of SLMVs in neu-

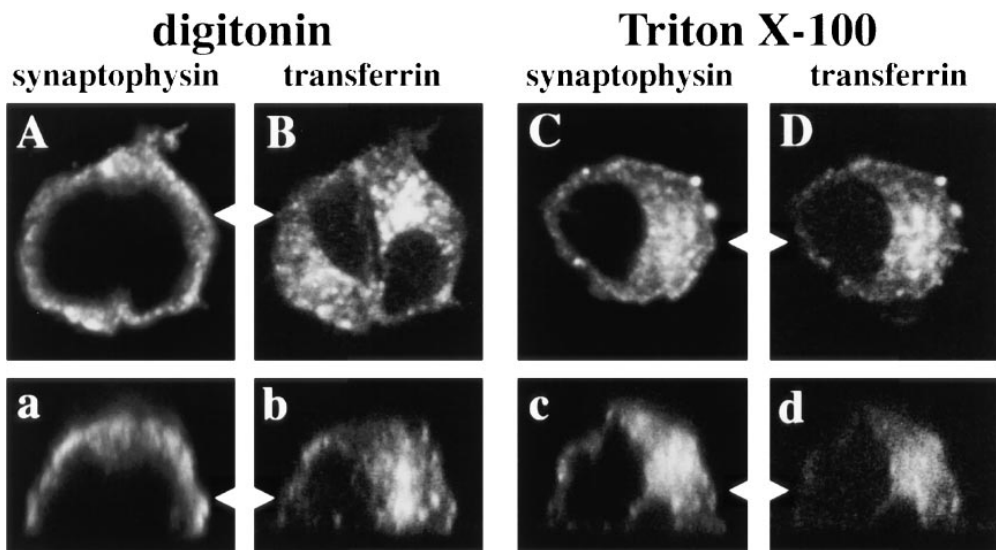


Figure 11. Double fluorescence analysis of PC12 cells for synaptophysin and internalized transferrin. PC12 cells were incubated with Texas red-labeled transferrin for 30 min at 37°C, fixed with paraformaldehyde, permeabilized with either digitonin (A, B, a, and b) or Triton X-100 (C, D, c, and d), and labeled with anti-synaptophysin followed by Cy2-labeled secondary antibody. Cells were analyzed by confocal laser scanning microscopy for synaptophysin immunoreactivity (A, a, C, and c) or internalized transferrin (B, b, D, and d). Single optical sections in the xy (A–D) or xz (a–d) planes of the

same cells are shown. The white triangular indentations at the margins of the panels indicate the approximate positions of the respective corresponding plane. The same confocal microscope parameters were used for the image pairs shown in panels A and C, B and D, a and c, and b and d. Note that upon permeabilization with digitonin, synaptophysin immunoreactivity is detected at the cell periphery but not in the perinuclear area which, however, shows transferrin fluorescence. After permeabilization with Triton X-100, the synaptophysin immunoreactivity at the cell periphery is decreased concomitant with its appearance in the perinuclear area, where it is largely colocalized with transferrin.

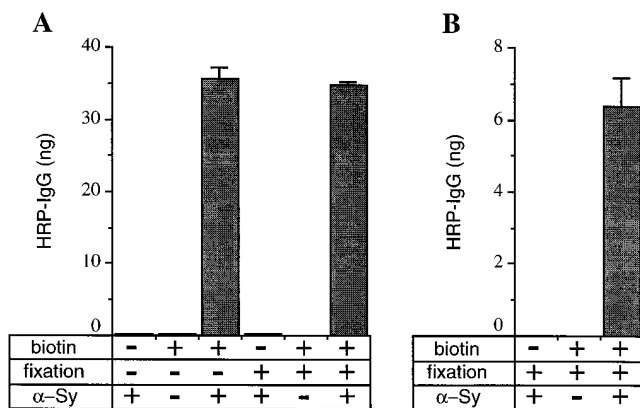


Figure 12. Synaptophysin biotinylated at 18°C is quantitatively extracted from paraformaldehyde-fixed PC12 cells by Triton X-100 (A) and is accessible to anti-synaptophysin after digitonin permeabilization of fixed cells (B). (A) PC12 cells were incubated without (–) or with (+) sulfo-NHS-LC-biotin for 30 min at 18°C, chased for 5 min at 18°C in the presence of glycine, and fixed (+) or not fixed (–), and Triton X-100 extracts were subjected to streptavidin-agarose adsorption. Specific immunoreactivity due to the binding of biotinylated synaptophysin to streptavidin-agarose was determined by incubating the beads without (–) or with (+) anti-synaptophysin antibody (α -Sy) followed by HRP-conjugated goat anti-mouse IgG antibody. Data are the mean of values obtained from three coverslips; bars indicate SD. (B) PC12 cells were incubated without (–) or with (+) sulfo-NHS-LC-biotin for 30 min at 18°C, chased for 5 min at 18°C in the presence of glycine, fixed, permeabilized with digitonin, incubated without (–) or with (+) anti-synaptophysin (α -Sy), and extracted with Triton X-100. The Triton extracts were subjected to streptavidin-agarose adsorption, and anti-synaptophysin bound to the beads via biotinylated synaptophysin was detected by HRP-conjugated goat anti-mouse IgG antibody. Data are the mean of values ob-

roendocrine cells, which is illustrated by the model shown in Fig. 14. According to our results, SLMVs originate from a subplasmalemmal tubulo-cisternal membrane system, referred to as SLMV donor compartment, that is (a) distinct from the transferrin receptor-containing endosome and (b) connected to the plasmalemma via a narrow membrane continuity.

The SLMV Donor Compartment Is Connected to the Plasma Membrane

The key observation, which provided the basis for the identification and characterization of the SLMV donor compartment, was that upon cell surface biotinylation at 18°C, a temperature which blocked the appearance of synaptophysin in SLMVs, all of the biotinylated synaptophysin was present in avidin-protected membranes, the majority of which were accessible to MesNa and therefore in continuity with the plasmalemma. This allowed us to show, by chasing such biotinylated synaptophysin following MesNa treatment at 37°C, that SLMVs originate from the latter membranes. Further evidence is provided by our experiments in which PC12 cells were biotinylated at 18°C, treated with avidin or MesNa, and then perforated to perform cell-free SLMV biogenesis; MesNa but not avidin per-

tained from three coverslips; bars indicate SD. The lower synaptophysin immunoreactivity in B than A (compare ordinate scales) presumably reflects incomplete accessibility of the anti-synaptophysin to its epitope when added to digitonin-permeabilized fixed cells as compared with anti-synaptophysin addition after Triton X-100 extraction of synaptophysin and its adsorption to streptavidin-agarose beads.

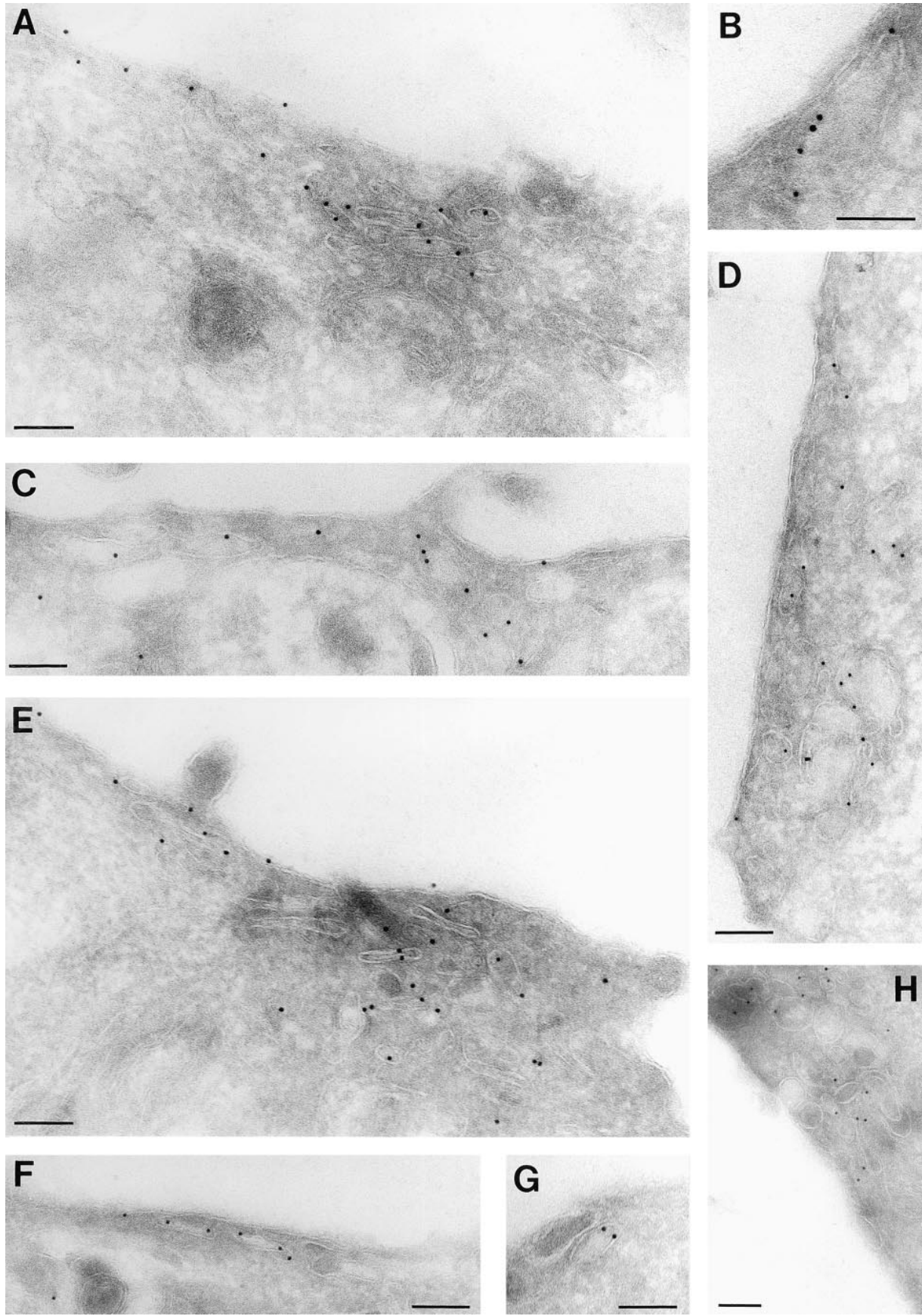


Figure 13. Morphology of the SLMV donor compartment. PC12 cells incubated at 18° (A–D) or 37°C (E–H) with sulfo-NHS-LC–biotin for 30 min followed by a 5-min chase were fixed at 4°C. Ultrathin cryosections were immunogold-labeled for synaptophysin and analyzed by electron microscopy. Note the synaptophysin immunoreactivity associated with tubulo-cisternal membrane structures beneath the plasma membrane. Little synaptophysin immunoreactivity is found at the plasma membrane itself. Bars, 100 nm.

treatment abolished the appearance of biotinylated synaptophysin in SLMVs (Schmidt, A., and W.B. Huttner, manuscript in preparation).

SLMVs originated from membranes in continuity with the plasmalemma not only upon reversal of the 18°C block, but also when cells were maintained at physiological temperature. After cell surface biotinylation at 37°C for 2 min followed by a 10 min chase, some (~5% in Fig. 9 A) of the biotinylated synaptophysin had already appeared in SLMVs, but the vast majority (>90% in Fig. 9, B and C) was still present in the faster sedimenting membranes from which these SLMVs must have been originating. The latter synaptophysin was protected from avidin but largely sensitive to MesNa.

The membrane connection between the SLMV donor compartment and the plasma membrane must be fairly narrow at 18°C because biotinylated synaptophysin was protected from avidin added at this temperature. This connection may, however, allow passage of other proteins (with less crosslinking potential than avidin) into the SLMV donor compartment given that Desnos et al. (1995) observed SLMV biogenesis upon reversal of a 15°C temperature block during which VAMP/synaptobrevin, tagged with a monoclonal antibody, had been internalized. Further constriction of this membrane connection appears to occur upon cooling cells to 4°C; during biotinylation at 18°C, biotinylated synaptophysin accumulated in the SLMV donor compartment, whereas sulfo-NHS-LC-biotin added at 4°C to cells preincubated at 18°C did not reach the synaptophysin in this compartment.

In two out of seven experiments, ~60% of the synaptophysin biotinylated at 18°C was found to be accessible to avidin (data not shown). While we do not know the reason for this variability of results (which may be due to differences in the batch of cells, passage number, state of confluency, batch of growth medium, etc.), it raises the possibility that the membrane connection between the SLMV donor compartment and the plasma membrane is subject to dynamic changes and may open and close, similar to the membrane dynamics of caveolae during potocytosis (Anderson, 1993). Perhaps, as a means of regulation, the SLMV donor compartment even pinches off from the plasma membrane and reconnects with it. This would be reminiscent of the acid-secreting gastric parietal cell, for which the reversible connection of an internal membrane compartment (rather than an exocytic-endocytic vesicle) with the plasma membrane has been hypothesized (Forte and Yao, 1996; Pettitt et al., 1996).

The SLMV Donor Compartment: A Tubulo-cisternal Membrane System beneath the Plasma Membrane

A characteristic feature of the SLMV donor compartment at the light microscopic level was the synaptophysin immunofluorescence at the cell cortex. Visualization of this synaptophysin required the use of the mild detergent digitonin as permeabilizing agent on fixed cells, which allowed the selective detection of the peripheral but not the perinuclear synaptophysin. Use of the stronger detergent Triton X-100 resulted in the quantitative extraction of the peripheral synaptophysin from fixed cells. Consistent with these immunofluorescence data, synaptophysin labeled

in the SLMV donor compartment by biotinylation at 18°C followed by fixation could be tagged with an antibody upon digitonin permeabilization and was quantitatively extracted by Triton X-100.

At the electron microscopic level, the correlate of the peripheral synaptophysin immunofluorescence, and hence of the SLMV donor compartment, was a tubulo-cisternal membrane system beneath the plasmalemma, observed in both cells maintained at physiological temperature and cells cooled to 18°C. Our observations confirm and extend those of other investigators who described synaptophysin-immunoreactive subplasmalemmal membrane structures in PC12 cells (Johnston et al., 1989); occasionally, these were seen in continuity with the plasma membrane, consistent with our biochemical results.

The narrow connection of the SLMV donor compartment to the plasma membrane and its tubular structure would slow down the entry into and spreading within this compartment of extracellularly added fluid phase markers. This provides an explanation why HRP internalized for 5 min had been detected in SLMVs after long (3 h) but not short (7 min) chase, an observation previously taken to indicate the origin of SLMVs from an endosomal compartment distinct from the plasma membrane (Bauerfeind et al., 1993). Given its structure, the SLMV donor compartment is also likely to become physically separated from the plasma membrane upon homogenization of cells. This would explain why the SLMV donor membranes from PC12 cells incubated at 15°C sedimented differently from the plasma membrane (Desnos et al., 1995).

By both immunoblotting and immunofluorescence, PC12 cells were found to lack caveolin (data not shown), and hence their subplasmalemmal tubulo-cisternal membrane system is distinct from the caveolin-positive caveolae. The morphological appearance of the synaptophysin-immunoreactive membrane structures seen in cells incubated at 18°C clearly speaks against the possibility that the SLMV donor compartment corresponded to nascent SLMVs that had not yet pinched off from the plasma membrane. This possibility was also contradicted by the observation that the vast majority (>80%) of the synaptophysin biotinylated at 18°C was recovered, upon chase at 37°C, in membranes larger than SLMVs, as revealed by their sedimentation properties.

Segregation of Synaptophysin and the Transferrin Receptor

SLMVs are devoid of transferrin receptor (Clift-O'Grady et al., 1990; Cameron et al., 1991). Given the SLMV donor compartment described here, where does the segregation of synaptophysin and transferrin receptor occur? A key observation of the present study was that the SLMV donor compartment did not contain detectable levels of biotinylated transferrin receptor, as judged from its inaccessibility to MesNa at both 37° and 18°C. This suggests that the transferrin receptor and synaptophysin segregate at the plasma membrane, with transferrin receptor being internalized directly from the plasma membrane via endocytic vesicles and synaptophysin moving into the SLMV donor compartment by lateral mobility and/or membrane invagination (Fig. 14).

compartment described here for PC12 is related to the compartment implicated in synaptic vesicle biogenesis in nerve cells. Neurons are thought to contain at least two types of endosomes, the somatodendritic, transferrin receptor-containing, "housekeeping" endosome and the axonal, transferrin receptor-lacking, "specialized" endosome thought to be involved in synaptic vesicle biogenesis and recycling (Cameron et al., 1991; Kelly, 1993a; Mundigl et al., 1993). Our data showing the absence of detectable levels of transferrin receptor in the SLMV donor compartment of PC12 cells further support its close relationship to the axonal endosome. Moreover, our data support the notion (Kelly, 1993a) that the somatodendritic and axonal types of endosomes can coexist in the absence of obvious cell polarity.

Recently, Takei et al. (1996) proposed, on the basis of electron microscopic observations of K^+ -depolarized neurons in culture and isolated nerve terminals, that the axonal endosome may be in continuity with the plasma membrane, and that the reformation of synaptic vesicles after exocytosis may occur in a single vesicle budding step from these deep membrane invaginations of the presynaptic plasma membrane. Our results concerning the donor compartment of SLMVs are fully consistent with the observations of Takei et al. (1996). Together, the latter report and the present study suggest that a plasma membrane-connected SLMV/synaptic vesicle donor compartment may have a central role in both SLMV/synaptic vesicle de novo biogenesis and reformation.

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