Biogenesis of Synaptic Vesicle-like Structures in a Pheochromocytoma Cell Line PC-12

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Abstract. The presence of unique proteins in synaptic vesicles of neurons suggests selective targeting during vesicle formation. Endocrine, but not other cells, also express synaptic vesicle membrane proteins and target them selectively to small intracellular vesicles. We show that the rat pheochromocytoma cell line, PC12, has a population of small vesicles with sedimentation and density properties very similar to those of rat brain synaptic vesicles. When synaptophysin is expressed in nonneuronal cells, it is found in intracellular organelles that are not the size of synaptic vesicles. The major protein in the small vesicles isolated from PC12 cells is found to be synaptophysin, which is also the major protein in rat brain vesicles. At least two of the minor proteins in the small vesicles are also known synaptic vesicle membrane proteins. Synaptic vesicle-like structures in PC12 cells can be shown to take up an exogenous bulk phase marker, HRP. Their proteins, including synaptophysin, are labeled if the cells are surface labeled and subsequently warmed. Although the PC12 vesicles can arise by endocytosis, they seem to exclude the receptor-mediated endocytosis marker, transferrin. We conclude that PC12 cells contain synaptic vesicle-like structures that resemble authentic synaptic vesicles in physical properties, protein composition and endocytotic origin.

SYNAPTIC vesicles were first identified when electron micrographs of nerve terminals revealed clusters of spherical vesicles with remarkably constant diameters. Generation of antibodies to purified synaptic vesicles showed that they contained unique proteins (Carlson and Kelly, 1980; Jones et al., 1981). To understand how synaptic vesicles are made therefore, we need to know how unique synaptic vesicle proteins are targeted selectively to the synaptic vesicle, and how uniform diameters are generated.

A cell line containing synaptic vesicles would facilitate the study of synaptic vesicle biogenesis. Endocrine cell lines are good candidates since they express four of the known synaptic vesicle membrane proteins: p65 (Matthew et al., 1981), SV2 (Buckley and Kelly, 1985), synaptophysin or p38 (Jahn et al., 1985; Wiedenmann and Franke, 1985), and synaptobrevin (Baumert et al., 1989). Although some of these proteins can be detected in dense core secretory granules (Lowe et al., 1988; Obendorf et al., 1988), there is general consensus that in the pheochromocytoma cells line, PC12, the majority of the proteins are in small, electron-lucent vesicles of unknown function (Navone et al., 1986; Wiedenmann et al., 1988). The relationship of PC12 cell vesicles to authentic brain synaptic vesicles is not clear. Johnston et al. (1989) have recently suggested that the small PC12 vesicles are pleiomorphic, larger than synaptic vesicles, and are involved in transferrin transport. On the other hand, synaptophysin-containing vesicles from PC12 cells cannot be distinguished from authentic rat brain synaptic vesicles on sizing columns (Wiedenmann et al., 1988). If the vesicles in PC12 cells are valid analogues of brain synaptic vesicles, and if they can be isolated, then analysis of membrane protein targeting to PC12 vesicles should clarify how synaptic vesicles are generated.

We can identify in PC12 cells, vesicles that are similar to authentic rat brain synaptic vesicles by several criteria. They have the same sedimentation velocity and buoyant density and contain at least two of the other synaptic vesicle membrane proteins, p65 and SV2. We can isolate them and show that their major protein appears to be synaptophysin, as is true for authentic synaptic vesicles. By two techniques we show that PC12 vesicles arise by endocytosis. The PC12 vesicles can therefore be considered valid analogues of brain synaptic vesicles by four criteria: size, density, protein composition, and endocytotic origin. They may not be completely analogous, however, because we have failed to demonstrate neurotransmitter retention.

Since PC12 vesicles can be readily isolated, it is possible to compare targeting data generated by biochemical analysis to earlier morphological studies. The distribution of synaptophysin and endocytosed transferrin overlapped in immunofluorescence studies of both PC12 cells and also fibroblasts transfected with DNA encoding synaptophysin (Johnston et al., 1989). The conclusion was that a significant fraction of the synaptophysin is targeted to an organelle common to

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all cells, namely the early endosome. In contrast, the endocytotic vesicles we isolate do not appear to participate in transferrin endocytosis. Furthermore, when we analyze fibroblast cells transfected with DNA-encoding synaptophysin, we cannot detect the characteristic small vesicle population. Since the small vesicles are cell type specific, while the endocytotic pathway is universal, it appears unlikely that the small vesicles function in the early endosome cycle.

The data we present here lead us to propose that the vesicles we isolate are not transferrin transport intermediates and that the capacity to generate homogeneous diameter synaptic vesicle structures is a property restricted to neural cells and at least some endocrine cells. Knowledge of how membrane proteins are selectively targeted to these vesicles and how the vesicles acquire such uniform diameters should illuminate the biogenesis of synaptic vesicles.

Materials and Methods

Cell Culture and Organelle Isolation

A pheochromocytoma cell line transfected with human growth hormone (hGH), PC12pMT:hGH, was grown as described in Lowe et al. (1988) in 10% CO_2 at 37°C and feeding medium consisting of DME H-21 containing 10% FBS, 5% horse serum, penicillin (100 U/ml), streptomycin (100 U/ml), and Geneticin (G418; Gibco Laboratories, Grand Island, NY) at 0.25 mg/ml. When required, 7S nerve growth factor (Calbiochem-Behring Corp., La Jolla, CA) was added at 75 ng/ml.

The standard procedure for vesicle isolation was as follows. Two confluent 15-cm plates of PC12 cells were rinsed once with buffer A (150 mM NaCl, 10 mM Hepes, pH 7.4, 1 mM EGTA, 0.1 mM $MgCl_2$), and using a cell scraper (Costar Data Packaging Corp., Cambridge, MA), the cells were removed into 25 ml buffer. Cells were centrifuged at 300 *g* for 7 min, the supernatant discarded, and the pellet resuspended by gentle trituration in 1 ml buffer A containing protease inhibitors (pepstatin, chymostatin, leupeptin, and aprotinin at 10 ng/ml; 1 mM PMSF, 1 μg/ml *o*-phenanthroline, and 10 μM benzamidine). Homogenization was performed using five passes across a ball bearing in a Cell Cracker (European Molecular Biological Laboratory) (12 μm clearance). The homogenate was centrifuged in 4 ml polycarbonate tubes in an SS-34 rotor at 10,000 *g* for 5 min. The low-speed supernatant (S1) was collected and centrifuged at 27,000 *g* for 35 min. The S1, the high-speed supernatant (S2), and the pellet (P2), resuspended in buffer A, were analyzed as described in the text.

Glycerol velocity gradients were as described earlier (Carlson et al., 1978) except that buffer A was used. 200–250-μl samples were layered on 5–25% glycerol gradients in buffer A and centrifuged at 48,000 rpm in a SW50 or SW55 (Beckman Instruments, Inc., Palo Alto, CA) for 1 h at 5°C in a polyallomer tube. In some experiments, fast sedimenting material was trapped on a pad (0.4 ml) of 50% sucrose in buffer A. Samples were collected from the bottom of the tubes. When the centrifuge conditions were modified, the modifications are noted in the figure legends. To purify the synaptic vesicle-like (SVL)[1] structures further, peak fractions from the glycerol gradients were diluted slightly and run on 4-ml preformed linear gradients of sucrose (10–50% wt/vol) in buffer A. All gradients were centrifuged in SW50 or SW55 rotors, at 48,000 rpm, 5°C for at least 5 h (see figure legends). The time was chosen knowing the sedimentation rate of the vesicles, to allow the vesicles to reach their equilibrium density. The presence of SVL vesicles in PC12 cell homogenates was not dependent on the presence of nerve growth factor in the medium, nor the particular form of homogenization used, since the Dounce homogenizer (Kontes Glass Co., Vineland, NJ) was also effective.

Cell Labeling

For cell surface labeling, a membrane-impermeant iodinated reagent, [^{125}I]sulfosuccinimydyl(hydroxyphenyl)propionate ([^{125}I]sulfo-SHPP) was used (Thompson et al., 1987) as described. Cells were metabolically labeled with [^{35}S]Translabel (ICN K & K Laboratories Inc., Irvine, CA). To label the cytoplasmic stores of catecholamine, cells were grown in [^{3}H]norepinephrine (NE, l-[7,8^{3}H]norepinephrine, 39 mCi/mM; Amersham Corp., Arlington Heights, IL) as described by Lowe et al. (1988). To examine the effect of ATP on storage of [^{3}H]NE, cells were homogenized in the presence of 1 mM ATP, and an ATP regenerating system consisting of creatine phosphokinase (100 μg/ml), creatine phosphate (10 mM), and 1 mM $MgCl_2$, with the usual buffers and protease inhibitors. To avoid NE leakage during isolation, the velocity gradient contained 1 mM ATP and 1 mM $MgCl_2$.

1. *Abbreviations used in this paper*: hGH, human growth hormone; NE, norepinephrine; SVL, synaptic vesicle-like.

Rat Brain Synaptic Vesicle Preparations

We developed a novel technique of synaptic vesicle isolation that avoided hypotonic lysis of synaptosomes. In addition, isolation was performed at physiological ionic strengths to minimize adsorption of soluble proteins to the vesicles. Brains from 21 rats, decapitated after carbon dioxide narcosis, were homogenized in 110 ml of ice-cold buffer A for 2 min using a Waring blender (Waring Products Division Dynamics Corp. of America, New Hartford, CT). All subsequent steps of the preparation were done at 4°C. The homogenate was centrifuged (SS34; Sorvall Instruments Div., Newton, CT) for 15 min at 18,000 rpm. The supernatant was layered on 20 ml of buffer A containing 20% D_2O and centrifuged in Oakridge tubes in a rotor (45 Ti; Beckman Instruments, Inc.) for 2–4 h at 44,000 rpm. The pellet (P2) was homogenized in 30 ml buffer A using 12 strokes of a glass-Teflon homogenizer. 7.5 ml of resuspended P2 was layered on a step gradient consisting of 1.5 ml each of 70, 85, and 100% D_2O containing 320 mM sucrose, 4 mM Hepes (pH 7.4). The gradients were centrifuged in an SW 41 (Beckman Instruments, Inc.) for 70 min at 40,000 rpm. The discrete band at the 70% D_2O interface was collected, pooled, and concentrated by diluting threefold with buffer A and pelleting for at least 4 h in a 45 Ti rotor at 44,000 rpm. The resulting pellets (P3) were resuspended to 1.6 ml with 12 strokes of a 2-ml glass-Teflon homogenizer. 0.4 ml of the resuspended P3 was layered on continuous density gradients (20% D_2O in buffer A to 100% D_2O, 320 mM sucrose, 4 mM Hepes, pH 7.4) and centrifuged in an SW 41 Ti for 3 h at 40,000 rpm. A broad band is observed through the center of the gradient with a smaller band at a lighter density. The broad band was collected, pooled, and concentrated as before by pelleting. The pellets were resuspended in buffer A (P4) and immediately centrifuged in an Eppendorf 5415 microcentrifuge (Brinkman Instruments Co. Division of Sybron Corp., Westbury, NY) for 4 min at top speed (16,000 *g*). The supernatant (S5) was carefully removed to a new tube. The vesicles in the S5 eluted from a S-1000 Sephacryl sizing column (Pharmacia, Uppsala, Sweden) in a single peak and had a polypeptide composition similar to the synaptic vesicles isolated by Huttner et al. (1983). In the text this is referred to as procedure A.

We also isolated rat brain synaptic vesicles using a procedure (procedure B) similar to that developed for PC12 vesicles. A resuspended P2 from the rat brain homogenate was centrifuged on a 5–25% glycerol gradient (buffer A) in a rotor (SW55; Beckman Instruments, Inc.) for 1 h, 48,000 rpm at 5°C. The peak fractions were pooled, and centrifuged on a 10–50% (wt/vol) sucrose gradient (buffer A) for 20 h at 48,000 rpm, 5°C (SW55; Beckman Instruments, Inc.). Fractions of highest antigenicity were pooled, dialyzed, and used as rat brain synaptic vesicle markers. Procedures A and B yielded rat brain synaptic vesicles that had identical sedimentation properties on glycerol density gradients and the same buoyant density.

Assays of Synaptic Vesicle Antigens

Three techniques were used to determine the level of synaptic vesicle antigenicity in fractions. The first was the dot blot assay described by Wiedenmann et al. (1988). The second was the solid-phase immunoadsorbent assay described by Carlson et al. (1980) using the synaptophysin mAb, SY38 (Boehringer Mannheim, Mannheim, FRG), at 0.5 μg/ml. For Fig. 1, two other mAbs were used, anti-p65 kindly provided by L. Reichardt (University of California at San Francisco), and anti-SV2 (Buckley and Kelly, 1985).

The third technique involved immunoprecipitation of samples from metabolically labeled cells, followed by polyacrylamide gel electrophoresis. To 150 μl of each gradient fraction was added 2 μl of a polyclonal anti-rat brain synaptic vesicle serum and 850 μl of buffer B (66 mM EDTA, 1% NP-40, 0.4% deoxycholate, 10 mM Tris (pH 7.4), and 0.3% SDS. After an overnight incubation at 4°C, 50 μl of fixed *Staphylococcus aureus* immunoadsorbent (Zymed Laboratories, S. San Francisco, CA) was added for 30 min at room temperature. The immunoadsorbent was washed through two 30% sucrose pads made up with buffer B, washed once with buffer B alone, and then suspended in sample buffer. Samples were then counted or analyzed by SDS-gel electrophoresis.

Immunoadsorption of Organelles

The procedure followed was similar to that of Lowe et al. (1988) with the following minor modifications. Membrane fractions were preincubated in fivefold dilution of normal rabbit serum to block nonspecific antibody binding sites. Dynabeads M-450, magnetic polystyrene beads coated with goat anti-mouse IgG (Dynal Inc., Great Neck, NY), were incubated either with SY38 (antisynaptophysin monoclonal) or, as a control, an excess of nonspecific mouse immunoglobulin. An alternative control was the incubation of the vesicle fraction in a 1:5 dilution of a rabbit antisynaptic vesicle antiserum. At such concentrations subsequent binding of free antisynaptophysin mAb (SY38) was blocked by >95% (data not shown).

Fluid Phase Uptake of HRP

The uptake of HRP into PC12-pMT:hGH cells was assayed according to the method of Steinman et al. (1974) and Steinman and Cohn (1972) with the following modifications. Two 15-cm dishes of cells were incubated for 2 h in culture medium containing HRP at a concentration of 10 mg/ml. The cells were then cooled to 0°C and washed 10 times with ice-cold serum-free media. After a final wash with buffer A, cells were scraped off the plates and prepared for the subcellular fractionation procedure as previously described.

Triton X-100 was added to 200 μl of each gradient fraction to a final concentration of 0.05% (vol/vol). Each fraction was then added to 0.8 ml of substrate and the change in absorbance at 460 nm with respect to time was measured on a spectrophotometer (Perkin-Elmer Corp., Oakbrook, IL) attached to a chart recorder. The cells exhibited no endogenous peroxidase activity.

Expression of Synaptophysin by DNA Transfection

To obtain a clone coding for the entire length of synaptophysin, a λgt10 rat hippocampal library was screened with a synthetic oligonucleotide corresponding to nucleotides 1-70 of the synaptophysin coding sequence (Leube et al., 1987; Sudhof et al., 1987). Two overlapping synaptophysin cDNA clones (−84 to 213 and 9 to 1164; numbering of base pairs starts at first base in the initiation codon) subcloned into Bluescript (Stratagene, La Jolla, CA) were joined at a Pvu II site in the overlapping region. After confirmation by sequencing, this construct was cloned into the expression vector

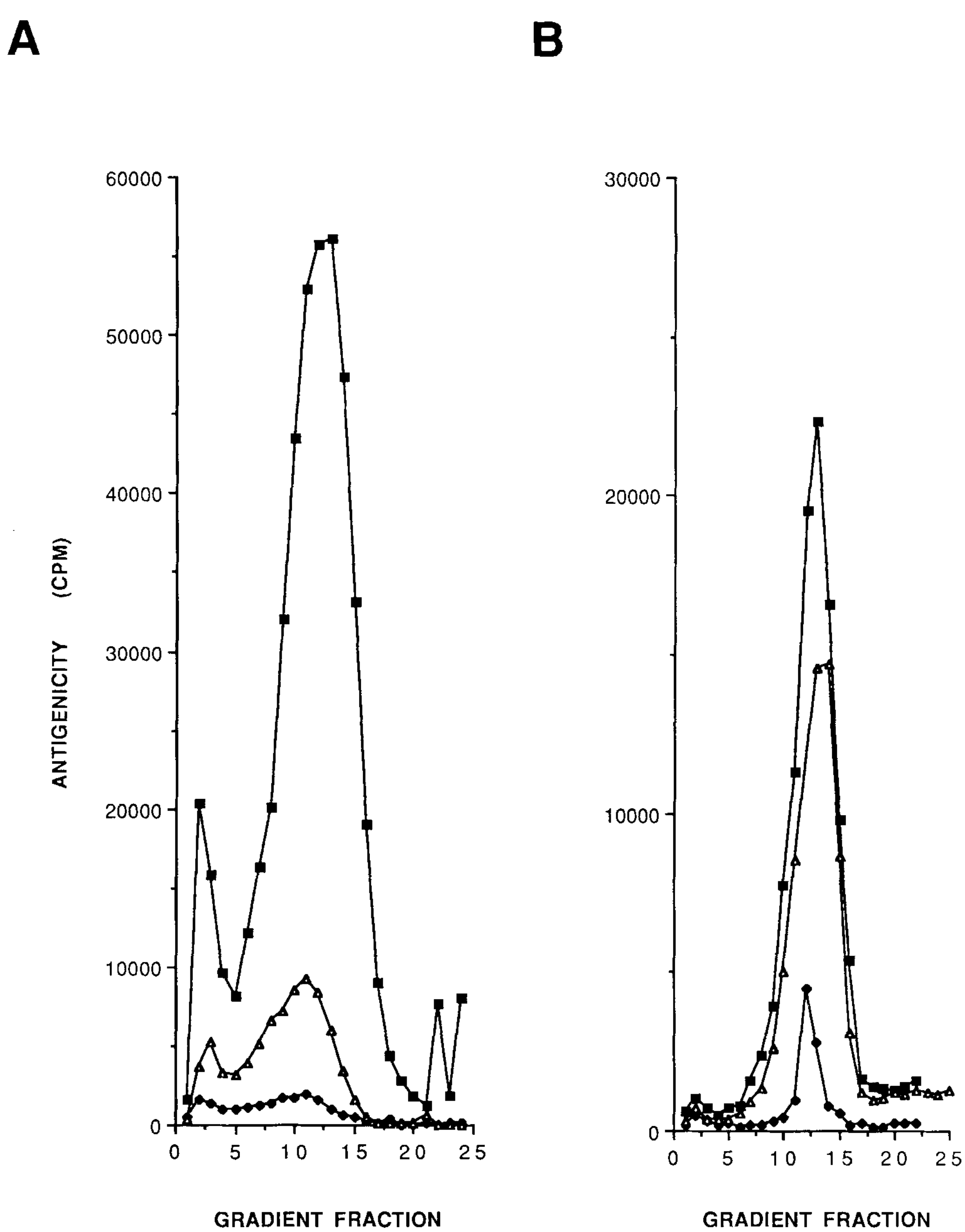

Figure 1. Sedimentation rates of synaptic vesicle proteins from PC12 cells (*A*) and rat brain (*B*). Membranes were centrifuged on a 5–25% glycerol gradient with a pad of 50% sucrose. Fractions were collected and assayed by the solid phase immunoadsorbent assay for three synaptic vesicle antigens, synaptophysin (■), SV2 (△), and p65 (◆). The samples analyzed were (*A*) a high-speed supernatant (27,000 *g*, 35 min) from a PC12 homogenate, and (*B*) rat brain synaptic vesicles. The rat brain synaptic vesicles used in this experiment were purified by procedure B (see Materials and Methods). Synaptic vesicles isolated by procedure A had identical sedimentation characteristics. Antigenicity in this and subsequent figures is in arbitrary units. The ratio of antigenicity reflects relative abundance in the membrane fractions.

pSM (M. H. Brodsky and D. Littman, University of California at San Francisco, unpublished observations) which contains the SV40 early promoter. pSM-p38 was transiently introduced into COS cells using the lipid-mediated transfection procedure of Felgner et al. (1987). The transfected cells in two confluent 15-cm dishes were metabolically labeled with 2 mCi [^{35}S]Translabel 12 h before harvesting. The homogenization and centrifugation conditions were the same as described above for the experiments with PC12 cells.

Hormone Assay

hGH was assayed by RIA utilizing a murine mAb (Allegro TM hGH Immunoassay System; Nichols Institute, San Juan Capistrano, CA).

Transferrin Uptake

Mouse Apo transferrin (Cappel Laboratories, Westchester, PA) was iron saturated (Karin and Mintz, 1980) and freshly iodinated with Iodogen (Pierce Chemical Co., Rockford, IL). [^{125}I]transferrin was added to a 15-cm dish of PC12 cells at 15 μg/ml in 8 ml of PBS/1% BSA/0.1% glucose at 37°C for 2 h. Titration of transferrin concentration demonstrated 50% receptor occupancy at 15 μg/ml transferrin concentration. Subsaturating conditions were used to enhance receptor-mediated endocytosis relative to bulk phase. After uptake, transferrin that had not been internalized was removed from cell surface receptors with two washes in buffer A followed by two washes in low pH buffer (10 mM citrate, 150 mM NaCl, 0.1 mM $MgCl_2$, 1 mM EGTA, pH 5.0), and two additional washes with buffer A. With such washing, the ratio of transferrin taken up in 10 min at 37°C, to transferrin bound at 0°C was 20:1. The cells were then fractionated as previously described.

Results

Identification of a Class of Small Vesicles in PC12 Cells

When we homogenized PC12 cells and monitored the synaptic vesicle protein, synaptophysin, during fractionation we found that about half the synaptophysin antigenicity could be recovered in a high-speed (27,000 *g*, 35 min) supernatant. When the high-speed supernatant was centrifuged on a glycerol velocity gradient, the majority of the synaptophysin sedimented as a single peak with a sedimentation velocity close to that of authentic rat brain synaptic vesicles (Fig. 1). Since synaptic vesicles have dimensions of 30–60 nm we assume that the PC12 vesicles have a similar size. Two other well-established synaptic vesicle proteins, p65 (Matthew et al., 1981) and SV2 (Buckley and Kelly, 1985), cosedimented with the synaptophysin (Fig. 1 *A*). Although the antigens were in the same relative abundance in PC12 vesicles as in rat brain synaptic vesicles (Fig. 1 *B*), the ratio of one antigen to another was not identical. The ratio of synaptophysin to the SV2 antigen in PC12 vesicles was about five times greater than would be predicted from the brain synaptic vesicle measurements. We conclude that in PC12 cells a large fraction of the synaptic vesicle membrane proteins is in vesicles with sedimentation properties similar to rat brain synaptic vesicles.

Since the position of the vesicle peak in the gradient is a linear function of time (data not shown), the glycerol gradients are separating by sedimentation rate and not by equilibrium density. Electric organ synaptic vesicles (Carlson et al., 1978), which have a diameter of 80 nm (Sheridan et al., 1966), sediment under these conditions ∼1.4 times faster than the PC12 vesicles. Using the known sedimentation rates of electric organ vesicles (Carlson et al., 1978) we estimate the PC12 vesicles to be ∼80S. We do not know if the 80S vesicle peak is identical to the small electronlucent vesicles seen in the electron microscope (Wiedenmann and Franke, 1985; Navone et al., 1986) or the vesicles isolated by permeation chromatography (Wiedenmann et al., 1988). It is possible that the 80S vesicles are a subclass of the small vesicle population detected, for example, by Johnston et al. (1989). Until the function of the 80S vesicles is known we call them SVL vesicles, to emphasize the biochemical similarities between SVL vesicles and the synaptic vesicles. The SVL vesicles can also be detected in other endocrine cell lines such as the pituitary line, AtT-20 (L. Matsuuchi, A. Linstedt, and R. Kelly, unpublished observations).

Protein Composition of the SVL Vesicles

To identify proteins in PC12 SVL vesicles, PC12 cells were labeled with [^{35}S]-labeled methionine and cysteine. Fractions from a glycerol velocity gradient with the highest synaptophysin antigenicity were pooled and analyzed by equilibrium centrifugation on sucrose density gradients (Fig. 2 *A*). A peak of synaptophysin antigenicity coincided with a peak of radioactivity at a density of 1.113 ± 0.003 g·cm^{-3} (five measurements). Rat brain synaptic vesicles had a buoyant density of 1.118 g·cm^{-3} under these conditions. When fractions across the gradient were analyzed by SDS gel electrophoresis, synaptophysin was found to be the major labeled protein (Fig. 2 B) in the SVL vesicle population. Using a silver stain to identify proteins, synaptophysin is also the major protein in purified rat brain synaptic vesicles (Fig. 2 *C*, lane *4*). In addition, seven smaller polypeptides (*asterisks*) copurified with synaptophysin (Fig. 2 *B*). The inability to detect p65 is probably due to its relative scarcity (Fig. 1). The SV2 antigen is also difficult to detect both because of its low abundance and because it forms a diffuse band, presumably due to glycosylation (Buckley and Kelly, 1985; Pfeffer and Kelly, 1985). Other minor bands are in contaminating membranes that do not peak at the density of the SVL vesicles.

An alternative means of identifying components of vesicles is to isolate them by immunoadsorption using an antibody to the cytoplasmic domains of the vesicle proteins. The [^{35}S]-labeled SVL vesicle pool from a glycerol velocity gradient was mixed with magnetic beads to which antisynaptophysin antibody had been attached (Lowe et al., 1988). About 30% of the radioactivity was specifically adsorbed from the pool. When the immunoadsorbed radioactivity was analyzed by SDS gel electrophoresis, several polypeptides in addition to synaptophysin could be identified. Synaptic vesicle proteins identified by both immunoabsorption and comigration with synaptophysin on density gradients are indicated by asterisks in Fig. 2 C. The minor protein at 18 kD might be synaptobrevin (Baumert et al., 1989). The other six minor polypeptides of sizes ranging from 22 to 32 could be breakdown products although protease inhibitors were present during the isolation. Since the SVL vesicles resemble rat brain synaptic vesicles both in physical properties and protein composition, we suggest that the endocrine cell line, PC12, has the capacity to make synaptic vesicles. Although the physiological significance, if any, of this capacity is obscure, it allows vesicle biogenesis and vesicle protein targeting to be studied in a cell line.

Endocytotic Origin of SVL Vesicles

Since synaptic vesicle membranes recycle at the nerve terminal, many, if not all of the vesicles in neurons have arisen by endocytosis. If SVL vesicles can arise by endocytosis it

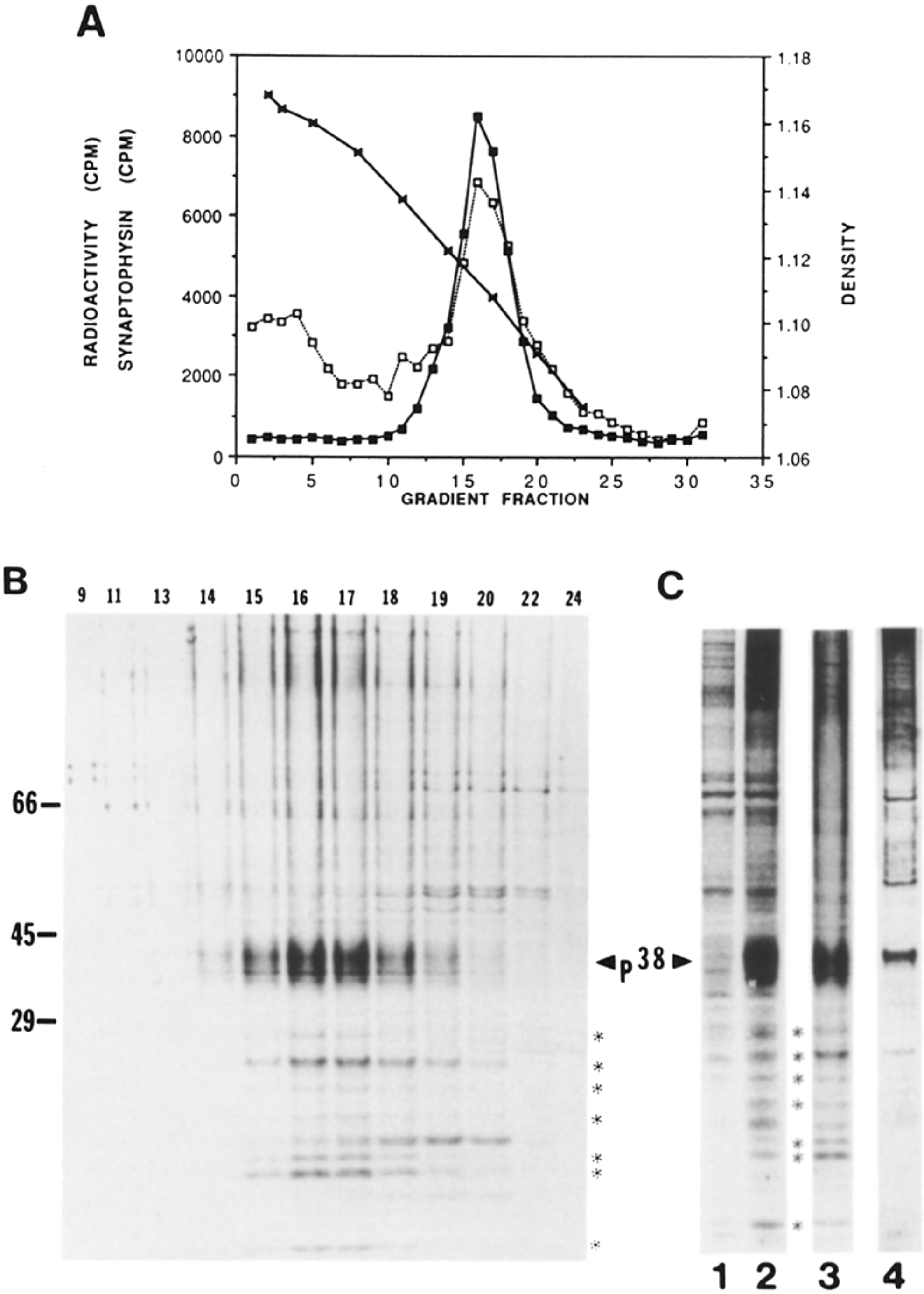

Figure 2. Isolation of PC12 SVL vesicles by equilibrium density centrifugation. Cells were labeled for 16 h with [^{35}S]methionine and cysteine, and homogenized. The high-speed supernatant (27,000 *g*, 35 min) was centrifuged on a 5–25% glycerol velocity gradient and the fractions corresponding to the SVL vesicle peak were pooled, diluted 1.5-fold, layered on a 10–50% sucrose density gradient, and centrifuged to equilibrium at 48,000 rpm for 11.5 h (*A*) Fractions were analyzed for synaptophysin antigenicity (■), [^{35}S] radioactivity (□), and density was measured by refractive index (*). (*B*) Selected fractions were analyzed by SDS-PAGE. Synaptophysin (p38) is indicated. Other polypeptides copurifying with synaptophysin are indicated by asterisks. (*C*) Comparison of SVL vesicles isolated by immunoadsorption or by centrifugation with rat brain synaptic vesicles. The polypeptide distribution in fraction number 17 (Fig. 2 *B*) is compared (lane *3*) to proteins immunoadsorbed from a glycerol gradient SVL vesicle peak using antisynaptophysin (lane *2*) or control antibodies (lane *1*). Lane *4* shows the polypeptide composition of purified rat brain synaptic vesicles (procedure A, see Materials and Methods) for comparison, after electrophoresis and silver staining. Synaptophysin (p38) is indicated. The same proteins are asterisked as in Fig. 2 *B*.

should be possible to detect endocytotic markers comigrating with SVL vesicles on velocity and density gradients. To detect fluid phase endocytosis, cells were incubated for 2 h in HRP, homogenized, and a high-speed supernatant was analyzed by velocity sedimentation. Most of the HRP was detected by enzyme assay as soluble enzyme at the top of the gradient. A small fraction of HRP (Fig. 3 *A*) consistently sedimented with the synaptophysin antigenicity. When the high-speed supernatant was analyzed by equilibrium density centrifugation, the enzyme activity was again recovered in fractions containing the peak synaptophysin antigenicity (Fig. 3 *B*). When the enzyme activity in the vesicle peak was compared to the total membrane-associated enzyme in a P2 fraction (see Materials and Methods), ~2% was in the vesicles, suggesting that they are only a minor endocytotic compartment in terms of volume. Their small internal volume may explain why endocytotic vesicles with these sedimentation properties have not previously been reported, to our knowledge.

As a second marker of endocytosis, cells were surface labeled with an ^{125}I-labeled membrane-impermeant reagent, [^{125}I]sulfo-SHPP (Thompson et al., 1987). Homogenates were prepared from cells kept at 0°C to inhibit endocytosis and from cells returned to incubation medium for 1 h at

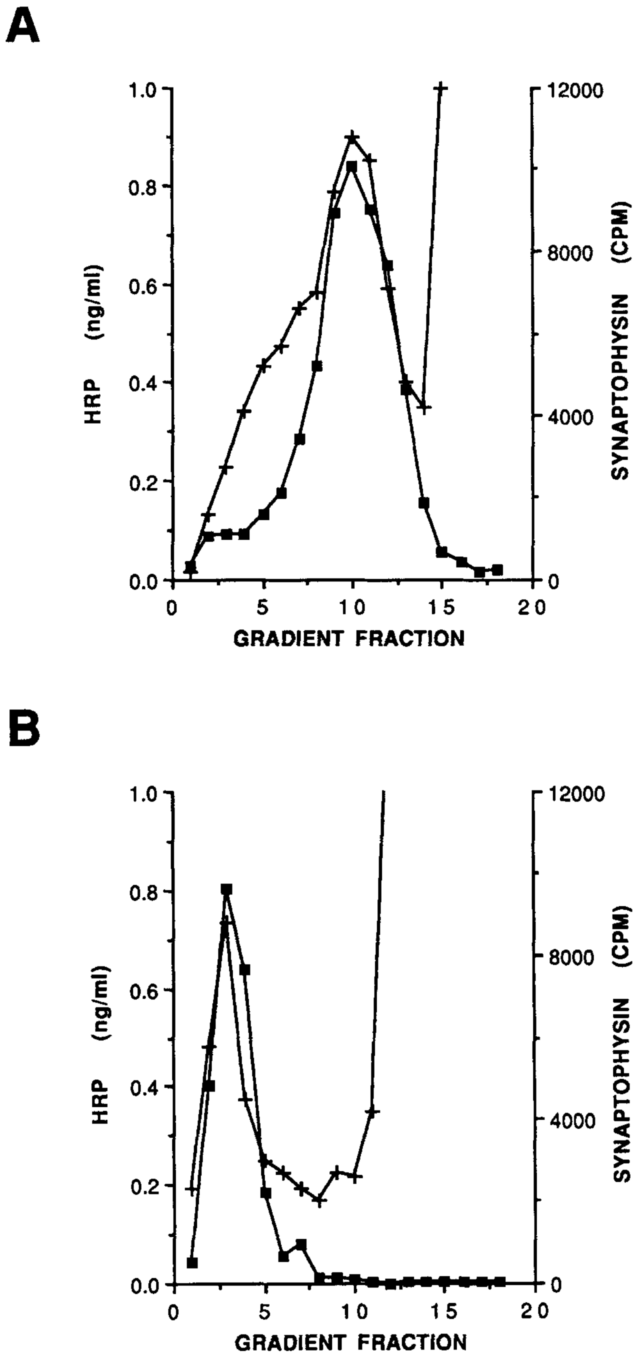

Figure 3. A fluid phase marker comigrates with the SVL vesicles. Cells incubated in 10 mg/ml HRP for 2 h were homogenized, and the high-speed supernatant (27,000 *g*, 35 min) was analyzed by (*A*) velocity sedimentation on a 5–25% glycerol gradient with no sucrose pad, or (*B*) equilibrium density centrifugation on a 50–800 mM sucrose gradient, with a 400-μl, 50% sucrose pad. Samples were analyzed for HRP (+), and synaptophysin antigenicity (■). The velocity centrifugation was at 48,000 rpm for 1 h, and the equilibrium centrifugation was for 5 h at 48,000 rpm. From refractive index measurements, the density of the synaptophysin-containing membranes in *B* is 1.119 $g \cdot cm^{-3}$.

37°C. Analysis of the high-speed supernatants by velocity sedimentation showed a peak of surface-labeled material sedimenting with synaptophysin antigenicity in the warmed cells, but not in the cells kept at 0°C (Fig. 4 *A*). A labeled protein in the position of synaptophysin was found after SDS-PAGE, only in the fractions containing synaptophysin antigenicity (Fig. 4 *B*). When the SVL vesicle peak was pooled and analyzed by equilibrium density centrifugation, again, the antigenicity and the radioactivity coincided (Fig. 5 *A*). Analysis of the protein by SDS gel electrophoresis showed that one of the labeled proteins was synaptophysin (Fig. 5 *B*). These data indicate that at least some of the vesicles with the size and density of synaptic vesicles arise by endocytosis, and that the proteins of the SVL vesicles can be derived from the plasma membrane.

Synaptophysin-containing vesicles from surface-labeled cells warmed to 37°C were isolated by immunoadsorption to magnetic beads coated with antisynaptophysin antibody. When the immunoadsorbed proteins from the high-speed supernatant were examined by gel electrophoresis, labeled synaptophysin was readily detected (Fig. 6, lane *2*), but not in the control (lane *3*). Immunoadsorption was also performed on isolated SVL vesicles. A high-speed supernatant was fractionated by velocity sedimentation as in Fig. 4 *B*, and the SVL vesicle fractions were pooled. Immunoadsorption from the pooled fractions removed specifically 30–40% of the radioactivity. When the bound material was analyzed by SDS gel electrophoresis, labeled synaptophysin could again be readily detected (Fig. 6, lane *4*), confirming that some of the synaptophysin in the SVL vesicles had at some time been exposed on the cell surface.

Some of the other labeled proteins in the high-speed supernatant were immunoadsorbed selectively by antisynaptophysin antibodies. Specifically coprecipitating with synaptophysin in surface-labeled SVL membranes was a protein doublet (M_r = 46,000; 49,000) and three proteins of M_r in the range of 22,000–35,000 kD. (Fig. 6). The latter three proteins may correspond to proteins in this size range identified by metabolic labeling (Fig. 2). The strongly labeled doublet also comigrates with synaptophysin-containing membranes in velocity (Fig. 4 *B*) and equilibrium (Fig. 5 *B*) centrifugation. The two polypeptides could be novel synaptic vesicle proteins, readily labeled by surface iodination, but not so easily detected by $[^{35}S]$methionine labeling (Fig. 2).

Transferrin in Small Endocytotic Vesicles

Internalization of labeled transferrin by receptor-mediated endocytosis can be used to identify the early endosome and compartments involved in shuttling between early endosome and plasma membrane. To determine if SVL vesicles are involved in transferrin transport, cells were incubated with iodinated transferrin at a concentration that does not saturate the receptors. To maximize the fraction of transferrin associated with endocytotic vesicles, transferrin that remained on the surface after incubation at 37°C was removed by washing. To detect transferrin in the SVL vesicles, the high-speed supernatant was analyzed by velocity sedimentation on a glycerol density gradient. Little of the labeled transferrin sedimented faster than free transferrin, and the sedimentable transferrin consistently sedimented slightly faster than the SVL vesicles (Fig. 7 *B*). A more dramatic demonstration

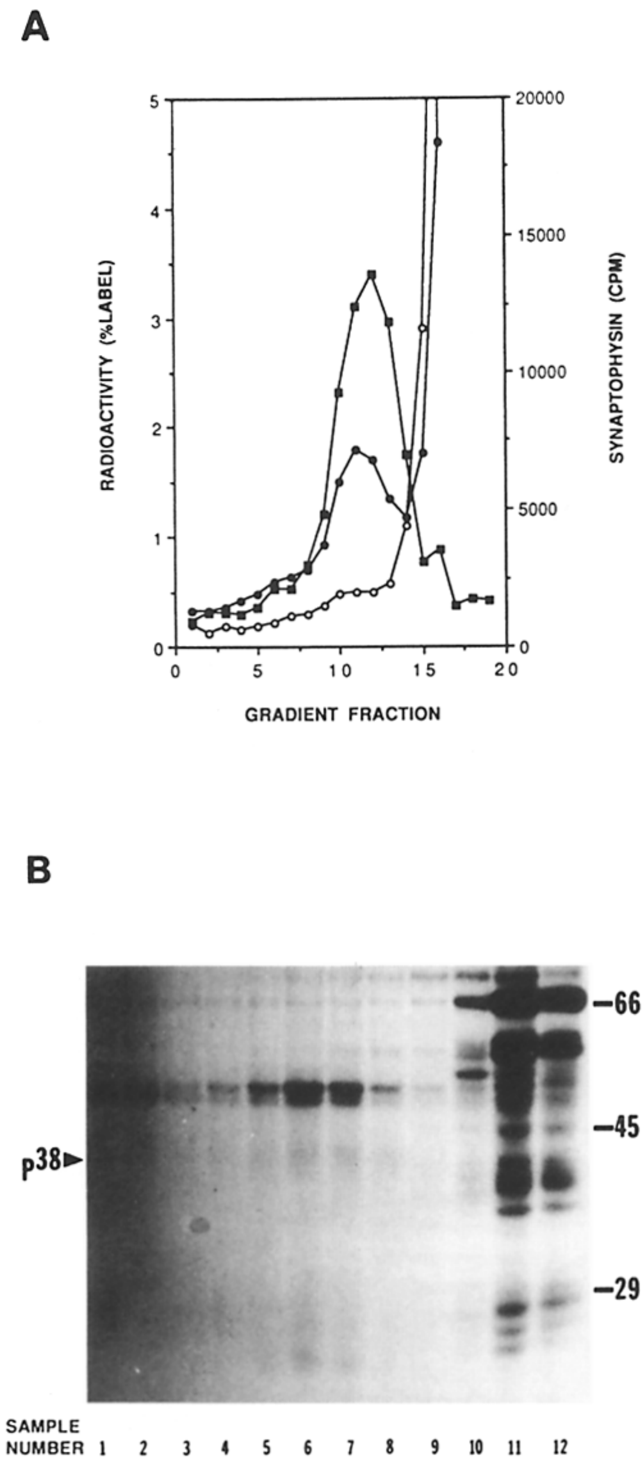

Figure 4. Synaptophysin labeled using a membrane-impermeant reagent comigrates with SVL vesicles during velocity sedimentation. PC12 cells were labeled with an [^{125}I]sulfo-SHPP at 0°C for 30 min. One plate of cells was kept at 0°C while another was warmed to 37°C for 1 h. (*A*) The high-speed supernatants from homogenates of the 0°C cells (○) and the 37°C cells (●) were analyzed by velocity sedimentation on glycerol gradients without sucrose pads, and fractions were counted and analyzed for synaptophysin antigenicity (■). Only the antigenicity of fractions from the 37°C material is given. The abscissa is plotted as percent label since more radioactivity is consistently recovered in the 0°C fraction. In this experiment 162,000 cpm of high-speed supernatant was layered on the 0°C gradient and 72,000 cpm was layered on the 37°C gradient. (*B*) A high-speed supernatant from an experiment identical to the above in which cells were warmed for 1 h at 37°C was analyzed as in *A*. Twelve fractions were collected from the gradient and analyzed by SDS-PAGE followed by autoradiography. The peak of labeled synaptophysin in fractions 5–7 corresponded to the peak of SVL vesicles, measured by synaptophysin antigenicity (not shown). The positions of synaptophysin (p38) and molecular weight markers are given.

of the difference between the transferrin-containing compartments and the synaptophysin-rich ones was obtained by analyzing a low-speed supernatant (10,000 *g*, 10 min) by velocity sedimentation (Fig. 7 *A*). About six times more sedimentable transferrin was recovered in the low-speed supernatant and the distribution of transferrin-containing membranes was clearly different from that of synaptophysin-containing membranes. We conclude that transferrin and synaptophysin are mainly in different compartments in a 10,000 *g* supernatant and that little, if any, of the labeled transferrin in endocytotic compartments is in the SVL vesicles.

The data in Fig. 7 were generated by incubating the cells for 2 h at 37°C in labeled transferrin. Essentially identical results were obtained using a 10-min incubation (data not shown). In similar experiments using iodinated β-very low density lipoprotein, we detected none of this endocytotic marker in SVL vesicles (data not shown). Thus, although SVL vesicles can arise by endocytosis, they appear to exclude at least two conventional markers of receptor-mediated endocytosis.

Targeting of Synaptophysin in Fibroblasts

If SVL vesicles existed in all cells they would presumably be the targets of synaptic vesicle proteins introduced by DNA transfection techniques. However, when fibroblast or epithelial cell lines were transfected with DNA-encoding synaptophysin, it did not accumulate in vesicles of the correct sedimentation velocity. When a low-speed supernatant (10,000 *g*, 10 min) from COS cells expressing synaptophysin transiently was analyzed by velocity sedimentation synaptophysin-containing vesicles were observed, but the majority of synaptophysin was in membranes of higher sedimentation velocity (Fig. 8 *A*). Similar results were obtained with stably transfected Madin-Darby canine kidney cells and 3T3 cells (not shown). In comparison, an identical low-speed supernatant from PC12 cells has ~50% of the synaptophysin antigenicity in the SVL vesicle peak (Fig. 8 *A*). The distribution of synaptophysin antigenicity in COS cells was confirmed by immunoprecipitation from cells labeled in their sulfur-containing amino acids (Fig. 8 *B*). Thus, although synaptophysin can enter membrane vesicles in transfected cells, the vesicles have properties quite different from synaptic vesicles. The biochemical differences between endocrine cells and fibroblasts cannot be detected by immunofluorescence microscopy. When the synaptophysin distribution in transfected cells and in PC12 cells was compared, a considerable amount of the fluorescence in all the cell types was distributed in cytoplasmic punctate structures (not shown) as was reported by Johnston et al. (1989). We conclude that SVL vesicles, defined by their velocity and equilibrium sedimen-

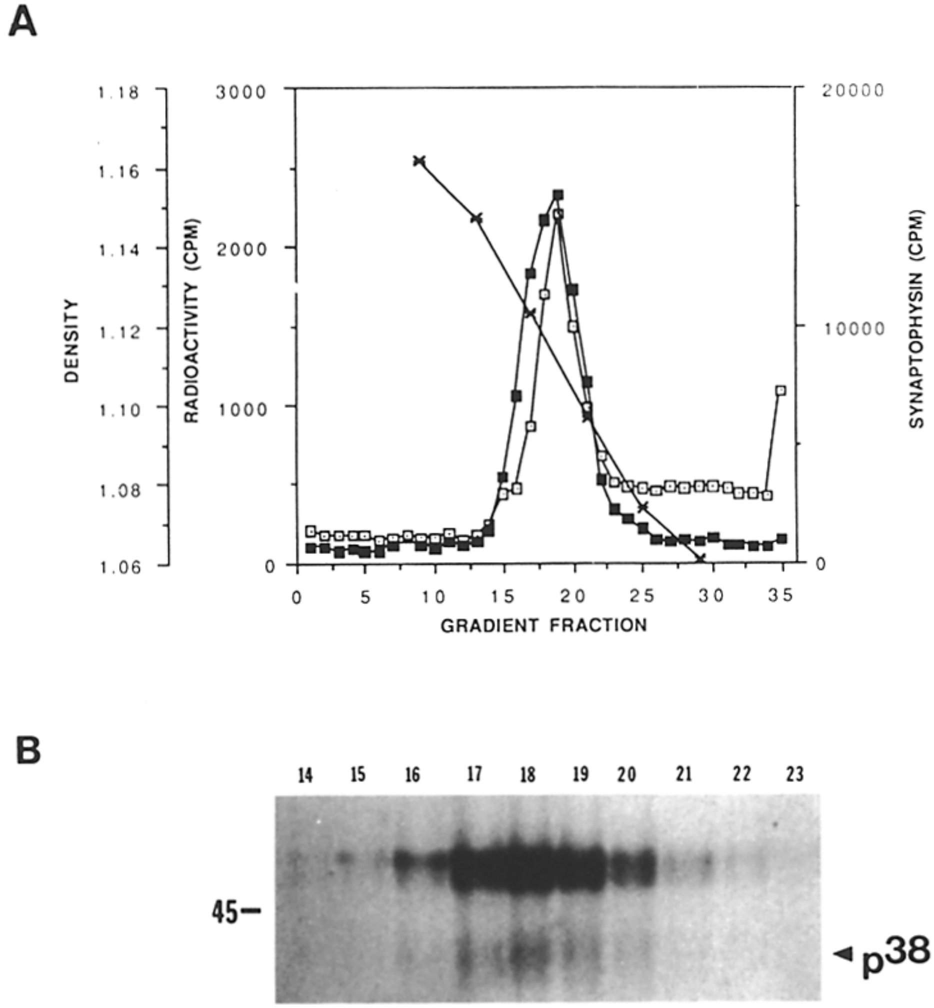

Figure 5. Synaptophysin, labeled with a membrane-impermeant reagent (^{125}I-sulfoSHPP), is recovered in SVL vesicle fractions after velocity then equilibrium density centrifugation. (*A*) Fractions from a glycerol velocity gradient with the highest levels of synaptophysin antigenicity were pooled, diluted, and analyzed on a 10–50% sucrose density gradient in buffer A. Centrifugation was at 48,000 rpm for 14 h. Fractions were analyzed for synaptophysin antigenicity (■), [^{125}I] radioactivity (⊡), and density measured by refractive index (*). (*B*) Samples from the indicated fractions were analyzed for polypeptide composition after SDS-PAGE. The position of synaptophysin (p38) is marked.

tation characteristics and as targets of synaptic vesicle proteins, are not detectable in cells other than endocrine and neuronal cells.

Distribution of [^{3}H]NE in Subcellular Fractions

If the SVL vesicles were identical to synaptic vesicles they should be able to take up and retain neurotransmitters. The NE and acetylcholine content of PC12 cells has been localized to vesicles of two different size classes (Schubert and Klier, 1977). Both types of vesicles had, however, dense cores in the electron microscope and were significantly denser than authentic synaptic vesicles on equilibrium centrifugation. In case the SVL fraction was missed in the earlier study, PC12 cells were labeled for 1 h with [^{3}H]NE before homogenization. No detectable radioactivity was observed cosedimenting with the SVL vesicles isolated from a high-speed supernatant (Fig. 9). To try to minimize leakage of neurotransmitter, cells were homogenized in 1 mM ATP and an ATP regenerating system and centrifuged in glycerol gradients containing 1 mM Mg-ATP. Still no [^{3}H]NE was detectable in the SVL vesicle peak. Less than 1% of the [^{3}H]NE in a P2 fraction (see Materials and Methods) was recovered in SVL vesicles. The organelles containing [^{3}H]NE could be identified when a low-speed (10,000 *g*, 5 min) supernatant was analyzed by velocity sedimentation under appropriate conditions (Fig. 9 *B*). The rapidly sedimenting [^{3}H]NE-containing vesicles shown in Fig. 9 *B* have the properties expected of dense core secretory granules. They sedimented ~25 times faster than the SVL vesicles, contained ATP, and their content of [^{3}H]NE dropped by ~40% on stimulation (not shown). We conclude that the SVL vesicles package or retain [^{3}H]NE poorly compared to dense secretory granules. If the content after isolation reflects physiological content, then >95% of the stored [^{3}H]NE is in dense vesicles. Either the machinery for uptake and storage of [^{3}H]NE is not adequately expressed in the SVL vesicles, or the vesicles lose their content quickly during isolation, even in the presence of ATP.

The cell line used in these experiments was one that also expresses hGH. No hGH could be detected in the SVL vesicles (not shown), but it could be readily detected in more rapidly sedimenting compartments.

Note that there is no peak of synaptophysin cosedimenting with the dense secretory vesicle peak in Fig. 9 *B*. This is consistent with other data, both biochemical (Wiedenmann et al., 1988) and electron microscopic (Navone et al., 1986) that dense granule membranes are relatively poor in synaptic

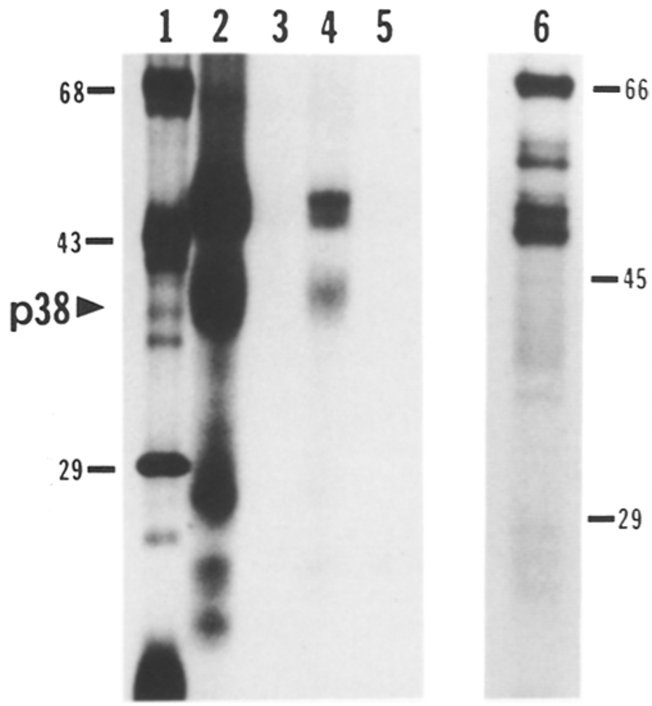

Figure 6. Proteins labeled using a membrane impermeant reagent are immunoadsorbed by antisynaptophysin antibodies attached to beads. A high speed supernatant (27,000 *g*, 35 min) was immunoadsorbed using antisynaptophysin (lane *2*), or control antibody (lane *3*), and the adsorbed material analyzed by PAGE. The high-speed supernatant was also fractionated by glycerol velocity sedimentation as in Fig. 4, the SVL vesicle fractions were pooled and labeled vesicles isolated from the pool by immunoadsorption using antisynaptophysin antibodies (lane *4*) or control antibodies (lane *5*). Lane *6* is the entire high-speed supernatant. Lane *1* is labeled molecular weight standards. The position of synaptophysin (p38) is indicated. The positions of unlabeled molecular weight standards are given on the right.

vesicle membrane proteins, compared to the SVL vesicles. Stimulation of the cells did not affect detectably the amount of synaptophysin in the SVL vesicles.

Since there is no evidence that the SVL vesicles can store and secrete neurotransmitter, it is not yet clear what role, if any, they play in PC12 cells.

Discussion

Synaptic vesicles are so unique in their diameters and in their abundance that their presence is often used to identify a cell as a neuron. Such unique structures could be generated by a pathway that is entirely neuron specific or one that is a modification of a pathway that exists in most cells. In the hope of obtaining clues to how vesicles are generated, the expression of synaptic vesicle membrane proteins has been studied both in transfected nonneuronal cells and also in endocrine cells, which for some unknown reason express all known synaptic vesicle membrane proteins. By both strategies, when fluorescent transferrin is used as an early endosome label, considerable amounts of synaptophysin are found associated with endosomes (Johnston et al., 1989). The simplest interpretation of these data is that synaptic vesicle proteins are targeted to the pathway that recycles between the early endosome and the plasma membrane.

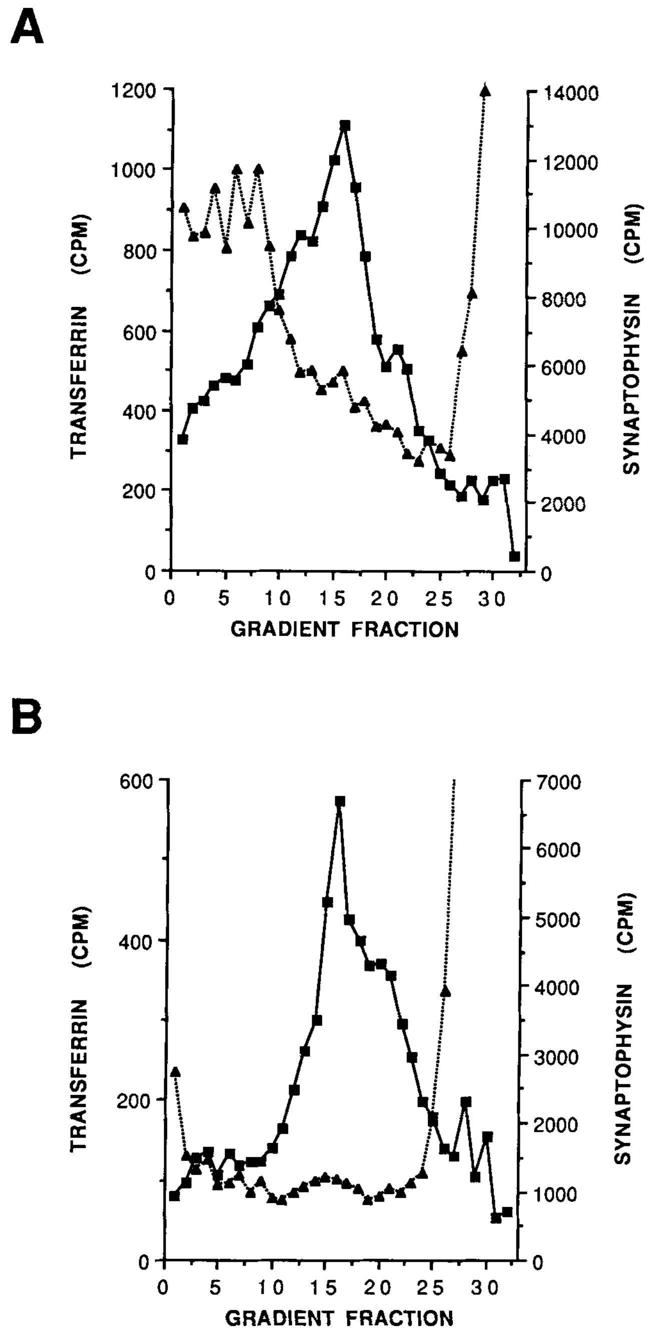

Figure 7. Endocytosed [^{125}I]transferrin is recovered in small vesicles that do not migrate with SVL vesicles. Cells were incubated for 2 h in the presence of subsaturating amounts of [^{125}I]transferrin, washed to remove surface transferrin, and then homogenized. (*A*) A low-speed supernatant (10,000 *g*, 5 min) was fractionated on a glycerol velocity gradient and analyzed for the distributions of [^{125}I]transferrin (▲) and synaptophysin (■). (*B*) An equal volume of a high-speed supernatant (27,000 *g*, 35 min) was analyzed in the same way. Note that about half the synaptophysin in *A* is recovered in *B*, and that the ratio of the two scales in *A* and *B* is the same.

The element of the endosome recycling pathway that superficially most resembles the synaptic vesicle is the small transport vesicles that carry receptors from the plasma membrane to the endosome, and back again. Transport inter-

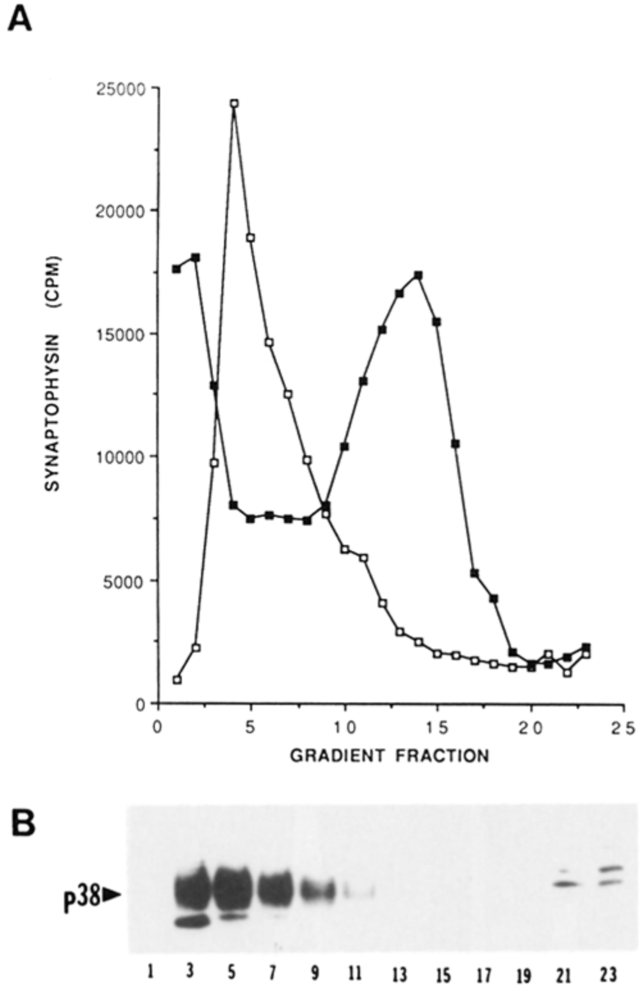

Figure 8. Synaptophysin recovered in transfected COS cells does not accumulate in SVL vesicles. (*A*) Cultures of control PC12 cells (■) and COS cells transfected with synaptophysin-expressing DNA (□) were homogenized and aliquots of a low-speed supernatant (10,000 *g*, 5 min) analyzed by velocity sedimentation using a sucrose pad. Samples were assayed for synaptophysin antigenicity. No signal was seen in an untransfected control. (*B*) The transfected COS cells were labeled for 12 h with [^{35}S]methionine and cysteine. Fractions from the gradient of transfected cell material were analyzed by SDS-PAGE after immunoprecipitation with a polyclonal antiserum that recognizes synaptophysin. The fraction numbers are given below and the position of p38 is indicated.

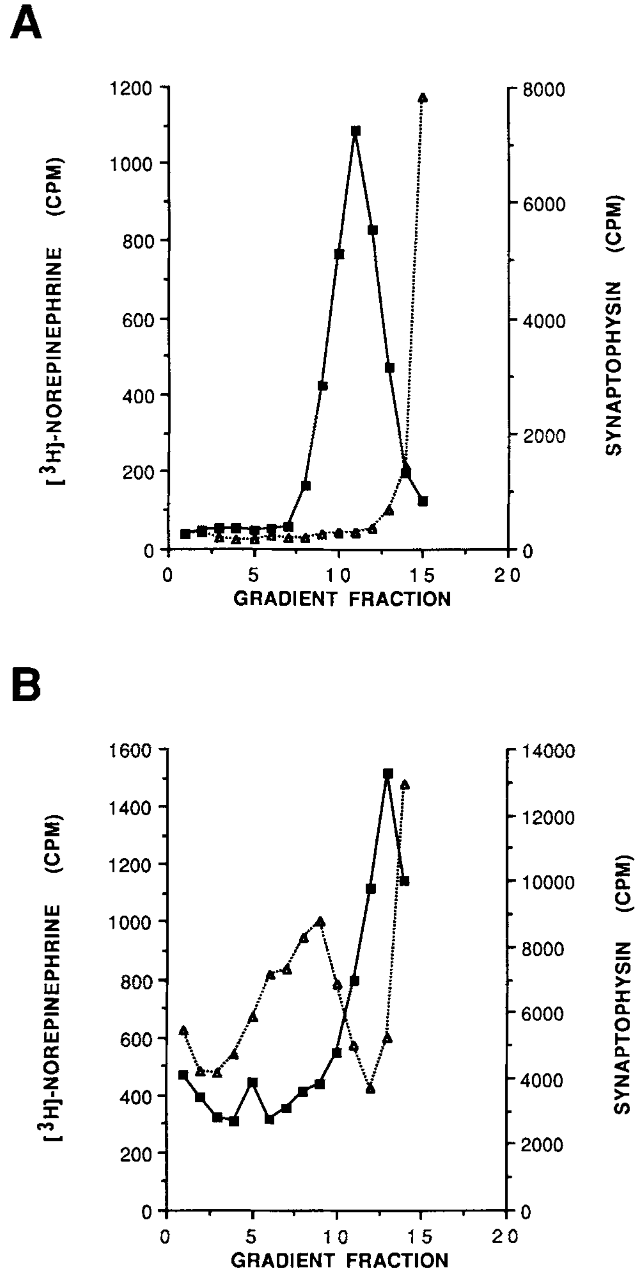

Figure 9. Recovery of [^{3}H]NE in membrane fractions. Cells were incubated in [^{3}H]NE for 1 h, washed, and homogenized. The high-speed supernatant (*A*) and the P2 fraction (see Materials and Methods) (*B*) were analyzed for [^{3}H]NE (△) and synaptophysin antigenicity (■). Note that the centrifugation conditions in *A* are standard, 48,000 rpm for 1 h, whereas in B samples were analyzed by centrifuging at only 25,000 rpm for 10 min. The P2 fraction is known from the work of Lowe et al. (1988) to contain dense secretory granules.

mediates between cellular compartments have been identified and shown to be small membranous vesicles (Bennett et al., 1988; de Curtis and Simons, 1989). The data presented here make the possibility that synaptic vesicle proteins are selectively targeted to endocytotic transport intermediates less attractive. No transferrin or β-very low density lipoproteins could be found in the SVL vesicles, whereas nonspecific bulk phase and surface markers of endocytosis were readily detected. Furthermore, SVL vesicles are not found in fibroblast cells, which presumably have endocytotic transport vesicles. Targeting of vesicle proteins to endocytotic transport intermediates is unattractive also on purely biological grounds. The majority of the synaptic vesicles in the nerve terminal are recycling very slowly. If the synaptic vesicle membrane proteins changed transport vesicles into synaptic vesicles, normal endocytotic cycling would be severely perturbed.

An alternative hypothesis for SVL formation in neurons and PC12 cells is more appealing. If synaptic vesicle proteins recycle from the plasma membrane to the endosome, as suggested by the data of Johnston et al. (1989), then they could selectively associate with one another in the endosome to form an aggregate that excludes other membrane proteins. Proteins in such an aggregate might then bud off from the endosomal membrane into a vesicle of the correct dimensions. Synaptic vesicle protein aggregation and vesicle budding could occur in intracellular locations other than the endosome however. The plasma membrane is one alternative site; another is the Golgi apparatus, if vesicles of synaptic vesicle dimensions can form biosynthetically as well as by endocytosis.

The association and budding model can be extended to explain the difference we have noted between PC12 cells and transfected fibroblasts. In transfected fibroblasts, synaptophysin is targeted to the early endosome pathway (Johnston et al., 1989). In the absence of other synaptic vesicle proteins, synaptophysin can aggregate only with itself in the endosome and so will bud off in a vesicle that is almost exclusively synaptophysin (Leube et al., 1989). Since such vesicles do not have the dimensions of an SVL vesicle (Fig. 8), synaptophysin alone is not sufficient to generate a vesicle of the correct dimensions. One explanation of why PC12 cells, but not fibroblasts, form SVL vesicle-sized structures is that PC12 cells express other synaptic vesicle proteins in addition to synaptophysin. The other synaptic vesicle proteins, alone or in combination, might determine vesicle dimensions.

It is now possible to analyze what signals target synaptic vesicle membrane proteins to endosomes and to the SVL vesicles. Furthermore, when more synaptic vesicle proteins are cloned and sequenced it will be possible to ask which proteins confer on synaptic vesicles their highly characteristic dimensions.

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