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Fractionation of synaptophysin-containing vesicles from rat brain and cultured PC12 pheochromocytoma cells

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Synaptophysin is a transmembrane glycoprotein of neuroendocrine vesicles. Its content and distribution in subcellular fractions from cultured PC12 cells, rat brain and bovine adrenal medulla were determined by a sensitive dot immunoassay. Synaptophysin-containing fractions appeared as monodispersed populations similar to synaptic vesicles in density and size distribution. Membranes from synaptic vesicles contained ≈100-times more synaptophysin than chromaffin granules. In conclusion, synaptophysin is located almost exclusively in vesicles of brain and PC12 cells which are distinct from dense core granules.

Synaptophysin; Synaptic vesicle; Dense core vesicle; Dot immunoassay

1. INTRODUCTION

Synaptophysin is a major integral membrane protein of synaptic vesicles of the CNS [1,2] which occurs as a homooligomer and binds Ca^{2+} on its cytoplasmic domain [3]. The primary structure of this protein has recently been derived from cDNA clones ([4], see also [5,6]). Synaptophysin seems to be intimately involved in neurotransmitter storage and neurotransmission, and its developmental expression in the CNS coincides with synaptogenesis [7,8]. Synaptophysin is widely distributed in neoplastic neuroendocrine tissues and cell lines including the cell line PC12 derived from rat pheochromocytoma [3,10,12]. Immunoelectronmicroscopical studies have suggested that synaptophysin occurs at synapses [1,2] as well as in normal [2,11] and neoplastic [10,12] neuroendocrine cells in a distinct type of small (40–80 nm diameter) vesicles with an electron-microscopically translucent content. However, several laboratories [13,14] recently provided evidence for the occurrence of synaptophysin in dense core granule membranes implying the participation of this polypeptide in at least two different secretory vesicle pathways. Because of this discrepancy between direct immunolocalization data and fractionation results, we now analyzed the synaptophysin content of vesicle fractions derived from rat and bovine brain, bovine adrenal medulla as well as cultured PC 12 cells by a newly developed dot immunoassay, which can detect ≥10 fmol synaptophysin.

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Abbreviations: BSA, bovine serum albumin; NC, nitrocellulose; PAGE, polyacrylamide gel electrophoresis; SCV, synaptophysin-containing vesicles

2. MATERIALS AND METHODS

2.1. *Cells*

PC12 cells [15] originally provided by Dr H. Thoenen (Max-Planck-Institut für Psychiatrie, Martinsried) were grown as described [3,12].

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2.2. *Materials*

Nitrocellulose (BA85) and blotting paper were purchased from Schleicher & Schüll (Dassel); Percoll, Sephacryl S-500, Sephacryl S-1000 and Sepharose 4B from Pharmacia (Freiburg); controlled pore glass and tunicamycin from Sigma (München); anti-mouse immunoglobulins coupled to horseradish peroxidase from Amersham-Buchler (Braunschweig); and anti-mouse or anti-rabbit IgG coupled to alkaline phosphatase from Promega (Madison, WI). Monoclonal antibody SY38 (subclass IgG_1) against synaptophysin [2], was purified from mouse peritoneal ascites fluid by HPLC. The punching apparatus was from Inotech (Wohlen, CH). Murine monoclonal antibody SG I against secretogranin I was a kind gift from Dr Wieland Huttner (EMBL, Heidelberg). Rabbit antiserum against bovine dopamine β-hydroxylase (DBH, EC 1.14.17.1) was kindly provided by Dr John H. Phillips (University of Edinburgh Medical School, Edinburgh).

2.3. *Dot immunoassay*

Synaptophysin was determined by an enzyme-linked solid-phase immunoassay modified from the method of Becker et al. [16]. Samples (100 or 200 μl, <10 μg protein) were mixed with an equal volume of 0.16 N NaOH in 40% methanol and absorbed onto nitrocellulose (NC) sheets in a 96-well dot-blot apparatus (Schleicher and Schuell). Air-dried sheets were incubated in blocking bluffer (buffer A) containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100 and 1% milk powder or 5% BSA. The NC was then incubated with antibody SY38 (about 2.0 μg/ml) in buffer A containing BSA for 1 h and washed three times for 5 min with buffer A. The sheet was incubated for 45 min with horseradish peroxidase coupled to goat anti-mouse (IgG) antibody diluted 1:500 in buffer A containing BSA. After three consecutive washes, for 5 min each, with buffer A and buffer containing 50 mM Tris-Cl (pH 8.0) and 150 mM NaCl, the dots carrying the samples were punched into a 96-well microwell plate. 150 μl per well of 100 mM citrate-phosphate buffer (pH 5.0) containing 0.5 mg/ml of *o*-phenylenediamine and 0.01% H_2O_2 were applied. The reaction was stopped by the addition of 50 μl of 25% (w/v) sulfuric acid per well. Color development was determined in an ELISA reader at 492 nm. All steps were performed at room temperature.

2.4. *Preparation of postnuclear supernatants*

Tissue from rat and bovine brain and bovine adrenal medulla was washed with ice-cold SEAT buffer [10 mM triethanolamine, 10 mM acetic acid (pH 7.4), 250 mM sucrose, 1 mM EDTA] and homogenized in 1.7 ml SEAT buffer per g tissue by repeated passage through a 5 ml syringe. The homogenate was squeezed once through a 17-gauge needle, diluted with SEAT buffer (1:1) and rehomogenized by pipetting through the blue tip of a Gilson pipette according to Miskimins and Shimizu [17]. In some cases, SEAT buffer was replaced by buffer B [10 mM Na-Hepes (pH 7.4), 60 mM KCl, 10 mM NaCl, 160 mM glycine, 0.1 mM EDTA]. The supernatant resulting from centrifugation at 550 × *g* for 5 min was used for further experiments.

PC12 cells were washed from the culture dish, suspended in 0.5 ml SEAT buffer and triturated 40 × with a fire-narrowed Pasteur-pipette tip. In some experiments, subcellular fractions of PC12 cells and rat brain were prepared with buffer B. After centrifugation for 5 min at approx. 550 × *g*, the supernatant was removed and the procedure repeated twice. The combined supernatants ('lysates') contained about 95% of the total synaptophysin as judged by Western and dot immunoblotting. All steps were performed at 4°C. In some cases, postnuclear supernatants were subjected to hypotonic lysis by adding approx. 15 vols cold water followed by gentle homogenization in a motor-driven Potter-Elvehjem homogenizer (1500 rpm, 10 mM Hepes, pH 7.4). After incubation for 30 min on ice, the homogenate was centrifuged at 17000 × *g* for 20 min, and the supernatant subjected to centrifugation at 150000 × *g* for 90 min. The resulting pellet was homogenized with a Dounce homogenizer ('shock-lysate') and subjected to Percoll centrifugation.

2.5. *Purification of membrane fractions*

Synaptic vesicles were isolated according to Huttner et al. [18], except that permeation chromatography was omitted. Inside-out vesicles derived from human red blood cells and chromaffin granule membranes derived from bovine adrenal medulla were prepared according to standard procedures [19,20].

2.6. *Electrophoresis and immunoblotting*

SDS-PAGE was performed according to Laemmli [21] using 10% gels. Blot transfer was done as described by Kyhse-Andersen [22], except that block and wash buffers and primary antibodies were the same as for the dot immunoassay. Binding of primary antibody was detected by either anti-mouse or anti-rabbit antibody (IgG) coupled to alkaline phosphatase.

2.7. *Protein determination*

Protein concentration was determined according to Bradford [23]. Relative protein concentrations of inside-out vesicles were determined by subjecting identical amounts of Percoll fractions to SDS-PAGE and Coomassie blue staining and using band III, the major integral membrane protein of inside-out vesicles, as a protein reference. The polypeptide band was excised, eluted with 1.0 ml of 50% isopropanol, and the eluent quantified photometrically at 595 nm.

3. RESULTS

3.1. *Dot immunoassay*

Based on an immunoassay for the postsynaptic glycine receptor [16], an immunoassay for synaptophysin of higher specificity and sensitivity than previously reported [7,24] was developed. This assay was calibrated using purified synaptophysin for reference (not shown). The sensitivity of the dot immunoassay critically depended on the conditions of sample application to the NC membrane. A solution of 0.08 N NaOH in 20% methanol gave the best results, and served as standard application medium, unless stated otherwise. The presence of

SDS (0.03%) abolished the immunosignal for synaptophysin. Triton X-100 (0.03%) reduced the signal to approx. 20% of that in detergent-free application buffer. Cholate, octylglucoside, or a mixture of octylglucoside and deoxycholate gave satisfactory results (not shown). Unspecific binding of antibody SY38 was assessed either by applying similar amounts of rat liver protein to the NC or by omitting the antibody. Background binding depended on the quality of secondary antibodies and usually accounted for <5% of total binding. Approx. 10 fmol synaptophysin could reliably be detected in up to 100 μg (total protein) crude brain lysate or 10 ng (total protein) synaptic vesicles. A previously described dot immunoassay for synaptophysin [24] was approx. 100-fold less sensitive with the SY38 monoclonal antibody (not shown). This difference may be explained by the presence of both SDS and Triton X-100 at neutral pH in the absence of methanol. Percoll interfered with the dot immunoassay at concentrations >0.5%; e.g. the presence of 10% Percoll in the application buffer caused a 4-fold decrease in immunoreactivity (not shown).

3.2. *Synaptophysin content of synaptic vesicle and chromaffin granule membranes*

The synaptophysin content of purified synaptic vesicle and chromaffin granule membranes was compared by immunoblotting (fig.1a) and dot immunoassay (fig.1b) using monoclonal antibody SY38 against synaptophysin. Whereas dopamine β-hydroxylase was found solely in chromaffin granule membranes (fig.1a, lane 5′), synaptophysin was contained primarily in synaptic vesicles (fig.1a, lane 2′). However, membrane preparations of chromaffin granules also contained some synaptophysin (fig.1a, lane 3′). Synaptophysin immunoreactivity therefore was quantified by dot immunoassay. To obtain similar dot immunoreactivities for both membrane fractions, chromaffin granule membranes had to be applied in approx. 125-fold excess over synaptic vesicles from rat brain on a protein basis (fig.1b). Bovine synaptic vesicles showed an immunosignal about 25-times higher than that of bovine chromaffin granule membranes (not shown). This difference between rat and bovine may reflect variable purities of membrane preparations rather than species differences.

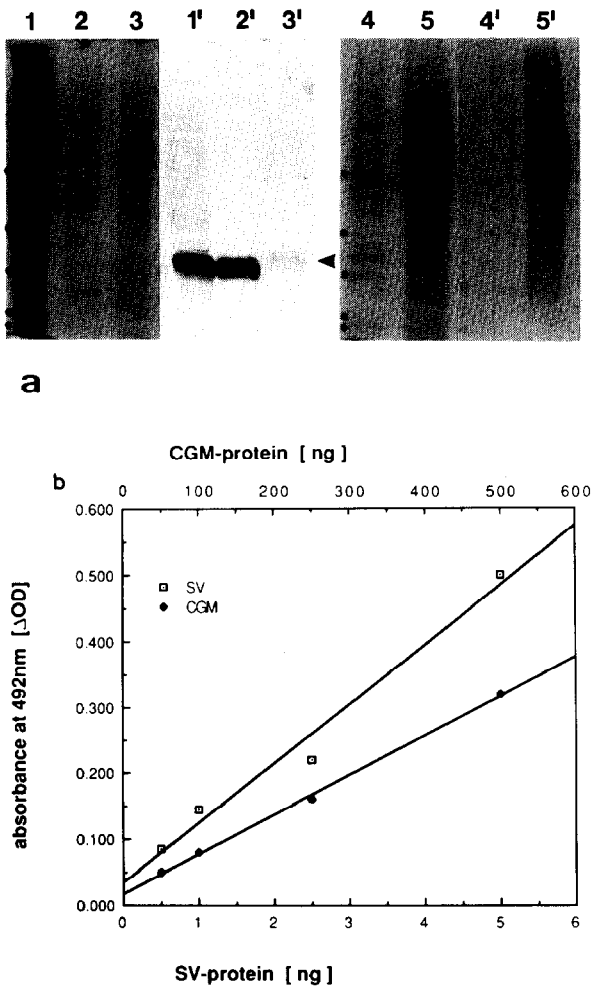

Fig.1. Synaptophysin content of synaptic vesicle and chromaffin granule membranes as determined by immunoblotting (a) and dot immunoassay (b). (a) A postnuclear supernatant of rat brain (lanes 1,1′; 30 μg protein), purified synaptic vesicle (lanes 2,2′ and 4,4′; 1.5 μg protein) and chromaffin granule (lanes 3,3′, 4 μg protein and lanes 5,5′, 9 μg protein) membranes were subjected to 12.5% SDS-PAGE, Lanes: 1–5, Coomassie blue-stained gels; 1′–5′, corresponding immunoreplicas using antibody SY38 (lanes 1′–3′) and antiserum against DBH (lanes 4′,5′). Arrowhead denotes a weakly immunoreactive polypeptide of 40 kDa, typical for synaptophysin in adrenal medulla [11]. The positions of molecular mass markers are indicated by dots on the left: bovine albumin, 66 kDa; egg albumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20.1 kDa. (b) Purified bovine chromaffin granule membranes [CGM (◆)] and rat synaptic vesicles [SV (■)] were analyzed by dot immunoassay using monoclonal antibody SY38 (for details see section 2). Note that similar immunosignals are obtained only with a 100-fold protein excess of chromaffin granule membrane protein.

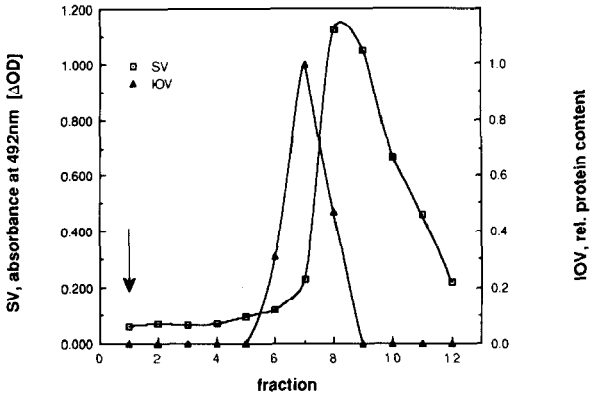

Fig.2. Percoll gradient analysis of purified vesicles. Purified bovine brain synaptic vesicles [SV (□), 250 μg total protein] were subjected to Percoll gradient centrifugation; fractions (1 ml) were analyzed by dot immunoassay for synaptophysin. Maximal immunoreactivity, determined at 492 nm, is found in fraction 8, corresponding to a density of 1.030 g/ml. Inside-out vesicles (▲), quantified as described in section 2, sediment in fraction 7. Purified bovine chromaffin granules and intact human red cells (arrow) form a visible band in fraction 1 of the gradient. Fraction 1, bottom; fraction 12, top of the gradient.

3.3. *Percoll gradient centrifugation of subcellular fractions of PC12 cells and brain tissue*

The density of SCV was determined by centrifugation on 20% (w/v) Percoll gradients. Gradients were calibrated with purified rat brain synaptic vesicles, inside-out vesicles of human erythrocytes, and chromaffin granules as well as intact red cells. Purified synaptic vesicles sedimented at a density of 1.030 g/ml similarly to inside-out vesicles (fig.2). Synaptic vesicles subjected to freeze-thawing migrated identically to unfrozen synaptic vesicles (not shown). Intact red

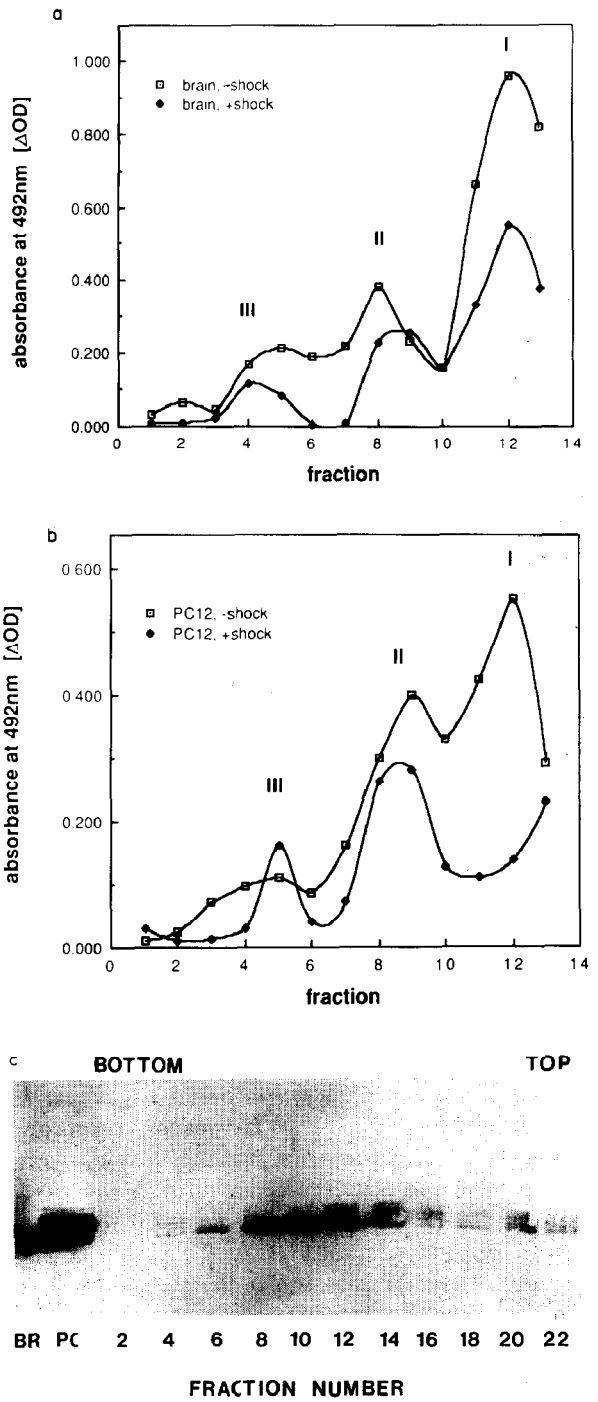

Fig.3. Percoll gradient analysis of synaptophysin-containing membrane fractions of rat brain and PC12 cells. Postnuclear supernatants of brain (a) and PC12 cells (b) in SEAT buffer, before (□) and after (♦) hypotonic shock lysis (for details see section 2) were subjected to Percoll gradient centrifugation. Fractions of 1 ml were analyzed for synaptophysin by dot immunoassay. Three immunoreactive peaks were observed. Peak I (fraction 12 in a,b) corresponds to synaptophysin, which is only partly sedimentable at 150000 × *g*. Note that ultracentrifugation of the osmotically shocked postnuclear supernatant reduces the immunosignal in peak I. Peak II (fraction 8 in a and fraction 9 in b) corresponds to densities of 1.030 and 1.027 g/ml, respectively. These densities are very similar to that of purified rat brain synaptic vesicles (see fig.2). For immunoblotting (c), equivalent amounts of postnuclear supernatant from brain and PC12 cells were mixed and centrifuged on a Percoll gradient in SEAT buffer as described in section 2. Aliquots of the postnuclear supernatant of brain (BR), PC12 cells (PC), and of the indicated fractions (0.6 ml) of the gradient were applied to 10% SDS-PAGE and analyzed by Western blotting as described.

cells and purified chromaffin granules were found as a visible band in the bottom fraction of the gradient (fig.2).

When postnuclear supernatants of PC12 cells or brain were subjected to centrifugation on Percoll density gradients, two major peaks of synaptophysin immunoreactivity were detectable by dot assay: peak I near the top of the gradient (fig.3a,b, fraction 12), and peak II at a density of 1.030 g/ml for brain (fig.3a, fraction 8) and 1.027 g/ml for PC12 cells (fig.3b, fraction 9). Osmotic shock and homogenization did not affect the positions of immunoreactive peaks of subcellular fractions from brain and PC12 cells on Percoll gradients (fig.3a,b). Neither the mode of application (i.e. sample applied on top or mixed with the Percoll prior to centrifugation) nor the method of tissue homogenization (i.e. Potter-Elvehjem homogenizer vs trituration) influenced the position of the peaks. When peak II was subjected to recentrifugation, the peak was recovered at the same density position as in the first run (not shown). The density of the vesicles determined by this method therefore reflects intrinsic qualities of the vesicles rather than conditions of vesicle preparation. However, differences in height of peaks I and III most likely reflect variable immunoreactivities in the supernatant after ultracentrifugation and interference by Percoll with immunodetection.

Synaptophysin molecules from brain and PC12 cells differ in their apparent molecular mass on SDS-PAGE [3]. Thus, SCV from brain and PC12 cells can be discriminated by Western blotting. Postnuclear supernatants from brain and PC12 cells were mixed and subjected to Percoll gradient centrifugation in SEAT buffer. As already observed by dot immunoassay for synaptophysin, SCV derived from PC12 cells showed a slightly lower density than those derived from brain upon sedimentation in SEAT buffer (fig.3c). However, these differences were not observed in buffer B (not shown).

3.4. *Size-exclusion chromatography of postnuclear supernatants of PC12 cells and rat brain*

To analyze the size relationship between purified synaptic vesicles and synaptophysin-containing subcellular fractions derived from brain and PC12 cells, we subjected postnuclear supernatants to gel-

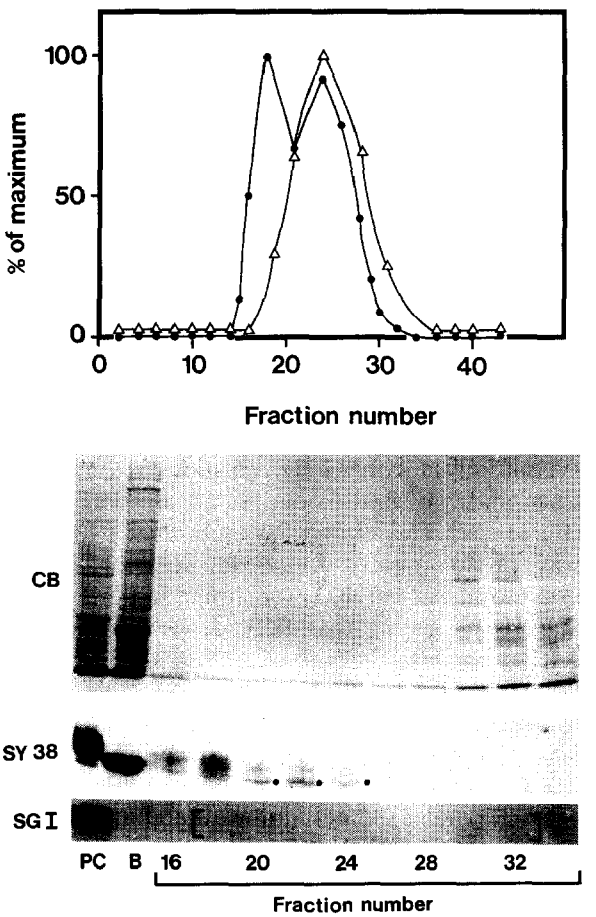

Fig.4. Gel-permeation chromatography of lysates from brain and PC12 cells. Equivalent amounts of postnuclear supernatants (•) from brain and PC12 cells were mixed and subjected to gel filtration. Fractions obtained were analyzed by dot immunoassay for synaptophysin (upper panel). Two immunoreactive peaks were observed. Note that purified synaptic vesicles (Δ) are confined to peak II only. Immunoreactive fractions were further analyzed by SDS-PAGE followed by Coomassie blue staining (second panel from top, CB) and immunoblotting using antibodies against synaptophysin (third panel from top, SY38) and secretogranin I (bottom panel, SG I). Lanes: 1, PC12 (PC); 2, rat brain lysate (B). Fractions with even numbers (16–34) were analyzed. Vertical brackets in panel SG I denote an immunoreactive polypeptide doublet of 113/115 kDa typical for secretogranin I. Dots in panel SY38 (fractions 20–24) demarcate a polypeptide of 38 kDa typical for brain synaptophysin.

permeation chromatography on columns of Sephacryl S-500, S-1000, Sepharose 2B and controlled pore glass, equilibrated with buffer B.

Columns of Sephacryl S-500 gave the best recovery and resolution and were used exclusively

thereafter. When a mixture of postnuclear supernatants from PC12 cells and rat brain was subjected to gel-permeation chromatography, two peaks of synaptophysin immunoreactivity were observed by dot immunoassay: peak I (fractions 15–20) was found in the void volume, whereas peak II (fractions 20–36) was included in the column (fig.4). Purified synaptic vesicles from rat brain, prepared according to Huttner et al. [18], were found in peak II only. Freeze-thawing of these vesicles did not alter their chromatographic properties (not shown). Further analysis of these two peaks by Western blotting, using monoclonal antibody SY38 against synaptophysin, showed that synaptophysin-containing fractions from PC12 cells and rat brain comigrated in both peaks (fig.4). Whereas 'brain' synaptophysin (38 kDa) was predominantly found in peak II, 'PC12' synaptophysin (40 kDa) was found in both peaks I and II, indicating that SCV derived from PC12 cells are at least in part of similar size to that of SCV from rat brain.

Secretogranin I (113/105 kDa), a marker for large dense core vesicles in PC12 cells, was identified by a specific monoclonal antibody [25] in peak I (void volume), indicating that secretory granules are excluded from the column (fig.3). Furthermore, secretogranin I was detected in fractions 30–40, known to contain soluble proteins.

4. DISCUSSION

Vesicles from neuroendocrine cells and tissues can be classified according to their synaptophysin content as determined by the combined use of a highly sensitive dot immunoassay and separation techniques of subcellular particles. (i) Synaptophysin can be recovered from CNS tissue in a monodisperse peak fraction, indicating that it is located in a distinct type of small vesicles. (ii) Synaptophysin is over 100-fold enriched in membranes of synaptic vesicles from brain but not of chromaffin granules from adrenal medulla. (iii) Neuroendocrine neoplastic cells (e.g. PC12 cells) possess synaptophysin-containing vesicles, similar in density and size to their normal neuronal counterparts. Thus, most of the synaptophysin present in neuroendocrine cells is enriched and stored in this distinct form of neurotransmitter vesicle. (iv) Synaptophysin-containing vesicles represent a vesicle subpopulation resistant to various kinds of preparative treatments such as hypotonic shock lysis, homogenization and freeze-thawing.

The present data are based on immunoblotting and a newly developed highly sensitive immunoassay for synaptophysin using monoclonal antibody SY38 [2], which has been widely employed as a broad immunohistochemical probe for the identification of normal and neoplastic neuroendocrine cells ([9,27]; review [26]). The assignment of synaptophysin to vesicle subpopulations has recently been subject to a controversy. Immunoelectron-microscopic data suggest that synaptophysin is present in small vesicles with an electron-translucent core but not dense core vesicles [2,11]. However, the use of fixatives during sample preparation may lead to alterations in the structure of vesicles and antigenic epitopes. In contrast, Lowe et al. [13], based on immunoprecipitation of various vesicle fractions, suggested that synaptophysin occurs in addition to chromaffin granules. This method, however, is limited by high background and does not permit a quantitative evaluation of the synaptophysin content of these membrane fractions, since even small amounts of antigen may lead to a precipitation. Therefore, we have studied the distribution of synaptophysin in membranes of native vesicle subpopulations by immunoassay. Compared to its content in small 'clear' vesicles, the amount of synaptophysin in large dense core vesicles is negligibly small. Although small amounts of synaptophysin may be found in other membranes, we suggest in view of the great differences shown here that synaptophysin is specifically sorted into the small clear vesicle pathway. The use of detection and separation methods reported here may lead to an identification of other polypeptides associated with synaptophysin-containing vesicles. This may help to corroborate further our hypothesis that synaptophysin is a specific marker of a distinct vesicle pathway.

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