Synaptophysin Binds to Physophilin, A Putative Synaptic Plasma Membrane Protein

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Abstract. We have developed procedures for detecting synaptic vesicle-binding proteins by using glutaraldehyde-fixed or native vesicle fractions as adsorbent matrices. Both adsorbents identify a prominent synaptic vesicle-binding protein of 36 kD in rat brain synaptosomes and mouse brain primary cultures. The binding of this protein to synaptic vesicles is competed by synaptophysin, a major integral membrane protein of synaptic vesicles, with half-maximal inhibition seen between 10^{-8} and 10^{-7} M synaptophysin. Because of its affinity for synaptophysin, we named the 36-kD synaptic vesicle-binding protein physophilin (*φυσα, greek* = bubble, vesicle; *φιλος, greek* = friend). Physophilin exhibits an isoelectric point of ~7.8, a Stokes radius of 6.6 nm, and an apparent sedimentation coefficient of 5.6 S, pointing to an oligomeric structure of this protein. It is present in synaptic plasma membranes prepared from synaptosomes but not in synaptic vesicles. In solubilization experiments, physophilin behaves as an integral membrane protein. Thus, a putative synaptic plasma membrane protein exhibits a specific interaction with one of the major membrane proteins of synaptic vesicles. This interaction may play a role in docking and/or fusion of synaptic vesicles to the presynaptic plasma membrane.

NEUROTRANSMISSION between a presynaptic nerve terminal and a postsynaptic target cell involves quantal discharge of transmitter molecules. It is commonly thought that quantal release is mechanistically based on the storage of neurotransmitter in specialized organelles, synaptic vesicles, and on the fusion of these vesicles with the presynaptic plasma membrane (Reichardt and Kelly, 1983). Neurotransmitter release is triggered by depolarization-induced calcium influx into the nerve terminal (Augustine et al., 1987). The time interval between opening of presynaptic calcium channels and postsynaptic response lies in the range of a few hundred microseconds, indicating that synaptic vesicles have to be kept in close association with the plasma membrane in order to assure rapid release (Reichardt and Kelly, 1983; Smith and Augustine, 1988). Indeed, many synaptic vesicles are anchored near release sites by the cytoskeleton, and some appear to be directly docked to the plasma membrane (Kelly, 1988).

The specific functions of synaptic vesicles, therefore, include uptake and storage of neurotransmitter (Maycox et al., 1990), interaction with the cytoskeleton (Walker and Agoston, 1987; Landis et al., 1988; Südhof et al., 1989), docking and fusion with the plasma membrane (Heuser et al., 1979; Heuser and Reese, 1981), and retrieval into the cytoplasm (Ceccarelli and Hurlbut, 1980; Torri-Tarelli et al., 1987; Valtorta et al., 1988). Specialized vesicle proteins are likely to be involved in all these processes, although protein involvement is often only inferred by analogy with nonneuronal systems. Protein-mediated membrane fusion has been best characterized with viral spike glycoproteins (for review see Stegmann et al., 1989). The earliest event in cell fusion mediated by a viral fusion protein is the sudden formation of a narrow pore sharing similarities with proteinaceous ion channels (Spruce et al., 1989). A somewhat similar "fusion pore" has also been observed during exocytosis in mast cells (Breckenridge and Almers, 1987). The fusion events during intravesicular transport in mammalian cells require (besides other factors) an *N*-ethylmaleimide-sensitive protein, which is found in both membrane-bound and soluble forms (Malhotra et al., 1988; Schatz, 1989). A specific interaction of secretory vesicles with their fusion sites via complementary recognition before fusion has been initially suggested by Palade (1975). A putative 51-kD docking protein for chromaffin granules of adrenal medulla was identified recently by intracellular application of antibodies (Schweizer et al., 1989). Furthermore, based on ultrastructural data calpactin I has been proposed to cross-link vesicles and plasma membranes in chromaffin cells (Nakata et al., 1990).

We have focused our attention on synaptophysin, one of the major integral membrane proteins of small clear synaptic vesicles (Wiedenmann and Franke, 1985; Jahn et al., 1985; for review see Wiedenmann and Huttner, 1989). Synaptophysin is a hexameric molecule composed of an *N*-glycosylated protein of 38 kD (Rehm et al., 1986; Thomas et al., 1988), whose primary structure has been elucidated by cDNA

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sequencing (Leube et al., 1987; Südhof et al., 1987; Buckley et al., 1987). Each 38-kD subunit spans the synaptic vesicle membrane four times, with both its NH_2 and COOH termini exposed to the cytosol (Leube et al., 1987; Johnston et al., 1989). After reconstitution into artificial liposomes and fusion with planar lipid bilayers, synaptophysin exhibits voltage-dependent channel activity in vitro (Thomas et al., 1988). However, the in vivo function of this synaptic vesicle membrane protein is still obscure. Based on biochemical data, in vitro reconstitution and comparison with gap junction proteins, we have suggested that synaptophysin may dock to a complementary protein in the presynaptic plasma membrane of active zones, possibly followed by formation of a fusion pore structure initiating neurotransmitter release (Thomas et al., 1988; Thomas et al., 1989). In this report, we present evidence that synaptophysin binds to a presynaptic plasma membrane protein of 36 kD. The possible involvement of synaptophysin and this binding protein in the docking process of synaptic vesicles are discussed.

Materials and Methods

Materials

Materials were purchased from the following sources: PMSF, aprotinin, pepstatin, glutaraldehyde (grade I), *Escherichia coli* β-galactosidase, bovine liver catalase, rabbit muscle lactic dehydrogenase, pig heart mitochondrial malic dehydrogenase, horse heart cytochrome *c*, high and low molecular weight markers, and prestained molecular weight markers were from Sigma Chemical GmbH (München, FRG); ^{35}S-methionine (>1,000 Ci/mmol sp act) and ^{14}C-methylated proteins were from Amersham Buchler GmbH (Braunschweig, FRG); Sephacryl S-300, protein A-Sepharose CL-4B, Polybuffer exchanger PBE 94, Polybuffer 74, Polybuffer 96, and dextran blue were from Pharmacia (Freiburg, FRG); horseradish peroxidase-conjugated goat anti-mouse IgG was from Dianova (Hamburg, FRG); alkaline phosphatase-conjugated goat anti-mouse and goat anti-rabbit IgG were from Promega Biotec (Madison, WI); nitrocellulose membranes (pore size 0.45 μm) were from Schleicher & Schuell (Dassel, FRG); endoproteinase Lys-C, proteinase K, ATP, and GTPγS were from Boehringer Mannheim GmbH (Mannheim, FRG); and EN^{3}HANCE was from New England Nuclear (Braunschweig, FRG). All other chemicals used were of analytical grade.

Subcellular Fractionations

6-wk-old Wistar rats were obtained from Charles River Laboratories (Sulzfeld, FRG). Synaptosomes were prepared from freshly dissected rat brains according to the method of Wolf and Kapatos (1989). All solutions additionally contained 0.1 mM PMSF, 1 μg/ml pepstatin, and 0.02–0.03 U/ml aprotinin as protease inhibitors. The synaptosomal pellets were resuspended in PBS at a protein concentration of 10 mg/ml. A cytosolic fraction was obtained by 10-fold dilution of the synaptosomes into 5 mM Tris-HCl, pH 7.4, followed by homogenization at 1,500 rpm with five strokes of a Teflon pestle. The hypotonic lysate was left on ice for 30 min and then centrifuged at 100,000 *g* for 1 h. Synaptic plasma membranes were prepared from synaptosomes by hypotonic lysis (without sonication) and combined flotation-sedimentation density gradient centrifugation (Jones and Matus, 1974). The material banding at the 34%/28.5% (wt/vol) sucrose interphase was collected, centrifuged at 80,000 *g* for 20 min, and resuspended in 5 mM Hepes-NaOH, pH 7.4, 50 μM $CaCl_2$. A mitochondrial fraction was recovered from the pellet of the density gradient centrifugation (Jones and Matus, 1974) by resuspension in 10 mM KP_i, pH 7.4, 150 mM KCl. Synaptic vesicles were prepared as described (Huttner et al., 1983), except that the final gel filtration step was omitted. The vesicles were resuspended in 10 mM KP_i, pH 7.4, 150 mM KCl. Membrane fractions from rat liver were prepared as published by Becker et al. (1989). All subcellular fractions were stored in small aliquots at −70°C until use.

Solubilization of Subcellular Fractions

Unless indicated otherwise, subcellular fractions were solubilized at 5 mg protein/ml in the presence of 1.875% (wt/vol) Triton X-100 and 1 M KCl (final concentrations) with gentle shaking for 1 h at 4°C. Insoluble material was removed by centrifugation at 130,000 *g* for 20 min. Solubilization was carried out in the presence of protease inhibitors as specified above.

For alkaline extraction, synaptosomes were pelleted and taken up in aqueous NaOH of different pH values (Steck and Yu, 1973) at a protein concentration of 5 mg/ml. After 15 min on ice, membranes were centrifuged at 130,000 *g* for 20 min, and the supernatant was neutralized with 1 M Tris-HCl, pH 7.4. The pellet was washed with 10 mM KP_i, pH 7.4, 150 mM KCl, to neutrality, resuspended to the initial volume, and solubilized by adding Triton X-100 to 1.875% (wt/vol). After 1 h on ice, insoluble material was removed by centrifugation at 130,000 *g* for 20 min.

Purification of Synaptophysin and Synaptophysin Fusion Protein SCY-MS2

Synaptophysin was purified from solubilized rat brain synaptic vesicles by a combination of chromatofocusing and gel exclusion chromatography (Thomas et al., 1988). A bacterial fusion protein (SCY-MS2) encompassing the cytoplasmic carboxy-terminal tail of synaptophysin joined to MS2 polymerase was prepared as described (Knaus and Betz, 1990).

Preparation, Fractionation, and Solubilization of Primary Neuronal Cell Cultures

Total brains were dissected from fetal mice (C57BL/6J or BALB/c) at days 13 and 14 of gestation. Primary cultures of neuronal cells were prepared, maintained, and metabolically labeled as described by Hoch et al. (1989) for primary cultures of spinal cord. Specifically, cells (in 6-cm-diam culture dishes) were incubated after 16 d with 1 ml of methionine-free medium containing 1 mCi [^{35}S]methionine. After 17 h, the harvested cells were either solubilized immediately or subjected to hypotonic lysis and differential centrifugation. In the latter case, the washed cellular pellet of one culture dish was suspended in 1 ml of 20 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 0.1 mM PMSF, 1 μg/ml pepstatin, 0.02 U/ml aprotinin. In this hypotonic buffer, cells were left on ice for 10 min and then homogenized with a Teflon-glass homogenizer. The homogenate was sequentially centrifuged at 1,000 *g* for 10 min, at 20,000 *g* for 20 min, and at 130,000 *g* for 20 min. The resulting pellets are referred to as crude nuclear fraction (1,000 *g*), crude membrane fraction (20,000 *g*), and crude vesicular fraction (130,000 *g*), respectively. The final 130,000-*g* supernatant constituted the cytosolic fraction. For solubilization, either the washed cell pellet of one culture dish or the various pellets obtained by differential centrifugation were resuspended in 10 mM KP_i, pH 7.4, containing 150 mM KCl and the protease inhibitors specified above, adjusted to 1.875% (wt/vol) Triton X-100 and 1 M KCl, and gently shaken for 1 h at 4°C. Insoluble material was removed from the ^{35}S-labeled extracts by centrifugation at 130,000 *g* for 20 min.

Fixation of Organelles

Synaptic vesicles and mitochondria (3 mg protein/ml in 10 mM KP_i, pH 7.4, 150 mM KCl) were fixed with 2% (wt/vol) glutaraldehyde for 3 h at 4°C as described by Meyer and Burger (1979) for chromaffin granules. After termination of the fixation reaction by 0.1 M NH_4Cl, the organelles were dialyzed twice against PBS for 4 h. The fixed organelles were pelleted at 15,000 *g* for 15 min, resuspended to a nominal protein concentration of 3 mg/ml in PBS by using a Dounce homogenizer, and stored at 4°C.

Binding Assays

Chemically fixed organelles (25 μl) were incubated with nonlabeled solubilized subcellular fractions (25 μl) in 10 mM KP_i, pH 7.4, 150 mM KCl, 1 mM $CaCl_2$ under shaking for 30 min at room temperature (22°C; final volume 300 μl). A sucrose cushion (750 μl) consisting of 0.2 M sucrose, 40 mM Hepes-NaOH, pH 7.4, 1 mM $CaCl_2$, was overlaid with sample and centrifuged at 15,000 *g* for 15 min at 4°C. The supernatant was carefully removed, and 100 μl of gel electrophoresis sample buffer was added to the pellet. The pellet was resuspended by trituration with sample buffer, while care was taken not to rinse the walls of the tube above the sucrose cushion. The samples were boiled for 5 min at 95°C, shortly centrifuged in a microfuge, and the supernatant was stored at −20°C until analysis by SDS-PAGE.

With native organelles, the following standard procedure was used. Equal amounts of radioactivity of the solubilized fractions from primary neuronal cultures were diluted into buffer containing 10 mM KP_i, pH 7.4, 150 mM KCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$ (final volume 1 ml). Taking into

account the detergent concentration of the solubilized fractions as well as the amount of detergent contributed by additional compounds, Triton X-100 was adjusted to a final concentration of 0.035% (wt/vol) in all cases. After addition of 60 μg of either native synaptic vesicles or mitochondria from rat brain, the mixtures were incubated under shaking for 30 min at room temperature (22°C). Sucrose cushions consisting of 500 μl of 0.1 M sucrose, 40 mM Hepes-NaOH, pH 7.4, 1 mM $CaCl_2$ were carefully overlaid with the samples and centrifuged at 130,000 *g* for 20 min at 4°C using an ultracentrifuge (model TL-100; Beckman Instruments Inc., Palo Alto, CA). The supernatant was removed with a pipette, and the pelleted organelles were solubilized by trituration in 25 μl of gel electrophoresis sample buffer. Additionally, 30 μl of gel electrophoresis sample buffer were used to rinse the bottom of the tube and combined with the solubilized vesicles. The samples then were processed for SDS-PAGE as described above.

Column Chromatography

Chromatofocusing on a Polybuffer exchanger PBE 94 column (1 ml) was performed at different pH ranges. At pH 7–4, the column was equilibrated with 25 mM imidazole-HCl, pH 7.4, 0.05% (wt/vol) Triton X-100, and eluted with Polybuffer 74-HCl, pH 4.0, 0.05% (wt/vol) Triton X-100. At pH 9–6, the column was equilibrated with 25 mM ethanolamin-CH_3COOH, pH 9.4, 0.05% (wt/vol) Triton X-100, and eluted with Polybuffer 96-CH_3COOH, pH 6.0, 0.05% (wt/vol) Triton X-100. Radiolabeled fractions were supplemented with 0.5 mg/ml of solubilized unlabeled synaptic plasma membrane proteins before chromatofocusing. Ionic strength was ≤0.05. 1-ml fractions were collected and analyzed in the binding assay using native synaptic vesicles.

Gel exclusion chromatography on a Sephacryl S-300 column (62 ml) was performed using 10 mM KP_i, pH 7.4, 150 mM KCl, 0.05% (wt/vol) Triton X-100. The total homogenate of a primary neuronal culture (450 μl), marker proteins (*E. coli* β-galactosidase [6.84 nm], bovine liver catalase [5.21 nm], rabbit muscle lactic dehydrogenase [4.75 nm], pig heart mitochondrial malic dehydrogenase [4.32 nm], horse heart cytochrome *c* [1.87 nm]) and dextran blue were applied to the column in a total volume of 1 ml. 0.88-ml fractions were collected and analyzed in the binding assay using native synaptic vesicles. Marker proteins were detected by light absorption or enzymatic activity.

Sucrose Gradient Centrifugation

Linear sucrose gradients (5–20% [wt/vol], 13 ml) were prepared in 10 mM Hepes-NaOH, pH 7.4, 150 mM KCl, 0.05% (wt/vol) Triton X-100. Solubilized membrane fractions from labeled primary neuronal cultures were diluted to 150 mM KCl, and 500 μl was applied to each gradient and centrifuged in a rotor (model SW 40; Beckman Instruments, Inc.) at 39,000 rpm for 18.5 h at 4°C. 840-μl fractions were collected and analyzed in the binding assay using native synaptic vesicles. Marker proteins (*E. coli* β-galactosidase [15.93 S], bovine liver catalase [11.30 S], rabbit muscle lactic dehydrogenase [6.95 S], pig heart mitochondrial malic dehydrogenase [4.32 S], horse heart cytochrome *c* [1.80 S]) were detected in a parallel gradient by their light absorption or enzymatic activity.

Electrophoretic Procedures and Immunoblotting

SDS-PAGE on 10% (unless indicated otherwise) gels was performed as described (Laemmli, 1970). Silver staining was done according to Merril et al. (1982). For fluorography, gels were treated with EN^{3}HANCE according to the manufacturer's instructions and subsequently dried for 2 h at 70°C under vacuum. After drying, gels were exposed to preflashed Kodak X-Omat films for 1–7 d. The intensity of fluorographed or silver-stained bands was quantified by scanning with a Hirschmann Elscript 400 densitometer. Transfer of proteins to nitrocellulose membrane sheets was performed as described (Kyhse-Andersen, 1984). Marker proteins were visualized on the nitrocellulose with Ponceau S. Immunodetection of synaptophysin using either mAb SY38 (1 μg/ml) (Wiedenmann and Franke, 1985) or a polyclonal antiserum (1:240) (Knaus and Betz, 1990) was performed as described by Becker et al. (1989). Anti-mouse or anti-rabbit IgGs coupled to alkaline phosphatase (1:7,500) were used for enzymatic detection.

Protein Determination

Protein content of subcellular fractions was determined by a modification of the Lowry procedure (Larson et al., 1986). The concentration of purified synaptophysin was estimated after SDS-PAGE by comparing the intensity of the silver-stained bands with those of BSA standards (Boehringer Mannheim GmbH). The concentration of the latter was calibrated by measuring the absorption at 280 nm. The concentration of SCY-MS2 was determined by an enzyme-linked dot immunoassay (Wiedenmann et al., 1988) using purified synaptophysin as a standard.

Protease Treatment of Synaptic Vesicles

Synaptic vesicles (75 μg of protein) were treated in a total volume of 150 μl of 10 mM KP_i, pH 7.4, 150 mM KCl, either with 1 U of endoproteinase Lys-C, or 150 μg of proteinase K, for 2 h at 37°C. After centrifugation at 130,000 *g* for 20 min at 4°C, the vesicular pellets were washed twice with 10 mM KP_i, pH 7.4, 150 mM KCl, and resuspended to the initial volume. Aliquots were used for vesicle binding assays or SDS-PAGE.

Phase Separation in Triton X-114

For partitioning of proteins during Triton X-114 phase separation, the modification of Bennett et al. (1988) of the original procedure (Bordier, 1981) was used. A homogenate of a labeled primary neuronal culture (50 μl) was diluted into 1 ml of cold 1% (wt/vol) Triton X-114 in PBS (precondensed three times). After phase separation at 37°C for 5 min and centrifugation for 1 min in a microfuge, upper (aequeous) and lower (detergent) phases were collected and brought to 1 ml with cold PBS. Equal amounts of radioactivity were analyzed in the binding assay using native synaptic vesicles.

Miscellaneous Methods

Liquid scintillation counting was performed using Ready Solv (Beckman, München, FRG). IgG fractions were isolated from immune sera by using protein A–Sepharose CL-4B chromatography (Ey et al., 1978).

Results

Synaptophysin in Chemically Fixed Organelles Binds to a 36-kD Synaptosomal Protein

Glutaraldehyde-fixed chromaffin granules have been successfully used to identify a granule-binding protein from the plasma membrane of adrenal medulla (Meyer and Burger, 1979). The physiological relevance of this protein was recently demonstrated by inhibition of exocytosis after intracellular injection of specific antibodies (Schweizer et al., 1989). To analyze interactions between synaptic vesicle proteins and other synaptosomal components, we designed an assay with glutaraldehyde-fixed synaptic vesicles. These vesicles were incubated with solubilized synaptosomes from rat brain. Synaptic vesicles were subsequently separated from the synaptosomal extract by centrifugation through a sucrose cushion, and bound proteins were solubilized and subjected to SDS-PAGE. Control experiments confirmed that proteins of the fixed vesicles were not solubilized under our conditions, nor were proteins of the solubilized synaptosomes sedimented through the sucrose cushion without fixed vesicles being added. Furthermore, the binding reaction proved to be insensitive to the detergent Triton X-100 up to a concentration of 0.5% (wt/vol) (data not shown). Nonspecific binding or binding to ubiquitous proteins was monitored using glutaraldehyde-fixed mitochondria from rat brain as a control adsorbent. Comparison of the patterns of synaptosomal proteins binding to fixed mitochondria and synaptic vesicles, respectively (Fig. 1 *a*, lanes *2* and *3*), identified a prominent protein of 36 ± 1 kD ($n = 7$) that predominantly bound to synaptic vesicles but not to mitochondria.

Some residual binding to mitochondria of this protein was probably caused by contamination of our mitochondrial preparation with synaptic vesicles as judged from immunoblots of the synaptic vesicle marker, synaptophysin (Fig. 1

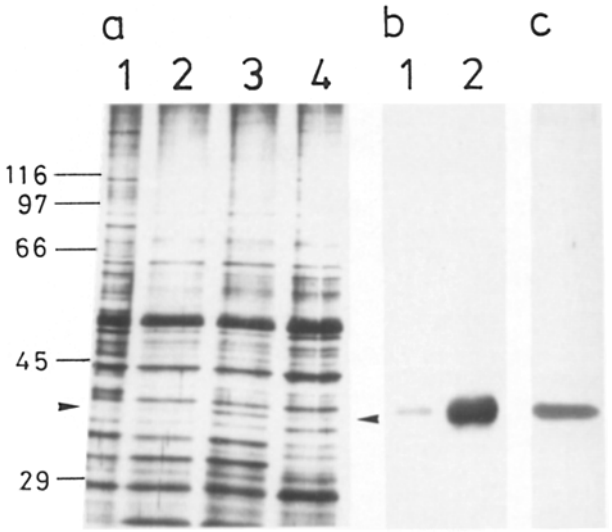

Figure 1. Binding assay with glutaraldehyde-fixed organelles. (*a*) A detergent/salt extract of rat brain synaptosomes (lane *1*) was diluted and incubated with glutaraldehyde-fixed organelles. The pattern of proteins binding to mitochondria (lane *2*) or synaptic vesicles (lanes *3* and *4*) was revealed by silver staining. Incubations were performed in the absence (lanes *2* and *3*) or presence (lane *4*) of purified synaptophysin added to a concentration of 2.5×10^{-7} M. Arrowheads mark the position of physophilin. Molecular masses (in kilodaltons) of marker proteins are shown on the left. (*b*) Immunodetection of synaptophysin in 5 μg of the mitochondrial (lane *1*) or synaptic vesicle (lane *2*) fractions. Subcellular fractions were used for SDS-PAGE before glutaraldehyde fixation and immunoblotted with mAb SY38. (*c*) Purity of synaptophysin (2 μg) used for competition experiments. The gel was stained with silver.

b). Note that the synaptic vesicle-binding protein of 36 kD, in contrast to most other synaptic vesicle- or mitochondria-binding proteins, was not a major component of the synaptosomal extract (Fig. 1 *a*, lane *1*), excluding nonspecific binding of an ubiquitous protein. When purified synaptophysin (for purity, see Fig. 1 *c*) was added in excess of the amount endogenously present in synaptic vesicles (Knaus et al., 1986), binding of the 36-kD protein was blocked to ~90% (Fig. 1 *a*, lane *4*). Addition of synaptophysin also inhibited the vesicle binding of some other proteins, in particular of a synaptosomal protein of 31 kD (Fig. 1, compare lanes *3* and *4*). However, the binding of these proteins is considered nonspecific, since all these proteins bound to mitochondria (Fig. 1 *b*) and represented abundant synaptosomal components (Fig. 1 *a*). Furthermore, these polypeptides were not detected as synaptophysin-displaceable vesicle-binding proteins when a different assay procedure was used (see below, and Fig. 3). We conclude that synaptophysin binds a synaptosomal protein of 36 kD for which we propose the name "physophilin" (φυσα, *greek* = bubble, vesicle; φιλος, *greek* = friend).

Because of their similar molecular masses, we compared the sizes of synaptophysin and physophilin. Fig. 2 shows that these two proteins could be separated by SDS-PAGE. Furthermore, no synaptophysin immunoreactivity below its position in synaptic vesicles was detected among the synaptic vesicle-binding proteins, using both mono- and polyclonal antibodies (data not shown). Hence, physophilin is not a degradation product of synaptophysin.

Synaptophysin Embedded in Native Organelles Binds to a 36-kD Protein of Primary Neuronal Cell Cultures

To exclude the possibility of artificially generating binding sites during glutaraldehyde fixation, we also used native vesicles as adsorbent to identify binding proteins. To this end, primary neuronal cell cultures from embryonic mouse brain were labeled metabolically with [^{35}S]methionine and used as a source for synaptic vesicle-binding proteins. Before incubation with native organelles, the detergent concentration in the extracts of primary cultures was diluted to 0.035% (wt/vol) Triton X-100, just above the critical micellar concentration of this detergent. Under this condition, the solubilized membrane proteins were still in solution, but added synaptic vesicle proteins were not solubilized as judged from a close to quantitative (>95%) recovery of synaptophysin in the pellet after centrifugation of the vesicles through a sucrose cushion. Furthermore, the protein pattern of synaptic vesicles and mitochondria did not detectably change under the conditions of the binding assay (data not shown). However, raising the concentration of Triton X-100 to 0.1% (wt/vol) results in the solubilization of >80% of synaptophysin (Wiedenmann and Franke, 1985; Jahn et al., 1985). In a solubilized crude membrane fraction from primary mouse brain cultures, the most prominent protein

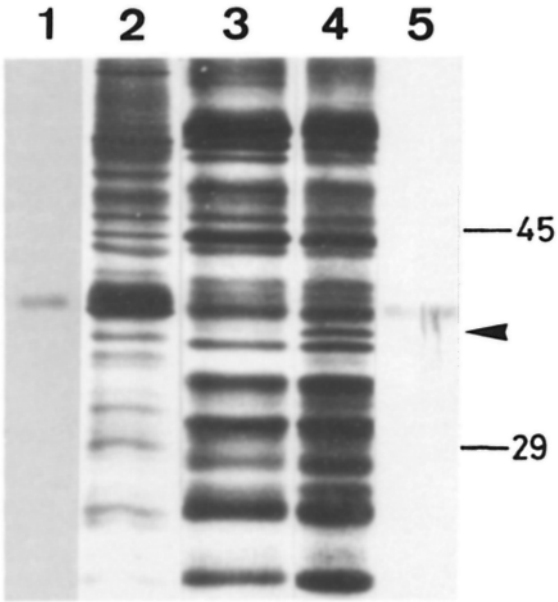

Figure 2. Size comparison of synaptophysin with physophilin. (Lane *1*) immunoblot of synaptic vesicles using monoclonal antibody SY38; (lane *2*) protein pattern of synaptic vesicles; (lane *3*) synaptosomal mitochondria-binding proteins; (lane *4*) synaptosomal synaptic vesicle-binding proteins; (lane *5*) purified synaptophysin (38 kD). The position of physophilin is indicated by an arrowhead. Note that in the molecular mass range of 38 kD, synaptic vesicles reveal a poorly resolved prominent doublet out of which the major upper band corresponds to synaptophysin. The samples were separated by SDS-PAGE and transferred to nitrocellulose (lane *1*) or stained with silver (lanes *2–5*). Molecular masses (in kilodaltons) of marker proteins are shown on the right.

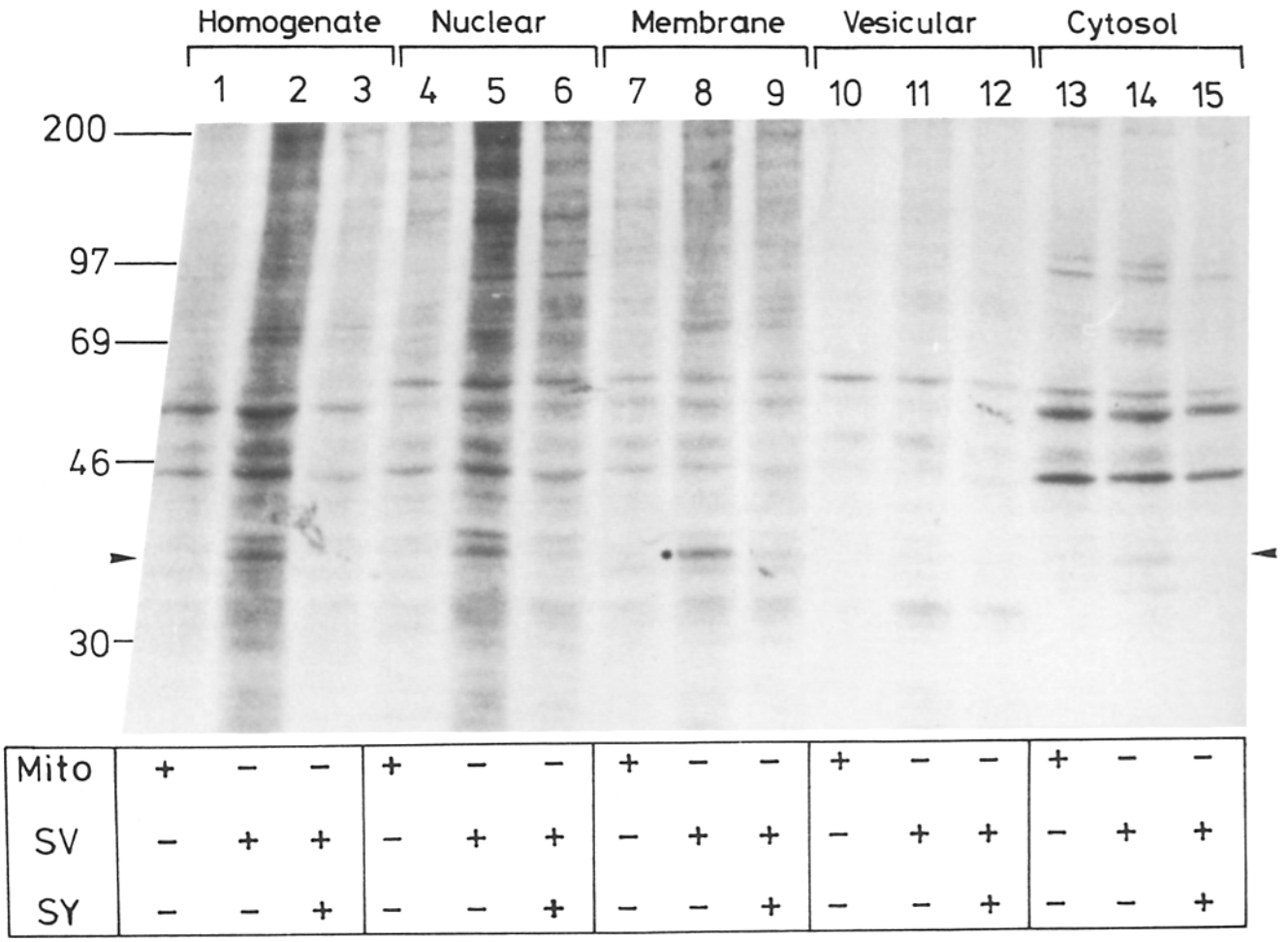

Figure 3. Binding assay with native organelles. A total homogenate (lanes *1–3*) as well as the extracts of the crude nuclear fraction (lanes *4–6*), of the crude membrane fraction (lanes *7–9*), of the crude vesicular fraction (lanes *10–12*), and the cytosolic fraction (lanes *13–15*) of [^{35}S]methionine-labeled brain cultures were used in a binding assay with native organelles as described in Materials and Methods. The patterns of mitochondria-binding proteins (lanes *1*, *4*, *7*, *10*, and *13*) and synaptic vesicle–binding proteins in the absence (lanes *2*, *5*, *8*, *11*, and *14*) or presence (lanes *3*, *6*, *9*, *12*, and *15*) of 9×10^{-8} M purified synaptophysin are shown. The dried gels were exposed for 4 d at −70°C. The position of physophilin is indicated by arrowheads as well as by a star in lane *8*. Molecular masses of ^{14}C-methylated marker proteins are given on the left. The presence (+) or absence (−) of mitochondria (*Mito*), synaptic vesicles (*SV*), or synaptophysin (*SY*) in each binding assay is indicated at the bottom.

which bound to nonfixed synaptic vesicles had a molecular mass of 36 ± 1 kD (n = 5) (Fig. 3, lane *8*). This synaptic vesicle–binding protein showed almost no affinity for native mitochondria (Fig. 3, lane *7*). Its binding to synaptic vesicles was blocked to about 70% by the addition of purified synaptophysin to a concentration of 9×10^{-8} M (Fig. 3, lane *9*; the addition of higher amounts of purified synaptophysin was not feasible because of raising the Triton X-100 concentration above 0.035% [wt/vol]). Obviously, the proteins of molecular mass of 36 kD identified by our binding assays behaved in an identical manner in rat synaptosomes and primary mouse brain cultures. Furthermore, their migration behavior in SDS-PAGE was indistinguishable when compared to prestained molecular weight markers that can be visualized on both silver-stained gels and gels dried for fluorography (data not shown). Therefore, the synaptic vesicle-binding proteins of 36 kD seen by both approaches are thought to be identical, i.e., physophilin.

Physophilin was found only in trace amounts in both the cytosolic (Fig. 3, lane *14*) and the crude vesicular (Fig. 3, lane *11*) fractions. It is noteworthy that two prominent synaptic vesicle-binding proteins, probably actin and tubulin, were greatly enriched in the cytosolic fraction, indicating an efficient separation of cellular components by differential centrifugation. Physophilin was also seen in the crude nuclear fraction (Fig. 3, lane *5*) and in a total homogenate (Fig. 3, lane *2*). These data suggest physophilin to represent a membrane-associated protein.

Characteristics of the Synaptophysin–Physophilin Interaction

To assess the affinity between synaptophysin and physophilin, we titrated the inhibitory effect of synaptophysin on the synaptic vesicle–binding activity of physophilin. Purified synaptophysin (see Fig. 1 *c*) in concentrations of up to 5.8×10^{-7} M inhibited the binding of physophilin to glutaraldehyde-fixed vesicles by >90% (Fig. 4). Because of limita-

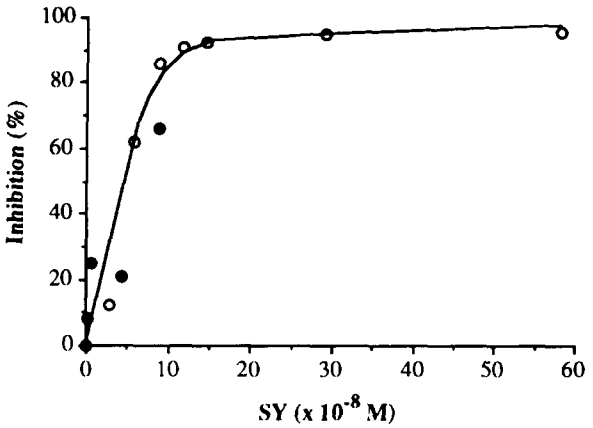

Figure 4. Competition of physophilin-binding by synaptophysin. Synaptosomal proteins or proteins of a crude membrane fraction of primary brain cultures were bound to glutaraldehyde-fixed synaptic vesicles (○) or nonfixed synaptic vesicles (●), respectively, in the presence of increasing concentrations of purified synaptophysin (*SY*). Inhibition was calculated by scanning silver-stained gels (○) or fluorograms (●) and normalizing the intensity of the physophilin band to the actin band. The ratio of physophilin to actin in the absence of purified synaptophysin served as control. Throughout the gel, the actin band used for normalization showed a silver staining approximately constant in absolute intensity. The native molecular mass of purified synaptophysin was taken as 228 kD (Thomas et al., 1988). The concentration of endogenous synaptophysin in solubilized synaptosomal or brain culture proteins was below 1×10^{-9} M in all assays.

tions with the detergent concentration, purified synaptophysin could only be used up to 8.8×10^{-8} M with nonfixed vesicles. This resulted in an ~70% inhibition of the binding reaction (Fig. 4). However, both binding assays yielded comparable results, indicating that the binding site for physophilin was not dramatically altered during the fixation of synaptic vesicles with glutaraldehyde. Half-maximal inhibition (IC_{50}) of physophilin-binding was seen at 10^{-8}–10^{-7} M synaptophysin (Fig. 4). These data show that the binding of synaptophysin to physophilin is of relatively high affinity.

To further validate the specificity of the synaptophysin–physophilin interaction, a variety of other proteins and protein mixtures were tested for competing synaptic vesicle binding of physophilin. Whereas 1×10^{-7} M synaptophysin resulted in an 80% inhibition (Fig. 4), bovine serum albumin (3×10^{-6} M), ovalbumin (3×10^{-6} M), immunoglobulin G (6×10^{-6} M), cytochrome *c* (1×10^{-5} M), hemoglobin (2×10^{-6} M), and tetanus toxin (0.5 mg/ml) all had no significant effect (inhibition ≤10%). Furthermore, binding of physophilin to synaptic vesicles was not affected by the addition of solubilized mitochondrial proteins or solubilized liver membrane proteins to a concentration of 0.2 mg/ml. Likewise, no inhibition was observed when solubilized synaptic vesicle proteins were added to the binding assay in an amount that raised the endogenous synaptophysin concentration to maximally 1×10^{-8} M (data not shown). However, when synaptic vesicles were solubilized and applied to chromatofocusing (the first step in our purification procedure for synaptophysin; see Thomas et al., 1988), the inhibitory effect of the resulting fractions on synaptic vesicle-binding of physophilin correlated with their synaptophysin content (Fig. 5). Some inhibition was also observed in fractions lacking synaptophysin. These effects were not very different from the normal variations (~10–15%) seen in the densitometric analysis of gels, in contrast to that caused by synaptophysin-containing fractions. However, the minor involvement of additional components of synaptic vesicles in the interaction between physophilin and these vesicles cannot be excluded presently. Because a variety of soluble as well as membrane proteins except synaptophysin showed no significant inhibitory effect, we conclude that the interaction between synaptophysin and physophilin is specific.

Since both neurotransmitter release and retrieval of synaptic vesicles into the cytoplasm are calcium-dependent processes (Augustine et al., 1987; Ceccarelli and Hurlbut, 1980), a possible influence of this cation has to be considered in any nerve terminal process. Initially, all our binding assays were performed in the presence of calcium (1 mM). Inclusion of calcium chelators (10 mM EGTA) in the assay buffers and sucrose cushions, however, showed no effect on the synaptic vesicle binding of physophilin to native vesicles (data not shown). In view of the emerging role of small GTP-binding proteins in vesicular transport (Barbacid, 1987; Segev et al., 1988; Bourne, 1988) and of the presence of a small GTP-binding protein, rab3, in synaptic vesicles (Mollard et al., 1990), the possible involvement of GTP-binding proteins in the association of synaptophysin with physophilin was investigated using native synaptic vesicles. However, physophilin binding was not dependent on GTPγS (50 μM). Likewise, addition of ATP (1 mM) during the binding reaction did not alter the results (data not shown).

A bacterial fusion protein, SCY-MS2, containing the cytoplasmic carboxy-terminal tail of synaptophysin following its fourth transmembrane domain (amino acids 218–307; see Knaus and Betz, 1990) was also tested for competition in our binding assays. Even at concentrations of 7×10^{-7} M SCY-MS2, we did not detect any inhibitory effect of the fusion construct (Fig. 6 *a*). Similarly, 5×10^{-4} M of a synthetic tetrapeptide (GYGP) that contains the repeated motif of the cytoplasmic tail of synaptophysin (Leube et al., 1987) showed no effect. This suggested that the carboxy-terminal cytoplasmic tail of synaptophysin may not be important for physophilin binding. Further support for this assumption is derived from binding assays in the presence of antisynaptophysin antibodies. Monoclonal antibody SY38 (Wiedenmann and Franke, 1985) binds to a flexible segment in the center of the cytoplasmic repeat structure of synaptophysin, and a polyclonal antisynaptophysin serum also preferentially recognizes epitopes that are located in this cytoplasmic tail region (Knaus and Betz, 1990). None of these anti-tail antibodies blocked the binding of physophilin to synaptophysin, regardless of whether native or glutaraldehyde-fixed vesicles were used as adsorbent (Fig. 6 *c*, lanes *4–6*, and data not shown). Furthermore, treatment of synaptic vesicles with endoproteinase Lys-C resulted in the loss of the carboxy-terminal repeat structure of synaptophysin (cleavage produced a 27–28-kD fragment and presumably occurred at lysine residue 237 [Fig. 6 *b*]; other cytoplasmic lysine residues located close to the transmembrane segments probably are inaccessible to the enzyme). Upon treatment of synaptic vesicles with endoproteinase Lys-C, the binding of physophilin was not altered. In contrast, no proteins were bound after digestion of synaptic vesicles with proteinase K (Fig. 6 *c*, lanes

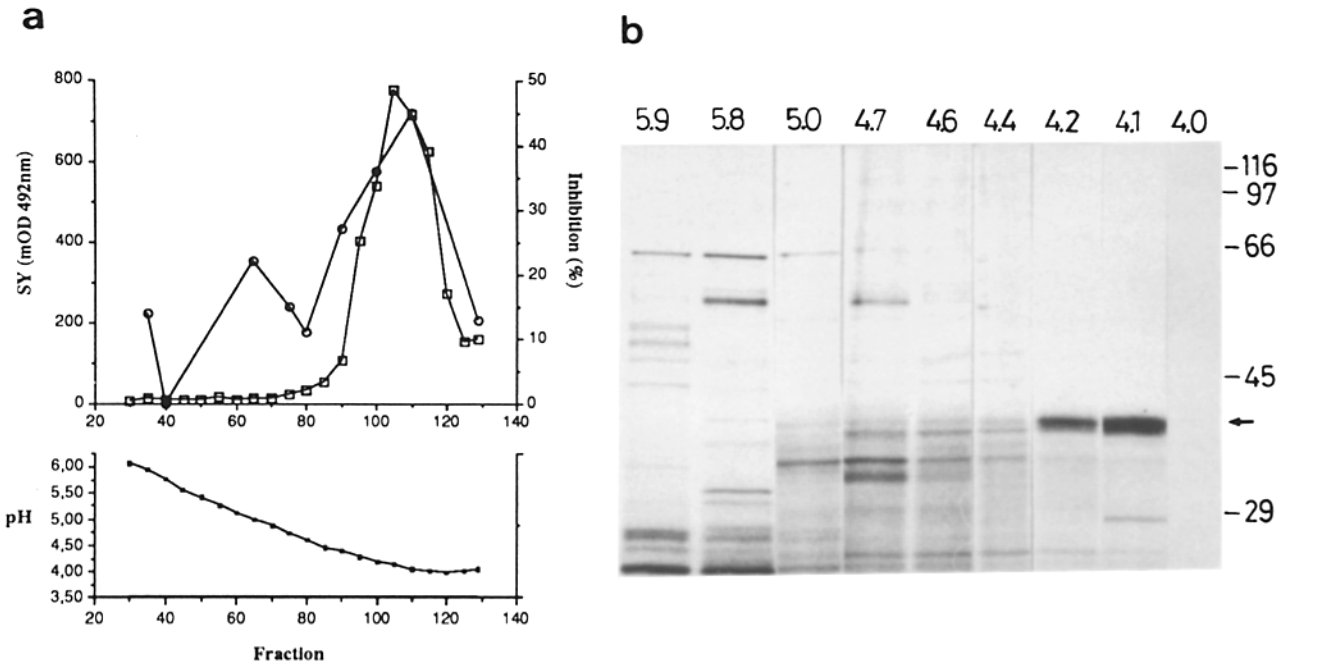

Figure 5. Fractionation of synaptic vesicle proteins. (*a*) Chromatofocusing of solubilized synaptic vesicles. The pH gradient (pH 6–4) is shown in the lower part of the figure. The elution profile of synaptophysin (*SY, squares*) was determined by measuring the optical density at 492 nm in an enzyme-linked dot immunoassay (Wiedenmann et al., 1988). Equal amounts of the neutralized fractions were tested for their ability to inhibit the binding of physophilin to glutaraldehyde-fixed vesicles. Relative inhibition (*circles*) was determined as described in Fig. 4. In the binding assay with the pH 4.1 fraction, the concentration of synaptophysin was estimated to be 5×10^{-8} M. This resulted in an inhibition of 45%, in agreement with the titration data presented in Fig. 4. (*b*) Protein pattern of the chromatofocusing fractions that were used for competition. Equal amounts were subjected to SDS-PAGE and stained with silver. The pH value of each fraction is indicated above the respective lane; the position of synaptophysin is marked with an arrow. Molecular masses (in kilodaltons) of marker proteins are shown on the right.

1–3). Because removal of the COOH-terminal 70 amino acids of synaptophysin did not alter physophilin binding, we conclude that these residues are not involved in the binding domain.

Physophilin Is an Oligomeric Basic Protein

Analysis of the sedimentation behavior of solubilized physophilin on sucrose density gradients indicated an apparent sedimentation coefficient of 5.6 S (Fig. 7). Thus, physophilin binds not in monomeric form to synaptophysin, but as a large complex. When a total homogenate from [^{35}S]methionine-labeled brain cultures was applied to gel exclusion chromatography, the synaptic vesicle–binding activity of physophilin eluted at a Stokes radius of 6.56 nm (data not shown). Again, this indicated a large quaternary structure. Furthermore, electrophoretic examination of the sedimentation and elution profiles obtained in these experiments revealed that no other synaptic vesicle–binding protein cofractionated precisely with physophilin (Fig. 7, and data not shown), suggesting that physophilin may be a major component of this complex.

To determine the isoelectric point of physophilin, the physophilin-containing fractions of a sucrose density gradient of solubilized crude membranes from radiolabeled brain cultures were pooled and applied to chromatofocusing in the range of pH 7 to pH 4. Proteins of isoelectric point within this range will bind to the column, whereas basic proteins will appear in the flowthrough fractions. Physophilin was detected in the flowthrough by our standard binding assay with native synaptic vesicles (data not shown). Subsequently, these flowthrough fractions were collected and re-applied to chromatofocusing in the range of pH 9 to pH 6. Elution of physophilin was maximal at pH 7.8 (data not shown). Thus, physophilin is a slightly basic protein, in contrast to synaptophysin, which is highly acidic (Wiedenmann and Franke, 1985; Thomas et al., 1988).

Subcellular Localization of Physophilin

Binding of proteins from primary brain cultures to nonfixed synaptic vesicles had revealed that physophilin was greatly enriched in a crude membrane fraction and nearly absent from crude vesicular and cytosolic fractions (see Fig. 3). To further substantiate the subcellular localization of physophilin, different cellular fractions from rat brain were investigated for the presence of physophilin (Fig. 8). Physophilin binding was detected in synaptosomes as well as in synaptic plasma membranes prepared from the latter, but not in synaptic vesicles (Fig. 8, compare lanes *1–3*). Furthermore, physophilin was not present in a cytosolic fraction (Fig. 8, lane *5*; minor amounts of physophilin occasionally seen in cytosolic fractions prepared from radiolabeled cells [see Fig. 3] probably reflected residual contamination resulting from a modified homogenization and centrifugation procedure). Control experiments with rat liver membranes also gave negative results (Fig. 8, lane *6*). Therefore, physophilin appears to be associated with synaptic plasma membranes.

A series of extraction experiments was carried out to de-

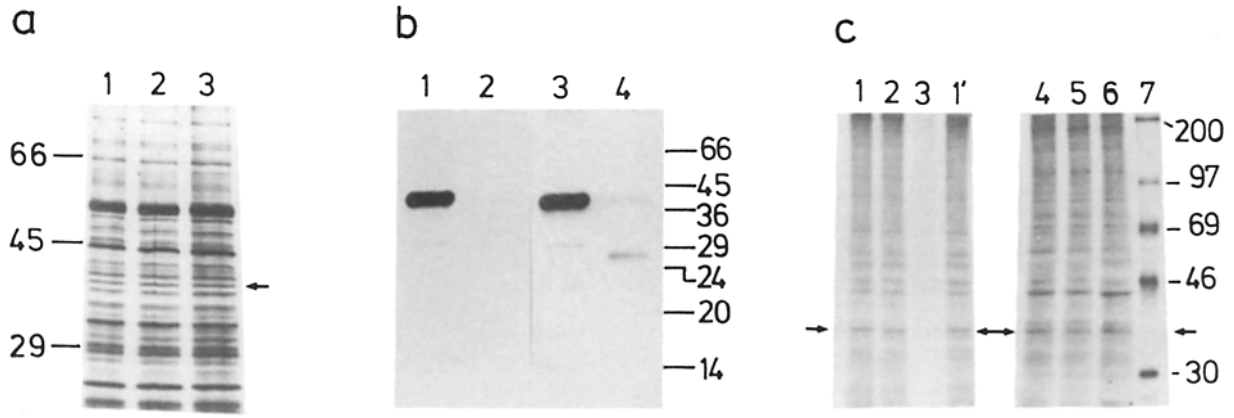

Figure 6. The COOH-terminal tail region of synaptophysin is not involved in physophilin binding. (*a*) Solubilized synaptosomal proteins were bound to glutaraldehyde-fixed synaptic vesicles in the absence (lane *1*) or presence of 7×10^{-7} M SCY-MS2 fusion protein (lane *2*), or 5×10^{-4} M tetrapeptide GYGP (lane *3*), respectively. Vesicle-binding proteins were separated by 10% SDS-PAGE and visualized by silver staining. The position of physophilin is indicated by an arrow; molecular masses (in kilodaltons) of marker proteins are shown on the left. (*b*) Native synaptic vesicles were incubated for 2 h at 37°C in the absence (lanes *1* and *3*) or presence (lanes *2* and *4*) of endoproteinase Lys-C. Aliquots were applied to 15% SDS-PAGE, transferred to nitrocellulose, and immunoreacted with mAb SY38 (lanes *1* and *2*) or with the IgG fraction of a polyclonal antisynaptophysin serum (lanes *3* and *4*). Molecular masses (in kilodaltons) are shown on the right. After proteolytic cleavage, the SY38 epitope (between residues 269 and 289; see Knaus and Betz [1990]) is almost completely lost (lane *2*), indicating that the cytoplasmic carboxy-terminal tail of synaptophysin has been removed. No intermediate products were observed at shorter times (data not shown), suggesting a single defined cleavage site (probably lysine 237). A membrane-bound degradation product of ~27–28 kD was revealed by the polyclonal antiserum (lane *4*). The weak staining seen is due to removal of the major antigenic epitope (see Knaus and Betz, 1990). (*c*) Solubilized crude membranes (lanes *1–3*) or a total homogenate (lanes *4–6*) from metabolically labeled primary neuronal cultures were analyzed for physophilin-binding activity using native synaptic vesicles as adsorbent. Incubations were carried out in the absence (lanes *1–4*) or presence of 6×10^{-7} M mAb SY38 (lane *5*) or 6×10^{-6} M of an IgG fraction of polyclonal antisynaptophysin antibodies (lane *6*), respectively, after preincubation of the vesicles with antibody for 30 min at 4°C. In case of solubilized crude membranes as donor fractions, vesicles had been preincubated in the absence (lanes *1*, and *1'*) or presence of endoproteinase Lys-C (lane *2*) or proteinase K (lane *3*), followed by two washing steps. Samples were analyzed by 8% SDS-PAGE and fluorography. ^{14}C-methylated marker proteins are shown in lane *7*, their molecular masses (in kilodaltons) are given on the right. The position of physophilin is indicated by arrows.

termine the nature of the association of physophilin with synaptic plasma membranes. The extracts were tested on glutaraldehyde-fixed synaptic vesicles for the presence of physophilin (data not shown). High salt alone (1 M KCl) was unable to solubilize physophilin. This could not be attributed to altered binding properties of the protein since it was routinely observed after our standard high salt/detergent extraction (1 M KCl, 1.875% [wt/vol] Triton X-100; see Materials and Methods). Alkaline extraction at pH 10, 11, or 12, known to remove peripheral membrane proteins from erythrocyte membranes (Steck and Yu, 1973), also failed to promote solubilization of physophilin. In contrast, physophilin-binding could be recovered in the pellet after alkaline pretreatment up to pH 11, arguing against inactivation of physophilin under these conditions. On the other hand, when Triton X-100 was solely used to extract physophilin from synaptic plasma membranes, efficient binding was observed after solubilization at detergent concentrations ≥1% (wt/vol). These results are consistent with physophilin being an integral membrane protein.

Partitioning of the proteins solubilized from a labeled primary neuronal culture with Triton X-114 (Bordier, 1981) did not reveal a well-defined separation behavior of physophilin binding activity (data not shown). However, distribution into both detergent and water phases has also been reported for other integral membrane proteins that do not have a particularly low polarity index (Maher and Singer, 1985; Schneider et al., 1985; Schmitt et al., 1987). In conclusion, the data described above support the view that physophilin indeed is an integral membrane protein of synaptic plasma membranes.

Discussion

In this report, we have described procedures for studying the interaction of synaptic vesicles with solubilized proteins from various sources. One method takes advantage of glutaraldehyde-fixed vesicles as an adsorbent to identify binding proteins. The fixed vesicles can be easily separated from the incubation mixture by centrifugation in a microfuge, thus allowing a selective solubilization of the adhering proteins only. The fixation procedure is similar to customary protocols used for sample fixation in immunoelectron microscopy where binding of antibodies to fixed epitopes is still observed. Chemically fixed chromaffin granules have previously been used to isolate a granule-binding protein from the plasma membrane of adrenal medulla (Meyer and Burger, 1979) that appears to be involved in exocytosis (Schweizer et al., 1989).

The synaptic vesicle binding of proteins depending on multivalent interactions, or conformational flexibility of mobile receptor sites, may not be detected in a binding assay with fixed vesicles. This limitation is overcome by the use of nonfixed synaptic vesicles and minimal amounts of detergent just above the critical micellar concentration. In this case, ultracentrifugation must be used to recover the synaptic

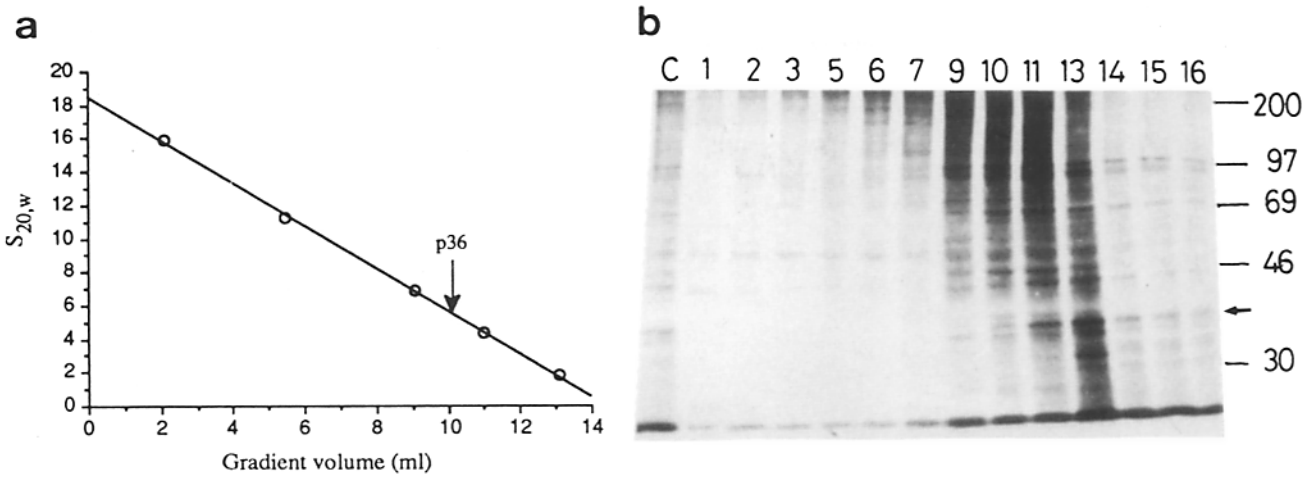

Figure 7. Sedimentation behavior of physophilin. (*a*) Solubilized crude membranes from [^{35}S]methionine-labeled brain cultures were centrifuged on a 5–20% sucrose gradient, and the apparent sedimentation coefficient of physophilin was determined by comparison to marker proteins as described in Materials and Methods. The position of physophilin is indicated as p36 in this graph. The gradient was fractionated from the bottom (*left*) to the top (*right*). (*b*) A 300-μl aliquot of each fraction was tested for the presence of physophilin. Gel electrophoretic evaluation of the binding assay using native synaptic vesicles is shown here for some of the fractions, numbered from the bottom (*left*) to the top (*right*). *C* denotes a binding assay with the solubilized crude membranes before sedimentation. The position of physophilin is indicated by an arrow. Molecular masses (in kilodaltons) of marker proteins are given on the right. The gel was exposed for 3 d.

vesicles, and the adhering proteins labeled in order to be distinguishable from synaptic vesicle proteins. This binding assay with nonfixed vesicles certainly represents a closer approach to the in vivo situation, although the presence of low detergent concentrations may render the vesicles leaky. The possibility that partner proteins loose their binding capacity upon membrane solubilization cannot however, be excluded.

Physophilin was the only brain protein detected that fulfilled all of the following criteria: first, it could be identified as a prominent vesicle-binding protein in both assay systems; second, it did not bind to mitochondria; third, its binding to synaptic vesicles was competed by synaptophysin but not by many other proteins. Therefore, we have concentrated on the characterization of this protein. It should be noted, however, that the assays described here additionally revealed the binding to synaptic vesicles of minor components that remain to be analyzed.

Previous attempts to isolate physophilin by affinity chromatography using immobilized synaptophysin as a ligand were unsuccessful (Thomas, L., unpublished results). Thus, either synaptophysin was coupled to the affinity matrix via its binding domains, or additional components of synaptic vesicles are necessary for the binding reaction. This underlines the advantage of working with whole vesicles instead of isolated proteins as long as the molecular details of vesicle–plasma membrane interaction are not understood.

The interaction between vesicle-bound synaptophysin and solubilized physophilin was only blocked by solubilized synaptophysin. A variety of other proteins including solubilized membrane proteins from subcellular fractions did not interfere with the synaptophysin–physophilin binding, pointing to the specificity of this interaction. Moreover, our titration data show an affinity in the submicromolar range between synaptophysin and physophilin. Together with the finding that this affinity is not influenced by calcium ions, GTPγS, or ATP, which often regulate short-term events, this suggests that in vivo the binding of synaptophysin to physophilin may not only be transient, but serve a more permanent interaction. In view of inherent limitations of our binding assay (e.g., presence of detergent) and the possible existence of additional factors not revealed by the latter, further studies will be required to elucidate this point.

Physophilin binding of synaptophysin was not affected by an excess of SCY-MS2 fusion protein and antisynaptophysin antibodies, or by endoproteinase Lys-C treatment of synaptic vesicles which cleaved the COOH-terminal tail of synaptophysin, presumably at lysine residue 237 (Fig. 6 *b*). We therefore suppose that the physophilin binding site is associated with cytoplasmic domains of synaptophysin not included in its characteristic carboxy-terminal repeat structure. Other synaptic vesicle proteins associated with synaptophysin could also contribute to physophilin binding. Interestingly, when amino acid substitutions between rat and Torpedo synaptophysin are considered, there are only 13% nonconservative exchanges (6 out of 46 intracellular residues) within the cytoplasmic stretches preceding lysine 237, but 34% nonconservative replacements (24 out of 70 carboxy-terminal residues) in the remaining cytoplasmic repeat region cleaved off by the endoproteinase (for substitutions and transmembrane topology, see Cowan et al., 1990). The substantially higher degree of evolutionary conservation argues for the functional importance of this assigned physophilin binding domain. Although the carboxy-terminal cytoplasmic tail containing the characteristic repeat structure appears not to be involved in physophilin binding, it may play a role in interactions with extravesicular proteins, e.g., cytoskeletal elements. Synaptic vesicles are known to be anchored to the cytoskeleton before they reach their release sites (Kelly, 1988).

The hydrodynamic parameters of solubilized physophilin indicate that this 36-kD protein is part of a large complex. At present, we cannot exclude the presence of other subunits within this complex, although the 36-kD protein appeared to be its major component. Tentatively assuming a homo-oligomeric structure for physophilin, a number of 4 to 5 sub-

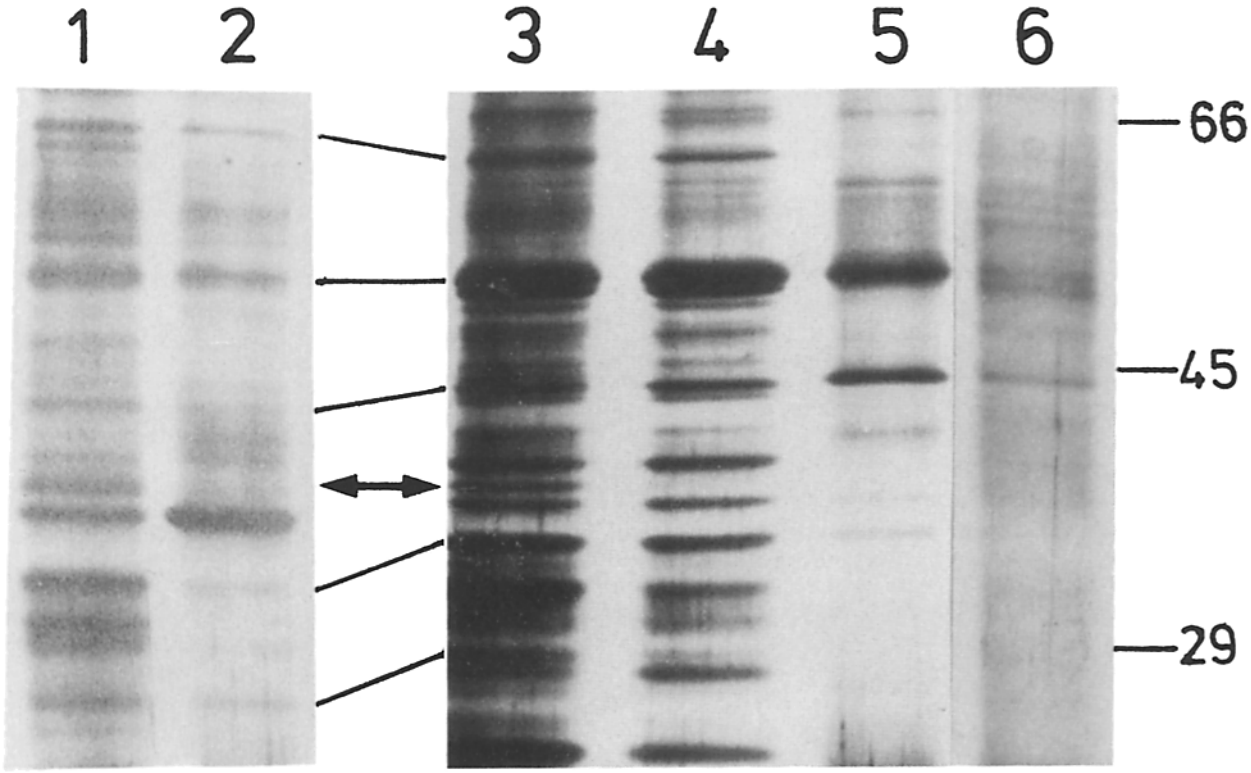

Figure 8. Subcellular localization of physophilin. Synaptic plasma membranes (lane *1*), synaptic vesicles (lane *2*), synaptosomes (lanes *3* and *4*), cytosol (lane *5*), and liver membranes (lane *6*) were prepared, solubilized, and analyzed for the presence of physophilin using glutaraldehyde-fixed synaptic vesicles (lanes *1–3*, *5*, and *6*) or mitochondria (lane *4*). The endogenous synaptophysin concentration in the binding assay with solubilized synaptic vesicle proteins (lane *1*) was below 1–2 × 10^{-8} M. The position of physophilin is indicated by a double-headed arrow between lanes *2* and *3*. Molecular masses (in kilodaltons) of marker proteins are given on the right. Gel bands were revealed by silver staining.

units is consistent with the Stokes radius and sedimentation coefficient determined here. A detailed structural analysis of purified physophilin awaits purification of this protein.

Binding activity of physophilin was found in synaptic plasma membrane fractions derived from synaptosomes. Because it was not detected in purified synaptic vesicle fractions, the presence of physophilin cannot be attributed to contamination by these vesicles. Furthermore, the solubilization data indicate that physophilin behaves as an integral membrane protein. Hence, this synaptophysin-binding protein appears to be specifically located in synaptic membranes different from the membranes of small synaptic vesicles. Immunofluorescence and immunoelectron microscopy will be required to show whether physophilin is indeed localized in the presynaptic plasmalemma or even in the plasma membrane of active zones.

Calpactin, a widespread calcium-dependent phospholipid and actin-binding protein that has been described to play a role in exocytosis in adrenal chromaffin cells (Ali et al., 1989; Nakata et al., 1990) possesses a subunit of 36 kD. However, it appears unlikely that physophilin is identical to calpactin. First, the binding of calpactin to chromaffin granules is calcium dependent (Creutz et al., 1983), whereas the binding of physophilin to synaptic vesicles is not. Second, the Stokes radius of calpactin is significantly smaller than that of physophilin (4.1 vs. 6.6 nm; see Gerke and Weber, 1984). Finally, in contrast to physophilin, calpactin can be solubilized by high salt extraction (Greenberg and Edelman, 1983).

The specific docking of secretory vesicles to the plasma membrane is assumed to represent a necessary step preceding exocytosis (Bourne, 1988; Kelly, 1988). Physophilin seems to be a good candidate for mediating such docking of synaptic vesicles to the presynaptic plasma membrane before quantal release. The vesicle recognition site for physophilin should comprise the abundant synaptic vesicle component synaptophysin, but other proteins might in addition be involved. The docking process results in correct positioning of vesicles in the ultimate vicinity of the release site, thus allowing neurotransmitter discharge to occur immediately upon stimulation. Whether the fusion process itself then is initiated by a proteinaceous fusion pore structure remains to be proven. If so, it appears logical to have the elements of specific recognition as well as fusion pore formation combined in one macromolecular complex.

The results presented here should help in isolating physophilin and further characterizing its localization and interaction with synaptophysin. With these two components in hand, the model outlined above then may be experimentally approached.

We thank B. Wiedenmann and C.-M. Becker for stimulating discussions. We also thank W. Hoch, C.-M. Becker, and I. Wolters for help with the primary brain cultures, P. Knaus for providing the SCY-MS2 construct, W. Mayser for his support of preparative work, E. Habermann for the kind gift of tetanus toxin, and our colleagues for critical reading of the manuscript. L. Thomas is a fellow of the Boehringer Ingelheim Fonds.

This work was supported by Deutsche Forschungsgemeinschaft (SFB

317 and Leibniz Programm), Bundesministerium für Forschung und Technologie (BCF 365/1), and Fonds der Chemischen Industrie.

Received for publication 11 May 1990 and in revised form 12 June 1990.

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