Interactions of Synapsin I with Small Synaptic Vesicles: Distinct Sites in Synapsin I Bind to Vesicle Phospholipids and Vesicle Proteins

Fabio Benfenati, Martin Bähler, Reinhard Jahn, and Paul Greengard

Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York 10021

Abstract. Synapsin I is a major neuron-specific phosphoprotein that is specifically localized to the cytoplasmic surface of small synaptic vesicles. In the present study, the binding of synapsin I to small synaptic vesicles was characterized in detail. The binding of synapsin I was preserved when synaptic vesicles were solubilized and reconstituted in phosphatidylcholine. After separation of the protein and lipid components of synaptic vesicles under nondenaturing conditions, synapsin I bound to both components. The use of hydrophobic labeling procedures allowed the assessment of interactions between phospholipids and synapsin I in intact synaptic vesicles. Hydrophobic photolabeling followed by cysteine-specific cleavage of synapsin I demonstrated that the head domain of synapsin I penetrates into the hydrophobic core of the bilayer. The purified NH_2-terminal fragment, derived from the head domain by cysteine-specific cleavage, bound to synaptic vesicles with high affinity confirming the results obtained from hydrophobic photolabeling. Synapsin I binding to synaptic vesicles could be inhibited by the entire molecule or by the combined presence of the NH_2-terminal and tail fragments, but not by an excess of either NH_2-terminal or tail fragment alone. The purified tail fragment bound with relatively high affinity to synaptic vesicles, though it did not significantly interact with phospholipids. Binding of the tail fragment was competed by holosynapsin I; was greatly decreased by phosphorylation; and was abolished by high ionic strength conditions or protease treatment of synaptic vesicles. The data suggest the existence of two sites of interaction between synapsin I and small synaptic vesicles: binding of the head domain to vesicle phospholipids and of the tail domain to a protein component of the vesicle membrane. The latter interaction is apparently responsible for the salt and phosphorylation dependency of synapsin I binding to small synaptic vesicles.

SYNAPSIN I is a neuron-specific synaptic vesicle-associated phosphoprotein (11, 13, 20, 32, 35). It has a collagenase-insensitive head domain containing a serine residue that can be phosphorylated by cAMP-dependent protein kinase or calcium/calmodulin-dependent protein kinase I (site 1) and an elongated collagenase-sensitive tail domain with two serine residues that can be phosphorylated by calcium/calmodulin-dependent protein kinase II (sites 2 and 3) (10, 19, 31, 39). The association of synapsin I with highly purified brain small synaptic vesicles has recently been partially characterized (34). Purified dephosphorylated synapsin I bound to synapsin I-depleted synaptic vesicles with high affinity (K_d = 10 nM at 40 mM NaCl) and saturability (B_{max} = 800 fmol/μg protein). Increasing the ionic strength of the medium, or phosphorylating the tail domain, decreased the affinity of this binding without altering the B_{max}. The amount of synapsin I rebound at saturation, representing ~6% of the total synaptic vesicle protein, was similar to the amount found in native synaptic vesicles (20, 34).

Dephosphorylated synapsin I has also been demonstrated to bundle F-actin (2). Both the bundling activity and the number of binding sites on F-actin were decreased by phosphorylation of synapsin I on the tail sites. These interactions may have a role in the regulation of vesicle traffic and neurotransmitter release.

A variety of physiological and pharmacological manipulations of intact neurons or nerve terminals have been shown to be effective in regulating the phosphorylation state of synapsin I (for review, see references 11 and 33). Moreover, in vivo injection of dephosphorylated synapsin I into the preterminal digit of the squid giant synapse reduced neurotransmitter release, whereas synapsin I phosphorylated on the tail sites was completely ineffective (25). In extruded axoplasm from the squid giant axon, synapsin I inhibited organelle movement in a phosphorylation-dependent manner (30). These data are consistent with an inhibitory action of synapsin I on neurotransmitter release either by anchoring the

Dr. Benfenati's present address is Department of Human Physiology, The University of Modena, Via Campi 287, 41100 Modena, Italy. Dr. Jahn's present address is Department of Neurochemistry, Max-Planck Institute for Psychiatry, Am Klopferspitz 18a, D-8033 Martinsried, Federal Republic of Germany.

vesicles to the cytoskeleton or by preventing vesicle fusion with the presynaptic membrane. Thus, it seemed to be of importance to identify and further characterize the nature of the interaction between synapsin I and small synaptic vesicles.

Binding of synapsin I to pure phospholipid vesicles with a composition similar to synaptic vesicles is reported by Benfenati et al. (6). In this paper, we have investigated the binding of synapsin I to intact and reconstituted synaptic vesicles, in an attempt to establish if the phospholipid interaction occurs in native synaptic vesicles and if a vesicle protein(s) is involved in synapsin I binding. The results presented here suggest the existence of two sites of interaction between synapsin I and synaptic vesicles. The head of the protein seems to be involved in phospholipid binding and bilayer penetration, whereas the tail domain appears to bind to a vesicle protein component, an interaction that is strongly modulated by phosphorylation and ionic strength conditions.

Materials and Methods

Materials

L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK)-trypsin and α-chymotrypsin were from Cooper Biomedical Inc. (Malvern, PA). 1-palmitoyl-2-[11-[4-[3-(trifluoromethyl)diazirinyl]phenyl] [2-^{3}H]undecanoyl]-*sn*-glycero-3-phosphocholine ([^{3}H] PTPC/11,[1] 15 Ci/mmol), prepared following the procedure of Harter et al. (15), was kindly supplied by Drs. C. Harter and J. Brunner (Eidgenössische Technische Hochschule, Zürich, Switzerland). 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl) diazirine ([^{125}I]TID, 10 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Calmodulin and calcium/calmodulin-dependent protein kinase II purified as described (29, 40) were gifts of Drs. Fred Gorelick and Gerald Thiel of our laboratory. Synapsin I was purified under nondenaturing conditions from bovine brain as described (2, 34). Antibodies specific for synapsin I peptides containing phosphorylation site 1 (head) and phosphorylation site 3 (tail) (10) were raised in our laboratories by Dr. A. Czernik. Polyclonal antibodies against synaptophysin (p38) were raised in our laboratory by Dr. R. Jahn. All other materials were obtained as described (6).

Generation of Synapsin I Fragments

Cysteine-specific Chemical Cleavage of Synapsin I. Purified synapsin I was cleaved with 2-nitro-5-thiocyanobenzoic acid (NTCB) as described (3, 24, 27). The fragments obtained by the cysteine-specific cleavage were identified and aligned based on the structural model of synapsin I reported in Bähler et al. and Benfenati et al. (3, 6). The NH_2-terminal fragment (29 kD) and the tail fragment (35–40 kD doublet) were purified from the total digest as described (3). The purified tail fragment was phosphorylated in vitro by calcium/calmodulin-dependent protein kinase II as described for synapsin I (34) to near stoichiometry and repurified by batch adsorption to carboxymethylcellulose. Two-dimensional phosphopeptide maps of the tail fragment were obtained as described (19). No significant changes in the phosphopeptide pattern were observed, compared to holosynapsin I phosphorylated in the tail sites under the same conditions.

Limited Chymotryptic Digestion of Synapsin I. Purified dephosphorylated synapsin I (1 mg), containing a trace amount of [^{32}P]synapsin I phosphorylated at sites 2,3 by purified calcium/calmodulin-dependent protein kinase II, was incubated for 30 min at 37°C with α-chymotrypsin at an enzyme/synapsin I ratio of 1:200 (wt/wt) in a buffer containing 200 mM NaCl, 50 mM $NaHCO_3$, 25 mM Tris/HCl (pH 8.0), 1 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol, and 3 mM NaN_3. The reaction was stopped by adding PMSF to a final concentration of 0.5 mM and by chilling the sample to 0°C. The digest was then dialyzed against a buffer containing 175 mM NaCl, 25 mM Tris/HCl (pH 7.5), 0.1 mM EDTA and 3 mM NaN_3. Using this procedure, a major chymotryptic fragment of about 22 kD was generated. The NH_2 terminus of this fragment is located 42 aminoacids NH_2-terminal to phosphorylation site 2 (10).

Purification of Small Synaptic Vesicles and Removal of Endogenous Synapsin I

Small synaptic vesicles were purified from rat neocortex (male Sprague-Dawley; body weight, 150–200 g) through the step of chromatography on controlled pore glass as described by Huttner et al. (20). Vesicles were concentrated by ultracentrifugation for 2 h in a rotor (model Ti-60; Beckman Instruments Inc., Palo Alto, CA) at 50,000 rpm, and the pellet was resuspended by repeatedly forcing it through a 27-gauge syringe needle in 0.3 M glycine/5 mM Hepes (pH 7.4)/3 mM NaN_3 (glycine buffer) at a protein concentration of 2–3 mg/ml. The vesicle suspension ("unstripped vesicles") was kept on ice and used within a few hours of preparation to minimize endogenous synapsin I dissociation. To remove endogenous synapsin I, 0.4 M NaCl was added to an equal volume of controlled pore glass-purified vesicles in glycine buffer (pool of vesicles from the controlled pore glass-column, 10–20 μg protein/ml). The suspension was incubated on ice for 2 h and then centrifuged for 2 h in a rotor (model Ti-60; Beckman Instruments, Inc.) at 50,000 rpm. The resulting pellet was resuspended, as described above, in glycine buffer without NaCl and adjusted to a final protein concentration of 1 mg/ml (stripped vesicles).

Reconstitution of Synaptic Vesicles in Phospholipid Bilayers

Aliquots of purified stripped vesicles (usually 1–2 mg of total protein) were resuspended in "reconstitution buffer" containing 10 mM Tris/HCl (pH 7.4), 100 mM NaCl, and 3 mM NaN_3. The resuspended vesicles were solubilized by addition of octyl glucoside (3% wt/vol final concentration), or Na cholate (1.5% wt/vol, final concentration), and gentle stirring at 4°C for 20–30 min under N_2 (vesicle reconstitution by the two procedures gave similar results). The mixture was then centrifuged at 200,000 *g* for 20 min in an ultracentrifuge (model TL-100; Beckman Instruments, Inc.), the soluble fraction collected and the pellet discarded or reextracted. Phosphatidylcholine, containing a trace amount of [^{14}C]-phosphatidylcholine, and cholesterol (both dissolved in chloroform at a concentration of 10 mg/ml) were mixed in a 9:1 ratio, dried to a thin film in the cold under a gentle stream of N_2, and put under vacuum for at least 1 h to remove residual traces of organic solvent. Sucrose was dissolved into the vesicle extract to a final concentration of 300 mM and the mixture was then added to the dried lipid film, gently mixed, and stirred on ice for 15 min under N_2 to allow a complete solubilization of the exogenous lipids. The detergent/lipid molar ratio in the final solution was always kept over 10:1. Unilamellar reconstituted vesicles were obtained after removal of the detergent by gel filtration as described (6). The vesicles formed following this procedure had typical protein/lipid ratios of around 1:10 (wt/wt).

Separation of Protein and Lipid Components of Synaptic Vesicles

The procedure used for the separation of vesicle protein and lipid components under nondenaturing conditions was that described by Helenius and Simons (16) with slight modifications. Synaptic vesicles (1 mg of protein) were extracted in the presence of an excess of Na cholate (50:1 weight ratio with respect to vesicle proteins) in reconstitution buffer. A trace amount of [^{14}C]phosphatidylcholine was added to the vesicle extract. Protein micelles were separated from lipid micelles on a 1 × 50 cm Sephadex G-100 gel filtration column equilibrated with reconstitution buffer containing 20 mM of Na cholate. The delipidated protein pool was then concentrated by ultrafiltration and reconstituted in phosphatidylcholine/cholesterol (90:10), as described above. The lipid pool was collected and vesiculized by extensive dialysis without addition of exogenous phospholipids as described by Benfenati et al. (6).

Assay of Binding of Synapsin I or Synapsin I Fragments to Intact and Reconstituted Synaptic Vesicles

The binding of synapsin I and of synapsin I fragments to intact and recon-

1. *Abbreviations used in this paper*: NTCB, 2-nitro-5-thiocyanobenzoic acid; [^{3}H]PTPC/11, 1-palmitoyl-2-[11-[4-[3-(trifluoromethyl)diazirinyl]-phenyl] [2-^{3}H]undecanoyl]-*sn*-glycero-3-phosphocholine; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine.

stituted synaptic vesicles (5 μg total protein/sample) was carried out under standard assay conditions (40 mM NaCl equivalent) or at higher ionic strength (150 mM NaCl equivalent), as described by Benfenati et al. (6). The vesicle recovery in the pellet was determined by liquid scintillation spectrometry when reconstituted vesicles labeled with [^{14}C]phosphatidylcholine were used, and by dot immunobinding using antibodies against synaptophysin as described (21, 22) when intact synaptic vesicles were employed. The average vesicle recovery was always 50–70%.

Hydrophobic Labeling of Synaptic Vesicles

[^{125}I]TID Labeling. [^{125}I]TID was injected as an ethanol solution into the vesicle dispersion in glycine buffer, and the mixture was allowed to incubate for 5 min at room temperature before photolysis (~1 μCi/sample containing 100 μg protein; final ethanol concentration <0.5%).

[^{3}H]PTPC/11 Labeling. Liposomes were formed from pure [^{3}H]PTPC/11, dried under N_2, resuspended in glycine buffer, and vesiculized in a sonicating water bath for 15 min. The liposomes were spun at 300,000 *g* for 15 min to get rid of possible aggregates and multilamellar liposomes. The [^{3}H]PTPC/11 liposomes (~0.05–0.1 mCi/sample) were then added to synaptic vesicles (100 μg protein/sample). The incorporation of the label in the vesicle membrane was achieved by incubation at 37°C for 30 min with occasional mixing. The amount of reagent incorporated under these conditions was ~20%.

Photoactivation of these reagents was carried out as described (6). After photolysis, the samples were centrifuged once at 300,000 *g* for 15 min, and the pellets were resuspended in 200 μl of glycine buffer. The resuspended pellets were delipidated and analyzed by SDS-PAGE and autoradiography (6).

In some experiments, the resuspended pellets were solubilized by addition of 1% (wt/vol, final concentration) SDS and boiled for 1 min, followed by immunoprecipitation of synapsin I, as described by Goelz et al. (14) using *Staphylococcus aureus* cells. Synapsin I was desorbed by resuspension in stop solution (23) and boiling for 5 min or by incubation in 6 M guanidine chloride. Synapsin I desorbed in 6 M guanidine chloride was then reacted with NTCB for cysteine-specific cleavage, as described by Benfenati et al. (6).

Limited Proteolysis of Synaptic Vesicles

Reconstituted sucrose-loaded synaptic vesicles were used because of the higher recovery during the washings and the faster determination of vesicle recovery based on the amounts of phospholipids ([^{14}C]phosphatidylcholine). Vesicles (100 μg protein) were incubated in 200 mM glycine/300 mM sucrose/50 mM Hepes (pH 7.4) with increasing concentrations of TPCK-trypsin (trypsin/protein weight ratio = 1:5000, 1:1000, and 1:200) at room temperature for 60 min. An excess of soybean trypsin inhibitor (10 μg/ml) and 0.2 mM PMSF were added to stop the reaction and the samples were further incubated for 30 min at 4°C. The vesicles were then centrifuged at 300,000 *g* for 20 min and washed several times in 500 mM glycine/5 mM Hepes (pH 7.4) containing 10 μg/ml soybean trypsin inhibitor and 0.1 mM PMSF, incubated overnight in the presence of soybean trypsin inhibitor and resuspended in standard assay buffer for measurement of synapsin I and tail fragment binding. Additional samples containing vesicles and either synapsin I or tail fragment were incubated but not centrifuged to assess the survival of the total ligands in the incubation mixture in the presence of possible residual protease activity.

Miscellaneous Techniques

Purified synapsin I was iodinated using iodo beads as described (6). SDS-PAGE was performed according to Laemmli (23). The following proteins were used as molecular mass standards: phosphorylase b (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD) and α-lactalbumin (14 kD). Proteins in the gels were electrophoretically transferred to nitrocellulose, as described (37). The immunolabeling of nitrocellulose sheets was performed using anti-synapsin I or anti-synaptophysin polyclonal antibodies followed by [^{125}I]protein A overlay, autoradiography, and gamma counting (4). Saturation and inhibition curves were analyzed using the computer program RECEPT (5). Protein was determined according to Lowry et al. (26) or Bradford (7), using BSA as a standard. Phospholipid was determined by assaying phospholipid phosphorus following the method of Ames (1).

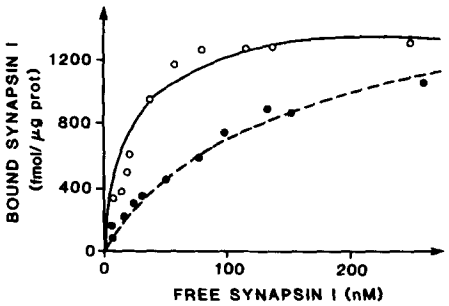

Figure 1. Binding of synapsin I to reconstituted synaptic vesicles. Synaptic vesicles were solubilized in 3% octyl glucoside and reconstituted with the addition of exogenous phosphatidylcholine and cholesterol as described in Materials and Methods. Reconstituted synaptic vesicles were incubated in standard assay buffer ([○] 40 mM NaCl equivalent) or under higher ionic strength conditions ([●] 150 mM NaCl equivalent) with various concentrations of synapsin I. Bound synapsin I was separated from the free protein by ultracentrifugation through a 5% (wt/vol) sucrose cushion and quantitated by dot immunobinding. Bound synapsin I was corrected for vesicle recovery. Free synapsin I was calculated by subtracting the corrected bound values from the total amount of synapsin I added to each sample. Data were fitted by using a nonlinear regression analysis following the simple one site model (computer program recept; 5). K_d, 17 nM, under standard conditions; K_d, 70 nM, at higher ionic strength; B_{max} in both conditions was ~1300 fmol synapsin I/μg protein.

Results

Synapsin I Binding after Separation and Reconstitution of Protein and Lipid Components from Synaptic Vesicles

To test if binding of synapsin I still occurred after reconstitution of synaptic vesicle proteins in phospholipid bilayers, highly purified synaptic vesicles depleted of endogenous synapsin I were solubilized by detergent and reconstituted after the addition of exogenous phosphatidylcholine and cholesterol to improve vesicle formation and protein incorporation. The protein pattern of reconstituted synaptic vesicles, as evaluated by SDS-PAGE and Coomassie staining, was very similar to the one of native synaptic vesicles. Binding of synapsin I to reconstituted synaptic vesicles showed a K_d of about 15 nM and a B_{max} of 1,300 fmol synapsin I/μg protein (Fig. 1). Increasing the ionic strength of the medium caused an approximate fivefold decrease in affinity without greatly affecting the saturation level, as has been observed in native synaptic vesicles (34).

To investigate the molecular components of synaptic vesicles involved in the interaction with synapsin I, we artificially separated vesicle proteins from vesicle lipids by means of solubilization in an excess of Na cholate, a detergent that generates very small micelles in solution (17). The difference in molecular size between protein and lipid micelles allowed their separation by gel filtration. The elution of the two components from the column was monitored by absorbance at 280 nm and [^{14}C]phosphatidylcholine radioactivity (Fig. 2). The protein peak eluted in the void volume and contained only 2% of the total phospholipid phosphorus present in the loaded sample. Lipids were virtually completely separated from the protein peak and eluted in the inclusion volume.

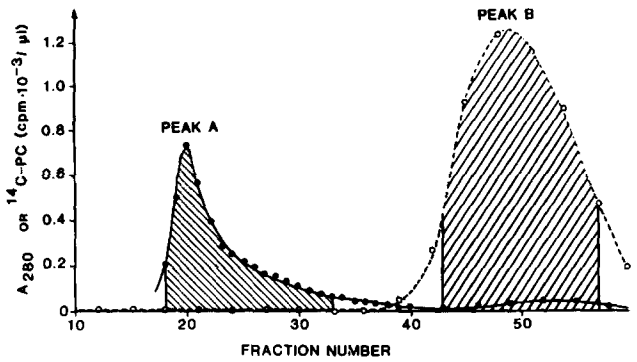

Figure 2. Separation of vesicle proteins and lipids by gel filtration after solubilization in sodium cholate. Purified synaptic vesicles (1 mg total protein) were depleted of synapsin I and solubilized in reconstitution buffer containing a trace amount of [^{14}C]phosphatidylcholine in the presence of a 50-fold excess of Na cholate with respect to protein (wt/vol). The extract was loaded onto a 1 × 50 cm Sephadex G-100 gel filtration column, previously equilibrated in reconstitution buffer containing 20 mM Na cholate. The elution profiles for absorbance at 280 nm (●) and [^{14}C] radioactivity (○) are shown. The pooled fractions used for reconstitution and synapsin I binding assay are shown as hatched areas. Phospholipid phosphorus determination performed on the loaded sample and on the protein pool (*PEAK A*) and lipid pool (*PEAK B*) showed that vesicle proteins were delipidated to ~98%.

The protein fraction was reconstituted in artificial vesicles by addition of exogenous cholesterol and phosphatidylcholine, which have been demonstrated not to interact with synapsin I (6), whereas the endogenous phospholipid fraction was vesiculized without the addition of exogenous lipids. The binding of synapsin I was assayed in these two fractions and in the parent intact synaptic vesicles. The binding data obtained in the different preparations were compared by using the protein/phospholipid ratio of 1:3 found in our synaptic vesicle preparation. Vesicle proteins and phospholipids both bound synapsin I with high affinity and to a significant extent (Fig. 3). The sum of the mean estimated B_{max} values of the single components (1,195 fmol/μg protein equivalent) was similar to the value found for total reconstituted vesicles (1,300 fmol/μg protein; Fig. 1) and slightly higher than the maximal amount of synapsin I bound by intact vesicles (912 fmol/μg protein). Presumably, the increased binding found in the reconstituted systems is attributable to the unmasking of potential binding sites that are not accessible to synapsin I on intact vesicles.

Hydrophobic Labeling of Synapsin I and Other Synaptic Vesicle Proteins Using [^{3}H]PTPC/11 or [^{125}I]TID

We tested the possible occurrence of synapsin I–phospholipid interactions in intact synaptic vesicles by means of hydrophobic labeling procedures. Either [^{125}I]TID or [^{3}H]-PTPC/11 was used to label synaptic vesicles containing endogenous synapsin I (unstripped vesicles) and synaptic vesicles that had been depleted of endogenous synapsin I (stripped vesicles) and then were incubated with purified synapsin I (rebound vesicles). Fig. 4 shows the results obtained from a typical experiment. The two hydrophobic probes gave similar labeling patterns. The autoradiograms show that several protein bands were intensely labeled by both reagents. It is likely that these bands represent primarily integral membrane proteins, since they were labeled to approximately the same extent in synaptic vesicles exposed to low salt (unstripped vesicles) and to high salt (stripped vesicles).

The major bands labeled by both probes included proteins of apparent molecular masses of 92, 65, 54, 38 kD, and at least five low molecular mass proteins migrating in the 35–19 kD region. Some of these labeled proteins represent major bands as seen on a Coomassie stain of the vesicle sample (92, 65, and 38 kD). Two of them have previously been characterized as synaptic vesicle-specific integral membrane proteins: the 65 kD protein corresponds to p65 described by Matthew et al. (28); the 38 kD protein is p38 (synaptophysin) described by Jahn et al. (22) and Wiedenmann and Franke (41), and could be quantitatively immunoprecipitated using specific antibodies (data not shown). From the primary sequence of synaptophysin (36), four transmembrane regions have been predicted, consistent with the intense labeling of this protein by the hydrophobic probes. Some other bands, such as the 54 kD protein and proteins migrating in the low molecular mass region, though markedly labeled, do not represent major components of the vesicles as seen by protein staining, suggesting that they are probably glycoproteins.

Synapsin I was labeled by this procedure to a significant extent in both unstripped and rebound vesicles as confirmed by immunoprecipitation using anti-synapsin I–specific antibodies, even though it is a nonintegral membrane protein

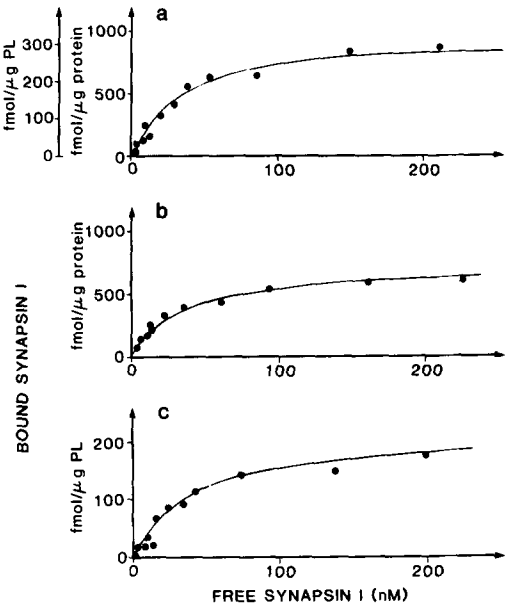

Figure 3. Synapsin I binding to intact synaptic vesicles (*a*), reconstituted vesicle proteins (*b*) and endogenous phospholipid liposomes (*c*). Endogenous vesicle lipids and proteins were separated as described in the legend to Fig. 2 and in Materials and Methods. The protein pool was reconstituted by the addition of exogenous phosphatidylcholine and cholesterol whereas the phospholipid pool was vesiculized by detergent removal. The binding of synapsin I was performed as described in the legend to Fig. 1. Synapsin I bound to vesicles is expressed as either fmol/μg protein or fmol/μg phospholipid. The B_{max} values were 912 fmol/μg protein or 304 fmol/μg phospholipid (*a*); 640 fmol/μg protein (*b*); and 185 fmol/μg phospholipid (*c*). The protein/phospholipid ratio determined in intact synaptic vesicles was 1:3 (wt/wt).

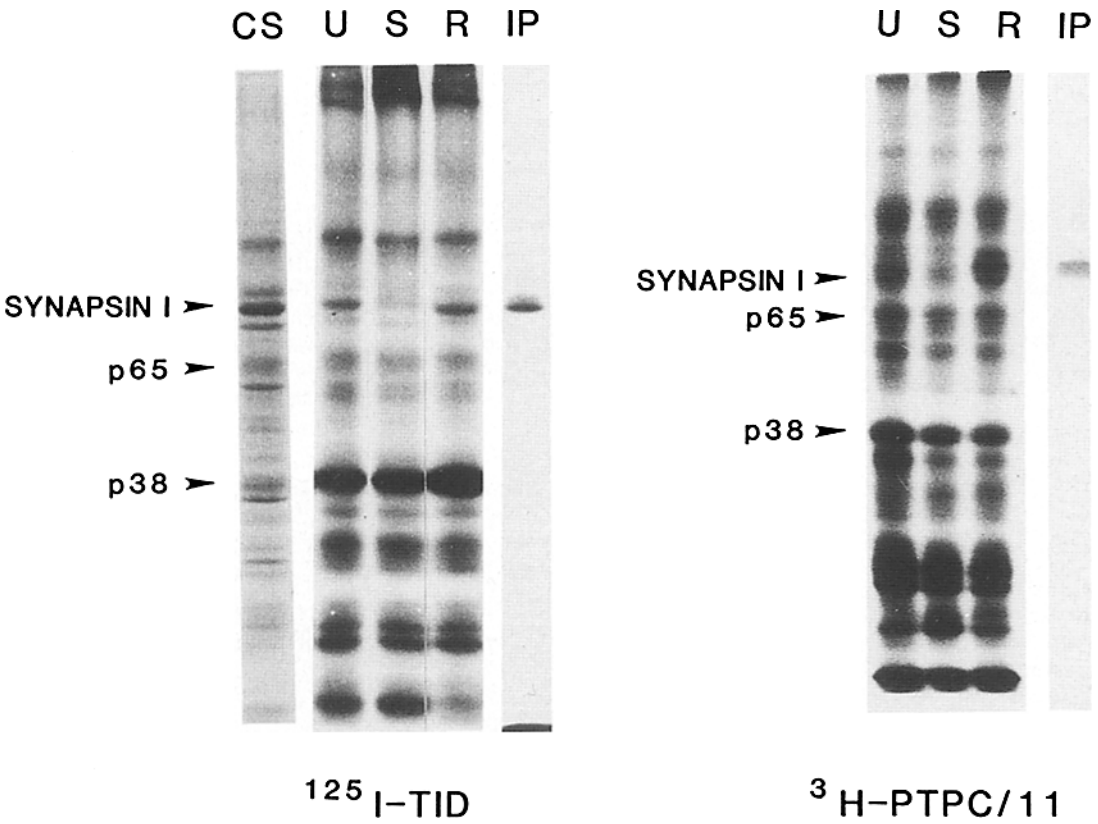

Figure 4. Hydrophobic labeling of intact synaptic vesicles with [^{125}I]TID or [^{3}H]PTPC/11. [^{125}I]TID or [^{3}H]PTPC/11 was incorporated into intact synaptic vesicles (unstripped vesicles, *U*), synapsin I–depleted synaptic vesicles (stripped vesicles, *S*) or synapsin I–depleted synaptic vesicles rebound in vitro with purified synapsin I (rebound vesicles, *R*) as described under Materials and Methods. The samples were transferred to pyrex tubes, irradiated with UV light, delipidated, and subjected to SDS-PAGE. The amount of radioactive reagent covalently bound to vesicle proteins was determined by autoradiography. On the left the Coomassie stain (*CS*) for the unstripped vesicle sample is shown for comparison. After photolabeling, an aliquot of unstripped vesicles was solubilized, and endogenous synapsin I was immunoprecipitated and analyzed by SDS-PAGE and autoradiography (*IP*).

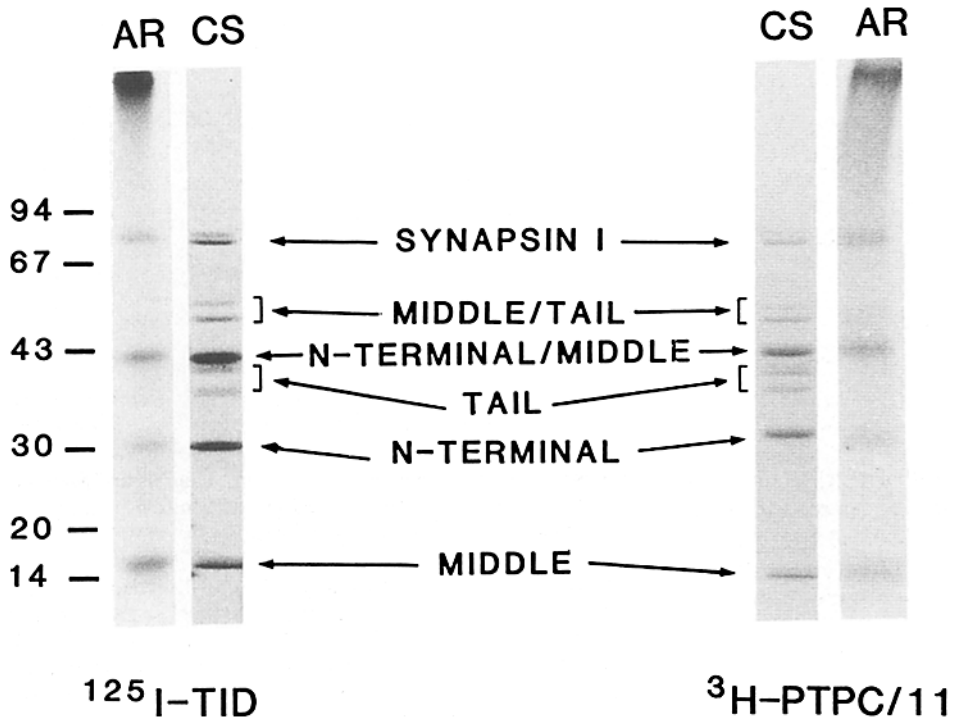

Figure 5. Specific domains of endogenous synapsin I hydrophobically labeled by [^{125}I]TID or [^{3}H]PTPC/11. [^{125}I]TID or [^{3}H]-PTPC/11 was incorporated into unstripped synaptic vesicles. After photolysis, samples were solubilized and synapsin I was immunoprecipitated and subjected to cysteine-specific cleavage with NTCB as described in Materials and Methods. The samples were analyzed by SDS-PAGE using a linear 7.5–15% polyacrylamide gradient and autoradiography. *AR*, autoradiography, and *CS*, Coomassie stain of synapsin I fragments. Molecular mass standards are shown on the left in kD. The labels indicate the regions of synapsin I from which the fragments were derived. The fragments derived from the head domain (*N-terminal*, *middle*, and *N-terminal/middle*) were labeled by the hydrophobic probes.

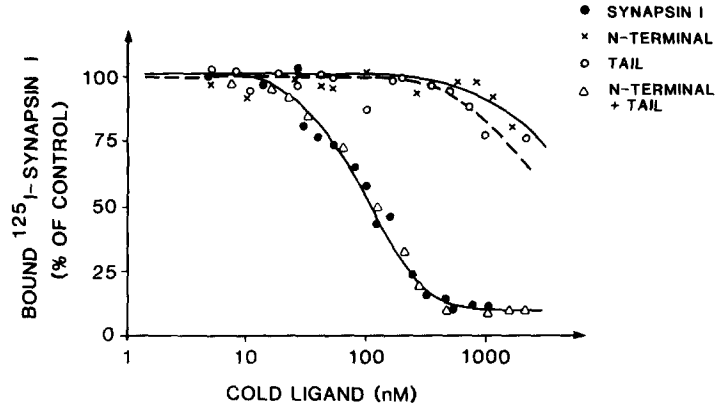

Figure 6. Displacement of [^{125}I]synapsin I bound to synaptic vesicles by synapsin I, NH_2-terminal fragment and/or tail fragment. Synapsin I-depleted synaptic vesicles were incubated with 35 nM [^{125}I]synapsin I in standard assay buffer in the absence or presence of various concentrations of either synapsin I (•), purified NH_2-terminal fragment (X), purified tail fragment (○), or NH_2-terminal plus tail fragments in equimolar amounts (△). The residual binding of [^{125}I]synapsin I is expressed in percent of the binding in the absence of competing ligand. IC_{50} = 100 nM; K_i = 22 nM for inhibition by synapsin I. IC_{50} = 102 nM; K_i = 23 nM for inhibition by the simultaneous presence of the two fragments.

(Fig. 4, *IP*). These results suggest that (*a*) synapsin I interacts with phospholipids in intact synaptic vesicles; (*b*) as previously demonstrated with liposomes (6), this association with phospholipids is accompanied by direct penetration of selected regions of the molecule into the hydrophobic core of the membrane; and (*c*) there are no detectable differences between the endogenous binding and the binding of exogenous synapsin I to synaptic vesicles. The specificity of the labeling was indicated by the lack of detectable labeling when UV irradiation was omitted (data not shown) and by the fact that PTPC/11, after incubation with vesicles and removal of the nonincorporated compound, becomes a structural component of the vesicle membrane with the photoactivatable group confined to the depth of the lipid bilayer. The similarity of the labeling patterns of [^{125}I]TID and [^{3}H]-PTPC/11 indicates that, at least under our conditions, [^{125}]TID is also a selective probe for the hydrophobic core of the membrane.

When synapsin I phosphorylated at both head and tail sites was incubated at saturating concentrations with synapsin I-depleted synaptic vesicles, the extent both of binding and of [^{3}H]PTPC/11 labeling were similar to that observed with saturating concentrations of bound dephosphorylated synapsin I (data not shown). Using [^{125}I]TID and lower synapsin I concentrations, the amount of phosphorylated synapsin I bound was lower than that of dephosphorylated synapsin I, and the label incorporation was similarly decreased. These results indicate that the labeling efficiency mainly depends on the amount of synapsin I bound to the bilayer and is not affected by its phosphorylation state (data not shown).

To define the regions of synapsin I mediating the hydrophobic interaction with the vesicle membrane, unstripped (Fig. 5) and rebound (not shown) synaptic vesicles were incubated with either [^{125}I]TID or [^{3}H]PTPC/11, photolyzed and solubilized; synapsin I was immunoprecipitated and subjected to cysteine-specific cleavage with NTCB, as described in Materials and Methods. The labeling pattern of synapsin I fragments was very similar with the two hydrophobic probes and with endogenous versus rebound synapsin I. The fragments selectively labeled were the NH_2-terminal fragment (29 kD), the middle fragment (15 kD), and the NH_2-terminal/middle fragment (40 kD), namely fragments belonging to the collagenase-resistant head domain of synapsin I. Sometimes a weak labeling of the middle/tail fragment was found with long exposure times, but the tail fragment was never labeled under these conditions. The greater amount of radioactivity associated with the middle fragment compared to the middle/tail fragment was attributable to the larger relative amount of the former produced by the digestion procedure. The synapsin I fragments labeled by the hydrophobic reagent on native synaptic vesicles correspond to the fragments demonstrated to bind and penetrate artificial phospholipid bilayers (6).

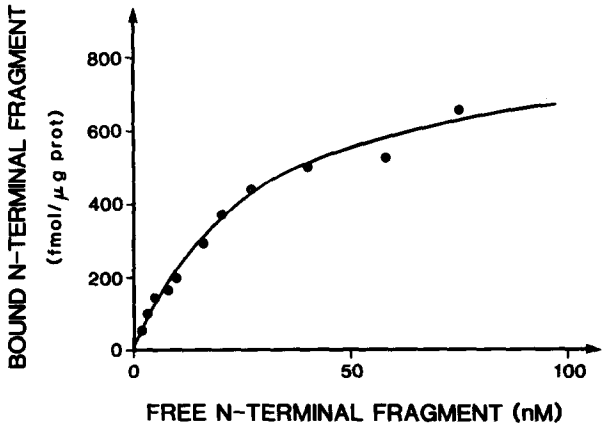

Figure 7. Binding of purified NH_2-terminal fragment of synapsin I to synaptic vesicles. Synaptic vesicles depleted of endogenous synapsin I were incubated in standard assay buffer with various concentrations of purified NH_2-terminal fragment (29 kD) for 60 min on ice. The binding was performed as described in Materials and Methods and in the legend to Fig. 1. The amount of NH_2-terminal fragment bound was determined by dot immunobinding employing an antibody specific for phosphorylation site 1. The K_d value was ~25 nM, and the B_{max} value was ~800 fmol/μg, protein (*prot*).

Specific Peptide Domains Involved in Synapsin I Binding to Synaptic Vesicles

The experiments described in the previous section supported the idea that the head domain of synapsin I is involved in the binding of synapsin I to synaptic vesicles. However, previous experiments involving collagenase treatment of synaptosomal lysates (38) and purified synaptic vesicles (20) suggested an involvement of the collagenase-sensitive tail domain in the binding of synapsin I to synaptic vesicles. Moreover, phosphorylation of synapsin I in the tail domain (but not in the head domain) altered the affinity of binding to synaptic vesi-

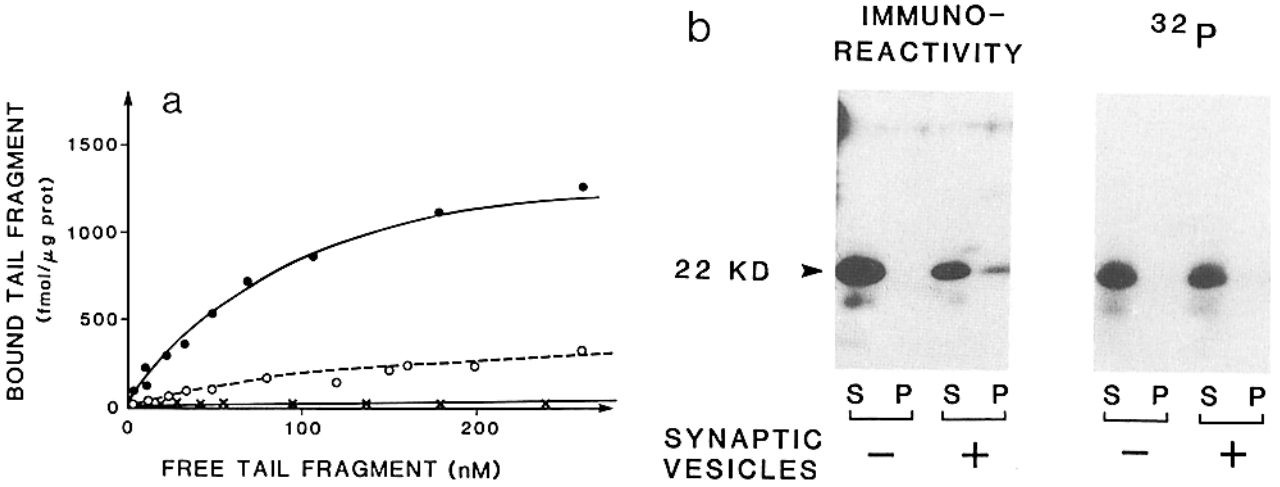

Figure 8. Binding of the tail fragment of synapsin I to synaptic vesicles. (*a*) Synapsin I-depleted synaptic vesicles were incubated with various concentrations of the dephosphorylated form of purified tail fragment (35–40 kD) in standard assay buffer (●, 40 mM NaCl equivalent) or in higher ionic strength buffer (X, 150 mM NaCl equivalent). Purified tail fragment that had been phosphorylated to near stoichiometry by calcium/calmodulin-dependent protein kinase II was also tested for binding to synaptic vesicles in standard assay buffer (○). The binding of the phosphorylated fragment at 150 mM NaCl equivalent was identical to that observed with the dephosphorylated fragment, and it is not shown in the figure. (*b*) A shorter fragment (22 kD) derived from the tail domain of synapsin I, and containing phosphorylation sites 2 and 3, was obtained in nondenaturing conditions by chymotryptic cleavage of dephosphorylated synapsin I in the presence of a trace amount of [^{32}P]synapsin I that had been phosphorylated at sites 2 and 3 by calcium/calmodulin-dependent protein kinase II. This 22-kD fragment (25 μg protein) was incubated in standard assay conditions in the absence or presence of synapsin I-depleted synaptic vesicles (250 μg protein). After separation of free and bound fragment by centrifugation at 200,000 *g* for 20 min, the pellet (*P*) and supernatant (*S*) fractions were analyzed by SDS-PAGE. Gels were either dried and autoradiographed to detect the [^{32}P]phosphorylated fragment or blotted onto nitrocellulose and analyzed for fragment immunoreactivity using antibodies specific for phosphorylation site 3 and [^{125}I]protein A overlay.

cles (34), but not to phospholipids (6). To test the possibility of a multi-site interaction between synapsin I and synaptic vesicles, the ability of purified NH_2-terminal fragment and purified tail fragment to inhibit synapsin I binding was analyzed. [^{125}I]synapsin I bound to intact synaptic vesicles was efficiently displaced by the holomolecule but not by either the NH_2-terminal fragment (29 kD) or the tail fragment (35–40 kD doublet). The simultaneous presence of equimolar concentrations of NH_2-terminal and tail fragments inhibited [^{125}I]synapsin I binding with a dose-response curve almost identical to that observed with cold holosynapsin I (Fig. 6). Similar inhibition curves, in terms of IC_{50} and residual [^{125}I]synapsin I binding, were obtained using equimolar mixtures of NH_2-terminal/middle and tail fragments or NH_2-terminal/middle and middle/tail fragments (data not shown). Using an antibody specific for rat synapsin I (M. Bähler and F. Benfenati, unpublished observations), we also demonstrated that the binding of endogenous synapsin I to rat synaptic vesicles was inhibited to the same extent by bovine holosynapsin I or by the combination of NH_2-terminal and tail fragments obtained from bovine synapsin I (data not shown). These various results suggested that both domains of synapsin I are involved in binding of synapsin I to synaptic vesicles and that a single fragment is not able to detach synapsin I from its binding sites.

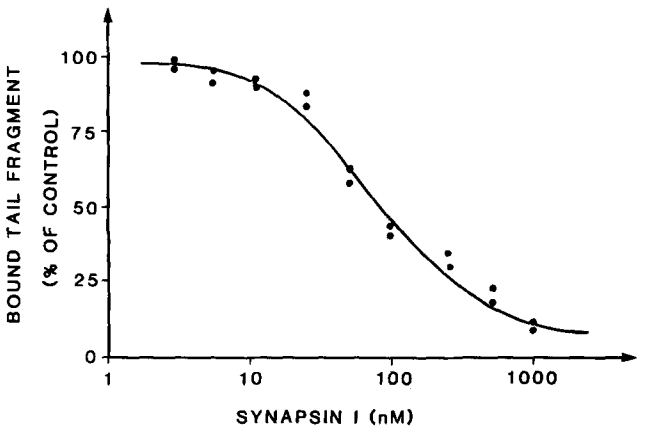

Figure 9. Inhibition by holosynapsin I of tail fragment binding to synaptic vesicles. Synapsin I-depleted synaptic vesicles were incubated with 40-nM purified unlabeled tail fragment in the absence or presence of various concentrations of synapsin I. The samples were centrifuged through a 5% (wt/vol) sucrose cushion, and the pellets were resuspended in stop solution. Tail fragment bound to vesicles was separated from synapsin I by SDS-PAGE, transferred to nitrocellulose sheets, and assayed by immunoblotting with anti-synapsin I polyclonal antibodies, [^{125}I]protein A overlay, and counting. The residual binding of the tail fragment is expressed in percent of the binding in the absence of synapsin I. IC_{50} = 65 nM, K_i = 43 nM.

To further characterize the specific peptide domains involved in synapsin I binding, the binding of the purified NH_2-terminal and tail fragments to synaptic vesicles was analyzed. The NH_2-terminal fragment bound to synaptic vesicles with high affinity (K_d = 25–30 nM) and apparent saturability at ~800 fmol/μg protein (Fig. 7).

When purified tail fragment (35–40 kD doublet) was incubated with synapsin I-depleted synaptic vesicles in standard assay buffer, high-affinity and saturable binding was also observed (Fig. 8 *a*). The K_d value was somewhat higher than that observed for holosynapsin I (~55 nM). The tail

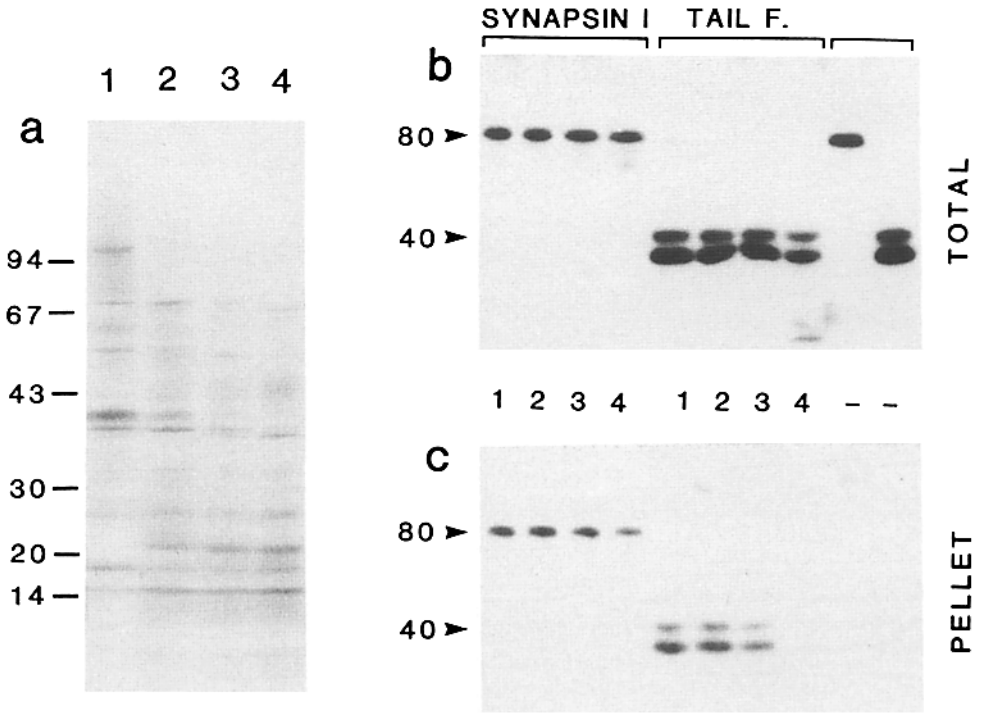

Figure 10. Effect of limited trypsinization of reconstituted synaptic vesicles on synapsin I binding and synapsin I tail binding. Reconstituted synaptic vesicles were subjected to treatment with various concentrations of TPCK-trypsin (lane *1*, control; lane *2*, 1:5000; lane *3*, 1:1000; lane *4*, 1:200 trypsin/vesicle protein ratio, wt/wt) for 60 min at room temperature. The reaction was stopped, and the vesicles were washed several times to remove any residual protease activity as described in Materials and Methods. An aliquot of each preparation of vesicles was subjected to SDS-PAGE and Coomassie staining (*a*). Molecular mass standards are shown on the left in kD. Other aliquots of the vesicles (10 μg of protein/sample) were incubated at 4°C for 60 min in 100 μl of standard assay buffer with synapsin I (20 nM) or purified tail fragment (50 nM). After the incubation, samples were either transferred into stop solution, boiled and subjected to SDS-PAGE, or centrifuged at 300,000 *g* for 20 min to separate bound and free ligand. Pellets were resuspended in stop solution, boiled, and subjected to SDS-PAGE. Proteins on the gels were electrophoretically transferred to nitrocellulose sheets, and synapsin I and tail fragment were detected by immunolabeling with antisynapsin I polyclonal antibodies and [^{125}I]protein A overlay followed by autoradiography. The autoradiograms are shown for total ligand present at the end of the incubation (*TOTAL*, *b*) and ligand bound to vesicles (*PELLET*, *c*). Samples containing the corresponding amount of synapsin I or tail in standard assay buffer, but without the addition of vesicles, are shown in the two lanes on the right of *b* and *c*.

fragment, although very basic, did not specifically interact with acidic phospholipids (6) and was not significantly labeled with [^{125}I]TID or [^{3}H]PTPC/11 when holosynapsin I was bound to intact synaptic vesicles. The binding capacity of native synaptic vesicles for the tail fragment (~1200 fmol/μg of protein) was somewhat higher than the B_{max} value for synapsin I. Stoichiometric phosphorylation of the tail fragment, performed in vitro with purified calcium/calmodulin-dependent protein kinase II, greatly decreased the binding to intact synaptic vesicles in terms of both affinity and maximal number of binding sites when the assay was performed under standard conditions (40 mM NaCl equivalent) (Fig. 8 *a*). The binding was almost abolished by increasing the ionic strength to 150 mM NaCl equivalent, for both phosphorylated and dephosphorylated forms, suggesting that the binding is mainly because of electrostatic interactions between the strongly basic tail fragment and some vesicle component negatively charged at physiological pH.

Results similar to those observed using the NTCB-cleaved tail fragment (35–40 kD) were also obtained with a shorter fragment (22 kD) derived from the tail domain of synapsin I under nondenaturing conditions. The total immunoreactivity, reflecting the dominant dephosphorylated form of the fragment, sedimented to some extent with synaptic vesicles, whereas [^{32}P] radioactivity, representing the trace amount of the phosphorylated form of the fragment, was restricted to the supernatant fraction (Fig. 8 *b*). These results further demonstrate that fragments derived from the tail domain of synapsin I are able to interact with synaptic vesicles and that this interaction is reduced by phosphorylation.

Synapsin I was able to inhibit the binding of purified tail fragment almost completely, indicating the specific nature of the tail binding (Fig. 9). The IC_{50} was about 84 nM corresponding to a K_i of 43 nM, close to the K_d of tail binding to synaptic vesicles (55 nM).

To determine the chemical nature of the site on synaptic vesicles involved in tail binding, reconstituted synaptic vesicles were subjected to partial proteolysis by incubation with various concentrations of trypsin (Fig. 10). The binding of the tail fragment could be reduced or abolished by exposure of vesicles to trypsin. In contrast, the binding of holosynapsin I could be weakened but not abolished, by the same treatment, indicating the existence of a residual nonprotein component for the association of holosynapsin I with synaptic vesicles, probably represented by endogenous acidic phospholipids.

Discussion

The results presented in this paper indicate that the specific association of synapsin I with synaptic vesicles is complex in nature. The following data are consistent with the possibility that the hydrophobic head domain of synapsin I interacts with the hydrophobic core of the phospholipid bilayer of syn-

aptic vesicles and that the hydrophilic tail domain binds to an integral membrane protein(s) present in synaptic vesicles:

(*a*) synapsin I bound both to protein and to lipid components of synaptic vesicles when they were separated and reconstituted in vitro;
(*b*) a hydrophobic region belonging to the head domain of synapsin I was able to penetrate the phospholipid bilayer of intact synaptic vesicles;
(*c*) [^{125}I]synapsin I bound to synaptic vesicles could be displaced by the entire synapsin I molecule or by the simultaneous presence of the NH_2-terminal fragment and the tail fragment, but not by either fragment alone;
(*d*) the purified NH_2-terminal fragment bound to synaptic vesicles with high affinity;
(*e*) the purified tail fragment interacted with high affinity with some nonlipid vesicle component; this interaction was competed by holosynapsin I, was dependent on the ionic strength of the medium and on the phosphorylation state of the peptide and was abolished by prior protease treatment of the vesicles.

The binding of the tail fragment was greatly impaired by either an increase in the ionic strength of the medium or by stoichiometric phosphorylation of this fragment. Either of these procedures would be expected to alter greatly the nature of electrostatic interactions between the positively charged tail and the synaptic vesicles. Under the same conditions (i.e., high ionic strength medium, or stoichiometric phosphorylation of the tail, or both) there was only a fivefold increase in the K_d for the binding of synapsin I to synaptic vesicles. This effect on holosynapsin I presumably reflects dissociation of the tail from its binding site, leaving only the hydrophobic head region to interact with the vesicles.

It will be of importance to establish whether the specific localization of synapsin I to small synaptic vesicles is attributable to binding to phospholipids or to proteins or to both. It will also be of importance to determine whether the binding of the tail domain to synaptic vesicles and its modulation by phosphorylation are physiologically relevant. It is of concern that at an ionic strength equivalent to 150 mM NaCl, the binding of the tail fragment is practically abolished; under the same conditions, the effect of phosphorylation on binding of holosynapsin I to synaptic vesicles is also abolished (34). However, the ionic strength of the intracellular milieu is below 150 mM NaCl equivalent, in part because of the partial dissociation of the intracellular K^+/proteinate buffer systems at physiological pH.

Further insight into the nature of the binding of synapsin I to synaptic vesicles comes from a comparison of synapsin I and protein III. Protein III is a major brain phosphoprotein that appears to be specifically localized, like synapsin I, on small synaptic vesicles (8, 11, 18). Protein III possesses a significant structural homology with synapsin I, but lacks the basic tail domain and the phosphorylation sites 2 and 3 for calcium/calmodulin-dependent protein kinase II (9, 18). Nevertheless, protein III is more tightly bound to synaptic vesicles than is synapsin I and cannot be released by increasing the ionic strength of the medium (M. Browning, R. Jahn, and F. Benfenati, unpublished observations). Moreover, purified protein III interacts with phospholipid bilayers and penetrates to some extent into the hydrophobic core of the membrane in analogy with the head domain of synapsin I (F. Benfenati and M. Bähler, unpublished observations). Thus, the synapsin I tail domain does not seem to be obligatory for the association with synaptic vesicles, but may have a crucial regulatory function in binding of synapsin I to synaptic vesicles, dependent on its phosphorylation state.

In our experiments, it has not been possible so far to exclude an interaction of the head domain of synapsin I with a protein component of synaptic vesicles. The possibility of such an interaction, in addition to the phospholipid binding, has to be further investigated, since it represents an attractive explanation for the specific subcellular localization of both synapsin I and protein III.

An ultimate goal of studying the cell biology of synapsin I is to understand the mechanism(s) by which dephosphorylated synapsin I, but not phosphorylated synapsin I, provides an inhibitory constraint to the release of neurotransmitter from the nerve terminals (25). One possible mechanism by which synapsin I might limit neurotransmitter release would be through cross-linking of small synaptic vesicles to the F-actin meshwork of the nerve terminal (2, 3). Phosphorylation of synapsin I on the tail domain, by decreasing its binding to synaptic vesicles (20, 34; and present results) and/or to F-actin (2), would impair this cross-linking, thereby increasing the number of vesicles available for fusion with the presynaptic membrane. A second possible mechanism would be through masking the fusion sites on the vesicle membrane. In this case, phosphorylation of synapsin I on the tail domain, by decreasing its affinity for synaptic vesicles, would unmask potential fusion sites. These two mechanisms are not mutually exclusive. At the present time there is insufficient information to favor either of these mechanisms or others that could be proposed for providing the cellular basis for the ability of synapsin I to regulate neurotransmitter release.

We thank Dr. W. Schiebler for initiating the reconstitution experiments on synapsin I binding to synaptic vesicles.

This research was supported by grants from the United States Public Health Service (MH-39327 and AA-06944 to P. Greengard). F. Benfenati was the recipient of a postdoctoral fellowship from the Fogarty International Center, Department of Health and Human Services, National Institutes of Health, Bethesda, MD 20892 (1 F05 TW03645-01 BI-5 AHR).

Received for publication 1 November 1988.

References

1. Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. *In* Methods in Enzymology. Vol. 8. E. F. Neufeld and V. Ginsburg, editors. Academic Press Inc., New York. 115–118.
2. Bähler, M., and P. Greengard. 1987. Synapsin I bundles F-actin in a phosphorylation-dependent manner. *Nature (Lond.)* 326:704–707.
3. Bähler, M., F. Benfenati, F. Valtorta, A. J. Czernik, and P. Greengard. 1989. Characterization of synapsin I fragments produced by cysteine-specific cleavage: a study of their interactions with F-actin. 108:1841–1849.
4. Batteiger, B., W. J. Newhall, and R. B. Jones. 1982. The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. *J. Immunol. Methods.* 55:297–307.
5. Benfenati, F., and V. Guardabasso. 1984. Basic concepts to analyze binding data using personal computers: the "RECEPT" program. *In* Principles and Methods in Receptor Binding. F. Cattabeni and S. Nicosia, editors. Plenum Publishing Corp., New York. 41–63.
6. Benfenati, F., P. Greengard, J. Brunner, and M. Bähler. 1989. Electrostatic and hydrophobic interactions of synapsin I and synapsin I fragments with phospholipid bilayers. 108:1851–1862.
7. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
8. Browning, M. D., C.-H. Huang, and P. Greengard. 1987. Similarities be-

tween Protein IIIa and Protein IIIb, two prominent synaptic vesicle-associated phosphoproteins. *J. Neurosci.* 7:847–853.
9. Deleted in proof.
10. Czernik, A. J., D. T. Pang, and P. Greengard. 1987. Amino acid sequences surrounding the cAMP-dependent and calcium/calmodulin-dependent phosphorylation sites in rat and bovine synapsin I. *Proc. Natl. Acad. Sci. USA.* 84:7518–7522.
11. De Camilli, P., and P. Greengard. 1986. Synapsin I: a synaptic vesicle-associated neuronal phosphoprotein. *Biochem. Pharmacol.* 35:4349–4357.
12. De Camilli, P., R. Cameron, and P. Greengard. 1983. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. I. Its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in frozen and plastic sections. *J. Cell Biol.* 96:1337–1354.
13. De Camilli, P., S. M. Harris, W. B. Huttner, and P. Greengard. 1983. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. II. Its specific association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. *J. Cell Biol.* 96:1355–1373.
14. Goelz, S. E., E. J. Nestler, B. Chehrazi, and P. Greengard. 1981. Distribution of Protein I in mammalian brain as determined by a detergent-based radioimmunoassay. *Proc. Natl. Acad. Sci. USA.* 78:2130–2134.
15. Harter, C., T. Bächi, G. Semenza, and J. Brunner. 1988. Hydrophobic photolabeling identifies BHA2 as the subunit mediating the interaction of bromelain-solubilized influenza virus hemagglutinin with liposomes at low pH. *Biochemistry.* 27:1856–1864.
16. Helenius, A., and K. Simons. 1972. The binding of detergents to lipophilic and hydrophilic proteins. *J. Biol. Chem.* 247:3656–3661.
17. Helenius, A., and K. Simons. 1975. Solubilization of membranes by detergents. *Biochim. Biophys. Acta.* 415:29–79.
18. Huang, C.-H., M. D. Browning, and P. Greengard. 1982. Purification and characterization of Protein IIIb, a mammalian brain phosphoprotein. *J. Biol. Chem.* 257:6524–6528.
19. Huttner, W. B., L. J. DeGennaro, and P. Greengard. 1981. Differential phosphorylation of multiple sites in purified Protein I by cyclic AMP-dependent and calcium-dependent protein kinases. *J. Biol. Chem.* 256: 1482–1488.
20. Huttner, W. B., W. Schiebler, P. Greengard, and P. De Camilli. 1983. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. III. Its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. *J. Cell Biol.* 96:1374–1388.
21. Jahn, R., W. Schiebler, and P. Greengard. 1984. A quantitative dot-immunobinding assay for proteins using nitrocellulose membrane filters. *Proc. Natl. Acad. Sci. USA.* 81:1684–1687.
22. Jahn, R., W. Schiebler, C. Ouimet, and P. Greengard. 1985. A 38,000-dalton membrane protein (p38) present in synaptic vesicles. *Proc. Natl. Acad. Sci. USA.* 82:4137–4141.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
24. Leto, T. L., and V. T. Marchesi. 1984. A structural model of human erythrocyte protein 4.1. *J. Biol. Chem.* 259:4603–4608.
25. Llinás, R., T. L. McGuinness, C. S. Leonard, M. Sugimori, and P. Greengard. 1985. Intraterminal injection of synapsin I or calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse. *Proc. Natl. Acad. Sci. USA.* 82:3035–3039.
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265–275.
27. Matsudaira, P., R. Jakes, L. Cameron, and E. Atherton. 1985. Mapping the cysteine residues and actin-binding regions of villin by using antisera to the amino and carboxyl termini of the molecule. *Proc. Natl. Acad. Sci. USA.* 82:6788–6792.
28. Matthew, W. D., L. Tsavaler, and L. F. Reichardt. 1981. Identification of a synaptic vesicle-specific membrane protein with a wide distribution in neuronal and neurosecretory tissue. *J. Cell Biol.* 91:257–269.
29. McGuinness, T. L., Y. Lai, and P. Greengard. 1985. Ca^{2+}/calmodulin-dependent protein kinase II. Isozymic forms from rat forebrain and cerebellum. *J. Biol. Chem.* 260:1696-1704.
30. Deleted in proof.
31. Nairn, A. C., H. C. Hemmings, Jr., and P. Greengard. 1985. Protein kinases in the brain. *Annu. Rev. Biochem.* 54:931–976.
32. Navone, F., P. Greengard, and P. De Camilli. 1984. Synapsin I in nerve terminals: selective association with small synaptic vesicles. *Science (Wash. DC).* 226:1209–1211.
33. Nestler, E. J., and P. Greengard. 1984. Protein Phosphorylation in the Nervous System. John Wiley & Sons Inc., New York. 394 pp.
34. Schiebler, W., R. Jahn, J.-P. Doucet, J. Rothlein, and P. Greengard. 1986. Characterization of synapsin I binding to small synaptic vesicles. *J. Biol. Chem.* 261:8383–8390.
35. Sieghart, W., J. Forn, R. Schwarcz, J. T. Coyle, and P. Greengard. 1978. Neuronal localization of specific brain phosphoproteins. *Brain Res.* 156:345–350.
36. Südhof, T. C., F. Lottspeich, P. Greengard, E. Mehl, and R. Jahn. 1987. A synaptic vesicle protein with a novel cytoplasmic domain and four transmembrane regions. *Science (Wash. DC).* 238:1142–1144.
37. Towbin, L., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
38. Ueda, T. 1981. Attachment of the synapse-specific phosphoprotein Protein I to the synaptic membrane: a possible role of the collagenase-sensitive region of Protein I. *J. Neurochem.* 36:297–300.
39. Ueda, T., and P. Greengard. 1977. Adenosine 3′:5′-monophosphate-regulated phosphoprotein system of neuronal membranes. I. Solubilization, purification, and some properties of an endogenous phosphoprotein. *J. Biol. Chem.* 252:5155–5163.
40. Watterson, D. M., W. J. Harrelson, Jr., P. M. Keller, F. Sharief, and T. C. Vanaman. 1976. Structural similarities between the Ca^{2+}-dependent regulatory proteins of 3′:5′-cyclic nucleotide phosphodiesterase and actomyosin ATPase. *J. Biol. Chem.* 251:4501–4513.
41. Wiedenmann, B., and W. W. Franke. 1985. Identification and localization of synaptophysin, and integral membrane glycoprotein of M_r 38,000 characteristic of presynaptic vesicles. *Cell.* 41:1017–1028.