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

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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 NH₂-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 NH₂-terminal and tail fragments, but not by an excess of either NH₂-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 (Kᵦ = 10 nM at 40 mM NaCl) and saturability (Bᵦᵦ = 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 Bᵦᵦ. 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
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]- sn-glycero-3-phosphocholine (["H"]TPPC1, 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). ["H"]PCPC (75 Ci/mmol) was obtained from American C.H. Instruments Inc., Palo Alto, CA)

**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 by Bährle et al. and Benfenati et al. (3, 6). The NH2-terminal fragment (29 kDa) and the tail fragment (35–40 kDa 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 (29, 40) were gifts of Drs. Fred Gordenick 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) 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 by Bährle et al. and Benfenati et al. (3, 6). The NH2-terminal fragment (29 kDa) and the tail fragment (35–40 kDa 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 (29, 40) were gifts of Drs. Fred Gordenick 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) 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).

**Limited Chymotryptic Digestion of Synapsin I.** Purified dephosphorylated synapsin I (1 mg), containing a trace amount of ["H"]TPPC1 synapsin I phosphophorylated 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 NaHCO3, 25 mM Tris/HC1 (pH 8.0), 1 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol, and 3 mM NaN3. 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/HC1 (pH 7.5), 0.1 mM EDTA and 3 mM NaN3. Using this procedure, a major chymotryptic fragment of about 22 kDa was generated. The NH2 terminus of this fragment is located 42 aminoacids NH2-terminal to phosphorylation site 2 (10).

**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 NaN3. The resuspended vesicles were solubilized by addition of octyl glycoside (3 wt/vol final concentration), or Na cholate (3.5 wt/vol, final concentration), and gentle stirring at 4°C for 20–30 min under N2 (vesicle reconstitution by the two procedures gave similar results). The mixture was then centrifuged at 200,000 g for 20 min in a ultracentrifuge (model TL-100; Beckman Instruments, Inc., the soluble fraction collected and the pellet discarded or reextracted. Phosphatidylcholine, containing a trace amount of [14C]-phosphatidylcholine, and cholesteryl (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 N2, 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 N2 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 [14C]phosphatidylcholine was added to the vesicle extract. Protein micelles were separated from lipid micelles on a 1 × 50 cm Sephacryl 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-
ststituted 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 after reconstituted vesicles labeled with [3H]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**

**[3H]ITID Labeling.** [3H]ITID was injected as an ethanol solution into the vesicle dispersion in glycerol 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%).

**[3H]TPPCII Labeling.** Liposomes were formed from pure [3H]TPPCII, dried under N₂, resuspended in glycerol buffer, and vesiculized in a sonication water bath for 15 min. The liposomes were spun at 300,000 g for 15 min to get rid of possible aggregates andmultilamellar liposomes. The [3H]TPPCII 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%.

Photolysis 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 glycerol 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 amount of phospholipids ([14C]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), carboxylic anhydrase (30 kD), trypsin inhibitor (20 kD), and β-lactalbumin (14 kD). Proteins in the gels were electrochemically transferred to nitrocellulose, as described (37). The immunolabeling of nitrocellulose sheets was performed using anti-synapsin I or anti-synaptophysin polyclonal antibodies followed by [125I]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).

**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 phosphatidylycholine 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 Kᵦ of about 15 nM and a Bₘᵦ 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 [14C]phosphatidylycholine 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.
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 Bmax 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 [3H]PTPC/11 or [3H]TID**

We tested the possible occurrence of synapsin I–phospholipid interactions in intact synaptic vesicles by means of hydrophobic labeling procedures. Either [3H]TID or [3H]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 kDa, and at least five low molecular mass proteins migrating in the 35–19 kDa region. Some of these labeled proteins represent major bands as seen on a Coomassie stain of the vesicle sample (92, 65, and 38 kDa). Two of them have previously been characterized as synaptic vesicle-specific integral membrane proteins: the 65 kDa protein corresponds to p65 described by Matthew et al. (28); the 38 kDa 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 kDa 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 4. Hydrophobic labeling of intact synaptic vesicles with \(^{125}\text{I}\)-TID or \(^{3}\text{H}\)PTPC/11. \(^{125}\text{I}\)-TID or \(^{3}\text{H}\)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 \(^{125}\text{I}\)-TID or \(^{3}\text{H}\)PTPC/11. \(^{125}\text{I}\)-TID or \(^{3}\text{H}\)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.
specific domains involved in synapsin I binding to synaptic vesicles (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 [125I]TID and [3H]PTPC/11 indicates that, at least under our conditions, [125I]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 [125I]synapsin I binding to synaptic vesicles was very similar with the two hydrophobic probes and with endogenous versus rebound synapsin I. The fragments selectively labeled were the NH2-terminal fragment (29 kD), the middle fragment (15 kD), and the NH2-terminal plus tail fragments in equimolar amounts (a). The residual binding of [125I]synapsin I is expressed in percent of the binding in the absence of competing ligand. IC50 = 100 nM; Kd = 22 nM for inhibition by synapsin I, IC50 = 102 nM; Kd = 23 nM for inhibition by the simultaneous presence of the two fragments.

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 [125I]TID or [3H]PTPC/11, photolyzed and solubilized; synapsin I was immunoprecipitated and subjected to cysteine-specific cleavage with NT-2CB, 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 NH2-terminal fragment (29 kD), the middle fragment (15 kD), and the NH2-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).

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 vesicles.

Figure 7. Binding of purified NH2-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 NH2-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 NH2-terminal fragment bound was determined by dot immunobinding employing an antibody specific for phosphorylation site 1. The Kd value was ~25 nM, and the Bmax value was ~800 fmol/μg, protein (prot).
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 [32P]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 [32P]phosphorylated fragment or blotted onto nitrocellulose and analyzed for fragment immunoreactivity using antibodies specific for phosphorylation site 3 and [125I]protein A overlay.

To further characterize the specific peptide domains involved in synapsin I binding, the binding of the purified NH2-terminal and tail fragments to synaptic vesicles was analyzed. The NH2-terminal fragment bound to synaptic vesicles with high affinity (Kd = 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. 8a). The Kd 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 [125I]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.

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 synapsin I, although very basic, did not specifically interact with acidic phospholipids (6) and was not significantly labeled with [125I]ITID or [3H]PTPC/II 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 Bmax 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 [32P] 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 IC50 was about 84 nM corresponding to a KI of 43 nM, close to the KI 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.
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 constituted 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) [32P]synapsin I bound to synaptic vesicles could be displaced by the entire synapsin I molecule or by the simultaneous presence of the NH2-terminal fragment and the tail fragment, but not by either fragment alone;
(d) the purified NH2-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 this fragment). Either of these could be expected to increase in the Ks for the binding of synapsin I to synaptic vesicles; 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 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 mutally 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.

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