Electrostatic and Hydrophobic Interactions of Synapsin I and Synapsin I Fragments with Phospholipid Bilayers

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Abstract. Synapsin I, a major neuron-specific phosphoprotein, is localized on the cytoplasmic surface of small synaptic vesicles to which it binds with high affinity. It contains a collagenase-resistant head domain and a collagenase-sensitive elongated tail domain. In the present study, the interaction between synapsin I and phospholipid vesicles has been characterized, and the protein domains involved in these interactions have been identified. When lipid vesicles were prepared from cholesterol and phospholipids using a lipid composition similar to that found in native synaptic vesicle membranes (40% phosphatidylcholine, 32% phosphatidylethanolamine, 12% phosphatidylserine, 5% phosphatidylinositol, 10% cholesterol, wt/wt), synapsin I bound with a dissociation constant of 14 nM and a maximal binding capacity of about 160 fmol of synapsin I/µg of phospholipid. Increasing the ionic strength decreased the affinity without greatly affecting the maximal amount of synapsin I bound. When vesicles containing cholesterol and either phosphatidylcholine or phosphatidylcholine/phosphatidylethanolamine were tested, no significant binding was detected under any conditions examined. On the other hand, phosphatidylcholine vesicles containing either phosphatidylserine or phosphatidylinositol strongly interacted with synapsin I. The amount of synapsin I maximally bound was directly proportional to the percentage of acidic phospholipids present in the lipid bilayer, whereas the $K_d$ value was not affected by varying the phospholipid composition. A study of synapsin I fragments obtained by cysteine-specific cleavage showed that the collagenase-resistant head domain actively bound to phospholipid vesicles; in contrast, the collagenase-sensitive tail domain, though strongly basic, did not significantly interact. Photolabeling of synapsin I was performed with the phosphatidylcholine analogue 1-palmitoyl-2-[11-[4-[3-(trifluoromethyl)diazirinyl]phenyl]-[2-3H]undecanoyl]-sn-glycero-3-phosphocholine; this compound generates a highly reactive carbene that selectively interacts with membrane-embedded domains of membrane proteins. Synapsin I was significantly labeled upon photolysis when incubated with lipid vesicles containing acidic phospholipids and trace amounts of the photoactivatable phospholipid. Proteolytic cleavage of photolabeled synapsin I localized the label to the head domain of the molecule. The results suggest that synapsin I, which has an amphiphilic character and local regions of positive charge, interacts with artificial membranes containing acidic phospholipids, and this surface interaction, presumably of electrostatic nature, is followed by a penetration of the head domain into the hydrophobic core of the membrane.

Protein phosphorylation represents a process of paramount importance in the nervous system and is involved in signal transduction and information processing (45). Synapsin I, a collective name for two closely related peptides (Ia and Ib) with apparent molecular weights of 86,000 and 80,000 on SDS-PAGE, is a major neuron-specific phosphoprotein (52) and was purified to homogeneity from bovine and rat brain (26, 51, 55). It is a very basic, acid-soluble protein composed of two major domains, namely an elongated collagenase-sensitive COOH-terminal domain rich in proline and glycine (tail), and a collagenase-resistant NH$_2$-terminal domain rich in hydrophobic amino acids (head) (45, 55). Synapsin I is an excellent substrate for at least three distinct protein kinases in brain and undergoes multisite phosphorylation on serine residues (15, 26, 43). The head domain can be phosphorylated by either cAMP-dependent protein kinase or calcium/calmodulin-dependent protein kinase I (site 1). The tail domain can be phosphorylated on two serine residues by calcium/calmodulin-dependent protein kinase II (sites 2, 3).

In the nerve terminal, synapsin I is specifically localized on the cytoplasmic surface of small synaptic vesicles (16, 17, 27, 44), to which it binds with high affinity ($K_d = 10$ nM).
(51). Binding of synapsin I with somewhat lower affinity to various cytoskeletal elements, namely F-actin, spectrin, neurofilaments, and microtubules, has also been demonstrated (2, 4, 5, 21, 53). The binding of synapsin I to both synaptic vesicles (51) and F-actin (2) is significantly weakened by phosphorylation in the tail region. These interactions are thought to have physiological relevance in the regulation of vesicle traffic at the nerve terminal, as well as in the modulation of neurotransmitter release. A variety of physiological stimuli affecting synaptic function have been shown to regulate the state of synapsin I phosphorylation (for reviews, see references 18, 45, 46). Moreover, in vivo injections of dephosphorylated synapsin I into the squid giant axon significantly reduced neurotransmitter release, whereas the phosphorylated forms were ineffective under the same experimental conditions (37).

The high affinity association with synaptic vesicles and a remarkable tendency to self-associate (55) suggested the possibility that synapsin I has a high surface activity. Thus, the possibility of an interaction between synapsin I and lipid vesicles (51) and F-actin (2) is significantly weakened by phosphorylation (2, 4, 5, 21, 53). The binding of synapsin I to both synaptic vesicles (51) and F-actin (2) is significantly reduced neurotransmitter release, whereas the phosphorylated forms were ineffective under the same experimental conditions (37).

The high affinity association with synaptic vesicles and a remarkable tendency to self-associate (55) suggested the possibility that synapsin I has a high surface activity. Thus, the possibility of an interaction between synapsin I and lipid bilayers that represent an ideal amphiphilic environment has been considered. Moreover, the identification and characterization of such an interaction may be helpful in elucidating the nature of the high affinity binding of synapsin I to synaptic vesicles. We have now investigated the interaction of purified synapsin I with artificial phospholipid membranes. To get further insight into the mechanism(s) governing the association of synapsin I with phospholipid bilayers, labeling experiments using the photoactivatable phospholipid analogues [2-3H]undecanoyl-sn-glycero-3-phosphocholine; [125I]TID, 3-(trifluoromethyl)iodophenyl)diazirine (J22)[TID] (11, 12) were carried out. Both compounds selectively label the membrane-embedded domains of integral membrane-associated proteins revealing the existence of interactions between selected protein domains and the hydrophobic core of the membrane.

The results described here demonstrate that synapsin I interacts, with high affinity and saturability, with phospholipid bilayers when acidic phospholipids, namely phosphatidylserine and/or phosphatidylinositol, are included. This interaction is probably initiated by a surface electrostatic attraction and followed by the penetration of the hydrophobic regions of the head domain of synapsin I into the hydrophobic core of the bilayer.

Materials and Methods

Materials

[^32P]ATP (2.90 Ci/mmol), [14C]phosphatidylcholine (0.1 Ci/mmol), [125I]NaI (17 Ci/mg) and [125I]-labeled protein A (90 μCi/μg) were from New England Nuclear (Boston, MA). [125I]TID (10 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL). [8H]PTPC/11 (15 Ci/mmol) was prepared according to the procedure of Harter et al. (23). Phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL), stored at −20°C in the dark, and used within two months.

1. Abbreviations used in this paper: NTB, 2-nitro-5-thiocyanobenzoic acid; [8H]PTPC/11, 1-palmitoyl-2-[4-[3-(trifluoromethyl)diazirinyl]phenyl]-[2-H]undecanoyl-sn-glycero-3-phosphocholine; [125I]TID, 3-(trifluoromethyl)-3-(m-[125I]iodophenyl)diazirine.

Cholesterol, cholic acid, octyl glucoside, NP-40, Triton X-100, and 2-nitro-5-thiocyanobenzoic acid (NTCB) were purchased from Sigma Chemical Co. (St. Louis, MO); Sephadex G-50, G-100, Sepharose 2B, and Sephacryl S-200 were from Pharmacia Fine Chemicals (Piscataway, NJ); Iodo Beads were from Pierce Chemical Co. (Rockford, IL); hydrolase mix was from Bio-Rad Laboratories (Richmond, CA); PYM membranes for ultratransmission were from Amicon Corp. (Danvers, MA); carboxymethylcellulose was from Whatman Inc. (Clifton, NJ); Staphylococcus aureus V8 protease was from Miles Laboratories Inc. (Elkhart, IN); ultra pure sucrose was from Bethesda Research Laboratories (Gaithersburg, MD); and nitrocellulose sheets (pore size 0.2 μm) were from Schleicher & Schuell, Inc. (Kodak, N.Y.). HEPES, the n-polyphosphate of cAMP-dependent protein kinase; and (c) sites 1, 2, and 3 (in the tail domain) using calcium/calmodulin-dependent protein kinase II, purified as described (40, 57), were gifts of Drs. Fred Gorelick and Gerald Thiel of our laboratory. Synapsin I was purified under nondenaturing conditions from bovine brain as previously described (2, 51). Polyclonal antibodies against synapsin I were obtained by injecting rabbits with purified protein in complete Freund's adjuvant. Antibodies specific for synapsin I peptides containing phosphorylation site 1 (head) or phosphorylation site 3 (tail) (15) were raised in our laboratory by Dr. A. Czerwick. All other chemicals, obtained from Sigma Chemical Co. or Serva Fine Biochemicals Inc. (Garden City Park, NY), were of analytical grade.

Phosphorylation and Iodination of Synapsin I

Purified bovine dephosphorylated synapsin I was phosphorylated to near stoichiometry at (c) site 1 (in the head domain) using the catalytic subunit of cAMP-dependent protein kinase; and (c) sites 1, 2, and 3 (in the tail domain) using calcium/calmodulin-dependent protein kinase II, purified as described (40, 57), were gifts of Drs. Fred Gorelick and Gerald Thiel of our laboratory. Synapsin I was purified under nondenaturing conditions from bovine brain as previously described (2, 51). Polyclonal antibodies against synapsin I were obtained by injecting rabbits with purified protein in complete Freund's adjuvant. Antibodies specific for synapsin I peptides containing phosphorylation site 1 (head) or phosphorylation site 3 (tail) (15) were raised in our laboratory by Dr. A. Czerwick. All other chemicals, obtained from Sigma Chemical Co. or Serva Fine Biochemicals Inc. (Garden City Park, NY), were of analytical grade.

Preparation of Phospholipid Vesicles

"Pure phosphatidylcholine vesicles" were prepared from phosphatidylcholine/cholesterol (90:10). "Mixed phospholipid vesicles," mimicking the lipid composition of the synaptic vesicle membrane (42, 59, 60), were prepared from phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine/phosphatidylinositol/cholesterol (40:32:12:5:10). Phospholipid vesicles were formed according to Mimms et al. (41) with slight modifications. Briefly, phospholipids and cholesterol (dissolved in chloroform at a concentration of 10 mg/ml) were mixed in a glass test tube and a trace amount of [14C]phosphatidylcholine was added. Phospholipid vesicles were then 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. The dried lipid film was then dissolved in 0.5-1.0 ml of "reconstitution buffer" containing either 25 mM Tris/HCl (pH 7.4), 3 mM NaN3, 3 mM NaCl, or 10 mM Tris/HCl (pH 7.4), 300 mM sucrose, 100 mM NaCl, 3 mM NaN3 (sucrose/reconstitution buffer for sucrose-loaded vesicles) in the presence of 3% (wt/vol) octyl glucoside. The samples were gently stirred at room temperature for 20 min under N2. The detergent/lipid molar ratio in the final solution was always kept over 10:1. Unilamellar vesicles were obtained by removing the detergent either by extensive dialysis against cold N2-saturated reconstitution buffer or by gel filtration on a 1.5 × 20 cm Sephadex G-50 column equilibrated with the same buffer. In the latter procedure, the fractions corresponding to the radioactivity/turbidity peak were pooled and concentrated by vacuum dialysis. The vesicle samples were then dialyzed against a low ionic strength buffer containing either 300 mM glycine, 5 mM Hepes (pH 7.4), 3 mM NaN3 (unloaded vesicles), or 300 mM sucrose, 200 mM glycine, 5 mM Hepes (pH 7.4), 3 mM NaN3 (sucrose-loaded vesicles). Phospholipid vesicles were kept on ice, used within 2-3 d and diluted with the appropriate buffer immediately before use. Vesicles made according to this procedure had di-
Assay of Synapsin I Binding to Phospholipid Vesicles

The binding of synapsin I to phospholipid vesicles (15–25 μg of phospholipid) was carried out in "standard assay solution" containing 250 mM glucose, 30 mM sucrose, 30 mM NaCl, 5 mM Tris/HCl, 4 mM Hepes (pH 7.4), 20 μM EDTA, 0.22 mM Na2SO4, and 100 μg/ml of BSA. For assays at higher ionic strength, the assay solution contained 20 mM glucose, 30 mM sucrose, 97 mM KCl, 46 mM NaCl, 1.6 mM MgCl2, 0.9 mM NaHCO3, 5 mM Tris/HCl, 4 mM Hepes (pH 7.4), 20 μM EDTA, 0.22 mM Na2SO4, and 100 μg/ml of BSA. Synapsin I (or synapsin I fragments) present at 200,000 g for 15 min in a rotor (model TLA-100.2; Beckman Instruments, Inc., Palo Alto, CA) to remove aggregates was added last using precoated pipette tips. Aliquots added with the coated pipette tips were analyzed for the amounts of synapsin I added to the samples. A typical curve included 10–12 different synapsin I concentrations ranging from 5 to 200–300 nM.

After addition of synapsin I, samples were incubated on ice for 60 min. Synapsin I bound to vesicles was separated from free synapsin I by ultracentrifugation as follows. Each sample was layered on top of 100 μl of 5% sucrose (wt/vol) in glycine buffer and centrifuged at 42,000 rpm for 30 min in a rotor (model T1 42.2; Beckman Instruments, Inc.). The supernatants were carefully aspirated and the pellets resuspended in 40 μl of "spot buffer" containing 160 mM NaCl, 10 mM NaPO4 (pH 7.4), and 1.4% (vol/vol) Triton X-100. During the resuspension procedure, care was taken not to wet the tube wall above the sucrose cushion to prevent contamination by free synapsin I, since it was reversibly bound with a very low dissociation constant. The recovery of the vesicles labeled with [3H]phosphatidylcholine was determined by liquid scintillation spectrometry, with phosphatidylcholine or by phospholipid phosphorus determination following the method of Ames (1).

Hydrophobic Labeling with [3H]PTPC/II and [3H]TID

[3H]PTPC/II. Mixtures of phospholipids and cholesterol including 0.2–0.5% (wt/wt) of [3H]PTPC/II in chloroform were dried and vesiculized as described above. Usually 10 μg of synapsin I (or 20 μg of NTGB digest) were incubated with 200 μg of phospholipid vesicles in 500 μl of standard assay solution at 4°C for 60 min. The samples were then transferred to pyrex tubes, flushed with N2, sealed and photolyzed for 5 min at room temperature using a 300-W medium pressure mercury lamp (Ace Glass, Inc., Vineyard, NJ). To screen out infrared and shortwave UV light, a filter of circulating cold water (15 mm) and a filter of saturated solution of copper sulfate (20 mm) were interposed between the lamp tube and the samples. After photolysis, 3 vol of chloroform/methanol (1:2 vol/vol) were added to the samples to extract any noncovalently bound [3H]PTPC/II. Samples were incubated for 30 min at room temperature and centrifuged for 5 min in a Savant Instruments, Inc. (Hicksville, NY) minifuge at maximal speed. The supernatants were carefully aspirated and the pellets resuspended in 100 μl of 20 mM Tris/HCl, 10 mM NaPO4 (pH 7.4), and 1.4% (vol/vol) saline for fluorography. After drying under vacuum and heat, gels were exposed to film (X-OMAT, Eastman Kodak Co., Rochester, NY) at −70°C for various amounts of time.

[3H]TID. When labeled TID was used as hydrophobic reagent, unlabled sucrose-loaded phospholipid vesicles were incubated with synapsin I for 60 min at 4°C. At the end of the incubation, 1 μl of [3H]TID in ethanol was injected (final concentration of ethanol in the sample <0.5%), and the samples were allowed to incubate for an additional 5 min at room temperature before photolysis. After photolysis, liposomes were sedimented by centrifugation at 30,000 g for 20 min, and the pellets were resuspended and extracted with chloroform/methanol as described above. After electrophoresis, gels were stained, destained, dried, and exposed to Kodak X-OMAT film at −70°C with an intensifying screen. In some experiments, synapsin I, precipitated after chloroform/methanol extraction, was dissolved in 6 M guanidine/HCl and subjected to cysteine-specific cleavage with NTGB as described (5).

Miscellaneous Techniques

SDS-PAGE was performed according to Laemmli (34). Gels were silver stained following the procedure of Wray et al. (61). 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 alphalactalbumin (14 kD). Limited proteolysis gels were run as described (25). Proteins in the gels were electrophoretically transferred to nitrocellulose as reported (54). The immunolabeling of nitrocellulose sheets was performed using anti-synapsin I polyclonal antibodies followed by [125I]protein A overlay (6). Saturation and inhibition curves were analyzed using the computer program RECEPT (7). Protein was determined according to Lowry et al. (38) or Bradford (10), using BSA as a standard. Autoradiograms were scanned using a soft laser beam densitometer, and the integrated areas under the peaks of the densitometric traces used as a measure of radiolabel incorporation.

Results

Characterization of Synapsin I Binding to Phospholipid Vesicles

Saturation curves in both low (40 mM NaCl equivalent) and high (150 mM NaCl equivalent) ionic strength conditions for the binding of synapsin I to pure phosphatidylcholine vesicles and to mixed phospholipid vesicles are shown in Fig. 1. In the case of pure phosphatidylcholine vesicles, it was not
Figure 1. Binding of synapsin I to pure phosphatidylcholine vesicles (a) or mixed phospholipid vesicles (b) as a function of synapsin I concentration. Sucrose-loaded phospholipid vesicles (15 μg phospholipid/sample) were incubated with various amounts of synapsin I for 60 min on ice. Bound synapsin I was separated from the free protein by ultracentrifugation through a 5% (wt/vol) sucrose cushion and quantitated by dot immunobinding. The bound values, expressed as fmol/μg phospholipid, were 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. (●) binding under standard conditions (40 mM NaCl equivalent); (○) binding at higher ionic strength (150 mM NaCl equivalent). The data in b were fitted using a nonlinear regression procedure following the simple model of one ligand, one noncooperative binding site; $K_d$ under standard conditions = 10 nM, $K_d$ at higher ionic strength = 62 nM, and $B_{max}$ in both conditions was ~160 fmol synapsin I/μg total phospholipid.

Possible to detect a saturable binding of synapsin I under either set of ionic strength conditions. The amount of synapsin I found in the pellet was always very low and increased only slowly along with the free synapsin I concentration. On the other hand, after using mixed phospholipid vesicles that approximated the phospholipid composition of native synaptic vesicles, a high affinity and saturable binding of synapsin I was observed under low ionic strength conditions. Nonlinear regression analysis of saturation curves revealed a $K_d$ of $107 \pm 1.5$ nM and a $B_{max}$ of ~167 ± 4 fmols of synapsin I/μg phospholipid, which are values in the same range as those found for native synaptic vesicles. Increasing the ionic strength of the medium significantly decreased the affinity of the binding ($K_d = 63.3 \pm 6.6$ nM), and only slightly decreased the maximal amount of synapsin I bound (144 ± 8 fmol/μg) phospholipid. The effect of ionic strength seemed to be independent of the ion species present in the medium, since a comparable effect was obtained by substituting glutamate− for Cl− at comparable conductivity values (data not shown).

The possible effect of size of phospholipid vesicles on the binding of synapsin I was examined by using two differently sized vesicle populations, formed by cholate solubilization/removal (diameter 30-40 nm) (13) or by octyl glucoside solubilization/removal (diameter 200-240 nm) (41). The two procedures gave similar $K_d$ and $B_{max}$ values (Fig. 2). The binding of synapsin I to mixed phospholipid vesicles was not affected by the absence of cholesterol in the lipid mixture (data not shown).

The binding of synapsin I to mixed phospholipid vesicles had a broad pH dependency, being maximal around pH 6.5-7.5. Below pH 5.5 and above pH 8.0, the interaction was considerably weakened with a residual binding lower than 50% of that observed at optimal pH (Fig. 3). This pH-dependency did not seem to be related to isoelectric precipitation, since the pI of synapsin I was above pH 11 (18, 55).

To study the influence of the phosphorylation of synapsin I on its interaction with pure phosphatidylcholine and mixed

Figure 2. Binding of synapsin I to mixed phospholipid vesicles as a function of vesicle size. Two populations of vesicles with the same phospholipid composition were analyzed. (a) Vesicles with a diameter of 30-40 nm were formed by lipid solubilization using sodium cholate followed by detergent removal with gel filtration, (b) Vesicles with a diameter of 200-240 nm were obtained with the standard octyl glucoside solubilization/removal procedure described under Materials and Methods. (a and b, top) Elution profiles of vesicles after Sepharose 2B chromatography as evaluated by the radioactivity of [14C]phosphatidylcholine added in trace amounts to the phospholipid mixture. The shaded areas indicate the fractions that were pooled for the binding studies. (a and b, bottom) The corresponding phospholipid vesicle samples (20 μg phospholipid/sample) were incubated in the presence of various concentrations of synapsin I. The saturation curves were carried out and analyzed as described in the legend to Fig. 1.
Figure 3. Binding of synapsin I to mixed phospholipid vesicles as a function of pH. Mixed phospholipid vesicles were incubated with synapsin I (10 nM) at various pH values. The pH of the medium was adjusted by using buffers with different pKs (acetate, Mes, Hepes, Tris, and borate buffers), so that each buffer was used at a pH = pK ± 0.7, keeping constant the total ionic strength of the assay solution (40 mM NaCl equivalent).

phospholipid vesicles, the protein was phosphorylated on its different sites using [γ-32P]ATP and purified protein kinases, as described in Materials and Methods. Table I summarizes the results of the binding of the different phosphorylated forms of synapsin I to mixed phospholipid vesicles under low and high ionic strength conditions. In contrast to the data obtained using native synaptic vesicles, the changes in the phosphorylation state of synapsin I did not significantly affect its binding to mixed phospholipid vesicles under either low or high salt conditions. The $K_d$ values of the different phosphorylated forms under low ionic strength conditions were higher than the corresponding value of dephosphorylated synapsin I, but this effect was not statistically significant. The $B_{max}$ values of the phosphorylated forms were in the same range as the corresponding value for the dephosphorylated form.

To identify the component(s) in the phospholipid mixture responsible for the interaction with synapsin I, the effect of varying the phospholipid composition on synapsin I binding was examined (Fig. 4). When vesicles contained 100% phosphatidylcholine or 50% phosphatidylcholine/50% phosphatidylethanolamine (wt/wt), it was not possible to detect significant binding under any condition. On the other hand, vesicles containing 50% phosphatidylcholine and 50% acidic phospholipid (wt/wt) (either phosphatidylserine or phosphatidylinositol) strongly interacted with synapsin I, showing affinity and $B_{max}$ values (relative to the acidic phospholipid) similar to those found using mixed phospholipid vesicles. The amount of synapsin I maximally bound was directly proportional to the percentage of acidic phospholipid present in the lipid bilayer (Fig. 5), whereas the $K_d$ value was not affected by the different phospholipid compositions. Thus, the two acidic phospholipids, phosphatidylserine and phosphatidylinositol, seem to account for the binding of synapsin I to mixed phospholipid vesicles, having a similar effective-

Table I. Binding of Dephosphorylated and Phosphorylated Synapsin I to Mixed Phospholipid Vesicles

| Conditions                        | $K_d$ (nM) | $B_{max}$ (fmol synapsin I/μg phospholipid) |
|-----------------------------------|-----------|-------------------------------------------|
| Standard assay (40 mM NaCl equivalent) |           |                                           |
| Dephosphorylated Synapsin I       | 10.7 ± 1.5| 167 ± 4                                   |
| Phosphorylated Synapsin I (site 1)| 13.5 ± 4.1| 159 ± 5                                   |
| Phosphorylated Synapsin I (sites 2| 13.7 ± 3.9| 165 ± 8                                   |
| and 3)                            |           |                                           |
| Phosphorylated Synapsin I (sites 1| 13.2 ± 5.7| 171 ± 8                                   |
| and 3)                            |           |                                           |
| High ionic strength (150 mM NaCl equivalent) |       |                                           |
| Dephosphorylated Synapsin I       | 63.3 ± 6.6| 144 ± 8                                   |
| Phosphorylated Synapsin I (site 1)| 71.6 ± 5.2| 134 ± 5                                   |
| Phosphorylated Synapsin I (sites 2| 65.7 ± 7.2| 155 ± 13                                  |
| and 3)                            |           |                                           |
| Phosphorylated Synapsin I (sites 1| 78.3 ± 6.0| 141 ± 15                                  |
| and 3)                            |           |                                           |

Binding was carried out as described in Materials and Methods and in the legend to Fig. 1. $K_d$ and $B_{max}$ values are given as means ± SEM (n = 4) in nM and fmol synapsin I/μg phospholipid, respectively.

![Figure 4](image-url)

Figure 4. Phospholipid specificity of synapsin I binding. Sucrose-loaded phospholipid vesicles (composed of cholesterol plus either 100% [wt/wt] phosphatidylcholine [a], 50% phosphatidylcholine/50% phosphatidylethanolamine [b], 50% phosphatidylserine/50% phosphatidylcholine [c], or 50% phosphatidylinositol/50% phosphatidylcholine [d]) (20 μg total phospholipid/sample) were incubated with various concentrations of synapsin I in either standard assay buffer (40 mM NaCl equivalent, ✔) or higher ionic strength buffer (150 mM NaCl equivalent, ◼). The corresponding saturation curves are shown. For further details, see the legend to Fig. 1.
Figure 5. Dependency of synapsin I binding to phospholipid vesicles on the relative amounts of acidic phospholipids. a and b. Sucrose-loaded phospholipid vesicles formed from increasing fractional amounts of phosphatidylserine (a) or phosphatidylinositol (b) in phosphatidylcholine were incubated with various concentrations of synapsin I. The saturation curves corresponding to the different phospholipid compositions are shown. Acidic phospholipid, expressed as a fraction of total phospholipid, was 0.05 (●), 0.10 (X), 0.25 ( ), and 0.50 (○). For further details, see the legend to Fig. 1. In c, synapsin I bound at saturation is plotted as a function of the fractional amount of phosphatidylserine (PS), phosphatidylinositol (PI), or phosphatidylethanolamine (PE). Values represent means ± SEM (n = 4).

Figure 6. Synapsin I binding to endogenous lipids extracted from brain small synaptic vesicles. Small synaptic vesicles were purified from rat neocortex and endogenous lipids were extracted either with chloroform/methanol or by solubilization in a molar excess of sodium cholate followed by separation of the lipid-containing micelles using gel filtration. In both cases, lipid vesicles were formed by detergent removal and exposed to various concentrations of synapsin I. Saturation curves are plotted for detergent (●) and chloroform/methanol-extracted (○) synaptic vesicle lipids. Endogenous phospholipids were collectively assayed using phospholipid phosphorus determination (20 μg total phospholipid/sample). The mean values for Kd and Bmax were 27 nM and 178 fmol/μg phospholipid, respectively.

Specific Peptide Domains Involved in Synapsin I–Phospholipid Interaction

To identify the protein domain(s) responsible for the synapsin I–phospholipid interaction, the fragments of synapsin I...
generated by cysteine-specific cleavage were tested for binding by a sedimentation assay using sucrose-loaded mixed phospholipid vesicles. The results of this experiment are shown in Fig. 7. When mixed phospholipid vesicles were incubated with a total NTCB digest, the majority of the fragments sedimented with the vesicles and only the tail fragment was present in significant amounts in the supernatant fraction, suggesting that the collagenase-resistant head of synapsin I is mainly involved in the interaction with phospholipids. When the prespun total NTCB digest was incubated in standard assay solution in absence of vesicles and subjected to ultracentrifugation, none of the fragments was found in detectable amounts in the pellet analyzed by SDS-PAGE and silver stain.

To substantiate these findings, the binding of purified NH2-terminal and purified tail fragments to pure phosphatidylcholine and mixed phospholipid vesicles was investigated. The results of this experiment are shown in Fig. 8. The purified NH2-terminal fragment bound with high affinity and apparent saturability to mixed phospholipid vesicles (Fig. 8 a), exhibiting values for $K_d$ (24 nM) and $B_{max}$ (200 fmol/µg phospholipid) similar to those obtained for the binding of holosynapsin I. However, because of the high tendency of the fragments derived from the head domain of synapsin I to self-associate, the $K_d$ value may be overestimated because fragment microaggregates may not be able to interact with the phospholipid vesicles. The increase in the ionic strength of the medium from 40 mM to 150 mM NaCl equivalent caused a 58% increase in the $K_d$ value without altering the $B_{max}$ (data not shown).

The tail fragment (Fig. 8 b) and the middle/tail fragment (not shown) bound to some extent to mixed phospholipid vesicles, but the binding showed a lack of saturability at the concentrations tested. These results demonstrate that, even though the tail region of synapsin I is strongly basic and hence positively charged at physiological pH, and the low salt conditions maximize the occurrence of electrostatic interactions, this is not sufficient to bring about a high affinity interaction with vesicles containing negatively charged phospholipids. As with the findings for intact synapsin I, no significant interaction with pure phosphatidylcholine vesicles was observed for the purified NH2-terminal or tail fragments (Fig. 8), or for the middle/tail fragment.

Further proof that the head domain and not the tail domain of synapsin I is involved in the phospholipid interaction came from competition experiments using mixed phospholipid vesicles (Fig. 9). $[^{125}]$synapsin I, demonstrated in preliminary experiments to be as effective as the native protein in binding to phospholipids, was used as a ligand. Binding of $[^{125}]$synapsin I was competed by increasing amounts of cold synapsin I. The IC$_{50}$ was about 80 nM at a $[^{125}]$synapsin I concentration of 35 nM; this value gave a $K_d$ of $\sim$22 nM, which is in the same range as the $K_d$ determined for phospholipid vesicles of identical composition by saturation curves using cold synapsin I as a ligand. The NH2-terminal fragment was as effective as the holomolecule in inhibiting $[^{125}]$synapsin I binding in terms of both IC$_{50}$ and percentage of residual binding. In contrast, when the tail fragment was used, no significant inhibition of $[^{125}]$synapsin I binding was detected at concentrations up to the micromolar range.

**Hydrophobic Labeling of Synapsin I Using $[^{3}H]$PTPC/II and $[^{125}]$TID**

To investigate whether the interaction of synapsin I with
Sucrose-purified tail fragment. Sucrose-loaded mixed phospholipid vesicles were incubated in standard assay buffer with various concentrations of cold synapsin I (o), purified NH2-terminal fragment (a) or tail fragment (b). Fragments were obtained by cysteine-specific cleavage and purified as described under Materials and Methods. The amounts of bound ligands were determined by dot immunobinding using antibodies specific for the NH2-terminal fragment/middle fragment and, to a lesser extent, in the middle/tail fragment (which includes part of the collagenase-resistant head domain). Sometimes a weak labeling of the active diazirine group and possible hydrophobic pockets in the protein molecule. The topographical selectivity of the labeling was guaranteed by the fact that [3H]PTPC/ll is a structural component of the membrane with the photoactivatable group confined to the hydrophobic core of the lipid bilayer. The amount of the radioactive photoaffinity reagent covalently bound to synapsin I, however, was always very low compared to the total amount of reagent in the bilayer. The relatively low labeling efficiency is largely attributable to the strong reactivity of the photogenerated carbene toward any kind of neighboring chemical groups, which include not only amino acid side chains but also phospholipid acyl chains and cholesterol present in huge molar excess with respect to the amount of synapsin I bound to the phospholipid vesicle.

To specify the protein domain of synapsin I interacting with the phospholipid bilayer, two complementary approaches were taken: (a) the mixture of fragments generated after NTCB-digestion of synapsin I was incubated with mixed phospholipid vesicles and subsequently photolyzed (predigestion; Fig. 11, lane I); or (b) intact synapsin I was incubated with mixed phospholipid vesicles, and then, after photolysis and delipidation, subjected to cysteine-specific cleavage (postdigestion, Fig. 11, lane 2). The results obtained from these two approaches were qualitatively similar. It is apparent that, with both digestion protocols, the labeling occurred in both the NH2-terminal fragment and the NH2-terminal/middle fragment and, to a lesser extent, in the middle fragment; i.e., the portions of synapsin I characterized by a high hydrophobicity and belonging to the collagenase-resistant head domain. Sometimes a weak labeling of the middle/tail fragment (which includes part of the collagenase-resistant head domain) was found with long exposure times, but the tail fragment was never labeled under these conditions. Thus, the fragments labeled by the hydrophobic reagent corresponded to those that were demonstrated to bind artificial bilayers involved the penetration of selected protein domains into the hydrophobic core of the membrane, hydrophobic photoactivatable lipid probes known to selectively label the membrane-embedded domains of integral proteins were used. Synapsin I was incubated for 60 min on ice with vesicles of different phospholipid compositions containing 0.2–0.5% (wt/wt) [3H]PTPC/ll in standard assay solution, followed by photolysis, delipidation of synapsin I with chloroform/methanol, SDS-PAGE, and fluorography as described in Materials and Methods. In each set of experimental conditions, photolysis was omitted in a control sample to rule out the possibility of nonspecific labeling. In all cases, no detectable labeling of synapsin I occurred in the absence of photolysis. Synapsin I was not labeled after incubation with pure phosphatidylcholine vesicles and was only slightly labeled after incubation with vesicles containing 50% phosphatidylcholine and 50% phosphatidylethanolamine (wt/wt). In contrast, the incubation of synapsin I with phosphatidylcholine vesicles containing increasing amounts of acidic phospholipids, namely phosphatidylserine and phosphatidylinositol, induced a progressive incorporation of the label into the synapsin I molecule (Fig. 10 a). The fluorograms were densitometrically scanned to obtain an estimate of the incorporated label (Fig. 10 b). The results were in agreement with the binding data (see Fig. 5 for comparison). The lack of detectable labeling with pure phosphatidylcholine vesicles ruled out the possibility of an extravesicular interaction between the active diazirine group and possible hydrophobic pockets in the protein molecule. The topographical selectivity of the labeling was guaranteed by the fact that [3H]PTPC/ll is a structural component of the membrane with the photoactivatable group confined to the hydrophobic core of the lipid bilayer. The amount of the radioactive photoaffinity reagent covalently bound to synapsin I, however, was always very low compared to the total amount of reagent in the bilayer. The relatively low labeling efficiency is largely attributable to the strong reactivity of the photogenerated carbene toward any kind of neighboring chemical groups, which include not only amino acid side chains but also phospholipid acyl chains and cholesterol present in huge molar excess with respect to the amount of synapsin I bound to the phospholipid vesicle.

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to phospholipid vesicles. In some digestion experiments, $[^{125}I]$TID was used to label the hydrophobic core of the liposome membrane (Fig. 11, lane 3). The results were virtually identical to those obtained using $[^{3}H]$PTPC/II even though TID is not as specific as PTPC in its topographical localization within the membrane.

**Discussion**

Synapsin I is a major synaptic vesicle-associated phosphoprotein. The investigation on the possible interactions between synapsin I and phospholipid vesicles was undertaken for several reasons. Synapsin I was found to specif-
phospholipids was determined using fragments generated by
cysteine-specific cleavage. The resulting fragments, identi-
"phospholipid site" for synapsin I on the lipid bilayer
is represented by acidic phospholipids such as phosphatidyl-
serine and phosphatidylinositol which are present in biologi-
cal membranes and are mainly concentrated in the cytoplas-
ic layer (59). The binding of synapsin I is in fact absent or
negligible with phosphatidylcholine or phosphatidylcholine/
phosphatidylethanolamine vesicles, whereas it is directly
proportional to the percentage of acidic phospholipids pre-
sent. The high affinity interaction, however, seems to show
some degree of specificity for phosphatidylserine and phos-
phatidylinositol, since the incorporation of the strongly
negative diacyl phosphate into phospholipid vesicles was
not able to support a high affinity interaction with synapsin I.
The stoichiometry of the binding was fairly low, ~1 synap-
sin I molecule/900 molecules of acidic phospholipids. This
value is not far from the stoichiometries reported in the liter-
ature for other protein-phospholipid interactions (20, 31,
35). Endogenous phospholipids extracted from highly puri-
fied small synaptic vesicles behaved like the commercial
phospholipids used in the experiments described here. How-
ever, this does not prove that such an interaction actually oc-
curs in the native vesicles, and, furthermore, it does not en-
tirely account for the binding of synapsin I to synaptic
vesicles, since the vesicle protein component is also able to
bind synapsin I (8) and synapsin I binding to intact vesicles
is sensitive to its phosphorylation state (51).
The domain of synapsin I involved in the interaction with
phospholipids was determined using fragments generated by
cysteine-specific cleavage. The resulting fragments, identi-
that the association of synapsin I with phospholipid vesicles is initiated by an electrostatic attraction involving the head domain. At this point, after a close contact with the amphiphilic environment of the membrane, a conformational change in the synapsin I molecule might occur with an increase of the amphiphilic α-helix in the head region, which contains significant amounts of hydrophobic amino acids. This conformational change may be followed by insertion of the hydrophobic surface into the bilayer with the helical axis roughly tangential to the vesicle surface at a depth allowing the formation of hydrophobic interactions. Such a model has been proposed for the interaction of amphiphilic peptides with phospholipid bilayers (31).

The possibility of a conformational change in synapsin I upon contact with amphiphilic environments is supported by data obtained from circular dichroism analysis of synapsin I secondary structure (M. Ho et al., manuscript in preparation). The analysis of synapsin I spectra indicated that there is a considerable increase in the alpha helical content upon going from aqueous buffer (25% alpha helix) to 50% trifluoroethanol, a solvent known to mimic the hydrophobic environment of the membrane (43% alpha helix). These results suggest that synapsin I has a significant potential to form ordered structures at membrane surfaces that may mediate strong hydrophobic interactions with the bilayer. In this view, the acidic phospholipids interacting with synapsin I may have a permissive role for the occurrence of the hydrophobic interaction, allowing a close contact with the bilayer through electrostatic forces; such a contact could trigger a conformational change followed by the penetration of the ordered hydrophobic domains of the molecule into the membrane. The existence of such hydrophobic interactions may be partly responsible for the high affinity of synapsin I association with phospholipid vesicles. The strongly charged tail fragment does not bind with high affinity to the phospholipid vesicles. Similarly, the weak positive charge of pure phosphatidylycholine vesicles may prevent the occurrence of a close contact between synapsin I and the bilayer, a prerequisite for the hydrophobic interaction.

Several proteins have been reported to interact with phospholipid bilayers and, in some cases, to penetrate the hydrophobic core of the membrane. A physiological role for the interaction of apolipoproteins, surfactant-associated protein SAP-35 and several cytoskeletal proteins with phospholipids has been suggested. In the case of apolipoproteins, the lipid binding coincides with the biological activity of carrying lipids in an aqueous environment (22, 31); in the case of SAP-35, it is probably involved in the extracellular organization of the surfactant phospholipids in a regular lattice on the alveolar surface (32, 49); in the case of cytoskeletal proteins, it may mediate their association with the cytoplasmic surface of the plasmalemma or of subcellular structures (28, 33, 47, 48, 50, 56).

Synapsin I has a peculiar molecular structure characterized by high surface activity (M. Ho et al., manuscript in preparation) and a very basic isoelectric point (55); it associates in vivo and in vitro with synaptic vesicles (16, 17, 27, 44) to which it binds in a phosphorylation-dependent manner (51), and it binds to F-actin in vitro exhibiting a remarkable phosphorylation-dependent bundling activity (2). In the nerve terminal, synapsin I is thought to be involved in the modulation of neurotransmitter release (37). The results of the present study demonstrate that the head domain of synapsin I is able to insert into lipid bilayers. It will be of importance to determine the extent to which this interaction contributes to the selective association with synaptic vesicles as well as the extent to which it is involved in mediating reversible interactions between vesicles and other structures such as the cytoskeleton and the presynaptic membrane.

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