Synapsin I–Mediated Interaction of Brain Spectrin with Synaptic Vesicles

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Abstract. We have established a new binding assay in which ^{125}I-labeled synaptic vesicles are incubated with brain spectrin covalently immobilized on cellulosic membranes in a microfiltration apparatus. We obtained saturable, high affinity, salt- (optimum at 50–70 mM NaCl) and pH- (optimum at pH 7.5–7.8) dependent binding. Nonlinear regression analysis of the binding isotherm indicated one site binding with a K_d = 59 μg/ml and a maximal binding capacity = 1.9 μg vesicle protein per μg spectrin. The fact that the binding of spectrin was via synapsin was demonstrated in three ways. (*a*) Binding of synaptic vesicles to immobilized spectrin was eliminated by prior extraction with 1 M KCl. When the peripheral membrane proteins in the 1 M KCl extract were separated by SDS-PAGE, transferred to nitrocellulose paper and incubated with ^{125}I-brain spectrin, 96% of the total radioactivity was associated with five polypeptides of 80, 75, 69, 64, and 40 kD. All five polypeptides reacted with an anti-synapsin I polyclonal antibody, and the 80- and 75-kD polypeptides comigrated with authentic synapsin Ia and synapsin Ib. The 69- and 64-kD polypeptides are either proteolytic fragments of synapsin I or represent synapsin IIa and synapsin IIb. (*b*) Pure synapsin I was capable of competitively inhibiting the binding of radioiodinated synaptic vesicles to immobilized brain spectrin with a K_I = 46 nM. (*c*) Fab fragments of anti-synapsin I were capable of inhibiting the binding of radioiodinated synaptic vesicles to immobilized brain spectrin. These three observations clearly establish that synapsin I is a primary receptor for brain spectrin on the cytoplasmic surface of the synaptic vesicle membrane.

NEUROTRANSMITTER release is a specialized instance of regulated exocytotic secretion (Kelly, 1988). Synaptic vesicles held close to the active zone of the presynaptic plasma membrane through interaction with the cytoskeleton are released upon nerve stimulation, dock at a release site on the cytoplasmic surface of the plasma membrane via interaction with a docking protein, and then fuse with the membrane releasing neurotransmitter (Kelly, 1988, DeCamilli et al., 1990). Two observations indicate that spectrin may play an important role in these early stages of neurotransmission. Spectrin has been found associated with the cytoplasmic surface of small spherical synaptic vesicles within the cytoplasm of the nerve terminal (Zagon et al., 1986), and molecules resembling spectrin have been demonstrated to link synaptic vesicles to the active zone of the presynaptic plasma membrane (Landis et al., 1988; Hirokawa et al., 1989).

Since the discovery of nonerythroid spectrin (Goodman et al., 1981), brain spectrin has become the most extensively studied member of this family of proteins (for review see Goodman et al., 1988). Mammalian neurons have been demonstrated to contain two distinct spectrin isoforms, both of which are $(\alpha\beta)_2$ tetramers with a 240-kD α subunit and a 235-kD β subunit (Reiderer et al., 1986). Brain spectrin (240/235) is found in axons and soma, while brain spectrin (240/235E) is located in soma and dendrites of neurons from various mammalian species (Riederer et al., 1986, 1988). Immunoelectronmicroscopic investigation of spectrin localization within mammalian neurons, indicated a high concentration of brain spectrin (240/235) in the presynaptic terminal associated with the cytoplasmic surface of 50-nm-diam spherical synaptic vesicles and the plasma membrane (Zagon et al., 1986). In addition, recent quick-freeze deep-etch electron microscopy of presynaptic terminals have demonstrated small spherical synaptic vesicles in contact with long thin strands (>100 nm long) which were thought to be brain spectrin (Landis et al., 1988; Hirokawa et al., 1989).

In this report, we describe that purified 50-nm synaptic vesicles bind to a single class of high affinity binding sites on immobilized spectrin (240/235) in vitro. We demonstrate that a high affinity attachment site on the vesicle surface is synapsin I; a synaptic vesicle protein previously suggested to play an essential role in the early events of synaptic transmission (for reviews see Goodman et al., 1988; DeCamilli et al., 1990). The phosphorylation of synapsin I has previously been correlated with membrane depolarization and neuro-

transmitter release under a variety of physiological conditions (for review see DeCamilli et al., 1990). The current report is the first demonstration that synapsin I represents a high affinity attachment site for brain spectrin on the cytoplasmic surface of the synaptic vesicle membrane.

Materials and Methods

Materials

^{125}I-labeled Bolton-Hunter Reagent and ^{125}I-protein A were from Dupont-New England Nuclear (Boston, MA); DTT and Aquacide II were from Calbiochem-Behring Corp. (San Diego, CA); diisopropylfluorophosphate (DFP), PMSF, leupeptin, pepstatin A, Triton X-100, Tween 20, BSA, and EGTA were from Sigma Chemical Co. (St. Louis, MO); Sephacryl S-500 and Sepharose 2B were from Pharmacia Inc. (Piscataway, NJ); controlled pore glass beads (Glyceryl-CPG GLY03000B) were from Electro-Nucleonics (International, SW Breda, Netherlands), cellulosic membranes containing aldehyde groups (Memtest, 0.65 μm) were from Memtek (Amicon Division, Beverly, MA); and nitrocellulose membranes (0.45 μm) were from Millipore Continental Water Systems (Bedford, MA). Antiserum against bovine synapsin was prepared in rabbit as described (Krebs et al., 1987). Fab fragments from rabbit antisynapsin serum and from mouse anti-bovine synaptophysin (Boehinger Mannheim Diagnostics, Inc., Houston, TX) were isolated with the use of immobilized protein A and immobilized papain (Pierce Chemical Co., Rockford, IL) according to manufacturer's instructions.

Procedures

Bovine Brain Spectrin Isolation. Bovine brains were obtained from the local slaughter house within 30 min of death, processed according to Bennett et al. (1986), and stored up to 8 wk at −70°C. Spectrin was purified according to the same authors with the exception that the last DEAE-cellulose column chromatography step was omitted. Usually 100 g of frozen tissue was used and the gel filtration was carried out with a Sephacryl S-500 column (3× 100 cm). The tetrameric spectrin peak contained only spectrin bands and minimal (~1%) proteolytic fragment of the alpha subunit (160 kD).

When isolated spectrin was to be immobilized on aldehyde group containing matrix, the pooled fractions were concentrated by dialysis against Aquacide II up to 300–350 μg/ml and dialyzed against 0.5 M Na_2CO_3 pH 9.3. If spectrin was to be labeled with ^{125}I-Bolton-Hunter reagent, 4 ml of the concentrated tetramer (~500 μg/ml in column buffer, pH 8.2; Bennett et al., 1986) was used for the reaction with ~3,000 kBq of the reagent. After labeling, the mixture was subjected to chromatography on a Sephacryl S-500 column (2.0 × 60 cm) equilibrated with 5 mM $NaPO_4$, 1 mM EGTA, 0.2 mM DTT, pH 7.5. The tetrameric spectrin peak was collected.

Isolation of Small Synaptic Vesicles. Small synaptic vesicles were isolated according to the published protocol of Huttner et al. (1983), with the exception that buffers contained DFP (400 μM) to prevent proteolysis. Fractions from controlled pore glass bead column chromatography were tested for synapsin I content in quantitative dot immunobinding assay according to Jahn et al. (1984), using rabbit antibovine synapsin I antiserum at 1:250–1:500 dilutions and isolated bovine brain synapsin I as a standard. We have determined that synapsin I constitutes ~3% of the synaptic vesicle protein. Synapsin I was isolated from bovine brain according to the published method (Krebs et al., 1986).

Labeling of Isolated Vesicles with ^{125}I-Bolton-Hunter Reagent. Isolated vesicles were first washed with 300 mM sucrose in 5 mM $NaPO_4$, 20 mM NaCl, 1 mM EGTA, pH 8.0. Vesicle suspension (0.2 ml, 600–800 μg protein) was added to 0.2 ml of the same buffer containing ^{125}I-Bolton-Hunter reagent (~3,000 kBq) and incubated on ice for 1 h. The mixture was then applied to a disposable column filled with Sepharose 2B (0.5 × 18 cm) equilibrated with the same buffer. The first peak containing labeled vesicles was collected. Labeled vesicles were added to unlabeled vesicles to obtain the desired concentration and specific radioactivity.

Brain Spectrin–Synaptic Vesicle Binding Assay. Memtek 65 μm membranes were washed briefly in distilled water and blotted dry on filter paper, soaked for 15 min in 0.5 M sodium carbonate pH 9.5, and blotted dry again. The membrane was then placed in a Bio-Dot apparatus (Bio-Rad Laboratories, Richmond, CA) and 5–20 μl of purified brain spectrin (250–500 μg/ml) dialyzed against 0.5 M sodium carbonate, pH 9.5, was added to the wells. After 2 h at room temperature the membrane was treated for 15 min with 50 μl per well 0.1% sodium borohydride in PBS (5 mM $NaPO_4$, 150 mM NaCl, pH 7.4). Typically half of the wells were treated identically, but without added spectrin. The membrane in these wells served as control matrix. To quantitate the binding of spectrin to the membrane, ^{125}I-labeled spectrin dialyzed against sodium carbonate pH 9.5 was used as a ligand in separate wells. The membranes were washed with PBS (3 × 200 μl), 1 M NaCl (2 × 200 μl), PBS (2 × 200 μl), PBS containing 0.5% Tween 20 (2 × 200 μl), and finally test buffer (2 × 200 μl) (either 5 mM $NaPO_4$ or Tris-HCl, pH 7.5 containing 1 mM EGTA, 0.2 mM DTT, and 20 μg/ml PMSF). The incubation mixture (total volume 200 μl) contained in 5 mM Tris or phosphate buffer: vesicle protein (2–40 μg), 65 mM NaCl, 50 μg/ml BSA, and 20 μg/ml PMSF. After 30 min at room temperature unbound vesicles were removed by suction and the membrane was washed twice with 50 μl of the test buffer without BSA. The apparatus was disassembled, the membrane was marked and cut into pieces containing the well area and counted in a gamma counter (Packard Autogamma 500C; Packard Instrument Company, Meriden, CT). All points were performed in duplicate, and controls (<30%) are routinely subtracted.

Extraction of Peripheral Membrane Proteins from the Synaptic Vesicles. Purified synaptic vesicles were resuspended in 200 μl extraction buffer (10 mM $NaPO_4$, pH 7.6, 1 M KCl, 1 mM EDTA, 0.2 mM DTT, 0.55 mM DFP) to a final vesicle protein concentration of 5 mg/ml. The vesicles were incubated for 2 h at 0°C in extraction buffer, and sedimented at 360,000 *g* (2 h, 2°C). This high salt extract found in the supernatant was used for subsequent Western blotting and spectrin blotting as described below.

SDS-PAGE and Western Blotting. SDS-PAGE was performed using the Laemmli (1970) buffer system, and protein was either stained with Coomassie blue (Fairbanks et al., 1971) or transferred to nitrocellulose paper for blotting experiments (Towbin et al., 1979).

The high ionic strength extract from synaptic vesicles (50 μg/lane) transferred to nitrocellulose paper was incubated at room temperature with a polyclonal rabbit antibody against synapsin I (1:1,000, overnight). We have previously published the characterization of this synapsin I antibody (Krebs et al., 1987). Before antibody incubation, the nitrocellulose filter was blocked (2–5 h) with 5% nonfat milk, in 50 mM Tris-Cl pH 7.4, 150 mM NaCl. The anti-synapsin I antibody was incubated in the same buffer + 0.1% Tween 20. After washing away unbound antibody, the filters were incubated with ^{125}I-protein A (3.7 kBq/ml) in the buffer given above for 3 h, washed, dried, and autoradiographed.

Spectrin Blotting. Nitrocellulose strips containing 50 μg high ionic strength extract from the synaptic vesicles were incubated overnight at room temperature in spectrin blotting buffer (10 mM $NaPO_4$, 55 mM NaCl, 1 mM EDTA, 1 mM NaN_3, 0.2% Triton X-100, 4% BSA, pH 7.6) containing 10 nM ^{125}I-bovine brain spectrin (150 × 10^6 cpm/nm). Controls contained 10 nM ^{125}I-bovine brain spectrin plus 0.5 μM unlabeled spectrin. After incubation the strips were washed with spectrin blotting buffer minus BSA (five times, 60 min/wash), spectrin blotting buffer minus BSA and Triton X-100 (three times, 60 min/wash), and were then dried and autoradiographed.

Protein Determinations. Protein was determined according to Bradford (1976) using Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories) and BSA as a standard.

Results

Binding of Synaptic Vesicles to Brain Spectrin

To quantitatively analyze the association of purified 50-nm-diam spherical synaptic vesicles with brain spectrin, it was essential to develop an in vitro assay in which free and bound vesicles were easily and quantitatively separated. Since differences in sedimentation properties were too small, conventional sedimentation methods were found to be inapplicable. However, the immobilization of purified brain spectrin on cellulosic membranes containing free aldehyde groups allowed the separation of bound and free vesicles, with as many as 96 samples run quickly and simultaneously using a microfiltration apparatus.

In preliminary studies the attachment of ^{125}I-labeled synaptic vesicles to immobilized brain spectrin was found to be rapid, with equilibrium reached within 1 min at 22°C (data not shown). The synaptic vesicle–spectrin interaction was ionic strength dependent (Fig. 1), with an optimum at 50–70

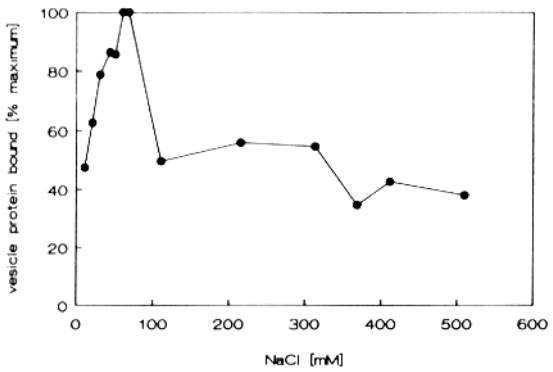


Figure 1. Effect of NaCl on binding of labeled ^{125}I small synaptic vesicles to immobilized spectrin. ^{125}I-vesicles (21 μg/ml, 3,750 cpm/μg) were incubated in 5 mM $NaPO_4$ containing 1 mM EGTA, 0.2 mM DTT, 50 μg/ml BSA 20 μg/ml PMSF, pH 7.5, with 1.09 μg immobilized brain spectrin. NaCl (1 M solution in the above buffer) was added to achieve indicated concentrations. Further steps and controls were as described in Materials and Methods.

mM NaCl, and ~50% optimal binding at 150 mM NaCl. It is of interest that quantitative immunodot assays indicate that under the conditions of our assay, 50–60% of the major synaptic vesicle protein synapsin I is released from the vesicles by 500 mM NaCl, correlating with the 50–60% inhibition of vesicle binding to spectrin. The pH optimum was at pH 7.5–7.8 (Fig. 2).

Using the optimal buffer conditions described above, we studied the concentration dependent binding of ^{125}I-synaptic vesicles to immobilized brain spectrin. A binding isotherm typical of eight independent experiments is presented in Fig. 3 *A*, with Scatchard analysis presented in Fig. 3 *B*. To rule out the possibility that vesicles containing the basic protein synapsin I on their surface are binding nonspecifically to the electronegative protein spectrin; we demonstrate in Fig. 3 *A* that two other electronegative proteins, immobilized bovine serum albumin and ovalbumin, did not bind ^{125}I-labeled synaptic vesicles. Both the Scatchard analysis and nonlinear regression analysis of the binding isotherm (Fig. 3 *A*) performed with a ENZFITTER computer program (R. J. Leatherbarrow, Biosoft, Incorporated, Milltown, NJ) indicated that our saturable binding isotherms best fit a one-site model. Because of the well known limitations of using Scatchard analysis to determine K_D and β max values for ligand–vesicle interactions, we have used the ENZFITTER computer program to perform nonlinear regression analysis on the data from binding isotherms such as that presented in Fig. 3 *A*. Nonlinear regression analysis of eight independent binding studies has indicated a mean K_D = 59 μg/ml vesicle protein and a maximal binding capacity (β max) = 1.9 μg vesicle protein bound/μg brain spectrin. As several vesicle proteins and lipids on the cytoplasmic surface of the synaptic vesicles would be ^{125}I labeled with Bolton Hunter reagent, the validity of our assay requires that the labeled components remain associated with the synaptic vesicles during the course of our incubations. When ^{125}I-labeled vesicles were incubated at room temperature for 30 min under assay conditions, and vesicles sedimented at 200,000 *g* for 30 min, 95–97.5% of the total ^{125}I label and synapsin I (calculated by quantitative immunodot assay) were found in the vesicle pellet. Therefore, the vesicles can be treated operationally as a pure homogenous ligand (despite the fact that several components are ^{125}I labeled) when calculating the vesicle affinity and maximal binding capacity for brain spectrin with binding isotherms.

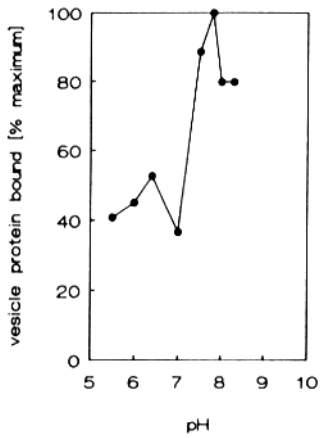


Figure 2. Effect of pH of the reaction buffer on binding of labeled synaptic vesicles to immobilized brain spectrin. ^{125}I-synaptic vesicles (56 μg/ml, 3,785 cpm/μg) were incubated in phosphate buffers of indicated pH with 1.09 μg of immobilized brain spectrin. Final concentrations of 45 mM $NaPO_4$, 7.0 mM NaCl, 1 mM EGTA, 0.2 mM DTT, and 50 μg/ml BSA, 20 μg/ml PMSF were used. Further steps and controls as described in Materials and Methods.

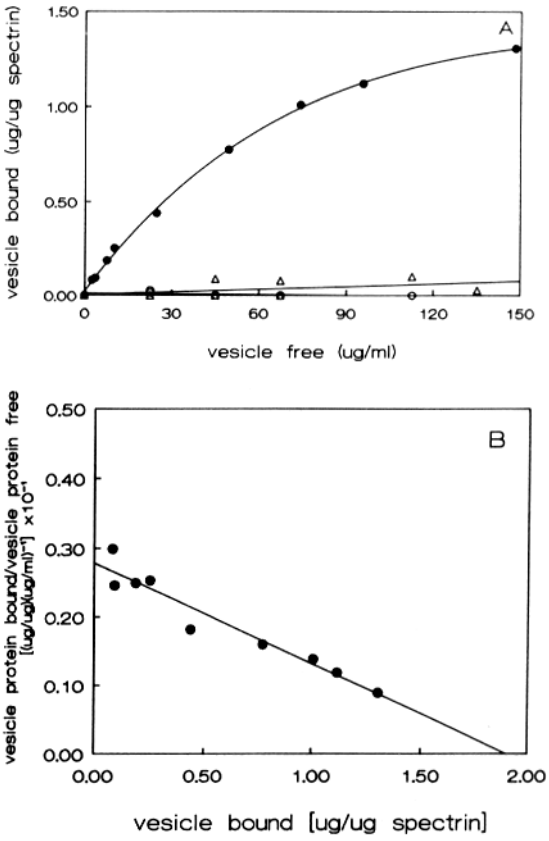


Figure 3. Binding of increasing concentrations of ^{125}I-labeled synaptic vesicles to immobilized brain spectrin. (*A*) Varying concentrations of ^{125}I-vesicles (sp act 3,785 cpm/μg) were incubated in a final volume of 200 μl of 5 mM Tris-HCl, 65 mM NaCl, 1 mM EGTA, 0.2 mM DTT, 50 μg/ml BSA 20 μg/ml PMSF, pH 7.5 in a Bio-Dot filtration apparatus with Memtest membrane containing 1.09 μg covalently attached brain spectrin (●), 0.75 μg bovine serum albumin (○), or 1.25 μg ovalbumin (△) per well. Further steps and controls as described in Materials and Methods. (*B*) Data from *A* transformed into Scatchard (1949) plot.

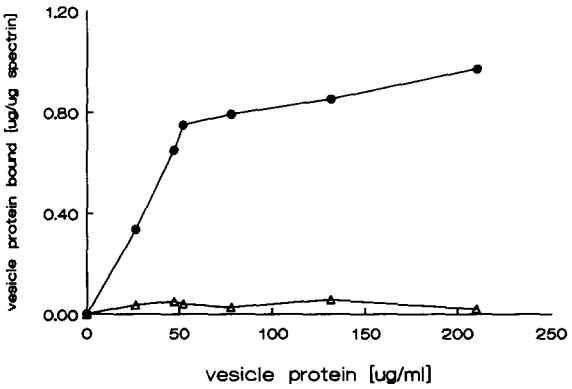


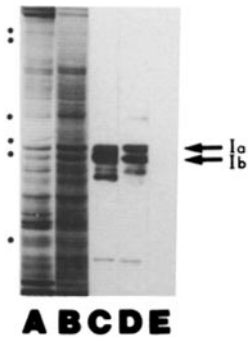
Figure 4. Comparison of binding of ^{125}I-labeled small synaptic vesicles (●) and 1 M KCl extracted vesicles (△) to immobilized bovine brain spectrin. The vesicles were incubated 30 min in a final volume of 200 μl of 5 mM $NaPO_4$, 20 mM NaCl, 1 mM EGTA, 0.2 mM DTT, 100 μg/ml BSA 20 μg/ml PMSF, pH 7.4, at room temperature with immobilized brain spectrin (2.6 μg/well) in a Bio-Dot apparatus. Unbound material was removed by suction and the wells were washed twice with 50 μl of the above buffer without BSA. For details see Materials and Methods.

Synaptic Vesicles Bind to Brain Spectrin Via Synapsin I

When we compared the binding of synaptic vesicles as isolated, to vesicles that were extracted with 1 M KCl, we obtained up to a 97% decrease of the binding of the salt extracted vesicles to immobilized spectrin (Fig. 4). This indicates that the binding site is a peripheral membrane protein.

We reasoned that the binding protein(s) responsible for spectrin's attachment to synaptic vesicles would be found in the 1 M KCl supernatant after sedimenting the salt depleted vesicles. We therefore separated the 1 M KCl extracted proteins by SDS-PAGE, transferred the proteins to nitrocellulose, and then probed the blots with 10 nM ^{125}I-brain spectrin (Fig. 5). Polypeptides of 95, 80, 75, 69, 64, and 40 kD bound ^{125}I-spectrin, and this binding was eliminated with excess (0.5 μM) unlabeled spectrin (Fig. 5, *top*, lanes *D* and *E*). The 80- and 75-kD polypeptides comigrated with authentic purified bovine synapsin Ia and Ib, and anti-synapsin I IgG reacted with the 80-, 75-, 69-, 64-, and 40-kD polypeptides (Fig. 5, *top*, lane *C*). Therefore, the 80- and 75-kD spectrin binding proteins are synapsin Ia and Ib; the 69- and 64-kD anti-synapsin reactive peptides are either proteolytic fragments of synapsin I or synapsin IIa and IIb which share >50% sequence homology to synapsin Ia and Ib (Sudhof et al., 1989); and the 40-kD polypeptide is a proteolytic fragment of synapsin I or synapsin II which retains spectrin binding activity. Scanning densitometry of the spectrin blot (Fig. 5, *bottom*) and integration of the peaks indicated that 96.0% of the ^{125}I-labeled spectrin bound is associated with the synapsin I related polypeptides (80, 75, 69, 64, and 40 kD). The only nonsynapsin I related polypeptide that bound spectrin was a 95-kD polypeptide, and only 2.2% of the ^{125}I-spectrin associated with the blotted proteins was associated with this protein.

The experiment described above indicated that synapsin I was a strong candidate as the attachment site for brain spectrin



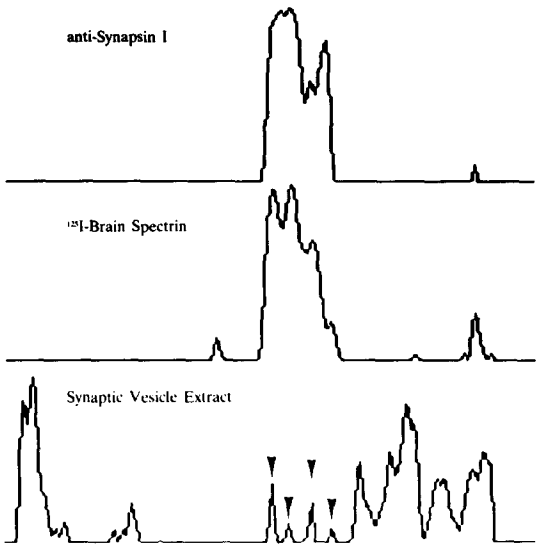


Figure 5. Spectrin blotting of synaptic vesicle peripheral membrane proteins separated by SDS-PAGE. (*Top*) The peripheral membrane proteins extracted from 50 nm spherical synaptic vesicles were separated on a 7% polyacrylamide gel by SDS-PAGE (Laemmli, 1970). 50 μg of protein were loaded per lane and (*A*) stained with Coomassie blue, or transferred to nitrocellulose paper and (*B*) stained with amido black. Alternatively the nitrocellulose filters were incubated with (*C*) anti-synapsin I + ^{125}I-protein A, (*D*) 10 nM ^{125}I-brain spectrin, or (*E*) 10 nM ^{125}I-brain spectrin + 0.5 μM unlabeled brain spectrin. Autoradiography at −70°C was for 30 (lane *C*), and 1 h (lanes *D* and *E*) using Kodak X-Omat XAR-5 film with a Dupont Cronex Lightning Plus intensifying screen. The five dots on the left indicate molecular mass markers: α rbc spectrin (240 kD), β rbc spectrin (220 kD), band 3 (95 kD), protein 4.1 (80 kD), protein 4.2 (72 kD), and actin (43 kD). The Ia and Ib on the right indicate the migration of purified bovine synapsin Ia and synapsin Ib from an adjacent lane (not shown). (*Bottom*) Lanes *A*, *C*, and *D* from the top were scanned and peaks integrated by a Zeneih Lazer Scanning Densitometer. Note that the synapsin I related polypeptides of 80, 75, 69, and 64 kD (shown with arrows) are the major ^{125}I-brain spectrin binding proteins. In addition a 40-kD synapsin related polypeptide (to the right of the scan), and a 95-kD nonsynapsin related polypeptide (to the left of the scan) are two minor spectrin binding proteins.

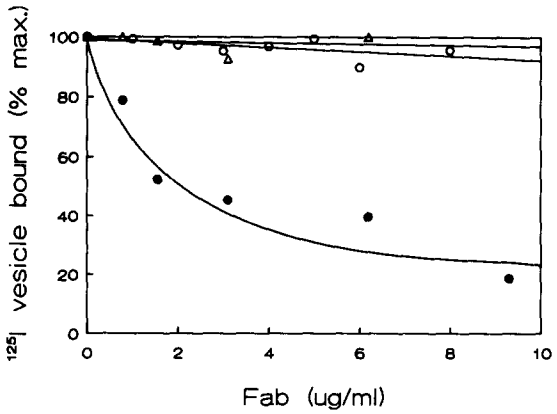


Figure 6. Inhibition of the interaction of ^{125}I-labeled vesicles with immobilized brain spectrin by Fab fragments purified from polyclonal rabbit anti-synapsin antiserum. Vesicles (30 μg/ml) were incubated with immobilized brain spectrin (1.6 μg) in the presence of indicated concentrations of anti-synapsin I Fab fragments (●), anti-synaptophysin Fab fragments (△), or in the presence of anti-synapsin I Fab fragments adsorbed for 2 h with synapsin I (20 μg) immobilized on nitrocellulose (○). The nitrocellulose strip was subsequently blocked with 1 mg/ml BSA in 5 mM $NaPO_4$, 65 mM NaCl, 0.2 mM DTT, 1 mM EGTA, 20 μg/ml PMSF, pH 7.5 for 1 h before use for an adsorption.

on the vesicle surface. To test this hypothesis, we asked whether Fab fragments of anti-synapsin I antibody could block the binding of ^{125}I-labeled vesicles to immobilized brain spectrin. As shown in Fig. 6, anti-synapsin I Fab almost completely abolished the binding of ^{125}I-labeled synaptic vesicles to immobilized brain spectrin, with half-maximal inhibition at ~3 μg/ml Fab. In a control experiment Fab preabsorbed with synapsin I had no ability to block this binding. To rule out the possibility that any Fab directed against a major component of the cytoplasmic vesicle surface might sterically interfere with the vesicle–spectrin interaction, we also demonstrate in Fig. 6 that Fab against the cytoplasmic domain of synaptophysin does not inhibit the binding of ^{125}I-synaptic vesicles to immobilized spectrin.

To directly ask whether synapsin I represented the spectrin attachment site we incubated three fixed concentrations of ^{125}I-labeled synaptic vesicles (18.0, 27.5, and 31.0 μg/ml) with immobilized brain spectrin (0.5 μg/well) in the presence of increasing concentrations of pure synapsin I (Fig. 7 *A*). Dixon analysis (1953) of this data indicates that synapsin I competitively inhibited the binding of ^{125}I-synaptic vesicles to immobilized spectrin with an apparent K_i = 46 nM (Fig. 7 *B*).

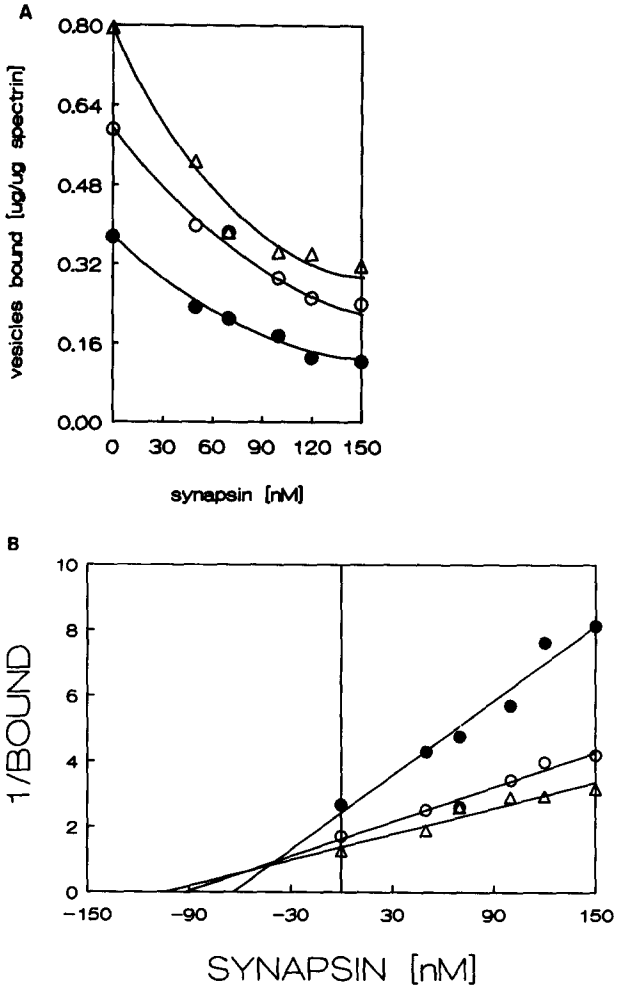


Figure 7. Inhibition of the small synaptic vesicle-spectrin interaction by isolated synapsin I. (*A*) Three concentrations of labeled vesicles: (●) 18.0, (○) 27.5, and (△) 31.0 μg/ml were incubated with indicated concentrations of isolated bovine synapsin I in 5 mM $NaPO_4$, 65 mM NaCl, 1 mM EGTA, 0.2 mM DTT, 50 μg/ml BSA, 20 μg/ml PMSF, pH 7.5, in a Bio-Dot apparatus containing cellulosic membrane with immobilized brain spectrin (0.5 μg/well). Further steps and controls are as specified in Materials and Methods. (*B*) Data presented in a Dixon (1953) plot.

Discussion

We demonstrated that 50-nm-diam synaptic vesicles bind to brain spectrin by a high affinity interaction with a single class of binding sites. The primary binding site on the cytoplasmic surface of the synaptic vesicles has been determined to be synapsin I by three criteria. (*a*) High ionic strength extraction of the vesicles eliminated their ability to bind to brain spectrin, suggesting that the binding site is a peripheral membrane protein. Spectrin blotting of the extracted peripheral membrane proteins demonstrated that 96% of the bound spectrin was associated with synapsin Ia, synapsin Ib, and synapsin related polypeptides of 69, 64, and 40 kD. (*b*) Anti-synapsin I Fab abolishes binding of synaptic vesicles to immobilized spectrin; while anti-synaptophysin Fab has no effect upon binding. (*c*) Purified synapsin I competitively inhibits the binding of synaptic vesicles to immobilized brain spectrin with K_i = 46 nm.

Now that we have demonstrated that synapsin I is the primary synaptic vesicle binding site for spectrin, we can calculate the affinity and stoichiometry of this interaction on the membrane surface. Returning to the binding isotherm, which indicated a K_D and maximal binding capacity of 59 μg/ml and 1.9 μg/μg, respectively, and having calculated that synapsin I represents 3% of the vesicle protein by quantitative immunodot assay, we have calculated a K_D = 24 nM synap-

sin I and a stoichiometry of 0.8 mol synapsin I/mol spectrin on the membrane surface (M_r = 75 kD [synapsin] and 1,000 kD [spectrin tetramer]). The K_D of 24 nM synapsin I on the membrane surface is only a little lower than the K_I of 46 nM for synapsin I inhibition of the synaptic vesicle–spectrin interaction, and could indicate a slightly tighter binding of these proteins on the membrane surface. The ~1:1 stoichiometry must be viewed with the understanding that immobilized spectrin tetramer may not have both of its potential binding sites sterically available for synaptic vesicle binding. Indeed, it is possible that other sites along the spectrin molecule may not be available because of adherence to the cellulosic membrane. However, there is no a priori reason to believe that any one site along the spectrin molecule would be preferentially linked to the free aldehyde groups on the cellulosic membrane.

Our finding that synapsin I represents the major binding site on the vesicle membrane surface for brain spectrin is consistent with several earlier observations. Brain spectrin had previously been demonstrated to bind synapsin I on nitrocellulose blots (Baines and Bennett, 1985) and in solution (Krebs et al., 1987). Our previous analysis of the binding of pure synapsin I to pure brain spectrin (240/235) was performed at 0°C and used a gel filtration assay (Krebs et al., 1987). The K_D value obtained in the earlier study was 700 nM, considerably higher than the K_I value obtained in the current study (46 nM). However, since our current study was performed at 22°C, the different values could be due to increased affinity at higher temperature, differences in assay conditions, or both. The significance of the current work is that it demonstrates for the first time that the interaction of brain spectrin with synapsin I is of biological significance on the synaptic vesicle surface. We have previously demonstrated by rotary shadowing and electronmicroscopy that synapsin I bindings to the ends of the spectrin tetramer at a site close to the actin binding site (Krebs et al., 1987). Furthermore we have shown that brain spectrin binds end-on to small spherical synaptic vesicles (Goodman et al., 1988). Because of the current work, we now appreciate that the end-on binding of spectrin to the synaptic vesicles is due to the attachment of synapsin to the ends of the spectrin tetramer.

An important question is the functional significance of the brain spectrin–synaptic vesicle interaction. We can think of two nonmutually exclusive possibilities. Immunoelectronmicroscopy has indicated a high concentration of brain spectrin associated with synaptic vesicles in the cytoplasm of the presynaptic terminal (Zagon et al., 1986), where synaptic vesicles are closely associated with actin filaments (Landis et al., 1988; Hirokowa et al., 1989). Synapsin I attaches the synaptic vesicles to brain spectrin (this report), at a site close to the actin binding site (Krebs et al., 1987; Goodman et al., 1988). Therefore, synapsin I may attach synaptic vesicles to a spectrin–actin meshwork in the cytoplasm (Goodman et al., 1988), until the Ca^{2+}-mediated phosphorylation allows release of the vesicles and movement towards the presynaptic membrane. Our new assay system for studying the interaction of purified 50-nm-diam synaptic vesicles with immobilized brain spectrin, gives us a direct way of observing the regulation of this interaction by synapsin I phosphorylation or Ca^{2+}-calmodulin in future studies. A second possible functional role is based upon the observation (Landis et al., 1988; Hirokawa et al., 1989) that filaments of the size of spectrin appear to link synaptic vesicles to the plasma membrane. It seems possible that spectrin which is associated with the presynaptic plasma membranes (Zagon et al., 1986) may play an important role in modulating the docking and fusion of the synaptic vesicles. Future studies must address these potential functional roles.

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