

# Interactions of Synapsin I with Phospholipids: Possible Role in Synaptic Vesicle Clustering and in the Maintenance of Bilayer Structures

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**Abstract.** Synapsin I is a synaptic vesicle-specific phosphoprotein composed of a globular and hydrophobic *head* and of a proline-rich, elongated and basic *tail*. Synapsin I binds with high affinity to phospholipid and protein components of synaptic vesicles. The *head* region of the protein has a very high surface activity, strongly interacts with acidic phospholipids and penetrates the hydrophobic core of the vesicle membrane. In the present paper, we have investigated the possible functional effects of the interaction between synapsin I and vesicle phospholipids. Synapsin I enhances both the rate and the extent of  $\text{Ca}^{2+}$ -dependent membrane fusion, although it has no detectable fusogenic activity per se. This effect, which appears to be independent of synapsin I phosphorylation and local-

ized to the *head* region of the protein, is attributable to aggregation of adjacent vesicles. The facilitation of  $\text{Ca}^{2+}$ -induced liposome fusion is maximal at 50–80% of vesicle saturation and then decreases steeply, whereas vesicle aggregation does not show this biphasic behavior. Association of synapsin I with phospholipid bilayers does not induce membrane destabilization. Rather,  $^{31}\text{P}$ -nuclear magnetic resonance spectroscopy demonstrated that synapsin I inhibits the transition of membrane phospholipids from the bilayer ( $L_{\alpha}$ ) to the inverted hexagonal ( $H_{II}$ ) phase induced either by increases in temperature or by  $\text{Ca}^{2+}$ . These properties might contribute to the remarkable selectivity of the fusion of synaptic vesicles with the presynaptic plasma membrane during exocytosis.

**S**YNAPSIN I is a major neuron-specific phosphoprotein specifically associated with the cytoplasmic side of synaptic vesicles, the organelles that store and release neurotransmitter (De Camilli et al., 1983a,b; Huttner et al., 1983; Navone et al., 1984; Nestler and Greengard, 1984; De Camilli et al., 1990). Synapsin I is composed of a globular and hydrophobic  $\text{NH}_2$ -terminal region (*head*) and of a proline-rich, elongated and basic  $\text{COOH}$ -terminal region (*tail*) (Ueda and Greengard, 1977). Synapsin I has a very high surface activity and forms stable monolayers at an air-water interface, properties which are mostly attributable to the presence of amphiphilic secondary structures within the *head* region of the protein (Südhof et al., 1989; Ho et al., 1991).

The binding of synapsin I to synaptic vesicles displays high affinity ( $K_d = 10$  nM), saturability and sensitivity to phos-

phorylation of the protein by  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaM kinase II)<sup>1</sup> on the *tail* region (Schiebeler et al., 1986). The binding appears to consist of multiple interactions of distinct sites of synapsin I with protein and phospholipid components of synaptic vesicles. While the *tail* region specifically binds the regulatory domain of synaptic vesicle-associated CaM kinase II and its binding is virtually abolished by phosphorylation (Benfenati et al., 1989a, 1992b), the *head* region strongly interacts with the acidic phospholipids of the cytoplasmic leaflet of the vesicles and penetrates the hydrophobic core of the vesicle membrane (Benfenati et al., 1989a,b). The major sites responsible for

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1. *Abbreviations used in this paper:*  $B_{\text{max}}$ , maximal binding capacity; CaM kinase II,  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II; cAMP, cyclic AMP; CF, carboxymethylfluorescein;  $H_{II}$ , inverted hexagonal phase;  $L_{\alpha}$ , extended bilayer phase; LRh-PE,  $L$ - $\alpha$ -phosphatidylethanolamine *N*-(lissamine rhodamine B sulfonyl); NBD-PE,  $L$ - $\alpha$ -phosphatidylethanolamine *N*-(4-nitrobenzo-2-oxa-1,3-diazole);  $^{31}\text{P}$ -NMR,  $^{31}\text{P}$  nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

such an interaction are represented by hydrophobic domains present in the NH<sub>2</sub>-terminal part of synapsin I.

The estimated number of synapsin molecules per vesicle (10–30; Schiebler et al., 1986; De Camilli et al., 1990) and the limiting surface area of one synapsin I molecule calculated by film-balance studies (150–180 nm<sup>2</sup>; Ho et al., 1991), suggest that synapsin I covers a large percentage of the vesicle surface. Therefore, it may be involved in the maintenance of the molecular structure and stability of the vesicle membrane and/or play some role in regulating the interactions of synaptic vesicles with other nerve terminal structures such as the actin-based cytoskeleton or the presynaptic membrane.

Synapsin I has the ability to bind actin filaments (Bähler and Greengard, 1987; Petrucci and Morrow, 1987) and to nucleate actin monomers (Valtorta et al., 1992b; Benfenati et al., 1992a) in a phosphorylation-dependent fashion, thereby reversibly cross-linking synaptic vesicles to the actin-based cytoskeleton of the nerve terminal (Ceccaldi, P. E., F. Grohovaz, F. Benfenati, E. Chierregatti, P. Greengard, and F. Valtorta, manuscript submitted for publication) which may make the vesicles unavailable for exocytosis. Phosphorylation of synapsin I by CaM kinase II, by decreasing the ability of synapsin I to bind to the vesicle membrane and to actin, is thought to free synaptic vesicles from the cytoskeletal constraint and to make them available for docking and fusing with the presynaptic membrane upon stimulation (Greengard et al., 1993).

Several lines of evidence suggest that these mechanisms play a role in the regulation of neurotransmitter release from nerve terminals. Injection of dephosphorylated synapsin I into intact neurons or isolated nerve terminals induces a marked inhibition of both spontaneous and evoked neurotransmitter release, due to a decrease in the number of synaptic vesicles available for fusion (Llinas et al., 1985; Lin et al., 1990; Hackett et al., 1990; Llinas et al., 1991; Nichols et al., 1992). This effect is totally absent when synapsin I which has been phosphorylated by CaM kinase II is used.

Synapsin I also appears to be involved in the biogenesis of synaptic vesicles and in the maturation of functional nerve terminals. Microinjection of synapsin I into *Xenopus* embryos accelerates the acquisition of mature quantal release mechanisms by neuromuscular synapses in culture (Lu et al., 1992). In addition, overexpression of synapsin II, a highly homologous protein sharing the *head* region with synapsin I, induces a dramatic increase in the number of varicosities and synaptic vesicles during differentiation of neuroblastoma × glioma hybrid cell lines (Han et al., 1991).

In this study we have investigated the effects of synapsin I on the aggregation and fusion of phospholipid vesicles and on the structure, integrity and permeability of the phospholipid bilayer, with the aim of identifying functional consequences of the interaction between synapsin I and synaptic vesicle phospholipids.

## Materials and Methods

### Materials

[ $\gamma$ -<sup>32</sup>P]ATP (2,900 Ci/mmol) and [<sup>14</sup>C]phosphatidylcholine (0.1 Ci/mmol) were from Amersham Intl. (Buckinghamshire, England). Bovine brain phosphatidylcholine (PC), bovine brain phosphatidylethanolamine (PE), bovine brain phosphatidylserine (PS), bovine liver phosphatidylinositol (PI), *N*-[4-nitrobenzo-2-oxa-1,3-diazole] *L*- $\alpha$ -phosphatidylethanolamine

(NBD-PE) and *N*-(lissamine rhodamine B sulfonyl) *L*- $\alpha$ -phosphatidylethanolamine (LRh-PE) were obtained from Avanti Polar Lipids (Alabaster, AL), stored at –20°C in the dark and used within 3 mo. Carboxymethyl-fluorescein (CF) was from Eastman Kodak Co. (Rochester, NY), calmodulin from Boehringer (Mannheim, Germany), cholesterol, *n*-octyl  $\beta$ -*D*-glucopyranoside (octyl glucoside), and all other chemicals of analytical grade were from Sigma Immunochemicals (St. Louis, MO). Sepharose 2B, Sephacryl S-200, and S-300 were from Pharmacia (Uppsala, Sweden), hydroxylapatite from Bio-Rad Labs (Hercules, CA), and carboxymethylcellulose (CM-52) from Whatman (Maidstone, England). Catalytic subunit of cAMP-dependent protein kinase and CaM kinase II were purified as described (Kaczmarek et al., 1980; McGuinness et al., 1985) from bovine heart and rat brain, respectively. Synapsin I was purified under non-denaturing conditions from bovine brain as previously described (Schiebler et al., 1986; Bähler and Greengard, 1987). The synapsin I COOH-terminal fragment (*tail* fragment) was obtained after cysteine-specific cleavage of the protein and purified as described (Bähler et al., 1989).

### Phosphorylation of Synapsin I

Purified synapsin I was phosphorylated to near stoichiometry at: (a) site 1 using the catalytic subunit of cAMP-dependent protein kinase; (b) sites 2,3 using CaM kinase II; (c) sites 1,2,3 using both kinases as described (Schiebler et al., 1986), except that detergent was omitted. After phosphorylation, synapsin I was repurified by batch adsorption to CM-cellulose. A trace amount of radioactive ATP was added to the reaction mixtures for determining the stoichiometry of phosphorylation.

### Preparation of Phospholipid Vesicles

Phospholipid vesicles of the following composition were prepared: (a) PC vesicles (weight ratio PC/cholesterol = 90:10); (b) mixed phospholipid vesicles (weight ratio PC/PE/PS/PI/cholesterol = 40:32:12:5:10), mimicking the phospholipid composition of synaptic vesicles (Westhead, 1987). Fluorescently labeled vesicles had the same lipid composition with the addition of the appropriate amounts (2% of the total lipid, wt/wt) of NBD-PE or LRh-PE either alone (single-labeled liposomes) or in combination (double-labeled liposomes). Phospholipids and cholesterol (dissolved in chloroform at a concentration of 10 mg/ml) were mixed in a glass test tube, dried to a thin film under a gentle stream of argon, and put under vacuum for at least 2 h to remove residual traces of organic solvent. Unilamellar vesicles were obtained by either of two methods. In one procedure, the dried lipid film was dissolved in 0.5–1.0 ml of a buffer containing 25 mM Tris/HCl (pH 7.4), 150 mM NaCl, 3 mM Na<sub>2</sub>N<sub>3</sub> (buffer A) in the presence of 3% (wt/vol) octyl glucoside (final phospholipid concentration, 3–5 mg/ml). The samples were gently stirred at room temperature for 20 min under argon. The detergent/lipid molar ratio in the final solution was always kept above 10:1. To remove the detergent, the samples were extensively dialyzed against cold, degassed, N<sub>2</sub>-saturated buffer A as described (Mimms et al., 1981; Benfenati et al. 1989a). The vesicle samples were then dialyzed against a low ionic strength isotonic buffer containing 300 mM glycine, 5 mM Hepes (pH 7.4), 3 mM Na<sub>2</sub>N<sub>3</sub> (buffer B). Alternatively, the dried lipid film was resuspended in buffer B by vigorous mixing and vesiculated by bath sonication (model G1128 P1T; Laboratory Supplies Co. Inc., Hicksville, NY) for 15 min at 25°C. The vesicle suspension was cleared from large multilamellar liposomes by high-speed centrifugation in a Beckman TLA-100.2 rotor (55,000 rpm, 20 min). Phospholipid vesicles were kept on ice, used within 2–3 d and diluted with the appropriate buffer immediately before use. Occasionally, phospholipid vesicles were checked by electron microscopy after negative staining or were sized by running them through a 1.5 × 25 cm Sepharose 2B gel filtration column equilibrated with buffer A. Phospholipid concentrations were determined based on the specific activity of the phospholipid mixture to which a trace amount of [<sup>14</sup>C]phosphatidylcholine had been added or by phospholipid phosphorus determination according to the method of Ames (1966). Carboxyfluorescein-containing liposomes were prepared as described above, by sonication in buffer B containing 50 mM CF. Before use, vesicles with encapsulated CF were separated from the medium by a fast gel filtration/centrifugation technique (Schiebler et al., 1986). Small columns were packed with Sephacryl S-300 (3 ml) equilibrated in buffer B and prespun for 1 min at 1,000 × g in a swing-out rotor. After loading of the sample (0.5 ml), the columns were spun at 1,000 g for 1.5 min, and the flow-through collected.

### Turbidity Assay

Changes in vesicle size were monitored by measuring spectrophotometri-

cally the apparent absorption (turbidity) of liposome suspensions. Turbidity was measured in samples containing 50  $\mu\text{g}$  of phospholipids in 600  $\mu\text{l}$  by reading the absorbance at 325 and 600 nm as a function of time on a Beckman DU-8 spectrophotometer. In this assay, the turbidity of a suspension varies as a function of particle size and there is no angular dependence involved (Chong and Colbow, 1976; Gibson and Strauss, 1984).

### Fluorescence Resonance Energy Transfer Assays

Transfer of electronic excitation energy between donor and acceptor fluorophores (fluorescence resonance energy transfer, FRET) incorporated into the membranes of donor and acceptor vesicle populations was used for monitoring vesicle clustering and/or fusion. The energy donor was NBD and the acceptor was LRh. Both fluorophores were covalently attached to PE, thus ensuring stable incorporation into the vesicle membrane. Since the transfer efficiency is inversely related to the sixth power of the distance between the donor and acceptor fluorophores, maximal energy transfer occurs when both donor and acceptor are on the same vesicle or when they are on distinct vesicles which have come into close contact or fused with each other. Minimal energy transfer occurs when donor and acceptor are on different vesicles which are not in contact or when the vesicles are disrupted by the addition of detergent. Fluorescence measurements (steady-state emission spectra and kinetic recordings at fixed wavelengths) were carried out at 22°C using a Perkin-Elmer LS-50 spectrofluorometer by exciting the donor at 470 nm and using excitation and emission slits of 2.5 and 5 nm, respectively. To reduce inner filter effects, the optical density of the sample was always kept below 0.1. At the end of the experiments, Triton X-100 (0.1–1% final concentration) was added to each sample to solubilize vesicles and eliminate energy transfer, allowing the determination of the concentrations of fluorophores and the efficiency of energy transfer. Two assays were used: in the **aggregation/fusion assay** (Hoekstra, 1982; Gibson and Strauss, 1984), the fluorescence donor and acceptor were incorporated into separate vesicle populations. Two populations of vesicles (100  $\mu\text{g}$  of phospholipid for each vesicle population, containing 2% labeled phospholipid) were mixed in a 2 ml cuvette with continuous stirring and energy transfer followed after the addition of synapsin I and/or  $\text{Ca}^{2+}$ . A close proximity between acceptor and donor due to any aggregation and/or fusion involving vesicles labeled with different fluorophores will result in energy transfer which can be followed as either a decrease in NBD emission at 520 nm (NBD quenching) or an increase in LRh emission at 590 nm. In the **fusion assay** (Struck et al., 1981), one population of vesicles containing both fluorophores in equimolar amounts (50  $\mu\text{g}$  phospholipid, 2% labeled phospholipids) was mixed with unlabeled vesicles (150  $\mu\text{g}$  phospholipid). Under these conditions, vesicle fusion induced by the addition of  $\text{Ca}^{2+}$  leads to intermixing of labeled and unlabeled membrane components, resulting in a decrease of the surface density of donor and acceptor fluorophores, i.e., in a decrease of the energy transfer efficiency. Therefore fusion is followed by an increase in NBD emission at 520 nm (NBD dequenching) or a decrease in LRh emission at 590 nm. The total extent of fusion (fluorescence plateau at time  $\infty$ ) and the fusion rate (fluorescence intensity at time 0.5 min) were calculated. In both assays, the evaluation of energy transfer underestimates the extent of aggregation and/or fusion, since it does not detect "silent" aggregation or fusion events involving vesicles belonging to the same population.

### Carboxyfluorescein Leakage Assay

Release of vesicle contents due to membrane destabilization and/or fusion was measured by following the fluorescence of a soluble fluorophore encapsulated within the phospholipid vesicles (Düzgünes et al., 1981). The fluorescence of CF in samples containing CF-loaded vesicles (50  $\mu\text{g}$  phospholipid) was measured at excitation and emission wavelengths of 470 and 520 nm, respectively. Release of CF from the highly concentrated intravesicular solution into the medium, due to destabilization of the lipid bilayer, decreases the intense self-quenching of the fluorophore and can be followed as an increase in fluorescence emission. The release of CF induced by the addition of various proteins and/or  $\text{Ca}^{2+}$  was calculated by subtracting the spontaneous release and expressed in percent of the maximal fluorescence determined by vesicle solubilization with Triton X-100 (1% final concentration).

### $^{31}\text{P}$ Nuclear Magnetic Resonance Spectroscopy

Phospholipid dispersions were prepared as follows. Mixtures of PE-PS or PE-PC (20–40 mg phospholipid) in chloroform were dried to a thin film under argon and traces of organic solvent removed by exposure to vacuum

for several hours. The dried phospholipid mixtures were resuspended in 2 ml of either buffer A (high salt buffer) or buffer B (low salt buffer) containing 25% (vol/vol)  $\text{D}_2\text{O}$  by vigorous vortexing above the gel-to-liquid crystalline transition temperature and by cycles of freeze-thawing (dry ice and 45°C water bath). Alternatively, phospholipids were dissolved at room temperature for 20 min in buffer A containing 3% octyl glucoside, followed by detergent removal with extensive dialysis at 4°C. Synapsin I and/or  $\text{Ca}^{2+}$  were added slowly to the lipid dispersion in plastic tubes with gentle mixing. The samples were incubated for 10 min at room temperature before being transferred to nuclear magnetic resonance (NMR) tubes (10-mm diam; Wilmad Glass Company, Buena, NJ).  $^{31}\text{P}$ -nuclear magnetic resonance ( $^{31}\text{P}$ -NMR) spectra were obtained as previously described on a Bruker AMX400 spectrometer at 161.98 MHz with a broad-band multinuclear probe which was tuned to the sample. A total of 1,200–2,000 scans were accumulated by using a 60° pulse (10  $\mu\text{s}$ ), a 29.4 kHz-sweep width and a 1-s interpulse delay. The power of the proton decoupler (WALTZ216 broad band decoupling sequence) was set to the appropriate level during acquisition and decreased during the relaxation delay to prevent sample heating. Before Fourier transformation, an exponential multiplication was applied to the accumulated free induction decays, resulting in a 50 Hz linebroadening. Spectra were collected at constant temperature obtained by the use of an electrical heater and a liquid nitrogen cooler. All spectra were scaled to the height of the highest peak. The zero level of the p.p.m. scale was calibrated using an external phosphoric acid standard. In some cases,  $^{31}\text{P}$ -NMR spectral analysis was performed on sonicated phospholipid vesicles obtained as described above, and used to assess isotropic peak broadening due to vesicle aggregation (Taraschi et al., 1982).

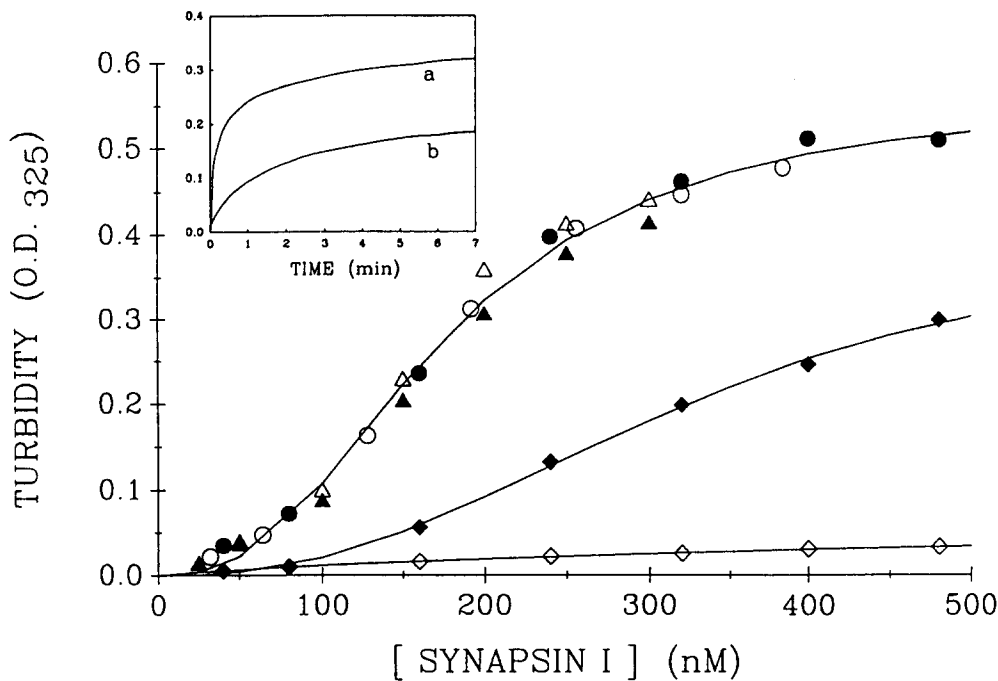
### Other Procedures

The concentration of synapsin I was determined as described (Lowry et al., 1951) using bovine serum albumin as standard. The analysis of the dose-response curves was carried out by using the computer program ALLFIT (De Lean et al., 1978). In some cases, the total concentrations of synapsin I were transformed into percentages of saturation of vesicle sites as previously described (Benfenati et al., 1991).

### Results

Addition of synapsin I to mixed phospholipid liposomes induced a dose-dependent increase in the turbidity of the solution, indicative of a marked increase in the particle size. The effect was already apparent at 25 nM and it approached a plateau at 400 nM, exhibiting an apparent  $\text{ED}_{50}$  of 175 nM synapsin I (Fig. 1). Increasing the ionic strength of the solution from 40 mM to 150 mM NaCl equivalent considerably decreased the magnitude of the effect. No detectable increase in turbidity was observed after the addition of synapsin I to liposomes not containing acidic phospholipids, in agreement with the reported specificity of the binding of synapsin I for PS and PI and with its inability to interact with liposomes composed only of PC or PC/PE. The purified COOH-terminal fragment (*tail*), although strongly basic, did not significantly increase the turbidity of suspensions of mixed phospholipid vesicles at concentrations below 250 nM (not shown). Site-specific phosphorylation of synapsin I had no effect on the synapsin I-induced increase in turbidity of mixed phospholipid liposomes. These observations are compatible with the presence of specific phospholipid-binding sites in the *head* region of synapsin I and with the negligible effects of synapsin I phosphorylation on its binding to liposomes (Benfenati et al., 1989a). The formation of aggregates in the vesicle suspension after the addition of synapsin I was also suggested by the marked broadening of the narrow symmetrical peak typical of phospholipids experiencing isotropic motion (Cullis and Hope, 1979; Cullis and de Kruijff, 1979), as observed in  $^{31}\text{P}$ -NMR spectra (data not shown).

To further investigate this phenomenon, we labeled sepa-



**Figure 1.** Dose-dependent effects of synapsin I on the turbidity of a phospholipid vesicle suspension. Dephosphorylated synapsin I (●), synapsin I phosphorylated on the *head* site by cAMP-dependent protein kinase (○), synapsin I phosphorylated on the *tail* sites by CaM kinase II (▲), or synapsin I phosphorylated by both kinases (△) was added in the final concentrations reported on the X-axis to a sample of mixed phospholipid vesicles (50  $\mu$ g of phospholipid/600  $\mu$ l) in buffer B supplemented with 40 mM NaCl. The steady-state turbidity values, recorded after reaching a stable plateau, are shown on the Y-axis as optical density at 325 nm. The effects of the interactions of dephosphorylated synapsin I with mixed phospholipid vesicles under high

ionic strength (buffer A, ◆) and with pure phosphatidylcholine vesicles under low salt conditions (buffer B plus 40 mM NaCl, ◇) are also shown. (*Inset*) Time-course of the increase in turbidity induced by the addition of 200 nM dephospho-synapsin I to mixed phospholipid vesicles under low (curve *a*) and high (curve *b*) salt conditions.

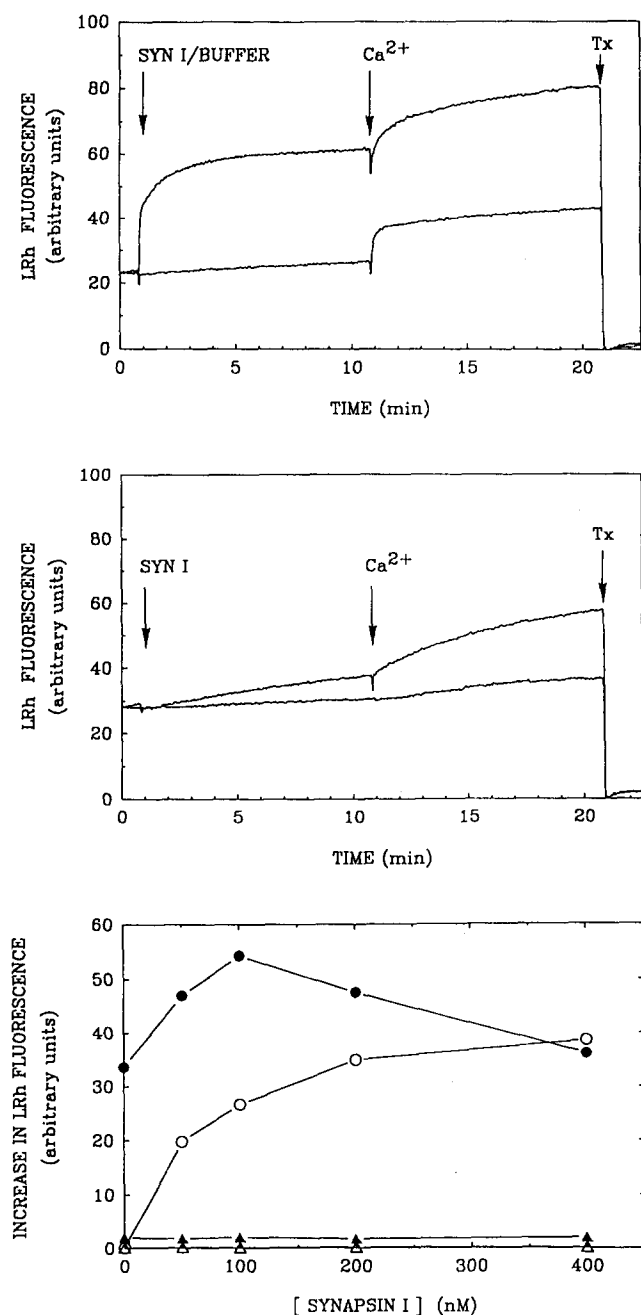
rate populations of vesicles of the same phospholipid composition with a trace amount of either NBD-PE or LRh-PE before mixing them in equimolar amounts in the absence or presence of synapsin I and/or  $\text{Ca}^{2+}$ . The addition of synapsin I (100 nM) to mixed phospholipid vesicles induced a marked decrease in NBD emission accompanied by a concomitant increase in LRh emission, suggesting that the two probes had come into close contact. The subsequent addition of  $\text{Ca}^{2+}$  (3 mM) further increased this effect, whereas solubilization of the vesicles completely removed the excitation of LRh by NBD (Fig. 2, *top*). The time-course of the increase in LRh fluorescence due to synapsin I was very rapid and the final level of LRh emission reached after the subsequent addition of  $\text{Ca}^{2+}$  was much higher than that observed in samples treated with  $\text{Ca}^{2+}$  alone. Increasing the ionic strength strongly decreased the effects of both synapsin I and  $\text{Ca}^{2+}$  on the increase in LRh fluorescence, but did not abolish the potentiation of the  $\text{Ca}^{2+}$ -triggered fluorescence increase induced by synapsin I (Fig. 2, *middle*). Consistent with the results observed in the turbidity assay, the effect of synapsin I was dose dependent, approaching saturation at concentrations above 200 nM (Fig. 2, *bottom*). The increase in LRh fluorescence induced by  $\text{Ca}^{2+}$  exhibited a biphasic pattern, with a maximal effect at 100 nM synapsin I. No detectable effects of either synapsin I or  $\text{Ca}^{2+}$  were observed when pure PC fluorescently labeled vesicles were used.

Such changes induced by synapsin I can in principle be ascribed either to extensive clustering of vesicles which maintain their structural integrity or to extensive membrane fusion giving rise to large vesicular structures. To gather further information on the occurrence of aggregation and/or membrane fusion phenomena in the liposome suspension, we used the vesicle fusion assay in which a population of phospholipid vesicles labeled with equimolar amounts of

both NBD-PE and LRh-PE (and therefore exhibiting maximal NBD to LRh energy transfer) are mixed with unlabeled vesicles. At variance with the previous assay, in this case pure aggregation of vesicles is silent, as it does not affect the surface density of the two fluorophores, whereas fusion of labeled and unlabeled vesicles decreases the energy transfer and increases the emission of NBD.

The upper panel of Fig. 3 depicts the increase in NBD fluorescence observed upon addition of synapsin I and/or  $\text{Ca}^{2+}$  to mixed phospholipid vesicles at low ionic strength. Calcium, by triggering vesicle fusion, increased NBD emission. The addition of synapsin I did not alter fluorescence emission but greatly potentiated the effect of a subsequent addition of  $\text{Ca}^{2+}$ , both in terms of plateau level and of rate of increase. Such an effect was also present when synapsin I was added after  $\text{Ca}^{2+}$ , although in this case the rate of fluorescence increase was not as high as in the samples pretreated with synapsin I. Similarly to what was observed in the previous assay, the effect of synapsin I was decreased by increasing the ionic strength of the medium and was absent when pure PC liposomes were used as unlabeled vesicles. The effect was attributable to the *head* region of synapsin I, since the purified *tail* fragment was totally ineffective and did not alter the effect of  $\text{Ca}^{2+}$  (not shown). Consistent with the turbidity data, phosphorylation of synapsin I by purified protein kinases did not significantly affect the potentiation of  $\text{Ca}^{2+}$ -induced vesicle fusion (not shown).

The effect of the incubation of the vesicles with synapsin I was quantitatively analyzed over a wide range of  $\text{Ca}^{2+}$  concentrations. Synapsin I potentiated  $\text{Ca}^{2+}$ -induced fusion by markedly increasing the sensitivity of the vesicles to the fusogenic action of  $\text{Ca}^{2+}$  (detectable fusion occurred in the presence of synapsin I at  $\text{Ca}^{2+}$  concentrations which were ineffective per se) and by elevating five- to sixfold the rate



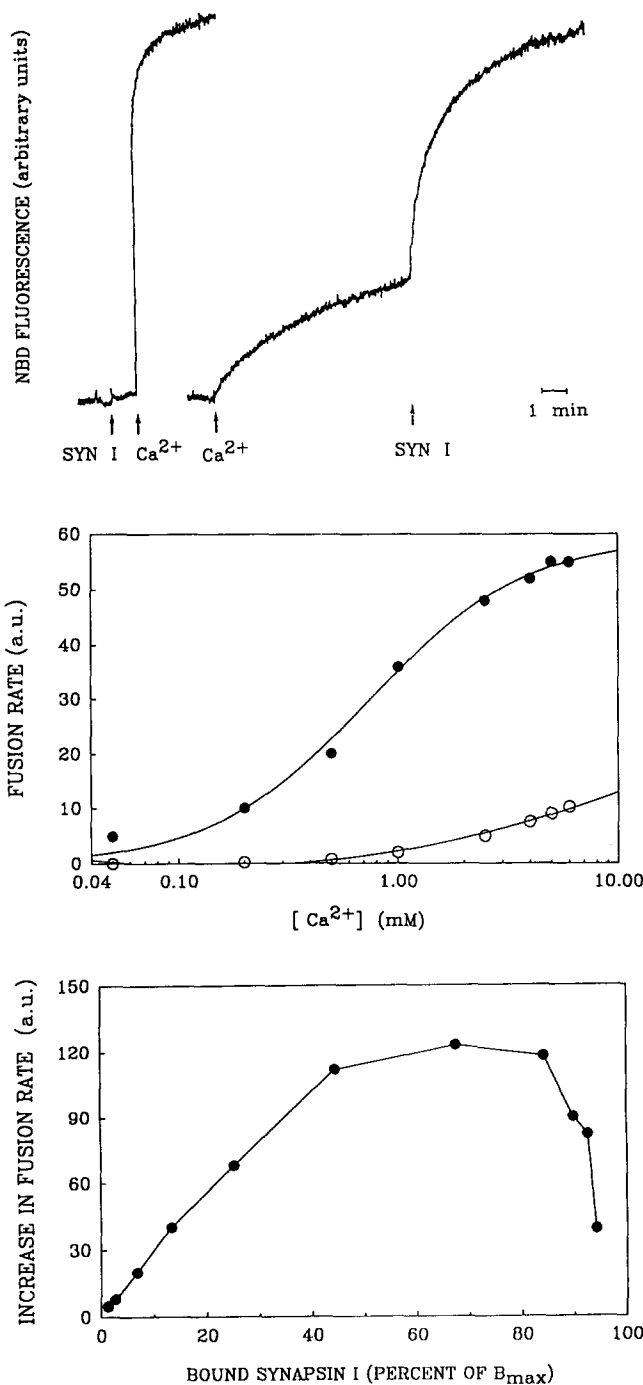
**Figure 2.** Effects of synapsin I and Ca<sup>2+</sup> on the aggregation/fusion of mixed phospholipid vesicles. Synapsin I (50–400 nM) and Ca<sup>2+</sup> (3 mM) were added sequentially to samples of NBD-labeled vesicles (100  $\mu$ g of phospholipid, 2% labeled) and LRh-labeled vesicles (100  $\mu$ g of phospholipid, 2% labeled) mixed in equimolar amounts. Fluorometric analysis was performed by exciting NBD at 470 nm and by recording the LRh emission intensity at 590 nm. (*Top and middle*) The increase in fluorescence of LRh upon addition of 100 nM synapsin I was followed as a function of time under low (buffer B/40 mM NaCl) and high (buffer A) salt conditions, respectively. The lower traces in both panels refer to samples incubated only with synapsin I storage buffer before the addition of Ca<sup>2+</sup>. The minimal energy transfer between NBD and LRh was determined at the end of each time-driven recording by adding 1% Triton X-100 (Tx) to the samples. (*Bottom*) Effects of increasing concentrations of dephosphorylated synapsin I on the plateau levels of fluorescence of samples containing equimolar amounts of NBD- and LRh-labeled liposomes composed either of mixed phospholipids ( $\circ$ ) or of pure PC ( $\Delta$ ). The filled symbols refer to the fluorescence plateau levels reached after the subsequent addition of 3 mM Ca<sup>2+</sup>.

and the extent of vesicle fusion (Fig. 3, *middle*; and not shown). The dependence of the effect of synapsin I on the dose exhibited a biphasic pattern. Based on the concentrations of phospholipids used in the assays and on the binding constants of synapsin I to liposomes of known composition (Benfenati et al., 1989b), it can be calculated that the synapsin I effect exhibited an ED<sub>50</sub> at  $\sim$ 25% of vesicle saturation and reached an apparent plateau at 50–80% of vesicle saturation. At higher degrees of saturation, the effect of synapsin I decreased steeply (Fig. 3, *bottom*).

To test whether the facilitation of Ca<sup>2+</sup>-induced fusion by synapsin I was due to destabilization of the vesicle bilayer or to a pure aggregating activity, the effects of synapsin I on CF leakage from phospholipid vesicles and on the <sup>31</sup>P-NMR spectra of phospholipid bilayers were investigated. In the absence of Ca<sup>2+</sup>, neither synapsin I nor its strongly basic tail fragment significantly increased the release of the water-soluble CF trapped in high concentration within mixed phospholipid vesicles, at variance with the results obtained with the very basic protein protamine. The subsequent addition of Ca<sup>2+</sup>, by triggering fusion, induced an increase in CF release which was not significantly different in the synapsin I-pretreated samples. No further increase in CF release was observed from the vesicles pretreated with protamine, suggesting that this protein had already induced extensive vesicle fusion (Fig. 4).

The analysis of the NMR spectra of <sup>31</sup>P, the naturally occurring isotope of phosphorus, in artificial membranes, allows a non-invasive detection of the molecular organization of the phospholipid molecules (Seelig, 1978; Cullis and de Kruijff, 1978). Hydrated phospholipid species can exhibit three major line shapes in the <sup>31</sup>P-NMR powder pattern. Phospholipids organized in extended bilayer structures exhibit a powder pattern characterized by a high field peak with a broad low field shoulder, hexagonal (H<sub>II</sub>) phase forming lipids (particularly PE) give rise to a powder pattern with a low field peak and a narrower high field shoulder (because of additional motional averaging of the <sup>31</sup>P tensor due to lateral diffusion around the central aqueous channels), whereas phospholipids experiencing isotropic motion (e.g., in vesicular or micellar structures) display a narrow, symmetrical peak.

At low ionic strength, phospholipid membranes composed of 80% PE and 20% PS gave rise to a spectrum which was characterized by a similar weight of the low and high field peaks at 5°C and by a progressive increase of the low field peak at increasing sample temperatures, consistent with a temperature-dependent shift from a partial bilayer to a totally hexagonal organization. Preincubation of the phospholipid membranes with synapsin I (1  $\mu$ M) markedly decreased the contribution of the low field peak at low temperature (5–25°C), although it was unable to counteract the bilayer-to-hexagonal transition at higher temperature (Fig. 5). The effect of synapsin I in the temperature range 5–25°C was more dramatic when the incubations were carried out at high ionic strength (Fig. 6, A and B). As a negative control, PE-PC membranes already organized in extended bilayers and lacking high-affinity binding for synapsin I (Benfenati et al., 1989a) were used. In this case the incubation with synapsin I was totally ineffective (Fig. 6 C). The ratio between the high and the low field peaks, expressing the prevalence of the bilayer structure over the H<sub>II</sub> phase, as a function of tem-



**Figure 3.** Effects of synapsin I on the Ca<sup>2+</sup>-triggered fusion of mixed phospholipid vesicles. 50  $\mu$ g of mixed phospholipid vesicles containing both NBD-PE and LRh-PE in equimolar amounts (2% of total phospholipid) were added to 150  $\mu$ g of unlabeled mixed phospholipid vesicles. Samples were excited at 470 nm and the fluorescence emission followed at 520 nm. Vesicle fusion was followed as the increase in fluorescence due to dequenching of NBD emission upon fusion of labeled vesicles with unlabeled ones. (Top): synapsin I (40 nM) was added to the sample either 1 min prior to the addition of 3 mM Ca<sup>2+</sup> (left trace) or 8 min after the addition of the same concentration of Ca<sup>2+</sup> (right trace). (Middle): the rate of vesicle fusion observed at increasing concentrations of Ca<sup>2+</sup> in the absence (○) or presence (●) of 40 nM synapsin I was evaluated as the level of fluorescence (in arbitrary units, a.u.) reached 30 s after the addition of Ca<sup>2+</sup>. Dose-response curves

perature and synapsin I concentration, is given in Fig. 7. The dependence of this effect on the synapsin I concentration had a similar shape under both low and high ionic strength conditions, with scarce effects below 70% of saturation of the membrane sites.

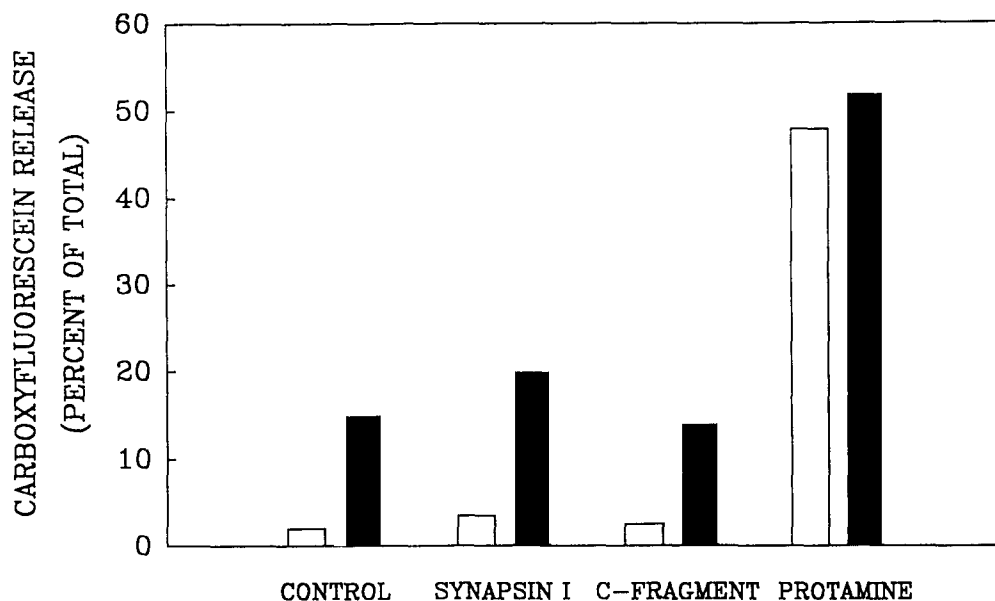
Since Ca<sup>2+</sup> is known to induce bilayer to inverted hexagonal transition in phospholipid dispersions of defined composition (Seelig, 1978; de Kruijff and Cullis, 1980), it was of interest to ascertain whether synapsin I was also able to inhibit the effect of Ca<sup>2+</sup>. Indeed, incubation with synapsin I not only virtually abolished the low field peak exhibited by the phospholipid mixture in basal conditions (low ionic strength, 5°C), but also inhibited the increase induced by the addition of 1 mM Ca<sup>2+</sup> (Fig. 8).

## Discussion

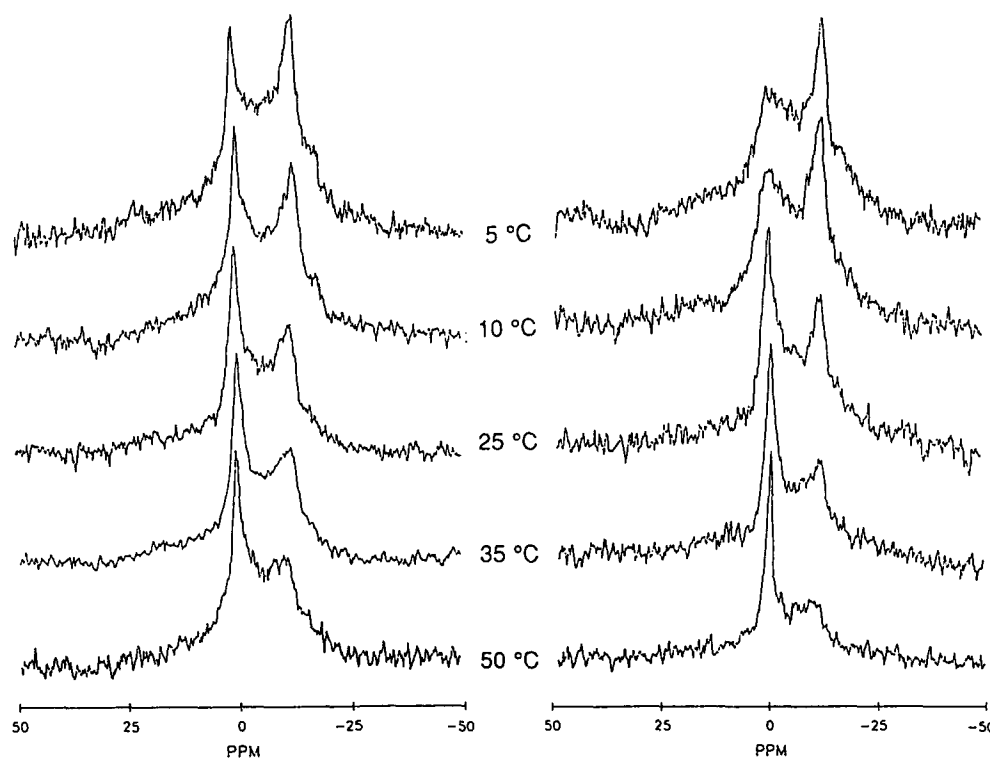
Synapsin I is a neuron-specific phosphoprotein which is involved in regulating the trafficking of synaptic vesicles within the nerve terminal and their availability for exocytosis (Valtorta et al., 1992; Greengard et al., 1993). These effects appear to be achieved through reversible interactions of synapsin I both with the vesicle membrane and with the actin-based cytoskeleton (Bähler et al., 1989; Benfenati et al., 1989a, 1991; Ceccaldi, P. E., F. Grohovaz, F. Benfenati, E. Chiergatti, P. Greengard, and F. Valtorta, manuscript submitted for publication). The high affinity ( $K_d$ , 10 nM) binding of synapsin I to the synaptic vesicle membrane is complex in nature and involves the phospholipid and protein components of synaptic vesicles. Because of its extremely high surface activity (Ho et al., 1991), synapsin I has a very high affinity for amphiphilic environments such as the membrane-water interface; it binds specifically to acidic phospholipids and penetrates the hydrophobic core of the vesicle membrane (Benfenati et al., 1989a,b). In the present paper, we have investigated the possible functional consequences of these properties of synapsin I, by studying its effects on the aggregation of phospholipid vesicles, on their ability to undergo Ca<sup>2+</sup>-induced fusion, and on the molecular organization of the phospholipid molecules.

The results suggest that the tight interaction of synapsin I with phospholipids induces vesicle clustering without destabilizing the bilayer structure of the vesicle membrane. Experiments performed on liposomes mimicking the phospholipid composition of native synaptic vesicles have shown that synapsin I induces vesicle aggregation, but has no detectable fusogenic activity per se. However, since a close contact between membranes is a prerequisite for the actual fusion process (Cullis and Hope, 1978; Wilschut and Hoekstra, 1984; White, 1992), synapsin I greatly potentiates the rate and extent of Ca<sup>2+</sup>-dependent membrane fusion. This

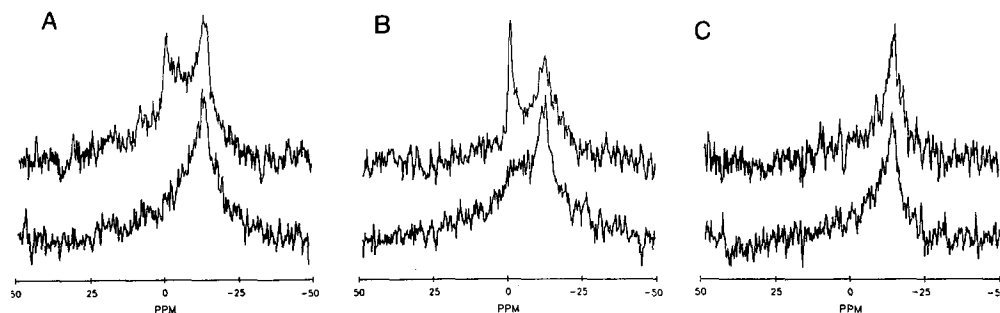
were fitted using the program ALLFIT (De Lean et al., 1978). (Bottom) Effects of the pretreatment of mixed phospholipid vesicles with increasing concentrations of synapsin I on the rate of fusion triggered by 3 mM Ca<sup>2+</sup>. The X-axis represents the degree of saturation of the vesicle sites expressed in percent of the maximal binding capacity ( $B_{max}$ ) and calculated from the total synapsin I concentrations and the known binding constants (see Materials and Methods). The Y-axis represents the differences between fusion rates observed in the presence and absence of synapsin I.



**Figure 4.** Leakage of the aqueous content from mixed phospholipid vesicles. Mixed phospholipid vesicles (50  $\mu$ g of phospholipid/1.5 ml) containing 50 mM CF were incubated for 3 min at 22°C in the absence (*control*) or presence of synapsin I (10  $\mu$ g), synapsin I tail fragment (5  $\mu$ g; C-fragment) or protamine (10  $\mu$ g) in the absence (□) or presence (■) of 3 mM Ca<sup>2+</sup>. The stimulated release of CF is expressed as percent of the total release measured after solubilization of the vesicles with 1% Triton X-100.

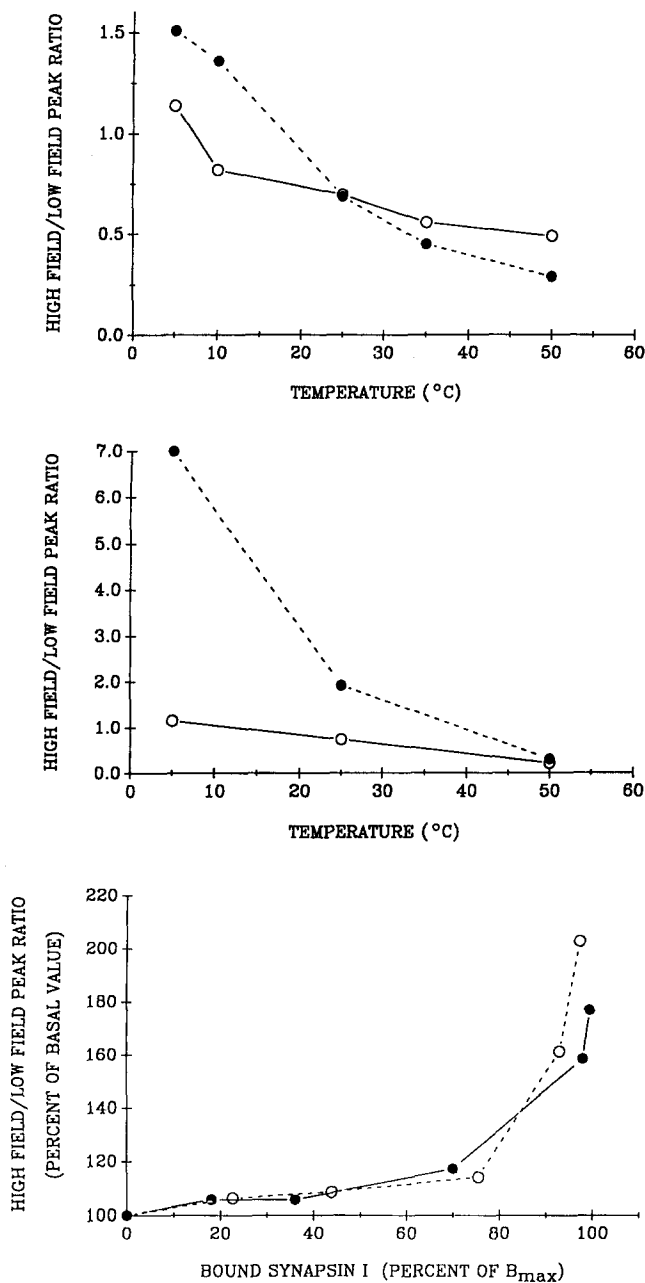


**Figure 5.** Effect of synapsin I on proton decoupled <sup>31</sup>P-NMR spectra of phospholipid membranes at low ionic strength. Aqueous dispersions of 20 mg PE/PS (weight ratio, 80:20) in 2 ml of buffer B containing 20 mM NaCl and 25% (vol/vol) D<sub>2</sub>O were incubated for 10 min at room temperature in the absence (spectra on the left) or presence (spectra on the right) of 1  $\mu$ M synapsin I, before collecting spectra at temperatures increasing stepwise from 5° to 50°C. The temperature-induced changes in the spectra were fully reversible when the sample temperature was brought back from 50° to 5°C (not shown). The high field peak corresponds to the contribution of the bilayer phase, whereas the low field peak refers to the presence of H<sub>II</sub> hexagonal structures.



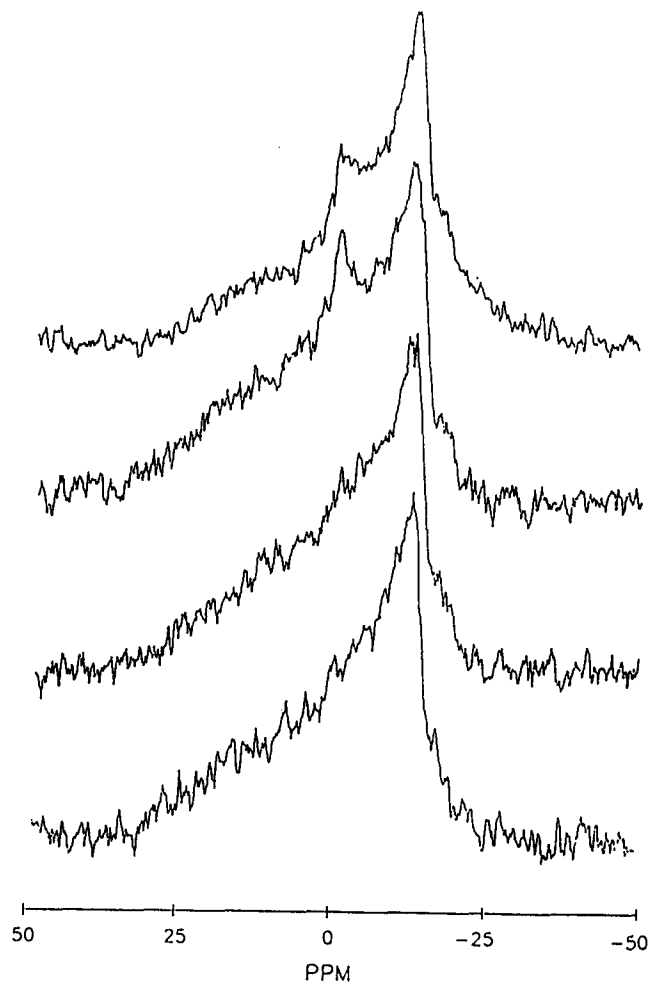
**Figure 6.** Effect of synapsin I on proton decoupled <sup>31</sup>P-NMR spectra of phospholipid membranes at high ionic strength. (A and B) Aqueous dispersions of 20 mg PE/PS (weight ratio, 80:20) in 2 ml of buffer A containing 25% (vol/vol) D<sub>2</sub>O were incubated for 10 min at room temperature in the absence (upper spectra) or presence (lower spectra) of 1  $\mu$ M synapsin I, before collecting spectra at 5°C (A) and 25°C (B). (C) Lack of effect of synapsin I (1  $\mu$ M) on the <sup>31</sup>P-NMR spectrum of aqueous dispersions of PE/PC (weight ratio, 80:20) at 5°C. Under these conditions, no apparent low field peak is present, suggesting that the bilayer phase dominates.

collecting spectra at 5°C (A) and 25°C (B). (C) Lack of effect of synapsin I (1  $\mu$ M) on the <sup>31</sup>P-NMR spectrum of aqueous dispersions of PE/PC (weight ratio, 80:20) at 5°C. Under these conditions, no apparent low field peak is present, suggesting that the bilayer phase dominates.



**Figure 7.** Quantification of the synapsin I-induced changes in proton decoupled  $^{31}\text{P}$ -NMR spectra of phospholipid membranes. (*Top and middle*) The ratios between the high field and the low field peaks in  $^{31}\text{P}$ -NMR spectra of aqueous dispersions of 20 mg PE/PS (weight ratio, 80:20) were calculated in the absence ( $\circ$ ) or presence ( $\bullet$ ) of 1  $\mu\text{M}$  synapsin I as a function of the sample temperature under low (*top*) or high (*middle*) ionic strength conditions. (*Bottom*) The ratios between the high field and the low field peaks were calculated at various degrees of membrane saturation by synapsin I (corresponding to total synapsin I concentrations ranging from 0.25 to 4.0  $\mu\text{M}$ ) under low ( $\bullet$ ) or high ( $\circ$ ) ionic strength conditions and expressed as percent changes with respect to the ratio obtained in the absence of synapsin I.

activity, which appears to be independent of synapsin I phosphorylation, is attributable to the clustering of adjacent vesicles which enhances the ability of  $\text{Ca}^{2+}$  to destabilize the membrane bilayer and to trigger the hydrophobic contact between the two facing membranes (Wilschut and Hoekstra,



**Figure 8.** Synapsin I counteracts the effects of  $\text{Ca}^{2+}$  on proton decoupled  $^{31}\text{P}$ -NMR spectra of phospholipid membranes. Spectra were collected from aqueous dispersions of 20 mg PE/PS (weight ratio, 70:30) in 2 ml of buffer B containing 20 mM NaCl and 25% (vol/vol)  $\text{D}_2\text{O}$  incubated for 15 min at 5°C with either (from top to bottom) buffer,  $\text{Ca}^{2+}$  (1 mM),  $\text{Ca}^{2+}$  (1 mM) plus synapsin I (2  $\mu\text{M}$ ), or synapsin I (2  $\mu\text{M}$ ).

1984; Papahadjopoulos et al., 1990; Allen et al., 1990). With artificial membranes, such as those used in this assay,  $\text{Ca}^{2+}$  is known to exert its fusogenic activity at concentrations in the millimolar range (Papahadjopoulos et al., 1990). The presence of synapsin I at subsaturating concentrations decreases the  $\text{Ca}^{2+}$  requirement by approximately one order of magnitude. However, the facilitation of the  $\text{Ca}^{2+}$ -dependent liposome fusion occurs only in a limited range of synapsin I concentrations (and of occupancy of vesicle sites), showing a biphasic behavior, whereas the aggregating ability of synapsin I does not show this biphasic behavior and reaches a plateau at higher synapsin I concentrations.

In principle, the synapsin I-induced cross-linking of vesicles with each other can be brought about either by the existence of multiple phospholipid binding sites in one synapsin I molecule or by self-association of two or more molecules of synapsin I, each bearing a single phospholipid binding site. Experimental evidence suggests that both hypotheses may hold true: several amino acid stretches predicted to form amphiphilic secondary structures (Kaiser and Kezdy,



1984) with the potential to bind to phospholipid membranes exist within the *head* region of synapsin I (Südhof et al., 1989; Ho et al., 1991). On the other hand, synapsin I has a high tendency to self-associate, due to its high surface activity and to the strongly hydrophobic character of the *head* region (Ueda and Greengard, 1977). The different behavior of the dose dependence of aggregation and fusion suggests that synapsin I concentrations higher than 100 nM (which, in the experimental system used, correspond to ~85% of membrane saturation) inhibit Ca<sup>2+</sup>-triggered membrane fusion while they still induce membrane aggregation. It is possible that, at saturating concentrations of synapsin I such as those present within nerve terminals (Schiebler et al., 1986; Sihra et al., 1989), the majority of the vesicle surface is covered by synapsin I molecules which stabilize the bilayer and inhibit the contact with Ca<sup>2+</sup>. Indeed, computer modeling of the interaction of synapsin I with synaptic vesicles predicts that, in the nerve terminal, dephosphorylated synapsin I saturates over 90% of the vesicle sites (Benfenati et al., 1991).

The association of synapsin I with the phospholipid bilayer is not accompanied by membrane destabilization or permeabilization. Although the binding of synapsin I involves penetration of the *head* region into the hydrophobic core of the membrane (Benfenati et al., 1989a), the incubation of synapsin I with liposomes in the absence of Ca<sup>2+</sup> did not change the release of their aqueous content into the external medium. Rather, <sup>31</sup>P-NMR spectroscopy demonstrated that synapsin I, at concentrations above 0.5 μM, is capable of preventing the transition of membrane phospholipids from the bilayer (L<sub>α</sub>) to the inverted hexagonal (H<sub>II</sub>) phase induced by either increases in temperature or addition of Ca<sup>2+</sup>, thereby increasing the probability for vesicle phospholipids to assume a bilayer organization both under basal conditions and in the presence of destabilizing stimuli. It is questionable whether the H<sub>II</sub> phase represents an obligatory intermediate in the steps leading to membrane fusion (Wilschut and Hoekstra, 1984; Allen et al., 1990; Papahadjopoulos et al., 1990; Hui et al., 1988). However, it has been shown that increased propensity of phospholipid membranes towards the formation of non-bilayer structures correlates well with increased membrane fusion (Hui et al., 1981; Cullis and Hope, 1978; Ellens et al., 1989; Eastman et al., 1992). The ability to raise the L<sub>α</sub> to H<sub>II</sub> phase transition temperature of PE-containing phospholipid vesicles and the absence of membrane permeabilization observed in the CF leakage assay suggest that synapsin I may possess a membrane-stabilizing activity.

The cytoplasmic leaflet of the synaptic vesicle membrane is enriched in PE, a typical nonbilayer structure-preferring lipid which tends to form concave surfaces at the lipid/water interface (Killian et al., 1992). It is possible that the stabilizing effect is due to the localization of synapsin I at the bilayer surface, which decreases the probability of curling of the PE-containing leaflet and of forming nonbilayer structures. In this respect, synapsin I resembles the pore-forming bacterial toxins colicins, which also insert into the membrane and have a membrane-stabilizing effect (Géli et al., 1992). In contrast, highly hydrophobic peptides such as gramicidin, melittin or signal peptides, by partitioning into the hydrophobic core of the membrane, induce membrane destabilization and the formation of nonbilayer structures (van Echteld et al., 1981; Batemburg et al., 1987a,b; Killian et al., 1990).

These data support a surface localization of the *head* region of synapsin I upon binding to synaptic vesicle phospholipids, with the highly charged *tail* region preventing stronger hydrophobic interactions and allowing a prompt reversibility of the vesicle binding (Benfenati et al., 1989a).

Synapsin I is a neuron-specific protein. In other secretory systems, the existence of phospholipid binding proteins has also been reported. A class of homologous proteins, the annexins, share the ability to associate with acidic membrane phospholipids and to promote the aggregation of liposomes as well as of secretory granules (Creutz, 1992). Like synapsin I, synexin (annexin VII, the first annexin to be characterized; Creutz et al., 1978) and the other annexins are not fusogenic proteins, but enhance the Ca<sup>2+</sup>-dependent fusion of phospholipid vesicles by accelerating the rate-limiting membrane aggregation process (Hong et al., 1981; Meers et al., 1988). Although synexin possesses an NH<sub>2</sub>-terminal hydrophobic domain with the potential to form amphipathic β-sheets (Burns et al., 1989), the phospholipid interaction is mediated by the highly conserved COOH-terminal region which is shared by the other annexins and contains the Ca<sup>2+</sup>-binding sites (Creutz, 1992). In the case of synexin (and of all the other annexins), vesicle aggregation is strictly Ca<sup>2+</sup> dependent (Hong et al., 1981; Creutz, 1992) and is probably achieved through self-association of annexin molecules at the membrane surface (Creutz et al., 1979; Zaks and Creutz, 1991). Similarly to synapsin I, the annexins are thought to form monolayers at the membrane surface with a cytoplasmic hydrophilic face and a hydrophobic portion embedded in the membrane (Brisson et al., 1991). Although synexin has been reported to form ion channels (Burns et al., 1989), its penetration into the membrane bilayer has never been directly demonstrated. Based on the structure of the proteins, it has been suggested that the annexins do not fully integrate into membranes, but interact with them by a mechanism involving profound membrane rearrangements (Brisson et al., 1991). Indeed, the potentiation of Ca<sup>2+</sup>-induced fusion by synexin is accompanied by destabilization and permeabilization of the membrane (Meers et al., 1988).

It is tempting to speculate that the unusual physicochemical properties of synapsin I are responsible, at least in part, for some distinctive features of the secretory process in neurons, which include: (a) the presence of clusters of synaptic vesicles close to the fusion sites, which minimizes their diffusion and the possibility of random fusion with the presynaptic plasma membrane (Ceccarelli and Hurlbut, 1980; Hirokawa et al., 1989; Valtorta et al., 1990); (b) the remarkable uniformity of the synaptic vesicle size (40–50 nm in diameter) (Ceccarelli and Hurlbut, 1980; Valtorta et al., 1990); (c) the existence of a reserve pool of synaptic vesicles (over 95% of total vesicles in certain terminals) which are not available for neurotransmitter release (Greengard et al., 1993); (d) the decrease in the Ca<sup>2+</sup>-independent, spontaneous fusion of synaptic vesicles with the presynaptic membrane induced by the microinjection of synapsin I into intact nerve terminals (Llinas et al., 1985; Lin et al., 1990; Hackett et al., 1990; Llinas et al., 1991); (e) the dissociation of synapsin I from actin filaments and synaptic vesicles induced by CaM kinase II phosphorylation under conditions of enhanced neurotransmitter release (Schiebler et al., 1986; Bähler and Greengard, 1987; Sihra et al., 1989; Torri-Tarelli et al., 1992).

The present results suggest that, in addition to linking syn-

aptic vesicles to the cytoskeleton, synapsin I is also able to induce vesicle clustering within the nerve terminal, a mechanism which may contribute to the sequestration of synaptic vesicles in the reserve pool and prevent their diffusion away from their functional compartment. In this respect, it is noteworthy that the injection of synapsin I into *Xenopus* embryos accelerates the morphological and functional maturation of neuromuscular synapses in culture, by inducing a correct compartmentalization of synaptic vesicles within the developing nerve terminal (Lu et al., 1992; Valtorta, F., N. Iezzi, F. Benfenati, B. Lu, M.-m. Poo, and P. Greengard, manuscript in preparation). Interestingly, in synapsin I-injected neurons, synaptic vesicles appear to be organized in clusters close to the presynaptic membrane (Valtorta, F., N. Iezzi, F. Benfenati, B. Lu, M.-m. Poo, and P. Greengard, manuscript in preparation). Moreover, in ribbon synapses of the retina, which lack the synapsins, synaptic vesicles do not appear to be clustered, but rather aligned along the "ribbon" (Usukura and Yamada, 1987; Mandell et al., 1990).

It has been reported that phospholipid vesicles of small diameter can experience phospholipid packing defects which may lead to membrane fusion and leakage of vesicular contents (Hoekstra, 1982; Hui and Sen, 1989; Nam et al., 1989). In the case of small synaptic vesicles, the smallest secretory organelles within cells, the ability of synapsin I to stabilize phospholipids in the bilayer arrangement may be important in the maintenance of vesicle integrity and uniform size, as well as in the prevention of random fusion events with neighboring vesicles or the presynaptic membrane (Nir et al., 1982). This possibility is strengthened by the fact that synapsin I is quite abundant in the nerve terminal with an average concentration of about 10  $\mu$ M and that, due to the very high limiting surface area and the stoichiometry of 10–30 molecules per vesicle, synapsin I may cover a large portion of the synaptic vesicle surface (Schiebler et al., 1986; De Camilli et al., 1990; Ho et al., 1991). The selectivity of the binding of synapsin I for synaptic vesicles (Huttner et al., 1983; Schiebler et al., 1986), whose molecular bases have been only partially clarified (Benfenati et al., 1989a; 1992b), may prevent the protein from acting on other organelles. It seems feasible that under resting conditions the vast majority of synaptic vesicles are either cross-linked to the cytoskeleton or clustered with other vesicles via synapsin I cross-bridges. This hypothesis is supported by the observation that in quick-frozen, deep-etched nerve terminals, synaptic vesicles appear to be linked to each other by rod-like molecules, presumably synapsin I (Landis et al., 1988; Hirokawa et al., 1989).

The partial dissociation of synapsin I from the vesicle membrane which occurs during stimulation (up to 60% desaturation in recycled vesicles after high-frequency electrical stimulation; Torri-Tarelli et al., 1992), besides contributing to the release of synaptic vesicles, may be associated with a decrease in the synapsin I stabilizing effect on the vesicle bilayer and result in an increased synaptic vesicle reactivity toward the presynaptic membrane. In conclusion, the interaction of synapsin I with vesicle phospholipids, in addition to contributing to its high-affinity binding to synaptic vesicles, may play an important functional role in the formation of clusters of synaptic vesicles in the proximity of the release sites, in the preservation of the structural integrity of the vesicle membrane and in the regulation of its reactivity toward other membranes. Since the synapsins also stimulate the morphological and functional maturation of nerve termi-

nals with their complement of synaptic vesicles (Han et al., 1991; Lu et al., 1992), an additional fascinating possibility is that those proteins, through their complex interactions with membrane phospholipids, also play a role in vesicle biogenesis.

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## References

- Allen, T. M., D. Hong, and D. Papahadjopoulos. 1990. Membrane contact, fusion and hexagonal ( $H_{II}$ ) transitions in phosphatidylethanolamine liposomes. *Biochemistry*. 29:2976–2985.
- Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. In *Methods in Enzymology*. Vol. 8. E. F. Neufeld, and V. Ginsburg, editors. Academic Press, New York. 115–118.
- Bähler, M., and P. Greengard. 1987. Synapsin I bundles F-actin in a phosphorylation-dependent manner. *Nature (Lond.)*. 326:704–707.
- Bähler, M., F. Benfenati, F. Valtorta, A. J. Czernik, and P. Greengard. 1989. Characterization of synapsin I fragments by cysteine-specific cleavage: a study of their interactions with F-actin. *J. Cell Biol.* 108:1841–1849.
- Batemburg, A. M., J. C. L. Hibbeln, A. J. Verkleij, and B. de Kruijff. 1987a. Melittin induced  $H_{II}$  phase formation in cardiolipin model membranes. *Biochim. Biophys. Acta*. 903:142–154.
- Batemburg, A. M., J. C. L. Hibbeln, and B. de Kruijff. 1987b. Lipid specific penetration of melittin into phospholipid model membranes. *Biochim. Biophys. Acta*. 903:155–165.
- Benfenati, F., M. Bähler, R. Jahn, and P. Greengard. 1989a. Interactions of synapsin I with small synaptic vesicles: distinct sites in synapsin I bind to vesicle phospholipids and vesicle proteins. *J. Cell Biol.* 108:1863–1872.
- Benfenati, F., P. Greengard, J. Brunner, and M. Bähler. 1989b. Electrostatic and hydrophobic interactions of synapsin I and synapsin I fragments with phospholipid bilayers. *J. Cell Biol.* 108:1851–1862.
- Benfenati, F., F. Valtorta, and P. Greengard. 1991. Computer modeling of synapsin I binding to synaptic vesicles and F-actin: implications for regulation of neurotransmitter release. *Proc. Natl. Acad. Sci. USA*. 88:575–579.
- Benfenati, F., F. Valtorta, E. Chieriegatti, and P. Greengard. 1992a. Interaction of free and synaptic vesicle-bound synapsin I with F-actin. *Neuron*. 8:377–386.
- Benfenati F., F. Valtorta, J. L. Rubenstein, F. Gorelick, P. Greengard, and A. J. Czernik. 1992b. Synaptic vesicle-associated  $Ca^{2+}$ /calmodulin-dependent protein kinase II is a binding protein for synapsin I. *Nature (Lond.)*. 359:417–420.
- Brisson A., G. Mosser, and R. Huber. 1991. Structure of soluble and membrane-bound annexin V. *J. Mol. Biol.* 220:199–203.
- Burns, A. L., K. Magendzo, A. Shirvan, M. Srivastava, E. Rojas, M. R. Aluani, and H. B. Pollard. 1989. Calcium channel activity of purified human synexin and structure of the human synexin gene. *Proc. Natl. Acad. Sci. USA*. 86:3798–3802.
- Ceccarelli, B., and W. P. Hurlbut. 1980. Vesicle hypothesis of the release of quanta of acetylcholine. *Physiol. Rev.* 60:396–441.
- Chong, C. S., and K. Colbow. 1976. Light scattering and turbidity measurements on lipid vesicles. *Biochim. Biophys. Acta*. 436:260–282.
- Creutz, C. E., C. J. Pazoles, and H. B. Pollard. 1978. Identification and purification of an adrenal medullary protein (synexin) that causes calcium-dependent aggregation of isolated chromaffin granules. *J. Biol. Chem.* 253:2858–2866.
- Creutz, C. E., C. J. Pazoles, and H. B. Pollard. 1979. Self-association of synexin in the presence of calcium. *J. Biol. Chem.* 254:553–558.
- Creutz, C. E. 1992. The annexins and exocytosis. *Science (Wash. DC)*. 256:924–931.
- Cullis, P. R., and M. J. Hope. 1978. Effect of fusogenic agent on membrane structure of erythrocyte ghosts and the mechanism of membrane fusion. *Nature (Lond.)*. 271:672–674.
- Cullis, P. R., and B. de Kruijff. 1979. Lipid polymorphism and the functional role of lipids in biological membranes. *Biochim. Biophys. Acta*. 559:399–420.
- De Camilli, P., R. Cameron, and P. Greengard. 1983a. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein: I. Its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in frozen and plastic sections. *J. Cell Biol.* 96:1337–1354.
- De Camilli, P., S. M. Harris, W. B. Huttner, and P. Greengard. 1983b. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein: II. Its specific as-

- sociation with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. *J. Cell Biol.* 96:1355-1373.
- De Camilli, P., F. Benfenati, F. Valtorta, and P. Greengard. 1990. The synapsins. *Annu. Rev. Cell Biol.* 6:433-460.
- de Kruijff, B., and P. R. Cullis. 1980. The influence of poly(L-lysine) on phospholipid polymorphism. Evidence that electrostatic polypeptide-phospholipid interactions can modulate bilayer/non-bilayer transitions. *Biochim. Biophys. Acta.* 601:235-240.
- De Lean, A., P. J. Munson, and D. Rodbard. 1978. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.* 235:E97-E102.
- Düzgünes, N., J. Wilschut, R. Fraley, and D. Papahadjopoulos. 1981. Studies on the mechanism of membrane fusion. Role of head-group composition in calcium- and magnesium-induced fusion of mixed phospholipid vesicles. *Biochim. Biophys. Acta.* 642:182-195.
- Eastman, S. J., M. J. Hope, K. F. Wong, and P. R. Cullis. 1992. Influence of phospholipid asymmetry on fusion between large unilamellar vesicles. *Biochemistry.* 31:4262-4268.
- Ellens, H., D. P. Stegel, D. Alford, P. L. Yeagle, L. Boni, L. J. Lis, P. J. Quinn, and J. Bentz. 1989. Membrane fusion and inverted phases. *Biochemistry.* 28:3692-3703.
- Géli, V., M. C. Koorengevel, R. A. Demel, C. Lazdunski, and J. A. Killian. 1992. Acidic interaction of the colicin A pore-forming domain with model membranes of *Escherichia coli* lipids results in a large perturbation of acyl chain order and stabilization of the bilayer. *Biochemistry.* 31:11089-11094.
- Gibson, S. M., and G. Strauss. 1984. Reaction characteristics and mechanisms of lipid bilayer vesicle fusion. *Biochim. Biophys. Acta.* 769:531-542.
- Greengard, P., F. Valtorta, A. J. Czernik, and F. Benfenati. 1993. Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science (Wash. DC).* 259:780-785.
- Hackett, J. T., S. L. Cochran, L. J. Greenfield, D. C. Brosius, and T. Ueda. 1990. Synapsin I injected presynaptically into goldfish Mauthner axons reduces quantal synaptic transmission. *J. Neurophysiol.* 63:701-706.
- Han, H. Q., R. A. Nichols, M. R. Rubin, M. Bähler, and P. Greengard. 1991. Induction of formation of presynaptic terminals in neuroblastoma cells by synapsin IIb. *Nature (Lond.).* 349:697-700.
- Hirokawa, N., K. Sobue, K. Kanda, A. Harada, and H. Yorifuji. 1989. The cytoskeletal architecture of the presynaptic terminal and molecular structure of synapsin I. *J. Cell Biol.* 108:111-126.
- Ho, M., M. Bähler, A. J. Czernik, W. Schiebler, F. J. Kezdy, E. T. Kaiser, and P. Greengard. 1991. Synapsin I is a highly surface-active molecule. *J. Biol. Chem.* 266:5600-5607.
- Hoekstra, D. 1982. Role of lipid phase separations and membrane hydration in phospholipid vesicle fusion. *Biochemistry.* 21:2833-2840.
- Hong, K., N. Düzgünes, and D. Papahadjopoulos. 1981. Role of synexin in membrane fusion. *J. Biol. Chem.* 256:3641-3644.
- Hui, S. W., T. P. Stewart, L. T. Boni, and P. L. Yeagle. 1981. Membrane fusion through point defects in bilayers. *Science (Wash. DC).* 212:921-923.
- Hui S.-W., S. Nir, T. P. Stewart, L. T. Boni, and S. K. Huang. 1988. Kinetic measurements of fusion of phosphatidylserine-containing vesicles by electron microscopy and fluorometry. *Biochim. Biophys. Acta.* 941:130-140.
- Hui S.-W., and A. Sen. 1989. Effects of lipid packing on polymorphic phase behavior and membrane properties. *Proc. Natl. Acad. Sci. USA.* 86:5825-5829.
- Huttner, W. B., W. Schiebler, P. Greengard, and P. De Camilli. 1983. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. III. Its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. *J. Cell Biol.* 96:1374-1388.
- Kaczmarek, L. K., K. R. Jennings, F. Strumwasser, A. C. Nairn, U. Walter, F. D. Wilson, and P. Greengard. 1980. Microinjections of catalytic subunit of cyclic AMP-dependent protein kinase enhances calcium action potentials of bag cell neurons in cell culture. *Proc. Natl. Acad. Sci. USA.* 77:7487-7491.
- Kaiser, E. T., and F. J. Kezdy. 1984. Amphiphilic secondary structure: design of peptide hormones. *Science (Wash. DC).* 223:249-255.
- Killian, J. A., A. M. Ph. de Jong, J. Bijvelt, A. J. Verkleij, and B. de Kruijff. 1990. Induction of non-bilayer lipid structures by functional signal peptides. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:815-819.
- Killian, J. A., C. H. J. Fabrie, W. Baart, S. Morein, and B. de Kruijff. 1992. Effects of temperature variation and phenethyl alcohol addition on acyl chain order and lipid organization in *Escherichia coli* derived membrane systems. A <sup>2</sup>H- and <sup>31</sup>P-NMR study. *Biochim. Biophys. Acta.* 1105:253-262.
- Landis, D. M. D., A. K. Hall, L. A. Weinstein, and T. S. Reese. 1988. The organization of the cytoplasm at the presynaptic active zone of a central nervous system synapse. *Neuron.* 1:201-209.
- Lin, J.-W., M. Sugimori, R. R. Llinas, T. L. McGuinness, and P. Greengard. 1990. Effects of synapsin I and calcium/calmodulin-dependent protein kinase II on spontaneous neurotransmitter release in the squid giant synapse. *Proc. Natl. Acad. Sci. USA.* 87:8257-8261.
- Lindblom, G., and L. Rilfors. 1989. Cubic phases and isotropic structures formed by membrane lipids - possible biological relevance. *Biochim. Biophys. Acta.* 988:221-256.
- Llinas, R., T. L. McGuinness, C. S. Leonard, M. Sugimori, and P. Greengard. 1985. Intraterminal injection of synapsin I or calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse. *Proc. Natl. Acad. Sci. USA.* 82:3035-3039.
- Llinas, R., J. A. Gruner, M. Sugimori, T. L. McGuinness, and P. Greengard. 1991. Regulation by synapsin I and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II of transmitter release in squid giant synapse. *J. Physiol.* 436:257-282.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Lu, B., P. Greengard, and M.-m. Poo. 1992. Exogenous synapsin I promotes functional maturation of developing neuromuscular synapses. *Neuron.* 8:521-529.
- Mandell, J. W., E. Townes-Anderson, A. J. Czernik, R. Cameron, P. Greengard, and P. De Camilli. 1990. Synapsins in the vertebrate retina: absence from ribbon synapses and heterogeneous distribution among conventional synapses. *Neuron.* 5:19-33.
- McGuinness, T. L., Y. Lai, and P. Greengard. 1985. Ca<sup>2+</sup>/calmodulin-dependent protein kinase II. Isozymic forms from rat forebrain and cerebellum. *J. Biol. Chem.* 260:1696-1704.
- Meers, P., J. Bentz, D. Alford, S. Nir, D. Papahadjopoulos, and K. Hong. 1988. Synexin enhances the aggregation rate but not the fusion rate of liposomes. *Biochemistry.* 27:4430-4439.
- Mimms, L. T., G. Zampighi, Y. Nozaki, C. Tanford, and J. A. Reynolds. 1981. Phospholipid vesicle formation and transmembrane protein incorporation using octyl glucoside. *Biochemistry.* 20:833-840.
- Nam, K.-Y., S. Kimura, H. Fujiki, and Y. Imanishi. 1989. Effects of phorbol ester and teleocidin on Ca<sup>2+</sup>-induced fusion of liposomes. *Biochem. Biophys. Res. Commun.* 165:1256-1261.
- Navone, F., P. Greengard, and P. De Camilli. 1984. Synapsin I in nerve terminals: selective association with small synaptic vesicles. *Science (Wash. DC).* 226:1209-1211.
- Nestler, E. J., and P. Greengard. 1984. Protein Phosphorylation in the Nervous System. John Wiley and Sons, Inc., New York. 398 pp.
- Nichols, R. A., T. J. Chilcote, A. J. Czernik, and P. Greengard. 1992. Synapsin I regulates glutamate release from rat brain synaptosomes. *J. Neurochem.* 58:783-785.
- Nir, S., J. Wilschut, and J. Bentz. 1982. The rate of fusion of phospholipid vesicles and the role of bilayer curvature. *Biochim. Biophys. Acta.* 688:275-278.
- Papahadjopoulos, D., S. Nir, and N. Düzgünes. 1990. Molecular mechanisms of calcium-induced membrane fusion. *J. Bioenerg. Biomembr.* 22:157-179.
- Petrucci, T. C., and J. S. Morrow. 1987. Synapsin I: an actin-binding protein under phosphorylation control. *J. Cell Biol.* 105:1355-1363.
- Schiebler, W., R. Jahn, J.-P. Doucet, J. Rothlein, and P. Greengard. 1986. Characterization of synapsin I binding to small synaptic vesicles. *J. Biol. Chem.* 261:8383-8390.
- Seelig, J. 1978. <sup>31</sup>P nuclear magnetic resonance and the head group structure of phospholipids in membranes. *Biochim. Biophys. Acta.* 515:105-140.
- Sihra, T. S., J. K. T. Wang, F. S. Gorelick, and P. Greengard. 1989. Translocation of synapsin I in response to depolarization of isolated nerve terminals. *Proc. Natl. Acad. Sci. USA.* 86:8108-8112.
- Struck, D. K., D. Hoekstra, and R. E. Pagano. 1981. Use of resonance energy transfer to monitor membrane fusion. *Biochemistry.* 20:4093-4099.
- Südhof, T. C., A. J. Czernik, H.-T. Kao, K. Takei, P. A. Johnston, A. Horuchi, S. D. Kanazir, M. A. Wagner, M. S. Perin, P. De Camilli, and P. Greengard. 1989. Synapsins: mosaics of shared and individual domains in a family of synaptic vesicle phosphoproteins. *Science (Wash. DC).* 245:1474-1480.
- Taraschi, T. F., A. T. M. van der Steen, B. de Kruijff, C. Tellier, and A. J. Verkleij. 1982. Lectin-receptor interactions in liposomes: evidence that binding of wheat germ agglutinin to glycoprotein-phosphatidylethanolamine vesicles induces non-bilayer structures. *Biochemistry.* 21:5756-5764.
- Torri-Tarelli, F., M. Bossi, R. Fesce, P. Greengard, and F. Valtorta. 1992. Synapsin I partially dissociates from synaptic vesicles during exocytosis induced by electrical stimulation. *Neuron.* 9:1143-1153.
- Ueda, T., and P. Greengard. 1977. Adenosine 3':5'-monophosphate-regulated phosphoprotein system of neuronal membranes. I. Solubilization, purification, and some properties of an endogenous phosphoprotein. *J. Biol. Chem.* 252:5155-5163.
- Usukura, J., and E. Yamada. 1987. Ultrastructure of the synaptic ribbons in photoreceptor cells of *Rana catesbeiana* revealed by freeze-etching and freeze-substitution. *Cell Tissue Res.* 247:483-488.
- Valtorta, F., R. Fesce, F. Grohovaz, C. Haimann, W. P. Hurlbut, N. Iezzi, F. Torri-Tarelli, A. Villa, and B. Ceccarelli. 1990. Neurotransmitter release and synaptic recycling. *Neuroscience.* 35:477-489.
- Valtorta, F., F. Benfenati, and P. Greengard. 1992a. Structure and function of the synapsins. *J. Biol. Chem.* 267:7195-7198.
- Valtorta F., P. Greengard, R. Fesce, E. Chiergatti, and F. Benfenati. 1992b. Effects of the neuronal phosphoprotein synapsin I on actin polymerization. I. Evidence for a phosphorylation-dependent nucleating effect. *J. Biol. Chem.* 267:11281-11288.
- van Echteld, C. J. A., R. van Stigt, B. de Kruijff, J. Leunissen-Bijvelt, A. J. Verkleij, and J. de Gier. 1981. Gramicidin promotes formation of the hexagonal H<sub>II</sub> phase in aqueous dispersion of phosphatidylethanolamine and phosphatidylcholine. *Biochim. Biophys. Acta.* 648:287-291.
- Westhead, E. W. 1987. Lipid composition and orientation in secretory vesicles. *Annu. N.Y. Acad. Sci.* 493:92-99.
- Wilschut, J., and D. Hoekstra. 1984. Membrane fusion: from liposomes to biological membranes. *Trends Biochem. Sci.* 9:479-483.
- White, J. M. 1992. Membrane fusion. *Science (Wash. DC).* 258:917-924.
- Zaks, W. J., and C. E. Creutz. 1991. Ca<sup>2+</sup>-dependent annexin self-association on membrane surfaces. *Biochemistry.* 30:9607-9615.