Effects of synaptic vesicles on actin polymerization

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Received 6 September 1996; revised version received 18 October 1996

Abstract We have analyzed the effects of synaptic vesicles on actin polymerization by using a time-resolved spectrofluorometric assay. We have found that synaptic vesicles have complex effects on the kinetics of actin polymerization, which vary depending on whether the synaptic vesicle-specific phosphoprotein synapsin I is absent or present on their membrane. Synapsin I bound either to synaptic vesicles or to pure phospholipid vesicles exhibits phosphorylation-dependent actin-nucleating activity. Synaptic vesicles depleted of endogenous synapsin I decrease the rate and the final extent of actin polymerization, an effect which is not observed with pure phospholipid vesicles. Thus, the state of association of synapsin I with synaptic vesicles, which is modulated by its state of phosphorylation, may affect actin assembly and the physico-chemical characteristics of the synaptic vesicle microenvironment.

Key words: Synaptic vesicle; Synapsin; Nerve terminal; Protein phosphorylation; Cytoskeleton; Actin

1. Introduction

Synapsin I is a synaptic vesicle-associated phosphoprotein which is implicated in the regulation of neurotransmitter release [1,2]. In microinjection studies it has been demonstrated that dephosphorylated synapsin I inhibits neurotransmitter release and that this effect is abolished upon synapsin I phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) [3–5], an enzyme which has been shown to be present on the synaptic vesicle membrane as a binding protein for synapsin I [6]. Synapsin I is able to bind and bundle actin filaments in vitro [7,8]. The interaction of synapsin I with actin monomers promotes nucleation and polymerization of actin into filaments, and both effects are decreased upon synapsin I phosphorylation by CaM kinase II [9–12]. Phosphorylation of synapsin I by CaM kinase II also affects its interaction with synaptic vesicles, reducing its affinity for their membrane [13,14].

Because of these interactions with actin and synaptic vesicles [15–17] and of their regulation by phosphorylation of synapsin I, synapsin I has been hypothesized to cross-link reversibly synaptic vesicles to the nerve terminal cytoskeleton, thus regulating the availability of synaptic vesicles for exocytosis [18,19]. Indeed, videomicroscopy experiments carried out in a reconstituted system with purified components have shown that synapsin I is able to cross-link synaptic vesicles to actin [20]. In addition, genetically engineered mice lacking synapsin I exhibit changes in the number and distribution of synaptic vesicles, alterations in selected phenomena of synaptic plasticity and a decreased maximal neurotransmitter release [21–23].

In the present paper we have analyzed the effects of synaptic vesicles on the kinetics of actin polymerization and the effects of synapsin I when bound to the vesicle membrane or to artificial phospholipid bilayers. We have observed that in its membrane-bound state synapsin I maintains its phosphorylation-dependent activities on actin nucleation and elongation. These activities, albeit qualitatively similar, appear quantitatively reduced with respect to those of free synapsin I. The effects of membrane-bound dephosphorylated synapsin I largely account for the effects of intact synaptic vesicles on actin nucleation. However, synaptic vesicles depleted of synapsin I exhibit a residual inhibitory effect on filament elongation and on the total amount of polymerized actin, an effect which cannot be ascribed to the lipidic content of vesicles.

These results indicate that the interactions of synaptic vesicles with the actin cytoskeleton are complex, and are consistent with the idea that synapsin I plays a major role in regulating these interactions, which can be important in the modulation of vesicle traffic in the nerve terminal.

2. Materials and methods

2.1. Purification and phosphorylation of synapsin I

Synapsin I was purified from bovine brain as described by Schiebler et al. [14] and modified by Bähler and Greengard [7], and stored at −80°C in 200 mM NaCl, 25 mM Tris-HCl, pH 8.0. Purified dephosphorylated synapsin I was phosphorylated by CaM kinase II as described by Schiebler et al. [14].

2.2. Actin purification and derivatization with N-(1-pyrenyl)iodoacetamide

Actin was prepared from an acetone powder of rabbit skeletal muscle in buffer A (0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM NaN_3, 0.5 mM β-mercaptoethanol, 2 mM Tris-HCl, pH 8.0) as described by Spudich and Watt [24] and further purified by gel filtration on a Sephadex G-150 column (Pharmacia, Sweden) as described by MacLean-Fletcher and Pollard [25].

Pyrenyl actin was prepared by a modification of the procedure described by Cooper et al. [26]. Briefly, G-actin was dialyzed against buffer A without β-mercaptoethanol and NaN_3, diluted to a concentration of 1 mg/ml, and polymerized by adding KCl and $MgCl_2$ to final concentrations of 100 and 1 mM, respectively. *N*-(1-Pyrenyl)-iodoacetamide (final concentration: 0.3 mg/ml) was dissolved in dimethylformamide and added to polymerized actin, and the mixture was rotated in the dark at 4°C for 8 h. The actin filaments were collected by centrifugation in a Beckman TLA 100.3 rotor at 100000 rpm for 15 min; the pellet was resuspended in buffer A, dialyzed against the same buffer for 48 h and clarified by centrifugation in a Beckman TLA 100.3 rotor at 100000 rpm for 15 min.

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Abbreviations: CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II

PII S0014-5793(96)01240-9

2.3. Purification of synaptic vesicles

Synaptic vesicles were purified from rat forebrain through the step of chromatography on controlled-pore glass beads as described by Huttner et al. [13]. Purified synaptic vesicles were centrifuged for 2 h at 175 000 × *g* and resuspended at a protein concentration of 1–2 mg/ml in glycine buffer (0.3 M glycine, 5 mM HEPES-NaOH, pH 7.4; 0.02% NaN_3).

Endogenous synapsin I was removed from synaptic vesicles by diluting these vesicles immediately after elution from the controlled-pore glass column with an equal volume of 0.4 M NaCl. After 2 h incubation on ice, the synaptic vesicles were centrifuged for 2 h at 175 000 × *g* and resuspended in glycine buffer as described above. By this procedure synapsin I is quantitatively removed from the vesicle membrane [13,14].

Synapsin I was rebound to synapsin I-depleted synaptic vesicles by incubating the vesicles with purified bovine synapsin I at concentrations expected to yield almost maximal binding to the synaptic vesicle membrane. The amount of synapsin I to be added was calculated as the sum of the amount corresponding to 6% of the synaptic vesicle protein (i.e. the binding capacity of the vesicles) and the amount necessary to give an additional 100 nM free synapsin I under high-salt conditions, or 20 nM free dephosphosynapsin I/100 nM phosphosynapsin I under low-salt conditions (i.e. 2-fold the K_d of the binding of synapsin I to the vesicle membrane).

2.4. Preparation of phospholipid vesicles

Mixed phospholipid vesicles were prepared from a mixture mimicking the phospholipid composition of synaptic vesicles (phosphatidylcholine : phosphatidylethanolamine : phosphatidylserine : phosphatidylinositol : cholesterol = 40:32:12:5:10, w/w) [27]. 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 nitrogen and placed under vacuum for at least 2 h to remove residual traces of organic solvent. The dried lipid film was dissolved in a buffer containing 25 mM Tris-HCl, pH 7.4; 150 mM NaCl, 3 mM NaN_3 (buffer B), in the presence of 3% (w/v) octyl glucoside to a final phospholipid concentration of 5 mg/ml. The sample was gently stirred at room temperature for 20 min. The detergent: lipid molar ratio in the final solution was always kept above 10:1. To remove the detergent, the sample was extensively dialyzed against cold, degassed, N_2-saturated buffer B as described [17,28]. The vesicle sample was then dialyzed against glycine buffer.

2.5. Fluorescence measurements

Fluorescence data were obtained using an LS50 spectrofluorometer (Perkin-Elmer, UK). The excitation wavelength was 365 nm and the emission wavelength was 407 nm, with excitation and emission slits set at 2.5 and 10 nm, respectively. The sample temperature was maintained at 25°C by using a water-jacketed cuvette holder and a circulating water bath. The samples were allowed to equilibrate in the cuvette for 10 min and the polymerization of actin was monitored by recording the enhancement of pyrenyl-actin fluorescence. Polymerization was triggered by the addition of nucleating salts (30 mM KCl and 1 mM $MgCl_2$) and/or synaptic vesicles in either the absence or presence of synapsin I. The data were plotted as arbitrary fluorescence values relative to the fluorescence of the sample before polymerization. Polymerization curves run under the same conditions were highly reproducible within the same experimental session. Experiments were repeated at least three times with different batches of freshly purified vesicles with similar results, although the absolute values of fluorescence varied slightly from session to session.

2.6. Sedimentation assays

At the end of the fluorescence measurements, the fraction of polymerized actin was determined by centrifugation of the samples in a Beckman TLA 100.2 rotor at 100 000 rpm for 10 min, as described by Grazi [29]. The pellets and supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie Blue staining and densitometric scanning of the gels (Molecular Dynamics, USA) or by Western blotting with anti-synapsin I antibody followed by ^{125}I-protein A (Amersham, Italy).

2.7. High-shear viscosity assays

High-shear viscosity was measured in Ostwald-type viscometers (Cannon Instruments, USA) at 25°C. The samples were prepared as described for the pyrenyl-actin assays and the kinetics of actin polymerization was followed by measuring the viscosity of the solution at 2-min intervals. Results are expressed as specific viscosity (η_{sp}), which is defined as the flow time of the sample divided by the flow time of the appropriate control (an identical solution containing no actin), minus 1 as follows:

$$\eta_{sp} = (t_{sample}/t_{buffer}) - 1$$

2.8. Other procedures

Protein concentrations were determined using the method of Bradford [30] or spectrophotometrically using an $E^{1\%}_{290nm}$ of 6.5 for actin [31] and an $E^{1\%}_{277nm}$ of 6.7 for synapsin I [32]. The concentration and labeling stoichiometry of pyrenyl-actin were determined as outlined by Cooper et al. [26]. Mathematical processing of the digitized curves of pyrenyl actin polymerization was performed on a personal computer using PC-MATLAB software (Rapid Data Inc., UK).

3. Results

Actin polymerization was measured by monitoring the increase in fluorescence of pyrenyl-actin which occurs when actin monomers are incorporated into filaments [33]. Fig. 1 shows the results obtained by incubating pyrenyl-actin with synaptic vesicles and/or dephosphosynapsin I under high ionic strength conditions (85 mM NaCl, 30 mM KCl, 1 mM $MgCl_2$). In agreement with previously reported data [9,10], purified free dephosphosynapsin I abolished the lag phase and increased the initial rate of polymerization. In addition, in the presence of free dephosphosynapsin I, the plateau levels of fluorescence reached were slightly higher than in control samples.

Similarly to free dephosphosynapsin I (Fig. 1b), native synaptic vesicles containing endogenous synapsin I reduced the lag phase of polymerization (Fig. 1c). However, at variance with free dephosphosynapsin I which increases the amount of F-actin at steady state, native vesicles slowed down polymerization and decreased considerably the fluorescence level reached after 60 min. When the vesicles were depleted of endogenous synapsin I before incubation with actin, both the rate of polymerization and the final fluorescence values were markedly decreased (Fig. 1d). When saturating concentrations of purified dephosphosynapsin I were added to these vesicles, the polymerization curves were similar to those obtained with purified intact synaptic vesicles (Fig. 1e).

In order to clarify the molecular basis of the inhibition of polymerization exerted by synapsin I-depleted synaptic vesicles, we added vesicles to actin solutions at various time points after the addition of polymerizing salts. Fig. 2 (upper panel) shows the results of one such experiment. In this experiment, the vesicles were added 8 min after the nucleating salts, i.e. at a time point when the values of pyrenyl-actin fluorescence in the samples containing only actin were higher than those reached in samples which contained synaptic vesicles since the beginning. The addition of synapsin I-depleted synaptic vesicles did not lead to decreases in fluorescence readings. Indeed, fluorescence continued to increase also after the addition of synapsin I-depleted synaptic vesicles, although the rate of increase was lower than that of control samples, and similar to that observed in the corresponding sample shown in Fig. 1d.

The possibility that the inhibition of polymerization observed with synapsin I-depleted synaptic vesicles was due to the phospholipid component of the vesicles was evaluated by

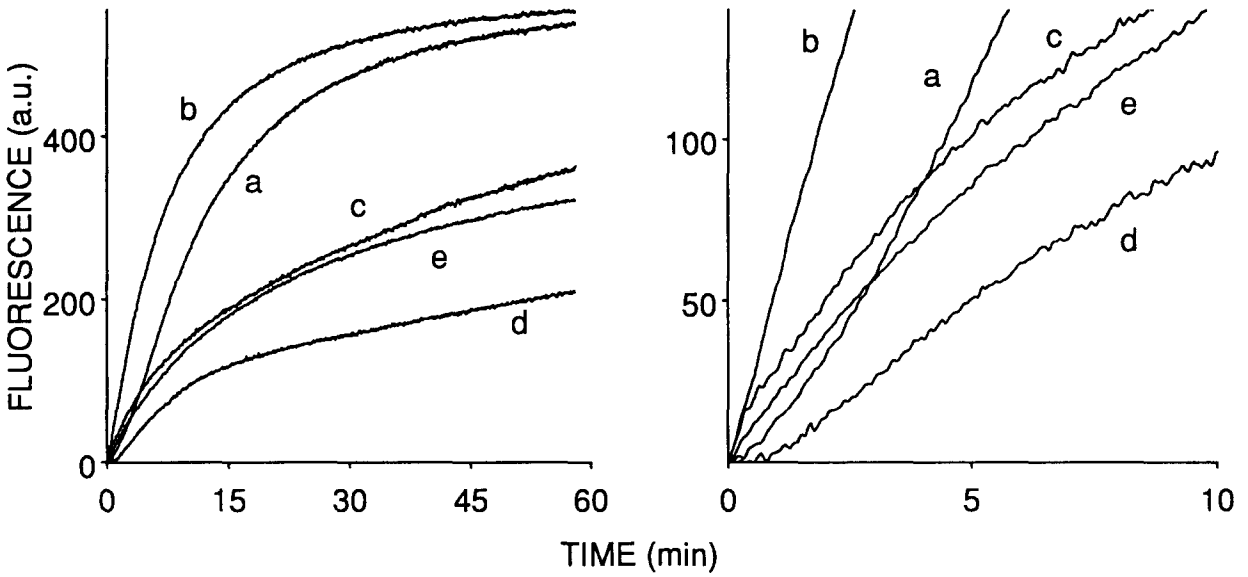

Fig. 1. Effect of synaptic vesicles and synaptic vesicle-bound synapsin I on actin polymerization triggered by KCl and $MgCl_2$. The polymerization of pyrenyl-G-actin (5 μM; 5% labeled) triggered by the addition of KCl and $MgCl_2$ was followed at 25°C by monitoring the increase in the fluorescence quantum yield. a, actin alone; b, actin plus 316 nM dephosphosynapsin I; c, actin plus untreated synaptic vesicles (0.3 mg protein/ml); d, actin plus synaptic vesicles depleted of endogenous synapsin I; e, actin plus synaptic vesicles (0.3 mg protein/ml) depleted of endogenous synapsin I and subsequently preincubated with 316 nM dephosphosynapsin I. The right panel shows the first 10 min of the experiment on an expanded scale. The experiments were performed under physiological ionic strength conditions. The final composition of the medium was: 85 mM NaCl, 30 mM KCl, 1 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM $CaCl_2$, 2.8 mM Tris-HCl, 100 mM glycine, 1.6 mM HEPES-NaOH, pH 7.4. Synapsin I and/or synaptic vesicles were added to the samples immediately prior to the addition of the nucleating salts.

testing the effects of pure lipid vesicles with a phospholipid composition similar to that of synaptic vesicles, i.e. enriched in acidic phospholipids such as phosphatidylserine and phosphatidylinositol [17]. At variance with artificial membranes bearing a net positive charge which were reported to favour actin polymerization [34], synaptic vesicle-like artificial vesicles altered neither the kinetics nor the final level of actin polymerization in the absence of synapsin I, independently of whether the vesicles were present from the beginning of the experiment (data not shown) or were added at later time points (Fig. 2, lower panel).

In order to characterize further the effect of synaptic vesicles, we employed two additional independent assays of polymerization, i.e. a high-speed sedimentation assay and a high-shear viscometry assay. The inhibitory effect of synaptic vesicles on the final extent of polymerization was corroborated by the finding that synaptic vesicles were able to decrease the amount of actin pelletable at high speed, an effect which was only partially reversed by the presence of dephosphosynapsin I bound to the vesicle membrane (Fig. 3). In agreement with what had been observed with the pyrenyl actin assay, synaptic vesicles decreased the steady-state values of viscosity in a dose-dependent fashion, with a more pronounced effect in the case of synapsin I-depleted synaptic vesicles (data not shown).

Given the complexity of the effects of synaptic vesicles on the polymerization of actin, it appeared useful to extrapolate the net effect of synapsin I. In order to achieve this, the curves of pyrenyl actin polymerization obtained with samples which did not contain synapsin I were subtracted from those of the corresponding samples containing synapsin I. The results of this analysis indicated that, in the absence of synaptic vesicles, the initial phases of polymerization were driven to a great extent by free synapsin I, whereas at later time points the contribution of the protein was quantitatively reduced. (Fig. 4a). These results are in agreement with the previously reported effects of dephosphosynapsin I on the nucleation of actin, and with the temporal analysis of the actin monomer binding rate in the presence of synapsin I [9,10]. In contrast, the effect of synaptic vesicle-bound synapsin I appeared biphasic. Thus, the initial actin nucleating effect appeared depressed with respect to the effect of free dephosphosynapsin I. However, at later time points, a contribution of dephosphosynapsin I to actin polymerization, which was not present with the protein free in solution, became apparent (Fig. 4b). The effect of dephosphosynapsin I bound to pure phospholipid vesicles was also analyzed. Under this condition, the contribution of synapsin I to polymerization appeared qualitatively similar to that of synaptic-vesicle bound dephosphosynapsin I, although quantitatively less pronounced in the second component of the curve (Fig. 4c).

Due to its actin-nucleating activity, free synapsin I is able to trigger the formation of actin filaments and bundles also in the absence of nucleating salts, i.e. under conditions in which actin polymerization is highly unfavoured [9]. Thus, it was of interest to determine whether synapsin I retains this ability when bound to membranes. Also in this case, it appeared useful to analyze the net effect of synapsin I by pairwise subtraction of the curves. In the absence of nucleating salts, the contribution made by free dephosphosynapsin I to actin polymerization appeared to increase monotonically with time (Fig. 5a). Dephosphorylated synapsin I bound to synaptic vesicles was still able to induce actin polymerization in the absence of K^+ and Mg^{2+}, although this activity was depressed with respect to that of the free form of the protein (Fig. 5b). In agreement with what had been observed with the free protein (data not shown and [5]), synapsin I stoichiometrically phosphorylated by CaM kinase II and subsequently bound to syn-

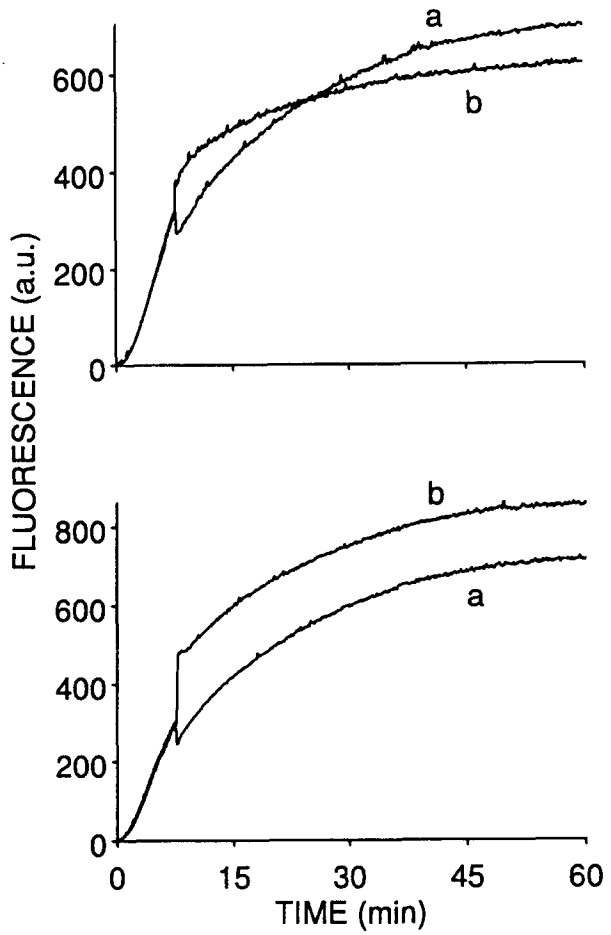

Fig. 2. Effect of synaptic vesicles and of pure phospholipid vesicles on the kinetics of actin filament elongation in the absence of synapsin I. The polymerization of G-actin (5 μM; 5% labeled) was triggered by the addition of KCl and $MgCl_2$. After 8 min either glycine buffer (a, upper and lower panels) synaptic vesicles depleted of endogenous synapsin I (0.3 mg protein/ml) (b, upper panel) or pure phospholipid vesicles (0.75 mg phospholipid/ml) (b, lower panel) were added to the cuvette and the incubation was continued for another 52 min. Ionic conditions were as described in the legend to Fig. 1. Experiments were performed under continuous stirring of the solution in the cuvette. Note the shifts in the fluorescence values due to autofluorescence of phospholipids and/or to dilution of the samples.

aptic vesicles lost its ability to drive actin polymerization (Fig. 5c). When dephosphorylated synapsin I was bound to pure phospholipid vesicles, actin polymerization reached a plateau level similar to that induced by synaptic vesicle-bound synapsin I (Fig. 5d), confirming that the interaction with the phospholipid bilayer partially interferes with the ability of synapsin I to trigger actin polymerization.

4. Discussion

In the present paper we have studied the effects of synaptic vesicles and of synaptic vesicle-bound synapsin I on the kinetics of actin polymerization by using a fluorometric technique that allows one to monitor actin polymerization by following the increase in fluorescence quantum yield of actin covalently labeled with pyrene. This technique offers the advantage of being non-perturbing, very sensitive, and of allowing a good degree of temporal resolution [33].

The data indicate that synaptic vesicles are able to influence the kinetics of polymerization. A major effect is due to the presence on the vesicle membrane of synapsin I, a protein which, in its soluble form, has previously been shown to exert a phosphorylation-dependent actin-nucleating effect [9–11]. The main results can be summarized as follows:

1. synapsin I bound to synaptic vesicles stimulates actin nucleation with an effect that is qualitatively similar to that observed for the protein free in solution albeit quantitatively smaller;
2. the partial decrease in actin polymerizing ability of synaptic vesicle-bound synapsin I is attributable to the hydrophobic interaction with membrane phospholipids of synaptic vesicles, as it is mimicked by the interaction of synapsin I with pure phospholipid vesicles;
3. the effect of membrane-bound synapsin I is also maintained under conditions in which actin polymerization is unfavoured and remains sensitive to phosphorylation by CaM kinase II which abolishes the ability of synapsin I to drive actin polymerization;
4. synaptic vesicles depleted of synapsin I decrease the rate and the steady-state level of actin polymerization triggered by polymerizing salts, an effect which is attributable to protein components and not to membrane phospholipids;
5. synapsin I bound to vesicles in dephosphorylated form can partially rescue actin polymerization from synaptic vesicle-mediated inhibition, exhibiting a facilitatory effect also on the later phases of polymerization which is not apparent when synapsin I is not bound to the membrane.

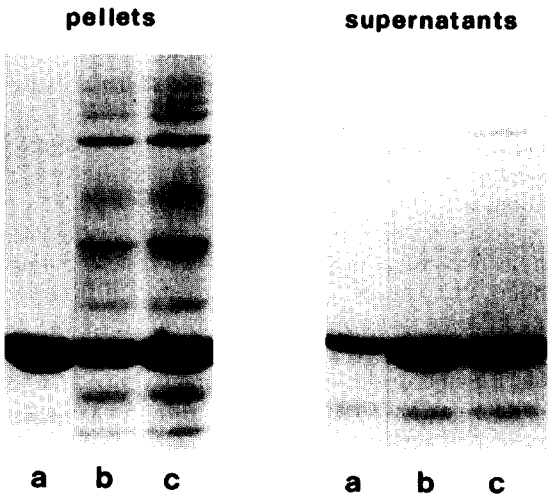

Fig. 3. Effect of synaptic vesicles and synaptic vesicle-bound synapsin I on the high-speed sedimentation of actin. 5 μM G-actin was polymerized for 2 h at 25°C in the absence or presence of synaptic vesicles. Ionic conditions were as described in the legend to Fig. 1. The amount of polymerized actin was determined by high-speed sedimentation (10 min at 100 000 rpm in a Beckman TLA 100.2 rotor), followed by analysis of the pellets and supernatants by Coomassie Blue staining of sodium dodecyl sulfate, 10% polyacrylamide gels. a, actin alone; b, actin plus synapsin I-depleted synaptic vesicles (0.3 mg protein/ml); c, actin plus synaptic vesicles (0.3 mg protein/ml) depleted of endogenous synapsin I and preincubated with 316 nM purified dephosphorylated synapsin I.

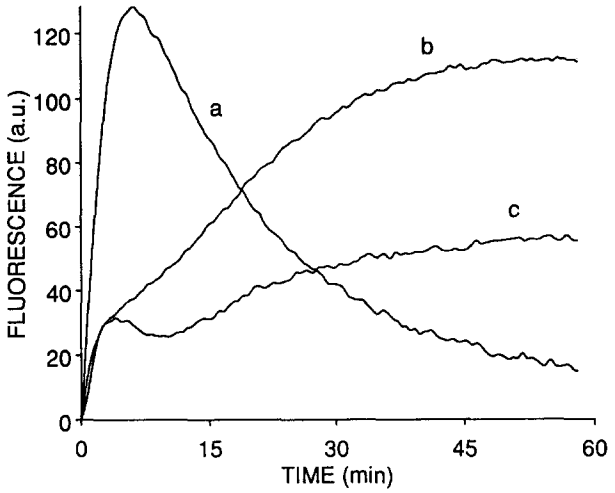

Fig. 4. Net effects of free and vesicle-bound synapsin I on actin polymerization triggered by KCl and $MgCl_2$. The polymerization of G-actin (5 μM; 5% labeled) was evaluated in the absence or presence of dephosphosynapsin I (316 nM) and/or synaptic vesicles (0.3 mg protein/ml) depleted of endogenous synapsin I or pure phospholipid vesicles (0.75 mg phospholipid/ml). Ionic conditions were as described in the legend to Fig. 1. The net effect of synapsin I was extrapolated by pairwise subtraction of the curves obtained in the presence and absence of the protein. a, effect of free dephosphosynapsin I ([actin+synapsin I]−[actin alone]); b, effect of dephosphosynapsin I bound to synaptic vesicles ([actin+synapsin I-depleted synaptic vesicles+synapsin I]−[actin+synapsin I-depleted synaptic vesicles]); c, effect of dephosphosynapsin I bound to pure phospholipid vesicles ([actin+phospholipid vesicles+synapsin I]−[actin+phospholipid vesicles]).

Two potential binding sites for F-actin have been described in the NH_2-terminal (domain C) and COOH-terminal (domain D) regions of synapsin I, respectively [15]. The NH_2-terminal region of synapsin I is also responsible for the interaction with actin monomers [9]. It is not clear yet whether the actin binding site is the same, although it has been suggested that synapsin I may remain attached to the sides of the growing filament after formation of actively elongating pseudonuclei [9]. The NH_2-terminal region of synapsin I is also involved in the interaction with the synaptic vesicle membrane and in the penetration of the phospholipid bilayer [16,17]. The reduced potency of vesicle-bound synapsin I with respect to the free protein might therefore be related to the simultaneous interaction with membrane phospholipids, which may reduce the mobility of synapsin I and its possibility to interact with actin monomers. The fact that synapsin I retains its ability to interact with actin monomers is in agreement with the rapid formation of actin filaments and bundles occurring when dephosphorylated synapsin I is added to a mixture of G-actin and synapsin I-depleted synaptic vesicles, as observed by videomicroscopy [20].

Synaptic vesicles depleted of endogenous synapsin I exhibit an inhibitory effect on the rate of actin polymerization and on the concentration of actin filaments at steady state. Since artificial vesicles sharing the same phospholipid composition with synaptic vesicles do not interfere with actin polymerization, a protein component with an intrinsic capping/severing activity may be responsible for the effects of synaptic vesicles on actin polymerization. When synapsin I is bound to synaptic vesicles, it may counteract these effects either directly or by preventing the interaction of this vesicle component with actin. Experiments are in progress aimed at determining the molecular identity of such protein component.

Taken together, the results indicate that: (a) the interaction of synapsin I with the synaptic vesicle membrane and the stimulation of actin polymerization are not mutually exclusive and support the hypothesis that synapsin I regulates the trafficking of synaptic vesicles during the exo-endocytotic cycle [19] and (b) synaptic vesicles interact in a complex manner with cytoskeletal actin of the nerve terminal: in synapsin I-free form they possess an intrinsic inhibitory activity on actin assembly and polymerization which is counteracted and transformed into a facilitatory activity upon association with dephosphorylated synapsin I. Since synapsin I also has a stabilizing effect on the vesicle membrane [35], dissociation of synapsin I triggered by CaM kinase II phosphorylation might be followed by both actin cytoskeleton disassembly and increased propensity for fusion.

In conclusion, the assembly of the actin cytoskeleton in the nerve terminal may depend not only on the state of phosphorylation of synapsin I, but also on its degree of physical association with synaptic vesicles. The two phenomena, which are interrelated, are sensitive to the levels of second messengers and to the physical state of the nerve terminal and can be important for the regulation of synaptic vesicle traffic and hence of the rate of the exocytotic process.

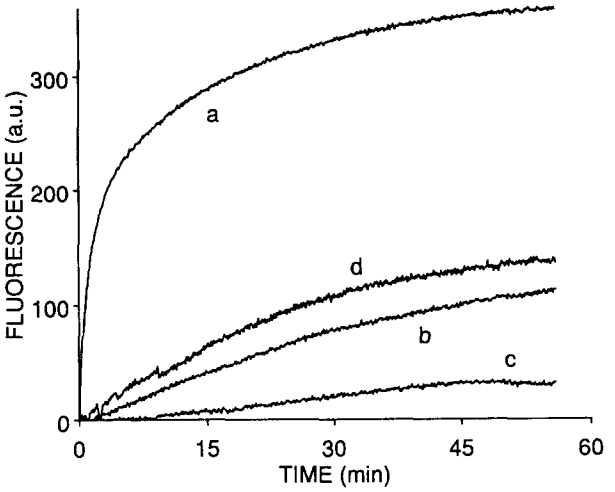

Fig. 5. Net effects of free and vesicle-bound synapsin I on actin polymerization in the absence of KCl and $MgCl_2$. G-actin (5 μM; 5% labeled) was incubated under low ionic strength conditions (15 mM NaCl), in a buffer containing 0.1 mM ATP, 0.1 mM $CaCl_2$, 2.8 mM Tris-HCl, 100 mM glycine, 1.6 mM HEPES-NaOH, pH 7.4. Polymerization was triggered by the addition of either synaptic vesicles (0.3 mg protein/ml) depleted of endogenous synapsin I or pure phospholipid vesicles (0.75 mg phospholipid/ml) in the absence or presence of synapsin I (236 nM dephosphorylated synapsin I or 316 nM synapsin I phosphorylated by CaM kinase II). The net effect of synapsin I was extrapolated by pairwise subtraction of the curves obtained in the presence and absence of the protein. a, effect of free dephosphosynapsin I ([actin+dephosphosynapsin I]−[actin alone]); b, effect of dephosphosynapsin I bound to synaptic vesicles ([actin+synapsin I-depleted synaptic vesicles+dephosphosynapsin I]−[actin+synapsin I-depleted synaptic vesicles]); c, effect of synapsin I phosphorylated by CaM kinase II bound to synaptic vesicles ([actin+synapsin I-depleted synaptic vesicles+phosphosynapsin I]−[actin+synapsin I-depleted synaptic vesicles]); d, effect of dephosphosynapsin I bound to pure phospholipid vesicles ([actin+phospholipid vesicles+synapsin I]−[actin+phospholipid vesicles]).

Acknowledgements: We wish to thank Dr. Riccardo Fesce for help in mathematical processing of the polymerization curves, Dr. Andrea Menegon for photographic assistance and Dr. Paul Greengard for continuous support. The work presented was supported by grants from Telethon to F.V. and F.B.. P.E.C. was the recipient of a fellowship from the European Molecular Biology Organization.

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