THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1992 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 267, No. 16, Issue of June 5, pp. 11281-11288, 1992
Printed in U.S.A.

Effects of the Neuronal Phosphoprotein Synapsin I on Actin Polymerization

I. EVIDENCE FOR A PHOSPHORYLATION-DEPENDENT NUCLEATING EFFECT*

(Received for publication, August 15, 1991)

Flavia Valtorta‡§, Paul Greengard¶, Riccardo Fesce‡, Evelina Chieregatti‡, and Fabio Benfenati‖

From the ‡"Bruno Ceccarelli" Center for the Study of Peripheral Neuropathies, Department of Medical Pharmacology, National Research Council Center of Cytopharmacology, San Raffaele Scientific Institute, University of Milano, 20129 Milano, Italy, the ¶Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, New York 10021-6399, and the ‖Institute of Human Physiology, University of Modena, 41100 Modena, Italy

Synapsin I is a synaptic vesicle-specific phosphoprotein which is able to bind and bundle actin filaments in a phosphorylation-dependent fashion. In the present paper we have analyzed the effects of synapsin I on the kinetics of actin polymerization and their modulation by site-specific phosphorylation of synapsin I. We found that dephosphorylated synapsin I accelerates the initial rate of actin polymerization and decreases the rate of filament elongation. The effect was observed at both low and high ionic strength, was specific for synapsin I, and was still present when polymerization was triggered by F-actin seeds. Dephosphorylated synapsin I was also able to induce actin polymerization and bundle formation in the absence of KCl and $MgCl_2$. The effects of synapsin I were strongly decreased after its phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II. These observations suggest that synapsin I has a phosphorylation-dependent nucleating effect on actin polymerization. The data are compatible with the view that changes in the phosphorylation state of synapsin I play a functional role in regulating the interactions between the nerve terminal cytoskeleton and synaptic vesicles in various stages of the exo-endocytotic cycle.

Synapsin I is a neuronal phosphoprotein primarily associated with the cytoplasmic surface of synaptic vesicles. Synapsin I undergoes multisite phosphorylation: one serine residue in the NH_2-terminal part of the molecule is phosphorylated by cAMP-dependent protein kinase and by Ca^{2+}/calmodulin-dependent protein kinase I, whereas two other serine residues, located in the COOH-terminal region, are phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II)[1] (for review, see De Camilli *et al.*, 1990). The binding of synapsin I to the synaptic vesicle membrane is characterized by high affinity and saturability and is modulated by phosphorylation, the binding affinity being reduced 5-fold upon phosphorylation of synapsin I by CaM kinase II (Huttner *et al.*, 1983; Schiebler *et al.*, 1986).

In *in vitro* systems, synapsin I has been shown to bind to the sides of actin filaments and to bundle them (Bähler and Greengard, 1987; Petrucci and Morrow, 1987). The bundling activity is decreased after phosphorylation by the catalytic subunit of cAMP-dependent protein kinase and virtually abolished after phosphorylation by CaM kinase II. In addition, phosphorylation by CaM kinase II induces a 50% decrease in the number of binding sites for synapsin I on actin filaments (Bähler and Greengard, 1987).

A variety of physiological and pharmacological manipulations known to stimulate neurotransmitter release have been shown to increase the state of phosphorylation of synapsin I (Nestler and Greengard, 1984). A role of synapsin I phosphorylation in the regulation of neurotransmitter release is also supported by experiments in which synapsin I was injected into the presynaptic digit of the squid giant synapse. In these experiments, it was observed that dephosphosynapsin I inhibits stimulus-evoked and spontaneous neurotransmitter release and that these effects are prevented by phosphorylation of synapsin I by CaM kinase II (Llinas *et al.*, 1985, 1991; Lin *et al.*, 1990). Since the binding of synapsin I to synaptic vesicles and to F-actin is reversible and is modulated by phosphorylation, it has been proposed that synapsin I regulates neurotransmitter release by forming a dynamic link between synaptic vesicles and the F-actin-based cytoskeletal network of the nerve terminal, thereby modulating the availability of synaptic vesicles for exocytosis (Bähler *et al.*, 1990; Benfenati *et al.*, 1991).

In recent years, the idea has emerged that exocytosis in nerve terminals as well as in other secretory systems involves the reorganization or disassembly of the subplasmalemmal network of actin filaments (for review, see Linstedt and Kelly, 1987; Valtorta *et al.*, 1990). In fact, actin filaments are highly dynamic structures which are able to undergo rapid cycles of polymerization and depolymerization. The state of polymerization of actin is believed to be a major factor in determining cytoplasmic viscosity and may therefore play a role in the regulation of organelle movement.

We have examined the possibility that synapsin I, in addi-

* This work was supported by Consiglio Nazionale delle Ricerche Progetto Strategico "Meccanismi di Release dei Neurotrasmettitori e loro Controllo" (to F. B.), Progetto Speciale "Meccanismi Molecolari di Trasduzione del Segnale" (to F. V.), Progetto Finalizzato "Invecchiamento" (to R. F., code 921131), NATO Collaborative Grant 0039/89 (to F. B. and P. G.), and by United States Public Health Service Grant MH 39327 (to P. G.). The support of Telethon (to F. V., F. B., and R. F.) is also acknowledged. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be sent: "B. Ceccarelli" Center, Dept. of Medical Pharmacology, University of Milano, Via Vanvitelli 32, I-20129 Milano, Italy. Tel.: 39-276110224; Fax: 39-27490937.

[1] The abbreviations used are: CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; G-actin, globular actin; F-actin, filamentous actin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

tion to binding to the sides of actin filaments, also affects the kinetics of actin polymerization, thereby regulating the assembly of actin within the nerve terminal. We have found that synapsin I is able to promote actin polymerization and that this property is modulated by phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—The catalytic subunit of cAMP-dependent protein kinase and CaM kinase II, purified as described by Kaczmarek *et al.* (1980) and by McGuinness *et al.* (1985), were gifts of A. Horiuchi and A. Nairn (The Rockefeller University, New York). *N*-(1-Pyrenyl)iodoacetamide was from Molecular Probes (Eugene, OR). Sephadex G-150 was from Pharmacia (Uppsala, Sweden). All other chemicals were of reagent grade and were obtained from standard commercial suppliers.

Purification and Phosphorylation of Synapsin I—Synapsin I (80–86 kDa) was purified from bovine brain as described by Schiebler *et al.* (1986) and modified by Bähler and Greengard (1987) and stored in 200 mM NaCl, 25 mM Tris-HCl, pH 8.0 at −80 °C. Purified dephosphorylated synapsin I was phosphorylated either by the catalytic subunit of cAMP-dependent protein kinase (site 1) or by CaM kinase II (sites 2 and 3) as described by Schiebler *et al.* (1986). The average phosphorylation stoichiometries were 0.89 mol of phosphate/mol of synapsin I for site 1 and 2.15 mol of phosphate/mol of synapsin I for sites 2 and 3. The COOH-terminal fragment of synapsin I (35–40 kDa) was generated by cysteine-specific cleavage of synapsin I with 2-nitro-5-thiocyanobenzoic acid and purified as described by Bähler *et al.* (1989).

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 (1971) and further purified by gel filtration on a Sephadex G-150 column following the procedure outlined by MacLean-Fletcher and Pollard (1980).

Pyrenyl-actin was prepared by a modification of the procedure described by Cooper *et al.* (1983). 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, added to polymerized actin, and the mixture was rotated in the dark at 4 °C for 8 h. Actin filaments were collected by centrifugation in a Beckman TLA 100.3 rotor at 100,000 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 100,000 rpm for 15 min. The pyrene/actin molar ratio varied between 0.65 and 0.9 in the various preparations. G-actin was kept on ice under continuous dialysis against buffer A and used within 2–3 days after the purification.

Fluorescence Measurements—Fluorescence data were obtained using an LS50 spectrofluorometer (Perkin-Elmer, Great Britain). The excitation wavelength was 365 nm, and the emission wavelength was 407 nm. The excitation and emission slits were 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 then the polymerization reaction was initiated by the addition of salts (KCl and $MgCl_2$) and/or synapsin I or synapsin I storage buffer. The sample size was 1.5 ml. The data are plotted as arbitrary fluorescence values relative to the fluorescence of the sample before polymerization. Polymerization curves run in the same conditions were highly reproducible within the same experimental session. Polymerization was generally faster on the first day, with freshly purified G-actin, and slowed down on the following days.

In the experiments in which the Ca^{2+}-dependence of the effect of synapsin I on actin polymerization was to be monitored, the free Ca^{2+} concentration was buffered by the addition of appropriate amounts of EGTA or EDTA as described by Martell and Smith (1974). EGTA and/or EDTA were added to the samples 10 min before polymerization was started by the addition of synapsin I and/or KCl and $MgCl_2$.

Sedimentation Assays—In order to determine the amount of polymerized actin, samples containing 5 μM G-actin were allowed to polymerize at 25 °C for 2 h under various ionic conditions in the presence or absence of synapsin I. The samples were then centrifuged in a Beckman TLA-100 rotor at 95,000 rpm for 10 min, as described by Grazi (1985), and the pellets and supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie Blue staining and densitometric scanning of the gels (Ultroscan XL laser densitometer, LKB, Bromma, Sweden).

Electron Microscopy—Actin-containing samples were prepared by polymerizing monomeric actin in the presence or absence of synapsin I for 30 min at room temperature in solutions of the same composition as those used for the polymerization assay. The samples (20 μl) were applied to carbon-coated copper grids which had been glow-discharged immediately before use in order to make them hydrophilic. After 40 s, the grids were rinsed with 10 drops of either 30 mM KCl, 1 mM $MgCl_2$, 0.5 mM ATP, pH 7.4, or 0.5 mM ATP, pH 7.4, followed by staining with 1% uranyl acetate for 30 s. The droplet of stain was then drawn off with filter paper and the grids were allowed to dry before observing them in a Hitachi H-7000 electron microscope.

Other Procedures—Protein concentrations were determined by a modification (Markwell *et al.*, 1978) of the method of Lowry (Lowry *et al.*, 1951) or spectrophotometrically using an $E^{1\%}_{290\ nm}$ of 6.5 for actin (Pollard, 1976) and an $E^{1\%}_{277\ nm}$ of 6.7 for synapsin I (Ueda and Greengard, 1977). The concentration and labeling stoichiometry of pyrenyl-actin were determined as outlined by Cooper *et al.* (1983). Free Ca^{2+} concentrations were estimated by a computer program using the stability constants described by Martell and Smith (1974).

RESULTS

Effects of Synapsin I on the Polymerization of Actin Induced by KCl and $MgCl_2$—Solutions containing 5 μM G-actin (5% labeled with *N*-(1-pyrenyl)iodoacetamide) were polymerized by the addition of 30 mM KCl and 1 mM $MgCl_2$. Polymerization was monitored by recording the enhancement of pyrenyl-actin fluorescence. The addition of synapsin I immediately prior to the nucleating salts caused a remarkable change in the kinetics of actin polymerization. Synapsin I virtually abolished the lag phase (corresponding to the activation and nucleation of actin monomers) and induced a dose-dependent acceleration of the initial rate of polymerization (Fig. 1, *upper panel*). In addition, the highest concentration of synapsin I tested (300 nM) also induced a decrease in the rate of filament elongation (see also Fig. 8). However, when the polymerization curves were followed to completion (3–4 h), the final levels of fluorescence of the synapsin I-containing samples were comparable with those of the control samples (data not shown).

Due to the relatively low ionic strength conditions used (15 mM NaCl, 30 mM KCl, 1 mM $MgCl_2$) and to the very basic isoelectric point of synapsin I, the observed effects might be due to nonspecific interactions between actin and synapsin I. In order to address this question, the effects of other basic proteins on actin polymerization kinetics were evaluated under the same ionic conditions. Cytochrome *c*, a protein with an isoelectric point similar to that of synapsin I, and the COOH-terminal fragment of synapsin I, which is entirely responsible for the basicity of the whole molecule, were analyzed. As shown in Fig. 1 (*lower panel*), neither cytochrome *c* nor the COOH-terminal fragment of synapsin I elicited effects similar to those of synapsin I. However, whereas in the presence of cytochrome *c* the actin polymerization curve was indistinguishable from that of actin alone, the COOH-terminal fragment of synapsin I induced a decrease in the elongation rate similar to that observed in the presence of the same concentration of holosynapsin I. Under the same ionic conditions, synapsin I was shown to decrease the critical concentration for actin polymerization in a dose-dependent fashion (Benfenati *et al.*, 1992).

Since the actions of many nucleating proteins are Ca^{2+}-dependent, the effects of synapsin I on actin polymerization were also analyzed at a buffered Ca^{2+} concentration below 10^{-9} M. The chelation of free Ca^{2+} *per se* shortened the lag phase and increased the rate of actin polymerization, in agreement with previous data indicating that in the presence

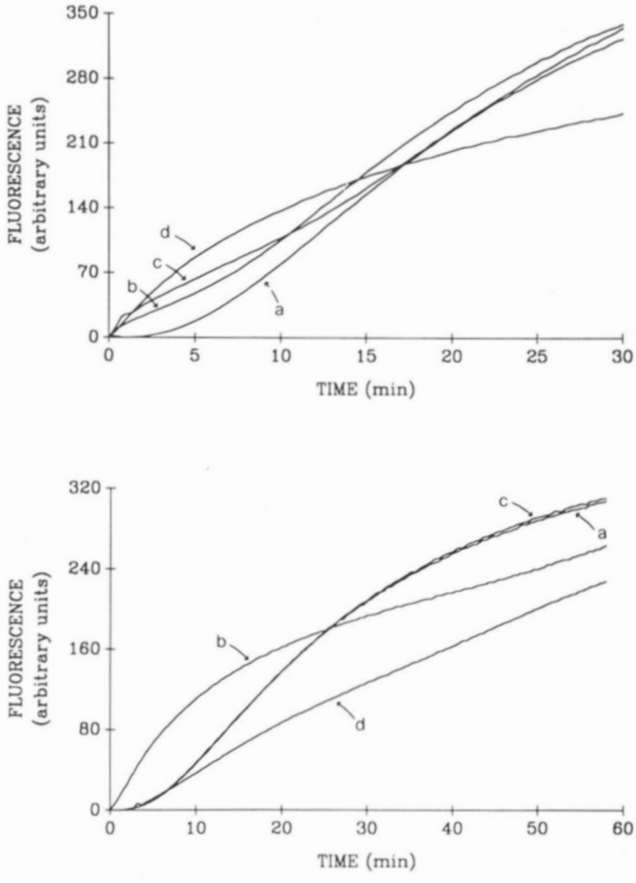

FIG. 1. **Synapsin I accelerates the kinetics of actin polymerization.** The polymerization of pyrenyl G-actin (5 μM; 5% labeled) was followed at 25 °C by monitoring the increase in fluorescence occurring after the addition of KCl and $MgCl_2$ at time 0. *Upper panel*, curves obtained in the absence (*a*) or presence of 50 nM (*b*), 100 nM (*c*), or 300 nM (*d*) dephosphorylated synapsin I are shown. The experimental conditions were: 15 mM NaCl, 30 mM KCl, 1 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, 0.5 mM NaN_3, 4.5 mM Tris-HCl, pH 7.6. *Lower panel*, the specificity of the effect was tested under the same ionic conditions by following the polymerization of actin in the presence of various basic proteins (*a*, actin alone; *b*, 300 nM synapsin I; *c*, 300 nM cytochrome *c*; *d*, 300 nM COOH-terminal fragment of synapsin I). The various proteins were added to the incubation mixture immediately before the addition of K^+ and Mg^{2+}.

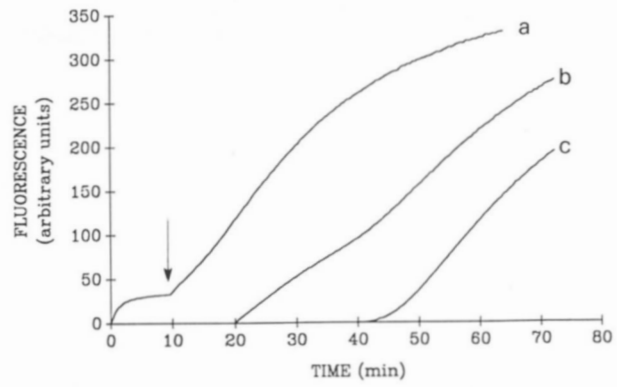

FIG. 2. **Synapsin I triggers actin polymerization in the absence of KCl and $MgCl_2$.** *a*, 100 nM dephosphorylated synapsin I was added to 5 μM pyrenyl-G-actin (5% labeled) at time 0. At time 10 min (*arrow*), 30 mM KCl and 1 mM $MgCl_2$ were added to complete polymerization. *b*, 100 nM dephosphorylated synapsin I, 30 mM KCl, and 1 mM $MgCl_2$ were added to 5 μM pyrenyl G-actin (5% labeled) at time 20 min. *c*, actin polymerization was triggered by the addition of 30 mM KCl and 1 mM $MgCl_2$ at time 40 min in the absence of synapsin I. Buffer conditions were: 15 mM NaCl, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, 0.5 mM NaN_3, 4.5 mM Tris-HCl, pH 7.6.

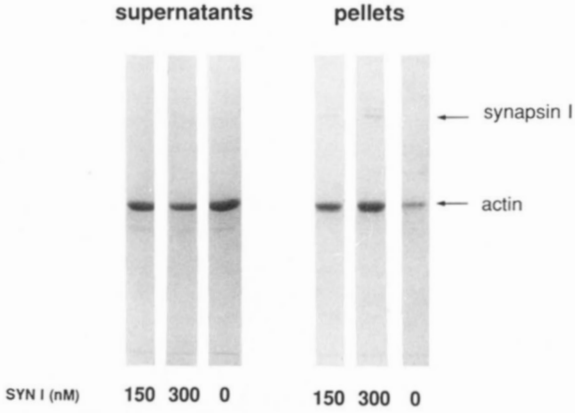

FIG. 3. **Effect of synapsin I on the high-speed sedimentation of actin.** 5 μM G-actin was incubated for 2 h at 25 °C in the absence of K^+ and Mg^{2+} (15 mM NaCl present) with or without synapsin I. The amount of polymerized actin was determined by high-speed sedimentation (10 min at 95,000 rpm in a Beckman TLA 100 rotor), followed by analysis of Coomassie Blue-stained sodium dodecyl sulfate, 10% polyacrylamide gels.

of Ca^{2+} nucleation is preceded by divalent cation exchange (Frieden *et al.*, 1980; Tobacman and Korn, 1983). However, in the absence of Ca^{2+} the addition of synapsin I was still effective in abolishing the lag phase and in inducing a further increase in the polymerization rate (see Fesce *et al.*, 1992 (accompanying paper)). Additional data on the effects of synapsin I on the actin critical concentration and on the number of filament ends confirm that the effects of synapsin I are not dependent on the presence of Ca^{2+} (Benfenati *et al*, 1992).

Effects of Synapsin I in the Absence of K^+ and Mg^{2+}—It was of interest to examine whether synapsin I could activate actin polymerization in the absence of K^+ and Mg^{2+}. Fig. 2 shows the results of such an experiment. When 100 nM synapsin I was added to G-actin in the absence of K^+ and Mg^{2+} (*curve a*, initial part), a fast increase in pyrenyl-actin fluorescence occurred, and the polymerization curve exhibited a hyperbolic shape. The initial rate, as well as the level of fluorescence reached, were proportional to the concentration of synapsin I present (see below). A subsequent addition of KCl and $MgCl_2$ triggered a further increase in fluorescence, with a shape similar to that induced by the polymerization of actin alone (*curve c*), except that no lag phase was present. When 100 nM synapsin I and nucleating salts were added simultaneously (*curve b*), the overall shape of the polymerization curve appeared to result from the simultaneous occurrence of the two processes.

In this experiment, the synapsin I storage buffer contributed 15 mM NaCl to the medium. However, the addition of 15 mM NaCl to G-actin in the absence of synapsin I did not induce any noticeable increase in fluorescence during the first 20 min of the experiment (see Fig. 7, *curve a*). At this time point, the amount of actin recovered in the high-speed pellet was significantly increased by synapsin I in a dose-dependent fashion. The concentrations of nonpelletable actin in samples containing 5 μM actin and 0, 150, and 300 nM synapsin I were 4.71, 4.20, and 3.75 μM, respectively. After a 2-h incubation under the same ionic conditions, these values decreased to 4.47, 2.39, and 1.46 μM, respectively (Fig. 3).

Polymerization curves obtained in the absence of K^+ and Mg^{2+} with various concentrations of synapsin I (150 and 300

nM) and of G-actin (1, 2.5, 5, and 7.5 μM) are shown in Fig. 4 (*upper* and *middle panels*). The hyperbolic curves reached plateaus which were proportional to the synapsin I concentration and appeared to be relatively independent of the actin concentration. At 7.5 μM actin a noticeable slope appeared, which was superimposed on the plateau. The plateau levels of fluorescence, calculated by logasymptotic extrapolation of curves performed with concentrations of actin ranging from 0.062 to 7.5 μM in the presence of 150 and 300 nM synapsin I, are plotted *versus* the actin/synapsin I molar ratio in the *lower panel* of Fig. 4. The maximal levels of fluorescence increased linearly with the actin concentration up to an apparent actin/synapsin I ratio of 3.5–4.15. Beyond this point, the samples run in the presence of 150 nM synapsin I exhibited constant levels of fluorescence up to 5 μM actin. At 7.5 μM actin, a considerable deviation from the general behavior was observed, suggesting that under these conditions some degree of further polymerization can occur even in the absence of K^+ and Mg^{2+}. In the presence of 300 nM synapsin I, a clear-cut plateau was not apparent, and further polymerization seemed to occur at lower actin concentrations (2.5–5 μM).

Analysis at the electron microscope after negative staining (Fig. 5) showed that in the presence of K^+ and Mg^{2+} synapsin I induced the formation of thick bundles of actin filaments (compare *panels a* and *b*). At higher magnification cross-bridges arranged in a regular fashion between adjacent actin filaments were revealed (*inset*). Fig. 5, *panel c*, is from a sample in which G-actin was incubated with synapsin I in the absence of K^+ and Mg^{2+}. Tiny actin filaments, bundles (*ar-*

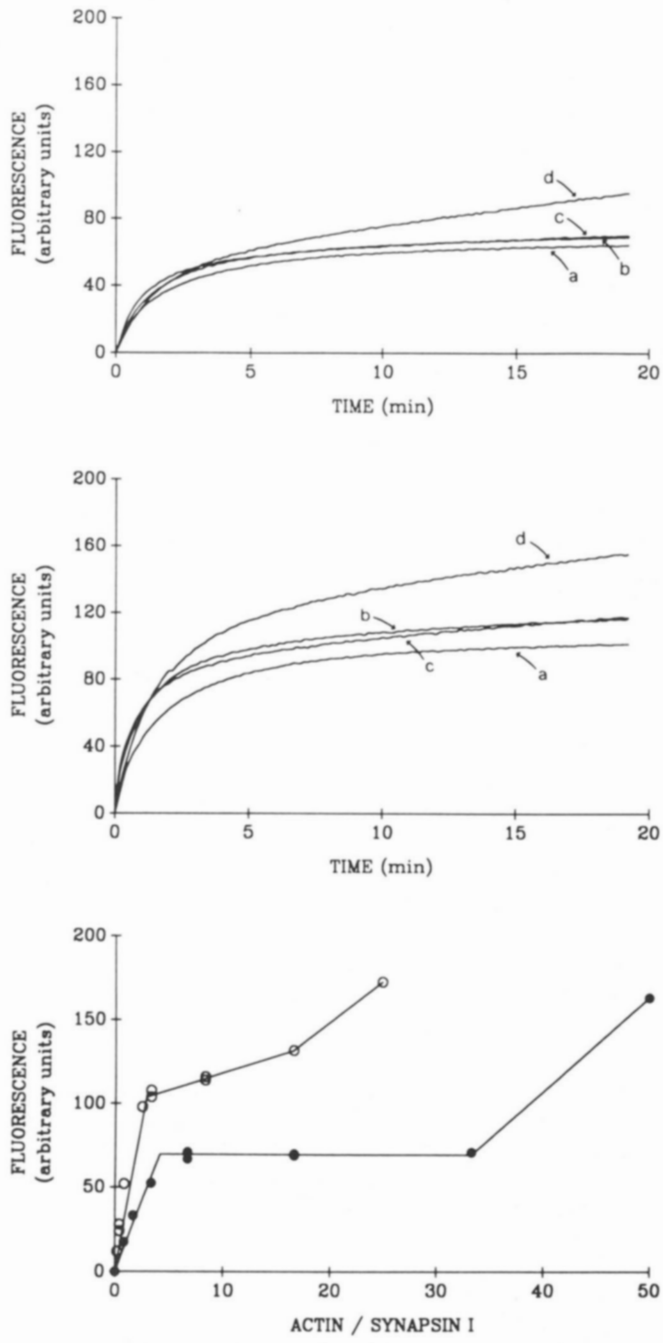

FIG. 4. **Dose-dependent effect of synapsin I on actin polymerization in the absence of K^+ and Mg^{2+}.** The polymerization of various concentrations of pyrenyl G-actin (0.062–7.5 μM) triggered by the addition of 150 nM (*upper panel*) or 300 nM (*middle panel*) synapsin I was measured in a buffer containing 15 mM NaCl, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, 0.5 mM NaN_3, 4.5 mM Tris-HCl, pH 7.6. For clarity, only some of the curves are shown. Actin concentrations: 1 μM (*a*), 2.5 μM (*b*), 5 μM (*c*), 7.5 μM (*d*). *Lower panel*, the extrapolated plateau levels of fluorescence obtained from the experiments performed at 150 (●) and 300 (○) nM synapsin I are plotted as a function of the actin/synapsin I molar ratio.

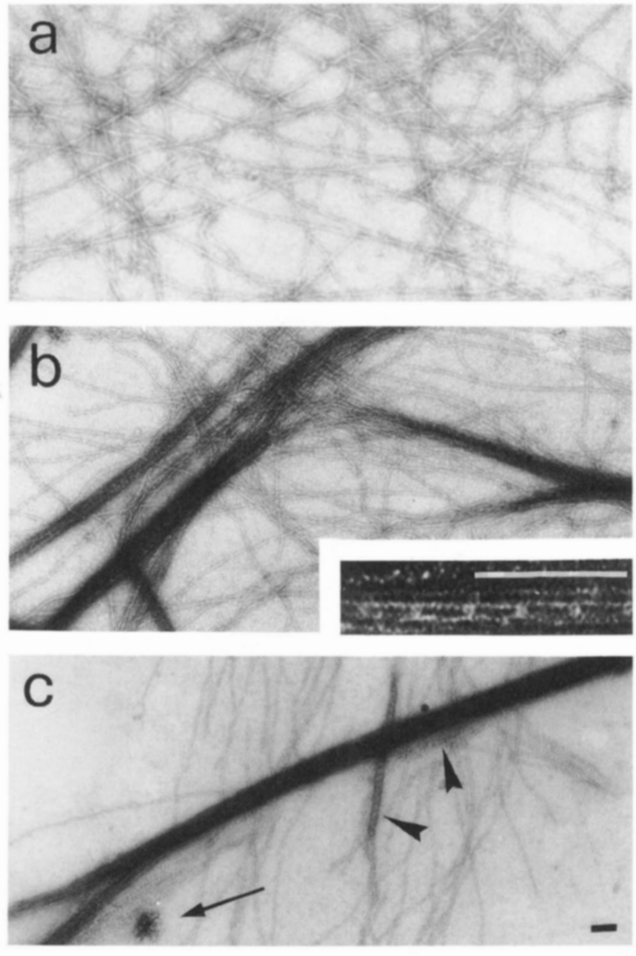

FIG. 5. **Electron microscopy of negatively stained actin samples.** Panels *a* and *b* show preparations of actin (5 μM) polymerized by the addition of 30 mM KCl and 1 mM $MgCl_2$ in the absence (*a*) or presence (*b*) of 300 nM dephosphorylated synapsin I. Buffer conditions were as described in the legend to Fig. 1. The *inset* shows a higher magnification of adjacent actin filaments bundled by regularly spaced synapsin I cross-bridges. *Panel c* shows a preparation in which 300 nM dephosphorylated synapsin I was added to 5 μM actin in the absence of K^+ and Mg^{2+} (*arrows*, aggregates from which actin filaments originate; *arrowheads*, bundles of actin filaments). Calibration *bars*, 0.5 μm.

rowheads), as well as aggregates from which actin filaments originate (*arrows*), are visible. Control samples containing G-actin incubated for 30 min at room temperature in the absence of synapsin I but with 15 mM NaCl were completely negative for the presence of filaments and/or bundles (not shown).

Modulation of the Effects of Synapsin I by Ionic Strength and Phosphorylation—Raising the concentration of NaCl from 15 to 85 mM in the presence of K^+ and Mg^{2+} did not noticeably affect the critical concentration for actin polymerization which in the various preparations ranged from 0.25 to 0.30 μM, in agreement with previously reported results (Benfenati *et al.*, 1992).

The increase in ionic strength potentiated the facilitating effect of synapsin I on actin polymerization, instead of abolishing it as it would be expected if the interaction between synapsin I and actin were nonspecific (compare Figs. 1 and 6). The initial rate of polymerization was strongly increased, the lag phase was abolished and the polymerization curve assumed a hyperbolic shape, suggesting that, under these conditions, most of the nuclei formed by the action of synapsin I actively elongate.

Site-specific phosphorylation of synapsin I seemed to selectively modulate this effect (Fig. 6). Stoichiometric phosphorylation of synapsin I either on site 1 by the catalytic subunit of cAMP-dependent protein kinase (*curve c*) or on sites 2 and 3 by CaM-kinase II (*curve d*) virtually abolished its ability to suppress the lag phase and to increase the initial rate of actin polymerization. The polymerization curves obtained under high ionic strength conditions with the phosphorylated forms of synapsin I had the usual sigmoidal shape observed for actin alone, and polymerization was only slightly faster than without synapsin I. The effects were particularly marked when synapsin I was phosphorylated on sites 2 and 3 by CaM kinase II. Under low ionic strength conditions, the effect of synapsin I phosphorylation was less pronounced than under high ionic strength conditions: the synapsin I-induced acceleration of actin polymerization was reduced, but the lag phase was still absent (data not shown).

Since phosphorylation of synapsin I by CaM kinase II markedly altered its effect on actin polymerization triggered by K^+ and Mg^{2+}, we investigated whether a similar effect was present also when actin polymerization was initiated by the addition of dephosphorylated synapsin I, or synapsin I phosphorylated by CaM kinase II, in the absence of K^+ and Mg^{2+}. We found that phosphorylation by CaM kinase II decreased the ability of synapsin I to trigger actin polymerization and that the phosphorylation dependence of the effect was more pronounced under high ionic strength conditions (Fig. 7).

Effects of Synapsin I on the Kinetics of Actin Polymerization Nucleated by Preformed Actin Filaments—The polymerization of actin induced by the addition of preformed nuclei (seeds) was analyzed in the absence or presence of synapsin I at low ionic strength (30 mM KCl, 1 mM $MgCl_2$, 15 mM NaCl) (Fig. 8). Under these conditions, the polymerization of actin exhibited no lag phase, a very fast initial rate and a hyperbolic shape. In the presence of synapsin I, a further increase in the initial rate of polymerization was observed. This effect was accompanied by a marked decrease in the rate of elongation, as already suggested by the experimental curves reported in Fig. 1.

Fig. 9 shows the results obtained when actin seeds were added at very low K^+ and Mg^{2+} concentrations (3 mM KCl, 0.1 mM $MgCl_2$) under either low (15 mM NaCl; *upper panel*) or high (85 mM NaCl; *lower panel*) ionic strength conditions. Sedimentation assays indicated that under these conditions, the critical concentrations for actin polymerization are 3.50

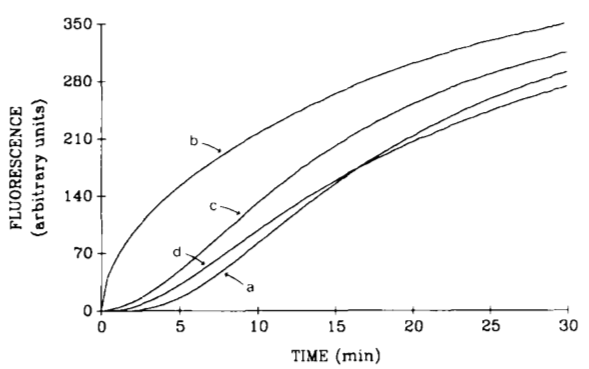

FIG. 6. **Effects of site-specific phosphorylation of synapsin I on actin polymerization at high ionic strength.** The polymerization of pyrenyl G-actin (5 μM; 5% labeled) triggered by the addition of KCl and $MgCl_2$ was analyzed in the absence of synapsin I (*a*) or in the presence of 300 nM dephosphorylated synapsin I (*b*), 300 nM synapsin I phosphorylated by cAMP-dependent protein kinase (*c*), or 300 nM synapsin I phosphorylated by CaM kinase II (*d*). The various forms of synapsin I were added to the samples at time 0, immediately before K^+ and Mg^{2+}. The experimental conditions were: 85 mM NaCl, 30 mM KCl, 1 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, 0.5 mM NaN_3, and 4.5 mM Tris-HCl, pH 7.6.

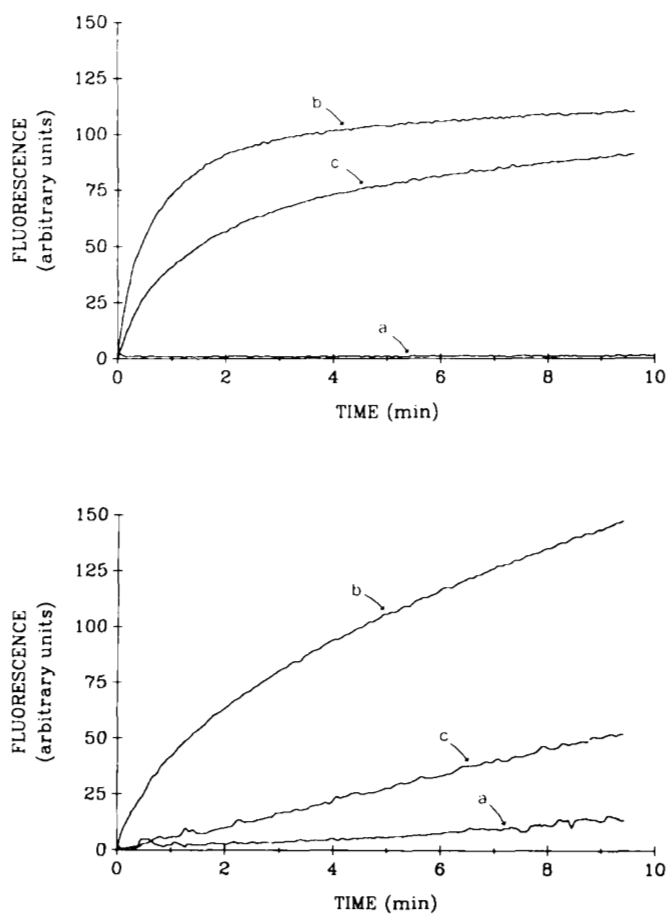

FIG. 7. **Effect of CaM kinase II phosphorylation on synapsin I-induced stimulation of actin polymerization.** The polymerization of pyrenyl-G-actin (5 μM; 5% labeled) was triggered by the addition at time 0 of synapsin I storage buffer (*a*), 300 nM dephosphorylated synapsin I (*b*), or 300 nM synapsin I phosphorylated by CaM kinase II (*c*) in the absence of KCl and $MgCl_2$. The experiment was performed at either low (15 mM NaCl; *upper panel*) or high (85 mM NaCl; *lower panel*) ionic strength conditions in a buffer containing 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, 0.5 mM NaN_3, and 4.5 mM Tris-HCl, pH 7.6.

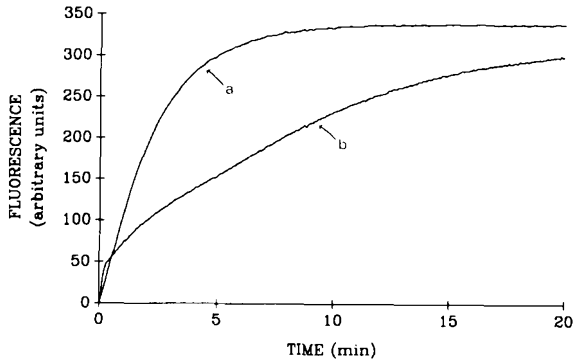

FIG. 8. **Effect of synapsin I on actin polymerization in the presence of F-actin seeds.** The polymerization of pyrenyl G-actin (5 μM; 5% labeled) triggered by the addition of F-actin seeds was measured at 30 mM KCl, 1 mM $MgCl_2$ in the absence (*a*) or presence (*b*) of 300 nM dephosphorylated synapsin I. In order to prepare actin seeds, 125 μg/ml G-actin was polymerized in the presence of 300 mM KCl and 10 mM $MgCl_2$. After 1 h at 25 °C, the filaments were sheared by sonication (Branson B-15 Sonicator, power 5, 10 pulses) and immediately added to the sample in a 1:10 ratio (v/v) at time 0. Experimental conditions were the same as described in the legend to Fig. 1.

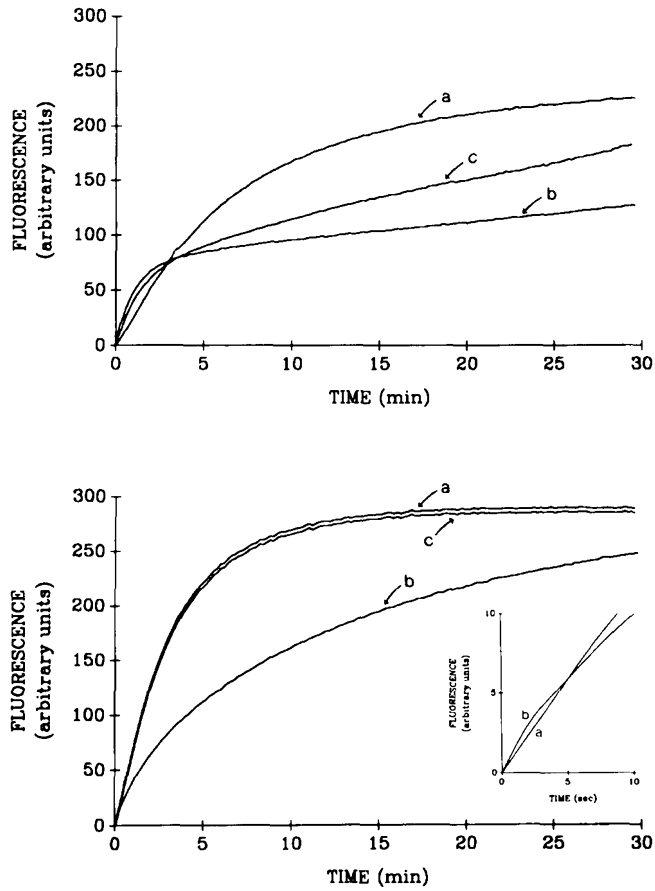

FIG. 9. **Effect of synapsin I on the polymerization of actin triggered by F-actin seeds at low KCl and $MgCl_2$.** The polymerization of pyrenyl G-actin (5 μM; 5% labeled) triggered by the addition of F-actin seeds was measured at 3 mM KCl, 0.1 mM $MgCl_2$, 15 mM NaCl (*upper panel*) or at 3 mM KCl, 0.1 mM $MgCl_2$, 85 mM NaCl (*lower panel*). Experimental conditions were the same as described in the legend to Fig. 8, except that the seeds were prepared at 30 mM KCl, 1 mM $MgCl_2$ (*a*, actin alone; *b*, actin + 300 nM dephosphosynapsin I; *c*, actin + 300 nM synapsin I phosphorylated by CaM kinase II). The *inset* in the *lower panel* shows the initial 10 s of curves *a* and *b*. Note that in the presence of dephosphosynapsin I, the initial rate of polymerization is higher with respect to the preparation containing actin only.

μM (at low ionic strength) and 2.02 μM (at high ionic strength). In the presence of 300 nM synapsin I, the concentration of nonpelletable actin was decreased to 1.40 and 0.59 μM under low and high ionic strength conditions, respectively. Under these ionic conditions, actin nucleation is not prevented, but occurs very slowly (see Attri *et al.*, 1991). In the absence of seeds and synapsin I, during the first 30 min either no noticeable increase or a rather small and slow increase in fluorescence was observed under low and high salt conditions, respectively (data not shown). However, when seeds (which function as nuclei) were added, the filaments promptly started to actively elongate. Under low ionic strength conditions, dephosphorylated synapsin I further increased the initial rate of actin polymerization and decreased the elongation rate, an effect similar to that observed in Fig. 8. Both effects were decreased after phosphorylation of synapsin I by CaM kinase II. Under high ionic strength conditions, the effect of dephosphorylated synapsin I on the initial rate of polymerization was much less pronounced. This effect, as well as the decrease of the elongation rate, were completely abolished after phosphorylation of synapsin I by CaM kinase II.

DISCUSSION

In the present paper pyrene-labeled actin was utilized to study the influence of synapsin I on the kinetics of actin polymerization. Since the finding by Kouyama and Mihashi (1981) that actin covalently labeled with pyrene increases in fluorescence about 20-fold upon polymerization, this assay has been widely used to study the kinetics of polymerization of actin under a variety of experimental conditions. The assay is particularly useful because of its high sensitivity, lack of shearing of the sample, and dependence on the concentration of polymerized actin, but not on filament length (Cooper *et al.*, 1983).

We found that synapsin I abolishes the lag phase and increases the initial rate of actin polymerization. The lack of a noticeable lag in the polymerization reaction, which is usually required for the condensation of monomers to form actin nuclei (for review, see Korn, 1982; Pollard and Cooper, 1986), suggests that the interaction between synapsin I and actin monomers is extremely fast (synapsin I was added to the incubation medium immediately before the addition of K^+ and Mg^{2+}) and that the synapsin I-G-actin complex may serve as a nucleus for the subsequent elongation step.

The synapsin I-driven polymerization of actin does not require the presence of K^+ and Mg^{2+}. However, in the absence of salts, the final levels of fluorescence reached were lower than those obtained in the presence of K^+ and Mg^{2+}, being essentially proportional to the synapsin I concentration. The subsequent addition of salts elicited a further fluorescence increase to plateau levels comparable with those observed with K^+/Mg^{2+}-induced actin polymerization. When synapsin I and K^+/Mg^{2+} were added at the same time, the overall shape of the polymerization curve appeared to result from the simultaneous occurrence of the two processes, indicating that the initial rate of polymerization is predominantly determined by synapsin I, whereas K^+ and Mg^{2+} drive the subsequent elongation and a delayed nucleation phase. In agreement with this interpretation, the steepening of the polymerization curve observed when salts were added after termination of the synapsin I effect suggests that further nucleation occurred, *i.e.* that synapsin I does not promote a generalized nucleation, but induces nucleation and elongation of a fraction of filaments whose magnitude depends on the synapsin I concentration and is inversely related to the total actin concentra-

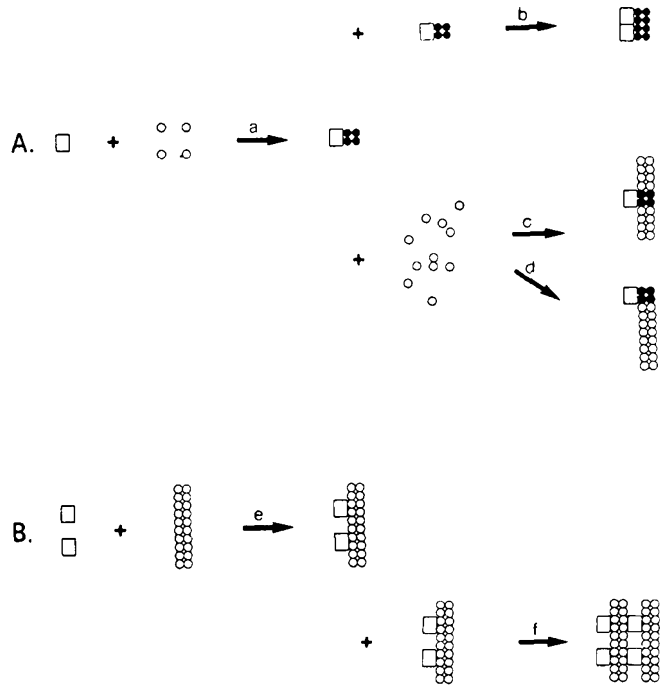

FIG. 10. **Schematic model of some possible mechanisms of interaction between synapsin I and actin.** *A*, synapsin I is able to bind to actin monomers, giving rise (*a*) to the formation of complexes. These complexes may associate with each other (*b*) and, above the critical concentration, actively elongate on both sides (*c*) or on one side (*d*) of the complex. *B*, synapsin I can also bind to the sides of actin filaments (*e*), leading to their association into bundles (*f*). □, synapsin I; ○, actin monomer; ●, synapsin I-complexed actin.

tion. The smaller initial slope exhibited by synapsin I-induced polymerization in the presence of K^+ and Mg^{2+} (compared with that observed in their absence) suggests that the presence of Mg^{2+} (and possibly of K^+) might interfere with the synapsin I-G-actin interaction.

The possibility that the fluorescence increase induced by synapsin I does not represent real polymerization but only reflects a higher quantum yield of the pyrene fluorophore when pyrenyl-actin is bound to synapsin I has been ruled out by experimental data obtained using various approaches. Actin pelleting experiments demonstrated that the synapsin I-induced fluorescence increase is associated with significant amounts of actin which can be sedimented at high speed. Moreover, under the same conditions, actin filaments and bundles were visualized by electron microscopy and videomicroscopy with fluorescein-labeled G-actin.[2] In spite of the high sensitivity of both techniques, no filaments were detectable under these conditions in the absence of synapsin I.

The relationship between the initial rate of polymerization and the concentration of synapsin I (see Fesce *et al.*, 1992 (accompanying paper)) indicates that the concentration of nuclei which are formed depends on a stoichiometric interaction between actin monomers and synapsin I molecules. In the presence of 150 nM synapsin I, the fluorescence increased linearly with the actin concentration until an actin/synapsin I molar ratio of about 4:1 and then remained constant for actin concentrations below the critical concentrations. These data suggest the presence of a tight binding between synapsin I and monomeric actin, with a stoichiometry of about 4 mol of actin/mol of synapsin I. This experimentally determined value corresponds to the stoichiometry determined by the curve fitting technique (see Fesce *et al.*, 1992 (accompanying paper)). In this respect, synapsin I is different from the classical "actin-nucleating proteins," which are generally active at very low stoichiometries (*e.g.* see Glenney *et al.*, 1981). In fact, the effect of synapsin I starts to be detectable at a 1:100 synapsin I/actin ratio, as opposed to villin which is markedly active at stoichiometries as low as 1:1000 (Glenney *et al.*, 1981). The high stoichiometry that we find is compatible with a model in which synapsin I, in addition to forming a complex with actin and initiating the formation of a filament, also binds to the sides of the growing filament and stabilizes it. Indeed, it has previously been shown that synapsin I is able to bind to preformed filaments with a 1:10 stoichiometry and to bundle them (Bähler and Greengard, 1987; Petrucci and Morrow, 1987; Bähler *et al.*, 1989).

When actin polymerization is triggered by actin seeds, an acceleration of the initial rate of polymerization can still be detected in the synapsin I-containing samples. Moreover, in the synapsin I-containing samples a decrease in the rate of filament elongation is observed. This effect cannot be ascribed to a barbed-end capping activity of synapsin I, since synapsin I does not compete with cytochalasin B for binding to the filament and decreases, rather than increasing, the critical concentration for actin polymerization in a dose-dependent fashion (Benfenati *et al.*, 1992). One possible explanation for the inhibitory effect on filament elongation is that the formation of bundles of actin filaments under these conditions (Bähler and Greengard, 1987) slows down the further addition of monomers at their free ends. It should be noticed that a decrease in the elongation rate is also caused by the COOH-terminal fragment of synapsin I, which does not seem to nucleate actin filaments, but which is able to induce the formation of bundles to some extent under low salt conditions (data not shown). We cannot exclude an interaction of synapsin I with the ends of the filaments, possibly resulting in changes in the on/off rates. However, this possibility seems unlikely, since synapsin I does not compete with cytochalasin B for binding to the barbed end (Benfenati *et al.*, 1992).

Many actin-binding proteins vary in their activities depending on the concentration of Ca^{2+} in the medium. In the case of synapsin I, its effects on critical concentration, number of filament ends, and kinetics of actin polymerization do not seem to be modulated by the presence of Ca^{2+} (Bähler and Greengard, 1987; Benfenati *et al.*, 1992; Fesce *et al.*, 1992 (accompanying paper)). However, both the actin-bundling and nucleating activities of synapsin I are inhibited upon its phosphorylation by CaM kinase II, and this modulation is more pronounced at physiological salt concentrations. In this manner, intracellular Ca^{2+} levels may modulate the effects of synapsin I on actin polymerization and assembly indirectly, through the activation of specific protein kinases.

The *in vitro* interaction of synapsin I with actin is complex in nature. Synapsin I is able to nucleate actin filaments as well as to bind to the sides of preformed filaments (Fig. 10). Nucleation presumably occurs through the formation of synapsin I-actin complexes, which can self-associate and/or actively elongate, when the actin concentration is higher than the critical concentration. At steady state, binding of synapsin I to the sides of the filaments prevails, causing bundling and possibly stabilization of the filaments.

The nucleating activity of dephosphorylated synapsin I might induce the growth of microfilaments from the vesicle membrane in the presence of sufficient levels of cytosolic monomeric actin. Indeed, the concentration of unassembled actin in the cytoplasm is generally high and undergoes rapid changes in response to a variety of stimuli (Stossel, 1989; Bernstein and Bamburg, 1989). Moreover, purified synaptic

[2] P. E. Ceccaldi and F. Grohovaz, personal communication.

vesicles from *Torpedo marmorata* have been shown to contain noticeable amounts of actin associated with their membrane (Tashiro and Stadler, 1978).

It is conceivable that the dephosphorylation of synapsin I promotes both the binding of the vesicles to preformed actin filaments and the growth of new actin filaments from the vesicle membrane. In both cases the vesicles would be rapidly caged in a cytoskeletal network and would not be available for exocytosis. Such a possibility is compatible with the inhibition of spontaneous and evoked neurotransmitter release observed after microinjection of dephosphorylated synapsin I into the squid giant axon (Llinas *et al.*, 1985, 1991; Lin *et al.*, 1990). In this respect, the nucleating activity of dephosphosynapsin I might be functionally important to recruit recycled synaptic vesicles and to quickly tether them to the actin-based cytoskeleton, thereby regulating vesicle traffic and turnover at the nerve terminal.

An effect of synapsin I phosphorylation by CaM kinase II would be that of inhibiting the growth of actin filaments from the vesicles. This event, associated with a decrease in the binding to preformed actin filaments (Bähler and Greengard, 1987), would facilitate the release of the vesicles from the cytoskeleton and increase their availability to undergo the exo-endocytotic cycle.

In addition, the nucleating and actin-stabilizing activity of synapsin I might also play a role in organizing the nerve terminal cytoskeleton during synaptogenesis. Indeed, a role of the synapsins in nerve terminal development has recently been demonstrated (Han *et al.*, 1991; Lu *et al.*, 1992).

Future experiments on the effects of synaptic vesicle-associated synapsin I on actin polymerization and on the dynamics of actin in living nerve terminals will be required in order to test these hypotheses.

Acknowledgments—We thank Dr. J. Meldolesi for critical reading of the manuscript and Drs. P. E. Ceccaldi, F. Onofri, and G. Stefani for their help in performing some of the experiments. We also acknowledge the assistance of Dr. G. Arslan and N. Iezzi for the electron microscopy experiments.

REFERENCES

Attri, A. K., Lewis, M. S., and Korn, E. D. (1991) *J. Biol. Chem.* **266,** 6815–6824

Bähler, M., and Greengard, P. (1987) *Nature* **326,** 704–707

Bähler, M., Benfenati, F., Valtorta, F., Czernik, A. J., and Greengard, P. (1989) *J. Cell Biol.* **108,** 1841–1849

Bähler, M., Benfenati, F., Valtorta, F., and Greengard, P. (1990) *BioEssays* **12,** 259–263

Benfenati, F., Valtorta, F., and Greengard, P. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 575–579

Benfenati, F., Valtorta, F., Chieregatti, E., and Greengard, P. (1992) *Neuron* **8,** 377–386

Bernstein, B. W., and Bamburg, J. R. (1989) *Neuron* **3,** 257–265

Cooper, J. A., Walker, S. B., and Pollard, T. D. (1983) *J. Muscle Res. Cell Motil.* **4,** 253–262

De Camilli, P., Benfenati, F., Valtorta, F., and Greengard, P. (1990) *Annu. Rev. Cell Biol.* **6,** 433–460

Fesce, R., Benfenati, F., Greengard, P., and Valtorta, F. (1992) *J. Biol. Chem.* **267,** 11289–11299

Frieden, C., Lieberman, D., and Gilbert, H. R. (1980) *J. Biol. Chem.* **255,** 8991–8993

Glenney, J. R., Kaulfus, P., and Weber, K. (1981) *Cell* **24,** 471–480

Grazi, E. (1985) *Biochem. Biophys. Res. Commun.* **128,** 1058–1063

Han, H. Q., Nichols, R. A., Rubin, M. R., Bähler, M., and Greengard, P. (1991) *Nature* **349,** 697–700

Huttner, W. B., Schiebler, W., Greengard, P., and De Camilli, P. (1983) *J. Cell Biol.* **96,** 1374–1388

Kaczmarek, L. K., Jennings, K. R., Strumwasser, F., Nairn, A. C., Walter, U., Wilson, F. D., and Greengard, P. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 7487–7491

Kouyama, T., and Mihashi, K. (1981) *Eur. J. Biochem.* **114,** 33–38

Korn, E. D. (1982) *Physiol. Rev.* **62,** 672–737

Lin, J. W., Sugimori, M., Llinas, R., McGuinness, T. L., and Greengard, P. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 8257–8261

Linstedt, A. D., and Kelly, R. B. (1987) *Trends Neurosci.* **10,** 446–448

Llinas, R., McGuinness, T. L., Leonard, C. S., Sugimori, M., and Greengard, P. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 3035–3039

Llinas, R., Gruner, J. A., Sugimori, M., McGuinness, T. L., and Greengard, P. (1991) *J. Physiol. (Lond.)* **436,** 257–282

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275

Lu, B., Greengard, P., and Poo, M. M. (1992) *Neuron*, in press

MacLean-Fletcher, S., and Pollard, T. D. (1980) *Biochem. Biophys. Res. Commun.* **96,** 18–27

Martell, A. E., and Smith, R. M. (1974) *Critical Stability Constants*, Plenum Press, New York

Markwell, M. A. K., Haas, J. M., Bieber, L. L., and Tolbert, N. E. (1978) *Anal. Biochem.* **87,** 206–210

McGuinness, T. L., Lai, Y., and Greengard, P. (1985) *J. Biol. Chem.* **260,** 1696–1704

Nestler, E. J., and Greengard, P. (1984) *Protein Phosphorylation in the Nervous System*, John Wiley & Sons, New York

Petrucci, T. C., and Morrow, J. (1987) *J. Cell Biol.* **105,** 1355–1363

Pollard, T. D. (1976) *J. Cell Biol.* **68,** 579–601

Pollard, T. D., and Cooper, J. A. (1986) *Annu. Rev. Biochem.* **55,** 987–1035

Schiebler, W., Jahn, R., Doucet, J.-P., Rothlein, J., and Greengard, P. (1986) *J. Biol. Chem.* **261,** 8383–8390

Spudich, J. A., and Watt, S. (1971) *J. Biol. Chem.* **246,** 4866–4871

Stossel, T. P. (1989) *J. Biol. Chem.* **264,** 18261–18264

Tashiro, T., and Stadler, H. (1978) *Eur. J. Biochem.* **90,** 479–487

Tobacman, L. S, and Korn, E. D. (1983) *J. Biol. Chem.* **258,** 3207–3214

Ueda, T., and Greengard, P. (1977) *J. Biol. Chem.* **252,** 5155–5163

Valtorta, F., Fesce, R., Grohovaz, F., Haimann, C., Hurlbut, W. P., Iezzi, N., Torri Tarelli, F., Villa, A., and Ceccarelli, B. (1990) *Neuroscience* **35,** 477–489