Dephosphorylated Synapsin I Anchors Synaptic Vesicles to Actin Cytoskeleton: An Analysis by Videomicroscopy

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Abstract. Synapsin I is a synaptic vesicle-associated protein which inhibits neurotransmitter release, an effect which is abolished upon its phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II). Based on indirect evidence, it was suggested that this effect on neurotransmitter release may be achieved by the reversible anchoring of synaptic vesicles to the actin cytoskeleton of the nerve terminal. Using video-enhanced microscopy, we have now obtained experimental evidence in support of this model: the presence of dephosphorylated synapsin I is necessary for synaptic vesicles to bind actin; synapsin I is able to promote actin polymerization and bundling of actin filaments in the presence of synaptic vesicles; the ability to cross-link synaptic vesicles and actin is specific for synapsin I and is not shared by other basic proteins; the cross-linking between synaptic vesicles and actin is specific for the membrane of synaptic vesicles and does not reflect either a non-specific binding of membranes to the highly surface active synapsin I molecule or trapping of vesicles within the thick bundles of actin filaments; the formation of the ternary complex is virtually abolished when synapsin I is phosphorylated by CaM kinase II. The data indicate that synapsin I markedly affects synaptic vesicle traffic and cytoskeleton assembly in the nerve terminal and provide a molecular basis for the ability of synapsin I to regulate the availability of synaptic vesicles for exocytosis and thereby the efficiency of neurotransmitter release.

THE past few years have witnessed a surge of new information relevant to the molecular basis of neurotransmitter release (for review, see Kelly, 1993; Jessel and Kandel, 1993; Thomas-Reetz and De Camilli, 1994; Valtorta and Benfenati, 1995). This complex process comprises a series of steps, including targeting, docking at the release sites, and exocytosis of synaptic vesicles. It is believed that in the nerve terminal only a small fraction of synaptic vesicles is readily available for release ("releasable pool"). The vast majority of synaptic vesicles belong to a "reserve pool", from which they can be recruited upon activity into the releasable pool (e.g., Martin, 1966). One model to account for the existence of a reserve pool of synaptic vesicles holds that this pool is formed by vesicles which are tethered to the nerve terminal cytoskeleton (Greengard et al., 1993). Thus, it appears important to identify the proteins which mediate the interaction of synaptic vesicles with the cytoskeleton and to study how this interaction is modulated.

Synapsin I is a phosphoprotein associated with the cytoplasmic surface of synaptic vesicles (for review see De Camilli et al., 1990; Valtorta et al., 1992*a*). In nerve terminals, the phosphorylation of synapsin I by Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II)[1] occurs promptly and to high stoichiometries during physiological activity (Nestler and Greengard, 1984). In addition, CaM kinase II is associated with synaptic vesicles and binds to the COOH-terminal region of synapsin I through its autoinhibitory domain (Benfenati et al., 1992*b*). Phosphorylation of synapsin I by CaM kinase II alters its conformation (Benfenati et al., 1990) and this change in structure is accompanied by changes in its biological properties. For instance, the affinity of synapsin I for the synaptic vesicle membrane is reduced upon its phosphorylation by CaM kinase II (Huttner

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1. *Abbreviations used in this paper*: CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; F-actin, actin filaments; FRET, fluorescence resonance energy transfer.

et al., 1983; Schiebler et al., 1986). Synapsin I is also able to bind actin filaments and monomers and these effects, too, are reduced upon phosphorylation by CaM kinase II. The interaction with actin filaments leads to bundle formation (Bähler and Greengard, 1987; Petrucci and Morrow, 1987), whereas the interaction with actin monomers promotes nucleation and polymerization of the monomers into filaments (Benfenati et al., 1992*a*; Fesce et al., 1992; Valtorta et al., 1992*b*; Ceccaldi et al., 1993). Microinjection studies indicate that dephosphorylated synapsin I inhibits neurotransmitter release. This effect is also abolished upon phosphorylation of synapsin I by CaM kinase II and is attributable to a decrease in the number of quanta released by a constant stimulus (Llinas et al., 1985, 1991; Nichols et al., 1990, 1992; Hackett et al., 1990; Lin et al., 1990).

This collection of data has prompted a model in which the inhibitory effect of synapsin I on neurotransmitter release is achieved by reversibly cross-linking synaptic vesicles to the actin cytoskeleton of the nerve terminal, thereby sequestering synaptic vesicles and making them unavailable for release (Greengard et al., 1993). Fragment analysis has localized the binding sites for the vesicle membrane and for actin in regions of the synapsin I molecule which are at least partially separate (Bähler et al., 1989; Benfenati et al., 1989), thus raising the possibility of a simultaneous interaction of synapsin I with synaptic vesicles and actin. However, a direct demonstration of the occurrence of such interaction has not been provided thus far. In quick-frozen, deep-etched nerve terminals images suggestive of synaptic vesicles cross-linked with actin filaments via synapsin I molecules have been observed (Landis et al., 1988; Hirokawa et al., 1989). However, in those studies no means of identifying synapsin I was provided. By using video-enhanced microscopy to directly visualize purified components labeled with fluorescent probes, we have now demonstrated that synapsin I is able to cross-link synaptic vesicles to the actin cytoskeleton under conditions of physiological ionic strength, and that this activity is virtually abolished upon phosphorylation of synapsin I by CaM kinase II.

Materials and Methods

Protein Purification and Isolation of Synaptic Vesicles

Synapsin I was purified from bovine brain as described by Schiebler et al. (1986), and modified by Bähler and Greengard (1987). It was stored at −80°C in 200 mM NaCl/25 mM Tris-Cl, pH 8.0. Purified dephosphorylated synapsin I was phosphorylated by CaM kinase II (purified according to the procedure described by McGuinness et al., 1985) as described by Schiebler et al. (1986). The phosphorylation stoichiometry ranged between 1.9 and 2.2 mol phosphate/mol synapsin I. The COOH-terminal fragment of synapsin I, generated by cysteine-specific cleavage, was purified as previously described (Bähler et al., 1989).

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-Cl, 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). Recombinant human T plastin, purified as described (Arpin et al., 1994), was a gift of M. Arpin and F. Vernel (Institut Pasteur, Paris, France).

Synaptic vesicles were purified from rat forebrain through the step of chromatography on controlled-pore glass beads as described by Huttner et al. (1983). Immediately after elution from the column, synaptic vesicles (10–20 μg protein/ml) were diluted with an equal volume of 0.4 M NaCl. After a 2 h incubation on ice, the vesicles were centrifuged for 2 h at 175,000 *g* and resuspended in 0.3 M glycine/5 mM Hepes-Na, pH 7.4. This procedure quantitatively removes synapsin I from the vesicle membrane (Huttner et al., 1983; Schiebler et al., 1986).

Labeling of Proteins and of Membrane Fractions

For labeling, G-actin was dialyzed against 2-mercaptoethanol-free buffer and polymerized by the addition of 100 mM KCl/1 mM $MgCl_2$. Then, 5-fluorescein-iodoacetamide (0.5 mg/ml final concentration; Molecular Probes, Eugene, OR) dissolved in acetone was added to the actin filaments and the sample incubated at 4°C in the dark for 10 h under constant rotation. The reaction was terminated by the addition of 0.5 mM β-mercaptoethanol and actin filaments were recovered by high speed centrifugation (400,000 *g* for 20 min). The actin pellet was resuspended in buffer A, extensively dialyzed against the same buffer and clarified by centrifugation. The labeling stoichiometry ranged between 1 and 1.2 mol fluorescein/mol actin monomer. The polymerization and synapsin I binding activity of labeled actin were indistinguishable from those of non-modified actin or pyrene-labeled actin (not shown). In some experiments, unlabeled actin was decorated with Bodipy FL-phallacidin (Molecular Probes).

Synaptic vesicles (1.8–2 mg/ml) were fluorescently labeled by incubation with 5-fluorescein-iodoacetamide (0.5 mg/ml) for 4 h in the dark. The sample was centrifuged for 4 min in an Eppendorf Microfuge to remove undissolved reagent. Membranes were recovered by high speed centrifugation (30 min at 350,000 *g*), washed three times, resuspended in glycine buffer (300 mM glycine/3 mM NaN_3/5 mM Hepes-Na, pH 7.4), stored in ice and used within 2–3 d. Fluorescein-labeled synaptic vesicles bound synapsin I with affinity and binding capacity comparable to unlabeled synaptic vesicles (not shown).

For the experiments on fluorescence resonance energy transfer (FRET), dephosphorylated synapsin I (1–1.4 mg/ml) was dialyzed for 36 h at 4°C against 100 mM NaCl, 10 mM Tris-Cl, pH 8.0. Tetramethyl-rhodamine iodoacetamide (Molecular Probes) was dissolved in dimethylsulfoxide and added to the protein at a final concentration of 0.5 mg/ml. Incubation was carried out for 4 h in the dark at 4°C under nitrogen. The reaction was quenched by the addition of 2-mercaptoethanol (0.5 mM final concentration), and unbound rhodamine was removed by dialysis for 48 h against 200 mM NaCl/25 mM Tris-Cl, pH 8.0. The concentration of rhodaminated synapsin I was determined spectrophotometrically, and was in the range 1 mg/ml, with a rhodamine/synapsin I molar ratio of about 0.3.

No unbound fluorochromes were detected in any of the labeled preparations after separation on SDS-PAGE and UV analysis of the unstained gels.

Video-enhanced Microscopy

For the F-actin experiments, samples containing synapsin I, labeled F-actin, and labeled synaptic vesicles were incubated at room temperature for 20 min in a buffer containing 100 mM KCl/1 mM $MgCl_2$/2 mM Tris-Cl/2 mM Hepes-Na/60 mM glycine/4 mM NaCl/0.2 mM ATP, pH 7.6. For the G-actin experiments, the buffer contained 70 mM NaCl and no KCl or $MgCl_2$. The concentration of synapsin I added to the vesicle samples was such as to achieve an almost complete saturation of the vesicle sites and a free concentration 2–3 times the K_d value (Schiebler et al., 1986). After incubation, a small aliquot of each sample (8 μl) was transferred to a glass slide which was sealed with a coverslip and observed with a digital fluorescence-imaging microscopy system built around an inverted IM 35 light microscope equipped with a silicon intensifier target video-camera. The images were digitized and processed using an Argus 100 image analysis system as previously described (Grohovaz et al., 1991). Digitized video-frames were averaged, cleared of background and transferred to a Motorola 68020 host computer for further processing or storage in hard disks.

Fluorescence Resonance Energy Transfer (FRET)

The kinetics of the binding of rhodaminated synapsin I to fluoresceinated actin and fluoresceinated synaptic vesicles was followed by observing the quenching in the fluorescein emission peak induced by rhodamine. Fluorescence measurements were performed in a LS-50 spectrofluorometer (Perkin Elmer, Beaconsfield, England) as previously described (Ceccaldi et al., 1993). The samples in 1.5 ml quartz cuvettes were excited at 450 nm and emission was monitored at 520 nm, which corresponds to the peak of emission of fluoresceinated actin. Excitation and emission slit widths were set at 5 and 10 nm, respectively. Readings were taken every 2 s, and all measurements were performed under continuous stirring.

Results

Interaction of Synaptic Vesicles with F-Actin

Incubation of fluorescein-labeled actin filaments (F-actin) with unlabeled dephosphorylated synapsin I induced the formation of intensely fluorescent actin bundles. In contrast, synapsin I phosphorylated by CaM kinase II did not have any effect on the organization of the F-actin network (Fig. 1, *A–C*). When purified synaptic vesicles depleted of endogenous synapsin I and fluorescently labeled with fluorescein-iodoacetamide were incubated with fluorescein-labeled actin filaments in the absence of exogenous synapsin I, they appeared randomly scattered in the visual field and did not alter actin assembly (Fig. 1 *D*). Upon addition of dephosphorylated synapsin I (Fig. 1 *E*), but not of synapsin I which had been phosphorylated by CaM kinase II (Fig. 1 *F*), virtually all synaptic vesicles were associated with actin filaments, which became organized into large bundles. The pattern observed in the presence of synaptic vesicles, synapsin I and F-actin was the same independent of the order of addition of the reagents (not shown). Binding of synaptic vesicles to actin bundles was confirmed by confocal microscopy, using synaptic vesicles and actin filaments labeled with different fluorochromes and incubated with dephosphorylated synapsin I (data not shown).

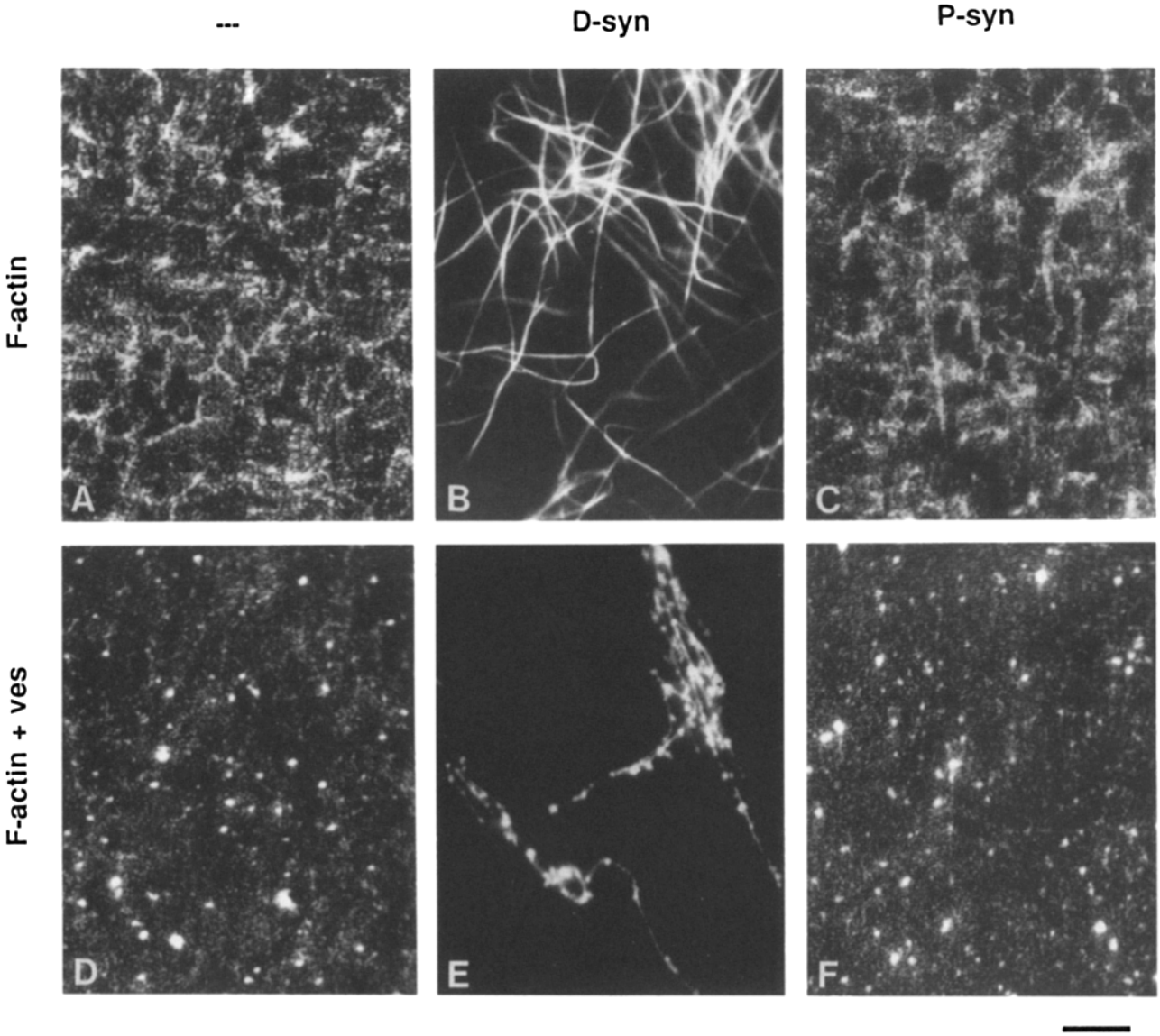

Figure 1. Dephosphorylated but not phosphorylated synapsin I cross-links synaptic vesicles to actin filaments. (*A–C*) Fluorescein-labeled actin filaments (2.5 μM actin) were incubated either in the absence (*A*) or presence of dephosphorylated synapsin I (0.3 μM; *B*) or of synapsin I which had been phosphorylated by CaM kinase II (0.3 μM; *C*). (*D–F*) Fluorescein-labeled actin filaments (2.5 μM actin) were incubated with fluorescein-labeled, synapsin I-depleted synaptic vesicles (0.2 mg/ml) either in the absence (*D*) or presence of dephosphorylated synapsin I (0.3 μM; *E*) or of synapsin I which had been phosphorylated by CaM kinase II (0.3 μM; *F*). (*D-syn*, dephosphorylated synapsin I; *P-syn*, synapsin I phosphorylated by CaM kinase II; *ves*, synapsin I-depleted synaptic vesicles). Samples were analyzed by video-enhanced microscopy as described in Materials and Methods. Bars: (*A* and *C*) 2 μm; (*B* and *E*) 20 μm; (*D* and *F*) 4 μm.

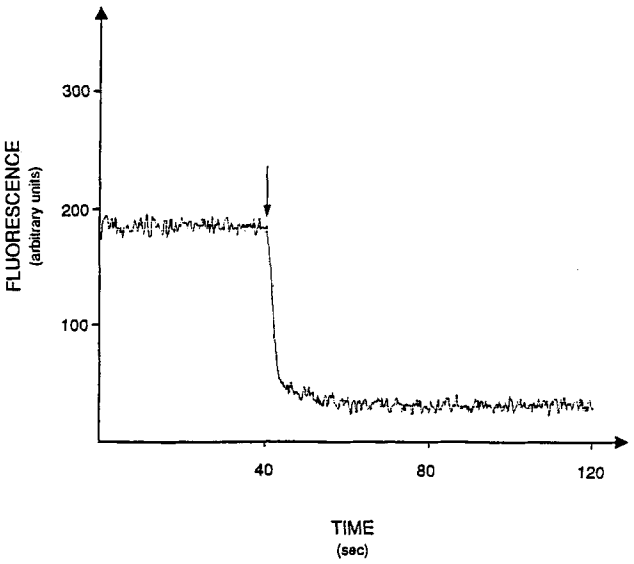

Figure 2. Time-course of the binding of synapsin I to synaptic vesicles and F-actin as evaluated by FRET. Fluorescein-labeled actin filaments (750 nM) and synapsin I-depleted fluoresceinated synaptic vesicles (0.07 mg/ml) (donors) were equilibrated for 1 h at 25°C in a quartz cuvette under continuous stirring. After a stable baseline was recorded for about 40 s, rhodaminated dephosphosynapsin I (acceptor, 150 nM) was added (*arrow*). The quenching of the donor fluorescence was followed as a function of time by monitoring the fluorescein emission at 520 nm (excitation wavelength: 450 nm; sampling intervals: 2 s; excitation and emission slits: 5 and 10 nm, respectively).

Kinetic Analysis of the Interaction of Synapsin I with Synaptic Vesicles and F-Actin

Although it was not possible to follow the kinetics of the association of synaptic vesicles with actin filaments by video-enhanced microscopy due to fading of the fluorescent probes, the binding of synaptic vesicles to actin filaments appeared to occur rapidly upon the addition of dephosphorylated synapsin I to the solution. The kinetics of the interaction of synapsin I with actin filaments and synaptic vesicles was quantitatively analyzed by measuring the energy transfer which occurs when two suitable fluorophores are brought into close contact (Jovin and Arndt-Jovin, 1989). Thus, the addition of rhodaminated synapsin I (acceptor) to a solution of fluoresceinated synaptic vesicles and fluoresceinated actin filaments (donors) was quickly followed by a marked decrease in the emission peak of fluorescein, as a result of the quenching induced by rhodamine (Fig. 2). The effect was specific for synapsin I, since other rhodaminated proteins such as ovalbumin or cytochrome c were ineffective (not shown). The half-time of the reaction was in the order of 3 s at 25°C and the quenching reached an apparent plateau within 20 s after the addition of rhodaminated synapsin I.

Specificity of the Interaction between Synaptic Vesicles and F-Actin

The ability of synapsin I to cross-link synaptic vesicles to actin filaments and to organize the filaments in bundles was not attributable to the basic character of its COOH-terminal region. Under the high ionic strength conditions used, neither the purified basic COOH-terminal fragment of synapsin I (compare Fig. 3 *A* with Fig. 1 *E*), nor cytochrome c, another very basic protein (not shown), were effective in bundling actin filaments or in attaching the vesicles to them. The observed interactions were not attributable to the chemical modification of actin and synaptic vesicles, since experiments performed using unlabeled components under the same conditions and analyzed by Nomarsky differential interference contrast videomicroscopy gave similar results (Bähler, M., F. Benfenati, F. Valtorta , and D. G. Weiss, unpublished results).

The specificity of the interaction among synaptic vesicles, synapsin I and actin filaments was further tested by incubating actin filaments and synapsin I in the presence of a fluorescein-labeled brain membrane fraction virtually devoid of synaptic vesicles (the flow-through fraction of the controlled-pore glass chromatography step obtained in the course of the purification of synaptic vesicles; Huttner et al., 1983). In contrast to synaptic vesicles, the fluorescent membranes did not bind to the actin bundles (compare Fig. 3 *B* with Fig. 1 *E*).

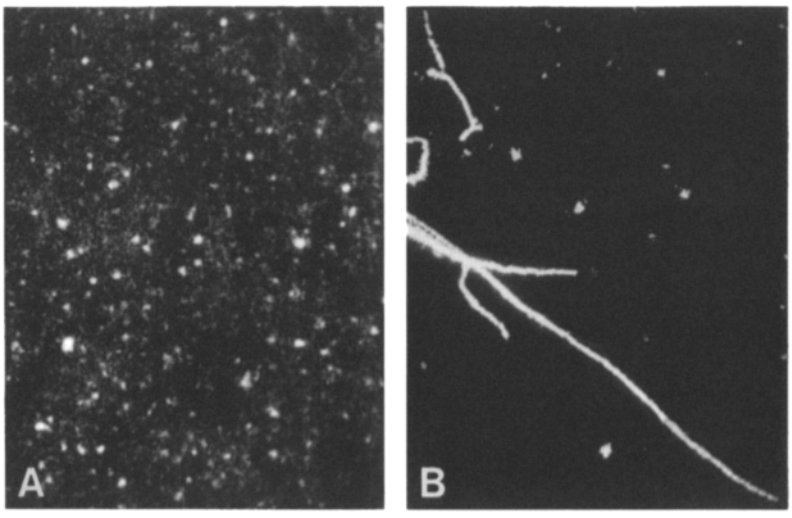

Figure 3. Specificity of the interaction between actin filaments, synapsin I, and synaptic vesicles. (*A*) Fluoresceinated actin filaments (2.5 μM) were incubated with synapsin I-depleted fluoresceinated synaptic vesicles (0.2 mg/ml) in the presence of the purified COOH-terminal fragment of synapsin I (0.3 μM). (*B*) Fluoresceinated actin filaments (2.5 μM) were incubated in the presence of dephosphorylated synapsin I (0.3 μM) with a brain membrane fraction virtually devoid of synaptic vesicle contamination (flow-through fraction from controlled-pore glass chromatography, 0.2 mg/ml) which had been labeled with 5-fluorescein-iodoacetamide by the same procedure used for labeling synaptic vesicles. For further details, see Materials and Methods. Bars: (*A*) 4 μm; (*B*) 15 μm.

The ability of synapsin I to cross-link synaptic vesicles to actin filaments was not attributable to its actin bundling activity causing non-specific trapping of synaptic vesicles within large actin bundles. Actin bundles were produced in the presence of plastin (Goldstein et al., 1985), an actin bundling protein which does not interact with synaptic vesicles (Ceccaldi, P. E., unpublished results). The incubation of plastin with actin filaments and synapsin I–depleted synaptic vesicles promoted the formation of extensive bundles, but did not affect the random distribution of the vesicles in the sample field (Fig. 4 *A*). In the presence of preformed bundles, addition of dephosphorylated synapsin I promoted the association of synaptic vesicles with actin filaments (Fig. 4 *B*).

Interaction of Synaptic Vesicles with G-Actin

When fluoresceinated monomeric actin (G-actin) was incubated for 20 min in a high ionic strength medium which does not favor spontaneous polymerization (70 mM NaCl in the absence of KCl and $MgCl_2$; Valtorta et al., 1992*b*), no fluorescent filaments were detectable (Fig. 5 *A*). Upon addition of dephosphorylated synapsin I (Fig. 5 *B*), but not of synapsin I phosphorylated by CaM kinase II (Fig. 5 *C*), the rapid formation of actin bundles was observed. Synapsin I–depleted synaptic vesicles did not induce any detectable formation of actin polymers in the absence of synapsin I (Fig. 5 *D*). However, when exogenous dephosphorylated synapsin I was present, the vesicles associated with newly formed actin bundles (Fig. 5 *E*). In fact, as soon as actin polymers could be detected, they were decorated with synaptic vesicles. When the incubation was performed in the presence of synapsin I which had been phosphorylated by CaM kinase II, actin polymers were never observed and synaptic vesicles were randomly scattered (Fig. 5 *F*) as observed for the samples incubated in the absence of synapsin I (Fig. 5 *D*).

Discussion

Synapsin I, a phosphoprotein reversibly associated with the cytoplasmic side of small synaptic vesicles, interacts with several cytoskeletal proteins including actin, one of the major components of the nerve terminal cytomatrix (for review see De Camilli et al., 1990; Valtorta et al., 1992*a*). Since most of the nerve terminal synapsin I is bound to synaptic vesicles (Huttner et al., 1983; Schiebler et al., 1986), it was of interest to ascertain whether synapsin I can be bound to actin and to synaptic vesicles simultaneously. However, standard biochemical techniques are not suitable for assessing this possibility and only indirect evidence on the interactions among synaptic vesicles, synapsin I, and actin has been available thus far (Benfenati et al., 1992*a*). Videomicroscopy allows a direct and sensitive analysis of molecular interactions with a high degree of morphological resolution. By using this non-perturbing technique, we have now provided a direct demonstration that synapsin I mediates the attachment of synaptic vesicles to the actin-based cytoskeleton.

The interactions between synaptic vesicles and actin were virtually abolished by CaM kinase II phosphorylation of synapsin I. This effect of phosphorylation was much more dramatic than that observed in in vitro assays of synapsin I binding either to synaptic vesicles or to actin (Schiebler et al., 1986; Bähler and Greengard, 1987), underlining the value of using a non-perturbing method for the analysis of the complex interactions between macromolecules and organelles.

It was reported earlier that dephosphorylated synapsin I is able to bind to and bundle actin filaments (Bähler and Greengard, 1987; Petrucci and Morrow, 1987; Bähler et al., 1989). In the present study, we were able to confirm these observations by videomicroscopy, as well as to demonstrate that the effects of synapsin I occurred in the presence of synaptic vesicles (see Fig. 1). The interaction of synapsin I with synaptic vesicles and actin is rapid and its kinetics is similar to that of the binding of free synapsin I to actin filaments (Ceccaldi et al., 1993). Based on the experimentally determined binding constants for the interaction between synapsin I and synaptic vesicles (Schiebler et al., 1986), it can be calculated that, under the conditions used for the experiments shown in Figs. 1 and 5, about 2/3 of synapsin I was bound to the vesicles in the synaptic vesicle–containing samples and only 1/3 was free. A limitation of the videomicroscopy technique is that it is impossible to ascertain the order of association and

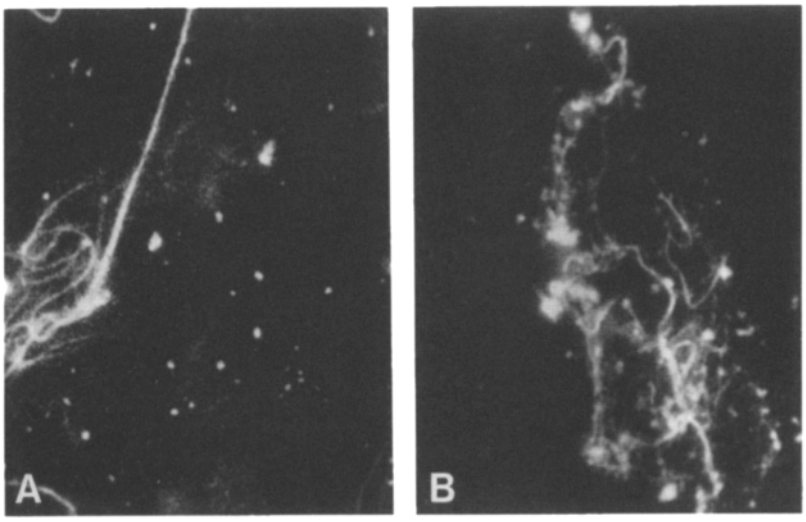

Figure 4. Interaction among actin filaments, synapsin I, and synaptic vesicles is not attributable to synapsin I–induced bundling of actin filaments. Actin filaments (2.5 μM) labeled with Bodipy-phallacidin were preincubated with the actin-bundling protein plastin (1 μM) and synapsin I–depleted fluoresceinated synaptic vesicles (0.2 mg/ml), followed by incubation in the absence (*A*) or presence (*B*) of dephosphorylated synapsin I (0.3 μM). The extent of bundle formation is larger in panel B due to the simultaneous presence of the two actin bundling proteins. For further details, see Materials and Methods. Bar, 6 μm.

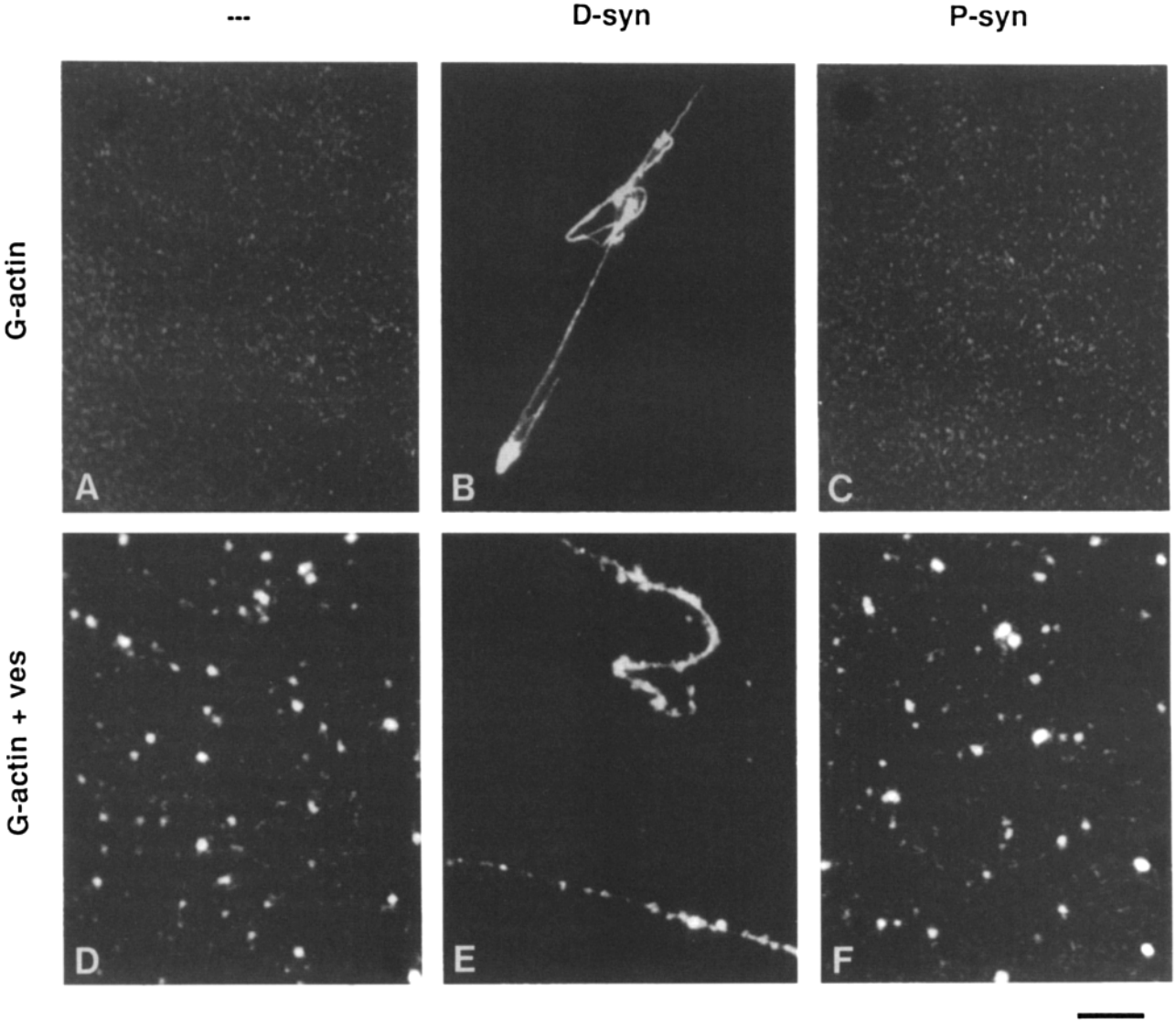

Figure 5. Dephosphorylated but not phosphorylated synapsin I promotes actin polymerization and cross-links synaptic vesicles to actin. (*A–C*) Fluorescein-labeled actin monomers (2.5 μM actin) were incubated either in the absence (*A*) or presence of dephosphorylated synapsin I (0.3 μM; *B*) or of synapsin I which had been phosphorylated by CaM kinase II (0.3 μM; *C*). (*D–F*) Fluorescein-labeled actin monomers (2.5 μM actin) were incubated with fluorescein-labeled synapsin I–depleted synaptic vesicles (0.2 mg/ml) either in the absence (*D*) or presence of dephosphorylated synapsin I (0.3 μM; *E*) or of synapsin I which had been phosphorylated by CaM kinase II (0.3 μM; *F*). (*D-syn*, dephosphorylated synapsin I; *P-syn*, synapsin I phosphorylated by CaM kinase II; *ves*, synapsin I–depleted synaptic vesicles). Samples were incubated for 20 min at room temperature under the conditions described in the legend to Fig. 1, except that the buffer contained 70 mM NaCl and no KCl or $MgCl_2$. Bars: (*A* and *C*) 2 μm; (*B* and *E*) 20 μm; (*D* and *F*) 3 μm.

dissociation of synaptic vesicles, synapsin I and actin filaments in the ternary complex. In addition, with contrast-enhancement techniques all objects whose actual diameter is below the resolution limit of the light microscope (0.2 μm) will be identical in apparent size. Since synaptic vesicles are 40–60 nm in diameter, it is not possible to state whether a fluorescent dot represents a single vesicle rather than a small cluster of vesicles. However, it is likely that small clusters are present in our preparation, also in view of the vesicle-aggregating ability of synapsin I (Benfenati et al., 1993).

Previous studies demonstrated that dephosphorylated synapsin I nucleates actin monomers and triggers the formation of actin filaments, and that those effects are reduced by CaM kinase II phosphorylation of synapsin I (Valtorta et al., 1992*b*; Fesce et al., 1992). This activity is likely to be physiologically relevant since it is known that, in living cells, as much as 50% of the total actin is present in the unpolymerized form (G-actin) and that the ratio between polymerized and unpolymerized actin strongly influences cytoplasmic viscosity and organelle motility and is therefore tightly regulated (Fechheimer and Zigmond, 1993). It was also reported earlier that, in the presence of synaptic vesicles, dephosphorylated synapsin I is able to increase the number of actin filaments at steady-state as evaluated by cytochalasin B binding, an effect attributable to the synapsin I–induced nucleation of actin monomers (Benfenati et al., 1992*a*). In the present study we were able to confirm these observations by direct visualization. The results of the previous studies to-

gether with the present observations indicate that synapsin I is able to bind to, nucleate and polymerize G-actin, as well as to bundle the newly formed actin filaments. Unfortunately it cannot presently be ascertained at which stage in this sequence of events synaptic vesicles become attached to synapsin I. However, according to the binding constants for the interaction of synapsin I with synaptic vesicles and with actin reported earlier (Schiebler et al., 1986; Bähler and Greengard, 1987; Benfenati et al., 1991; Fesce et al., 1992), we speculate that, under the experimental conditions used, synapsin I was in fact associated with synaptic vesicles both when it bound to F-actin (see Fig. 1) and when it stimulated actin nucleation and polymerization (see Fig. 5). Regardless of the precise sequence of interactions of the three components, the final effect achieved is that of promoting actin polymerization, thus embedding the vesicles in a cytoskeletal meshwork.

The present data support a model in which the processes of synapsin I dephosphorylation and phosphorylation switch on and off the interactions between synaptic vesicles and the actin-based cytoskeleton of the nerve terminal, thus triggering the transitions of the vesicles between a pool restrained to the cytoskeleton and a pool available for exocytosis (Greengard et al., 1993). Genetically altered mice lacking either synapsin I (Rosahl et al., 1993) or the α-subunit of CaM kinase II (Silva et al., 1992) show abnormal paired-pulse facilitation, a form of presynaptic plasticity involving an increase in interterminal Ca^{2+} and an augmented availability of synaptic vesicles for exocytosis (Zucker, 1989). The experimental results obtained with these two types of genetic deletion further support the proposal (Greengard et al., 1993) that the synapsin I/CaM kinase II system plays a key role in regulating the efficiency of the neurotransmitter-releasing machinery in the nerve terminal.

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