Coordinated Regulation of Synapsin I Interaction with F-Actin by Ca^{2+}/Calmodulin and Phosphorylation: Inhibition of Actin Binding and Bundling†

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ABSTRACT: The synapsins are a family of synaptic vesicle phosphoproteins whose role seems to be to limit the availability of small synaptic vesicles for exocytosis by linking them to the cytoskeleton. One member of the family, synapsin I, has been shown to bind calmodulin in a Ca^{2+}-dependent manner. In this study, we have examined whether or not calmodulin can regulate one of the activities of synapsin I, namely, its interaction with F-actin. Synapsin I is an actin bundling protein: this activity is controlled by phosphorylation. Here we show that calmodulin in the presence of Ca^{2+} is a competitive inhibitor of both actin binding and bundling by synapsin I. Under the conditions of our assay (0.45 μM synapsin I, 4 μM F-actin), half-maximal inhibiton of actin binding and bundling by unphosphorylated synapsin I was found with 4.3 and 3.7 μM calmodulin, respectively. The actin binding activity of synapsin I phosphorylated by cAMP-dependent protein kinase or by calmodulin-dependent protein kinase II showed similar sensitivity to calmodulin inhibition to unphosphorylated synapsin I. However, inhibition of bundling was potentiated. Half-maximal inhibition of bundling by synapsin I phosphorylated by cAMP-dependent kinase was achieved at approximately 0.5 μM calmodulin. Half-maximal inhibition of bundling by synapsin I phosphorylated by calmodulin-dependent protein kinase II was achieved at less than 0.2 μM calmodulin, although the maximum binding under the conditions of the assay was lower. Synapsin I phosphorylated by both cAMP-dependent kinase and calmodulin-dependent protein kinase II was half-maximally inhibited from actin binding by less than 100 nM calmodulin, although the binding and bundling characteristics of this form of synapsin I were weak even in the absence of calmodulin. We conclude that covalent (phosphorylation) and noncovalent (calmodulin) forms of regulation act together in regulating one of the activities of synapsin I.

The synapsins are a family of four phosphoproteins concentrated on small exocytotic vesicles in nerve synapses (De Camilli et al., 1990). In mature synapses, their role seems to be to limit the availability of synaptic vesicles to undergo exocytosis in response to nerve depolarization: this is illustrated by alterations to the short term synaptic plasticity of neurons in transgenic mice in which there is a null mutation the synapsin I gene (Rosahl et al., 1993). In addition to their role in synaptic exocytosis, evidence from studies on developing *Xenopus*, cultured neurons, and neuroblastoma cells indicates roles for members of the synapsin family in axon growth and synapse formation (Ferreira et al., 1994; Han et al., 1991; Schaeffer et al., 1994) Observations of synapsin I gene expression, which is initiated upon the onset of terminal differentiation of neurons, support a role for synapsins in early events in the formation of the nervous system (Melloni & Degennaro, 1994).

The two synapsin genes, I and II, produce four gene products by differential splicing of mRNA, giving synapsin Ia (polypeptide chain size 74 kDa), synapsin Ib (70 kDa), synapsin IIa (63 kDa), and synapsin IIb (52 kDa) (Sudhof et al., 1989). In functional synapses they act as linker molecules between small synaptic vesicles and various elements of the cytoskeleton (Bahler & Greengard, 1987; Baines & Bennett, 1985, 1986; Goldenring et al., 1986; Petrucci & Morrow, 1987; Sikorski et al., 1991). Upon nerve depolarization, the interactions of synapsin I (the collective term for synapsins Ia and Ib) with both the cytoskeleton and the vesicle are diminished, apparently freeing the vesicle for use in exocytotic reactions.

There is strong evidence for phosphorylation of synapsins being an important element in the control of synapsin I activity. Depolarization activates two Ca^{2+}/calmodulin-dependent protein kinases (kinases I and II) which phosphorylate synapsin I (Huttner et al., 1981; Kennedy et al., 1983). Kinase I phosphorylates serine 9 (phosphorylation site I), whereas kinase II phosphorylates serines 568 and 605 (phosphorylation sites II and III) (Czernik et al., 1987). Synapsin I can be phosphorylated in response to stimuli other than those that elevate cytoplasmic Ca^{2+}: for example, activation of cAMP-dependent protein kinase results in the phosphorylation of site I (Huttner et al., 1981). A fourth protein kinase, which phosphorylates a serine in a serine−proline motif, also phosphorylates synapsin I (Vulliet et al., 1989).

Phosphorylation of synapsin I at sites I, II, and III has been studied extensively. Site I is close to sites of binding to a number of cytoskeletal proteins, including actin and tubulin (Bahler et al., 1989; Bennett & Baines, 1992; Bennett et al., 1991; Petrucci & Morrow, 1991); sites II and III are close to a site of interaction with synaptic vesicles (Benfenati et al., 1992). Little evidence has been found for major alterations to the activity of synapsin I phosphorylated at

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site I, but phosphorylation at sites II and III has notable effects. The affinity of synapsin I for small synaptic vesicle membranes is reduced by phosphorylation by kinase II (Schiebler et al., 1986). The interaction of synapsin I with F-actin is reduced by kinase II phosphorylation (Bahler & Greengard, 1987; Petrucci & Morrow, 1987). Synapsin I has several actions on F-actin. It will bind laterally along actin filaments, and it will cross-link them into bundles. Synapsin I will also promote the polymerization of G-actin into F-actin filaments (Fesce et al., 1992; Valtorta et al., 1992). The effect of kinase II phosphorylation is to reduce the B_{max} value for synapsin I on actin: it has little or no effect on the affinity (Bahler & Greengard, 1987; Petrucci & Morrow, 1987). The alteration in B_{max} is reflected in a reduction of the bundling activity.

It is still open to debate whether phosphorylation alone can be the sole regulator of synapsin activity. Synapsins IIa and IIb do not contain serine or threonine residues that can be phosphorylated by kinase II (Sudhof et al., 1989) and yet must be presumed to play a regulated part in the exocytotic process. Synapsins IIa and IIb can only be phosphorylated at site I (by kinase I or cAMP-dependent protein kinase), but this is rather ineffective as a regulator of the known synapsin interactions, having only moderate effects on binding characteristics, for example (Bahler & Greengard, 1987; Schiebler et al., 1986). Furthermore, Sihra et al. (1989) have noted a translocation of synapsins Ia and Ib from the membrane/cytoskeletal compartment to a soluble compartment on depolarization of synaptosomes. However, the effect of kinase II on synapsins Ia and Ib is to reduce their affinity for vesicle membranes by only 5-fold: Benfenati et al. (1991) have doubted that this is enough to account for such a translocation.

Given this background, it is of considerable importance for understanding the process of neurotransmission to evaluate the potential of additional mechanisms of regulation of synapsin activity, which might act in concert with phosphorylation. Okabe and Sobue (1987) described an additional activity of synapsins Ia and Ib, binding to Ca^{2+}/calmodulin. We have characterized the interaction further and have found evidence for two high affinity binding sites for Ca^{2+}/calmodulin in the N-terminal 453 residues (the "head" region) (Goold & Baines, 1994; Hayes et al., 1991). The binding of calmodulin to these sites was essentially unaffected by phosphorylation at site I, sites II and III, or sites I, II, and III together; the K_d for binding was approximately 30 nM. The high local concentrations of both synapsins ($\geq$12 μM (Benfenati et al., 1991)) and calmodulin (50−60 μM (Blackshear, 1993; Klee & Vanaman, 1982)) at nerve endings suggest that in the presence of Ca^{2+} the calmodulin-binding sites on synapsin I should be extensively occupied. Clearly, there may be competition from other calmodulin-binding proteins, but synapsins Ia and Ib are among the most abundant calmodulin-binding proteins in nerve termini. The lack of any discernible effect of phosphorylation of synapsin I on calmodulin binding indicated that calmodulin should bind to the synapsin I head region in depolarized synapses irrespective of the phosphorylation state of the synapsin I. This is important because it indicates a potential for binding of calmodulin to modulate synapsin I activities in a way that perhaps complements phosphorylation.

The nature of any regulation of synapsin I activities by calmodulin has not been investigated in detail. In recent work we showed that calmodulin could inhibit both binding and bundling of F-actin by synapsin I (Goold & Baines, 1994), but the effect was not quantified, nor was the effect of phosphorylation examined. In this study, we have addressed these issues.

MATERIALS AND METHODS

Preparation of Synapsin I. Synapsin I (a mixture of synapsins Ia and Ib) was prepared from sheep brains collected at the point of slaughter by methods described previously (Bennett et al., 1985). These methods were originally developed for beef brain synapsin I, but were used without modification for the preparation of sheep brain protein (Goold & Baines, 1994). The protein was >95% pure by the criterion of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE).[1] Sheep brain was used in the present report because we have recently been unable to obtain beef brain (for which the synapsin I sequences are known) because of bovine spongiform encephalopathy in the U.K. The properties of sheep brain synapsin I that have been investigated so far are indistinguishable from those of the bovine protein: this includes the mass on SDS−PAGE, peptide map generated by cysteine cleavage, the presence of one cAMP-dependent protein kinase site in the head region and two calmodulin-dependent protein kinase II sites in the tail region, and antigenic cross-reactivity with several monoclonal antibodies (Goold & Baines, 1994). Synapsin I concentrations were estimated spectrophotometrically (Ueda & Greengard, 1977).

Phosphorylation of Synapsin I at Sites I, II, and III. Native sheep brain synapsin I has been shown to have little covalently bound phosphate (Goold & Baines, 1994) and was used in this study as unphosphorylated synapsin I without additional phosphatase treatment. Methods for phosphorylation of synapsin I at site I, sites II and III, or sites I, II, and III were as described elsewhere (Goold & Baines, 1994). The protein kinases used were as follows. For phosphorylation at site I, cAMP-dependent protein kinase catalytic subunit (Sigma P-2645) was used. For sites II and III, a recombinant fragment of calmodulin-dependent protein kinase II (kinase II-α315) was used. This active kinase fragment contains residues 1−315 of the α subunit of calmodulin-dependent protein kinase II: it was derived from a cDNA described by Bulleit et al. (1988). Kinase II-α315 displays a Ca^{2+}/calmodulin-dependent protein kinase activity with a substrate specificity indistinguishable from the native kinase II (Takeuchi-Suzuki et al., 1992). Kinase II-α315 was expressed in insect cells and purified as described elsewhere (Goold & Baines, 1994; Takeuchi-Suzuki et al., 1992). For phosphorylation at sites I, II, and III, synapsin I was first phosphorylated at sites II and III and then at site I. Phosphorylated synapsin I was repurified by cation exchange chromatography on a Mono S HR5/5 column. Methods used for phosphorylation of each site gave full occupancy.

Preparation of Actin. Actin was prepared from rabbit muscle acetone powder as described in Spudich and Watt (1971) and purified further by gel filtration of the monomer (MacLean-Fletcher & Pollard, 1980). Actin concentrations were estimated using the Bio-Rad Coomassie blue protein reagent with bovine serum albumin as the standard.

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

Preparation of Calmodulin. Calmodulin was purified from sheep brain by standard methods that include the use of Ca^{2+}-dependent hydrophobic chromatography (Dedman & Kaetzel, 1983; Gopalakrishna & Anderson, 1982). It was purified further by anion exchange chromatography using a Mono Q HR5/5 column (Dedman & Kaetzel, 1983; Goold & Baines, 1994). Calmodulin concentrations were estimated spectrophotometrically (Jarrett & Penniston, 1977).

Assay for Actin Binding. Methods for this were taken from Bahler and Greengard (1987) and were as described in Goold and Baines (1994). Synapsin I (concentrations given in the text and figure legends) was mixed with F-actin (4 μM) and calmodulin (concentrations given in the text and figure legends) in a solution consisting of 20 mM Hepes/NaOH, pH 7.4, 75 mM KCl, 2 mM $MgCl_2$, 0.5 mM 2-mercaptoethanol, and 1.1 mM ATP, with either 1 mM $CaCl_2$ or 5 mM EGTA. The samples were incubated for 30 min at 23 °C. Total F-actin (with any synapsin I bound) was collected by centrifugation in a Beckman Airfuge at 138 kPa for 30 min. Bundles of F-actin were separated from unbundled F-actin by centrifugation at 11000*g* for 10 min in a MSE microcentrifuge. In each case, supernatants were removed and prepared for SDS−PAGE. Pellets were resuspended in SDS sample buffer to give the same volume as the original mixtures, and equal volumes of supernatants and pellets were analyzed by SDS gel electrophoresis.

The proportion of actin and synapsin I in pellet and supernatant fractions was determined by densitometry of the gels (see below). From these proportions, molar values of synapsin I and actin bound or unbound were calculated. For interpretation of the data, data points were fitted to equations using the computer program Ultrafit (Biosoft, Cambridge, U.K.). Error values were calculated for 95% confidence.

Data on actin binding and bundling by synapsin I in the presence of a fixed concentration of calmodulin were analyzed using a form of the Hill equation much as described previously for analysis of microtubule bundling by synapsin I (Bennett & Baines, 1992):

$$Y = \frac{Y_{max} S^n}{K^n + S^n} \quad (1)$$

where Y is (in the case of F-actin bundling) the proportion of actin incorporated into bundles *or* (in the case of F-actin binding) the synapsin I bound to actin (mol of synapsin I/mol of actin monomer), Y_{max} is the maximum proportion that can be bundled or bound, S is the concentration of synapsin I, K is the concentration of synapsin I required for half-maximal bundling or binding, and n is the Hill coefficient.

As an index of the effectiveness of calmodulin as an inhibitor of synapsin I−F-actin interaction, the concentration of calmodulin required for 50% inhibition of bundling or binding was estimated. Data points shown in Figures 4 and 5 were fitted to the following equation:

$$Y = A + \frac{B - A}{1 + \exp(K(X - C))} \quad (2)$$

In this equation, A is the bottom of the curve, B is the top of the curve (maximum bundling or binding), C is the effective concentration of calmodulin required for 50% inhibition of bundling or binding (EC_{50}), X is the concentration of calmodulin added, and K is a constant. Synapsin I−F-actin−calmodulin interactions are complex and involve multisite interactions with synapsin I (which may be cooperative or noncooperative). This equation has therefore been used purely as a descriptive tool for the estimation of EC_{50} values.

Electron Microscopy. Samples of actin−synapsin I−calmodulin mixtures were settled on Formvar coated electron microscope grids, fixed with 3.75% glutaraldehyde, and stained with saturated uranyl acetate.

Gel Electrophoresis. The method of Laemmli (1970) was used for SDS−PAGE. Polyacrylamide gels (12.5% or 7.5%) were used, and they were stained with Coomassie brilliant blue R250. After complete destaining, protein bands were quantified by densitometry using the UVP SW 2000 densitometry system (UVP Ltd., Cambridge, U.K.). Synapsin I and actin were found to bind indistinguishable quantities of Coomassie blue per microgram of protein. Precautions were taken to ensure the linearity of the dye response in the densitometer readings.

RESULTS

Electron Microscopy of Mixtures of Synapsin I and F-Actin. In previous work (Goold & Baines, 1994), we showed that Ca^{2+}/calmodulin reduced the proportion of actin recoverable in the pellet after low speed centrifugation of actin−synapsin I mixtures. It also reduced the total amount of synapsin I bound to F-actin filaments. However, the effect on bundling was not shown by direct observation of synapsin I−F-actin mixtures in the electron microscope. Figure 1 shows electron microscopy of F-actin in the presence and absence of Ca^{2+}, calmodulin, and unphosphorylated synapsin I. Figure 1a shows actin filaments in the presence of Ca^{2+}; Figure 1b shows actin filaments in the presence of EGTA. In each case, the filaments were not bundled. Synapsin I bundled F-actin both in the presence of Ca^{2+} (Figure 1c) and in the presence of EGTA (Figure 1d). Calmodulin had no discernible effect on F-actin either in the presence of Ca^{2+} (Figure 1e) or in the presence of EGTA (Figure 1f). However, there was a substantial Ca^{2+}-dependent effect on actin bundling by synapsin I in the presence of calmodulin. Figure 1g shows that, in the presence of Ca^{2+} and calmodulin, synapsin I did not bundle F-actin filaments; Figure 1h shows that synapsin I bundled F-actin in the presence of calmodulin and EGTA. These effects were consistently observed in three separate experiments.

These data are consistent with previous data from pelleting assays and strongly suggest that synapsin I is an actin bundling protein under Ca^{2+}/calmodulin control.

Effects of Calmodulin on Actin Binding and Bundling by Synapsin I. While the data above introduce a novel aspect of synapsin I regulation, they do not quantify the effects of calmodulin on synapsin activity, nor do they address the relationship of calmodulin regulation to regulation by phosphorylation. To examine the first of these points, actin binding and bundling by various concentrations of unphosphorylated synapsin I were measured in the presence or absence of a fixed concentration of calmodulin.

Synapsin I (0−4.2 μM) was mixed with 4 μM F-actin in the presence and absence of 10 μM calmodulin. Synapsin I bound to F-actin was collected by high speed centrifugation. Figure 2 shows results of this experiment. In the presence and absence of both Ca^{2+} and calmodulin saturation curves

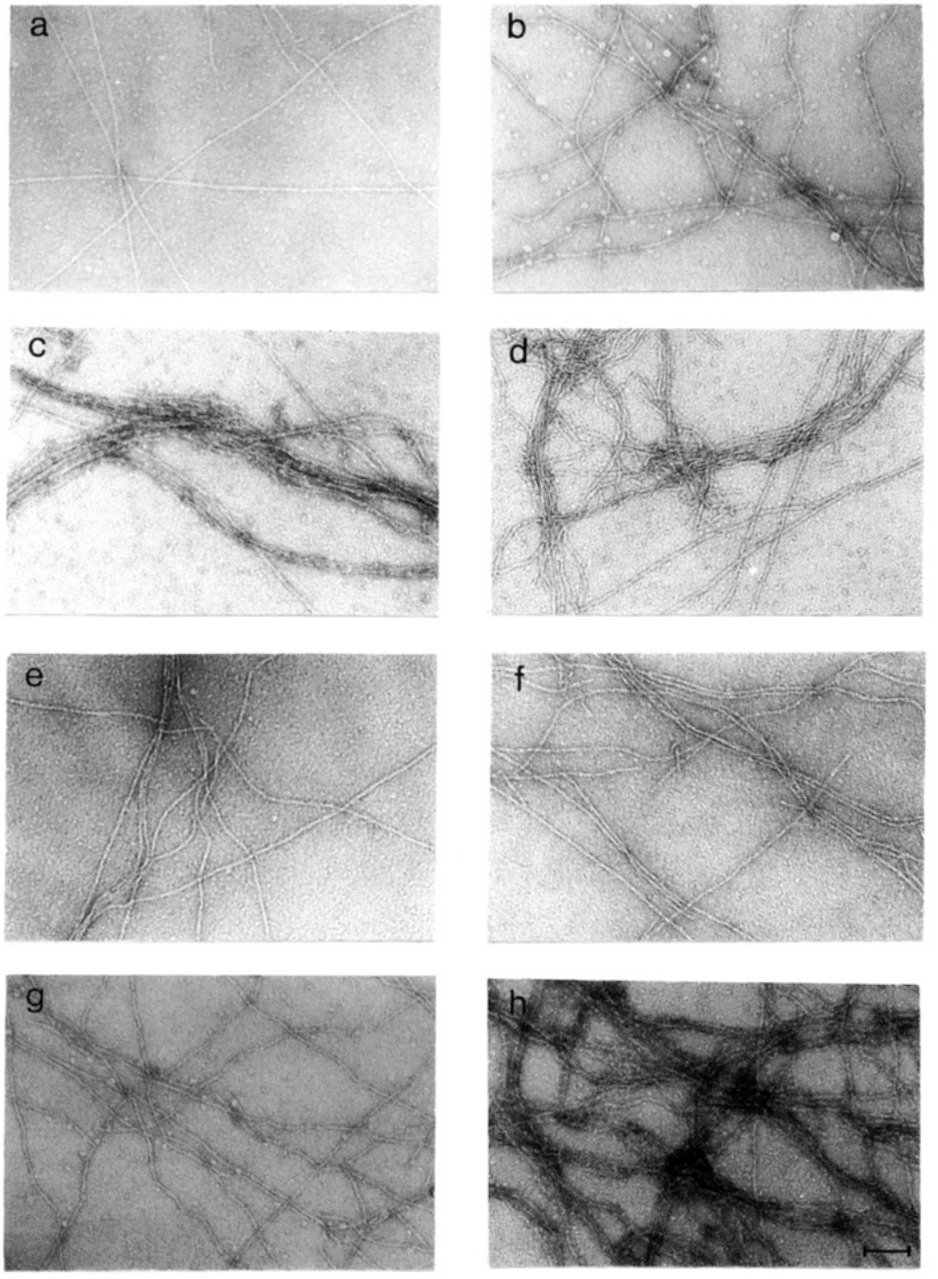

FIGURE 1: Electron microscopy of mixtures of F-actin in the presence and absence of synapsin I, Ca^{2+}, and calmodulin. The figure shows negative stain views of the following mixtures. (a) F-actin filaments in the presence of Ca^{2+}. (b) F-actin filaments in the presence of EGTA. (c) Synapsin I and F-actin in the presence of Ca^{2+}. (d) Synapsin I and F-actin in the presence of EGTA. (e) Calmodulin and F-actin in the presence of Ca^{2+}. (f) Synapsin I and F-actin in the presence of EGTA. (g) Calmodulin, synapsin I, and F-actin in the presence of Ca^{2+}. (h) Calmodulin, synapsin I, and F-actin in the presence of EGTA. Details of this experiment are given further in the text, but note that in (g) the filaments are not detectably bundled, whereas in (h) there are substantial bundles. Bar marker: 100 μm.

were obtained, the characteristics of which are summarized in Table 1. In EGTA, the presence or absence of calmodulin made no difference to the characteristics of synapsin I binding. With Ca^{2+} and calmodulin, the binding curve was significantly shifted to the right by comparison with the corresponding EGTA control: the concentration of synapsin I required for half-maximal binding was increased from about 0.6 to about 1.3 μM (Table 1). Whether this effect is solely due to the presence of active calmodulin is not clear. Addition of Ca^{2+} to binding mixtures in the absence of calmodulin raised the half-maximal requirement for synapsin I by comparison with the EGTA control. The error on the determinations makes it difficult to state that the binding characteristics in the presence of Ca^{2+} were different from those in EGTA, or in the presence of calmodulin.

Our data for the binding of synapsin I in the absence of Ca^{2+}/calmodulin are in reasonable agreement with previous estimates (Bahler & Greengard, 1987; Petrucci & Morrow, 1987) which put the K_d for the interaction at 1−2 μM. The small discrepancy with our data may arise from species differences (sheep synapsin I in this report, as against beef synapsin I in the others). Petrucci and Morrow (1987) differed from Bahler and Greengard (1987) in their estimates of B_{max} values for unphosphorylated synapsin I binding to F-actin. Our data (which give a stoichiometry of about 0.4 synapsin I per actin monomer) are in good agreement with those of Petrucci and Morrow (1987).

The effects of Ca^{2+}/calmodulin on actin bundling were also analyzed (Figure 3 and Table 2). Actin bundling by synapsin I was not affected by the presence of Ca^{2+}, EGTA,

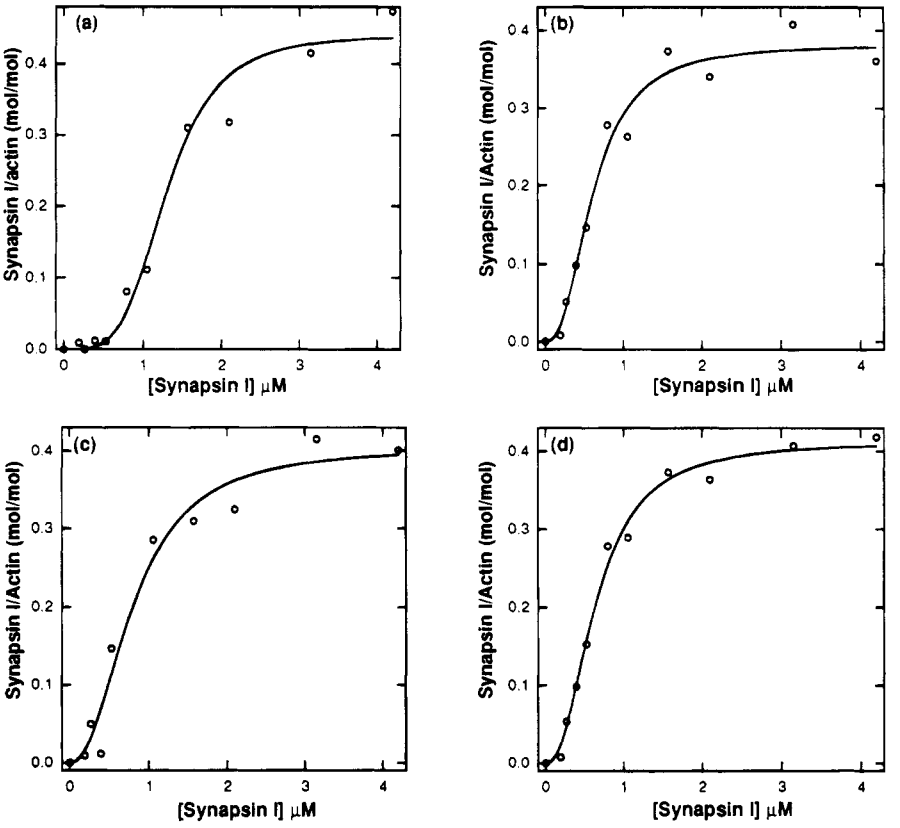

FIGURE 2: Binding of synapsin I to actin in the presence and absence of Ca^{2+} and calmodulin. Mixtures of synapsin I and F-actin in the presence and absence of Ca^{2+} and calmodulin were prepared as described in the text. The mixtures were incubated and centrifuged at high speed to recover the F-actin with any bound synapsin I. Synapsin I and actin in pellet and supernatant fractions were quantified as described in Materials and Methods. (a) Mixtures prepared with Ca^{2+} and calmodulin. (b) Mixtures prepared with Ca^{2+}. (c) Mixtures prepared with EGTA (d) Mixtures prepared with EGTA and calmodulin. The curves shown in this figure are drawn for (a) half-maximal binding = 1.3 μM, B_{max} = 0.44 mol/mol, n = 3.6; (b) half-maximal binding = 0.81 μM, B_{max} = 0.41mol/mol, n = 2.2; (c) half-maximal binding = 0.65 μM, B_{max} = 0.41 mol/mol, n = 2.3; and (d) half-maximal binding = 0.61 μM, B_{max} = 0.38 mol/mol, n = 2.4.

Table 1: Characteristics of Unphosphorylated Synapsin I Binding to F-Actin in the Presence and Absence of Ca^{2+} and Calmodulin

sample	half-maximal binding (μM synapsin I)	B_{max} (mol of synapsin I/ mol of actin)
+calmodulin and Ca^{2+}	1.30 ± 0.32	0.44 ± 0.08
+Ca^{2+}	0.81 ± 0.29	0.41 ± 0.08
+EGTA	0.65 ± 0.08	0.41 ± 0.03
+calmodulin and EGTA	0.61 ± 0.4	0.38 ± 0.01

or calmodulin in the presence of EGTA (half-maximal synapsin I for bundling in each case approximately 0.3−0.4 μM; maximum incorporation of actin into bundles approximately 80%). In the presence of Ca^{2+} and calmodulin, the maximal actin bundling was unchanged, but the half-maximal concentration of synapsin I required for bundling was raised nearly 3-fold (approximately 1.1 μM).

For comparison, using a low speed centrifugation assay similar to ours, Petrucci and Morrow (1987) found half-maximal bundling at <0.5 μM unphosphorylated synapsin I with 7 μM actin (exact value not reported). These data are consistent with our own.

Our data indicate that calmodulin in the presence of Ca^{2+} modulates the F-actin binding and bundling activity of unphosphorylated synapsin I, the most pronounced effects being on bundling.

Phosphorylation Potentiates the Inhibitory Effects of Calmodulin on Synapsin I−Actin Interaction. Synapsin I can be phosphorylated at specific sites in the head and tail regions; it was important to determine whether or not regulation of synapsin I activity by calmodulin changed with the phosphorylation state of synapsin I.

Synapsin I was prepared in different phosphorylation states: site I was phosphorylated using cAMP-dependent protein kinase; sites II and III were phosphorylated using an active fragment of the α subunit of calmodulin-dependent protein kinase II; and all three sites were phosphorylated using both kinases (see Materials and Methods for details). The inhibitory effect of calmodulin on actin binding and bundling by synapsin I in its different phosphorylation states was assessed by titrating fixed concentrations of synapsin I (0.45 μM) and F-actin (4 μM) with calmodulin over the range 0−10 μM. Figures 4 and 5 show the results of these experiments. Figure 4a shows the effect of increasing calmodulin concentrations on binding of unphosphorylated synapsin I to F-actin in the presence of Ca^{2+}. Synapsin I was progressively inhibited from binding to F-actin by increasing the calmodulin concentration. Half-maximal inhibition (EC_{50}) was achieved at about 4.0 ± 0.3 μM calmodulin. By contrast, Figure 4b shows that, in the presence of EGTA, no calmodulin inhibition of binding was detected. The inhibition profile of calmodulin on synapsin I phosphorylated at site I (PI-synapsin I; EC_{50} 4.3 ± 0.4 μM) was not greatly different (Figure 4c). Synapsin I phosphorylated at sites II and III (PII,III-synapsin I; EC_{50} 1.8 ± 0.2 μM) was more sensitive to inhibition than the other two forms (Figure 4d). However, synapsin I phosphorylated at

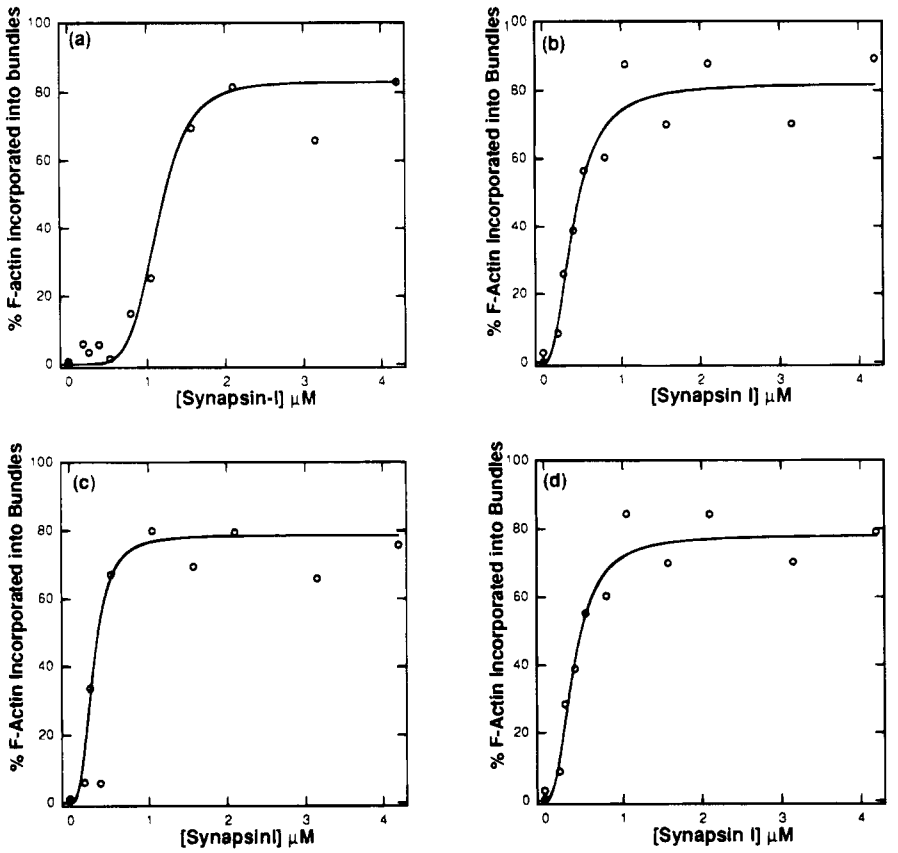

FIGURE 3: Bundling of actin by synapsin I in the presence and absence of Ca^{2+} and calmodulin. Mixtures of synapsin I and F-actin in the presence and absence of Ca^{2+} and calmodulin were prepared as described in the text. The mixtures were incubated and centrifuged at low speed to recover bundles of F-actin. Actin in pellet and supernatant fractions was quantified as described in Materials and Methods. (a) Mixtures prepared with Ca^{2+} and calmodulin. (b) Mixtures prepared with Ca^{2+}. (c) Mixtures prepared with EGTA (d) Mixtures prepared with EGTA and calmodulin. The curves shown in this figure are drawn for (a) half-maximal bundling = 1.1 μM, maximum bundling = 83%, n = 5; (b) half-maximal bundling = 0.32 μM, maximum bundling = 78%, n = 3; (c) half-maximal bundling = 0.37 μM, maximum bundling = 78%, n = 2.5; (d) half-maximal bundling = 0.40 μM, maximum bundling = 82%, n = 2.4

Table 2: Characteristics of F-Actin Bundling by Unphosphorylated Synapsin I in the Presence and Absence of Ca^{2+} and Calmodulin

sample	half-maximal bundling (μM synapsin I)	max actin incorpn into bundles (%)
+calmodulin and Ca^{2+}	1.14 ± 0.16	83 ± 10
+Ca^{2+}	0.32 ± 0.17	78 ± 11
+EGTA	0.37 ± 0.09	78 ± 9
+calmodulin and EGTA	0.40 ± 0.11	82 ± 11

sites I, II, and III (PI,II,III-synapsin I) was inhibited from binding to actin by lower calmodulin concentrations: EC_{50} was 0.2 ± 0.1 μM calmodulin, although in this case the value for uninhibited binding to F-actin was lower, most likely because the B_{max} value for F-actin binding by PI,II,III-synapsin I is relatively low (Bahler & Greengard, 1987).

The effect of increasing concentrations of calmodulin on actin bundling are shown in Figure 5. Unphosphorylated synapsin I was inhibited from bundling F-actin with a half-maximal value of about 3.7 ± 0.3 μM. By contrast with the binding data, PI- and PII,III-synapsin I were more easily inhibited from bundling actin by calmodulin. The EC_{50} for PI-synapsin I was 0.5 ± 0.1 μM (Figure 5b). The EC_{50} for PII,III-synapsin I was less than 0.2 μM (Figure 5c). PI,II,-III-synapsin I is a very weak actin bundling protein (Bahler & Greengard, 1987): no bundling by this form of synapsin I was observed in our assay conditions, and calmodulin had no observable effect on this.

In summary, calmodulin is a general inhibitor of synapsin I–F-actin interaction. Phosphorylation of synapsin I at specific sites selectively potentiates the susceptibility of synapsin I to calmodulin inhibition.

DISCUSSION

In this paper, we have shown two novel properties of the calmodulin–synapsin I interaction. First, calmodulin can inhibit the binding and/or bundling of F-actin by any of three different phosphorylated forms of synapsin I, as well as the unphosphorylated form. Second, site-specific phosphorylation of synapsin I by two different protein kinases differentially potentiates the inhibitory effect of calmodulin. These data introduce the concept that covalent and noncovalent mechanisms of regulation act together to control the activities of synapsin I.

Electron microscopy confirmed our earlier observation (Goold & Baines, 1994) that calmodulin in the presence of Ca^{2+} inhibits actin bundling by unphosphorylated synapsin I (Figure 1). Calmodulin appeared to be a competitive inhibitor of the synapsin I–F-actin interaction: it reduced the apparent affinity of synapsin I for actin in both bundling and binding assays, but maximal binding or bundling was unchanged (Figure 2). Moreover, addition of sufficient calmodulin in the presence of Ca^{2+}, but not in its absence, reduced binding and bundling to essentially undetectable levels (Figures 3–5).

It is notable that, depending on the phosphorylation state

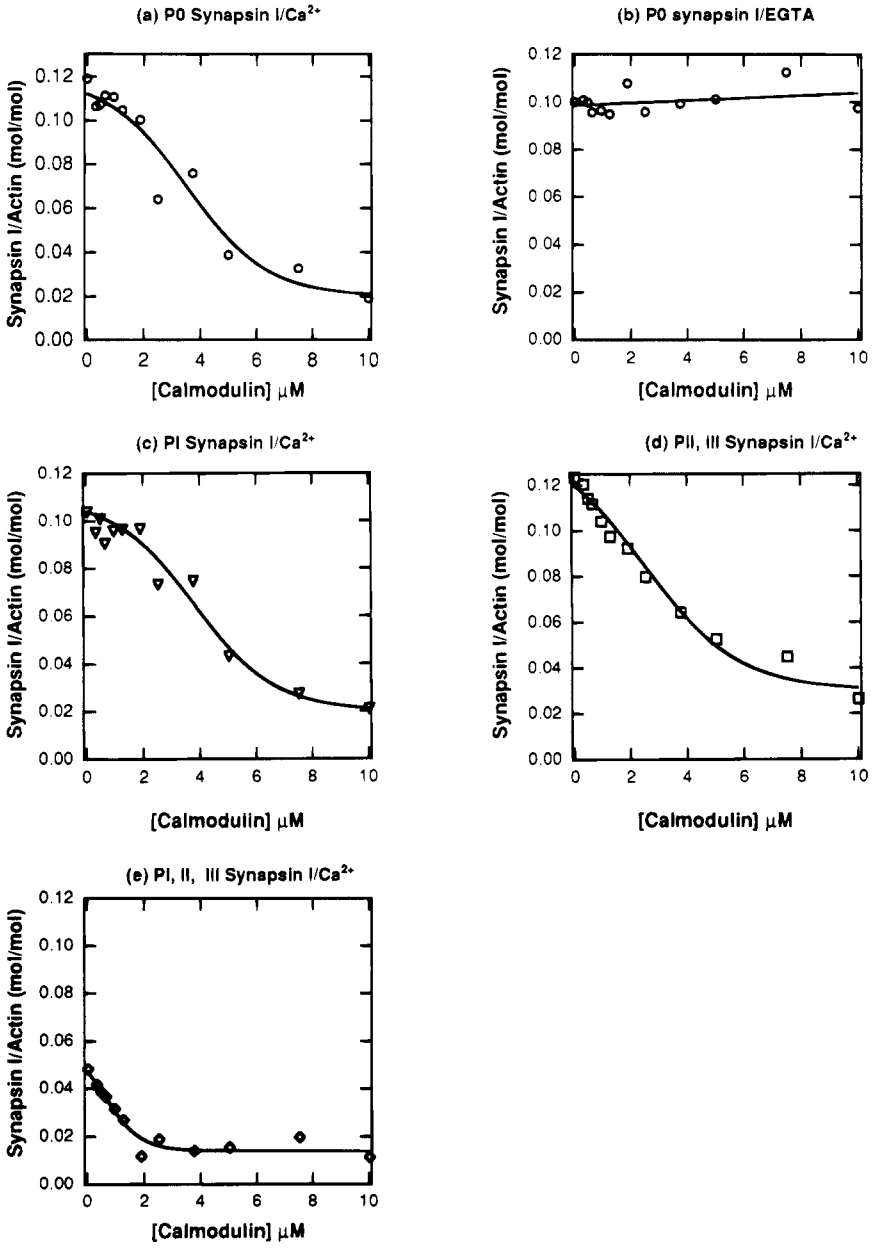

FIGURE 4: Calmodulin inhibition of actin binding by phosphorylated forms of synapsin I. Mixtures of 0.45 μM synapsin I in different phosphorylation states and 4 μM F-actin with various concentrations of calmodulin were prepared as described in the text. The mixtures were incubated and centrifuged at high speed to recover the F-actin with any bound synapsin I. Synapsin I and actin in pellet and supernatant fractions were quantified as described in Materials and Methods. (a) Mixtures prepared with unphosphorylated synapsin I in the presence of Ca^{2+}. (b) Mixtures prepared with unphosphorylated synapsin I in the presence of EGTA. (c) Mixtures prepared with synapsin I phosphorylated at site I in the presence of Ca^{2+}. (d) Mixtures prepared with synapsin I phosphorylated at sites II and III in the presence of Ca^{2+}. (e) Mixtures prepared with synapsin I phosphorylated at sites I, II, and III in the presence of Ca^{2+}.

of the synapsin I used in our assays, two different states that varied in their susceptibility to calmodulin inhibition were observed. One state was moderately susceptible to calmodulin inhibition: this was the state of unphosphorylated synapsin I in which both bundling and binding were half-maximally inhibited under the conditions of our assay by approximately 2−4 μM calmodulin. The other state was sensitive to calmodulin inhibition at lower concentrations. It seems likely that this type of inhibition is mediated by the high affinity binding of calmodulin to two nonoverlapping sites in the head region (K_d ~30 nM). PI-synapsin I and PII,III-synapsin I are moderately sensitive to inhibition by calmodulin of their actin binding activity (EC_{50} ~3.5 μM), but more sensitive to inhibition of bundling (EC_{50} ~0.5 μM). PI,II,III-synapsin I was highly sensitive to calmodulin inhibition of actin binding (EC_{50} ~0.2 μM), but bundles actin so weakly that no further effect of calmodulin was noted in our assays. The K_i for calmodulin in this process should be an indication of the affinity of calmodulin for the inhibitory sites; however, calculating a true K_i for competition between the noncooperative synapsin I−calmodulin interaction and the cooperative synapsin I−F-actin interaction is uncertain at best. (Assuming the simplest possible competition equilibria, under the conditions of our assay an EC_{50} ~3.5 μM would be consistent with a K_i in the micromolar range; an EC_{50} ~0.2 μM would be consistent with a K_i in the region of 50 nM.)

The existence of these two states was not predicted from studies of direct binding of calmodulin to synapsin I (Goold & Baines, 1994). In each of the phosphorylation states

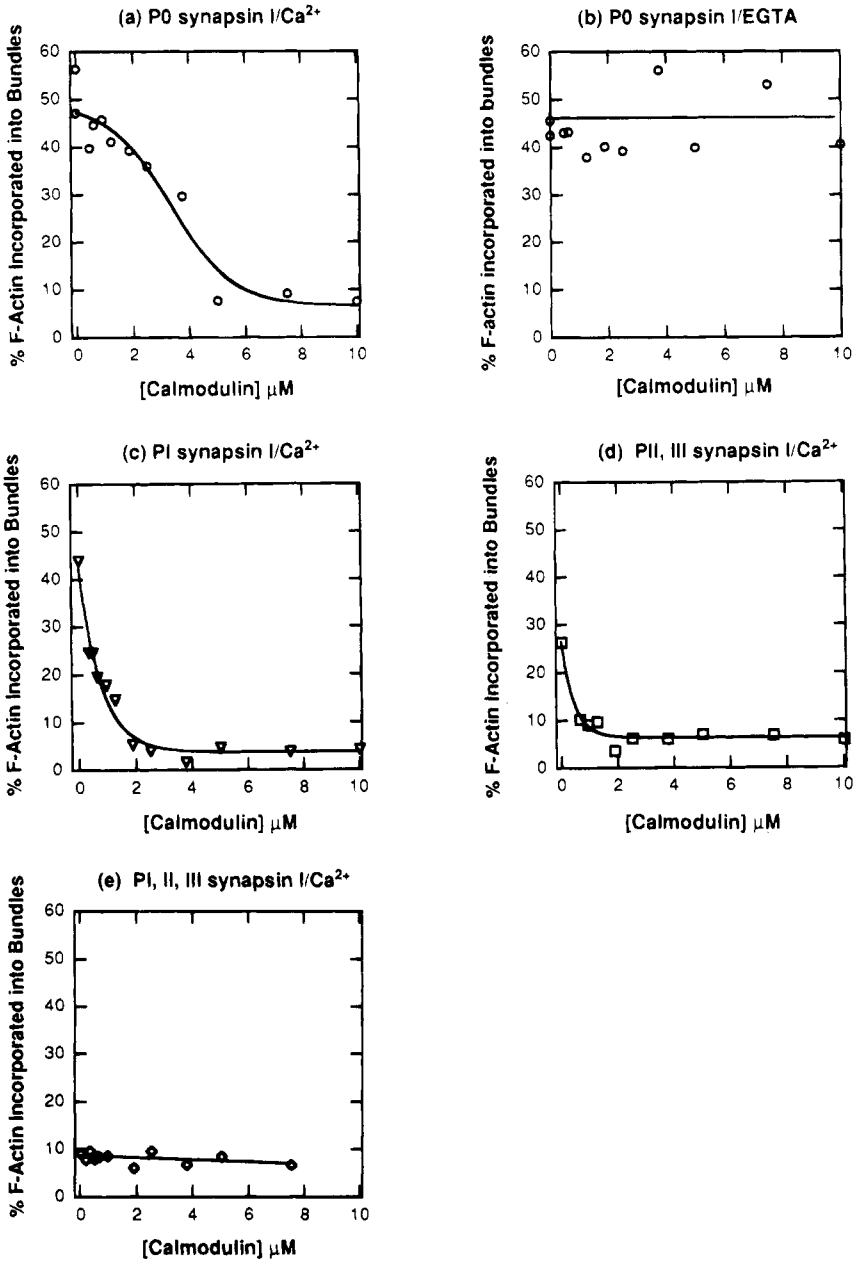

FIGURE 5: Calmodulin inhibition of actin bundling by phosphorylated forms of synapsin I. Mixtures of 0.45 μM synapsin I in different phosphorylation states and 4 μM F-actin with various concentrations of calmodulin were prepared as described in the text. The mixtures were incubated and centrifuged at low speed to recover bundles of F-actin. Actin in pellet and supernatant fractions was quantified as described in Materials and Methods. (a) Mixtures prepared with unphosphorylated synapsin I in the presence of Ca^{2+}. (b) Mixtures prepared with unphosphorylated synapsin I in the presence of EGTA. (c) Mixtures prepared with synapsin I phosphorylated at site I in the presence of Ca^{2+}. (d) Mixtures prepared with synapsin I phosphorylated at sites II and III in the presence of Ca^{2+}. (e) Mixtures prepared with synapsin I phosphorylated at sites I, II, and III in the presence of Ca^{2+}.

examined, synapsin I bound calmodulin labeled with acetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine with indistinguishable affinity. The simplest view of the highly sensitive states of synapsin I is that when calmodulin binds to the high affinity sites on them, it can act as an inhibitor. By contrast, when calmodulin is bound at the high affinity sites of the relatively insensitive synapsin I, it does not act as an inhibitor. A prediction from this would be that another, lower affinity, class of binding site for calmodulin exists on synapsin I which can also inhibit actin binding/bundling. However, no direct evidence for this site exists beyond the inhibition data. Lower affinity binding was not detected in *in vitro* binding assays with labeled calmodulin (Goold & Baines, 1994), although the conditions used in those assays (500 mM NaCl) might have reduced those interactions below the limits of detection. It seems unlikely that actin itself interacted with calmodulin: no calmodulin was detectable in the pellets of our binding assays—see Figure 8 in Goold and Baines (1994). No gross effect on F-actin was observed in the electron microscope (Figure 1). Previous reports indicate no direct actin–calmodulin interaction (Wallace & Piazza, 1987).

Our data indicate that the role of phosphorylation at site I is to potentiate the effect of calmodulin as an inhibitor of actin bundling. The interplay between phosphorylation and calmodulin binding reveals for the first time the importance of phosphorylation at site I in the regulation of synapsin I–F-actin interaction. This is the only phosphorylation site conserved in synapsins I and II, and it would therefore be

presumed to be of considerable importance. Despite the fact that this site is stoichiometrically filled upon nerve terminal depolarization, no clear role has emerged previously for this phosphorylation.

Facilitation of neuronal exocytosis either by repetitive stimulation of a synapse (e.g., paired pulse facilitation) or by facilitatory input from another neuron are examples of situations in which the phosphorylation of site I might be significant. Phosphorylation of site I would yield a form of synapsin I more sensitive to calmodulin inhibition of its interaction with F-actin, but which would remain bound to actin until full activation of calmodulin. It might be imagined that facilitatory input would establish a pool of presensitized synapsin I, more rapidly releasable from actin than unphosphorylated synapsin I. Conversely, inhibitory inputs, which reduced the site I phosphorylation, would reduce the sensitivity of synapsin I to calmodulin inhibition.

Phosphorylation of synapsin I by calmodulin-dependent protein kinase II modulates its activities *in vitro* (Bahler & Greengard, 1987; Benfenati et al., 1992; Petrucci & Morrow, 1987; Schiebler et al., 1986) and reduces the limitation that synapsin I imposes on synaptic exocytosis (Lin et al., 1990; Llinas et al., 1985; Nichols et al., 1992, for example). In this study, we have found that kinase II potentiates the inhibitory effect of calmodulin on actin bundling, and it is the more effective of the two kinases at sensitizing synapsin I to calmodulin inhibition of actin binding. Our data are consistent with the view that kinase II is a key element in the *in vivo* regulation of synapsin I activity.

It remains to be resolved whether or not calmodulin inhibits the actions of synapsin I on actin polymerization: as with bundling and binding, the principal covalent modification that regulates this aspect of synapsin I is catalyzed by kinase II (Fesce et al., 1992; Valtorta et al., 1992). Furthermore, it will be important to examine potential calmodulin regulation of synapsin II–F-actin interaction: it appears that synapsins I and II interact with actin in different ways (Chilcote et al., 1992).

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