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Phosphorylation-Dependent Inhibition by Synapsin I of Organelle Movement in Squid Axoplasm

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Synapsin I, a neuron-specific, synaptic vesicle-associated phosphoprotein, is thought to play an important role in synaptic vesicle function. Recent microinjection studies have shown that synapsin I inhibits neurotransmitter release at the squid giant synapse and that the inhibitory effect is abolished by phosphorylation of the synapsin I molecule (Llinas et al., 1985). We have considered the possibility that synapsin I might modulate release by regulating the ability of synaptic vesicles to move to, or fuse with, the plasma membrane. Since it is not yet possible to examine these mechanisms in the intact nerve terminal, we have used video-enhanced microscopy to study synaptic vesicle mobility in axoplasm extruded from the squid giant axon. We report here that the dephosphorylated form of synapsin I inhibits organelle movement along microtubules within the interior of extruded axoplasm and that phosphorylation of synapsin I on sites 2 and 3 by calcium/calmodulin-dependent protein kinase II removes this inhibitory effect. Phosphorylation of synapsin I on site 1 by the catalytic subunit of cAMP-dependent protein kinase only partially reduces the inhibitory effect. In contrast to the inhibition of movement along microtubules seen within the interior of the axoplasm, movement along isolated microtubules protruding from the edges of the axoplasm is unaffected by dephospho-synapsin I, despite the fact that the synapsin I concentration is higher there. Thus, synapsin I does not appear to inhibit the fast axonal transport mechanism itself. Rather, these results are consistent with the possibility that dephospho-synapsin I acts by a crosslinking mechanism involving some component(s) of the cytoskeleton, such as F-actin, to create a dense network that restricts organelle movement. The relevance of the present observations to regulation of neurotransmitter release is discussed.

Synapsin I is a neuron-specific phosphoprotein that is concentrated in presynaptic nerve terminals, where it appears to be associated primarily with the cytoplasmic surface of small synaptic vesicles (Ueda et al., 1973; Ueda and Greengard, 1977; De Camilli et al., 1979, 1983a, b; Huttner et al., 1983; Navone et al., 1984). It is a major substrate for cAMP-dependent protein kinase as well as calcium/calmodulin-dependent protein kinases I and II (CaM kinases I and II) (Huttner and Greengard, 1979; Sieghart et al., 1979; Huttner et al., 1981; Kennedy and Greengard, 1981; Czernik et al., 1987; Nairn and Greengard, 1987). cAMP-dependent protein kinase and CaM kinase I both phosphorylate a single serine residue (site 1) located in the collagenase-resistant head region of the synapsin I molecule, CaM kinase II phosphorylates a pair of serine residues (sites 2 and 3) located in the collagenase-sensitive tail region of the molecule. A variety of studies using intact nerve cell preparations have shown that physiological and pharmacological agents that affect synaptic function also produce alterations in the state of phosphorylation of synapsin I (for reviews, see Nestler and Greengard, 1984; Greengard et al., 1987). *In vitro* studies have shown that, under certain conditions, phosphorylation of the tail region of synapsin I by CaM kinase II reduces the affinity of synapsin I for the synaptic vesicle membrane (Huttner et al., 1983; Schiebler et al., 1986; Benfenati et al., 1989). Taken together, these studies led to the hypothesis that synapsin I might be involved in the regulation of some aspect of synaptic vesicle function.

The possibility that synapsin I might regulate neurotransmitter release was tested directly by injection of synapsin I into the preterminal digit of the squid giant synapse (Llinas et al., 1985). Injection of the dephosphorylated form of synapsin I decreased neurotransmitter release, whereas injection of synapsin I that had been phosphorylated at sites 2 and 3 in the tail region by CaM kinase II was without effect. Moreover, injection of CaM kinase II itself enhanced release. More recent studies (R. Llinas et al., unpublished observations) have expanded these initial findings and have shown that phosphorylation of site 1 in the head region of synapsin I by cAMP-dependent protein kinase is less effective than tail phospho-synapsin at reducing the ability of synapsin I to inhibit transmitter release at the squid giant synapse.

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We believe that synapsin I is more likely to be involved in modulating than in mediating release, since the fusion-release process per se can occur in less than 200 μsec (Llinas et al., 1981), and the turnover rates for the protein kinases are on the order of tens of milliseconds. Furthermore, it is unlikely that synapsin I, which is enriched only in neurons, regulates the exocytotic event itself, a process that is common to all secretory cells. Finally, within nerve terminals, synapsin I appears to be associated only with small (40–60 nm diameter) and not with large (>60 nm diameter) synaptic vesicles (De Camilli et al., 1983a, b; Huttner et al., 1983; Navone et al., 1984; De Camilli and Greengard, 1986). Thus, we think that synapsin I is most likely to be involved in modulating some prefusion property that is unique to small synaptic vesicles.

We have proposed that phosphorylation of synapsin I might regulate the availability of synaptic vesicles for release and that it might do so by regulating the ability of the vesicles to move to or fuse with the plasma membrane (Llinas et al., 1985). Since it is not yet possible to examine these mechanisms in the intact nerve terminal, a number of model systems are currently being used to study the mechanism of action of synapsin I. One of these systems, the extruded axoplasm of the squid giant axon, is the focus of the present report. The present studies were designed to test the possibility that synapsin I regulates the availability of synaptic vesicles for release by affecting the ability of the vesicles to move within the cytoplasm. This required a relatively intact system in which we could directly and clearly visualize vesicle movement. We therefore chose to use video-enhanced contrast-differential interference contrast (VEC-DIC) microscopy techniques to examine the effect of synapsin I on vesicle movement in the extruded squid axoplasm.

VEC-DIC microscopy techniques (Allen et al., 1981; Inoue, 1981) make it possible to detect structures as small as one-tenth the traditional limit of resolution of the light microscope. With these techniques, one can observe subcellular structures the size of individual small synaptic vesicles and microtubules (Allen et al., 1981; Allen and Allen, 1983). The extruded axoplasm preparation from the squid giant axon permits one to perform a variety of manipulations with macromolecules that do not ordinarily cross the plasma membrane and to use VEC-DIC microscopy to observe directly the effects of these manipulations on organelle movement (Brady et al., 1982, 1984, 1985). In addition, the extruded axoplasm preparation provides a useful means for determining if interactions between proteins and subcellular organelles that are suggested on the basis of reconstitution studies with purified components can occur within the axonal milieu. The present results indicate that synapsin I can restrict the ability of vesicles to move within the axoplasm and that this effect is dependent on the state of phosphorylation of the synapsin I molecule. A preliminary report of this work has been published (McGuinness et al., 1987).

Materials and Methods

Preparation of proteins. Synapsin I was purified in the dephosphorylated form from bovine brain by a modification (Bahler and Greengard, 1987) of the procedure of Schiebler et al. (1986). CaM kinase II was purified from rat forebrain as described by McGuinness et al. (1985), with the addition of hydroxylapatite chromatography inserted between the DEAE-cellulose chromatography and ammonium sulfate precipitation steps. Purified CaM kinase II (0.4–0.5 mg/ml) was dialyzed extensively against buffer A (10 mM HEPES, pH 7.2, 150 mM potassium aspartate, 5 mM $MgSO_4$) and stored at −70°C. The catalytic subunit of cAMP-dependent protein kinase was purified as described by Kaczmarek et al. (1980). Calmodulin was purified as described by Grand et al. (1979). Protein determinations were performed by the method of Peterson (1977), using BSA as standard.

Synapsin I (0.35 mg/ml) was phosphorylated by incubation for 30 min at 30°C in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.4 mM EGTA, 1 mM dithioerythritol, 10 mM $MgCl_2$, 100 μM ATP, with trace amounts of γ-^{32}P-ATP, with either CaM kinase II (3.4 μg/ml), 0.7 mM $CaCl_2$, 30 μg/ml calmodulin or the catalytic subunit of cAMP-dependent protein kinase (60 nM), 0.1% Nonidet-P40. "Mock"-phospho-synapsin I was treated as above except that both kinases, $CaCl_2$, calmodulin, and Nonidet-P40 were added, and ATP was omitted from the reaction mixture. Phosphorylation reactions were terminated by the addition of EDTA to a final concentration of 20 mM. One- and 2-dimensional peptide mapping (Kennedy and Greengard, 1981; Huttner et al., 1981; Kennedy et al., 1983) revealed that synapsin I was phosphorylated to a stoichiometry of 1.0 mol/mol on site 2 and on site 3 by CaM kinase II and to a stoichiometry of 0.95 mol/mol on site 1 by the catalytic subunit of cAMP-dependent protein kinase.

Some of the phosphorylated and dephosphorylated synapsin I preparations were fluorescently labeled with Texas red (Molecular Probes, Inc., Eugene, OR) according to the method of Titus et al. (1982) as described by Llinas et al. (unpublished obervations). The various forms of synapsin I were re-purified from kinases and/or unbound Texas red by CM-cellulose chromatography at pH 8.0 as described by Llinas et al. (unpublished observations), using buffer A for elution. The re-purified synapsin I was concentrated, dialyzed against buffer A, centrifuged in a Beckman TL 100 centrifuge at 450,000 *g* for 15 min to remove large aggregates, stored at 0–4°C, and used within 7 d of preparation or stored at −70°C and thawed immediately before use. Labeled and unlabeled synapsin I preparations were assayed for the ability to be phosphorylated by CaM kinase II using the procedure of McGuinness et al. (1985), to bind to purified synaptic vesicles using the procedure of Schiebler et al. (1986), to inhibit neurotransmitter release using the procedure of Llinas et al. (1985), and to inhibit organelle movement using the methods described in the present report.

Manipulation of axoplasm. Squid (*Loligo pealei*) having a mantle length of 10–20 cm were obtained daily from the Department of Marine Resources, Marine Biological Laboratory (Woods Hole, MA). Postganglionic squid giant axons were dissected, and each axoplasm was extruded onto a glass coverslip. The axoplasm was then covered with a second glass coverslip for viewing by VEC-DIC microscopy as previously described (Brady et al., 1985; Schroer et al., 1985). After recording baseline organelle movement, axoplasm was incubated with 20–40 μl of either buffer B (buffer A plus 1 mM ATP) alone or buffer B containing 1.3–10 μM synapsin I.

For the kinase injection experiments, axoplasm was extruded onto a glass coverslip, and CaM kinase II (0.4 mg/ml in buffer A) was injected from a microelectrode by means of short pressure pulses (20–65 psi, ~100 msec in duration) into a small region of the axoplasm. The axoplasm was then covered with a second coverslip for viewing by VEC-DIC microscopy. After recording baseline organelle movement, 20–40 μl of buffer B containing 1.3–10 μM dephosphosynapsin I, 40 μM $CaCl_2$, and 0.15 mg/ml calmodulin was applied to the chamber.

The rapid directed movement of membranous organelles within extruded axoplasm occurs in both the anterograde (from the cell body to the terminals) and the retrograde (from the terminals to the cell body) directions and, in neurons, is referred to as fast axonal transport (for review, see Grafstein and Forman, 1980). Fast transport has been shown, using VEC-DIC techniques, to occur along individual microtubules (Hayden et al., 1983; Allen et al., 1985; Schnapp et al., 1985). Recent evidence suggests that the translocating enzyme that may be responsible for anterograde transport is different from that responsible for retrograde transport (Vale et al., 1985 a, c; Paschal and Vallee, 1987; Paschal et al., 1987). In all the figures, the axoplasm was oriented horizontally such that the proximal or cell body end of the axoplasm is to the left and the distal or terminal end is to the right. Thus, movement from left to right is in the anterograde direction, and movement from right to left is in the retrograde direction.

Video-enhanced microscopy. Organelle movement was visualized using DIC optics on a Zeiss Axiomat microscope (with 100× objective, zoom set at 2.5×) using a Chalnicon videocamera (Hamamatsu Systems, Inc., Waltham, MA). A silicon-intensified target camera (Hamamatsu) and rhodamine filter set (Carl Zeiss, Inc., Thornwood, NY) were used for fluorescence imaging. Video images were processed with a Hamamatsu C-1440 or C-1966 Image Processor (Photonics Micros-

copy, Inc., Oak Brook, IL) to enhance contrast and to eliminate background optical noise. Video signals were recorded in real-time (30 frames/sec) on a Sony VO-5850 videotape recorder.

Depending on the particular biological preparation used, one can examine various parameters of fast axonal transport, including the number and types of organelles moving, their velocity, and the direction of movement. However, the intact extruded axoplasm preparation is not amenable to quantitation of the number of organelles moving per unit time. The number of moving particles in a single plane of focus within a healthy axoplasm is so numerous and the smallest organelles, which are the ones of interest in the present studies, have such low contrast that they are detected primarily as a continuous streaming across the screen. Because such organelles are well below the limit of resolution of the light microscope, it is physically impossible to distinguish these small moving particles as individual elements. Any estimate of the number of particles moving across even a short line drawn on the screen could be in error by orders of magnitude. In previous studies using intact axons (e.g., Allen et al., 1982; Adams and Bray, 1983; Forman et al., 1983; Koenig, 1986) or neurites in culture (e.g., Hollenbeck and Bray, 1987), only a fraction of the total number of moving particles was detected. In fact, the inability to detect the small synaptic vesicle-sized particles in these preparations reduced the total number of visible moving particles so that the small number of larger moving organelles could be counted. In previous studies using dissociated axoplasm (e.g., Allen et al., 1985; Schnapp et al., 1985; Vale et al., 1985b; Weiss, 1986), individual translocation events could be easily examined. For some of the intact and dissociated preparations, estimates were presented of the number of moving particles. However, no attempt was made in any of these studies to evaluate the sampling procedure to determine the population of organelles being counted or how these relate to the complete set of organelles in transport. Fortunately, in the present study, the effect of the dephosphorylated form of synapsin I on organelle movement was so dramatic that precise quantitation of the number of organelles affected was not critical.

Velocity measurements were performed with a Hamamatsu C-2117 video manipulator, which generates white pixels that move across the monitor. The direction and rate of these pixels were adjusted by eye to correspond alternately to the average velocity of particles moving in the anterograde or retrograde directions. Velocities are expressed as mean ± SEM.

The "trace" images were obtained using the Hamamatsu C-1966 Image Processor to extract a trace of moving objects by sequentially subtracting frames at specified intervals and accumulating the successive subtracted images. We had hoped to calculate the total intensities of the trace images and to use the intensity values as a quantitative measure of total movement. This could be done by using a root-mean-square average of the subtracted images as described by Forscher et al. (1987). However, several limitations inherent in the trace technique precluded its routine use in the present studies. Situations that limit the usefulness of this technique include too many moving particles for their trajectories to be resolved from one another, variation in intensity of illumination due to fluctuations of the light source or differences in the depth of focus, and particles moving in and out of the plane of focus. The first limitation is the reason why only the trajectories of the high-contrast, medium-sized and large organelles could be clearly seen in Figure 2*A*. Unfortunately, we were most interested in the movements of the low-contrast, small (synaptic vesicle-sized) organelles, which were lost in the background video noise. In addition, fluctuations in the illuminator and slight changes in the depth of focus during the course of a 1 hr experiment often made it impossible to compare directly the trace image or total intensity of the image before and after the experimental manipulation. As a result, this procedure provided a qualitative means of comparison but was not well suited for quantitative comparison of movement in axoplasm.

Results

Effect of dephospho-synapsin I on organelle movement within the interior of the axoplasm

In agreement with previous reports (Brady et al., 1982, 1984, 1985), in preparations that were not exposed to synapsin I, transport of organelles along microtubules was maintained for several hours after extrusion of the axoplasm. The situation in the presence of the dephosphorylated form of synapsin I is illustrated in Figure 1 and Table 1, where it can be seen that incubation of isolated axoplasm with dephospho-synapsin I resulted in an almost complete inhibition of directed organelle movement. Immediately before addition of dephospho-synapsin I, organelles of all sizes were seen moving in both anterograde and retrograde directions (Fig. 1, *A, B*). In contrast, by 1 hr after addition of dephospho-synapsin I to the axoplasm, virtually all directed organelle movement within the interior of the axoplasm came to a complete halt (Fig. 1, *C, D*). The smallest organelles appeared to be the most quickly and completely affected, but by 30 min to 1 hr after addition of dephospho-synapsin I, organelles of all sizes were clearly affected. In the absence of dephospho-synapsin I, the average velocity of particles moving in the anterograde and retrograde directions was 1.72 ± 0.098 μm/sec (n = 33) and 1.29 ± 0.042 μm/sec (n = 33), respectively (S. T. Brady and G. S. Bloom, unpublished observations; and the present report). After equilibration of the axoplasm with dephospho-synapsin I, the average velocity in both directions for most particles was essentially zero.

No detectable change in the structural organization of the axoplasm was noted on the addition of dephospho-synapsin I. For example, in contrast to results obtained with purified microtubules (Baines and Bennett, 1986), no dephospho-synapsin I-induced bundling of microtubules was detected in the intact axoplasm. Furthermore, there was no loss of order or increase in Brownian motion, which can be seen in disrupted axoplasm or axoplasm depleted of ATP (Brady et al., 1985). Instead, the axoplasm appeared to virtually freeze in the presence of dephospho-synapsin I, with many organelles undergoing repeated short "tugging" movements. This tugging behavior could represent constrained Brownian movement of particles trapped

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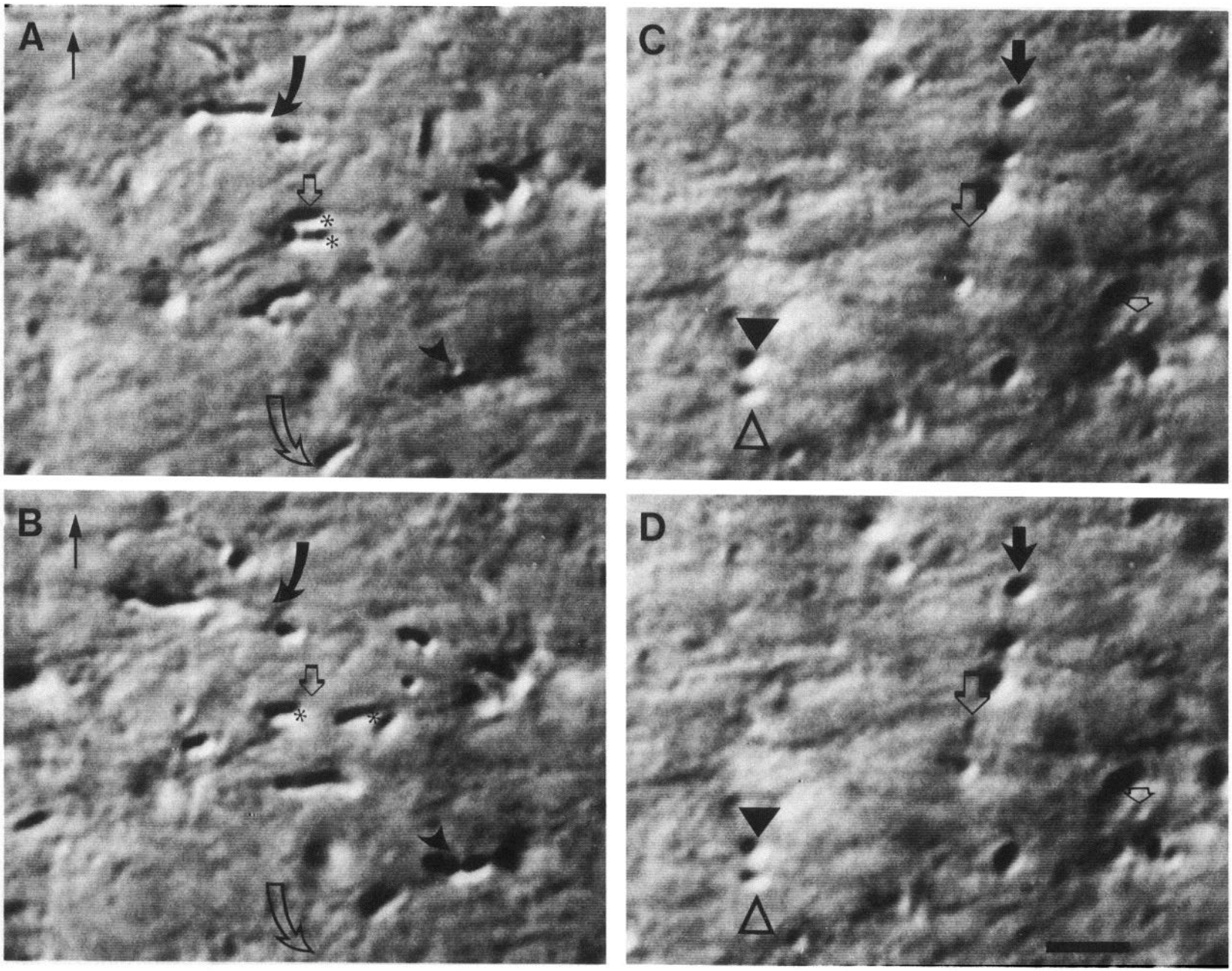
Figure 1. Effect of dephospho-synapsin I on organelle movement within the interior of the axoplasm. Stills from video records show organelle movement within the interior of extruded axoplasm immediately before (*A, B*) and 1 hr after (*C, D*) addition of 10 μM dephospho-synapsin I. *Arrows* and *arrowhead* in *A* point to a few of the numerous organelles that had moved during the 5.5 sec interval between frames *A* and *B*. The corresponding symbols in *B* point to the positions originally occupied by the respective organelles. *Solid, curved arrow* in *A* points to a mitochondrion that moved to the left, as seen in *B*. *Open, curved arrow* in *A* points to a large organelle that moved upward and to the right, as seen in *B*. *Solid, thin arrow* and *solid arrowhead* in *A* point to 2 smaller organelles that moved out of the field in *B*. *Open, straight arrow* in *A* points to 2 organelles that moved in opposite directions in *B*. These 2 organelles are marked with an * in both *A* and *B*; the top organelle moved in the retrograde direction, whereas the bottom organelle moved in the anterograde direction. In contrast to the movement observed between frames *A* and *B*, almost no organelles moved during the 5.5 sec interval between frames *C* and *D*. *Arrows* and *arrowheads* in *C* and *D* indicate just a few of the various-sized organelles that remained stationary in the presence of dephospho-synapsin I. Stills were made by playing the original videotape into the C-1966 Image Processor, performing a rolling average of 4 frames, and recording the averaged image onto an optical disc using a Panasonic TQ-2028F Optical Memory Disc Recorder. Single frames from the optical disc were photographed from a high-resolution monochrome monitor with a 35 mm camera onto Kodak Tech-pan film. Scale bar, 2 μm.


A
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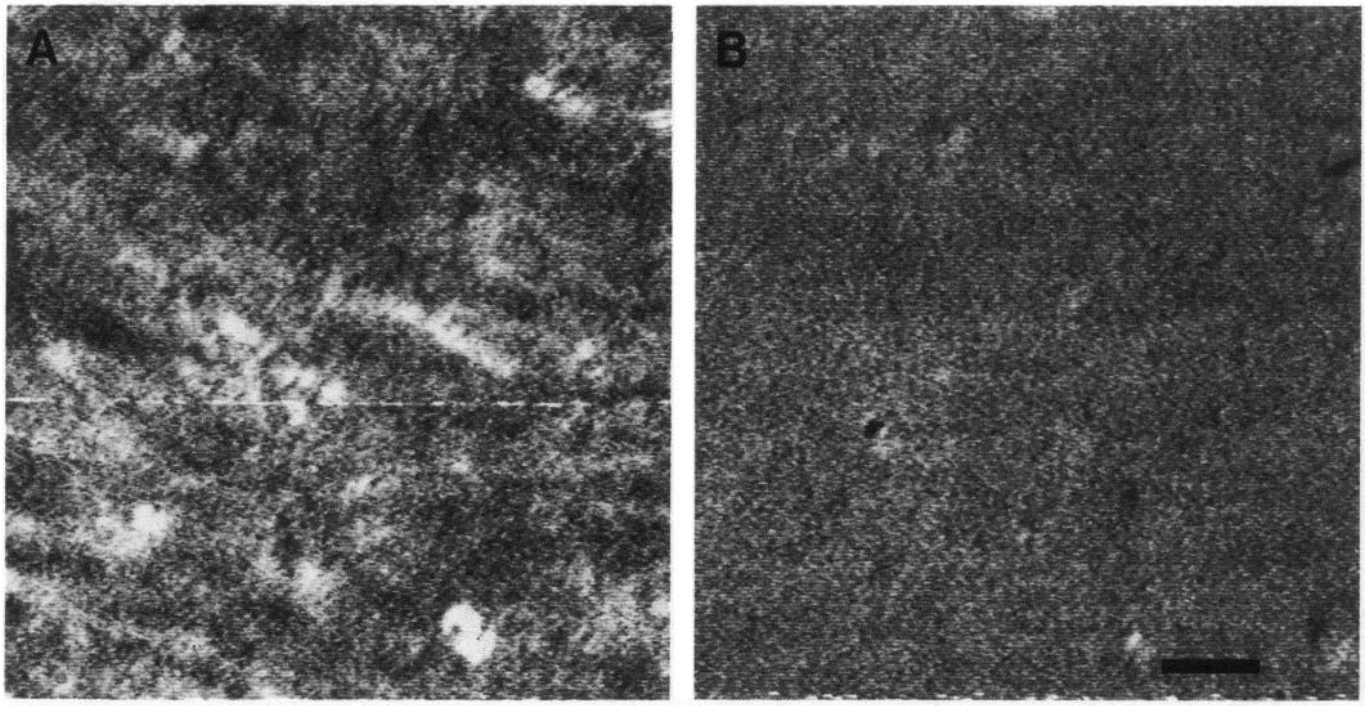


Figure 2. Trace images illustrating the effect of dephospho-synapsin I on organelle movement within the interior of the axoplasm. Traces were obtained by subtracting every third frame (0.1 sec intervals) and accumulating the subtracted images of 60 frames, as described in Materials and Methods. *A*, Linear paths produced by moving organelles within the axoplasm immediately before addition of dephospho-synapsin I. *B*, No such paths can be detected in the same axoplasm 1 hr after addition of 10 μM dephospho-synapsin I. Stills were made by copying the original videotape onto an optical disc and photographing single frames as described in the legend to Figure 1. Scale bar, 2 μm.

within the matrix of the axoplasm (Brady et al., 1985) or could represent attempted movement of organelles that are actually tethered to some component of the axoplasmic matrix. Replenishing the ATP did not reverse the inhibitory effect of dephospho-synapsin I on axonal transport.

Regions in which directed movement had ceased within the axoplasm were first detected within 5 min after dephospho-synapsin I addition and were detected throughout the axoplasm by 30 min. To monitor the diffusion of synapsin I into the axoplasm, the protein used for most of these experiments was fluorescently labeled with the rhodamine derivative Texas red. Labeled synapsin I was comparable to unlabeled synapsin I in terms of its ability to be phosphorylated by CaM kinase II, to bind to purified synaptic vesicles, to inhibit organelle movement (present report), and to inhibit neurotransmitter release (Llinas et al., unpublished observations). Fluorescent dephospho-synapsin I was found to penetrate the axoplasm with a time course similar to that found for the appearance of the inhibitory effect. This time course is consistent with that expected for diffusion of molecules the size of synapsin I in squid axoplasm (Brady et al., 1984, 1985; Morris and Lasek, 1984).

Table 1. Effect of dephospho-synapsin I and phospho-synapsin I on organelle movement within extruded axoplasm

Condition	Synapsin concentration (mg/ml)	Organelle movements (after 30 min)
Control	0	++++
Dephospho-synapsin I	0.1	+
	1.0	0
Mock phospho-synapsin I	0.1	+
	1.0	0
Tail phospho-synapsin I (calmodulin kinase II treated)	0.1	++++
	1.0	+++
Head phospho-synapsin I (cAMP-dependent protein kinase treated)	0.1	+++

Dynamic movement observed in real-time using video-enhanced microscopy cannot be adequately represented in still photographs. The isolated axoplasm preparation contains so many moving organelles that frames selected at 2 different times may look very similar because almost all organelles have moved in the interframe interval and other organelles have taken their place. Often, only the positions of medium-sized and large organelles can unequivocally be identified in successive still photographs. Furthermore, the ability to even detect the small synaptic vesicle-sized particles within the complex interior of the axoplasm is almost completely lost in the still picture. The changes in contrast generated by the movement of these particles contribute significantly to one's ability to study them.

Figure 2 represents an attempt to circumvent these limitations. The trace images in Figure 2 were obtained by sequentially subtracting DIC images captured at 0.1 sec intervals and accumulating 60 of the subtracted images. Trajectories of moving particles were visualized by changes in pixel brightness caused by movement during the interframe interval. The trajectories created by organelles moving within the axoplasm were clearly seen before addition of dephospho-synapsin I (Fig. 2*A*). In con-

trast, no trajectories were evident 1 hr after dephospho-synapsin I was applied to the axoplasm (Fig. 2*B*). This trace imaging technique provided a qualitative measure of the extent to which dephospho-synapsin I reduced movement of organelles.

Effect of dephospho-synapsin I on organelle movement at the periphery of the axoplasm

The most peripheral region of the extruded axoplasm preparation, i.e., the region nearest the buffer–axoplasm interface, has a less dense and less ordered organization than does the interior of the axoplasm proper (cf. Fig. 3, *A, B* to *C, D,* and see Brady et al., 1982; Miller and Lasek, 1985). In the periphery, organelle movement is not inhibited by dephospho-synapsin I. In contrast to the immobilization of particles produced by dephospho-synapsin I within the interior of the axoplasm (Fig. 3, *A, B*), organelle movement along microtubules near the boundary between the buffer and axoplasm (Fig. 3, *C, D*) was unaffected by dephospho-synapsin I. Typically, regions of the axoplasm more than 5–6 μm away from the boundary between buffer and axoplasm were clearly reduced in the amount of organelle movement within 10 min of dephospho-synapsin I addition. As the incubation continued, almost all organelle movement ceased within the interior of the axoplasm (Fig. 3, *A, B*). Throughout the course of the experiment, however, organelles of all sizes in the most superficial regions of axoplasm continued to move along easily resolved microtubules (Fig. 3, *C, D*). Similarly, organelle movement along isolated microtubules protruding from the edge of the axoplasm continued in the presence of dephospho-synapsin I (Fig. 4). Thus, axonal transport along individual microtubules in the periphery of the axoplasm and in the surrounding buffer continued unabated, despite the fact that the dephospho-synapsin I concentration was higher in these peripheral regions than in the interior of the axoplasm, as determined by fluorescent monitoring of Texas red-labeled synapsin I.

Effect of phospho-synapsin I on organelle movement

Phosphorylation of sites 2 and 3 in the tail region of the synapsin I molecule by CaM kinase II abolishes the ability of synapsin I to inhibit transmitter release at the squid giant synapse (Llinas et al., 1985; unpublished observations). Therefore, we examined whether phosphorylation of synapsin I by CaM kinase II would also reduce the ability of synapsin I to inhibit organelle movement in the squid giant axoplasm. As shown in the previous section, organelle movement was almost completely inhibited when axoplasm was incubated with 1.3–10 μM dephospho-synapsin I. In contrast, incubation of axoplasm with 1.3 μM tail phospho-synapsin I (synapsin I that had been phosphorylated on sites 2 and 3 in the tail region of the molecule by CaM kinase II) had no effect on organelle movement (see Table 1), and incubation with 10 μM tail phosphosynapsin I had only a slight inhibitory effect (Fig. 5). This minor effect at the higher concentration might have resulted from sites 2 and 3 being incompletely phosphorylated or partially dephosphorylated by endogenous squid protein phosphatase(s). The fact that 10 μM tail phospho-synapsin I had little effect on transport indicates that this concentration of dephospho-synapsin I, which abolished organelle movement, did not per se cause a nonspecific perturbation of axonal transport.

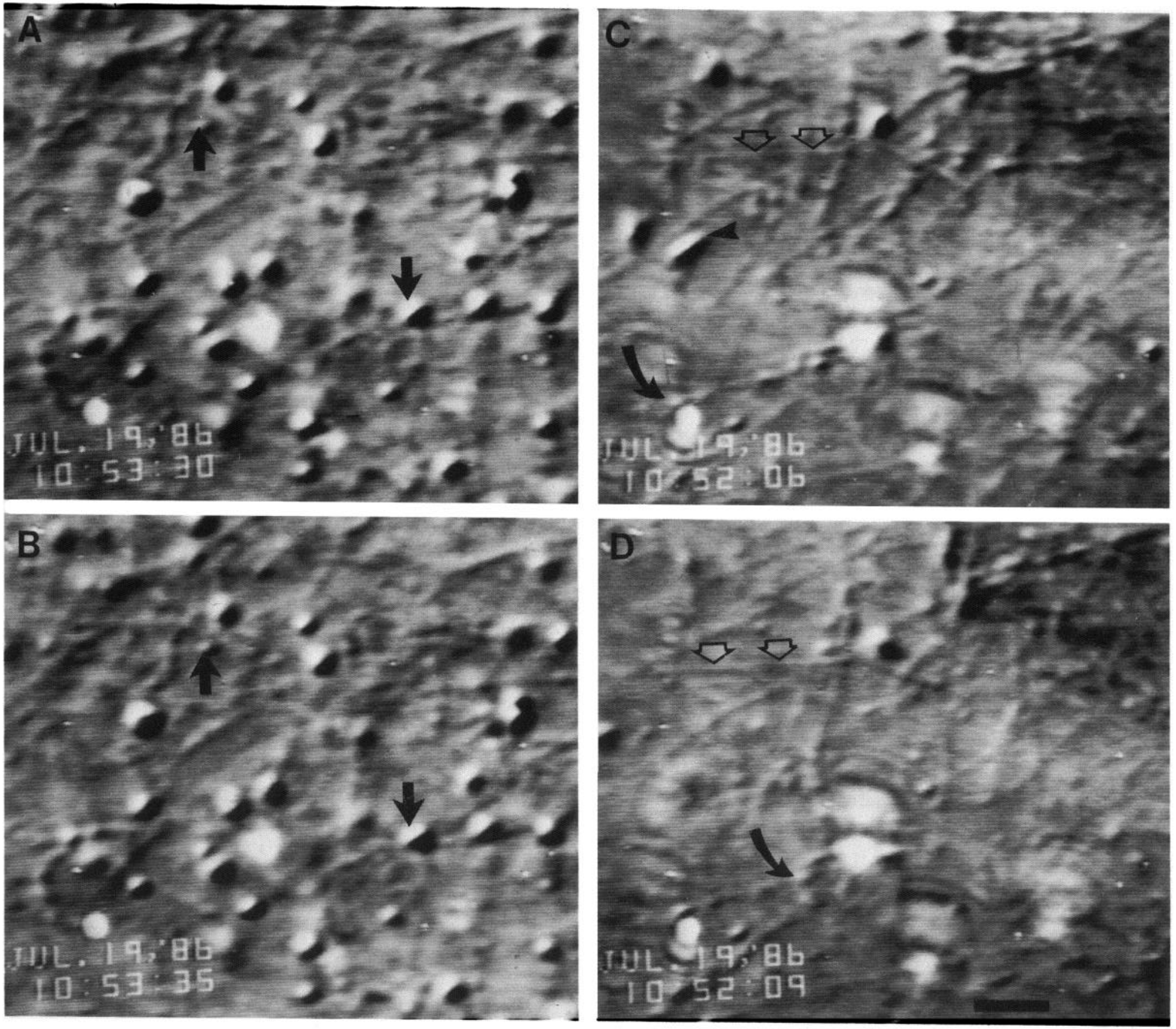
Phosphorylation of site 1 in the head region of the synapsin I molecule by cAMP-dependent protein kinase only partially reduced the ability of synapsin I to inhibit organelles from being transported along microtubules. Phosphorylation by cAMP-dependent protein kinase was not as effective as phosphorylation by CaM kinase II in preventing the inhibitory effect of synapsin I, but far more movement was seen in axoplasm treated with head phospho-synapsin I than if treated with dephospho-synapsin I (see Table 1).

The relative lack of effect of the phospho-synapsin I preparations was not due to nonspecific inactivation of the molecule by the phosphorylation conditions, since mock phospho-synapsin I (synapsin I that had been treated identically to phospho-synapsin I except that ATP was omitted from the phosphorylation reaction mixture) was indistinguishable from dephospho-synapsin I in its inhibitory effect. The relative lack of effect of the phospho-synapsin I preparations was also not due to poor penetration into the axoplasm, since fluorescence monitoring of Texas red-labeled synapsin I showed similar diffusion for the dephospho- and phospho-forms. The differences in transport blocking ability between dephospho- and phospho-synapsin I were also observed when the proteins were pressure-injected from a microelectrode directly into a small region of the axoplasm. In these experiments, dephospho-synapsin I inhibited movement in the region surrounding the injection site, whereas axonal transport proceeded normally at distances farther away. In contrast, phospho-synapsin I had no effect.

The microinjection technique was also used to test whether phosphorylation of synapsin I within the axoplasm itself by CaM kinase II would prevent the inhibitory action of synapsin I on organelle movement. CaM kinase II was pressure-injected into a small area at the distal end of the axoplasm. Organelle movement and the integrity of the axoplasm in the vicinity of the injection site were not noticeably different from that seen in other regions. The axoplasm was then incubated with buffer containing 1.3–10 μM dephospho-synapsin I in the presence of 40 μM calcium and 0.15 mg/ml calmodulin to promote phosphorylation. Incubation with these concentrations of calcium and calmodulin without dephospho-synapsin I had no detectable effect on organelle movement (Brady et al., 1984, 1985; and this study). Dephospho-synapsin I inhibited movement throughout the axoplasm except in the immediate vicinity of the kinase injection site. Near the injection site, normal organelle movement was observed. Decreases in organelle movement were seen with increasing distance from the site of injection. The distribution and intensity of the fluorescent synapsin I was the same at the injection site as in other regions. Thus, inhibition by dephospho-synapsin I can be prevented by CaM kinase II within the axoplasm itself.

Discussion

The present studies utilized the isolated axoplasm from the squid giant axon as one model for studying the mechanism by which synapsin I might regulate neurotransmitter release. These studies were undertaken to test the following hypothesis. If synapsin I regulates availability of synaptic vesicles for release by restricting the ability of vesicles to move within the cytoplasm of the presynaptic terminal, then adding concentrations of synapsin I that would be expected to exist within the terminal to the isolated axoplasm might inhibit vesicle movement in the axoplasm. This is, in fact, what was found (see Table 1). The dephosphorylated form of synapsin I virtually abolished all vesicle movement within the interior of the isolated axoplasm. Phosphorylation of synapsin I by CaM kinase II removed the inhibitory effect and phosphorylation by cAMP-dependent pro-


A
JUL. 19,'86
10:53:30
B
JUL. 19,'86
10:53:35
C
JUL. 19,'86
10:52:06
D
JUL. 19,'86
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tein kinase partially reduced the inhibitory effect on vesicle movement. These results are analogous to the inhibition of neurotransmitter release by dephospho-synapsin I and the reduction of that inhibition by phosphorylation of synapsin I. Thus, the results are consistent with the possibility that the mechanism by which synapsin I affects organelle movement in the isolated axoplasm is similar or identical to the mechanism by which it regulates synaptic vesicle availability in the presynaptic terminal.

The small vesicles in the axoplasm were clearly not the only organelles affected by synapsin I. Movement of all organelles was dramatically inhibited. This nonselective effect raises the possibility that synapsin I directly inhibits the microtubule-based motor mechanism for fast axonal transport. Several factors, however, suggest that synapsin I does not directly affect the transport mechanism itself. First, synapsin I had no apparent effect on the movement of organelles along microtubules in the periphery of the axoplasm or along isolated microtubules protruding into the buffer, where the concentration of synapsin I was the highest. Furthermore, the ability of synapsin I to inhibit organelle movement was critically dependent on a closely packed cytoskeletal matrix. For example, when the axoplasm was improperly extruded so that the original linear organization of the cytoskeletal network was disturbed, synapsin I was much less effective in inhibiting movement. Also, if a single axoplasm had highly organized regions adjacent to regions where the microtubules were disorganized and extending haphazardly in all directions, then significant inhibition was seen only in the organized regions. This ability of synapsin I to inhibit movement only within the interior of axoplasm in which the original cytoskeletal architecture has been maintained is not consistent with a direct effect on the fast axonal transport mechanism. It is also not consistent with the inhibitory effect depending solely on the concentration of a particular component that may have been diluted below a critical concentration in the buffer surrounding the axoplasm. Rather, it suggests that synapsin I may indirectly inhibit fast axonal transport by crosslinking neighboring axoplasmic structures, thus restricting the ability of organelles to move within the resulting network. However, the absence of an effect of dephospho-synapsin I on organelles moving along individual microtubules or in the periphery of the axoplasm suggests that the organelles are not crosslinked to the microtubules. Instead, some other axoplasmic structure must be required.

One possible mechanism for the inhibition of movement of membranous organelles reported here is the following. Dephospho-synapsin I inhibits synaptic vesicle mobility by crosslinking the vesicles to some component(s) of the cytoskeleton, thereby creating a dense meshwork or cage, which then indirectly restricts the movement of other organelles as well. Indeed, it has previously been proposed that synapsin I crosslinks synaptic vesicles to cytoskeletal structures and that this interaction is regulated by phosphorylation of the synapsin I molecule (De Camilli et al., 1983b; Huttner et al., 1983; Navone et al., 1984). In support of this proposal, synapsin I has been shown to bind with high affinity to isolated synaptic vesicles (Huttner et al., 1983; Schiebler et al., 1986; Steiner et al., 1987; Benfenati et al., 1989) and to interact *in vitro* with various cytoskeletal structures, including spectrin (Baines and Bennett, 1985; Kanda et al., 1986; Krebs et al., 1987), microtubules (Baines and Bennett, 1986; Goldenring et al., 1986), neurofilaments (Goldenring et al., 1986; Steiner et al., 1987), and F-actin (Bahler and Greengard, 1987; Petrucci and Morrow, 1987; Petrucci et al., 1988; Bahler et al., 1989).

Only in the synaptic vesicle (Huttner et al., 1983; Schiebler et al., 1986; Benfenati et al., 1989) and actin (Bahler and Greengard, 1987; Petrucci and Morrow, 1987; Bahler et al., 1989) studies was the effect of phosphorylation of synapsin I by cAMP-dependent protein kinase examined. Phosphorylation by cAMP-dependent protein kinase had only a slight effect on the interaction of synapsin I with purified vesicles (Schiebler et al., 1986) and with actin (Bahler and Greengard, 1987). It is not known whether these relatively minor effects are of physiological significance. Phosphorylation of synapsin I by CaM kinase II has been shown to decrease its interaction with purified synaptic vesicles (Huttner et al., 1983; Schiebler et al., 1986; Benfenati et al., 1989) and neurofilaments (Steiner et al., 1987) and to nearly abolish its ability to bundle F-actin (Bahler and Greengard, 1987; Petrucci and Morrow, 1987). Thus, phosphorylation-dependent interactions of synapsin I with any of these components could be responsible for the present findings on organelle movement.

Interaction with neurofilaments is unlikely to explain the effects of synapsin I in both the axoplasm and the presynaptic terminal, since neurofilaments are thought not to extend into the synaptic vesicle-containing region of the terminal (Roots, 1983; Walker et al., 1985). Interaction with microtubules is also unlikely to be the sole explanation for the effects of synapsin I, since no microtubule bundling was observed in the present experiments and phosphorylation of synapsin I, if anything, may enhance the interaction of synapsin I with microtubule proteins (Petrucci and Morrow, 1987). In addition, microtubules are thought not to extend into the synaptic vesicle region of the presynaptic nerve terminal (Roots, 1983; Walker et al., 1985), although evidence for some microtubules extending into the presynaptic terminal does exist (Hirokawa et al., 1989). Regardless, the absence of inhibition by dephospho-synapsin I when organelles are moving along individual microtubules in the absence of other structures (Fig. 4) makes it unlikely that interaction with microtubules is involved.

Interaction with F-actin, on the other hand, could explain both effects. The ability of synapsin I to bundle F-actin in *in vitro* systems (Bahler and Greengard, 1987; Petrucci and Morrow, 1987; Petrucci et al., 1988) is consistent with synapsin I

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Figure 3. Comparison of the effect of dephospho-synapsin I on organelle movement within the interior and near the periphery of the axoplasm. Stills from video records showing organelle movement within the interior (*A, B*) and near the periphery (*C, D*) of extruded axoplasm 20–22 min after addition of 1.3 μM dephospho-synapsin I. *Arrows* in *A* and *B* indicate 2 of the numerous organelles in the field that did not move during the 5 sec interval between frames *A* and *B*. *Open arrows* in *C* and *D* indicate a single microtubule. *Solid arrows* in *C* and *D* indicate the positions of a single organelle that moved along another microtubule during the 3 sec interval between frames *C* and *D*. In such peripheral regions, movement was generally active to the extent that few organelles remained in the field for more than 2 or 3 sec. *Arrowhead* in *C* indicates an organelle that is not seen in *D* because it had moved out of the field. Stills were made by accumulating 4 frames (0.13 sec) from the original videotape using the C-1966 Image Processor and photographing the accumulated image from the monitor onto Kodak TMAX 100 film. Scale bar, 2 μm.

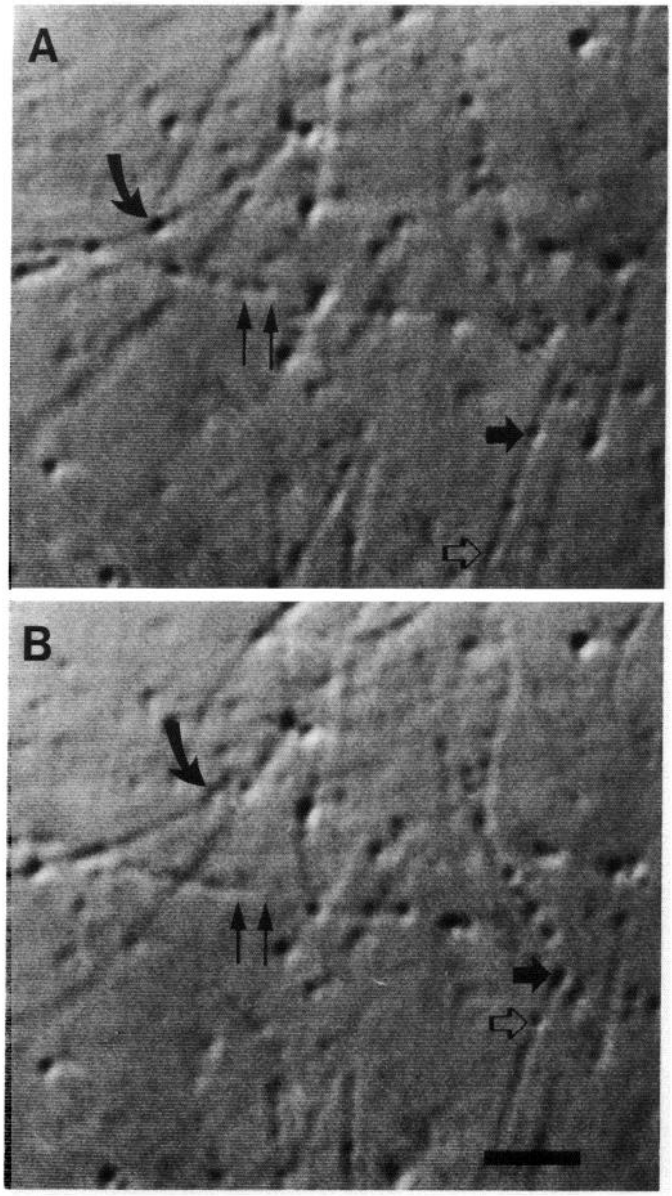

Figure 4. Effect of dephospho-synapsin I on organelle movement along isolated microtubules protruding from the axoplasm into the buffer. Stills from video records show that organelles continue to move along these isolated microtubules 1 hr after addition of 10 μM dephospho-synapsin I. *Arrows* in *A* point to organelles that moved during the 5.3 sec interval between frames *A* and *B*. *Solid, curved arrows* in *A* and *B* point to an organelle that moved upward to the left. *Thin double arrows* in *A* point to two smaller organelles that are not seen in *B* because they had moved out of the field. *Thin double arrows* in *B* point to the positions originally occupied by these organelles. *Short, solid* and *short, open arrows* in the lower right corners of *A* and *B* point to two organelles that moved upward along the indicated microtubule. Stills were made as described in the legend to Figure 1. The axoplasm used for this figure is the same axoplasm used for Figure 1. Scale bar, 2 μm.

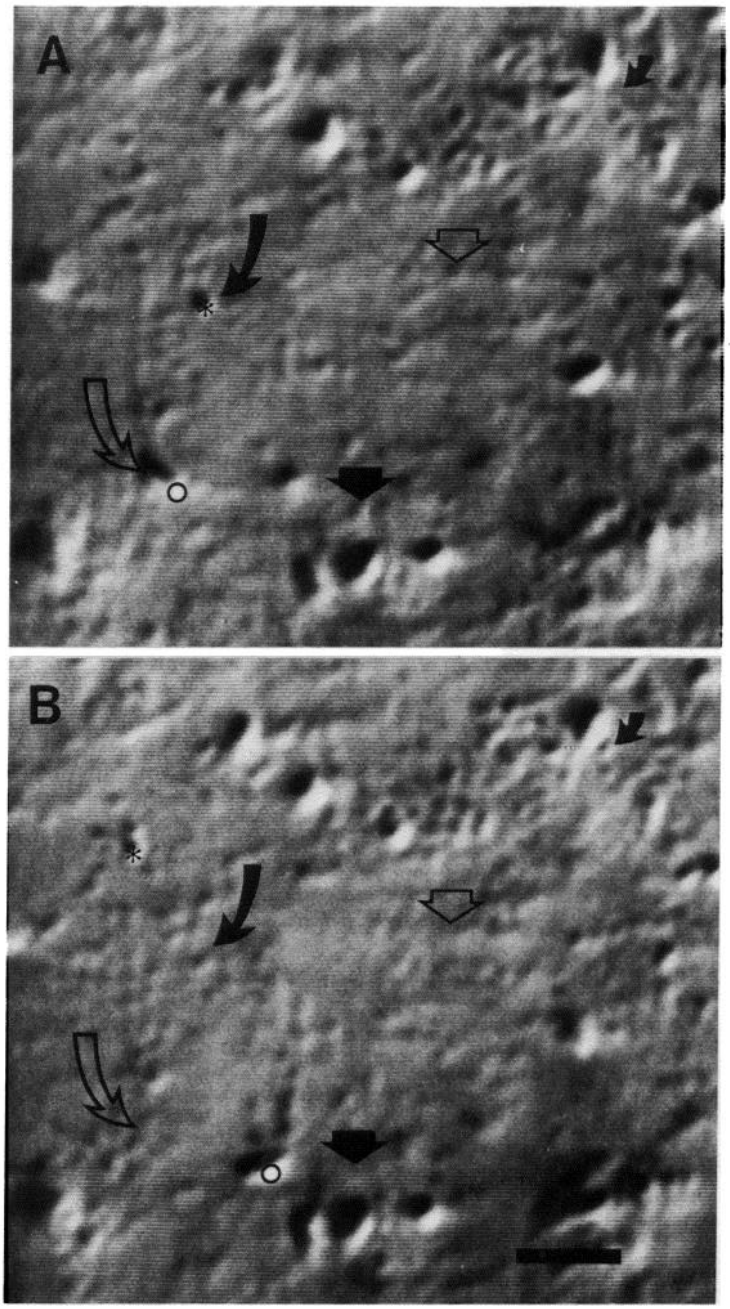

Figure 5. Effect of phosphorylation of synapsin I by CaM kinase II on organelle movement within the interior of the axoplasm. Stills from video records show organelle movement within the interior of isolated axoplasm 1 hr after addition of 10 μM tail phospho-synapsin I. *Straight arrows* and *large, curved arrows* in *A* point to a few of the many organelles that moved during the 5.5 sec interval between frames *A* and *B*. The corresponding symbols in *B* point to the positions originally occupied by the respective organelles. *Solid, curved arrow* in *A* pints to an organelle (*) that moved upward to the left, as seen in *B*. *Open, curved arrow* in *A* points to an organelle (o) that moved to the right in *B*. *Solid, straight,* and *open straight arrows* in *A* point to organelles that had moved out of the field in *B*. The *short, curved arrow* in the upper right corner of *B* points to an organelle that was not present in *A*. Stills were made as described in the legend to Figure 1. Scale bar, 2 μm.

affecting organelle mobility via a crosslinking mechanism involving actin microfilaments. The question as to whether actin-containing filaments are distributed predominantly under the axolemma (Chang and Goldman, 1973; Isenberg and Small, 1978; Metuzals and Tasaki, 1978; Hirokawa, 1982; Schnapp and Reese, 1982; Letourneau, 1983) or are also present in significant amounts in the central microtubule-containing regions of the axon or neurite (Yamada et al., 1971; LeBeux and Willemot, 1975; Jockusch et al., 1979; Kuczmarski and Rosenbaum, 1979; Spooner and Holladay, 1981; Papasozomenos and Payne, 1986; Fath and Lasek, 1988) has been the subject of

some dispute. However, actin is clearly present in high concentration in the extruded squid axoplasm (Morris and Lasek, 1984; Fath and Lasek, 1988) and is also found in the presynaptic nerve terminal (LeBeux and Willemot, 1975; Toh et al., 1976; Walker et al., 1985). Moreover, although actin is not necessary for fast axonal transport (Allen et al., 1985; Brady et al., 1985; Schnapp et al., 1985), several actin-binding and microfilament depolymerizing agents have been shown to disrupt fast axonal transport (Goldberg et al., 1980; Goldberg, 1982; Brady et al., 1984). Significantly, these microfilament depolymerizing agents are not effective at inhibiting organelle movement along individual microtubules (Brady, 1987), and the individual microtubules on the periphery do not contain detectable microfilaments (Schnapp et al., 1985; Brady, 1987). Further studies will be required to determine if synapsin I inhibits organelle movement within the squid axoplasm by crosslinking synaptic vesicles with F-actin, by crosslinking F-actin with F-actin, or by some mechanism involving other cytoplasmic component(s) and to determine if such interactions have physiological relevance to synaptic transmission.

Associations between synapsin I and various subcellular components that were produced under the *in vitro* conditions used in the present study may not occur in vivo. However, it should be noted that the axoplasm studies more nearly resemble physiologically relevant conditions than do studies that examine interactions between synapsin I and purified subcellular components. A variety of other proteins, including myosin fragments, calcium-free gelsolin, antitubulin antibodies, antimyosin antibodies, and calmodulin (Brady et al., 1985; Johnston et al., 1987; S. T. Brady, unpublished observations; and this report), had little or no effect on transport, indicating that the perturbation of axonal transport by synapsin I was highly specific. In support of the specificity of the action of synapsin I on transport, addition of only 2 phosphate groups to the tail region of the synapsin I molecule virtually abolish its inhibitory effect. Furthermore, it is unlikely that the inhibitory effect of dephosphosynapsin I is an artifact due to the overall surfactant (M. Ho et al., unpublished observations) or basic properties of the molecule, since phosphorylation of synapsin I has no detectable effect on its surface activity (M. Ho et al., unpublished observations) and has only a minimal effect on its isoelectric point. In addition, perfusion of axoplasm with the basic protein avidin had no detectable effect on movement of organelles (unpublished observations).

Synapsin I constitutes ~0.4% of mammalian cerebral cortex protein (corresponding to approximately 1.0% of total neuronal protein) (Goelz et al., 1981) and most of the synapsin I appears to be localized to presynaptic terminals (De Camilli et al., 1983a, b; Huttner et al., 1983). Indeed, synapsin I represents 6% of the total protein present in synaptic vesicle fractions isolated from nerve terminal endings (Huttner et al., 1983). Squid axoplasm contains approximately 25 mg/ml protein (Morris and Lasek, 1984); thus, the effective concentrations of synapsin I used in the present experiments, namely 1.3–10 μM, correspond to about 0.4–3.0% of total protein in the extruded axoplasm. These concentrations are probably higher than that which exists in axons, since synapsin I constitutes only 0.02% of total protein in the corpus callosum (Goelz et al., 1981). In addition, it appears that only a portion of the synapsin I present in the axon is associated with vesicles during their transport down the axon, with the remainder undergoing slow axonal transport independent of membranous organelles (Baitinger and Willard, 1987). Thus, the concentrations of synapsin I used in the present experiments are most likely reached only in synaptic terminals where synapsin I is specifically localized. Therefore, we do not propose that synapsin I regulates release by blocking fast axonal transport. In fact (Llinas et al., unpublished observations), disruption of microtubules or axonal transport could not account for the inhibition of neurotransmitter release by synapsin I. Instead, we suggest that the ability of synapsin I to block axonal transport might reflect its ability to modulate organelle movement in the nerve terminal. Such modulation might involve regulating release of the vesicles from the microtubules, targeting the released vesicles to a cytoskeletal (actin?) matrix, or regulating detachment of the vesicles from such a cytoskeletal matrix.

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