Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8108–8112, October 1989
Neurobiology

Translocation of synapsin I in response to depolarization of isolated nerve terminals

(phosphoproteins/synaptosomes/synaptic vesicles/neurotransmitter release)

TALVINDER S. SIHRA*, JAMES K. T. WANG†, FRED S. GORELICK‡, AND PAUL GREENGARD

Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Avenue, New York, NY 10021

Contributed by Paul Greengard, July 10, 1989

ABSTRACT **Depolarization of isolated nerve terminals (synaptosomes) has been shown to stimulate neurotransmitter release and to increase the phosphorylation state of a number of proteins, including synapsin I, in a Ca^{2+}-dependent manner. Synapsin I, a prominent nerve terminal phosphoprotein, interacts with the cytoplasmic surface of small synaptic vesicles and with cytoskeletal elements in a phosphorylation-dependent manner. In the present study we have found that depolarization of synaptosomes resulted in a rapid (2–5 sec) translocation of synapsin I from the particulate to the cytosolic (soluble) fraction. This translocation of synapsin I correlated with its phosphorylation state and was dependent on the presence of Ca^{2+} in the incubation medium. The stoichiometry of phosphorylation of soluble synapsin I was considerably higher than that of synapsin I in the particulate fraction, under both basal and depolarizing conditions. These data are consistent with the hypothesis that, *in situ*, the phosphorylation of synapsin I promotes its translocation from synaptic vesicles/cytoskeleton to the cytosol. This phosphorylation/translocation may be instrumental in regulating the release of neurotransmitter.**

Synapsin I, a major brain phosphoprotein (1), has been found in virtually all neurons and is specifically localized to the cytoplasmic surface of small synaptic vesicles (2–4). Binding studies show that the phosphorylation of purified synapsin I, at specific sites, decreases its affinity for isolated synaptic vesicles (4, 5). Additionally, *in vitro*, synapsin I interacts in a phosphorylation-dependent manner with actin (6) and associates with other cytoskeletal elements (7–10). Intraterminal injection of dephospho-synapsin I, but not phospho-synapsin I, inhibits the release of neurotransmitter at the squid giant synapse (11). It has been postulated that synapsin I may crosslink synaptic vesicles to the cytoskeleton in a phosphorylation-dependent manner and hence modulate neurotransmitter release by controlling the availability of synaptic vesicles for exocytosis (12). To date, the evidence supporting this hypothesis has been largely limited to *in vitro* studies (5, 6, 10). It is important, from a physiological viewpoint, to clarify the relationship between the phosphorylation of synapsin I and its subcellular distribution in an intact secretory system. In the present study, we have addressed this issue by using isolated nerve terminals (synaptosomes), which constitute a functional model for the study of neurotransmitter release (13).

MATERIALS AND METHODS

Materials. Leupeptin, antipain, chymostatin, and pepstatin were obtained from Chemicon. *Staphylococcus aureus* V8 protease was obtained from Miles. [^{32}P]Orthophosphate and ^{125}I-labeled protein A were obtained from DuPont/NEN. All other chemicals were of analytical grade and were obtained from standard commercial sources.

Preparation and Incubation of Synaptosomes. Synaptosomes were purified from rat cerebrocortices as described (14) with minor modifications (15). The final synaptosomal pellet was resuspended in standard incubation buffer (140 mM NaCl/5 mM KCl/5 mM $NaHCO_3$/1.2 mM NaH_2PO_4/1 mM $MgCl_2$/10 mM glucose/10 mM Hepes, pH 7.4) at a protein concentration of 3 mg/ml and preincubated for 5 min at 37°C. $CaCl_2$ (1 mM) was added and the synaptosomes were preincubated for a further 10 min prior to stimulation by depolarization or forskolin. Depolarization was achieved by the addition of a half-volume of high-[K^+] incubation buffer (35 mM NaCl/110 mM KCl/5 mM $NaHCO_3$/1.2 mM NaH_2PO_4/1 mM $MgCl_2$/10 mM glucose/10 mM Hepes, pH 7.4/1 mM $CaCl_2$) to obtain a final concentration of 40 mM K^+. Forskolin (final concentration, 50 μM) was added in a half-volume of standard incubation buffer containing 1 mM $CaCl_2$. Control synaptosomes received a half-volume of standard incubation buffer containing 1 mM $CaCl_2$. The final synaptosomal protein concentration was 2 mg/ml.

Synapsin I Fractionation. Synaptosomes were depolarized for the indicated periods of time and the incubation reactions were terminated by the disruption of synaptosomes, either by hypotonic lysis or by permeabilization with saponin. Hypotonic lysis was effected by the addition of 10 volumes of ice-cold lysis buffer [20 mM Hepes, pH 7.4/10 mM $Na_4P_2O_7$/1 mM EDTA/1 mM EGTA containing leupeptin (10 μg/ml), antipain (10 μg/ml), chymostatin (1 μg/ml), pepstatin (1 μg/ml), and aprotinin (0.1%)]. Disruption with saponin was achieved by the addition of one-third volume of 0.3% saponin solution (wt/vol in standard incubation buffer containing in addition 10 mM $Na_4P_2O_7$, 3 mM EDTA, 3 mM EGTA, and protease inhibitors as in lysis buffer) to give a final concentration of 50 μg of saponin per milligram of synaptosomal protein. After 10 min in the presence of saponin, cholesterol (final concentration, 80 μg/ml) was added to quench the effect of saponin.

Following hypotonic lysis or saponin disruption, samples were fractionated by centrifugation at 200,000 × *g* for 20 min (Beckman TL-100 ultracentrifuge with a TLA 100.2 fixed-angle rotor) in centrifuge tubes that had been treated with bovine serum albumin to minimize binding of synapsin I to the tube walls. Supernatants were aspirated, frozen in liquid nitrogen, and lyophilized. The lyophilized supernatants were resolubilized in 0.1% Triton X-100 (vol/vol in H_2O). Pellets were resuspended in 0.1% Triton X-100 (vol/vol in incubation buffer).

Synapsin I Quantitation. Samples either were treated with $NaDodSO_4$ sample buffer (16), subjected to $NaDodSO_4$/

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*To whom reprint requests should be addressed.
†Present address: Program in Neurosciences, Tufts University School of Medicine, Boston, MA 02111.
‡Present address: Department of Medicine, Yale University, West Haven, CT 06516.

PAGE, and electrotransferred onto nitrocellulose (17) or were spotted directly onto nitrocellulose for quantitation by dot immunobinding (18). The anti-synapsin I antibodies were raised against a 24-amino acid synthetic peptide derived from synapsin I (19) and reacted equally with dephospho- and phospho-synapsin I. Under the assay conditions employed, the ^{125}I-protein A labeling of bound antibodies was proportional to both synaptosomal protein and pure synapsin I standards (data not shown). Rat synapsin I was purified and iodinated as described (20, 21).

Synapsin I Phosphorylation. Synaptosomes (1 mg/ml) were prelabeled with [^{32}P]orthophosphate (1 mCi/ml; 1 mCi = 37 MBq) for 45 min in phosphate-free incubation buffer (22). Extrasynaptosomal [^{32}P]orthophosphate was removed by centrifugation of the synaptosomes at 1000 × *g* for 5 min. Subsequent incubation and fractionation were carried out as described above. Synapsin I in supernatants and pellets was immunoprecipitated (23) and subjected to NaDodSO$_4$/PAGE. ^{32}P-labeled synapsin I was located by autoradiography and gel bands were excised and quantitated by liquid scintillation spectrometry. The gel bands were then subjected to limited proteolysis using *S. aureus* V8 protease (24, 25).

Stoichiometry of Synapsin I Phosphorylation in Soluble and Particulate Fractions. The amount of synapsin I was determined in unlabeled synaptosomes and the extent of phosphorylation was determined in [^{32}P]orthophosphate-prelabeled synaptosomes incubated in parallel. Small aliquots of ^{32}P-labeled synaptosomes were removed from each sample and treated with 0.4 M perchloric acid immediately before the addition of lysis buffer to the remaining synaptosomes. The specific activity of [γ-^{32}P]ATP was determined in the supernatants of perchloric acid-treated aliquots of each sample. Nucleotides were separated by reverse-phase HPLC (absorbance detection at 254 nm) (26) and ATP was quantitated using pure ATP standards. [γ-^{32}P]ATP was determined in the HPLC ATP eluates by liquid scintillation spectrometry. ([β-^{32}P]ATP was estimated to represent <10% of the total ^{32}P-labeled ATP.) ^{32}P-labeled synapsin I in the soluble and particulate fractions was immunoprecipitated and quantitated as described above. The moles of $^{32}PO_4$ incorporated were calculated from the specific activity of the [γ-^{32}P]ATP.

All quantitative data represent the mean ± SEM of three replicate determinations. All results are representative of those found in each of three or four separate experiments.

RESULTS AND DISCUSSION

In one series of experiments, the distribution of synapsin I in cytosolic (soluble) and particulate fractions of synaptosomes was determined by using hypotonic lysis to disrupt the synaptosomes following incubation under control or depolarizing conditions. The separated fractions were subjected to NaDodSO$_4$/PAGE, electrotransferred onto nitrocellulose, and probed with affinity-purified anti-synapsin I antibodies. Depolarization of synaptosomes caused a rapid (within 2–5 sec) increase of synapsin I in the soluble fraction (Fig. 1*a*). Quantitation of the synapsin I translocated to the soluble fraction was carried out by a dot immunobinding assay (Fig.

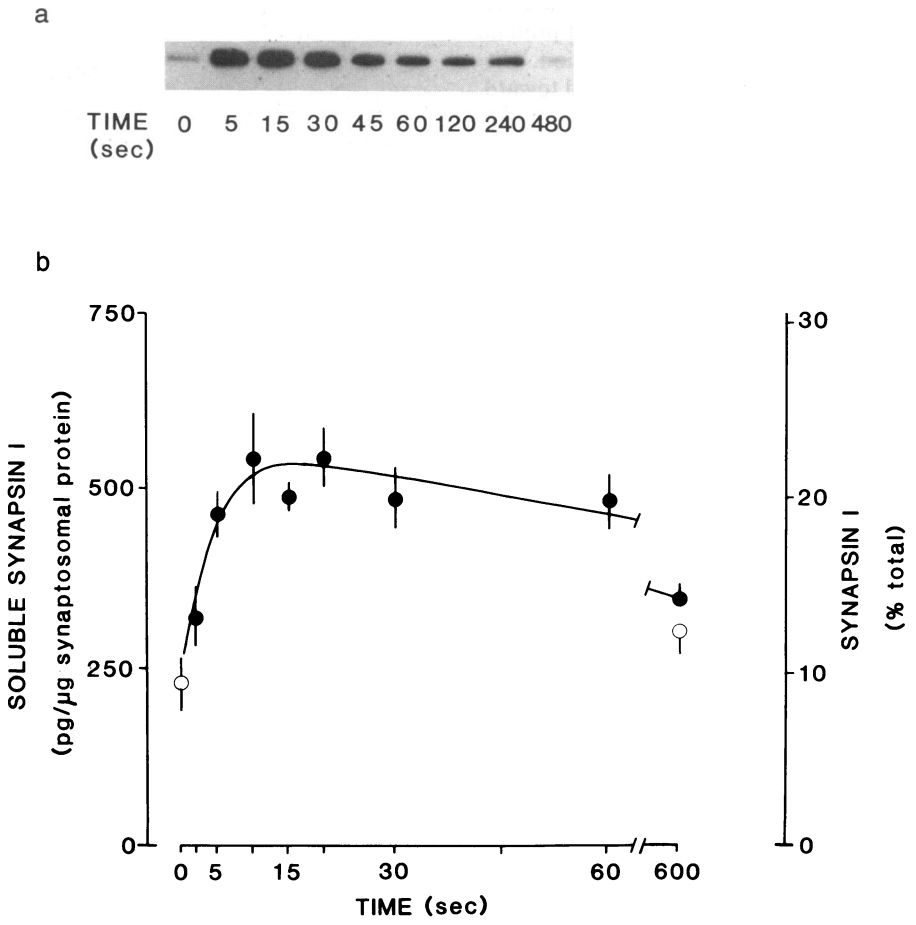

FIG. 1. Time course of translocation of synapsin I into the cytosolic (soluble) fraction following depolarization: analysis after hypotonic lysis. Synaptosomes were depolarized for the indicated times, hypotonically lysed, and fractionated as described in *Materials and Methods*. (*a*) Autoradiogram of an immunoblot of soluble synapsin I following NaDodSO$_4$/PAGE, illustrating the results of a typical experiment. (*b*) Dot immunobinding quantitation of soluble synapsin I from control (○) and depolarized (●) synaptosomes. Inverse changes were observed in the particulate fractions (data not shown).

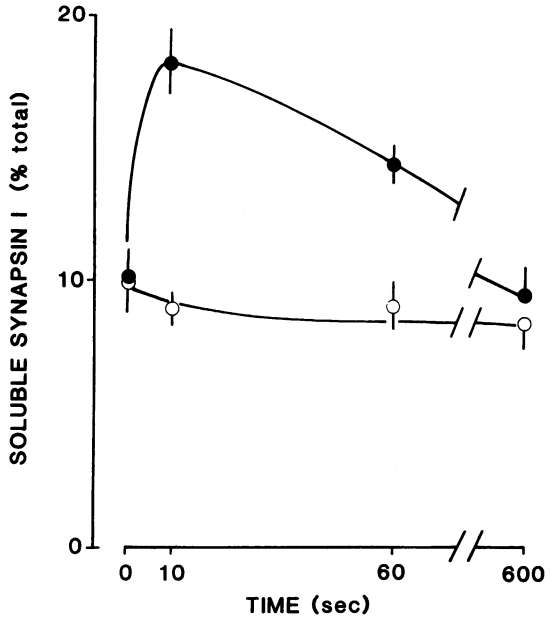

FIG. 2. Ca^{2+} dependence of depolarization-induced translocation of synapsin I. Synaptosomes were incubated in standard incubation buffer in the presence of 1 mM Ca^{2+}, in the absence (●) or presence (○) of 2 mM Na EGTA added 1 min prior to depolarization.

1*b*). The amount of soluble synapsin I increased from a basal level of 220 pg/μg of synaptosomal protein (9.7% of the total synapsin I) to a level of 500 pg/μg of synaptosomal protein (22% of total synapsin I) 10–15 sec after depolarization. The amount of synapsin I in the soluble fraction returned toward

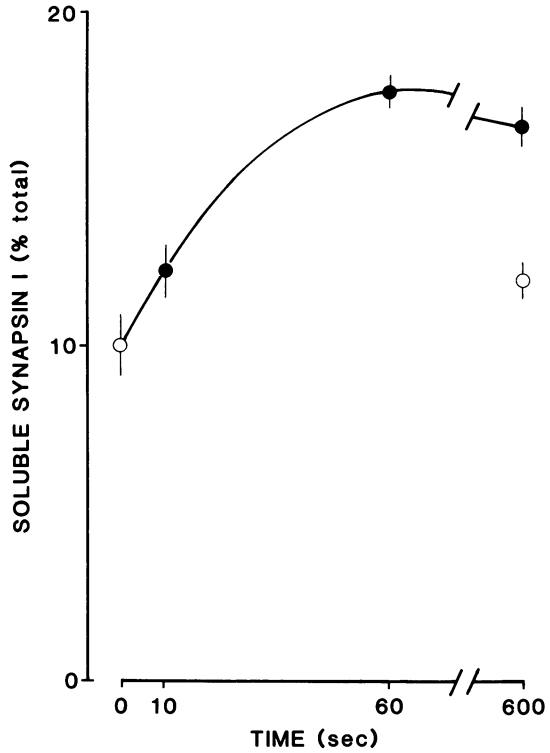

FIG. 3. Time course of translocation of synapsin I into the cytosolic (soluble) fraction following depolarization: analysis after saponin lysis. Synaptosomes were treated as described in the legend to Fig. 1, except that saponin lysis was used in place of hypotonic lysis to disrupt synaptosomes. Synapsin I in the soluble fractions was quantitated by dot immunobinding in control (○) and depolarized (●) synaptosomes.

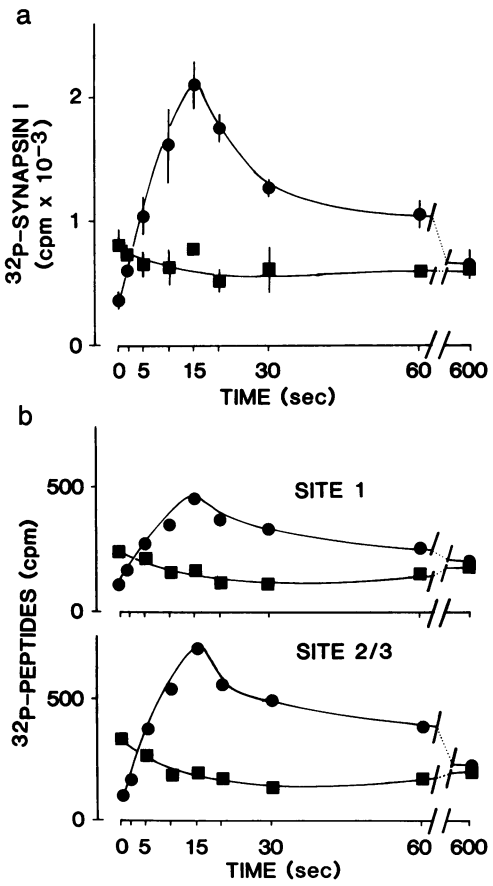

FIG. 4. Depolarization of synaptosomes leads to an increase in phospho-synapsin I in the soluble fraction. Synaptosomes were prelabeled with [^{32}P]orthophosphate as described in *Materials and Methods*. Synapsin I was immunoprecipitated from the soluble and particulate fractions and subjected to $NaDodSO_4$/PAGE. (*a*) ^{32}P-labeled synapsin I was visualized by autoradiography and quantitated by liquid scintillation spectrometry of the excised gel bands. Synapsin I phosphorylation in soluble (●) and particulate (■) fractions is shown as a function of depolarization time. (*b*) Gel bands were subjected to limited proteolysis by *S. aureus* V8 protease and the two synapsin I phosphopeptides were separated by $NaDodSO_4$/PAGE. ^{32}P-labeling of synapsin I peptides containing site I (*Upper*) and site 2/3 (*Lower*) is shown as a function of depolarization time for soluble synapsin I (●) and particulate synapsin I (■).

basal levels over the subsequent 10 min. In contrast, the levels of synaptophysin (p38), an integral membrane protein of small synaptic vesicles (27), remained unchanged in the particulate fractions from both control and depolarized synaptosomes (data not shown).

The redistribution of synapsin I was dependent on the presence of extrasynaptosomal Ca^{2+}, as no translocation was observed when 2 mM Na EGTA was present during depolarization (Fig. 2). Support for the interpretation that the translocation of endogenous synapsin I occurred in the intact synaptosomes (i.e., before lysis) came from experiments in which exogenous, ^{125}I-labeled dephospho-synapsin I was added to the lysates of control and depolarized synaptosomes. No difference in the distribution of ^{125}I-labeled synapsin I between soluble and particulate fractions was observed in the two conditions. These results suggest that the observed translocation does not reflect a post-lysis change in the binding of synapsin I related to an unidentified intraterminal change produced by depolarization.

In a second series of experiments, the distribution of synapsin I in soluble and particulate fractions was determined by using saponin lysis to disrupt synaptosomes following

Table 1. Stoichiometry of synapsin I phosphorylation in synaptosomes

Fraction	Parameter	Control	Depolarized*	Translocated†
Soluble	fmol of PO_4 per sample	318 ± 20	1080 ± 70	762
	fmol of synapsin I per sample‡	319 ± 42	544 ± 14	225
	mol of PO_4 per mol of synapsin I	1.00 ± 0.22	2.00 ± 0.10	3.39
Particulate	fmol of PO_4 per sample	490 ± 15	520 ± 44	
	fmol of synapsin I per sample	3250 ± 140	3130 ± 210	
	mol of PO_4 per mol of synapsin I	0.15 ± 0.03	0.17 ± 0.02	

*Synaptosomes were depolarized for 15 sec.
†Calculations were based on the assumption that the amount and specific activity of synapsin I present in the soluble fraction under control conditions were not altered upon depolarization.
‡Molecular weight of synapsin I was taken as 72,000.

incubation in control and depolarizing conditions. Low concentrations of saponin, a steroid glycoside, are known to produce focal discontinuities in plasma membranes without any apparent effect on intracellular membrane structures (28). With this permeabilization protocol, the depolarization-dependent translocation of synapsin I from the particulate to the soluble fraction was still observed (Fig. 3). Thus the depolarization-dependent redistribution of synapsin I appeared to be independent of the post-stimulation lysis procedure used.

The relationship between the phosphorylation and translocation of synapsin I was examined (Fig. 4*a*). Depolarization of synaptosomes caused a significant increase in ^{32}P-labeled synapsin I in the soluble fraction, which reached a maximum at 15 sec before returning to basal levels over the subsequent 10 min. There was little change in ^{32}P-labeled synapsin I in the particulate fraction. As was demonstrated previously (29), the chelation of Ca^{2+} resulted in the abolition of the depolarization-dependent phosphorylation of synapsin I (data not shown).

Synapsin I can be phosphorylated *in vitro* (24, 30, 31) and *in situ* (22, 25, 29) at three sites, site 1 ("head-region") being specific for either cyclic AMP-dependent protein kinase or Ca^{2+}/calmodulin-dependent protein kinase I, and site 2/3 ("tail-region") being specific for Ca^{2+}/calmodulin-dependent protein kinase II (24, 31). To analyze the phosphorylation of site 1 and site 2/3 separately, the ^{32}P-labeled synapsin I was subjected to limited proteolysis (24, 31). The time course of phosphorylation of site 1 and site 2/3 changed in parallel with the time course of total synapsin I phosphorylation (Fig. 4*b*). Moreover, the initial time course of synapsin I translocation from the particulate to the soluble fraction correlated well with the increase in phosphorylation of synapsin I (compare Figs. 1 and 4).

The stoichiometry of synapsin I phosphorylation was considerably higher in the soluble fraction (1.00 mol of PO_4 per mol of synapsin I) than in the particulate fraction (0.15 mol of PO_4 per mol of synapsin I) under basal conditions (Table 1). After nerve-terminal depolarization, there was an ≈3.4-fold increase in phosphorylation and an ≈1.7-fold increase in the amount of synapsin I in the soluble fraction, resulting in a 2-fold increase in the stoichiometry of phosphorylation of the soluble synapsin I. The specific activity of the newly translocated synapsin I can be calculated from the differences between the values obtained in control and depolarized synaptosomes and yields a stoichiometry of phosphorylation of about 3.4 mol of PO_4 per mol of synapsin I. In contrast, the stoichiometry of particulate synapsin I phosphorylation remained virtually unchanged upon depolarization. These results are consistent with a model in which the phosphorylation of synapsin I, at multiple sites, is responsible for its translocation from the particulate to the soluble fraction.

The data are consistent with the hypothesis that *in situ* phosphorylation of synapsin I underlies its translocation to the cytosolic fraction, but do not identify the individual site(s)—i.e., site 1 and/or site 2/3—that might be responsible. In an effort to address this issue, the effect of phosphorylation of site 1 alone on the translocation of synapsin I was examined. Forskolin, by increasing cyclic AMP levels and thereby activating cyclic AMP-dependent protein kinase,

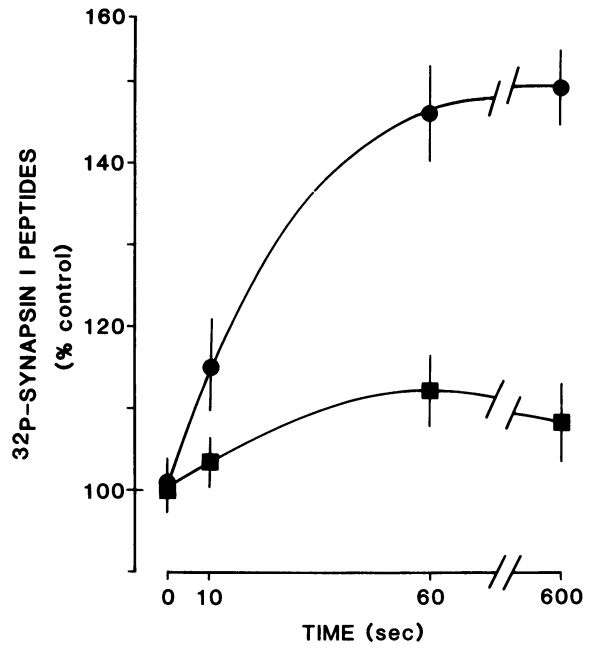

FIG. 5. Forskolin-induced phosphorylation of synapsin I: comparison of phosphorylation at site 1 (●) and site 2/3 (■).

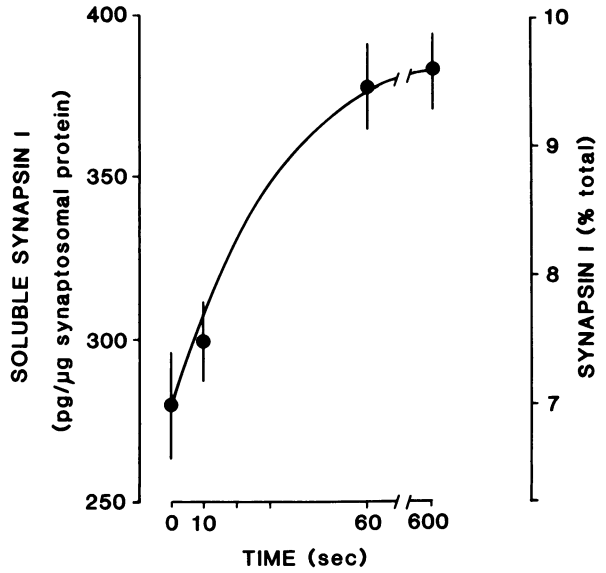

FIG. 6. Forskolin-mediated translocation of synapsin I.

stimulated the phosphorylation of synapsin I on site 1 but had little effect on site 2/3 phosphorylation (Fig. 5). Under these conditions, a translocation of synapsin I was observed (Fig. 6). However, the magnitude of the translocation response to forskolin was considerably less than that observed when K^+-induced depolarization was used to stimulate the phosphorylation at site 1 and site 2/3 (Figs. 1*b* and 6). These observations are of interest in relation to data indicating that the phosphorylation of site 1 alone brings about a small increase in the dissociation of synapsin I from synaptic vesicles *in vitro*. Unfortunately, we know of no procedure that would selectively stimulate phosphorylation on site 2/3.

Synapsin I has been shown to interact with synaptic vesicles and with actin. Synapsin I may, in its dephospho form but not in its phospho form, crosslink vesicles to actin (5, 6, 11, 12, 21, 32, 33). The phosphorylation-associated translocation of synapsin I to the cytosol in response to synaptosomal depolarization observed in the present study is consistent with a role for synapsin I in modulating the release of neurotransmitter (12) by controlling the availability of synaptic vesicles.

We thank Dr. A. Czernik for the supply of anti-synapsin I antibodies and Drs. R. Nichols, A. Nairn, S. Gandy, P. De Camilli, and R. Cameron for valuable discussions. This work was supported by U.S. Public Health Service Grants MH39327 and AA06944.

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