Phosphorylation-dependent Subcellular Translocation of a Ca^{2+}/Calmodulin-dependent Protein Kinase Produces an Autonomous Enyzme in *Aplysia* Neurons

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ABSTRACT We have shown previously that the subcellular distribution of a major calmodulin-binding protein is altered under conditions causing increased synthesis of cAMP in *Aplysia* neurons (Saitoh, T., and J. H. Schwartz, 1983, *Proc. Natl. Acad. Sci. USA*, 80:6708–6712). We now provide evidence that this M_r 55,000 protein is a subunit of a Ca^{2+}/calmodulin-dependent kinase: (*a*) both the M_r 55,000 calmodulin-binding protein and kinase activity are loosely attached to the membrane-cytoskeletal complex; (*b*) both kinase activity and the M_r 55,000 protein are translocated from the membrane-cytoskeleton complex to the cytoplasm under conditions that cause the change in the subcellular distribution of the M_r 55,000 calmodulin-binding protein; and (c) calmodulin-binding activity of the M_r 55,000 protein and the ability to carry out the Ca^{2+}/calmodulin-dependent phosphorylation of synapsin I are purified in parallel. The subcellular localization of the Ca^{2+}/calmodulin-dependent protein kinase appears to be under control of two second messengers: Ca^{2+} and cAMP. We find that the M_r 55,000 subunit is phosphorylated when the extracted membrane-cytoskeleton complex is incubated with Ca^{2+}, calmodulin, and ATP, with the concomitant release of this phosphorylated peptide from the complex. Previously, we had found that, when translocation occurs in extracts in the presence of cAMP and ATP (but in the absence of Ca^{2+}), there was no detectable phosphorylation of the M_r 55,000 subunit itself. The subcellular distribution of the subunit thus appears to be influenced by (*a*) cAMP-dependent phosphorylation, which, we infer, modifies some as yet unidentified structural component, causing the release of the enzyme; and (*b*) Ca^{2+}/calmodulin-dependent phosphorylation of the M_r 55,000 subunit. These studies also suggest that phosphorylation has an important regulatory consequence: during the Ca^{2+}/calmodulin-dependent translocation of the M_r 55,000 subunit, the kinase appears to be activated, becoming independent of added Ca^{2+}/calmodulin.

Influx of Ca^{2+} into the nerve terminal causes the release of neurotransmitter into the synaptic cleft. Release is thought to be brought about by the fusion of synaptic vesicles with the presynaptic membrane (see reference 27). Although the mechanisms responsible for neurotransmitter release are not yet completely understood, recent studies emphasize the possible involvement of protein phosphorylation (for examples, see references 17, 19, 28, and 31).

A protein kinase that is activated by Ca^{2+}/calmodulin has been identified in vertebrate nervous tissue and found to be the major constituent of the postsynaptic density (14, 15, 18, 20). Present throughout the nerve cell, however, it is recovered in the soluble cytoplasmic fraction, as well as in particulate fractions, and has been postulated to be in a dynamic subcellular equilibrium (3, 22). This kinase is composed of a major M_r 50,000 subunit and a minor M_r 60,000 subunit (3, 13, 36), both of which can bind calmodulin and be autophosphorylated. A similar enzyme has also been described in *Aplysia* nervous tissue (8, 29).

By using blot staining with ^{125}I-calmodulin, we previously have shown that there are at least 14 calmodulin-binding proteins in the *Aplysia* nervous system (32). A M_r 55,000 calmodulin-binding protein was found to be enriched in neurons and was shown to be loosely associated with the

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membrane-cytoskeleton complex. This calmodulin-binding protein was also found to have the interesting property of dissociating from the membrane cytoskeleton after exposure of ganglia to serotonin, a condition that increases synthesis of cAMP in *Aplysia* nervous tissue. DeRiemer et al. (8) also found that the major calmodulin-binding protein in *Aplysia* nervous tissue has a molecular weight of 50,000–55,000, and they suggested that it most probably is a subunit of a Ca^{2+}/calmodulin-dependent protein kinase. In this paper, we provide further evidence that the M_r 55,000 *Aplysia* calmodulin-binding protein is a subunit of the Ca^{2+}/calmodulin-dependent kinase and that this enzyme can dissociate from the membrane-cytoskeleton complex when phosphorylation takes place in a Ca^{2+}/calmodulin-dependent manner. Changes in both Ca^{2+}-dependent protein phosphorylation as well as in other molecular processes triggered by Ca^{2+} have been implicated in various forms of synaptic plasticity (1, 2, 7, 11, 16, 19, 24, 25, 28); it is therefore attractive to think that changes in the localization of this Ca^{2+}-dependent kinase might underlie modulation of synaptic function.

MATERIALS AND METHODS

Aplysia californica, weighing 150–250 g, were obtained from Pacific Bio-Marine Laboratories, Inc. (Venice, CA), and maintained at 15°C in artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH). Animals were anesthetized by injection of $MgCl_2$ and opened by a longitudinal incision through the foot. Isolated central ganglia were pinned to a silicone plastic (Sylgard, Dow Chemical Co., Midland, MI) and kept in high-Mg^{2+} seawater to block neurotransmitter release (5).

Tissue Fractionation

The membrane-cytoskeleton complex was separated from cytosol as previously described (30, 32). Neural components dissected from abdominal, cerebral, pedal, or pleural ganglia (12) were placed in 25 μl of stabilization buffer (2 M glycerol/0.2% saponin/1 mM $MgSO_4$/2 mM EGTA/0.02% Na azide/0.1 M K PIPES, pH 6.9), and centrifuged at 11,000 *g* for 10 min at 4°C (without homogenization). The amount of protein obtained from the neuronal components of an abdominal ganglion is ~0.2 mg (12); the total amount from the ganglia constituting the entire nervous systems is ~1 mg. The resulting pellet (membrane-cytoskeleton fraction) was resuspended in the buffer and centrifuged again. In some experiments (destabilizing conditions), glycerol was omitted and $MgSO_4$ was replaced by EDTA (destabilizing buffer).

In some experiments, samples were subjected to ammonium sulfate fractionation to remove endogenous calmodulin (35). Ammonium sulfate was added to bring samples to 55% (wt/vol) saturation at 4°C followed by centrifugation for 15 min at 11,000 *g*. Total kinase activity was precipitated at concentrations exceeding 40% saturation.

Measurement of Protein Kinase Activity

PROCEDURE I: Ca^{2+}/calmodulin-dependent kinase was assayed in extracts of ganglia containing 5 μg/μl protein or less at 25°C as described by Kennedy and Greengard (21) by phosphorylation of bovine synapsin I (the gift of Mary B. Kennedy, California Institute of Technology) with modifications in a final volume of 25 μl containing 50 mM Na borate buffer (pH 8.5), 10 mM magnesium acetate, 20 μg/ml bovine calmodulin (Calbiochem-Behring Corp., San Diego, CA), 0.1 mg/ml synapsin I, 5 or 50 μM [γ-^{32}P]ATP (1 μCi/tube, Amersham Corp., Arlington Heights, IL), either 0.4 mM EGTA (minus Ca^{2+}) or 0.4 mM EGTA/0.7 mM $CaCl_2$ (plus Ca^{2+}), 0.1 mg/ml bovine cAMP-dependent protein kinase inhibitor (Sigma Chemical Co., St. Louis, MO), 5 mM β-mercaptoethanol. After preincubation for 1 min, the reaction was initiated by addition of the ATP and terminated after 1 min by addition of 6.25 μl of fivefold SDS sample buffer (50% glycerol, 25% β-mercaptoethanol, 12% SDS, 0.31 M Tris-HCl, pH 6.8, 25 mM EDTA, and 0.1% bromphenol blue). We assessed incorporation of [^{32}P]phosphorous into the proteins separated by gel electrophoresis by cutting the phosphoproteins out from the dried gel for counting by liquid scintillation. Activity of the kinase was measured as the difference between the incorporation in the presence and in the absence of Ca^{2+}. Under these conditions, we were measuring the initial rate of the kinase reaction. Less than 10% was obtained in the absence of Ca^{2+}, and the bovine kinase inhibitor blocked 92% of cAMP-dependent kinase activity.

PROCEDURE II: In contrast to procedure I, in which the membrane-cytoskeleton complex is not preserved, this protocol permits assay of kinase activity under conditions that stabilize the membrane cytoskeleton (30) in 2 M glycerol, 0.2% saponin, 50 mM K PIPES (pH 6.8), 10 mM $MgSO_4$, 20 μg/ml calmodulin, 50 μM [γ-^{32}P]ATP (1 μCi/tube), either 0.4 mM EGTA (minus Ca^{2+}) or 0.4 mM EGTA/0.7 mM $CaCl_2$ (plus Ca^{2+}), 0.1 mg/ml of the bovine protein kinase inhibitor, and 0.02% sodium azide. After a 1-min preincubation at 25°C, the reaction was initiated by the addition of the ATP and stopped after 1 min as in procedure I. Where indicated, 1 mM unlabeled ATP was used. For maximum phosphorylation, the reaction was allowed to proceed for 30 min and the released components were separated from the membrane-cytoskeleton complex by centrifugation for 10 min at 11,000 *g* at 4°C. Inclusion of 0.1 mM trifluoperazine (TFP)[1] (Smith, Kline and French, Philadelphia, PA), together with the exclusion of Ca^{2+} from the phosphorylation reaction mixture and addition of EGTA, reduced the extent of phosphorylation of synapsin I by 91%, and of autophosphorylation of the M_r 55,000 subunit, by 96%. Glycerol at a concentration of 2 M did not alter Ca^{2+}/calmodulin-dependent phosphorylation.

Gel Electrophoresis, Blotting Procedures, and Autoradiography

We used discontinuous slab gels in SDS with a gradient from 6.5% to 12.5% acrylamide. The apparent molecular weight of the protein that we have designated 55,000 actually varies according to the gel system used. In our gradient gels, it migrates with a molecular weight of 55,000. With a lower percentage of acrylamide, this protein appears smaller (data not shown). (The vertebrate kinase subunit has an apparent molecular weight of 50,000 in an 8% gel [3], 52,000 in an 8.5% gel [13], and 55,000 in a 10% gel [36].) After the electrophoresis, we either stained gels with Coomassie Blue or blotted them onto sheets of nitrocellulose paper (Millipore Corp., Bedford, MA) for staining with bovine calmodulin iodinated as already described (32). Dried gels or blots were exposed to X-Omat AR film (Eastman Kodak Co., Rochester, NY) at −70°C for no longer than 2 d.

Column Chromatography

The proteins released from the membrane-cytoskeleton complex prepared from the nervous systems of 10 animals were precipitated with 40% saturated ammonium sulfate and resuspended in 3 ml 3 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, and 20 mM Tris-HCl (pH 7.4). This fraction was applied at a flow rate of 3 ml/h either to a DEAE-cellulose (DE 52, Whatman Chemical Separation Inc., Clifton, NJ, 0.3 × 5 cm) column or to a cellulose phosphate (Sigma Chemical Co., 0.3 × 5 cm) column (both equilibrated in the buffer). Both columns were washed with 5 ml of the buffer and then eluted at a flow rate of 4 ml/h with 24 ml of a linear gradient of 0–0.4 M NaCl. Affinity chromatography of proteins eluted from cellulose phosphate was carried out on columns (0.3 × 5 cm) of bovine calmodulin linked to Affigel (Bio-Rad Laboratories, Richmond, CA) (22).

RESULTS

Ca^{2+}/Calmodulin-dependent Kinase Activity Is Loosely Associated with the Membrane-Cytoskeleton Complex

A high concentration of glycerol has been used to stabilize the extracted membrane-cytoskeleton complexes (30), and we have previously used these conditions in *Aplysia* nervous tissue (32). In *Aplysia*, several calmodulin-binding proteins are released from this complex when destabilizing conditions are used, the major one having a molecular weight of 55,000 (32). Under stabilizing conditions, we now find that most of the Ca^{2+}/calmodulin-dependent kinase activity also is in the particulate (membrane cytoskeleton) fraction, either when assayed by phosphorylation of synapsin I or by phosphorylation of the endogenous M_r 55,000 substrate protein (Fig. 1*A*). In contrast, when destabilizing conditions were used to prepare the particulate and soluble fractions, Ca^{2+}/calmodulin-

[1] *Abbreviation used in this paper:* TFP, trifluoperazine.

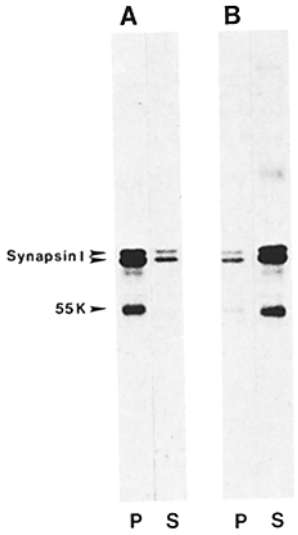

FIGURE 1 Subcellular distribution of Ca^{2+}/calmodulin-dependent protein kinase activity. Neuronal components from one abdominal ganglion were placed either in stabilization buffer (22.5 μl) (*A*) or destabilizing buffer (*B*) and centrifuged (see Materials and Methods). The pellets were washed either with stabilization buffer or with destabilizing buffer and then with 55% saturated ammonium sulfate (0.1 ml). The first and second resulting supernatants were combined and added to 55 μl of saturated ammonium sulfate (final concentration, 55% saturation). After 45 min at 4°C, samples were centrifuged again. The ammonium sulfate pellets were resuspended at 4°C overnight in the nonstabilizing buffer and their kinase activity assayed according to procedure I. *P*, pellet fraction; *S*, supernatant fraction. Under stabilizing conditions (*A*), in two independent experiments, 70% of kinase activity measured with synapsin I and 72% of the activity measured by phosphorylation of the endogenous M_r 55,000 protein (*55K*) were found in the pellet. Under destabilizing conditions (*B*), 24% of M_r 55,000 kinase activity and 21% of synapsin I kinase activity were found in the pellets.

dependent kinase activity was found predominantly in the supernatant fraction (Fig. 1 *B*).

Application of Serotonin Causes the Release of a Ca^{2+}/Calmodulin-dependent Kinase from the Membrane-Cytoskeleton Complex

Serotonin stimulates the synthesis of cAMP in *Aplysia* nervous tissue (6) and in specific identified neurons (4; see also reference 33). Application of serotonin, probably by increasing the concentration of cAMP, leads to the phosphorylation of certain cytoskeleton proteins (Bernier, L., J. Saitoh, and J. H. Schwartz, manuscript in preparation) and causes the translocation of a M_r 55,000 calmodulin-binding protein from the membrane-cytoskeleton complex to the cytoplasm (32). In control abdominal ganglia, from one-half to two-thirds of the Ca^{2+}/calmodulin-dependent protein kinase activity, measured using synapsin I as substrate, was associated with the membrane-cytoskeleton complex; only 30–40% was associated with the complex from ganglion treated with 50 μM serotonin for 2 min before extraction (Table I). Thus, it appears that the treatment causes the release of ~40% of the bound kinase from the membrane cytoskeleton.

Ca^{2+}/Calmodulin-dependent Phosphorylation Causes Dissociation of the M_r 55,000 Calmodulin-binding Protein from the Membrane-Cytoskeleton Complex

In the absence of Ca^{2+} and calmodulin, the M_r 55,000 calmodulin-binding protein remained associated with the membrane-cytoskeleton complex under phosphorylation conditions (Fig. 2 *A*). (In these experiments the complex has been separated from the cytoplasmic fraction and washed once before treatment under phosphorylation conditions. After the

TABLE I

Application of Serotonin to Aplysia Nervous Tissue Causes Dissociation of the Ca^{2+}/Calmodulin-dependent Protein Kinase from the Membrane-Cytoskeleton Complex

Treatment of ganglion	Experiment	Ca^{2+}/calmodulin kinase activity: Supernatant	Pellet	Remaining in pellet
		cpm		%
None	1	213	229	51.8
	2	65	170	72.4
	3	96	194	68.3
Serotonin	1	187	99	34.5
	2	171	123	41.8
	3	220	146	39.9

Neural components from abdominal ganglia were dissected out, fractionated, and processed for kinase assay as described in the legend to Fig. 1*A*. Some ganglia were exposed to 50 μM serotonin in supplemented artificial seawater (10) for 2 min before the dissection (32). Results from three independent experiments are presented; each value represents the kinase activity from an individual ganglion.

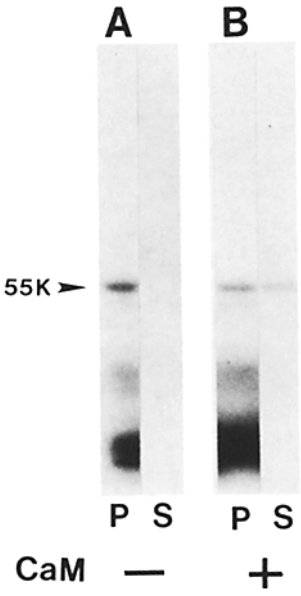

FIGURE 2 Blot with ^{125}I-calmodulin showing the Ca^{2+}/calmodulin/ATP-dependent dissociation of the M_r 55,000 calmodulin-binding protein (*55K*) from the isolated membrane-cytoskeleton complex. The membrane-cytoskeleton complex was prepared from four abdominal ganglia and was treated with 1 mM ATP under the stabilizing conditions (see Materials and Methods, procedure II) either in the presence (+) or in the absence (–) of Ca^{2+} and calmodulin. The proteins released were separated, blotted on nitrocellulose paper, and stained with ^{125}I-calmodulin.

treatment, any proteins released were separated by centrifugation.) About one-third of this calmodulin-binding protein was released when the complex was incubated in the presence of Ca^{2+}, calmodulin, and ATP, however (Fig. 2 *B*). Evidence that this binding protein is the same molecule as the M_r 55,000 protein that is phosphorylated in a Ca^{2+}/calmodulin-dependent manner was obtained by analyzing the distribution of the phosphorylated M_r 55,000 protein. A third of the total amount of this phosphorylated protein dissociated from the membrane-cytoskeleton complex after Ca^{2+}/calmodulin-dependent phosphorylation with procedure II (Table II). We did not detect the release of the phosphorylated M_r 55,000 protein when TFP was added during the incubation, which suggests that the release is mediated by calmodulin.

In these experiments, we presume that TFP is acting specifically to block the binding of calmodulin. The interpretation of these experiments depends on this pharmacologic specificity. Although it is possible that the drug has other actions under these conditions, we have previously shown

that TFP prevents the binding of Ca^{2+}/calmodulin to the M_r 55,000 subunit in crude extracts of *Aplysia* nervous tissue (32). Moreover, in another experiment, we incubated the isolated membrane-cytoskeleton complex under phosphorylation conditions (procedure II) in the presence of EGTA. After separating the released fraction from the pellet, we found that all the synapsin I kinase activity remained associated with the pellet fraction. Thus, TFP inhibited not only the phosphorylation of the M_r 55,000 protein, but also its release from the membrane cytoskeleton complex.

Release of the M_r 55,000 binding protein under these conditions does not seem to involve cAMP. Omission of the cAMP-dependent protein kinase inhibitor or the addition of cAMP to the phosphorylation reaction mixture did not stimulate either the phosphorylation or the release of the M_r 55,000 protein. We had previously found, however, that incubation of the total ganglion extract under cAMP-dependent phosphorylation conditions causes release of the M_r 55,000 calmodulin-binding protein from the membrane-cytoskeleton complex (32). This dissociation probably depends upon factors present in the cytoplasmic fraction, which is removed under the conditions of these experiments.

We obtained further evidence that the M_r 55,000 calmodulin-binding protein is a subunit of the kinase by partially purifying the supernatant fractions containing the enzyme activity which was dissociated by incubation of the membrane-cytoskeleton complex in the presence of Ca^{2+}/calmodulin and unlabeled ATP. Synapsin I kinase activity and the M_r 55,000 calmodulin-binding protein were eluted together from DEAE-cellulose (Fig. 3). (Since other kinases have been described that phosphorylate synapsin I [28], it is important to note that no other fractions from the DEAE column contained synapsin I kinase activity.) Neither the M_r 55,000 calmodulin-binding protein nor kinase activity was eluted from cellulose phosphate by 0.4 M NaCl, but both were eluted by 2 M NaCl. The two activities also were not separated during gel filtration on a column (1.4 × 60 cm) of Sephacryl S-400, emerging slightly after thyroglobulin (M_r 670,000, Pharmacia Fine Chemicals, Piscataway, NJ).

TABLE II

Release of the Phosphorylated M_r 55,000 Protein from the Membrane-Cytoskeleton Complex

Addition	Radioactivity in M_r 55,000 band		Proportion of total M_r 55,000 protein released
	Pellet	Supernatant	
	cpm		%
None	812	358	30.6
TFP	388	10	2.5

The membrane-cytoskeleton complex was isolated from six abdominal ganglia under stabilizing conditions. The complex was incubated for 10 min in the presence of Ca^{2+}, calmodulin, and [γ-^{32}P]ATP (see procedure II in Materials and Methods); where indicated, 10^{-4} M TFP was added. After the incubation, we separated the proteins released from the complex by centrifuging at 4°C. The M_r 55,000 protein is the major protein phosphorylated both in the presence and in the absence of TFP, and is the only phosphoprotein recovered in the released fraction. The values of radioactivity from this band are the average from two independent experiments, with values that differed by not >10%.

Released Ca^{2+}/Calmodulin-dependent Kinase Appears Independent of Ca^{2+}/Calmodulin

When the M_r 55,000 protein is phosphorylated and released from the membrane-cytoskeleton complex, the Ca^{2+}/calmodulin-dependent kinase is also released, and the released enzyme then appears to be Ca^{2+}/calmodulin independent.

We have shown that Ca^{2+}/calmodulin-dependent phosphorylation leads to the dissociation of a M_r 55,000 calmodulin-binding protein from the membrane-cytoskeleton (Fig. 2) and that, under the same conditions, a M_r 55,000 phosphorylated

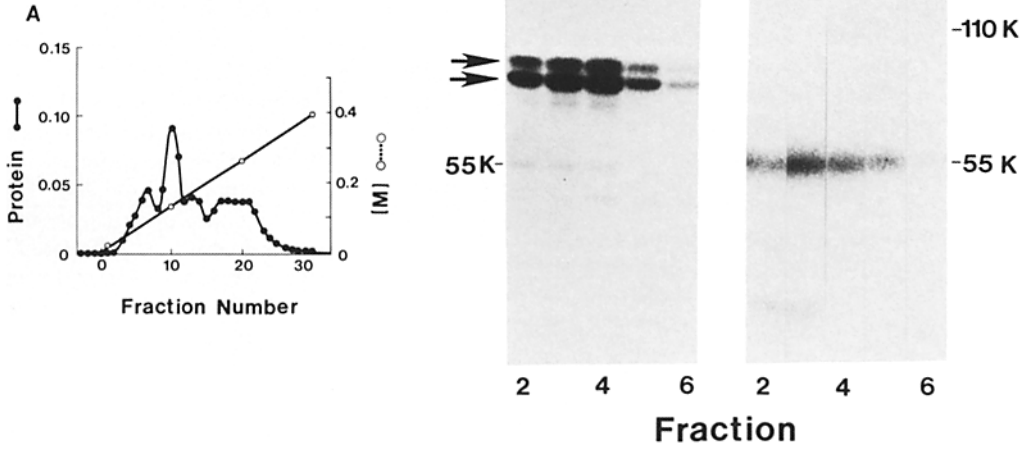

FIGURE 3 Chromatography of Ca^{2+}/calmodulin-dependent kinase activity and the M_r 55,000 calmodulin-binding protein (*55K*) on DEAE-cellulose. (*A*) Proteins released from the membrane-cytoskeleton complex isolated from ganglia dissected from 10 animals and containing ~15 mg protein were applied to the DE-52 column as described in Materials and Methods. The column was washed with 14 column volumes of column buffer: 71% of the sample protein was retained. At fraction 0 the salt gradient was started and 0.8-ml fractions were collected. (*B*) Samples (20 μl) from each of the fractions eluted were assayed for their kinase activity by procedure I with endogenous M_r 55,000 protein and added synapsin I (arrows). Autoradiograms corresponding to lanes containing fractions 2–6 are shown. We could not determine the purity of the preparation because there was an insufficient amount of protein on the gels to permit protein staining. Neither synapsin I kinase activity nor M_r 55,000 protein kinase activity was found in other fractions, however. (*C*) Samples (20 μl) from each of the same fractions were electrophoresed on a polyacrylamide gel, blotted onto nitrocellulose paper, and stained with ^{125}I-calmodulin. Autoradiograms of the nitrocellulose paper corresponding to lanes containing fractions 2–6 are shown. No M_r 55,000 calmodulin-binding protein was found in any other fractions.

protein is released (Table II). Because autophosphorylation of the M_r 55,000 subunit of the vertebrate Ca^{2+}/calmodulin-dependent kinase has been reported (3, 13), it is likely that the *Aplysia* protein that dissociates from the membrane-cytoskeleton is a subunit of a similar kinase. The membrane-cytoskeleton complex was incubated with unlabeled ATP and Mg^{2+} in the presence of Ca^{2+}/calmodulin under the phosphorylation conditions that preserve the cytoskeleton (procedure II). The proteins released were then separated from the membrane-cytoskeleton complex by centrifugation, and the kinase activity was assayed by procedure I using added synapsin I as substrate. As shown in Fig. 4*A*, much of the kinase activity was found to have been released into the supernatant fraction. This release was dependent on the presence of both Ca^{2+}/calmodulin and ATP (Table III), indicating the involvement of a Ca^{2+}/calmodulin-dependent phosphorylation.

The synapsin I kinase activity that is released from the membrane-cytoskeleton complex under conditions of Ca^{2+}/calmodulin-dependent phosphorylation appears to differ from the activity that is dissociated under nonstabilizing conditions in being independent of Ca^{2+} and calmodulin. Evidence for this independence is that the enzyme that becomes soluble after the treatment with Ca^{2+}/calmodulin and ATP (when subsequently precipitated by ammonium sulfate, a procedure that depletes calmodulin, reference 35) is active when later assayed in the presence of EGTA and TFP, a condition that should be both Ca^{2+}-free and calmodulin-free (Fig. 4*B*, supernatant, also see Table III). In contrast, the enzyme activity that remains bound to the complex, or is dissociated under nonstabilizing conditions, is Ca^{2+}/calmodulin-dependent (Fig. 4, compare the two particulate fractions; Table III).

Before treatment of the enzyme with Ca^{2+}, calmodulin, and unlabeled ATP (the conditions used above to release the kinase from the membrane-cytoskeleton complex), the two activities of the kinase (phosphorylation of synapsin I and autophosphorylation of the M_r 55,000 protein) correlated with one another (see Fig. 1). After the treatment, the ability of the M_r 55,000 protein to be phosphorylated does not parallel synapsin I kinase activity in either the pellet or the supernatant fractions (Fig. 4). In Fig. 4*A*, the most likely reason that the M_r 55,000 protein is not labeled after the treatment is that the treatment itself causes phosphorylation with the unlabeled ATP, and therefore the site normally phosphorylated in the assay with [^{32}P]ATP is already occupied.

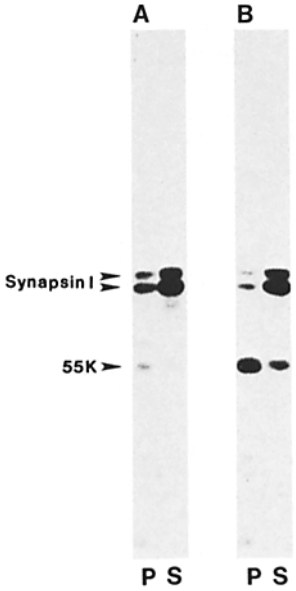

FIGURE 4 Ca^{2+}/calmodulin-dependent phosphorylation leads to the release of a kinase that is independent of Ca^{2+}/calmodulin. The membrane-cytoskeleton complex was incubated with unlabeled ATP in the presence of Ca^{2+}/calmodulin under stabilizing conditions (procedure II). After 30 min, the proteins released were separated from the membrane-cytoskeleton complex by centrifugation. Each fraction was precipitated with 55% saturated ammonium sulfate and its kinase activity assayed using procedure I either in the presence (*A*) or in the absence of Ca^{2+}/calmodulin (*B*). Minus Ca^{2+} (plus EGTA) tubes also received 0.1 mM TFP as an additional means of preventing the binding of any residual Ca^{2+}/calmodulin.

TABLE III

Release of Synapsin I Kinase Activity from the Membrane-Cytoskeleton Complex

Pretreatment of complex	Total activity measured with Ca^{2+}/calmodulin	Activity in supernatant	
		$+Ca^{2+}$/ calmodulin	$-Ca^{2+}$/ calmodulin
	%	%	
Nonstabilization buffer	100	80	26
Stabilization buffer			
$+Ca^{2+}$/CaM/ATP	96	74	53
$+Ca^{2+}$/CaM	53	17	9
+ATP	52	18	6
No addition	58	17	5

The membrane-cytoskeleton complex was isolated from the nervous systems of four animals under stabilizing conditions. About 70% of the total kinase activity in the extract was recovered in the membrane-cytoskeleton complex (see Table I). The membrane-cytoskeleton complex was resuspended in stabilization buffer (0.5 ml), divided into five portions, and sedimented again. This pellet was resuspended in 50-μl volumes either in nonstabilization buffer or in stabilization buffer supplemented with 10 mM $MgSO_4$. Where indicated, 0.7 mM $CaCl_2$/0.4 mM EGTA/20 μg/ml calmodulin with and without 1 mM ATP were added. After pretreatment for 30 min at 20°C, we separated the proteins released from the complex by centrifugation at 4°C. Each fraction was then precipitated with 40% saturated ammonium sulfate in the presence of bovine serum albumin (0.1 mg/ml) as a carrier. Kinase activity was assayed using procedure I either in the presence or in the absence of Ca^{2+}/calmodulin. Under these assay conditions (in the presence of the cAMP-dependent protein kinase inhibitor), only the larger polypeptide of synapsin I was phosphorylated (21), indicating that any cAMP-dependent kinase was completely inhibited. The results are expressed as the percentage (%) of the total synapsin I kinase activity that can be achieved after the disruption of cellular structure in nonstabilization buffer. The specific activity of the kinase under this condition, 5.5 pmol/mg protein per min, is set at 100% in the table. Values are the average of three experiments. Independent determinations did not differ by >20%.

It is surprising that in the extract pretreated with Ca^{2+}/calmodulin and ATP, the phosphorylation of the M_r 55,000 protein was actually greater in the presence of TFP (Fig. 4*B*). We need additional experiments to explain this paradoxically enhanced phosphorylation after calmodulin has been depleted by treatment with ammonium sulfate and the interaction of Ca^{2+}/calmodulin with the M_r 55,000 protein was further prevented by the addition of TFP. One attractive hypothesis, however, is that the M_r 55,000 protein has two types of sites that can be phosphorylated, one that is normally phosphorylated in the presence of Ca^{2+}/calmodulin, and the other that normally is cryptic in the presence of Ca^{2+}/calmodulin. Activation of the enzyme by a Ca^{2+}/calmodulin-dependent process followed by depletion of calmodulin and prevention of Ca^{2+}/ calmodulin binding by TFP would therefore result in the phosphorylation of the second sites.

DISCUSSION

Identification of the M_r 55,000 Binding Protein as a Subunit of the Aplysia Ca^{2+}/Calmodulin-dependent Kinase

DeRiemer et al. (8) suggested that the major calmodulin-binding protein in *Aplysia* nervous tissue is a subunit of a Ca^{2+}/calmodulin-dependent kinase. Our results support this idea. This M_r 55,000 protein is loosely associated with the membrane-cytoskeleton complex. Application of serotonin to

the intact ganglion brings about the dissociation of a portion both of the calmodulin-binding protein and of the Ca^{2+}/calmodulin-dependent kinase activity. Ca^{2+}/calmodulin-dependent phosphorylation causes the dissociation of the calmodulin-binding protein, the phosphorylated protein, and kinase activity. Finally, calmodulin binding, the phosphorylated protein, and kinase activity emerge together during chromatography on DEAE-cellulose and cellulose phosphate, or during gel filtration on Sephacryl S-400. Thus, the results presented in this paper indicate that all three properties reside in the same M_r 55,000 molecule. DeRiemer et al. (8) came to the same conclusion about a similar M_r 50,000–55,000 calmodulin-binding protein, from *Aplysia* nervous tissue, that they found cross-reacts with a monoclonal antibody produced against the bovine Ca^{2+}/calmodulin-dependent protein kinase.

Dual Control of the Subcellular Distribution of the Kinase by Two Second Messengers

We previously found that the M_r 55,000 calmodulin-binding protein can also be released from the membrane-cytoskeleton complex as a consequence of cAMP-dependent phosphorylation (32). When dissociation occurs in the presence of cAMP, however, there is no indication that the M_r 55,000 subunit itself is phosphorylated. We therefore suggested that the protein is released as a result of the cAMP-dependent phosphorylation of some unidentified component of the cytoskeleton whose modification would decrease the affinity of the M_r 55,000 protein for the cytoskeleton. In this paper, we show that Ca^{2+}/calmodulin-dependent phosphorylation also causes release of the Ca^{2+}/calmodulin-dependent kinase from the membrane-cytoskeleton complex. There are many instances in which cAMP and Ca^{2+}/calmodulin regulate the activity of the same effector molecule (reference 28). Intersection between second messenger systems permits more complex physiologic modulation and finer degrees of control.

Possible Activation of the Kinase

Loss of the dependence on added Ca^{2+}/calmodulin of synapsin I kinase activity of the enzyme translocated from the membrane-cytoskeleton complex is an unexpected result of subjecting the extract to conditions of Ca^{2+}/calmodulin-dependent phosphorylation. The evidence for this change in activity (which is presented in Fig. 4 and Table III) is tentative because we used the crude membrane-cytoskeleton complex. Thus, it is possible that the autonomy of synapsin I kinase activity that we observed was caused by activation of some contaminating kinase (for example, an activated lipid-dependent kinase) or by inactivation of inhibitors. Weighing against the presence of contaminating kinases is the observation presented in Fig. 3 that all of the synapsin I kinase activity in the fraction released from the membrane-cytoskeleton complex after treatment with Ca^{2+}/calmodulin and ATP was eluted as a single peak in the course of DEAE-cellulose chromatography.

We also showed that the independence of synapsin I kinase activity on Ca^{2+}/calmodulin could occur with the purified enzyme. A small amount of the kinase was purified from *Aplysia* ganglia by washing the membrane-cytoskeleton complex with low salt followed by cellulose phosphate column chromatography. The Ca^{2+}/calmodulin-dependent kinase was then further purified by affinity chromatography on a calmodulin Affigel column: this purified kinase is composed of the major M_r 55,000 subunit and several minor proteins with apparent molecular weights around 60,000 (Saitoh, T., and J. H. Schwartz, manuscript in preparation). Treatment of this purified preparation with Ca^{2+}/calmodulin and ATP, followed by reisolation after ammonium sulfate precipitation, yielded an amount of synapsin I kinase activity comparable with that present before the treatment; most important, the activity of this treated enzyme does not depend upon the subsequent addition of Ca^{2+}/calmodulin and is not affected by EGTA and TFP.

There is as yet insufficient information to specify the precise molecular mechanisms by which autonomy of enzyme activity might occur. The lack of dependence on Ca^{2+}/calmodulin of synapsin I kinase activity must result from some molecular change in the enzyme. One attractive idea is that the change in the properties of the kinase is caused by autophosphorylation of the M_r 55,000 subunit at one or more sites. Evidence for the presence of more than one site of phosphorylation is presented in Fig. 4. (Peptide mapping of the M_r 55,000 subunit phosphorylated under various conditions is now in progress.) It is also important to note that the molecular properties of the enzyme can be influenced by its subcellular distribution. We found that the kinase that remained with the membrane-cytoskeleton complex does not phosphorylate synapsin I as effectively as the released enzyme, and this is the reason for the apparently poor recovery of enzyme activity under some experimental conditions (Table III, "Total activity"). A possible explanation is unavailability of the added substrate protein. The enzyme was not inactivated under these conditions because it could phosphorylate the endogenous M_r 55,000 protein as efficiently as did the enzyme released under nonstabilizing conditions; moreover, after it is released by incubating the complex under the nonstabilizing conditions, this cryptic form of the enzyme becomes active with synapsin I as substrate (Fig. 1). The interaction of the kinase with the membrane-cytoskeleton complex might prevent efficient phosphorylation of exogenous synapsin I because of its relative inaccessibility as a substrate. Whatever the mechanism, release of the enyzme into the cytoplasm together with its independence from Ca^{2+}/calmodulin could be an important step in second messenger regulation by Ca^{2+}.

Physiologic Roles of the Ca^{2+}/Calmodulin-dependent Kinase

The physiologic functions of the Ca^{2+}/calmodulin-dependent kinase are not yet known in either vertebrates or invertebrates. Because it is concentrated in the postsynaptic density, the kinase might play some role in postsynaptic aspects of synaptic transmission. Although neither the nicotinic acetylcholine receptor nor the Na^+-channel is phosphorylated by this enzyme (see reference list of reference 28), it is possible that other receptors or channel proteins might be modified by the Ca^{2+}/calmodulin-dependent kinase. Because of its substrate specificity (see reference 28), the kinase might also regulate cytoskeletal structure, and thereby could influence the state of the postsynaptic neuron locally (see reference 26). Despite its concentration in the postsynaptic density, however, presynaptic functions are also possible for this kinase, since its principal substrate, synapsin I, is said to be a presynaptic marker (28) and the enzyme is also distributed through-

out the neuron. Another indication for functions that are not strictly postsynaptic is that the Ca^{2+}/calmodulin-dependent kinase exists in both free and bound forms within the cell. It has therefore also been suggested that this enzyme serves a presynaptic function (7).

The plasticity in the subcellular distribution of the Ca^{2+}/calmodulin-dependent kinase that we have observed fits well with the idea that this enzyme might regulate neurotransmitter release. Two possible mechanisms by which the enzyme might exert this control are the Ca^{2+}/calmodulin-dependent modulation of adenylate cyclase (16, 24) and the direct participation of Ca^{2+}/calmodulin phosphorylation in the final stages of transmitter release (7). Ca^{2+} has been implicated as an intracellular signal in the mechanism of associative learning that occur in *Aplysia* sensory neurons (1, 2, 16, 34). The Ca^{2+}/calmodulin-dependent kinase might mediate these effects of Ca^{2+} to enhance presynaptic facilitation in sensory neurons, because we found that the M_r 55,000 subunit is enriched in these neurons (32). There is also evidence that, during short-term sensitization of defensive reflexes in *Aplysia*, increases in cAMP-dependent protein phosphorylation are responsible for the facilitation of synaptic transmission from sensory neurons to motor neurons that underlies the enhancement of the reflexes. We found that conditions of sensitization alter the distribution of the enzyme in *Aplysia* nervous tissue (32). It is possible that increased Ca^{2+}/dependent phosphorylation might prolong presynaptic facilitation or even that it might underlie other forms of plasticity at these same synapses.

With the exception of the M_r 55,000 protein itself, we have not identified any of the many substrates of the Ca^{2+}/calmodulin-dependent kinase nor have we characterized their relationship to physiologic function. It therefore may be premature to specify how the translocation and activation of the enzyme affects the physiology of *Aplysia* neurons. In vertebrates, however, the Ca^{2+}/calmodulin-dependent kinase is a major constituent of the postsynaptic density fraction (14, 15, 18, 20). We suggest that in vertebrates, by analogy to *Aplysia*, the activated kinase might dissociate from the postsynaptic density (which can be found in presynaptic terminals that receive modulatory axo-axonic synaptic signals), translocate to the machinery involved in neurotransmitter release, and modulate release of transmitter through a phosphorylation step. In order to be effective physiologically, this mechanism would have to occur in nerve endings which themselves receive the modulatory synapses quite close to active zones (neurotransmitter release sites) (17, 23). Autoreceptors and receptors for neuroactive peptides (9) might also be associated with the kinase. Following an earlier suggestion (17), we would predict that terminals with modulatory axo-axonic input that activate the cAMP-dependent kinase or the Ca^{2+}/calmodulin-dependent kinase (or both) would be characteristic of areas of the brain implicated in synaptic plasticity, learning, and memory.

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REFERENCES

1. Abrams, T. W., L. Bernier, R. D. Hawkins, and E. R. Kandel. 1984. Possible roles of Ca^{2+} and cAMP in activity-dependent facilitation, a mechanism for associative learning in *Aplysia*. *Soc. Neurosci. Abstr.* 10:269. (Abstr.)
2. Abrams, T. W., T. J. Carew, R. D. Hawkins, and E. R. Kandel. 1983. Aspects of the cellular mechanism of temporal specificity in conditioning in *Aplysia*: preliminary evidence for Ca^{2+} influx as a signal of activity. *Soc. Neurosci. Abstr.* 9:168. (Abstr.)
3. Bennett, M. K., N. E. Erondu, and M. B. Kennedy. 1983. Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. *J. Biol. Chem.* 258:12735–12744.
4. Bernier, L., V. F. Castellucci, E. R. Kandel, and J. H. Schwartz, 1982. Facilitatory transmitter causes a selective and prolonged increase in adenosine 3′:5′-monophosphate in sensory neurons mediating the gill and siphon withdrawal reflex in *Aplysia*. *J. Neurosci.* 2:1682–1691.
5. Cedar, H., E. R. Kandel, and J. H. Schwartz. 1972. Cyclic adenosine monophosphate in the nervous system of *Aplysia californica*. I. Increased synthesis in response to synaptic stimulation. *J. Gen. Physiol.* 60:558–569.
6. Cedar, H., and J. H. Schwartz. 1972. Cyclic adenosine monophosphate in the nervous system of *Aplysia californica*. II. Effect of serotonin and dopamine. *J. Gen. Physiol.* 60:570–587.
7. De Lorenzo, R. J., S. D. Freedman, W. B. Yohe, and S. C. Maurer. 1979. Stimulation of Ca^{2+}-dependent neurotransmitter release and presynaptic nerve terminal protein phosphorylation by calmodulin-like protein isolated from synaptic vesicles. *Proc. Natl. Acad. Sci. USA.* 76:1838–1842.
8. DeRiemer, S. A., L. K. Kaczmarek, Y. Lai, T. McGuinness, and P. Greengard. 1984. Calcium/calmodulin-dependent protein phosphorylation in the nervous system of *Aplysia*. *J. Neurosci.* 4:1618–1625.
9. Duggan, A. W., and R. A. North. 1984. Electrophysiology of opioids. *Pharmacol. Rev.* 35:219–281.
10. Eisenstadt, M., J. E. Goldman, E. R. Kandel, H. Koike, J. Koester, and J. H. Schwartz. 1973. Intrasomatic injection of radioactive precursors for studying transmitter synthesis in identified neurons of *Aplysia californica*. *Proc. Natl. Acad. Sci. USA.* 70:3371–3375.
11. Fowler, V. M., and H. B. Pollard. 1982. Chromaffin granule membrane-F-actin interactions are calcium sensitive. *Nature (Lond.).* 295:336–339.
12. Giller, E. J., and J. H. Schwartz. 1971. Choline acetyltransferase in identified neurons of abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.* 34:93–107.
13. Goldenring, J. R., B. Gonzalez, J. S. McGuire, Jr., and R. J. DeLorenzo. 1983. Purification and characterization of a calmodulin-dependent kinase from rat brain cytosol able to phosphorylate tubulin and microtubule-associated proteins. *J. Biol. Chem.* 258:12632–12640.
14. Goldenring, J. R., J. S. McGuire, Jr., and R. J. DeLorenzo. 1984. Identification of the major postsynaptic density protein as homologous with the major calmodulin-binding subunit of a calmodulin-dependent protein kinase. *J. Neurochem.* 42:1077–1084.
15. Grab, D. J., R. K. Carlin, and P. Siekevitz. 1981. Function of calmodulin in postsynaptic densities. II. Presence of a calmodulin-activatable protein kinase activity. *J. Cell Biol.* 89:440–448.
16. Kandel, E. R., T. Abrams, L. Bernier, T. J. Carew, R. D. Hawkins, and J. H. Schwartz. 1983. Classical conditioning and sensitization share aspects of the same molecular cascade in *Aplysia*. *Cold Spring Harbor Symp. Quant. Biol.* 48:821–830.
17. Kandel, E. R., and J. H. Schwartz. 1982. Molecular biology of learning: modulation of transmitter release. *Science (Wash. DC).* 218:433–443.
18. Kelly, P. T., T. L. McGuinness, and P. Greengard. 1984. Evidence that the major postsynaptic density protein is a component of a Ca^{2+}/calmodulin-dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* 81:945–949.
19. Kennedy, M. B. 1983. Experimental approaches to understanding the role of protein phosphorylation in the regulation of neuronal function. *Annu. Rev. Neurosci.* 6:493–525.
20. Kennedy, M. B., M. K. Bennett, and N. E. Erondu. 1983. Biochemical and immunochemical evidence that the "major postsynaptic density protein" is a subunit of a calmodulin-dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* 80:7357–7361.
21. Kennedy, M. B., and P. Greengard. 1981. Two calcium/calmodulin-dependent protein kinases, which are highly concentrated in brain, phosphorylate protein I at distinct sites. *Proc. Natl. Acad. Sci. USA.* 78:1293–1297.
22. Kennedy, M. B., T. McGuinness, and P. Greengard. 1983. A calcium/calmodulin-dependent protein kinase from mammalian brain that phosphorylates Synapsin I: partial purification and characterization. *J. Neurosci.* 3:818–831.
23. Langer, S. Z. 1980. Presynaptic regulation of the release of catecholamines. *Pharmacol. Rev.* 32:337–362.
24. Livingstone, M. S., P. P. Sziber, and W. G. Quinn. 1984. Loss of calcium/calmodulin responsiveness in adenylate cyclase of *rutabaga*, a *Drosophila* learning mutant. *Cell.* 37:205–215.
25. Lynch, G., and M. Baudry. 1984. The biochemistry of memory: a new and specific hypothesis. *Science (Wash. DC).* 224:1057–1063.
26. Matus, A., G. Huber, and R. Bernhardt. 1983. Neuronal microdifferentiation. *Cold Spring Harbor Symp. Quant. Biol.* 48:775–782.
27. Miller, T. M., and J. E. Heuser. 1984. Endocytosis of synaptic vesicle membrane at the frog neuromuscular junction. *J. Cell Biol.* 98:685–698.
28. Nestler, E. J., and P. Greengard. 1984. Protein Phosphorylation in the Nervous System. John Wiley & Sons, New York. 398 pp.
29. Novak-Hofer, I., and I. B. Levitan. 1983. Ca^{++}/calmodulin-regulated protein phosphorylation in the *Aplysia* nervous system. *J. Neurosci.* 3:473–481.
30. Pallas, D., and F. Solomon. 1982. Cytoplasmic microtubule-associated proteins: phosphorylation at novel sites is correlated with their incorporation into assembled microtubules. *Cell.* 30:407–414.
31. Reichardt, L. F., and R. B. Kelly. 1983. A molecular description of nerve terminal function. *Annu. Rev. Biochem.* 52:871–926.

32. Saitoh, T., and J. H. Schwartz. 1983. Serotonin alters the subcellular distribution of a Ca^{2+}/calmodulin-binding protein in neurons of *Aplysia*. *Proc. Natl. Acad. Sci. USA.* 80:6708–6712.
33. Schwartz, J. H., L. Bernier, V. F. Castellucci, M. Palazzolo, T. Saitoh, A. Stapleton, and E. R. Kandel. 1983. What molecular steps determine the time course of the memory for short-term sensitization in *Aplysia*? *Cold Spring Harbor Symp. Quant. Biol.* 48:811–819.
34. Walters, E. T., J. H. Byrne, T. J. Carew, and E. R. Kandel. 1983. Mechanoefferent neurons innervating the tail of *Aplysia*. II. Modulation by sensitizing stimulation. *J. Neurophysiol.* 50:1543–1559.
35. Yamauchi, T., and H. Fujisawa. 1980. Evidence for three distinct forms of calmodulin-dependent protein kinases from rat brain. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 116:141–144.
36. Yamauchi, T., and H. Fujisawa. 1983. Purification and characterization of the brain calmodulin-dependent protein kinase (Kinase II), which is involved in the activation of tryptophan 5-monooxygenase. *Eur. J. Biochem.* 132:15–21.