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Quantitative Subcellular Localization of Calmodulin-Dependent Phosphatase in Chick Forebrain

Frank A. Anthony, Martin A. Winkler, Harold H. Edwards, and Wai Yiu Cheung

Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tennessee 38101

Using a radioimmunoassay, we have measured the level of calmodulin-dependent phosphatase (calcineurin) in various subcellular fractions from chick forebrain. Our results revealed high levels of the enzyme in the cytoplasm and microsomes. A considerable amount was also observed in synaptosomes, where it was found exclusively in the synaptoplasm, comprising 0.32% of the total synaptoplasmic protein. Immunocytochemical localization of the phosphatase in isolated synaptosomes supported the biochemical finding. Phosphatase was not detected in nuclei, myelin, synaptic vesicles, and mitochondria. These results suggest that myelin basic protein and histone H_1, widely used in biochemical characterization studies of the phosphatase, may not be physiological substrates, and that the cytoplasm, microsomes, and synaptoplasm may prove to be useful sources for the identification of physiological substrates.

Calcium plays an important role in many cellular functions, including such diverse processes as cell division and neurotransmitter release (Campbell, 1983); many of these functions are believed to be mediated through calcium-modulated proteins (Cheung, 1980; Kretsinger, 1980; Van Eldik et al., 1982). One of the best-characterized calcium-modulated proteins is calmodulin (Klee and Vanaman, 1982; Cheung, 1984; Babu et al, 1985), which regulates the activities of many enzymes, including several protein kinases and at least one protein phosphatase.

Calmodulin-dependent phosphatase, also termed calcineurin, is a heterodimer consisting of 2 subunits. Subunit A (M_r 60 kDa) possesses the catalytic site (Winkler et al., 1984) and binds calmodulin (Klee et al., 1979; Sharma et al., 1979); and subunit B (M_r 19 kDa) resembles calmodulin (Aitken et al., 1984), binds calcium (Klee et al., 1979), and regulates the activity of subunit A (Merat et al., 1985). The N-terminus of subunit B is blocked by myristic acid (Aitken et al., 1982), a posttranslational modification shared by several transformed gene products (Marchildon et al., 1984; Sefton and Hunter, 1984; Kamps et al., 1985) and the catalytic subunit of cAMP-dependent protein kinase (Carr et al., 1982).

Calmodulin-dependent phosphatase catalyzes the dephosphorylation of phosphoserine, phosphothreonine, and phosphotyrosine residues in proteins (Pallen and Wang, 1985; Pallen et al., 1985; Chan et al., 1986; Tallant and Cheung, 1986) and the dephosphorylation of several nonprotein substrates (Pallen and Wang, 1983; King and Huang, 1984; Li, 1984; Anthony et al., 1986). However, no *in vivo* substrates have yet been identified.

The phosphatase has been detected in many tissues (Wallace et al., 1980; Klumpp et al., 1983; Stewart et al., 1983; Tallant and Wallace, 1985); brain is a particularly rich source (Wallace et al., 1980). Immunocytochemical localization in mouse brain slices shows that the phosphatase is localized predominantly on postsynaptic densities (PSD) and dendritic microtubules (Wood et al., 1980). More recent studies have shown that the enzyme is found in chick brain and that its level increases during synaptogenesis (Tallant and Cheung, 1983). Also, the phosphatase is present in synaptic terminals of chick retina, where it is found in synaptoplasm, synaptic vesicles, synaptic membranes, and in subcellular fractions of synaptic plasma membranes and synaptic junctions (Cooper et al., 1985). Shields et al. (1985) have detected significant levels in cytosol and synaptosol of rat brain, but little if any in synaptic junctions or synaptic plasma membranes. Tallant and Cheung (1983) observed a considerable amount of phosphatase in a 100,000 × *g* supernatant of rat brain. Nevertheless, a detailed analysis of the subcellular distribution of the enzyme in any tissue has not been reported.

We have chosen chick brain for the quantitative subcellular localization of calmodulin-dependent phosphatase because chicks show little intraspecies variability, are easy to care for, and are inexpensive. Moreover, the subcellular fractions from chick have been well characterized (Babitch et al., 1976; Anthony and Babitch, 1985).

Materials and Methods

Materials. $Na^{125}I$ (13–17 mCi/μg) was purchased from Amersham. BioRad supplied the Enzymobead radioiodination reagent and SDS. Sigma supplied Triton X-100, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), leupeptin, aprotinin, polyethylene glycol, *n*-octylglucoside, sucrose, and BSA. Ficoll 400, Dextran T-500, and disposable Sephadex G-25 (PD-10) columns were obtained from Pharmacia. Auroprobe reagents for immunoblotting (GAR-G-BL), immunoelectron microscopy (GAR-G-LM), and a silver-enhancement kit were furnished by Janssen. New Zealand white rabbits were provided by a local supplier, newborn male Leghorn chicks by Stillwater Hatchery (Stillwater, OK), and bovine brains by Hernando Boneless Beef (Hernando, MS). Goat anti-rabbit immunoglobulin serum was a gift from Dr. William Walker, and rat oligodendrocytes were prepared by Dr. Yu-Yan Yeh, both of St. Jude Children's Research Hospital, Memphis, TN.

Iodination of calmodulin-dependent phosphatase. Calmodulin-dependent phosphatase was purified from bovine brain (Tallant et al., 1983) and stored in 50 mM Tris-HCl, pH 7.0, 0.5 mM DTT containing 60%

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Correspondence should be addressed to W. Y. Cheung at the above address.

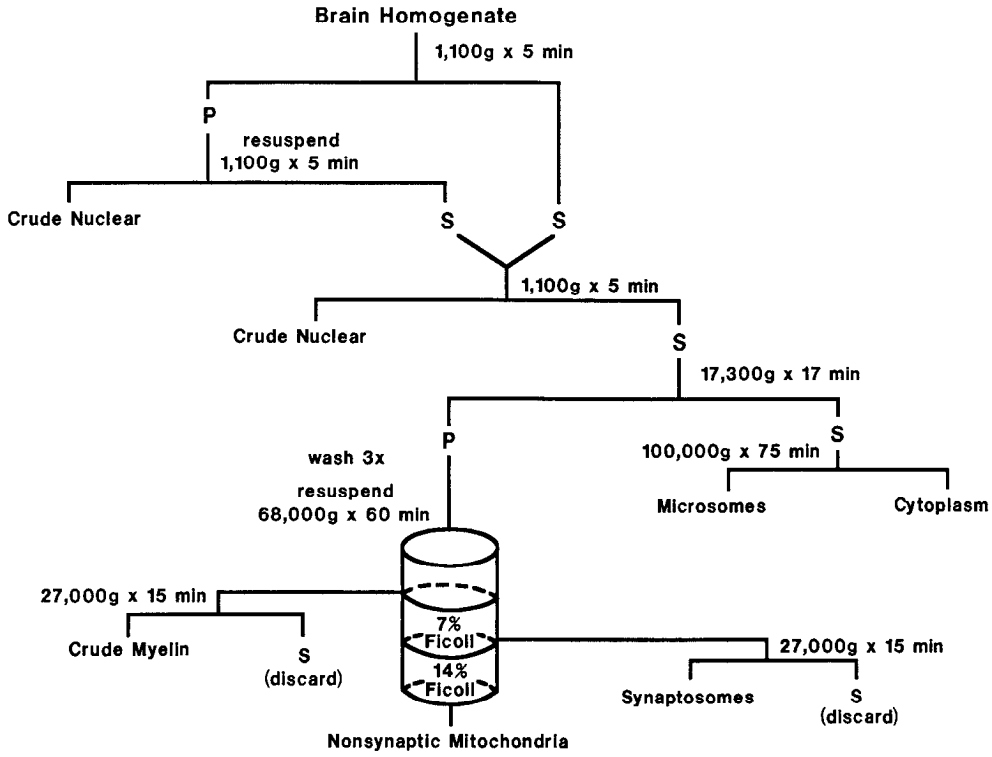

Figure 1. Diagrammatic scheme of the subcellular fractionation of chick forebrain homogenate.

glycerol at −20°C. The phosphatase was iodinated using the Enzymobead radioiodination reagent as described by the manufacturer, with minor modifications: 2 μg of phosphatase free of azide and DTT was reacted with 0.3 mCi of $Na^{125}I$ for 3–4 min. The reaction was stopped with 100 μl of 100 mM KI, and the unbound iodide was immediately separated from the labeled protein using a disposable Sephadex G-25 (PD-10) column, which had been equilibrated with 10 mg of BSA, washed exhaustively, and equilibrated with buffer A (20 mM sodium phosphate, 0.05% Triton X-100, and 0.15 *N* NaCl, pH 7.2). Labeled protein was eluted with buffer A. The phosphatase typically contained 0.5–1.2 mol of ^{125}I with a specific activity of 13–30 μCi/μg. Autoradiography indicated that both subunits A and B were iodinated. Aliquots of ^{125}I-phosphatase were stored in the presence of 0.1 mg/ml of BSA at −20°C.

RIA of phosphatase. Antisera against bovine brain calmodulin-dependent phosphatase were raised in rabbits according to a previous protocol (Wallace et al., 1980), with the exception that Freund's complete adjuvant was used only in the initial immunization. A detergent-based radioimmunoassay (RIA) developed for measuring calmodulin-dependent phosphatase was performed using whole antisera (Tallant et al., 1983). Assays were performed in BDS polymer tubes (Evergreen) using 5–1000 ng of bovine brain calmodulin-dependent phosphatase or 5–1000 μg of chick brain subcellular fractions, 1 μl of whole antiserum, and ^{125}I-phosphatase (20,000 cpm) in the presence of Triton X-100, SDS, and protease inhibitors. To precipitate the immune complex, 10 μl of goat anti-rabbit serum was added, and the final precipitate was collected by centrifugation at 20,000 × *g* for 20 min at 4°C. Data were corrected for nonspecific binding (counts bound in the presence of normal rabbit serum alone). Routinely, 3–6 protein concentrations of each sample were assayed to verify that the RIA curve was parallel to the standard. Each determination was replicated.

Preparation of subcellular fractions and synaptic subfractions. Subcellular fractions and synaptic subfractions were prepared from 6–10-d-old chick forebrains (cerebral hemispheres and optic lobes) in the presence of EDTA (Babitch et al., 1976); a diagrammatic scheme of the procedure is outlined in Figure 1. Crude nuclear pellets were further purified (McEwen and Zigmond, 1972). Crude myelin, isolated from the top of a Ficoll gradient, was osmotically shocked and then further purified on a sucrose density gradient (Norton and Poduslo, 1973). Synaptosomes were isolated from the 7%/14% Ficoll interface; pellets from the Ficoll gradients were used as the nonsynaptic mitochondrial fraction. The synaptosomes were osmotically lysed and centrifuged on a one-step sucrose density gradient. The pellet represented synaptic mitochondria; all other materials were collected, mixed, and centrifuged for 14 hr at 25,000 × g_{av}. The supernatant fluid represented synaptoplasm. The pellet was resuspended and centrifuged on a 5-step sucrose density gradient. The purest synaptic vesicles (SV) banded at the top of the 0.4 M sucrose layer of the 5-step sucrose density gradient, while the purest presynaptic membranes (SPM) banded at the 0.8/0.95 mM sucrose interface (Babitch et al., 1976). All steps were performed at 4°C.

PSD and pre- and postsynaptic membranes with attached postsynaptic densities (SM) were isolated in the absence of EGTA according to Gurd et al. (1982), or Cohen et al. (1977).

Protein determination. Protein concentration was determined according to Lowry (1951), using BSA as the standard.

Electron microscopy and immunocytochemistry. Synaptosomes were pelleted and fixed in 0.1 M sodium cacodylate, pH 7.3, 0.2 M sucrose, 0.5 mM $CaCl_2$, 2.6% glutaraldehyde, and 2% paraformaldehyde, rinsed in 0.1 M sodium cacodylate, pH 7.3, postfixed in 1% osmium tetraoxide in the cacodylate buffer, rinsed in 50% ethanol, and then stained *en bloc* with 4% uranyl acetate in 50% ethanol. After dehydration with an ascending series of ethanol concentrations, the sample was embedded in Spurr. Thin sections were stained with lead citrate (Venable and Coggshall, 1965).

For cryosectioning, the synaptosome pellet was fixed overnight at 4°C in 0.1 M K_2HPO_4, pH 7.3, 0.2 M sucrose, 0.5 mM $CaCl_2$, 0.05% glutaraldehyde, and 3% paraformaldehyde, and then immersed in 0.1 M PBS containing 7% sucrose for 3 hr at 4°C. The pellet was transferred to 0.1 M 1-4-piperazine-diethanesulfonic acid (PIPES), pH 7.0, containing 2.3 M sucrose for 3 hr at 4°C, frozen by liquid nitrogen-cooled copper-lined pliers, and stored under liquid nitrogen.

Frozen sections (100 nm) were cut on a Sorvall FS 1000 cryosectioning system at −100°C and placed on grids, which were inverted onto a plate of 2% gelatin and 0.75% agarose for 30 min. Subsequent manipulations were done at 22°C. The grids were floated up with 0.02 M Tris-HCl, pH 7.4, 0.9% NaCl (TBS) containing 0.01 M glycine for 15 min and conditioned on 3% gelatin in TBS for 15 min. One drop of phosphatase antiserum diluted 1:100 in TBS with 1% gelatin was allowed to interact with the cut sections on each grid for 60 min. The grids were rinsed twice for 5 min with TBS containing 0.1% gelatin. Goat anti-rabbit gold-labeled immunoglobulin (GAR-G-LM) diluted 1:20 in TBS containing 0.1% gelatin was placed on each grid and allowed to interact with the primary antibody for 60 min. Each grid was rinsed in TBS containing 0.1% gelatin twice for 5 min, and finally in water. Sections

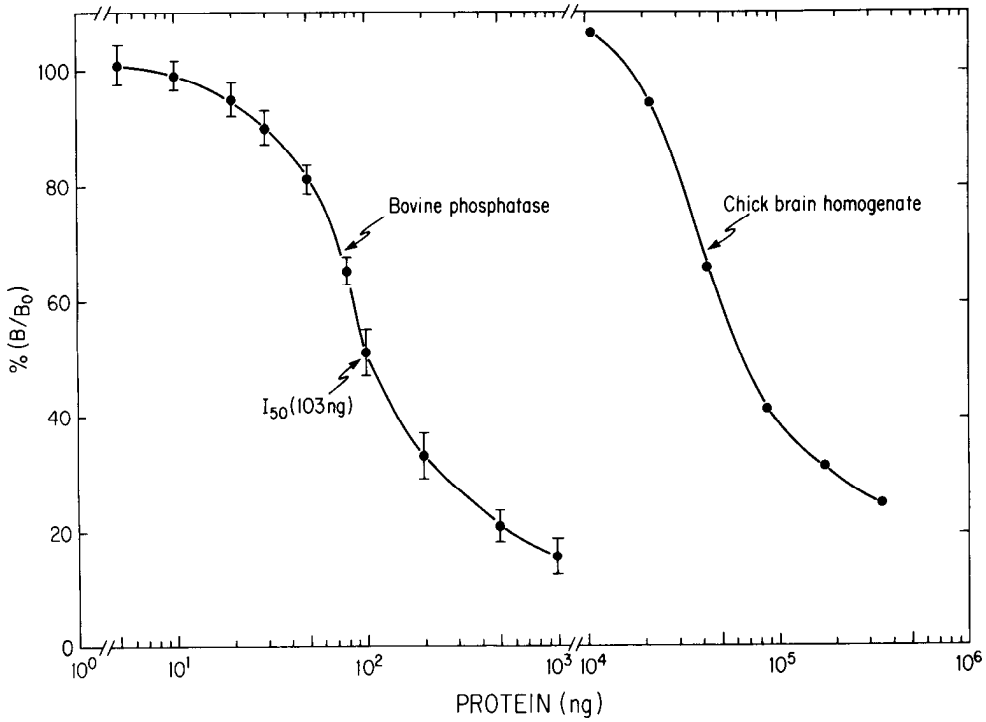

Figure 2. Radioimmunoassay of calmodulin-dependent phosphatase. Binding is represented as $\%(B/B_0)$, where B represents counts bound in the presence of increasing amounts of unlabeled antigen and B_0 represents counts bound in the absence of unlabeled antigen. For bovine brain phosphatase, the maximum B_0 varied depending upon the extent of iodination, but was usually about 30%. These data represent the mean ± SD from 10 different assays. The homogenate was prepared from chick forebrain. Other details are given under Materials and Methods.

were stained in a 1:1 mixture of 2% phosphotungstic acid and 1.5% Tylose 300 for 10 min, then rinsed quickly and embedded in 1.5% Tylose. Samples were screened using a Philips 301 electron microscope. Control grids had no first antibody.

Polyacrylamide gel electrophoresis and PAGE immunoblotting. Electrophoresis was performed as described by DeBlas et al. (1979). Protein samples were heated at 90°C for 2 min prior to electrophoresis, and a 20-well slab gel was run at 7 mA/gel.

After electrophoresis, the gel was incubated in transfer buffer (192 mM glycine, 25 mM Tris-HCl, pH 8.4, containing 20% methanol) for 30 min, and the proteins were transferred to a sheet of nitrocellulose at 100 mA for 2 hr (Towbin et al., 1979). The nitrocellulose was either stained with amido black or used for immunoblotting as described by Janssen (Piscataway, NJ). Briefly, after electrophoretic transfer, unoccupied protein-binding sites were saturated by incubating the membrane in 20 mM Tris-HCl, pH 8.2, 0.9% NaCl, 20 mM NaN_3, and 5% BSA for 45 min at 37°C. The membrane was then incubated in 2 ml of 20 mM Tris-HCl, pH 8.2, 0.9% NaCl, 1% normal goat serum, and 0.1% BSA containing 2 μl of phosphatase antiserum for 2 hr at 22°C. The membrane was washed 3 times (50 ml, 5 min/wash) in 20 mM Tris-HCl, pH 8.2, 0.9% NaCl, and 0.1% BSA, and then incubated in 2 ml of 20 mM Tris-HCl, pH 8.2, 0.9% NaCl, 20 mM NaN_3, 0.4% gelatin (wt/vol), and 0.1% BSA, containing a 1:100 dilution of gold-labeled goat anti-rabbit immunoglobulin (GAR-G-BL). The nitrocellulose was incubated overnight and washed twice in 20 mM Tris-HCl, pH 8.2, 0.9% NaCl, and 0.1% BSA. The blot was then silver-enhanced using a kit from Janssen.

Results

RIA of calmodulin-dependent phosphatase

A detergent-based RIA developed for bovine brain calmodulin-dependent phosphatase (Tallant et al., 1983) was used to measure the enzyme level in subcellular fractions isolated from chick forebrain. Because new antiserum was prepared for these studies, we first determined its cross-reactivity with chick brain phosphatase. Figure 2 shows a typical RIA in which unlabeled bovine brain phosphatase was added to compete with iodinated phosphatase for the antibody over a 500-fold concentration range. The amount of unlabeled protein displacing 50% of the iodinated phosphatase was about 100 ng, and the limit of detection was about 10 ng. Figure 2 also shows that a chick forebrain homogenate effectively displaced the iodinated phosphatase. The titration curves for bovine brain phosphatase and chick brain homogenate appeared to be parallel, indicating that the antibody against bovine phosphatase cross-reacted with the chick enzyme. These data established the utility of the antiserum for the measurement of calmodulin-dependent phosphatase in chick brain. Other experiments indicated that this antiserum is specific: Of the many proteins present in a crude extract of chick forebrain, the antibody recognized only subunits A and B of the phosphatase; it did not recognize any of the other proteins in the crude extract (see Fig. 4). Moreover, another polyclonal antibody against bovine brain calmodulin-dependent phosphatase, raised similarly in a rabbit, was extensively characterized and found to recognize only subunits A and B (Wallace et al., 1980); calmodulin, though sharing extensive homology with subunit B, was not recognized.

Distribution of phosphatase in subcellular fractions

Table 1 presents phosphatase levels measured by the RIA in subcellular fractions isolated from chick forebrain (Babitch et al., 1976). The level of enzyme detected in the homogenate was 1.8 ± 0.5 μg/mg of protein (mean ± SEM; $n = 4$). This value appeared to be higher than that observed previously for chick brain, but was within the range observed in other brain tissues (Tallant and Cheung, 1983; Shields et al., 1985). Only the cytoplasm and microsome fractions showed a higher level than that observed in the homogenate. Expressed per gram of tissue, the level of phosphatase was also highest in the cytoplasm and microsome fractions.

Although phosphatase was observed in the nuclear fraction, this fraction is usually heavily contaminated with cytoplasmic particles (McEwen and Zigmond, 1972) and was therefore further purified (see Materials and Methods). Examination of the

Table 1. Levels of calmodulin-dependent phosphatase in various subcellular fractions from chick forebrain

Fraction	Protein (mg/gm of tissue)	Phosphatase (μg/mg of protein)	Phosphatase (μg/gm of tissue)	Phosphatase (% Distribution[a])
Homogenate	106.2 ± 8.1	1.8 ± 0.5	196.3 ± 50	100
Cytoplasm	18.7 ± 1.9	2.6 ± 0.9	46.1 ± 12.6	21.4 ± 3.0
Microsome	15.1 ± 1.4	2.1 ± 0.6	29.8 ± 6.9	15.7 ± 1.5
Crude nuclear	11.3 ± 1.3	0.8 ± 0.3	7.7 ± 2.7	3.8 ± 0.3
Crude myelin	10.0 ± 1.0	1.0[b]	9.5[b]	4.1[b]
Synaptosome	5.5 ± 0.7	1.2 ± 0.4	6.4 ± 1.4	3.6 ± 0.8
Nonsynaptic mitochondria	2.9 ± 0.5	0.2[c]	0.9[c]	0.4[c]

The various subcellular fractions were prepared from chick forebrain, as described in Materials and Methods. Phosphatase was measured using an antiserum against bovine brain phosphatase. These data represent the mean ± SEM from 4 different preparations. Each determination was duplicated. The 3 washes of the crude synaptosomal pellet made prior to the Ficoll gradient accounted for 20% of the total phosphatase. Other washes in the fractionation account for 12% of the total phosphatase. Taking these values into account, the total phosphatase recovery was 81%; the protein recovery was 93%.

[a] Percentage distribution based on the μg/gm levels of phosphatase.

[b] Phosphatase was detected in only 1 of 4 different preparations.

[c] Phosphatase was detected in 2 of 4 different preparations.

purified nuclei by RIA did not reveal a detectable level of phosphatase.

To isolate myelin, synaptosomes, and mitochondria, a pellet consisting of a crude synaptosome fraction was washed 3 times and then separated on a Ficoll gradient (see Fig. 1). These washes, which reduce microsomal contamination in the crude synaptosome fraction from 50% to less than 15% (Gurd et al., 1974) contained a significant level of phosphatase. Therefore, the level of phosphatase in the microsome fraction may have been underestimated.

A small amount of phosphatase was observed in one of the 4 myelin preparations summarized in Table 1. To further examine whether the phosphatase was present in myelin, the crude myelin fraction was purified. Phosphatase was subsequently found to be below the level of detection. The absence of detectable phosphatase in the myelin fraction does not necessarily preclude its presence in oligodendrocytes, which produce myelin in the CNS. However, oligodendrocytes purified from rat brain did not show any detectable phosphatase.

The level of phosphatase in the synaptosome fraction was 6.4 ± 1.4 μg/gm tissue and represented 4% of the total phosphatase in chick forebrain. Synaptosomes isolated by this procedure have very few postsynaptic membranes and densities attached and thus represent mostly resealed presynaptic nerve endings (see Fig. 3*a* and Babitch et al., 1976).

Phosphatase was barely detected in the mitochondria fraction, suggesting that the trace amount may have resulted from contamination by other fractions.

Distribution of phosphatase in synaptic subfractions

Because of the presence of a considerable amount of phosphatase in the synaptosome, and its potential role in neurotransmitter release (Tallant and Cheung, 1986), this fraction was subfractionated further. Table 2 lists the distribution of the enzyme in

Table 2. Levels of calmodulin-dependent phosphatase in various synaptic subfractions

Fraction	Protein (mg/gm of tissue)	Phosphatase (μg/mg of protein)	Phosphatase (μg/gm of tissue)	Phosphatase (% Distribution[a])
Synaptosome	5.7 ± 1.0	1.1 ± 0.1	6.0 ± 0.3	100
Synaptoplasm	1.9 ± 0.3	3.2 ± 0.2	6.3 ± 0.8	99.9 ± 12
Synaptic mitochondria	1.6 ± 0.2	ND	ND	ND
Sample/0.4 M	0.1 ± 0.0	0.2[b]	<0.1[b]	0.2[b]
0.4/0.6 M	0.1 ± 0.0	0.3 ± 0.0	<0.1	0.3 ± 0.1
0.6/0.8 M	0.2 ± 0.1	0.2 ± 0.0	<0.1	1.0 ± 0.1
0.8/0.95 M	0.4 ± 0.3	0.3 ± 0.0	0.1 ± 0.0	2.0 ± 0.8
0.95/1.1 M	0.2 ± 0.1	0.3 ± 0.0	<0.1	0.9 ± 0.1
Pellet	0.2 ± 0.0	0.4[b]	<0.1[b]	1.5[b]

The various synaptic subfractions were prepared from synaptosomes, as described in Materials and Methods. The data represent the mean ± SEM of 5 separate determinations from 3 different preparations. Each determination was duplicated. The total protein recovery was 90% and the phosphatase recovery was usually 90–110%. ND, not detected.

[a] Percentage distribution based on the μg/gm levels of phosphatase.

[b] Values observed in only 1 of 5 different phosphatase determinations from 3 different synaptic subfractionation preparations.

Table 3. Levels of calmodulin-dependent phosphatase in various subcellular fractions obtained from the isolation of PSD

Fraction	Protein (mg/gm of tissue)	Phosphatase (μg/mg of protein)	(μg/gm of tissue)	(% Distribution[a])
Homogenate	101.6	2.5 ± 0.1	249.9	100
Supernatant 1	31.1	2.8 ± 0.0	88.6	35.4
Supernatant 2	16.8	4.4 ± 0.2	73.8	29.5
Crude nuclear	20.7	1.2 ± 0.1	17.2	6.8
Crude mitochondria	7.4	0.9 ± 0.1	6.4	2.5
Crude myelin	0.8	0.6 ± 0.0	0.5	0.2
Synaptic membranes	2.8	1.2 ± 0.0	3.4	1.4
PSD	0.4	0.2 ± 0.0	0.1	<0.1

Washes not listed in this table account for 10% of the total phosphatase. Supernatant 1 represents a mixture of cytoplasmic and microsomal material. Supernatant 2 was obtained after lysis of crude synaptosomes. The data represent the mean and SD from 4 determinations. The protein recovery was 98%, and the phosphatase recovery was 86%. PSD, postsynaptic densities.

[a] Percentage distribution based on the μg/gm levels of phosphatase.

these synaptic subfractions. The highest level of the enzyme was observed in synaptoplasm, where the enzyme represented 0.32% of the total synaptoplasmic protein. The relative concentration of phosphatase (in μg/mg protein) in the synaptoplasm was nearly 3 times that in the synaptosome fraction and almost twice that in the homogenate (see Table 1). This relative concentration was slightly enriched over the cytosol (Table 1) and was higher than that of any other subcellular fraction or synaptic subfraction.

Phosphatase was not detected in synaptic mitochondria, supporting the supposition that the enzyme is not intrinsic to the mitochondria (see also Table 1).

A trace level of phosphatase was detected in only one of several preparations of the fraction at the sample/0.4 M interface. This fraction is enriched with synaptic vesicles, and the lack of detectable phosphatase suggested that it was unlikely to be associated with synaptic vesicles.

Phosphatase levels were uniformly low in the synaptic subfractions isolated from the sucrose density-gradient interfaces of 0.4/0.6 M, 0.6/0.8 M, 0.8/0.95 M (purest presynaptic membranes), and 0.95/1.1 M sucrose. It is conceivable that these low levels represent contamination by synaptoplasm.

Electron microscopy of isolated synaptosomes

The present subcellular localization study suggests that, within the synaptosome, the phosphatase is localized exclusively in the synaptoplasm; therefore, we further investigated the synaptosomes by immunocytochemistry. Figure 3*a* is a micrograph of a typical preparation of synaptosomes after fixation, embedding, and staining. They contain synaptic vesicles, occasional mitochondria, and few or no attached postsynaptic membranes. Extrasynaptosomal mitochondria, synaptic membranes, and myelin are essentially absent. Figure 3*b* is a micrograph from the same preparation of synaptosomes that were lightly fixed, frozen, and cryosectioned prior to immunocytochemical staining for the phosphatase. The reaction product is not restricted to the synaptic plasma membrane, but is randomly distributed within the synaptosome, suggesting a synaptoplasmic localization. The morphology of each synaptosome is not as distinct as that in Figure 3*a* because of the reduced contrast associated with the cryosectioning procedure, and it is not possible to ascertain if the phosphatase is associated with any particular cellular organelle. In the control sample, only the secondary goat anti-rabbit antibody was used, and no reaction product was observed (Fig. 3*c*). Again, the morphology is not distinct because of the cryosectioning technique. These results are consistent with the RIA data indicating that the phosphatase was present in the cytoplasm of nerve terminals.

Measurement of phosphatase in postsynaptic densities

Immunocytochemical data have revealed that phosphatase is predominantly associated with PSD and dendritic microtubules (Wood et al., 1980). The procedures used for the isolation of subcellular fractions and synaptic subfractions described in Tables 1 and 2 did not yield PSD. Table 3 lists the various subcellular fractions obtained according to a different procedure (Gurd et al., 1982) and their levels of phosphatase, as measured by the RIA. Although some of the subcellular fractions in Table 3 bear labels similar to those in Tables 1 and 2, they should not be directly compared. Supernatant 1 (a mixture of cytoplasmic and microsomal proteins) and supernatant 2 (a mixture of proteins obtained following lysis of a crude synaptosome fraction) contained the highest levels of phosphatase. The crude nuclear and mitochondrial fractions contained minor levels of the enzyme, probably because of cross-contamination, and the crude myelin fraction contained even lower levels. The total level of phosphatase in these 3 fractions was less than 10% of the homogenate. Synaptic membranes (pre- and postsynaptic membranes with attached PSD) represented 1.4% of the total phosphatase. Further subfractionation of the synaptic membranes to obtain PSD indicated that the PSD accounted for less than 0.1% of the total phosphatase. Examination of this fraction using electron microscopy confirmed the presence of PSD (data not shown).

The PSD in Table 3 was obtained by using phase partitioning with polyethylene glycol and Dextran T-500 in the presence of *n*-octylglucoside, which could dissociate the phosphatase from the PSD during isolation. Therefore, another preparation of PSD was isolated by a more conventional procedure (Cohen et al., 1977). The crude synaptosome fraction was homogenized with Triton X-100, and the homogenate was centrifuged to obtain a

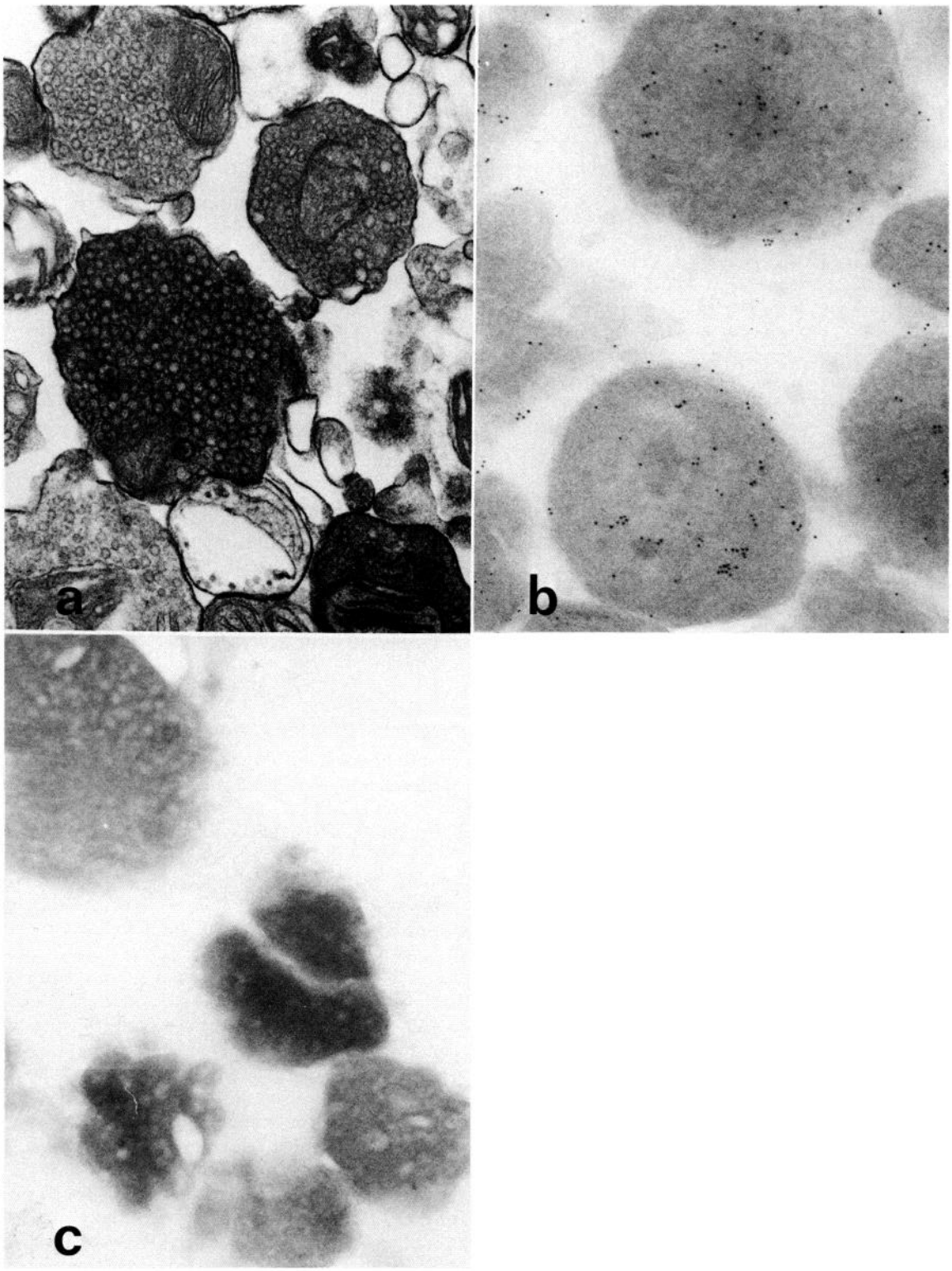

Figure 3. Electron micrographs of thin-sectioned synaptosomes isolated from chick forebrain. *a,* Spurr-embedded, unlabeled synaptosomes stained with lead citrate. ×49,500. *b,* Cryosection of synaptosomes first labeled with anti-calmodulin-dependent phosphatase and then with gold-labeled goat anti-rabbit immunoglobulin. ×49,400. *c,* Cryosection of synaptosomes treated only with gold-labeled goat anti-rabbit immunoglobulin. ×49,400.

Triton-insoluble pellet. The pellet was resuspended and then subfractionated on a sucrose density gradient to obtain PSD. Only 0.01% of the total phosphatase was observed in the synaptic membrane fraction, and phosphatase was below the level of detection in the PSD. Nevertheless, examination of this fraction using electron microscopy confirmed the presence of PSD (data not shown). Collectively, these results suggested that the PSD only contains a trace of the phosphatase.

PAGE immunoblots of PSD

In addition to using the RIA, we also attempted to detect phosphatase in the PSD using an immunoblotting procedure. This procedure is more sensitive than the RIA, and, with silver enhancement, can detect as little as 0.1 ng of protein (Moeremans et al., 1984). The procedure, however, is inherently not quantitative. Figure 4 shows the protein patterns on a nitrocellulose membrane after the proteins had been transferred from a polyacrylamide gel. The membrane was first incubated with rabbit anti-phosphatase serum and then with gold-labeled goat anti-rabbit immunoglobulin, and enhanced with silver. Figure 4, lane 1 contains 2 μg of bovine brain phosphatase and shows 2 positive reaction bands at 60 kDa (subunit A) and 16 kDa (subunit B, which migrates as a broad band at 16 kDa even though its m_r, according to amino acid sequence, is 19 kDa). Traces of a 45 and a 35 kDa band—presumably proteolytic products of subunit A (Winkler et al., 1984)—were also observed. These results indicated that the antibody recognized both subunits A and B, in agreement with previous data (Cooper et al., 1985). Figure 4, lane 2 contains 100 μg of a chick forebrain homogenate and reveals a weak positive reaction at 60 and 35 kDa and a strong reaction at 16 kDa. No other positive reactions were observed, indicating the specificity of the antisera. The weaker reaction at 60 kDa may be due to a less effective transfer of subunit A, presumably because of a higher molecular weight or a species variability of subunit A or B. The possibility of species variability cannot be excluded, but would appear unlikely in view of the nearly perfectly parallel titration curves of bovine brain calmodulin-dependent phosphatase and a chick brain homogenate (see Fig. 1). Another possibility is that the antibody recognizes subunit B better than subunit A of chick brain phosphatase. Figure 4, lane 3 contains 100 μg of chick PSD proteins and reveals a faint reaction product at 60 kDa and a strong reaction at 16 kDa. A control immunoblot, treated with non-immune rabbit antisera and then with gold-labeled goat anti-rabbit immunoglobulin or with gold-labeled goat anti-rabbit immunoglobulin alone, showed no reaction product. Another control immmunoblot indicated that the antiserum did not cross-react with calmodulin (data not shown). Inherent in electrophoretic transfer is the limitation that the procedure is not quantitative, and low levels of phosphatase might not be detected. In spite of this limitation, these results are consistent with the notion that the phosphatase is present as a minor component in the PSD.

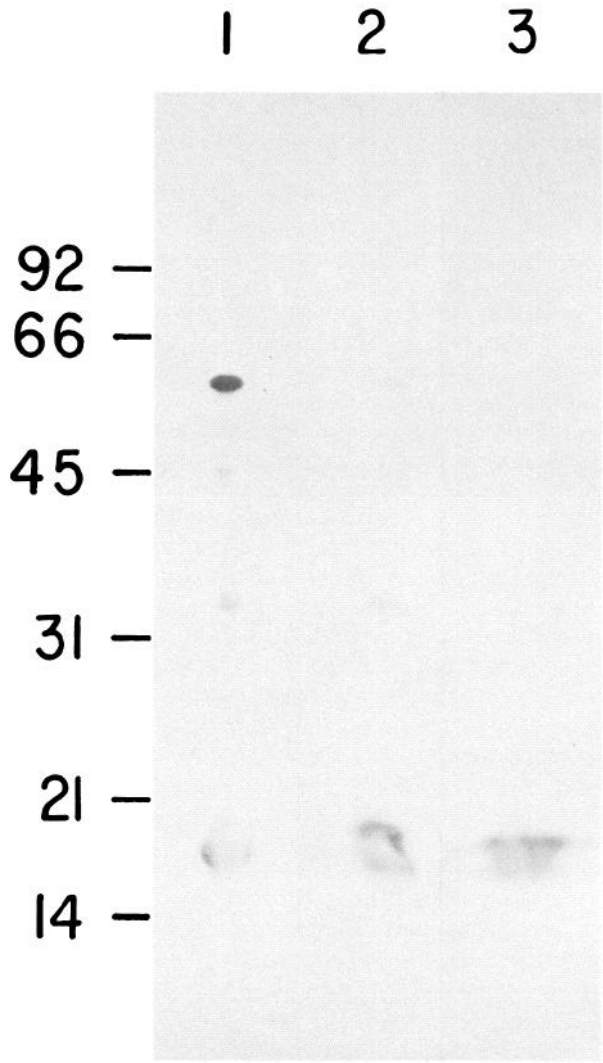

Figure 4. PAGE immunoblot of phosphatase and isolated PSD. A 12–18% linear gradient gel was run overnight at 7 mA/gel and the proteins were transfered to a nitrocellulose membrane. The resulting blot was incubated with antibodies and silver-enhanced as described under Materials and Methods. Lane *1*, 2 μg of bovine brain phosphatase; lane *2*, 100 μg of chick forebrain homogenate; lane *3*, 100 μg of chick PSD.

Discussion

Although calmodulin-dependent phosphatase has been found in many tissues and localized in a few, this report represents the first detailed quantitative localization of the enzyme in subcellular fractions isolated from any tissue. Inherent in any subcellular localization study are the limitations that the recovery of the enzyme is usually not quantitative and that the possibility of cross-contamination between fractions cannot be excluded. Thus, these results should be viewed with these limitations in mind.

We found that in chick brain the highest level of the enzyme occurred in the cytoplasm and microsomes. Further subcellular fractionation revealed that the synaptosome contained a considerable amount of the enzyme, which was localized exclusively to the synaptoplasm. A trace of phosphatase was detected in the PSD, and phosphatase was below the level of detection in mitochondria, synaptic vesicles, nuclei, and myelin.

Wood et al. (1980), Cooper et al. (1985), and Goto et al. (1986) have reported that the phosphatase is found only in neurons and not in any glial cells. We did not detect any phosphatase in chick brain myelin, nor did we detect it in brain nuclei. Kuret et al. (1986) also did not detect the enzyme activity in a rat liver nuclear lysate. The apparent lack of the phosphatase in myelin and nuclei implies that myelin basic protein and histone H_1, 2 protein substrates frequently used for *in vitro* characterization of the enzyme, may not be physiological substrates.

Using immunocytochemistry, Cooper et al. (1985) have observed pre- and postsynaptic localization of the phosphatase. Our results extend this study and show that more phosphatase is present presynaptically in the synaptoplasm than postsynaptically in the PSD. In the synaptoplasm, the phosphatase comprises 0.32% of the synaptoplasmic proteins, whereas it

accounts for a small amount in the synaptic membranes, and a trace level in the PSD.

The trace level of phosphatase found in the PSD appears to contrast with the immunocytochemical study of Wood et al. (1980), who found that phosphatase was predominantly localized at the PSD. Treatment of the brain tissue with fixative, a necessary step for immunocytochemical staining, could have cross-linked some soluble phosphatase to PSD; alternatively, some peroxidase reaction product could have precipitated at the PSD, giving rise to an apparently higher level of the enzyme. On the other hand, Somerville et al. (1984) have noted a high lability of PSD proteins, and, during our isolation procedure, some of the phosphatase might have dissociated from the PSD and not have been detected.

The finding that calmodulin-dependent phosphatase is concentrated in the cytosol of neurons and synaptosomes suggests that the enzyme may regulate protein dephosphorylation in nerve terminals. Synapsin is an *in vitro* substrate of the phosphatase and has been proposed as being involved in neurotransmitter release (Nestler and Greengard, 1984). Synapsin associated with synaptic vesicles is usually unphosphorylated, and when phosphorylated is dissociated from them (Schiebler et al., 1986), presumably facilitating the fusion of vesicles with the synaptic membrane and the release of neurotransmitter. Conceivably, phosphorylated synapsin would be accessible to the soluble synaptic phosphatase. DARPP-32 and G-substrate, which are cytosolic proteins dephosphorylated by the phosphatase *in vitro* (Nestler and Greengard, 1984), may likewise be potential *in vivo* substrates.

In summary, our results show that calmodulin-dependent phosphatase is primarily localized in the cytoplasm, microsomes, and synaptoplasm; these subcellular fractions may prove to be a useful source for the identification of physiological substrates.

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