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Calmodulin-Dependent Protein Phosphatase: A Developmental Study†

E. Ann Tallant and Wai Yiu Cheung*

ABSTRACT: Calmodulin-dependent protein phosphatase, one of the major calmodulin-binding proteins in bovine brain, dephosphorylates casein with a specific activity of 15 nmol mg^{-1} min^{-1} at 30 °C. The stimulation of phosphatase activity by calmodulin is reversed by ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid or trifluoperazine, a calmodulin antagonist. Antibodies raised in rabbit against the phosphatase inhibit the enzyme activity. The levels of the protein in brain extracts from various animals, determined by a radioimmunoassay, range from 20 μg/g of tissue in chick and fish brains to 143 μg in rat cerebrum. The ontogeny of the phosphatase was studied in nervous tissues from rat and chick, animals in which synaptogenesis takes place at different times during their development. The levels of the protein increased significantly in rat cerebrum and cerebellum and in chick brain and retina during the periods corresponding to major synapse formation. In rat cerebrum, the enzyme appeared to be equally distributed between the cytosol and the particulate fraction; the level in both compartments increased during the major period of synapse formation. Thus, the development of calmodulin-dependent protein phosphatase closely parallels synaptogenesis, implicating a role in some synaptic function.

Calmodulin, a multifunctional Ca^{2+}-binding modulator protein, is a major intracellular receptor of Ca^{2+}, regulating a wide spectrum of enzymatic activities [for review, see Wang & Waisman (1979), Cheung (1980), Klee et al. (1980), Brostrom & Wolff (1981), and Means et al. (1982)]. Calmodulin-dependent protein phosphatase is a major calmodulin-binding protein in brain extracts, and it dephosphorylates various proteins, including inhibitor 1, phosphorylase *b* kinase, casein, and histone (Stewart et al., 1982; Yang et al., 1982). This enzyme, previously referred to as calcineurin or CaM-BP_{80}, is particularly rich in the neostriatum (Wallace et al., 1980a) where it is localized principally at postsynaptic sites within neuronal somata and dendrites (Wood et al., 1980a). In chick retina, it has been found in both pre- and postsynaptic terminals (Cooper et al., 1982). The protein is a heterodimer, with the large subunit (M_r 60 000) binding calmodulin in a Ca^{2+}-dependent manner (Richman & Klee, 1978; Sharma et al., 1979; Wang et al., 1980) and the small subunit (M_r 16 500) binding four Ca^{2+} with high affinity ($K_d \leq 10^{-6}$ M) (Klee et al., 1979).

As part of our long-range goal to define the multifunctional roles of calmodulin in biological systems, we studied the ontogeny of calmodulin-dependent protein phosphatase in developing nervous tissues from rat and chick. The results of this investigation demonstrate that the phosphatase levels increase during the time of major synapse formation. A preliminary report of these results has appeared (Tallant & Cheung, 1982).

Materials and Methods

Materials. ^{125}I (16–20 mCi/μg) was purchased from Amersham and [γ-^{32}P]ATP (2 mCi/mmol) from New England Nuclear. Sodium dodecyl sulfate ($NaDodSO_4$)[1] and Affi-Gel Blue were obtained from Bio-Rad, and iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) was from Pierce Chemical Co. Sigma Chemical Co. supplied Triton X-100, phenylmethanesulfonyl fluoride, leupeptin, aprotinin, bovine serum

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; CaM, calmodulin; Tris, tris(hydroxymethyl)aminomethane.

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albumin, and dithiothreitol (DTT). Vitamin-free casein was purchased from ICN Nutritional Biochemicals. Porcine and bovine tissues were generous gifts of Fineberg Packing Co. of Memphis and were transported to the laboratory on ice. Other animals were obtained from the following sources: fertile White Leghorn chicken eggs from a local hatchery; pregnant Sprague-Dawley rats and Balb/c adult male mice from Harlan Sprague-Dawley; *Rana pipiens* frogs from Nasco. Tennessee catfish was a gift from Dr. Kent Gartner, Memphis State University, goat anti-rabbit IgG serum was generously supplied by Dr. William Walker, St. Jude Children's Research Hospital, and trifluoperazine was kindly provided by Dr. Harry Green of Smith, Kline and French Laboratories. All other reagents were of highest analytical grade.

Calmodulin was purified to apparent homogeneity by using fluphenazine–Sepharose affinity chromatography (Wallace et al., 1980b). Antisera against the phosphatase and calmodulin were raised in rabbits with native phosphatase (Wallace et al., 1980a) and dinitrophenylcalmodulin (Wallace & Cheung, 1979), respectively. Whole sera were used for the radioimmunoassays.

The catalytic subunit of cAMP-dependent protein kinase was isolated from bovine heart according to a procedure developed for bovine liver (Sudgen et al., 1976). Casein was labeled with $^{32}P_i$ by the catalytic subunit of cAMP-dependent protein kinase (Khandelwal et al., 1976).

Purification of Phosphatase. The enzyme was purified from bovine brain (Wallace et al., 1979) with some modifications which were necessitated primarily because the amount of chemically bonded dye in commercial Affi-Gel Blue varied with preparation. The Affi-Gel Blue column was eluted with buffer A [50 mM Tris-HCl (pH 7.8), 3 mM $MgSO_4$, 0.5 mM DTT, 1 mM EGTA, and 0.02% NaN_3]; the original buffer A contained 0.2 M NaCl, which was omitted to reduce the elution of contaminating proteins from the column. Peak enzyme fractions were pooled and loaded onto a calmodulin-affinity column as previously described (Wallace et al., 1979). The pooled fractions from the affinity column were then loaded onto a DEAE-cellulose column (5.0 × 0.7 cm), washed with 100 mL of buffer A, and eluted with a linear NaCl gradient (0–0.2 M) in 200 mL of buffer A. Phosphatase was eluted at approximately 0.08 M NaCl. The protein peak was concentrated by ultrafiltration on an Amicon PM-10 membrane and stored in small fractions at −70 °C. The overall yield of the protein was about 3–4 mg/kg of bovine cerebra. The DEAE-cellulose column improved the purity of the enzyme but at a considerable sacrifice in yield.

Phosphatase Assay. Phosphatase activity was measured by the release of $^{32}P_i$ from ^{32}P-labeled casein. The assay mixture (final volume 50 μL) contained 50 mM Tris-HCl (pH 7.0), 0.5 mM DTT, 0.1 mM $CaCl_2$, 15 μg of ^{32}P-labeled casein (about 40 000 cpm), 50 μg of bovine serum albumin, and an appropriate amount of enzyme. After a 5-min preincubation at 30 °C, the reaction was initiated with ^{32}P-labeled casein, and incubation was continued for 5–20 min at 30 °C. The reaction was terminated by the addition of 100 μL of ice-cold 25% trichloroacetic acid and 100 μL of bovine serum albumin (6 mg/mL). After a 15-min incubation on ice, the samples were centrifuged, and 0.15 mL of the supernatant fluid was counted by liquid scintillation spectrophotometry (Yang et al., 1980). The reaction was linear up to 15% phosphate released and was, therefore, kept within this limit. All of the ^{32}P radioactivity that was released by the phosphatase could be extracted into acid–molybdate, demonstrating that the ^{32}P radioactivity represented phosphate and not [^{32}P]phosphopeptides that might have been released by a trace contaminant of tissue proteases.

Tissue Preparation for Radioimmunoassay. Rat pups were chosen at ages from newborn to 30 days. The animals were killed by decapitation; their brains were quickly removed, frozen in liquid N_2, and stored at −70 °C until use. Unless indicated otherwise, the tissues were homogenized in 50 volumes of buffer A containing 0.25% $NaDodSO_4$ in a glass/Teflon tissue homogenizer; the homogenate was incubated in a boiling water bath for 10 min. A trace of unsolubilized tissue was removed by centrifugation at 25000*g* for 10 min at 4 °C. All samples were stored at −70 °C until use. Brain from other animals was processed similarly. White Leghorn chicken eggs were incubated in a humidified atmosphere at 37 °C. At appropriate times, embryos were removed, and the whole brain and retina were excised and processed as indicated. Embryo ages were confirmed by comparison with the staging of Hamburger & Hamilton (1951).

Iodination of Phosphatase and Calmodulin. Phosphatase was iodinated with iodogen, a chloramide insoluble in aqueous media, according to the procedure of Fraker & Speck (1978). The reaction mixture (25 μL) contained 5 μg of protein in 0.2 M sodium phosphate (pH 7.2) and 1.0 mCi of ^{125}I; iodination was initiated by transferring the reaction mixture into a tube (12 × 75 mm) coated with a thin film of iodogen (0.5 μg). After 60 min at 4 °C, the solution was quantitatively transferred to a Sephadex G-25 column (17 × 0.7 cm) to separate unreacted ^{125}I. The column had been equilibrated with phosphate-buffered saline (0.15 M NaCl and 20 mM sodium phosphate, pH 7.2) containing 0.05% Triton X-100. The phosphatase contained 1–2 mol of ^{125}I with a specific activity of 30–40 μCi/μg.

Calmodulin was iodinated similarly; the time of incubation was reduced to 10 min. Calmodulin contained 0.5 mol of ^{125}I with a specific activity of 60 μCi/μg.

The iodinated proteins were stored at −70 °C in small aliquots in the presence of 0.1 mg/mL bovine serum albumin.

Radioimmunoassay of Phosphatase and Calmodulin. Radioimmunoassays were performed in BDS polymer tubes (Evergreen) in a final reaction volume of 150 μL containing 50 mM Tris-HCl (pH 7.8), 3 mM $MgSO_4$, 4.3 mM EGTA, 66.7 μM phenylmethanesulfonyl fluoride, 6.7 μM leupeptin, and 10–20 milliunits of aprotinin. Triton X-100 was added to a final concentration of 1% to maintain a ratio of Triton X-100 to $NaDodSO_4$ in excess of 5:1 (Goelz et al., 1981). To the reaction mixture were added sequentially the following, with a 20-min incubation between each addition: sample or sample buffer (100 μL), antiserum or normal rabbit serum (0.5 μL), ^{125}I protein (20 000 cpm), and goat anti-rabbit IgG serum (5 μL). The mixture was incubated overnight at 4 °C with shaking. The precipitate was collected by centrifugation at 20000*g* for 10 min at 4 °C, and the supernatant fluid was removed by aspiration. The pellet was counted in a Nuclear Chicago γ spectrophotometer. Data were corrected for nonspecific binding, which accounted for about 3% of total input cpm. At least two and frequently four concentrations of each sample were assayed, to verify that the curve in the radioimmunoassay was parallel to the standard. Each determination was duplicated.

Protein Determination. Protein was determined according to Lowry et al. (1951), after the proteins had been precipitated with 10 volumes of cold 12.5% trichloroacetic acid. Bovine serum albumin was used as a standard.

Results and Discussion

Stimulation of Bovine Brain Protein Phosphatase by

Table I: Effect of Anti-Phosphatase Serum on the Activity of Calmodulin-Dependent Protein Phosphatase from Bovine Brain[a]

other additions	phosphatase activity (nmol of phosphate released mg^{-1} min^{-1})
none	0.42
calmodulin	17.11
normal rabbit serum	0.37
calmodulin + normal rabbit serum	17.30
anti-phosphatase serum	0.0
calmodulin + anti-phosphatase serum	5.02

[a] The reaction mixture contained the usual ingredients and other additions as indicated above. Calmodulin, 1.3 μM; normal whole rabbit serum, 10 μL; anti-phosphatase whole serum, 10 μL. The concentration of phosphatase was 3.8 nM. The enzyme was preincubated with the serum for 20 min at 30 °C before the addition of ^{32}P-labeled casein. Phosphatase activity was determined as described under Materials and Methods. The data are expressed in nanomoles of phosphate released per milligram of protein per minute, the background (with boiled phosphatase) having been substrated. Each determination was duplicated.

Calmodulin. The dependence of bovine brain protein phosphatase activity on Ca^{2+} and calmodulin has been shown previously with inhibitor 1 (Yang et al., 1982). In this study, we have used phosphorylated casein as the substrate. Our results show that in the presence of Ca^{2+} and calmodulin, phosphatase activity increased some 20-fold. In the absence of Ca^{2+}, calmodulin did not stimulate the enzyme. Trifluoperazine, an antipsychotic agent which antagonizes the biological activity of calmodulin (Levin & Weiss, 1976), did not affect the basal enzyme activity but abolished the calmodulin-stimulated activity. Troponin C, a close analogue of calmodulin, did not substitute for calmodulin, even at a concentration of 5-fold higher. These data support our earlier demonstration that the protein phosphatase from bovine brain is calmodulin dependent (Yang et al., 1982).

Effect of Anti-Phosphatase Serum on Bovine Brain Calmodulin-Dependent Protein Phosphatase Activity. Antibodies against the bovine brain phosphatase were raised in the rabbit. As shown in Table I, the antibodies suppressed both the basal and calmodulin-dependent phosphatase activities. In the control experiments, the normal serum did not show any effect on the two activities. Previous experiments have demonstrated that these antibodies were specific for the phosphatase (Wallace et al., 1980a).

Establishment of a Detergent-Based Radioimmunoassay. A radioimmunoassay was developed previously in this laboratory to quantify calmodulin-dependent phosphatase levels in various tissues (Wallace et al., 1980a). Those determinations were made in the soluble fraction of tissue extracts (100000*g* supernatant fluid). Subsequent studies (see Figure 2) indicated that a substantial fraction of the enzyme was associated with a 100000*g* particulate fraction. Therefore, the radioimmunoassay was modified as described below to allow measurement of this enzyme in the particulate fraction after having been solubilized in detergent.

Previous experiments showed that the presence of up to 1% of Triton X-100, a nonionic detergent, in the reaction mixture did not adversely affect the assay. However, this level of Triton X-100 did not solubilize all proteins from the particulate fraction. On the other hand, tissues are more readily solubilized in $NaDodSO_4$, an anionic detergent. Although the antigen–antibody complex is totally disrupted by 1% $NaDodSO_4$ alone, the presence of a 5-fold excess of Triton X-100 stabilizes the complex. Thus, the combined action of the two detergents allows more effective solubilization of the phosphatase in the particulate fraction and its determination by a radioimmunoassay. These findings are similar to those of Goelz et al. (1981) who described a detergent-based radioimmunoassay for protein I.

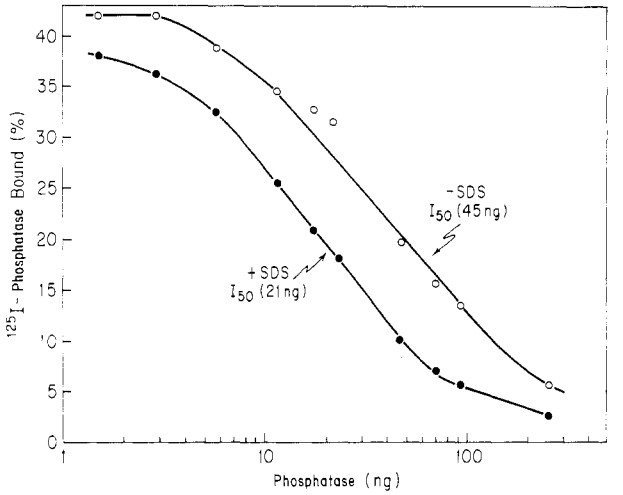

FIGURE 1: Radioimmunoassay of phosphatase in the presence or absence of $NaDodSO_4$. The final $NaDodSO_4$ concentration in the assay was 0.167%. Details are described under Materials and Methods. I_{50} refers to the concentration of the protein displacing 50% of ^{125}I-phosphatase.

Figure 1 shows the competition curves generated by the modified radioimmunoassay for the phosphatase; they were parallel in the presence or absence of $NaDodSO_4$. In fact, the presence of $NaDodSO_4$ shifted the curve to the left, indicating a greater sensitivity. Similar results were obtained for the radioimmunoassay of calmodulin (data not shown). These findings show the advantage of using $NaDodSO_4$ to solubilize proteins in a particulate fraction for radioimmunoassay.

Phosphatase Levels in Tissues of Various Animals. We have reported that calmodulin-dependent phosphatase is found primarily in brain tissues; the amount in nonnervous tissues is relatively low (Wallace et al., 1980a). Determination of the protein in nonnervous tissues by the radioimmunoassay generated competition curves which were not parallel to the standard curve; the possibility exists, however, that the phosphatase in the nonnervous tissues may be different and thus not be fully recognized by the antibodies which were raised in rabbits against the protein from bovine brain.

In this study, we have focused our experiments on two nervous tissues of various animals: brain and retina. Phosphatase was detected in all the brains examined, and the competition curves were parallel to the standard curve shown in Figure 1. Table II summarizes the levels of the protein in these brains; the amounts vary from 20 μg/g of tissue in fish brain to 143 μg/g of tissue in rat cerebrum. The levels in cerebellum are generally lower than those in the cerebrum, in agreement with an earlier study (Wallace et al., 1980a). Of the brains examined, rat exhibited the highest level of the enzyme, severalfold higher than that in fish and chick.

Phosphatase Levels in Developing Rat Brain. Rat brain is not fully developed at birth, and the majority of synaptogenesis takes place postnatally. In rat cerebrum, the number of synapses increases markedly between 1 and 4 weeks postnatally, and the number of synaptic vesicles increases with a similar time course (Aghajanian & Bloom, 1967; Hattori & McGeer, 1973; Armstrong-James & Johnson, 1970). Similarly, in rat cerebellum, histochemical and electrophysiological analyses (Woodward et al., 1969) indicate that rapid maturation occurs during the second week and that adult cells appear during the third and fourth weeks.

Table II: Level of Calmodulin-Dependent Protein Phosphatase in Various Animal Brains[a]

animal	phosphatase	
	μg/mg of protein	μg/g of tissue
bovine cerebrum	0.82 ± 0.04	55.7 ± 2.6
bovine cerebellum	0.45 ± 0.08	37.4 ± 3.2
rat cerebrum	2.89 ± 0.21	142.6 ± 16.3
rat cerebellum	1.00 ± 0.02	106.7 ± 18.9
porcine cerebrum	1.12 ± 0.11	60.2 ± 13.2
mouse whole brain	1.99 ± 0.07	78.2 ± 3.3
frog whole brain	0.80 ± 0.06	36.1 ± 0.7
fish whole brain	0.40 ± 0.03	20.1 ± 8.7
chick whole brain	0.37 ± 0.01	22.4 ± 1.4

[a] The level of phosphatase was measured in brain solubilized in buffer A containing 0.25% $NaDodSO_4$, as described under Materials and Methods. All brains were taken from adult animals with the exception of the chick, which was 3 days old. Data represent the mean ± standard deviation on tissues from two animals. Each determination was duplicated.

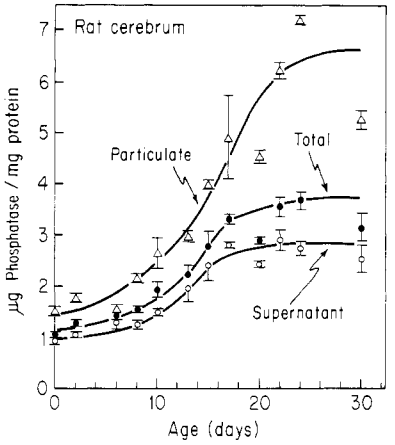

FIGURE 2: Level of phosphatase in supernatant and particulate fractions of rat cerebrum during postnatal development. The tissue was homogenized in 3 volumes of buffer A containing 100 μM phenylmethanesulfonyl fluoride in a glass/Teflon tissue homogenizer. The homogenate was centrifuged at 100000*g* for 1 h to obtain a supernatant fluid and a pellet. The pellet was rehomogenized and centrifuged similarly, and the second supernatant fluid was combined with the first. Repeated washing of the particulate fraction only released 1–2% more of the total phosphatase activity and was, therefore, not routinely performed. The pellet was then homogenized in buffer A containing 0.2% Triton X-100 in a volume equal to the first homogenization buffer; the suspension was gently shaken for 15 h at 4 °C and centrifuged at 100000*g* for 1 h. This supernatant fluid was designated as the particulate fraction. Total activity was obtained as the summed activities of the supernatant and particulate fractions. The concentration of Triton X-100 in the buffer only solubilized 40% of the phosphatase and other proteins. Since the data are expressed on the basis of milligrams of protein, this underestimation does not affect the data as presented in the figure. The radioimmunoassay was as described under Materials and Methods except that the reaction mixture contained no $NaDodSO_4$ and the concentration of Triton X-100 was reduced to 0.05%. Each point represents the mean ± standard deviation of tissues from two animals. Each determination was duplicated.

The developmental change of calmodulin-dependent phosphatase was first examined in rat cerebrum. A supernatant fraction and a particulate fraction were prepared from the tissue, and phosphatase was determined in both fractions. Figure 2 shows that the level of enzyme in the supernatant fraction was low in the newborn and increased severalfold between days 10 and 20, the period of major synapse formation. The level in the particulate fraction increased similarly. Note that the specific activity of the phosphatase in the particulate fraction is considerably higher than that in the

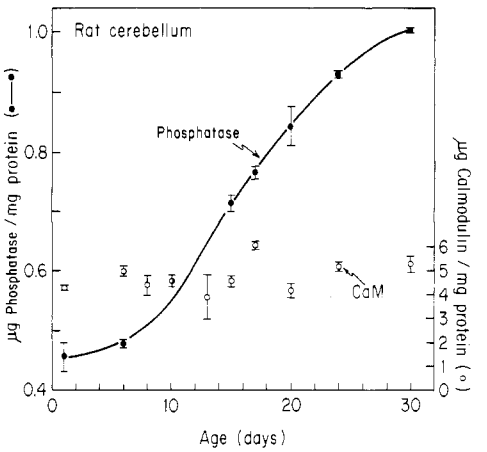

FIGURE 3: Level of phosphatase and calmodulin in rat cerebellum during postnatal development. Tissue preparation and radioimmunoassays are as described under Materials and Methods. The data are expressed per milligram of protein. Each point represents the mean ± standard deviation of tissues from two animals.

supernatant. It is conceivable that some of the activity in the supernatant fluid may have been solubilized during homogenization of the tissue, a subject deserving further study. When the levels of phosphatase are expressed on the basis of gram wet weight, the protein appears to be equally distributed between the soluble and particulate fractions (data not shown).

The high level of phosphatase in the particulate is not unexpected; a previous immunocytochemical study on mouse (Wood et al., 1980a) and rat (Wood et al., 1980b) showed its localization with postsynaptic densities and dendritic microtubules, structures normally found in the particulate fraction. Moreover, subcellular fractionation studies showed that isolated postsynaptic densities from dog contained this protein (Carlin et al., 1981).

In view of the considerable level of phosphatase in the particulate fraction, the extraction medium was modified in all subsequent studies to contain 0.25% $NaDodSO_4$. The inclusion of $NaDodSO_4$ solubilized essentially all the protein and thus measured more accurately the total tissue level of the protein. Moreover, as shown in Figure 1, this modification increased the sensitivity of the radioimmunoassay.

Using the modified procedure, we found that phosphatase levels in developing rat cerebellum increased markedly between days 10 and 20 postnatally, reaching a plateau at about day 30 (Figure 3). Thus, the developmental profile of the enzyme in the cerebellum is qualitatively identical with that in the cerebrum (Figure 2).

As a comparison, the calmodulin levels were measured in these samples. Our results did not reveal any dramatic change during this period, in agreement with a previous study in which calmodulin levels were measured by their ability to activate bovine brain phosphodiesterase (Smoake et al., 1974). The rather constant level of calmodulin in the developing cerebellum is of interest from another viewpoint: it serves as a "control" in that the marked increase of the phosphatase during the period of synaptogenesis is perhaps not fortuitous.

Figure 3 also shows that in rat cerebellum, the level of calmodulin is markedly higher than that of the phosphatase throughout the course of development—some 30-fold higher on a molar basis at day 30. The molecular weight of calmodulin (16700) is approximately one-fifth that of the phosphatase (80000). The finding of such a disparate molar ratio of the two proteins is not surprising; calmodulin regulates many enzymes, and calmodulin-dependent phosphatase is only one of them.

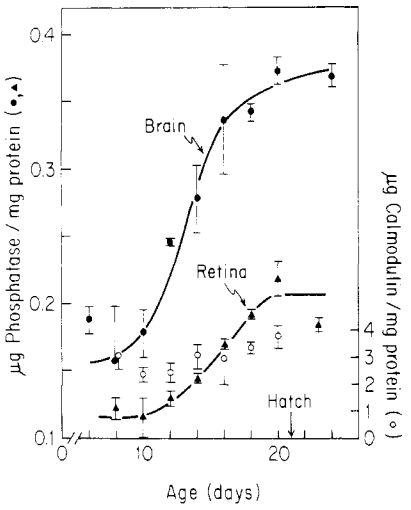

FIGURE 4: Level of phosphatase in chick brain and retina during embryonic development. Tissue preparation and radioimmunoassay are described under Materials and Methods. As a comparison, calmodulin levels in the retina were also measured. Phosphatase and calmodulin levels are expressed per milligram protein. Each point represents the mean ± standard deviation of tissue from two animals.

Phosphatase Levels in Developing Chick Nervous Tissues. Rats are born with their eyes closed and are capable of only limited activities, primarily because they are neurologically underdeveloped. In contrast, the chick is hatched with its eyes open and is fully alert in all aspects of activity, indicative of a fully developed neuronal system. Therefore, we extended the developmental studies to the chick. Figure 4 shows that in the embryonic chick brain, calmodulin-dependent phosphatase levels increased 2-fold between day 10 and the time of hatching at day 21, a period corresponding to major synaptogenesis.

In the embryonic chick, the retina is a most conspicuous organ and has been used extensively as an experimental system in many developmental studies. Figure 4 shows that the developmental pattern of the phosphatase in the retina is almost identical with that observed in the embryonic brain, again indicating a close correlation between synthesis of this protein and synaptogenesis.

As a further control, the level of calmodulin was determined in the retina and did not change appreciably during this period, reminiscent of the findings in rat cerebellum. Compared to rat cerebellum, the level of calmodulin relative to that of phosphatase is even higher in the retina, some 100-fold higher at the time of hatching.

The increase of the phosphatase in chick brain and retina is well correlated with the morphological development of synapse structure. In chick cerebellar cortex studied by electron microscopy, granule neurons begin to differentiate around day 11 of incubation, and the number of fibers increases until the time of hatching (Mugnaini, 1969). Similarly, photoreceptor synaptogenesis in embryonic and hatchling chicks has been studied by electron microscopy; photoreceptors line up around day 11 whereas synapses mature between days 17 and 21 (McLaughlin, 1976).

Comments on the Use of Radioimmunoassay for the Measurement of Calmodulin-Dependent Protein Phosphatase in Tissues. In addition to the radioimmunoassays, we measured calmodulin-dependent phosphatase in a bovine brain extract by an enzymatic assay using casein as a substrate. The extract contains a high level of phosphatase activity, about 60% of which appears to be calmodulin independent (data not shown), arbitrarily defined as the activity measured in the presence of trifluoperazine. Others have reported high levels of protein phosphatase activities in brain (Maeno & Greengard, 1972). Trifluoperazine, a hydrophobic compound, is not strictly specific for calmodulin and is known to affect the activities of various calmodulin-independent enzymes. Against a high level of phosphatase activities, the measurement of calmodulin-dependent phosphatase using trifluoperazine as an index is probably not accurate. Moreover, enzymatic activity associated with a particulate fraction—some 50% of the calmodulin-dependent phosphatase appears to be particulate—is frequently not fully accessible, and solubilization with a detergent invariably results in its inactivation. The use of a radioimmunoassay, which measures the level of the enzyme on the basis of its mass instead of enzymatic activity, would give a more accurate assessment of the tissue level of calmodulin-dependent protein phosphatase in tissue extracts.

Concluding Remarks. Phosphorylation of proteins by kinases and their subsequent dephosphorylation by phosphatases constitute a major cellular regulatory mechanism (Krebs & Beavo, 1979; Greengard, 1978). In the brain, calmodulin-dependent phosphorylation may control the chain of events involved in synaptic transmission from the synthesis of neurotransmitters to their subsequent release. Schulman & Greengard (1978) reported that brain contained an active calmodulin-dependent protein kinase which catalyzed the phosphorylation of a number of endogenous proteins, including protein I, which is exclusively associated with the membrane of vesicles (Bloom et al., 1979). DeLorenzo et al. (1979) found that a calmodulin-dependent kinase catalyzed the phosphorylation of certain presynaptic membrane proteins, believed to cause the release of neurotransmitters. Indeed, Yamauchi & Fujisawa (1979, 1980) demonstrated that a calmodulin-dependent protein kinase regulated tyrosine 3-monooxygenase and tryptophan 5-monooxygenase, enzymes involved in the synthesis of neurotransmitters such as dopamine, norepinephrine, epinephrine, and serotonin.

Protein phosphatase has been detected in the brain as in many other tissues. In the cytosol of rat cerebral cortex, three distinct protein phosphatases were found, distinguishable by their substrate specificity (Maeno & Greengard, 1972). Which, if any, of these phosphatases is dependent on calmodulin for activity is not known. Since the calmodulin-dependent protein phosphatase is found highly enriched in the brain, especially in certain regions such as neostriatum (Wallace et al., 1980a), where it is localized with postsynaptic densities and dendritic microtubules (Wood et al., 1980a), the possibility that it plays an important role in synaptic function appears likely. The close correlation between the synthesis of this enzyme and the development and differentiation of synapses further emphasizes its potential importance in some neuronal function.

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Registry No. Phosphoprotein phosphatase, 9025-75-6; trifluoperazine, 117-89-5; EGTA, 67-42-5.

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