Differential localization of calmodulin-dependent enzymes in rat brain: Evidence for selective expression of cyclic nucleotide phosphodiesterase in specific neurons

(calmodulin-binding proteins/calcinurin/immunocytochemistry)

RANDALL L. KINCAID*, CAREY D. BALABAN‡, AND MELVIN L. BILLINGSLEY‡

*Laboratory of Cellular Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892; and ‡Departments of Anatomy and Surgery and ‡Department of Pharmacology and The Cell and Molecular Biology Center, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033

Communicated by Karl H. Beyer, Jr., October 8, 1986

ABSTRACT High-affinity antibodies against calmodulin (CaM)-dependent cyclic nucleotide phosphodiesterase and protein phosphatase (calcineurin) were purified and characterized. Anti-calsequestrin antibody did not react with other phosphodiesterases or with the regulatory subunits of cAMP-dependent protein kinase. Affinity-purified goat anti-calsequestrin antibody recognized both the 61-kDa catalytic subunit and the 18-kDa Ca²⁺-binding subunit of the phosphatase. Neither antibody reacted with CaM, many CaM-binding proteins (calmodulin-dependent protein kinase, myosin light chain kinase, fodrin), or other cytoskeletal proteins from brain. The antibodies were used to compare the cellular localization of these two CaM-dependent enzymes in rat brain. Both calcineurin and phosphodiesterase were found predominantly in nerve cells; however, phosphodiesterase was restricted to very specific neuronal populations. Phosphodiesterase was prominent in the somatic cytoplasm and dendrites of regional output neurons—e.g., cerebellar Purkinje cells and hippocampal and cortical pyramidal cells. The extensive and uniform staining in the dendrites is consistent with postsynaptic localization and suggested an important function for this enzyme in neurons that integrate multiple convergent inputs. Calcineurin was present in virtually all classes of neurons, with immunoreactivity confined primarily to cell bodies. Both diffuse cytoplasmic staining and characteristic punctate staining of cell bodies were observed; the latter suggested compartmentalization of calcineurin at or near the plasma membrane. The results of this study demonstrate that calcineurin and phosphodiesterase are differentially localized in the central nervous system. Thus, the expression and compartmentalization of CaM-binding proteins may be highly regulated and specific for particular differentiated nerve cell types.

Our understanding of cyclic nucleotide and phosphoprotein metabolism relies heavily upon in vitro studies of enzymes isolated from specific tissues. However, without concomitant knowledge of their regional and subcellular distribution, the physiologic functions subserved by these enzymes are difficult to assess. It is known that cyclic nucleotide metabolism is important in mediating hormone-dependent cellular events in many tissues (1) and is thought to be involved in adrenergic neurotransmission in the central nervous system (2) and photoreception in mammalian rod outer segment (3). Interestingly, both the synthetic and degradative enzymes for cAMP metabolism have their highest specific activities in brain and are positively modulated by Ca²⁺ through the Ca²⁺-binding protein calmodulin (CaM) (4, 5). These facts suggest important and interactive roles for Ca²⁺ and cAMP-regulated systems in the function of nerve cells (6).

Perhaps equally important in control of nerve cell activity is the state of protein phosphorylation, which reflects the activities of several species of protein kinase and phosphatase (7, 8). Of particular interest has been the involvement of the CaM-dependent kinases and their putative substrates in neurotransmission. Increases in the activity of this enzyme have been correlated with increased electrical activity (9), and the kinase appears to be highly concentrated in synap-osomal preparations and postsynaptic densities (10, 11). Furthermore, it has now been established that a major CaM-binding protein in brain, calcineurin (12), is itself a protein phosphatase (13), suggesting that protein dephosphorylation is an important function controlled by Ca²⁺. While the preferred phosphoprotein substrate of this enzyme is not yet known, several neuron-specific proteins can serve as substrates of this enzyme (14).

It is tempting to speculate that Ca²⁺/CaM, cyclic nucleotides, and protein phosphorylation may interact in a concerted fashion to modulate neuronal activity. However, to support such a hypothesis, it is necessary to document and compare the regional and subcellular localization of such enzymes in nerve cells—i.e., are the CaM-dependent enzymes of cyclic nucleotide and phosphoprotein metabolism coordinately expressed in the same neurons or are they distributed in different populations of nerve cells? Since Ca²⁺-dependent events might vary greatly for different neurons, it is possible that only specific enzyme activities may be expressed in a particular differentiated cell type.

We have investigated these alternatives by using immunocytochemical localization of phosphodiesterase and calcineurin with high-affinity antibodies specific for these enzymes. These antibodies show no cross-reactivity with proteins of related function (i.e., CaM-binding proteins, phosphodiesterases) or with other cytosolic brain proteins. The present report compares the distribution of these proteins in brain and discusses their patterns of localization with regard to potential functional roles for these enzymes in the central nervous system.§

METHODS

Purification of CaM-Binding Proteins. Phosphodiesterase and calcineurin were purified to homogeneity from bovine brain as previously described (15).

Production and Purification of Antibodies. Adult male rabbits (The Jackson Laboratory, strain AJX-1) were injected

Abbreviation: CaM, calmodulin.

§A preliminary account of this work was presented at the 69th Annual Meeting of the Federation of American Societies for Experimental Biology in Anaheim, CA, April 21–26, 1985.
subcutaneously with phosphodiesterase (120 μg in complete Freund's adjuvant) and thereafter with 80 μg in incomplete Freund's adjuvant at 12- to 30-day intervals. Twelve days after the second injection, rabbits werebled via the marginal vein of the ear and serum was obtained by centrifugation after clotting. The IgG fraction was prepared by chromatography on a column of staphylococcal protein A-Sepharose (Pharmacia), 1 ml of bed volume per ml of undiluted serum, essentially as described by the manufacturer. After washing with 4 bed volumes of 0.02 M Tris- HCl, pH 8.0, containing 0.1 M NaCl and 0.1 mM EGTA (buffer A), the column was eluted with 0.1 M sodium acetate, pH 3.8, containing 10% (vol/vol) glycerol (buffer B), and the eluate was neutralized with 2 M Tris-HCl, pH 9.0, and adjusted to 2 mg of IgG per ml. For long-term storage this anti-phosphodiesterase IgG fraction was dialyzed overnight against 10 vol of buffer B containing 45% (vol/vol) glycerol and stored at -20°C.

An adult female goat was injected subcutaneously with purified calcineurin (1 mg in complete Freund's adjuvant) and thereafter with 1 mg in incomplete adjuvant at 30- to 40-day intervals. The animal was subjected to plasmaphoresis 7-10 days after injection and the plasma was stored at 4°C. Portions of the goat plasma (350 ml) were incubated overnight with 10 ml of calcineurin-Sepharose 4B (2 mg of protein per ml of gel) (16) with gentle mixing, after which the gel was transferred to a column (2.5 cm diameter). After washing with 60 ml of buffer A, the column was eluted with buffer B into a tube containing 0.1 bed volume of 2 M Tris, pH 9, and mixed. The eluate was chromatographed on a column of protein A-Sepharose (4 mg of eluted protein per ml of gel), as described above for phosphodiesterase antibody. This protein A eluate of affinity-purified antibody was used as the anti-calcineurin antibody.

**Results**

**Characterization and Specificity of Antibodies.** Antisera against bovine brain CaM-dependent cyclic nucleotide phosphodiesterase and protein phosphatase (calcineurin) were further purified by affinity chromatography on protein A-Sepharose and, in the case of calcineurin, by using antigen coupled to CNBr-activated Sepharose (see Methods). Phosphodiesterase antibody did not react with rod outer segment phosphodiesterase, purified liver cyclic nucleotide-stimulated phosphodiesterase, or the regulatory subunits of cAMP-dependent protein kinase (data not shown). Anti-phosphodiesterase antibody detected the 60-kDa peptide of the brain enzyme but did not react with other brain CaM-binding proteins (e.g., CaM-dependent protein kinase, fodrin, caldesmon, calcineurin) or with other proteins from whole brain supernatant (Fig. 1A). A faint immunoreactive peptide of approximately 70 kDa was observed in some immunoblots. This may represent a slightly larger form of the brain CaM-activated enzyme, which has been described (20).

The affinity-purified calcineurin antibody recognized both the 61- and 18-kDa subunits of the purified phosphatase and did not react with phosphodiesterase (Fig. 1B), other CaM-binding proteins, or other proteins from brain cytosol. Solid-phase immunoassays with immobilized calcineurin and phosphodiesterase showed no significant cross-reactivity of either antibody at dilutions of 1:200 or less (data provided to reviewers). The antibodies detected as little as 5 ng of phosphodiesterase or 10 ng of calcineurin present in blots of crude samples. Since the specificity and sensitivity of these antibodies had been established, comparison of the localization of these two enzymes in rat brain sections was carried out. Preimmune sera did not react with brain sections.

**Neuronal Distribution of Phosphodiesterase and Calcineurin in Rat Brain.** The number and pattern of cells immunoreactive for phosphodiesterase or calcineurin were quite different. Although both enzymes appeared to be absent from nonneuronal cell types, calcineurin was distributed widely, while only specific neuronal populations reacted with antibodies against phosphodiesterase. In some areas, the difference in immunoreactivity with these two enzymes was virtually all-or-none. For example, calcineurin immunoreactivity in hypothalamus was seen in all neurons of the paraventricular nucleus and adjacent structures (Fig. 2).

The 18-kDa Ca2+-binding subunit of calcineurin (12) was often difficult to detect in immunoblots of brain cytosol. Other Ca2+-binding proteins (CaM, S100 protein) are known to exhibit variable adsorption to nitrocellulose (21).
indicated on brain supernatant. 26 trypsinogen, Research (Bethesda to transferred electrophoresed on sodium dodecyl sulfate gels (7% acrylamide) and transferred to nitrocellulose. The positions of protein standards (Bethesda Research Laboratories) run in the same experiment are indicated on the ordinate (myosin, 200 kDa; phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 45 kDa; chymotrypsinogen, 26 kDa). Lane 1 was stained for protein with amido black. Lane 2 was allowed to react with biotinylated CaM (20), showing the many CaM-binding proteins in crude cytosol. Lanes 3 and 4 were incubated with antibodies against phosphodiesterase and calcineurin, respectively. (Incubation for 2.5 hr, 25°C at 1:1000 dilution of antibody.) (B) Immunoblot of purified proteins. Duplicate samples of phosphodiesterase (lanes 1 and 3; 1.2 μg) and calcineurin (lanes 2 and 4; 1.6 μg) were electrophoresed and transferred to nitrocellulose. One section was stained with amido black (lanes 1 and 2) and the other was incubated with anti-calcineurin antibody (lanes 3 and 4).

However, phosphodiesterase antibody staining was strikingly absent, being confined to the dorsal cap of the paraventricular nucleus, and a strand of cells lateral to the nucleus. Thus, although most neurons in the nucleus contained substantial CaM-dependent phosphatase, very low amounts of phosphodiesterase were present in the same cells.

FIG. 1. Specificity of immunoreactivity of anti-phosphodiesterase and anti-calcineurin antibodies. (A) Immunoblot of crude rat brain supernatant. Samples of rat brain cytosol (24 μg per lane) were electrophoresed on sodium dodecyl sulfate gels (7% acrylamide) and transferred to nitrocellulose. The positions of protein standards (Bethesda Research Laboratories) run in the same experiment are indicated on the ordinate (myosin, 200 kDa; phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 45 kDa; chymotrypsinogen, 26 kDa). Lane 1 was stained for protein with amido black. Lane 2 was allowed to react with biotinylated CaM (20), showing the many CaM-binding proteins in crude cytosol. Lanes 3 and 4 were incubated with antibodies against phosphodiesterase and calcineurin, respectively. (Incubation for 2.5 hr, 25°C at 1:1000 dilution of antibody.) (B) Immunoblot of purified proteins. Duplicate samples of phosphodiesterase (lanes 1 and 3; 1.2 μg) and calcineurin (lanes 2 and 4; 1.6 μg) were electrophoresed and transferred to nitrocellulose. One section was stained with amido black (lanes 1 and 2) and the other was incubated with anti-calcineurin antibody (lanes 3 and 4).

This indicated differential expression of these two CaM-dependent proteins in some neurons.

Although unreactive in many brain regions, the phosphodiesterase antibody produced intense immunoreactivity in specific populations of neurons in the neocortex (Fig. 3A), hippocampal formation (Fig. 3B), and cerebellar cortex (Fig. 3C). Phosphodiesterase was prominent in large pyramidal cells of the neocortex, the hippocampus, and Purkinje cells in the cerebellum; other nerve cell populations associated with these structures (e.g., cerebellar granule cells or basket cells) were unreactive. Furthermore, there was no significant staining of the many neurons (or axons) that impinge on these neuron populations. Characteristic of the immunoreactivity of these large cell types was the extensive staining produced in their dendritic fields (Fig. 3), although it was not observed at the level of dendritic spines (data not shown). Substantial reactivity also was associated with somata of these neurons, but axons showed no staining.

Calcineurin, on the other hand, appeared to be a marker for neurons in general, with the substantia nigra, corpus striatum, cerebral cortex, and hippocampus exhibiting the highest amounts of immunoreactivity. Areas such as cerebral cortex, which showed phosphodiesterase staining only in selected layers (III, V, VI), were uniformly reactive with calcineurin antibodies (data provided to reviewers). In contrast to the pattern seen for phosphodiesterase, immunoreactivity in all regions was characterized by dense punctate staining of the cell bodies, with little reaction in dendritic fields. Staining of the neuropil was observed in some regions, although it was weak compared to that seen in neuronal somata.

**Differences in Calcineurin and Phosphodiesterase Cellular Localization.** Striking differences in the cellular reactivity of calcineurin and phosphodiesterase were noted when viewed under higher magnification (oil-immersion optics). Photomicrographs of anti-phosphodiesterase reactivity suggested a relatively homogenous distribution in Purkinje cell somata and dendrites (Fig. 4A). The reaction product seen with the calcineurin antibody, though, was of two types; part of the immunoreactivity was uniformly distributed in the soma, while another was concentrated in small, dense deposits (Fig. 4B). This type of punctate immunoreactivity, which was often observed along the periphery of the cell body, is suggestive of a membrane association. This general pattern of staining also was observed in pyramidal cells of both the cerebral cortex and the hippocampus as well as cells in the corpus striatum. While occasional anti-calcineurin reactivity was seen in dendrites, this was usually found in regions just proximal to the cell body. It thus appears that there are clear
The high content of CaM (and several enzymes that it regulates) in brain has suggested a crucial role for Ca\(^{2+}\)-dependent events in nerve function. It has been shown that CaM is widely distributed in nerve cells throughout the brain, with especially high amounts in hippocampus and cortex (22, 23). In addition to its presence in the cytosol, it appears to be concentrated along microtubules and other cytoskeletal structures, suggesting an important role in modulating cell shape or plasticity. High concentrations of CaM have also been found in postsynaptic densities (24, 25), supporting a potential role in neurotransmission. To further elucidate the role of Ca\(^{2+}\)/CaM in the central nervous system, it is important to document the localization of CaM-dependent enzymes at inter- and intraneuronal level.

A major finding of the present study is that calcineurin is relatively ubiquitous throughout the central nervous system, while phosphodiesterase has a restricted distribution. This implies that the two enzymes are not colocalized in many neurons and argues against a simple scheme for concerted expression and regulation of CaM-dependent enzymes. Given the differences in their distribution, careful examination of immunocytochemical data may provide clues regarding functions for these enzymes in nerve cells.

The widespread immunoreactivity observed for calcineurin suggests a role of general importance for most neurons. At higher magnification the pattern of staining was quite distinctive, consisting of weaker cytoplasmic staining and dense punctate deposits that often appeared to be localized along the plasma membrane of the cell body. Such a dual distribution may reflect interactions with both cytosolic and membrane-bound phosphoprotein substrates. In this regard, neuron-specific substrates such as dopamine- and cAMP-regulated phosphoprotein (26) and G-substrate (27) are in the cytoplasm of cell bodies (corpus striatum and cerebellum, respectively), while others [synapsin I (28)] are particulate. Of interest is a report that both phosphorylation and dephosphorylation in rat synaptosomal preparations are modulated by Ca\(^{2+}\) (29), suggesting an association of calcineurin with synaptic structures. Wood et al. reported localization of calcineurin in postsynaptic densities of mouse basal ganglia (24), and it seems that the present findings might be compatible with such an explanation. However, the work of Shields et al. (30), as well as our own (unpublished observations), did not demonstrate high concentrations of calcineurin in defined synaptic fractions, the majority of reactivity being in cytoplasmic and synapticoplastic fractions. The inability to find intrinsically stable membrane-bound forms of the phosphatase may suggest that calcineurin can reversibly associate with particulate or cytoskeletal elements, accounting for the observed immunocytochemical results. Rigorous electron microscopic studies of the punctate calcineurin staining will be necessary to clarify the nature of such associations.

Phosphodiesterase, while it certainly may be expressed at low levels in many neurons, is present in highest concentrations in the soma and dendrites of certain nerve cells. The major phosphodiesterase-positive cell classes in the neocortex (layer III, V, and VI neurons), hippocampus (CA1 pyramidal cells), and cerebellar cortex (Purkinje cells) are all output neurons of their respective brain regions (32–34). It is of interest that both CA1 pyramidal cells [35, 36] and cerebellar Purkinje cells (37) display Ca\(^{2+}\)-dependent responses, which are also found in intradendritic recordings (38). The dendritic distribution of phosphodiesterase is consistent with sites of generation of calcium spikes, and hence, of Ca\(^{2+}\) influx into the cells during synaptic integration. Thus,

---

**Fig. 3.** Immunoreactivity of rat brain neurons for CaM-stimulated phosphodiesterase. (A) Layers II and III, neocortical pyramidal cells; the large arrows identify somatic cytoplasmic immunostaining, and the small arrows indicate the apical dendrites of the pyramidal cells. (B) Pyramidal cells of the CA1 region of the hippocampus. Staining was confined to the pyramidal cells in CA1-CA3; large solid arrows indicate somatic cytoplasmic staining, the large open arrow indicates an axon initial segment, and the small arrows indicate dendritic immunostaining. (C) Purkinje cells in the cerebellum. Large arrows indicate cell bodies; smaller arrows indicate the dendritic field in the molecular layer (mol). Note the absence of staining in the granule cell layer (gr).

**Fig. 4.** Patterns of phosphodiesterase (A) and calcineurin (B) immunoreactivities in cerebellar Purkinje cells at high magnification (oil-immersion photomicrographs). The somata are indicated by large solid arrows, dendritic processes by small arrows, and dense punctate immunoreactivity by large open arrows. In B, note the intense immunoreactive regions near the margin of the plasmalemma.

---

1. Recently, the major species of brain CaM-stimulated phosphodiesterase has been resolved into separate isozymes by using monoclonal antibodies (31). Since the polyclonal antibody used in our study recognizes all brain enzyme activity, it is not possible to distinguish which of these isozymes is expressed in particular neurons or if both are.
Ca\textsuperscript{2+}/CaM activation of phosphodiesterase (i.e., degradation of cyclic nucleotides) may play an important role in synaptic integration in these neurons. It should be noted that many neurons that exhibit Ca\textsuperscript{2+}-dependent activity appear to have low amounts of phosphodiesterase; hence, additional factors appear to be necessary to elicit maximal expression of the enzyme.

Cyclic nucleotide regulation has been implicated as a correlate of electrogenic behavior of these classes of output neurons. Treatment of cerebellar slices with a variety of depolarizing agents produced a marked increase in cAMP (39), and microiontophoretic application of either cAMP or dibutyryl-cAMP depressed electrical activity of cerebellar Purkinje cells (3, 40). Immunocytochemical localization of cAMP-dependent protein kinase subunits (41), GMP-dependent protein kinase (42, 43), and G-substrate (substrate for cGMP-dependent protein kinase) in Purkinje cells (27) further suggests a role for cyclic nucleotides in the mediation or sequelae of electrogenic events.

The high amounts of CaM-dependent phosphodiesterase in specific neurons might be compared to those of the light-activated cGMP phosphodiesterase found in rod outer segment of retina (44). In the retina, photic, rather than synaptic, stimulation causes increased production of cyclic nucleotide, which is rapidly inactivated by the phosphodiesterase. In both cell types, high concentrations of the phosphodiesterase may be required to effectively regulate cAMP (or cGMP) concentrations, thereby modulating integration of signal input. Since the afferent fibers would determine the amount of signal to output neurons, one might expect that damage to these could alter phosphodiesterase activity. In fact, selective chemical destruction of climbing fiber input to Purkinje cells virtually eliminates phosphodiesterase immunoreactivity in the dendrites and somata of these cells (45). This suggests that expression of brain phosphodiesterase in Purkinje cells is under transsynaptic regulation.

The basis for such selective production of phosphodiesterase is, of course, gene expression. Since only very few cells in brain express this enzyme in substantial amounts, the potential for expression may be very great and specific signals (e.g., synaptic activity) may dramatically increase the levels of this enzyme during neuronal development. Future studies on the regulation of this and other CaM-regulated enzymes may provide clues not only to control of gene expression but also perhaps to the process of nerve cell differentiation.

We thank Dr. Martha Vaughan for advice and encouragement and Drs. Jane Halpern and Mary Ann Danello for help in raising antisera. We also thank Carol Coulson, Maria Nightingale, Monique Gagnon, Keith Pennypacker, Carol Hoover, and Kyle Krady for excellent technical assistance. This work was supported by Public Health Service Research Career Development Award NS00891 to C.D.B. and a research grant from the International Life Science Institute Research Foundation to M.L.B.