

LOCALIZATION OF CALMODULIN IN RAT CEREBELLUM BY IMMUNOELECTRON MICROSCOPY

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ABSTRACT

Calmodulin, a multifunctional Ca^{++} -binding protein, is present in all eucaryotic cells. We have investigated the distribution of this protein in the rat cerebellum by immunoelectron microscopy using a Fab-peroxidase conjugate technique. In Purkinje and granular cell bodies, calmodulin reaction product was found localized both on free ribosomes and on those attached to rough endoplasmic reticulum (RER) and the nuclear envelope. No calmodulin was observed in the cisternae of RER or the Golgi apparatus. Calmodulin did not appear to be concentrated in the soluble fraction of the cell under the conditions used. Rather, peroxidase reaction product could be seen associated with membranes of the Golgi apparatus, the smooth endoplasmic reticulum (SER), and the plasma membrane of both cell bodies and neuronal processes. In the neuronal dendrites, calmodulin appeared to be concentrated on membranes of the SER, small vesicles, and mitochondria. Also, granular calmodulin was observed in the amorphous material. In the synaptic junction, a large amount of calmodulin was seen attached to the inner surface of the postsynaptic membrane, whereas very little was observed in the presynaptic membrane or vesicles. These observations suggest that calmodulin is synthesized on ribosomes and discharged into the cytosol, and that it then becomes associated with a variety of intracellular membranes. Calmodulin also seems to be transported via neuronal processes to the postsynaptic membrane. Calmodulin localization at the postsynaptic membrane suggests that this protein may mediate calcium effects at the synaptic junction and, thus, may play a role in the regulation of neurotransmission.

Changes in the level of Ca^{++} have been reported to be required in the regulation of a large number of key processes in the nervous system, including the general regulation of cellular metabolism. Cyclic nucleotide levels (2, 4, 15), protein phosphorylation (29, 34), and neurotubule (6, 21) and neurofilament function (5, 6) are Ca^{++} -dependent. Ca^{++} also controls a large number of activities restricted to the nervous system. It depresses nerve membrane excitability (10), stimulates K^+ conductance (26), and is involved in excitation-secre-

tion coupling at peripheral neuromuscular junctions (16) and central synapses (19). Electrical synapses are uncoupled by Ca^{++} as a result of increased permeability of gap junctions (20). Ca^{++} also modulates neurotransmitter synthesis (33) and is required for intracellular movement of substances by fast axonal transport (13).

A specific Ca^{++} -binding protein, calmodulin, has been shown to be present in μM concentrations in all eucaryotic cells (23). Because this protein binds 4 mol of Ca^{++} per mol of protein and

because its affinity for Ca^{++} is approximately equal to the estimated Ca^{++} concentration ($2 \mu\text{M}$) (7), it is likely that the physiologically active, intracellular, Ca^{++} -regulated events mentioned in the preceding paragraph require calmodulin as a Ca^{++} receptor. We have prepared a monospecific calmodulin antibody in goats and sheep (3, 8), and have used this antibody to localize calmodulin in tissue culture cells by indirect immunofluorescence microscopy (8, 31, 32). The purpose of this study was to localize calmodulin in the nervous system by immunoelectron microscopy to determine whether its ultrastructural localization was compatible with the locale of known Ca^{++} -requiring processes. Because the cerebellum is simpler than other central nervous system components, it was chosen for analysis. The localization of calmodulin in cerebellar Purkinje cells is compatible with a role for this protein in all of the known Ca^{++} -mediated events.

MATERIALS AND METHODS

Preparation of Fab-peroxidase Conjugate against Calmodulin

Calmodulin isolated from rat testis was used to immunize sheep as previously described (1). Isolation of Fab fraction from the sheep antiserum and conjugation with horseradish peroxidase was performed according to the method described by Lin et al. (19), with slight modifications. The IgG fraction was separated from the sheep antiserum by QAE-Sephadex A-50 chromatography (14) and digested with papain (28). The undigested IgG was separated from the reaction mixture by Sephadex G-100 gel filtration in 0.05 M Tris buffer containing 0.15 M NaCl, pH 8.0. The monospecific Fab fragments were purified by calmodulin Sepharose 4B affinity chromatography (18) (CNBr-activated Sepharose 4B was obtained from Sigma Chemical Co., St. Louis, Mo.). Finally, the Fab fragments were conjugated to horseradish peroxidase (Sigma Type VI) according to the methods of Nakane

and Kawao (27). The Fab-peroxidase conjugate was further purified by Sephadex G-100 gel filtration and was maintained at -79°C until used.

Tissue Preparation for Localization of Calmodulin by Electron Microscopy

The immunoelectron microscopic procedure used was similar to that described by Lin and Chang (18). Five male rats (each weighing 150 g) were anesthetized with chloral hydrate (35%, 1 ml/kg of body wt). Cerebella were removed and fixed immediately with 2% paraformaldehyde in 0.1 M PIPES buffer containing 0.02% CaCl_2 , pH 7.4, overnight at 4°C . Another five rats were anesthetized, and the brains were fixed with 500 ml of the same fixative by vascular perfusion. Cerebella were then removed and immersed in the fixative overnight at 4°C . After they had been washed in buffer for 1 h, 50- μm sections were prepared from each cerebellum with a vibratome. The brain sections were incubated with the Fab-peroxidase conjugate against calmodulin (0.2 mg/ml) for 1 h. After they had been washed with buffer, sections were refixed in 2.5% glutaraldehyde in 0.1 M PIPES buffer for 30 min at 25°C , followed by incubation with 0.05% 3,3'-diaminobenzidine-4 HCl in Tris-HCl buffer, pH 7.6, with 0.01% H_2O_2 for 7 min at 25°C . The sections were postfixated in 1% OsO_4 in the same buffer for 30 min. After dehydration, they were flat-embedded in Spurr's medium (30), and silanized glasses were used to press the sections. The positive reaction areas were marked under a light microscope and mounted on blank blocks. All thin sections were observed under the electron microscope without further staining. Three control experiments were performed: (a) the sections were incubated with goat Fab-peroxidase conjugate against chicken apo VLDL-II; (b) sheep Fab-peroxidase conjugate against calmodulin was treated with a fivefold molar excess of calmodulin before incubation with the section; (c) Horseradish peroxidase (1 $\mu\text{g}/\text{ml}$) alone was substituted for Fab-peroxidase conjugate against calmodulin.

Preparation of Subcellular Fractions

Rat cerebella were removed and homogenized in a solution containing 0.32 M sucrose, 1 mM NaHCO_3 , 1 mM MgCl_2 , and 0.5 mM CaCl_2 . The homogenate was fractionated as described by Beale et al. (1), and marker enzymes were used to assess the relative purity of each subcellular fraction (1). The enzymes assayed were adenylyl cyclase and 5'-nucleotidase (plasma mem-

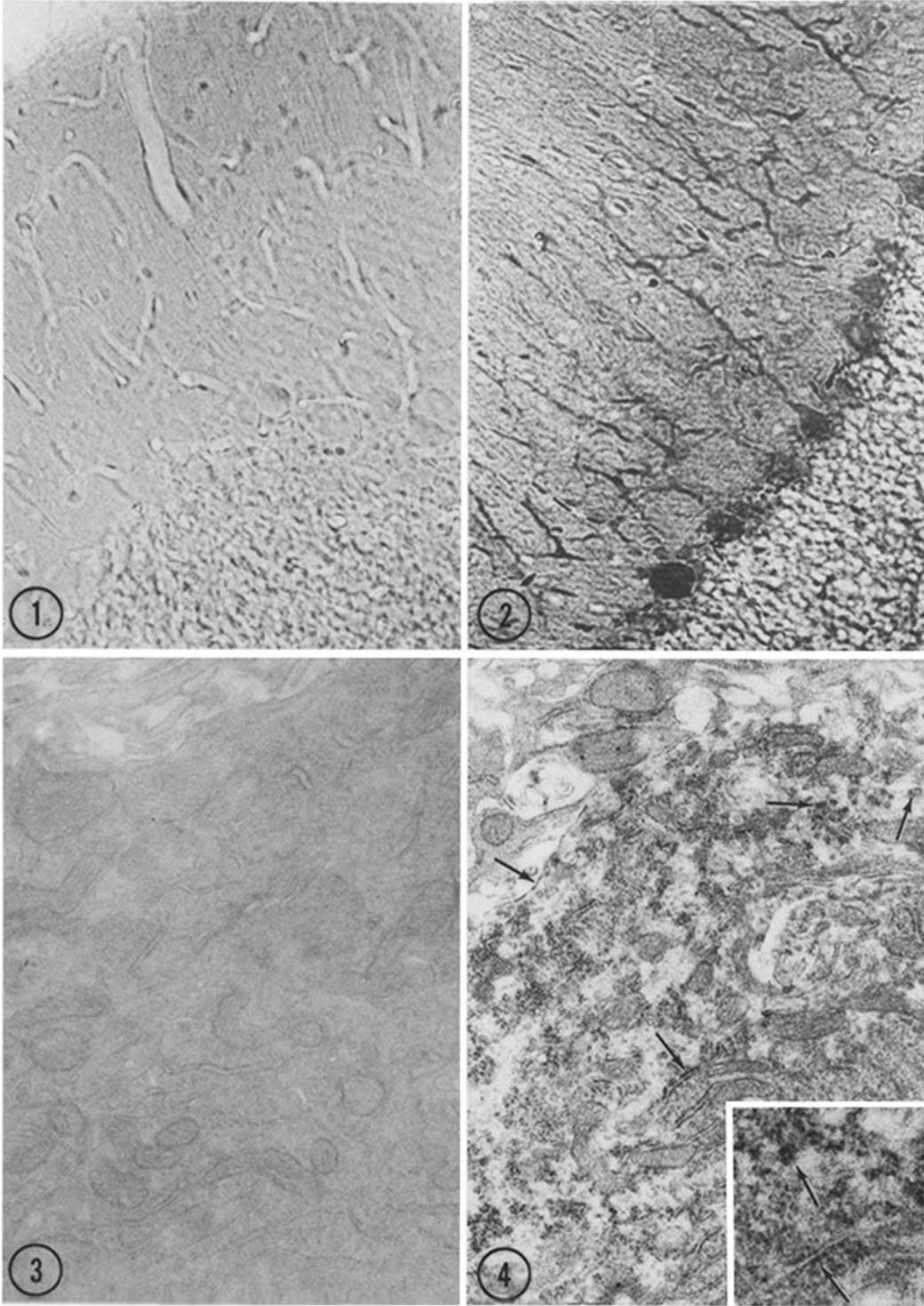
FIGURE 1 A control rat cerebellum treated with Fab-peroxidase conjugate against chicken apo VLDL-II shows no reaction product. $\times 1000$.

FIGURES 2-4 Rat cerebellum treated with Fab-peroxidase conjugate against calmodulin; no counterstain.

FIGURE 2 The reaction product (dark staining) is shown in the Purkinje cell bodies, their dendrites, and in the granular layer. $\times 1000$.

FIGURE 3 Rat cerebellum treated with preabsorbed Fab-peroxidase conjugate to calmodulin shows no staining. $\times 23,000$.

FIGURE 4 Part of a Purkinje cell showing electron-dense reaction product (arrows) on some free polysomes, bound ribosomes, SER, and plasma membrane. $\times 15,700$. *Inset*: high magnification from another area of the same Purkinje cell. The reaction product (arrows) is seen on free polysomes and bound ribosomes on endoplasmic reticulum. No reaction product is seen in the cisternae of RER. $\times 27,500$.



brane) (25), NADH cytochrome *c* reductase (mitochondria), triosephosphate isomerase (soluble) and RNA polymerase II (nuclear). All enzyme activities were determined in all subcellular fractions. In no case did cross-contamination account for >2% of the total enzyme activity in the respective fractions. Polyribosomes were prepared by the procedure of Means et al. (24), and the nascent peptide chains were released and separated from ribosomes as described by Means et al. (22). Neurotubules were isolated from cerebellum by the procedure of Marcum et al. (21). Synaptic plasma membranes and the postsynaptic densities derived from these membranes were kindly isolated and supplied by B. Lester and E. J. Peck, Jr., Department of Cell Biology, Baylor College of Medicine, Houston, Tex. (17).

RESULTS

When sections of the cerebellum that had been processed for Fab-peroxidase conjugate against calmodulin were examined by light microscopy, localization of brown reaction product was observed in Purkinje cell bodies, their dendrites, and the granular layer (Fig. 2). For comparison, a control section of rat cerebellum was treated with Fab-peroxidase conjugate against chicken apo VLDL-II. This section showed no reaction product (Fig. 1). When the sections were examined by electron microscopy, localization of calmodulin was found on free ribosomes as well as on ribosomes associated with rough endoplasmic reticulum (RER) (Fig. 4). Staining was also apparent on mitochondrial membranes, smooth endoplasmic reticulum (SER), and plasma membranes (Figs. 4 and 5). Although the membranes of the cristae and the inner and outer mitochondrial membranes were stained, no reaction product was seen in the matrix (Figs. 4-9). Likewise, the Golgi membranes were stained, but no staining was apparent in the cisternae. It was interesting to note that neurotubules of the dendrites also were unstained, whereas

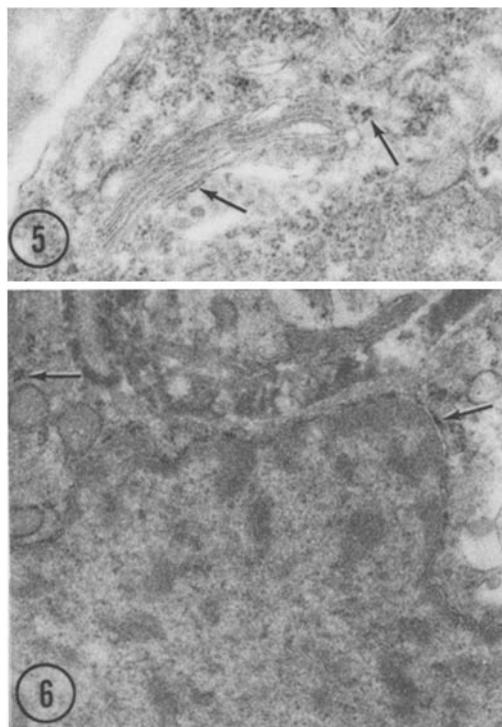


FIGURE 5-6 Rat cerebellum treated with Fab-peroxidase conjugate against calmodulin; no counterstain.

FIGURE 5 The cisternae of Golgi apparatus contain no reaction product, but one of the saccular membranes (arrow) and some free polysomes (arrow) are stained. $\times 20,500$.

FIGURE 6 A granular cell in the granular layer of rat cerebellum shows reaction product (arrows) on free polysomes and bound ribosomes attached to the nuclear envelope. $\times 18,000$.

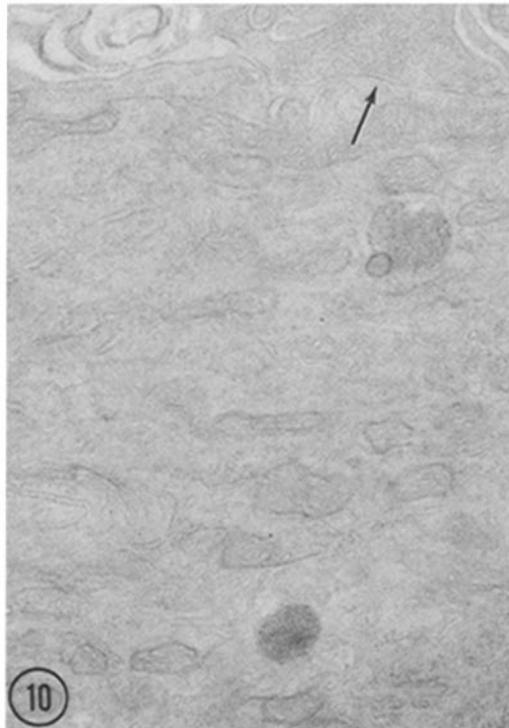
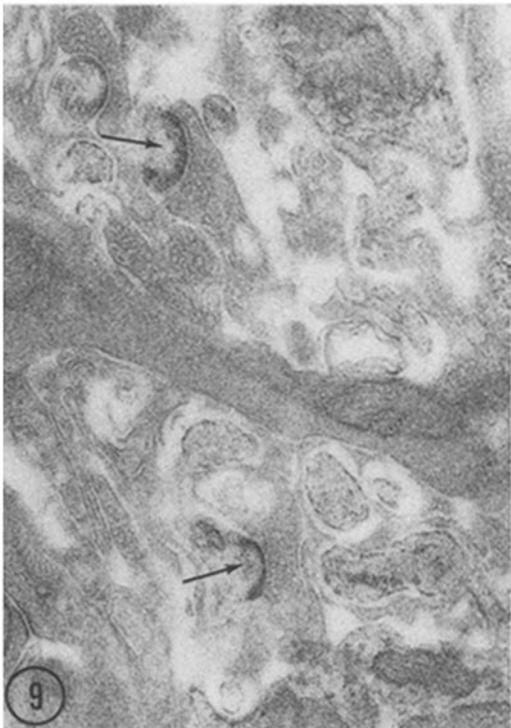
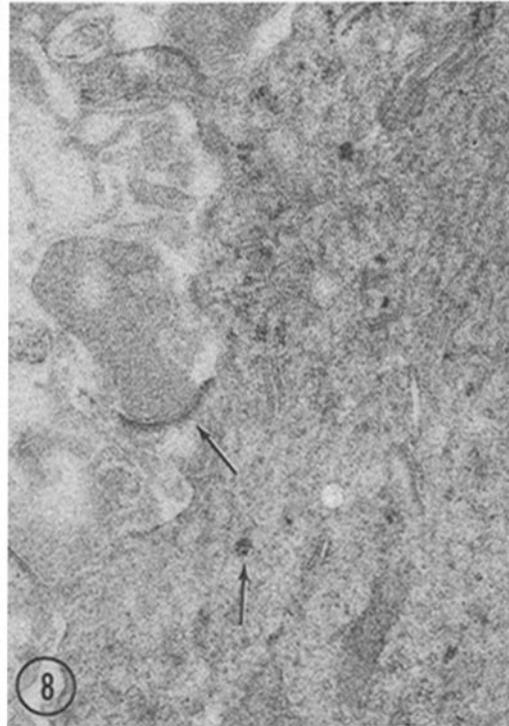
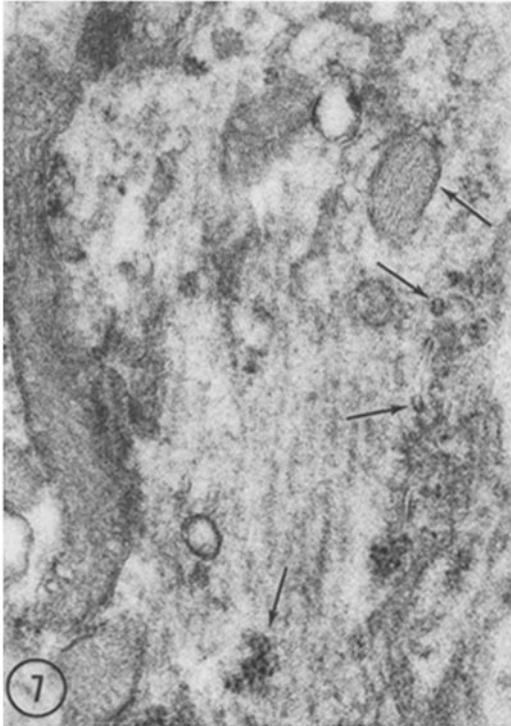
FIGURES 7-9 Rat cerebellum treated with Fab-peroxidase conjugate against calmodulin; no counterstain.

FIGURE 7 An oblique section of one neuronal dendrite shows reaction product (arrows) on SER, small vesicles, and outer and inner mitochondrial membranes. The reaction product is also present as granular particles (arrow) embedded in amorphous material. The neurotubules are rarely stained. $\times 34,000$.

FIGURE 8 Part of a neuronal dendrite reveals heavy reaction product (arrow) at postsynaptic membrane and in its vicinity. Scattered granular particles (arrow) of reaction product are also seen. The neurotubules are unstained. Some SER also show membrane-associated reaction product. $\times 22,000$.

FIGURE 9 Several axodendritic synapses (arrow) in the molecular layer of rat cerebellum show a heavy coating of reaction product at the inner surface of postsynaptic membranes and in their vicinity. $\times 39,500$.

FIGURE 10 Rat cerebellum treated with Fab-peroxidase conjugate against chicken apo VLDL-II. Part of a Purkinje cell with an axosomatic synapse (arrow) shows no reaction product in the cell body or at the postsynaptic membrane. $\times 23,000$.



other organelles of the processes, including SER, mitochondria, and granular particles in the amorphous material were stained (Figs. 7 and 8). These data were confirmed by assaying calmodulin in each subcellular fraction by radioimmunoassay (3).

At the synaptic junction, reaction products were heavily deposited at the inner surface of the post-synaptic membranes and in their vicinity (Figs. 8 and 9). Interestingly, the presynaptic nerve terminals, which contained many vesicles, showed little reaction product. Although much less reaction product was observed in the granular layer of the cerebellum, granular cells displayed some staining reaction on free ribosomes as well as on ribosomes bound to the outer nuclear envelope and the RER (Fig. 6). Occasionally, myelinated nerve fibers revealed small vesicles coated with reaction product (not shown).

Control tissue sections treated with Fab-peroxidase conjugate against chicken apo VLDL-II or exposed to preabsorbed Fab-peroxidase conjugate to calmodulin showed no staining (Figs. 1, 3, and 10). When the Fab-peroxidase conjugate was replaced with horseradish peroxidase, the reaction product was absent. Immersion fixation and perfusion fixation gave no significant difference in the distribution of reaction product, although perfusion fixation resulted in improved morphological preservation.

Table I shows the distribution of calmodulin in various fractions prepared from rat cerebellum. In all cases, calmodulin was quantitated by a sensitive radioimmunoassay (3). All subcellular fractions contained calmodulin except purified neurotubules and ribosomes that had been stripped of nascent peptide chains. Calmodulin values ranged from 0.4 to 2% of the total protein. The exception was in isolated postsynaptic densities (PSD), where calmodulin comprised 3% of the protein. These data agree with those reported by Grab et al. (11), who suggest a relative enrichment of calmodulin in the PSD fraction.

DISCUSSION

Calmodulin was localized in rat cerebellar Purkinje cells by immunoelectron microscopy using a Fab-peroxidase conjugate technique. Reaction product was found on both free and bound ribosomes but not in the cisternae of rough endoplasmic reticulum or Golgi apparatus, which is compatible with biochemical experiments that reveal

TABLE I
Calmodulin Distribution in Rat Cerebellum

Fraction	Calmodulin <i>μg/mg protein</i>
Cerebellar homogenate	9.5
Nuclei	6.7
Mitochondria	4.4
Microsomes	19.3
Plasma membranes	11.0
Postmicrosomal supernate	13.4
Polyribosomes	3.6
Nascent peptide chains	3.0
Ribosomes	<0.002
Neurotubules	<0.002
Synaptic plasma membranes	10.6
Postsynaptic densities	30.8

Calmodulin was assayed in all fractions by radioimmunoassay as described by Chafouleas et al. (3). Sample preparation is described in Materials and Methods.

calmodulin to be an intracellular protein that neither consists of a signal peptide chain (3) nor contains carbohydrate residues (7). This protein is not glycosylated and packaged in the Golgi apparatus because it is not destined for secretion. Rather, calmodulin was found to be associated with a variety of intracellular membranes and was found in granular particles that seemed to be in the soluble portion of the cell.

The data presented herein suggest that calmodulin is synthesized in cell bodies on both free and membrane-bound polyribosomes and that the nascent peptide chains are then released and become associated with a variety of intracellular membranes. In addition, the demonstration of granular calmodulin in the neurites suggests that it is transported from the cell body to the synaptic regions and that here it becomes integrated into the post-synaptic membrane. The data accumulated concerning the localization of calmodulin in Purkinje cells are positively correlated with information obtained from subcellular fractionation studies. As is shown in Table I, radioimmunoassay of calmodulin reveals the presence of this protein in each of the subcellular fractions identified by immunoelectron microscopy as being calmodulin positive. These fractions include plasma membrane, endoplasmic reticulum, polyribosomes, mitochondria, nuclei, and synaptosomes.

The biochemical and ultrastructural identification of calmodulin in the various subcellular fractions is also consistent with events in the nervous

system known to be regulated by calmodulin. Polysomes contain calmodulin that can be quantitatively released with the nascent chains, and the resultant monosomes do not contain calmodulin, which suggests that the localization of calmodulin on polyribosomes represents the site of synthesis. Plasma membrane-associated calmodulin is not a surprising finding inasmuch as these membranes contain phosphodiesterase (15), protein kinase (29), adenylyl cyclase (2), and $\text{Ca}^{++}\text{-Mg}^{++}$ ATPase (6). All of these enzymes have been shown to be calmodulin-dependent in mammalian brain. Similarly, Ca^{++} transport may be regulated by calmodulin in smooth ER and mitochondria. Localization on smooth ER is also consistent with a role for calmodulin in the regulation of tryptophan 5'-monooxygenase (33). The immunochemical data suggest the possibility that calmodulin may be associated only with the outer nuclear envelope of the nucleus. Indeed, bound ribosomes could be the positively stained organelles. However, our results do not rule out intranuclear localization. It should be pointed out that Harper et al. (12) argue that ACTH promotes an accumulation of nuclear calmodulin in adrenal tumor cells. In their study, calmodulin was visualized in frozen sections by an immunofluorescence technique.

The appearance of granular calmodulin in neuronal processes is consistent with the observation by Iqbal and Ochs (13) that Ca^{++} -calmodulin is involved in the regulation of fast axonal transport. Grab et al. (11) have isolated postsynaptic densities and have shown that calmodulin represents a major protein component. A functional role for the calcium-binding protein is suggested by the studies of DeLorenzo and colleagues (9), who report that calmodulin stimulates dopamine release from isolated synaptosomes. Our studies reveal an intense reaction product in the postsynaptic region of cerebellar synapses and, thus, support these biochemical and functional observations.

Our study reveals calmodulin localized in rat cerebellar Purkinje cells. The sites of positive reaction product complement the results of biochemical studies that show that calmodulin modulates a large number of events important in the regulation of nervous system function. It is likely that calmodulin mediates most (if not all) of the known functions of Ca^{++} in mammalian brain by acting as an intracellular receptor for this regulatory ion. Thus, changes in the activity of calmodulin would

be dictated by changes in the net flux or intracellular distribution of Ca^{++} promoted by substances that regulate chemical and electrical synaptic transmission.

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Note added in proof: While this manuscript was in press, Wood et al. (*J. Cell Biol.* **84**:66-76) reported the immunocytochemical localization of calmodulin on postsynaptic membranes of mouse brain basal ganglia.

REFERENCES

1. BEALE, E. G., J. R. DEDMAN, and A. R. MEANS. 1977. Isolation and regulation of the protein kinase inhibitor and the calcium-dependent cyclic nucleotide phosphodiesterase regulator in the Sertoli cell-enriched testis. *Endocrinology*. **101**:1621-1634.
2. BROSTROM, M. S., C. O. BROSTROM, B. MCL. BRECKENRIDGE, and D. J. WOLFF. 1976. Regulation of adenylyl cyclase from glial tumor cells by calcium and a calcium-binding protein. *J. Biol. Chem.* **251**:4744-4750.
3. CHAFOULEAS, J. G., J. R. DEDMAN, R. P. MUNJAAL, and A. R. MEANS. 1979. Calmodulin: development and application of a sensitive radioimmunoassay. *J. Biol. Chem.* **254**:10262-10267.
4. CHEUNG, W. Y. 1970. Cyclic 3',5'-nucleotide phosphodiesterase: demonstration of an activator. *Biochem. Biophys. Res. Commun.* **38**:533-538.
5. DABROWSKA, R., and D. J. HARTSHORNE. 1978. A Ca^{++} - and modulator-dependent myosin light chain kinase from non-muscle cells. *Biochem. Biophys. Res. Commun.* **85**:1352-1359.
6. DEDMAN, J. R., B. R. BRINKLEY, and A. R. MEANS. 1979. Regulation of microfilaments and microtubules by calcium and cyclic AMP. *Adv. Cyclic Nucleotide Res.* **11**:131-174.
7. DEDMAN, J. R., J. D. POTTER, R. L. JACKSON, J. D. JOHNSON, and A. R. MEANS. 1977. Physicochemical properties of rat testis Ca^{++} -dependent regulator protein of cyclic nucleotide phosphodiesterase: relationship of Ca^{++} -binding, conformational changes and phosphodiesterase activity. *J. Biol. Chem.* **252**:8415-8422.
8. DEDMAN, J. R., M. J. WELSH, and A. R. MEANS. 1978. Ca^{++} -dependent regulator: production and characterization of a monospecific antibody. *J. Biol. Chem.* **253**:7515-7521.
9. DELORENZO, J. R., S. D. FREEDMAN, W. B. YOHE, and S. C. MAURER. 1979. Stimulation of Ca^{++} -dependent neurotransmitter release and presynaptic nerve terminal protein phosphorylation by calmodulin and a calmodulin-like protein isolated from synaptic vesicles. *Proc. Natl. Acad. Sci. U. S. A.* **76**:1838-1842.
10. FRANKENHAUSER, B., and A. L. HODGKIN. 1957. The action of calcium on the electrical properties of squid axons. *J. Physiol. (Lond.)* **137**:218-244.
11. GRAB, D. J., K. BERZINS, R. S. COHEN, and P. SIEKEVITZ. 1979. Presence of calmodulin in postsynaptic densities isolated from canine cerebral cortex. *J. Biol. Chem.* **254**:8690-8698.
12. HARPER, J. F., W. Y. CHEUNG, R. W. WALLACE, H. HUANG, S. W. LEVINE, and A. L. STEINER. 1980. Localization of calmodulin in rat tissues. *Proc. Natl. Acad. Sci. U. S. A.* **77**:366-370.
13. IQBAL, F., and S. OCHS. 1978. Fast axoplasmic transport of a calcium-binding protein in mammalian nerve. *J. Neurochem.* **31**:409-418.
14. JOUSIA, J., and H. LUNDGREN. 1969. *Proides Biol. Fluids Proc. Colloq. Bruges*. **17**:511-515.
15. KAKIUCHI, S., and R. YAMAZAKI. 1970. Calcium dependent phosphodiesterase activity and its activating factor from brain. *Biochem. Biophys. Res. Commun.* **41**:1104-1110.

16. KATZ, B., and R. MILEDI. 1969. Tetrodotoxin-resistant electric activity in presynaptic terminals. *J. Physiol. (Lond.)* **203**:459-487.
17. LESTER, B. R., and E. J. PECK, JR. 1979. Kinetic and pharmacologic characterization of gamma-aminobutyric acid receptive sites from mammalian brain. *Brain Res.* **161**:79-97.
18. LIN, C. T., and J. P. CHANG. 1975. Electron microscopy of albumin synthesis. *Science (Wash. D. C.)* **190**:465-467.
19. LIN, C. T., J. P. CHANG, and J. P. CHEN. 1975. Ultrastructural localization of gamma chain and immunoglobulin G in human lymphocytes using enzyme-labeled Fab fragment. *J. Histochem. Cytochem.* **23**:624-631.
20. LOEWENSTEIN, W. R. 1976. Permeable Junctions. *Cold Spring Harbor Symp. Quant. Biol.* **40**:49-63.
21. MARCUM, J. M., J. R. DEDMAN, and A. R. MEANS. 1978. Control of microtubule assembly-disassembly by calcium-dependent regulator protein. *Proc. Natl. Acad. Sci. U. S. A.* **75**:3771-3775.
22. MEANS, A. R., I. B. ABRASS, and B. W. O'MALLEY. 1971. Protein biosynthesis on chick oviduct polyribosomes. I. Changes during estrogen-mediated tissue differentiation. *Biochemistry* **10**:1561-1570.
23. MEANS, A. R., and J. R. DEDMAN. 1980. Calmodulin: an Intracellular Calcium Receptor. *Nature (Lond.)*. In press.
24. MEANS, A. R., P. F. HALL, L. W. NICOL, W. H. SAWYER, and C. A. BAKER. 1969. Protein biosynthesis in the testis. IV. Isolation and properties of polyribosomes. *Biochemistry* **8**:1488-1495.
25. MEANS, A. R., and J. VAITUKAITIS. 1972. Peptide hormone "receptors." Specific binding of ³H-FSH to testis. *Endocrinology* **90**:39-46.
26. MEECH, R. W. 1974. The sensitivity of *Helix aspersa* neurons to injected calcium ions. *J. Physiol.* **237**:259-277.
27. NAKANE, P. K., and A. KAWAO. 1974. Peroxidase-labeled antibody: a new method of conjugation. *J. Histochem. Cytochem.* **22**:1084-1091.
28. PORTER, R. R. 1959. The hydrolysis of rabbit gammaglobulin and antibodies with crystalline papain. *Biochem. J.* **73**:119-126.
29. SCHULMAN, H., and P. GREENGARD. 1977. Stimulation of brain membrane protein phosphorylation by calcium and an endogenous heat-stable protein. *Nature (Lond.)* **271**:478-479.
30. SPURR, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**:31-43.
31. WELSH, M. J., J. R. DEDMAN, B. R. BRINKLEY, and A. R. MEANS. 1978. Calcium-dependent regulator protein: localization in mitotic apparatus of eucaryotic cells. *Proc. Natl. Acad. Sci. U. S. A.* **75**:1867-1871.
32. WELSH, M. J., J. R. DEDMAN, B. R. BRINKLEY, and A. R. MEANS. 1979. Tubulin and Calmodulin: effects of microtubule and microfilament inhibitors on localization in the mitotic apparatus. *J. Cell Biol.* **81**:624-634.
33. YAMAUCHI, T., and H. FUJISAWA. 1979. Activation of tryptophan 5-monooxygenase by calcium-dependent regulator protein. *Biochem. Biophys. Res. Commun.* **90**:28-34.
34. YAMAUCHI, T., and H. FUJISAWA. 1979. Most of the Ca²⁺-dependent endogenous phosphorylation of rat brain cytosol proteins requires Ca²⁺-dependent regulator protein. *Biochem. Biophys. Res. Commun.* **90**:1172-1178.