IMMUNOCYTOCHEMICAL LOCALIZATION OF CALMODULIN AND A HEAT-LABILE CALMODULIN-BINDING PROTEIN (CaM-BP$_{80}$) IN BASAL GANGLIA OF MOUSE BRAIN

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ABSTRACT

Antisera to calmodulin, a Ca^{2+}-dependent modulator protein, and a heat-labile calmodulin-binding protein have been used to localize these proteins in mouse caudate-putamen. The two proteins appear to be located at identical sites in this brain area. At the light microscopic level, calmodulin and calmodulin-binding protein are found within the cytoplasm and processes of large cells. At the electron microscopic level the proteins are associated with neuronal elements only, primarily at postsynaptic sites within neuronal somata and dendrites. Within the dendrites the immunocytochemical label is associated predominantly with the postsynaptic density and dendritic microtubules. These results are in accord with recent biochemical and immunohistochemical studies of calmodulin in brain and in dividing cells. Thus, calmodulin and the heat-labile calmodulin-binding protein may play a role in the nervous system at the site of neurotransmitter action and at the level of microtubular function.

KEY WORDS   calmodulin · immunocytochemistry · postsynaptic density · microtubules

Increasing evidence indicates that calmodulin, a Ca^{2+}-dependent modulator protein found in all eukaryotes examined, is an important mediator of calcium effects in cellular processes. The protein was originally discovered as an activator of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase (4, 5, 6, 7). In the presence of micromolar Ca^{2+}, calmodulin forms a complex with the apoenzyme of phosphodiesterase to give the active holoenzyme; lowering the Ca^{2+} concentration dissociates the holoenzyme into its components, returning the enzyme activity to its basal level (8, 22, 37). Since calmodulin is generally present in excess, the cellular flux of Ca^{2+} is believed to regulate the enzyme activity in vivo. Subsequently, calmodulin was found to regulate the activity of brain adenylate cyclase (3, 23, 24) and erythrocyte membrane Ca^{2+}-ATPase (12, 16, 17); the mechanism of stimulation appears identical to that previously described for phosphodiesterase (25).

More recently, calmodulin has been shown to regulate phosphorylase kinase (9), myosin light chain kinase (10, 34, 39, 47), plant NAD kinase (1), synaptosomal membrane Ca^{2+}-ATPase (35),

Ca^{2+}-transport in erythrocytes (15) and sarcoplasmic reticulum (18), phosphorylation of synaptic and cell membranes (30, 31), and the disassembly of microtubules (26). The mode of action of calmodulin on these systems has not been established.

In addition, calmodulin may be involved in other cellular reactions. Bovine brain contains several calmodulin-binding proteins (20); these may be additional calmodulin-regulated proteins, but their biological activities remain to be identified. One of these proteins, purified to apparent homogeneity and extensively characterized, suppressed calmodulin-supported activities of adenylate cyclase (40, 41), phosphodiesterase (19, 40, 41, 43, 44), and Ca^{2+}-ATPase (25), but not their basal activities. This calmodulin-binding protein (CaM-BP$_{80}$) is heat-labile, has a mol wt of 80,000, consists of two polypeptides of 60,000 and 18,500 daltons, and shows a pI of 6. CaM-BP$_{80}$ apparently exerts its effect by binding to calmodulin in direct competition with the apoenzymes. Another calmodulin-binding protein, isolated from bovine brain, is heat-stable and has a mol wt of 70,000 (32, 33). This protein (CaM-BP$_{70}$) also inhibits calmodulin-supported phosphodiesterase activity, presumably by a mechanism similar to that of the heat-labile protein (41, 42, 44).

Although many biochemical studies have been done with calmodulin and CaM-BP$_{80}$, relatively little is known about their localization at the cellular level. As a preliminary approach we have prepared antibodies directed against the two proteins, and have used immunocytochemical techniques to localize them in mouse brain at both the light and electron microscope levels. We found that in the basal ganglia both antibodies react in an identical pattern with the postsynaptic area and the dendritic microtubules.

MATERIALS AND METHODS

Horseradish peroxidase (Type VI, Sigma Chemical Co., St. Louis, Mo.), 3,3'-diaminobenzidine-4HCl (DAB) (Sigma), glutaraldehyde (Polysciences Inc., Warrington, Pa.), paraformaldehyde (Polysciences), and other chemicals were of reagent grade.

Preparation of Tissue

Adult C57Bl/6J mice (Southern Animal Farms, Prattsville, Ala.) were anesthetized by intraperitoneal injection of 3.5% chloral hydrate (1 ml/kg body weight) and perfused through the heart for 10 min with a fixative containing freshly prepared 4.0% paraformaldehyde and 0.1% glutaraldehyde in 0.12 M Millonig's phosphate buffer, pH 7.2 (29). After perfusion, the cerebral hemispheres were removed and stored overnight at 4°C in phosphate buffer containing 4.0% paraformaldehyde but not glutaraldehyde. Coronal slices (40–75 μm) of the fixed tissue were prepared with either a Sorvall TC-2 tissue sectioner (Ivan Sorvall, Inc., Newton, Conn.) or an Oxford vibratome (Ted Pella Co., Tustin, Calif.). The slices were collected in phosphate-buffered saline (PBS), and the best pieces were selected under a dissecting microscope for cytochemical study.

Preparation of Antibodies against Calmodulin and a Heat-Labile Calmodulin-Binding Protein in Rabbits

Calmodulin was purified to homogeneity from bovine brain (21) with a slight modification (42). The protein is a poor antigen, presumably because it is small, acidic, and lacks tissue and species specificity (7). Chemical incorporation of 3 dinitrophenyl (DNP$_3$) groups/calmodulin renders it highly antigenic. The DNP$_3$-calmodulin retains essentially all its biologic activity, and was used to immunize rabbits (42). Briefly, 1 ml of DNP$_3$-calmodulin (0.84 mg/ml) was mixed with 1 ml of Freund's complete adjuvant. The suspension was emulsified by brief sonication and injected subcutaneoulsy at 4–5 sites on the back just above the scapula on days 1, 17, 33, and 64. On day 72, each rabbit was bled from the marginal vein of the ear. The blood was allowed to clot and the whole sera were stored at −90°C.

CaM-BP$_{80}$, purified to homogeneity from bovine brain (41), was used without chemical modification to immunize rabbits. Briefly, 0.5 ml of CaM-BP$_{80}$ (1.0 mg/ml) was emulsified with 0.5 ml of Freund's complete adjuvant and injected as described for calmodulin on days 1, 21, and 135. On day 142, the rabbits were bled and the sera were prepared and stored as described above.

Immunocytochemical Procedures

The immunocytochemical procedures employed in this study were essentially the same as those described previously (46). All steps were performed with constant, gentle agitation. The tissue slices (40–75 μm) were immersed for 30–40 min at room temperature in rabbit sera directed against calmodulin or CaM-BP$_{80}$. The two sera were used at dilutions of 200- and 450-fold in PBS, respectively. In some experiments additional slices were incubated in 0.1% Triton X-100 in PBS for 15 min followed by incubation in antisera also containing 0.1% Triton X-100. The slices were washed with PBS (five changes) for 3 h, and incubated for 30–40 min at room temperature with a 1:600 dilution of peroxidase-labeled Fab fragments of purified goat anti-rabbit IgG (27, 28) prepared according to the two-step method of Avrameas (2). The slices were washed with PBS for 3 h to remove the excess second antiserum and then incubated for 15–20 min at 4°C in 50 ml of PBS containing 30 mg of DAB and 10 μl of 30% hydrogen peroxide (14). The slices were further washed with PBS for 30 min, and appropriate areas were dissected into separate pieces before postfixation with 2.0% osmium tetroxide in 0.12 M Millonig's buffer, pH 7.2. The slices were dehydrated through ethanol and propylene oxide, and flat-embedded in Epon-Araldite. Superficial ultrathin sections, cut parallel to the tissue face originally exposed to the reagents, were examined in a Philips EM 301 electron microscope with or without prior staining in uranyl acetate or lead citrate (38). Some of the osmicated slices were immersed overnight in 2.0% uranyl acetate in 0.1 M acetate buffer, pH 5.0, in darkness at 4°C, and dehydrated for embedding in plastic. The results reported here were obtained from slices of the mouse basal ganglia, the caudate-putamen and globus pallidus.

RESULTS

Light microscopy of the tissue slices (50–75 μm) treated with anti-calmodulin serum revealed a staining pattern characteristic of cells and processes in the caudate-putamen (Fig. 1 a). The profile obtained with anti-CaM-BP$_{80}$ serum appeared to be essentially identical (Fig. 2 a). The soma of large cells and the processes leaving the cell soma contained reaction product for peroxidase, but the nucleus did not (Figs. 1 a and 2 a). Labeled cell processes coursed in the region between cell somata, and a number of punctate-labeled profiles were also seen in this region (Figs. 1 a and 2 a). The islands of white matter localized in the caudate-putamen contained bundles of myelinated axons and showed no appreciable reaction product. Oligodendroglia could be identified by phase optics in the islands of white matter; these cells did not contain detectable reaction product. Tissue slices treated with nonimmune serum before exposure to the second antibody revealed no specific staining (data not shown). In addition, sections treated with preabsorbed immune serum also showed no staining with anti-calmodulin (Fig. 1 b), or greatly reduced staining with anti-CaM-BP$_{80}$ (Fig. 2 b).

At the electron microscopic level, brain slices treated with nonimmune or blocked immune serum contained no specific reaction product. In contrast, the slices treated with either antiserum revealed identical localization of reaction product within the cell somata and processes (Figs. 3–6). There was no difference in the labeling pattern with or without Triton X-100 treatment except that the detergent-treated slices were more washed out. The staining within the cell somata was distributed throughout the cytosol and was deposited on cellular organelles, especially on their membranes facing the cytoplasm (Figs. 4 b and 5 b). The reaction product was consistently greater at the cell periphery than in the perinuclear region, but the difference was not striking. All of the labeled cells received synaptic input and were therefore neurons. Oligodendroglia or astrocytes did not contain reaction product. A number of cell processes, cut in longitudinal or cross-section, contained label within them (Figs. 3–6). These labeled profiles were not myelinated axons. Many of these profiles were postsynaptic to unlabeled presynaptic terminals and thus appeared to be dendrites (Figs. 3–6) but we could not rule out the possibility that some of the smaller profiles were unmyelinated axons. The localization of these two antisera within the dendrites is in agreement with recent biochemical findings (see Discussion) that calmodulin is a component of postsynaptic densities (PSD). In areas where the PSD was localized, the staining was heavy, either with anti-calmodulin or with anti-CaM-BP$_{80}$ (Figs. 3–6). In most fields a few PSDs were not labeled. This may indicate a differential distribution of calmodulin and CaM-BP$_{80}$ at postsynaptic sites or it may be due to failure of penetration of antibody to all postsynaptic sites in a given region. We believe that the latter explanation is more likely, but it will take extensive immunocytochemical examination of brain regions containing identified synapses before this question may be resolved. Regardless of the explanation for this result, the unstained PSDs serve as a good internal morphological control for the positive immunocytochemical results. In tissue treated with DAB and osmium the PSD has an electron density even without heavy metal staining, but it is clear from Figs. 5 b and 6 a that this density is not comparable to the positive immunocytochemical staining.

Aside from the labeling at the PSD, the immunized sera also labeled the microtubules and the outer membrane of mitochondria (Figs. 3–6). In heavily labeled dendrites, the reaction product obscured the fine structural details within the process, making it difficult to identify other stained elements. In contrast, in lightly labeled profiles, the antibodies clearly decorated the microtubules (Figs. 3 and 5). The labeling of mitochondria was erratic, being usually associated with the membrane facing an area containing labeled microtubules and not with the membrane facing lightly labeled or unlabeled areas (Figs. 3 and 5).

DISCUSSION

Results presented in this communication indicate that both calmodulin and CaM-BP$_{80}$ are localized at postsynaptic sites in mouse basal ganglia neurons. Cyclic nucleotide phosphodiesterase (EC 3.1.4.17) is also localized at postsynaptic sites in brain (11). Since calmodulin and CaM-BP$_{80}$ regulate the activity of cyclic nucleotide phosphodiesterase in vitro (7, 19, 40, 43), these results support the contention that calmodulin plays a role in
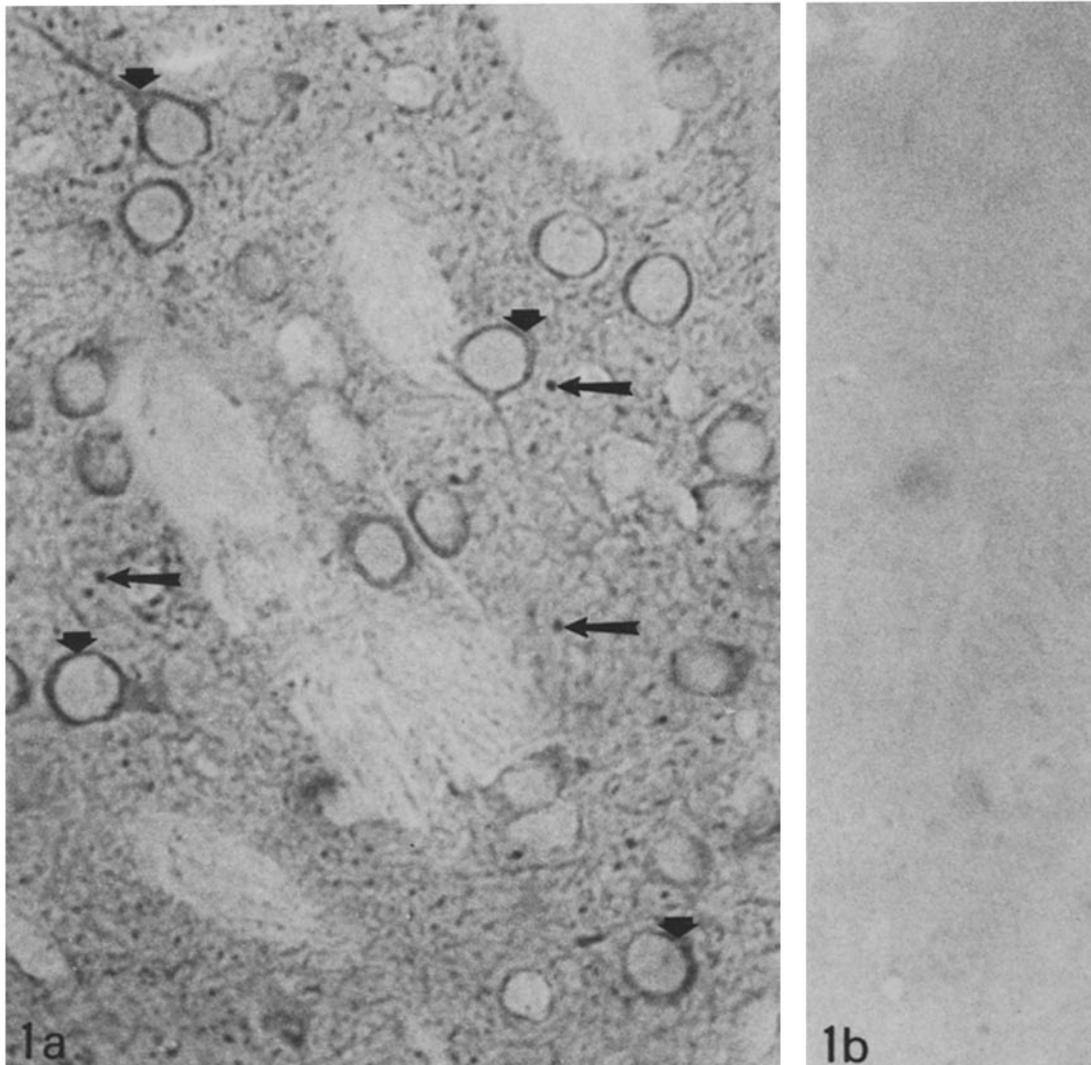
FIGURE 1 Light micrographs of mouse caudate-putamen stained with antiserum to calmodulin (a) or calmodulin antiserum preabsorbed with calmodulin (b). Specific anti-calmodulin staining shown in a is within cell somata and processes (short arrows). Numerous punctate labeled profiles are also observed (long arrows). The relatively clear areas are islands of white matter which do not label with antibody. No specific staining is seen with preabsorbed anti-calmodulin serum (b).

modulating the metabolism of cAMP involved in neurotransmission. It is not yet clear, however, that the phosphodiesterase activity detected biochemically in the PSD is regulated by calmodulin. A protein kinase associated with synaptosomal and PSD fractions does respond to calmodulin (13, 30, 31), suggesting that calmodulin may regulate a synaptic function other than cAMP metabolism.

Calmodulin and CaM-BP$_{80}$ form a complex in the presence of Ca^{2+} in vitro (19, 40, 41, 44); the identical localization of the two proteins in basal ganglia neurons suggests that a similar complex may be present in vivo under our experimental conditions. Our results should not be interpreted as indicating that calmodulin and CaM-BP$_{80}$ are localized only at postsynaptic sites; the possibilities exist that they may be present in other cellular
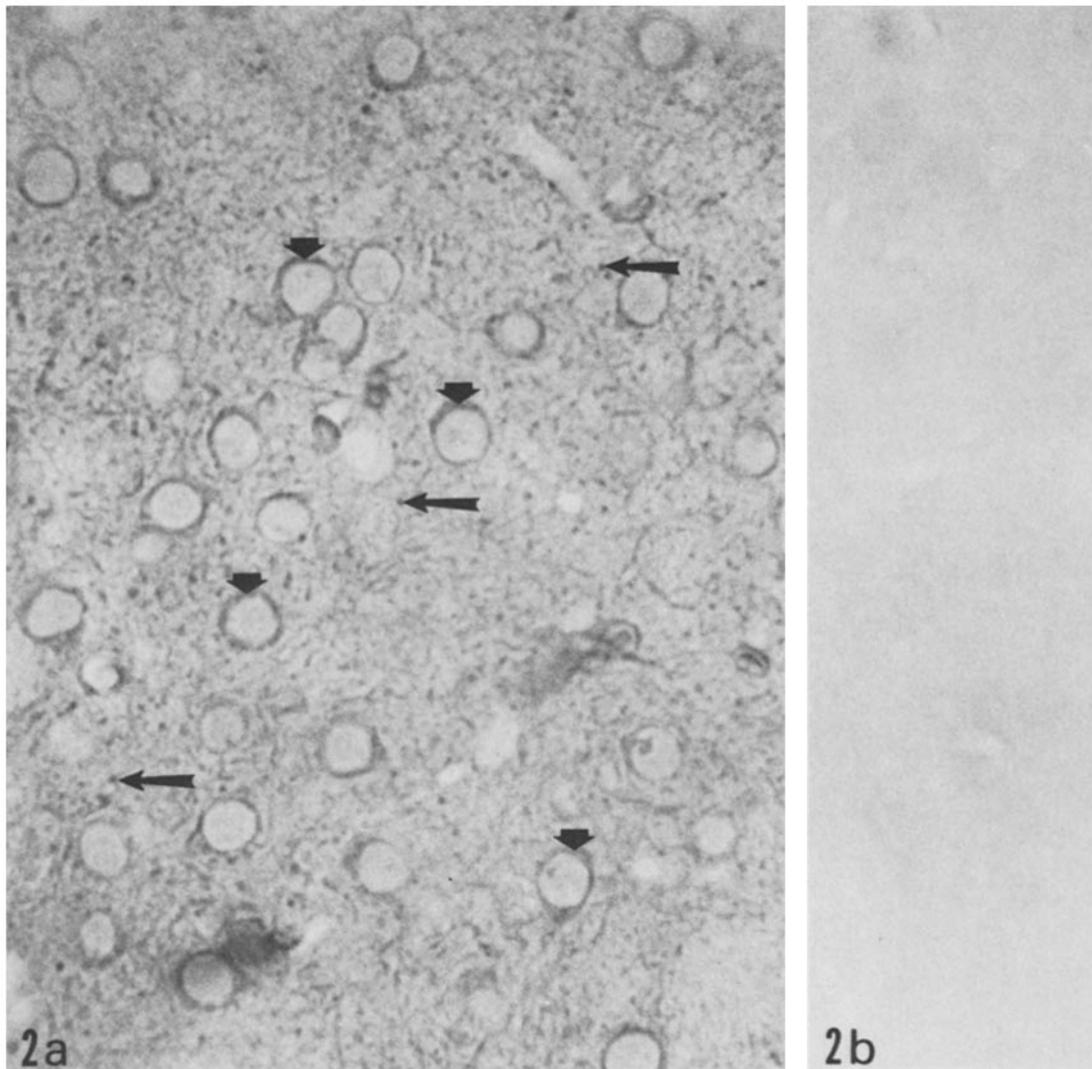
FIGURE 2 Light micrographs of mouse caudate putamen stained with antiserum to CaM-BP$_{80}$ (a) or CaM-BP$_{80}$ antiserum preabsorbed with CaM-BP$_{80}$ (b). Description of results and symbols are the same as for Fig. 1.

compartments but that either their levels are below detection by our methods or their antigenic sites are masked. Also, it must be pointed out that axons are smaller than dendrites and generally follow a more convoluted course in the tissue. Thus, axons would be less likely cut by the original tissue slice and would, therefore, be less accessible than dendrites to antibody reagents.

The association of calmodulin and CaM-BP$_{80}$ with specific organelles within the postsynaptic dendrites may imply a functional role in these organelles. A note of caution, however, should be mentioned. In our experience, immunocytochemical staining of brain tissue with high concentrations of certain rabbit antisera is often observed with the microtubules and PSD. Such a nonspecific staining in our tissues appears unlikely; we used antisera at dilutions that do not give nonspecific staining. Moreover, neutralization of the antiserum with the antigen effectively blocked the staining. In addition, the microtubules of axons contained no reaction product. Another possibility
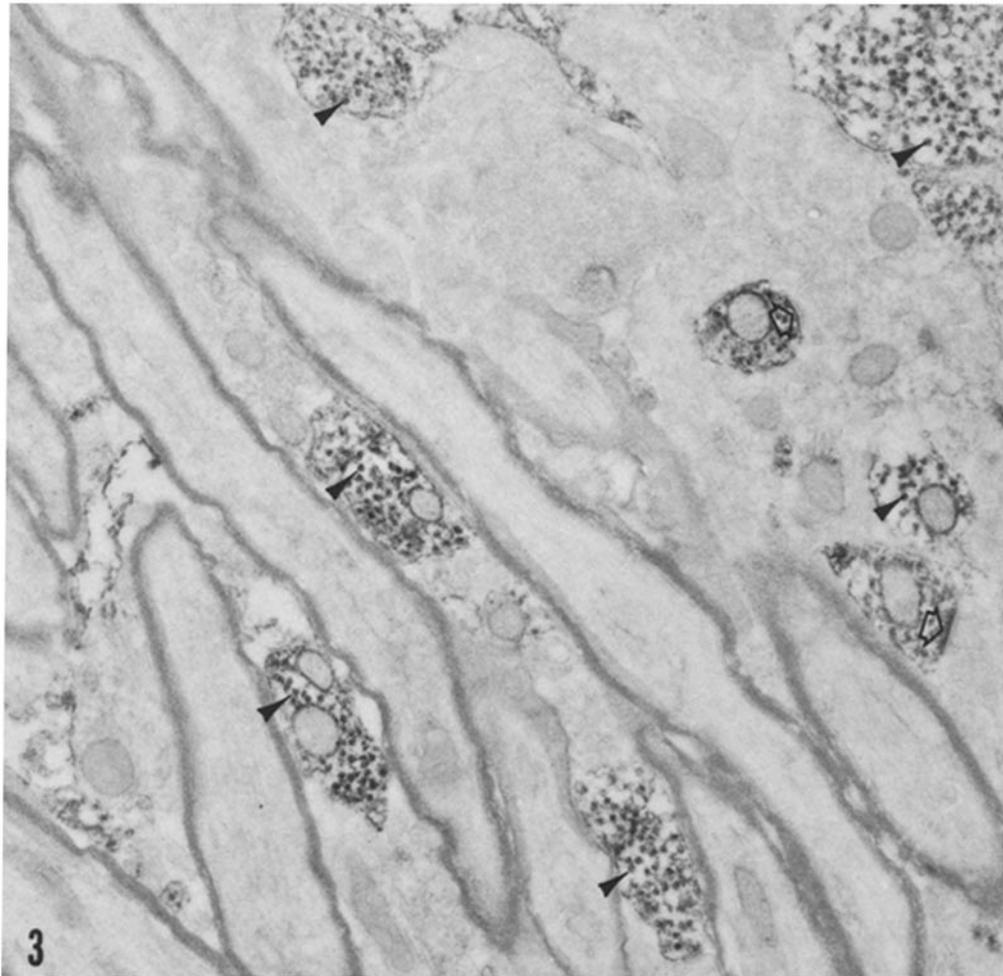
FIGURE 3 Anti-calmodulin labeling of the PSD (open arrows) and microtubules (long arrowhead) within apparent dendritic profiles. No label is observed within myelinated axons. × 24,000.

for the staining associated with the microtubules, mitochondria, and PSD is precipitation of soluble antigens to these organelles during aldehyde fixation, or precipitation of reactant product of peroxidase during cytochemical processing. This could explain why staining of the mitochondria is observed only on the membrane facing heavily labeled regions of the dendrite. If the two antigens were mitochondrial components, the staining should be present uniformly throughout the mitochondrial membrane regardless of its orientation.

Although it is difficult to eliminate the possibil-

ity that the microtubular and PSD staining results from crosslinking of soluble antigen or precipitation of peroxidase reaction product, several observations are consistent with the conclusion that calmodulin and CaM-BP$_{80}$ are associated with specific organelles. First, the immunocytochemical staining was always associated with the microtubule and PSD, and the staining was discrete and striking, even in profiles lightly labeled. If this represents crosslinking or precipitation reactions, they occur over a long distance in molecular terms. Second, the work of Grab et al. (13) clearly showed that calmodulin was associated with the PSD iso-
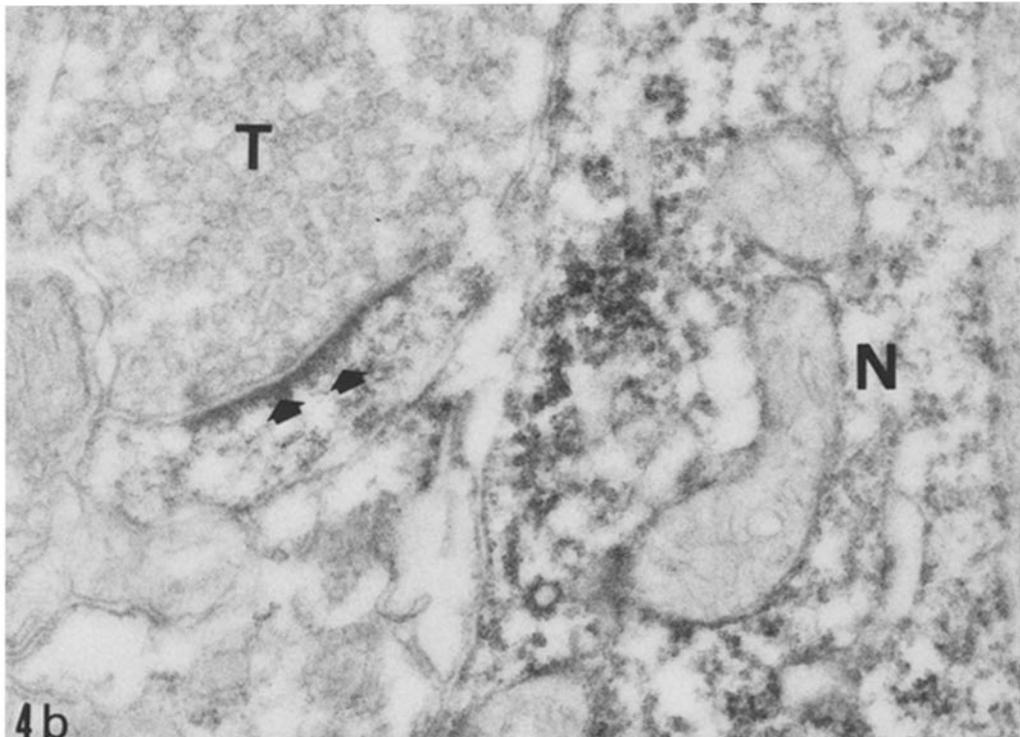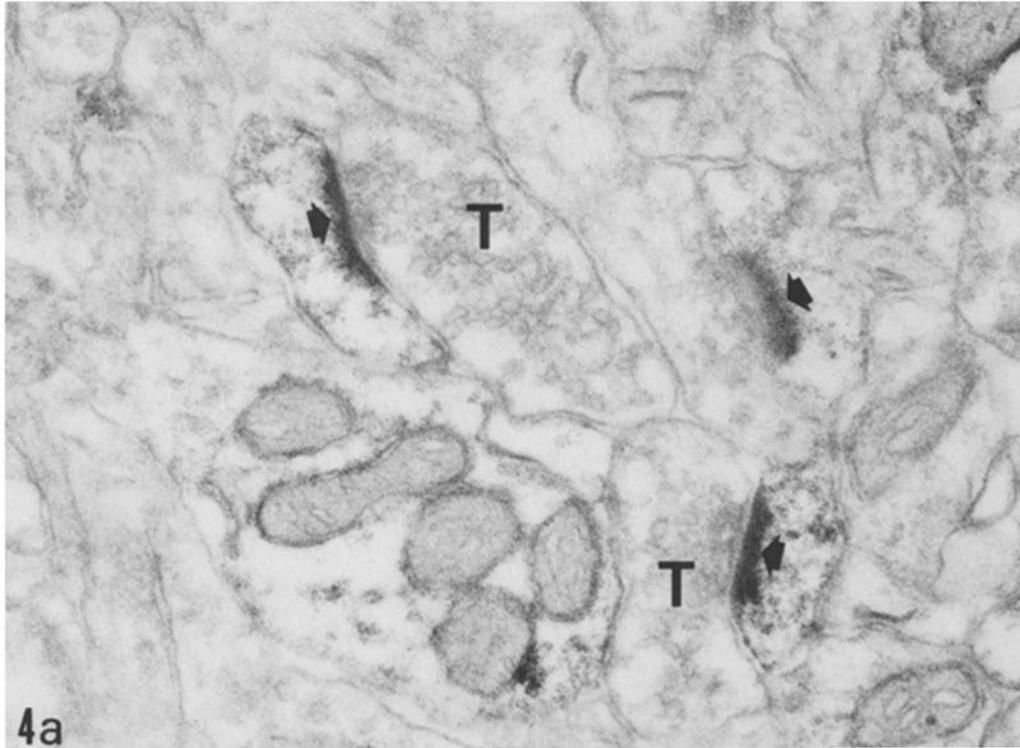
FIGURE 4 (*a* and *b*) Two examples of fields showing anti-calmodulin labeling of the PSD (short arrows). *T*, presynaptic terminal. *N*, neuron. The neuron contains label deposited on various cellular organelles. (*a*) × 70,200; (*b*) × 73,500.
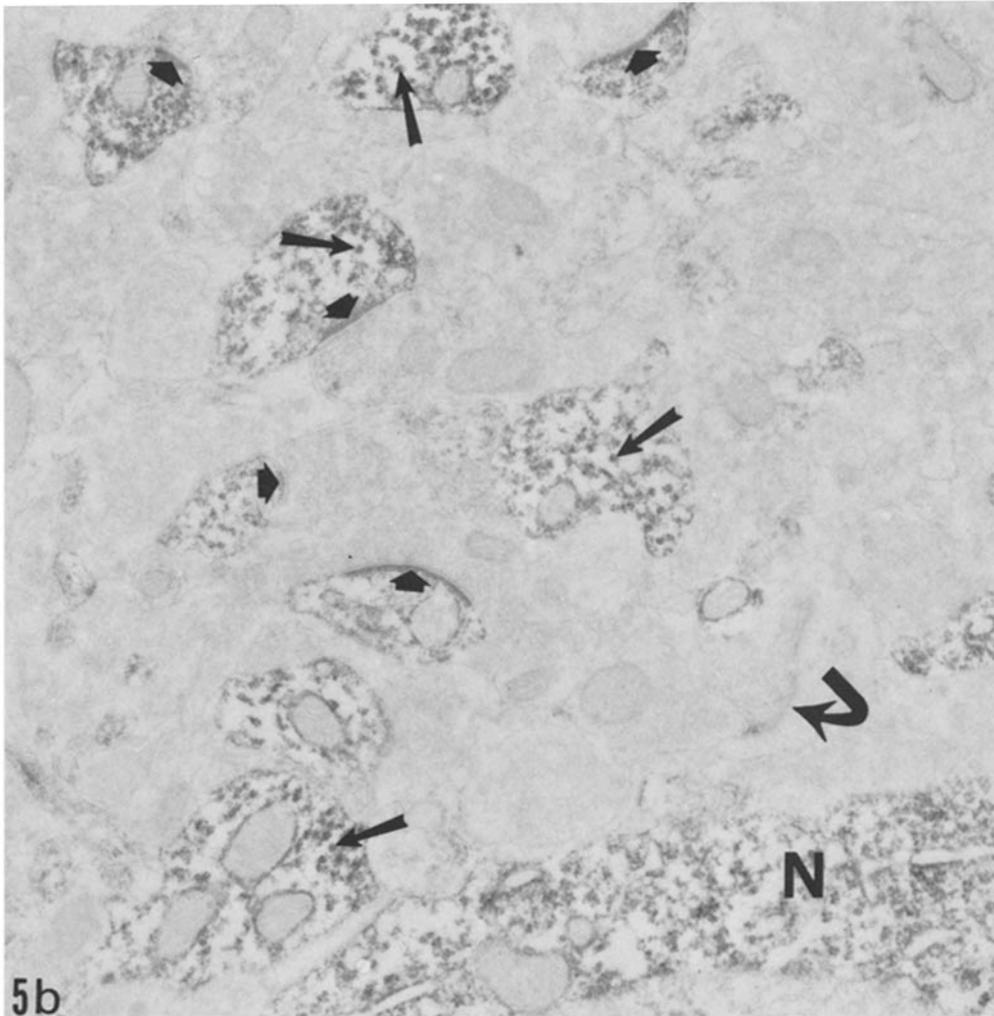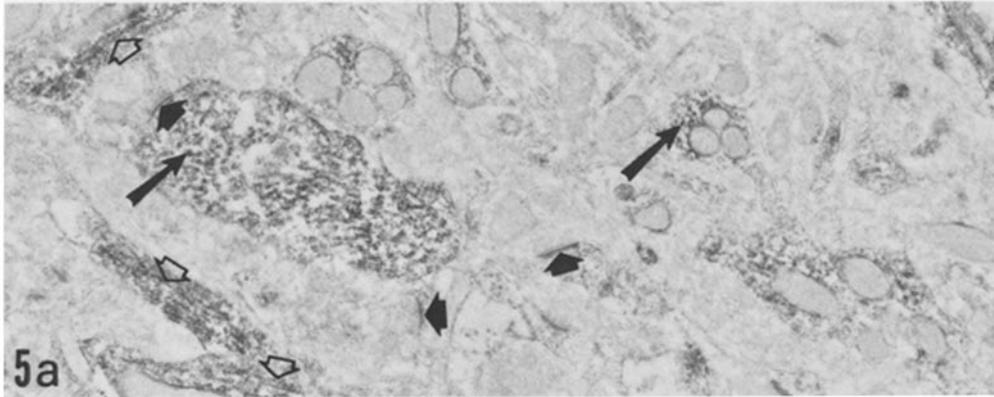
FIGURE 5 (*a* and *b*) Two examples of fields showing anti-CaM-BP$_{80}$ labeling of the PSD (closed short arrows) and microtubules cut in cross section (long straight arrows) or longitudinal section (short open arrows). Occasionally, an unlabeled PSD is observed (*b*, curved arrow) which serves as an internal control to compare to the appearance of labeled PSD. *N*, neuron which contains label deposited on various cellular organelles. (*a*) × 11,400; (*b*) × 22,800.

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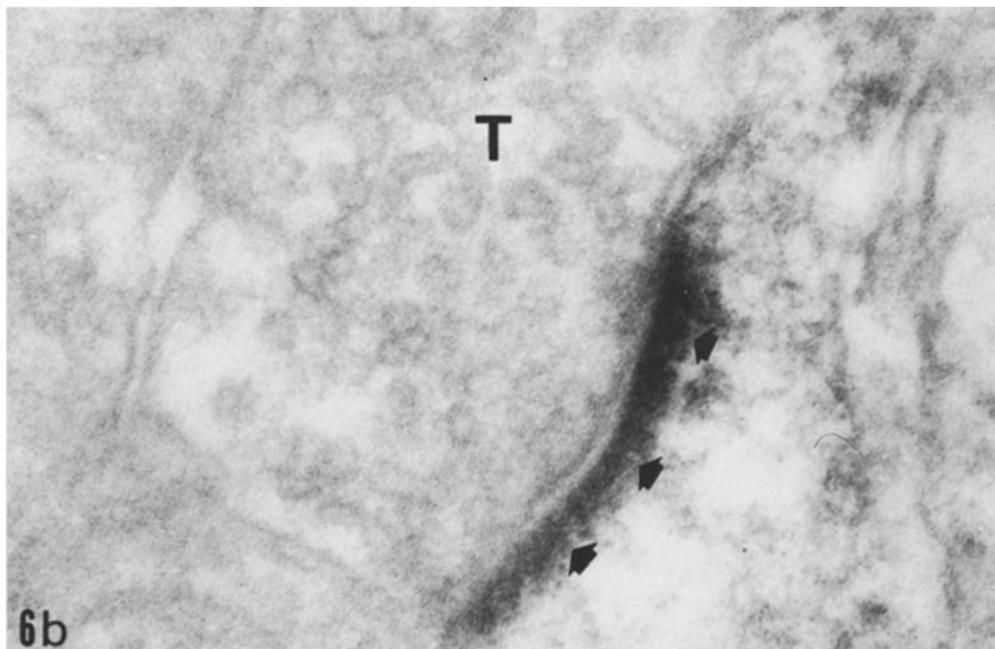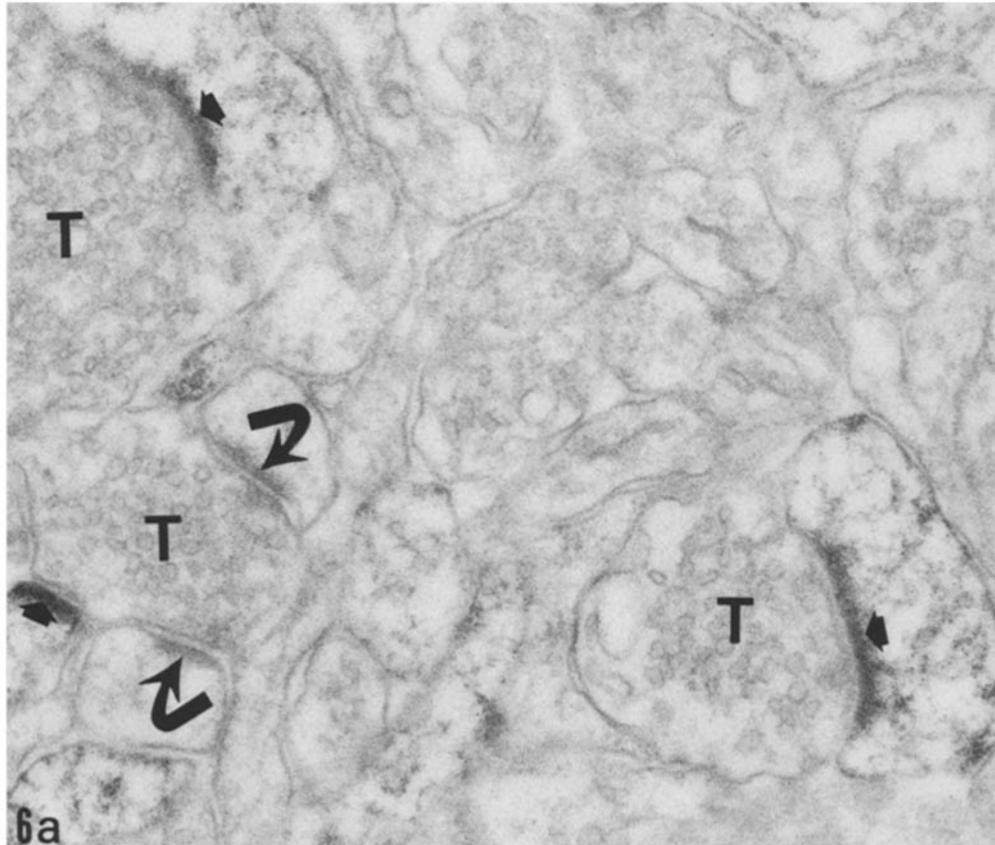
FIGURE 6 (a and b) Higher magnification examples of fields showing anti-CaM-BP$_{80}$ labeling of the PSD (short arrows). The curved arrows (a) point to very lightly labeled PSD which may be compared to the labeled PSD. T, presynaptic terminal. (a) × 68,800; (b) × 69,000.

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lated by subcellular fractionation. Third, Welsh et al. (45) demonstrated by immunofluorescence that calmodulin was associated with microtubules of the mitotic spindle of dividing cells. Finally, Marcum et al. (26) showed that calmodulin interfered with the assembly of microtubules in vitro. Collectively, these observations support the localization of calmodulin and CaM-BP$_{80}$ in the PSD and microtubules. This may imply a role for these proteins at the site of transmitter action and at the level of microtubular function. However, with the numerous effects potentially manifested by the regulatory influences of these two proteins, they may well have additional functions in neural tissue.

REFERENCES

1. ANDERSON, M. J., and J. M. CORMIER. 1978. Calcium-dependent regulator of NAD kinase in higher plants. *Biochem. Biophys. Res. Commun.* 84:595–602.
2. AVRAMEAS, S., and T. TERNYNCK. 1971. Peroxidase labeled antibody and Fab conjugates with enhanced intracellular penetration. *Immunochemistry.* 8:1175–1179.
3. BROSTROM, M. A., C. O. BROSTROM, B. M. BRECKENRIDGE, and D. J. WOLFF. 1976. Regulation of adenylate cyclase from glial tumor cells by calcium and a calcium-binding protein. *J. Biol. Chem.* 251:4744–4750.
4. CHEUNG, W. Y. 1967. Cyclic 3',5'-nucleotide phosphodiesterase: pronounced stimulation by snake venom. *Biochem. Biophys. Res. Commun.* 29:478–482.
5. CHEUNG, W. Y. 1969. Cyclic 3',5'-nucleotide phosphodiesterase: preparation of a partially inactive enzyme and its subsequent stimulation by snake venom. *Biochim. Biophys. Acta.* 191:303–315.
6. CHEUNG, W. Y. 1970. Cyclic 3',5'-nucleotide phosphodiesterase: demonstration of an activator. *Biochem. Biophys. Res. Commun.* 38:533–538.
7. CHEUNG, W. Y. 1970. Cyclic nucleotide phosphodiesterase. *In* Role of Cyclic AMP in Cell Function. P. Greengard and E. Costa, editors. Raven Press, New York. 51–65.
8. CHEUNG, W. Y., Y. M. LIN, Y. P. LIU, and J. A. SMOAKE. 1975. Regulation of bovine brain cyclic 3',5'-nucleotide phosphodiesterase by its protein activator. *In* Cyclic Nucleotides in Disease. B. Weiss, editor. University Park Press, Baltimore. 321–350.
9. COHEN, P., A. BURCHELL, J. G. FOULKES, P. T. W. COHEN, T. C. VANAMAN, and A. C. NAIRN. 1978. Identification of the Ca^{2+}-dependent modulator protein as the fourth subunit of rabbit skeletal muscle phosphorylase kinase. *FEBS Lett.* 92:287–293.
10. DABROWSKA, R., J. M. F. SHERRY, D. K. AROMATORIO, and D. J. HARTSHORNE. 1978. Modulator protein as a component of the myosin light chain kinase from chicken gizzard. *Biochemistry.* 17:253–258.
11. FLORENDO, N. T., R. J. BARRNETT, and P. GREENGARD. 1971. Cyclic 3',5'-nucleotide phosphodiesterase: cytochemical localization in cerebral cortex. *Science (Wash. D. C.).* 173:745–748.
12. GOPINATH, R. M., and F. F. VINCENZI. 1977. Phosphodiesterase protein
13. GRAB, D. J., K. BERZINS, R. S. COHEN, and P. SIEKEVITZ. 1978. Identification of a calcium dependent regulator protein (CDR) in the post-synaptic density (PSD). *J. Biol. Chem.* In press.
14. GRAHAM, R. C., and M. J. KARNOVSKY. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney. Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291–320.
15. HINDS, T. R., F. L. LARSEN, and F. F. VINCENZI. 1978. Plasma membrane Ca^{2+} transport: stimulation by soluble proteins. *Biochem. Biophys. Res. Commun.* 81:455–461.
16. JARRETT, H. W., and J. T. PENNISTON. 1977. Partial purification of the Ca^{2+}-Mg^{2+} ATPase activator from human erythrocytes: its similarity to the activator of 3':5'-cyclic nucleotide phosphodiesterase. *Biochem. Biophys. Res. Commun.* 77:1210–1216.
17. JARRETT, H. W., and J. T. PENNISTON. 1978. Purification of the Ca^{2+}-stimulated ATPase activator from human erythrocytes. *J. Biol. Chem.* 253:4676–4682.
18. KATZ, S., and M. A. REMTULLA. 1978. Phosphodiesterase protein activator stimulates calcium transport in cardiac microsomal preparations enriched in sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* 83:1373–1379.
19. KLEE, C. B., and M. H. KRINKS. 1978. Purification of cyclic 3',5'-nucleotide phosphodiesterase inhibitory protein by affinity chromatography on activator protein coupled to sepharose. *Biochemistry.* 17:120–126.
20. LAPORTE, D. C., and D. R. STORM. 1978. Detection of calcium-dependent regulatory protein binding components using ^{125}I-labeled calcium-dependent regulatory protein. *J. Biol. Chem.* 253:3374–3377.
21. LIN, Y. M., Y. P. LIU, and W. Y. CHEUNG. 1974. Cyclic 3':5'-nucleotide phosphodiesterase: Purification, characterization, and active form of an activator from bovine brain. *J. Biol. Chem.* 249:4943–4954.
22. LIN, Y. M., Y. P. LIU, and W. Y. CHEUNG. 1975. Cyclic 3',5'-nucleotide phosphodiesterase: Ca^{++}-dependent formation of bovine brain enzyme-activator complex. *FEBS Lett.* 49:356–360.
23. LYNCH, T. J., E. A. TALLANT, and W. Y. CHEUNG. 1976. Ca^{++}-dependent formation of brain adenylate cyclase-protein activator complex. *Biochem. Biophys. Res. Commun.* 68:616–625.
24. LYNCH, T. J., E. A. TALLANT, and W. Y. CHEUNG. 1977. Rat brain adenylate cyclase: further studies on its stimulation by a Ca^{2+}-binding protein. *Arch. Biochem. Biophys.* 182:124–133.
25. LYNCH, T. J., and W. Y. CHEUNG. 1979. Human erythrocyte Ca^{2+}-Mg^{2+}-ATPase: mechanism of stimulation by Ca^{2+}. *Arch. Biochem. Biophys.* 194:165–170.
26. MARCUM, J. M., J. R. DEDMAN, B. R. BRINKLEY, 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.
27. MENDELL, J. R., J. N. WHITAKER, and W. K. ENGEL. 1973. The skeletal muscle binding site of antistriated muscle antibody in myasthenia gravis: an electron microscopic immunohistochemical study using peroxidase conjugated antibody fragments. *J. Immunol.* 111:847–856.
28. MENDELL, J. R., and J. N. WHITAKER. 1978. Immunocytochemical localization studies of myelin basic protein. *J. Cell Biol.* 76:502–511.
29. MILLONIG, G. 1961. Advantages of a phosphate buffer for OsO$_4$ solutions in fixation. *J. Appl. Phys.* 32:1637. (Abstr.).
30. SCHULMAN, H., and P. GREENGARD. 1978. Stimulation of brain protein phosphorylation by calcium and an endogenous heat-stable protein. *Nature (Lond.).* 271:478–479.
31. SCHULMAN, H., and P. GREENGARD. 1978. Ca^{++} dependent protein phosphorylation system in membranes from various tissues, and its activation by "calcium dependent regulation." *Proc. Natl. Acad. Sci. U. S. A.* 75:5432–5436.
32. SHARMA, R. K., E. WIRCH, and J. H. WANG. 1978. Inhibition of Ca^{2+}-activated cyclic nucleotide phosphodiesterase reaction by a heat-stable inhibitor protein from bovine brain. *J. Biol. Chem.* 253:3575–3580.
33. SHARMA, R. K., R. DESAI, T. R. THOMPSON, and J. H. WANG. 1978. Purification of the heat-stable inhibitor protein of the Ca^{2+}-activated cyclic nucleotide phosphodiesterase by affinity chromatography. *Can. J. Biochem.* 56:598–604.
34. SHERRY, J. M. F., A. GORECKA, M. O. AKSOY, R. DABROWSKA, and D. J. HARTSHORNE. 1978. Roles of calcium and phosphorylation in the regulation of the activity of gizzard myosin. *Biochemistry.* 17:4411–4418.
35. SOUBE, K., S. ICHIDA, H. YOSHIDA, R. YAMAZAKI, and S. KAKIUCHI. 1979. Occurrence of a Ca^{2+}- and modulator protein-activatable ATPase in the synaptic plasma membranes of brain. *Biochem. Biophys. Res. Commun.* 99:199–202.
36. STEVENS, F. C., M. WALSH, H. C. HO, S. T. TEO, and J. G. WANG. 1976. Comparison of calcium binding proteins. *J. Biol. Chem.* 251: 4495–4500.
37. TESHINA, Y., and S. KAKIUCHI. 1974. Mechanism of stimulation of

Ca^{2+} + Mg^{2+}-dependent phosphodiesterase from rat cerebral cortex by the modulator protein and Ca^{2+}. *Biochem. Biophys. Res. Commun.* **56**:489–495.

38. VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**:407–408.

39. WAISMAN, D. M., T. J. SINGH, and J. H. WANG. 1978. The modulator-dependent protein kinase. *J. Biol. Chem.* **253**:3387–3390.

40. WALLACE, R. W., T. J. LYNCH, E. A. TALLANT, and W. Y. CHEUNG. 1978. An endogenous inhibitor protein of brain adenylate cyclase and cyclic nucleotide phosphodiesterase. *Arch. Biochem. Biophys.* **187**:328–334.

41. WALLACE, R. W., T. J. LYNCH, E. A. TALLANT, and W. Y. CHEUNG. 1979. Purification and characterization of an inhibitor protein of brain adenylate cyclase and cyclic nucleotide phosphodiesterase. *J. Biol. Chem.* **254**:377–382.

42. WALLACE, R. W., and W. Y. CHEUNG. 1979. Calmodulin: production of an antibody in rabbit and development of a radioimmunoassay. *J.*

Biol. Chem. **254**:6564–6571.

43. WANG, J. H., and R. DESAI. 1976. A brain protein and its effect on the Ca^{2+}- and protein modulator-activated cyclic nucleotide phosphodiesterase. *Biochem. Biophys. Res. Commun.* **72**:926–932.

44. WANG, J. H., and R. DESAI. 1977. Modulator binding protein. *J. Biol. Chem.* **252**:4175–4184.

45. WELSH, M. J., J. R. DEDMAN, B. R. BRINKLEY, and A. R. MEANS. 1978. Calcium-dependent regulator protein: localization in mitotic apparatus of eukaryotic cells. *Proc. Natl. Acad. Sci. U. S. A.* **75**:1867–1871.

46. WOOD, J. G., B. J. MCLAUGHLIN, and J. E. VAUGHN. 1976. Immunocytochemical localization of GAD in electron microscopic preparations of rodent CNS. *In* GABA in Nervous System Function. Roberts, Chase, and Tower, editors. Raven Press, New York. 133–148.

47. YAGI, K., M. YAZAWA, S. KAKIUCHI, and M. OHSHIMA. 1978. Identification of an activator protein for myosin light chain kinase as the Ca^{2+}-dependent modulator protein. *J. Biol. Chem.* **253**:1338–1340.