ABSTRACT Calcium/calmodulin-dependent protein kinase II (CaM kinase II) is a prominent enzyme in mammalian brain capable of phosphorylating a variety of substrate proteins. In the present investigation, the subcellular and regional distribution of CaM kinase II has been studied by light and electron microscopic immunocytochemistry using an antibody that recognizes the M, 50,000 and 60,000/58,000 subunits of the enzyme. Light microscopy demonstrates strong immunoreactivity in neuronal somata and dendrites and weak immunoreactivity in axons. Electron microscopy, in addition to confirming light microscopic observations, reveals moderate immunoreactivity in spines and weak immunoreactivity in nerve terminals. An accumulation of immunoreaction product is also present on postsynaptic densities. The presence of CaM kinase II in diverse structures throughout the neuron supports the view that this enzyme may be involved in mediating a variety of calcium-dependent physiological processes. CaM kinase II immunoreactivity is present in neurons throughout the brain, but a marked regional variation in the strength of the immunoreactivity exists. Overall, there is a gradient of staining intensity with the strongest immunoreactivity in the telencephalon and the weakest in the myelencephalon. The most heavily labeled regions of the telencephalon are the hippocampal formation, lateral septum, cortical regions, neostriatum, and amygdaloid complex.

Calcium/calmodulin-dependent protein kinase II (CaM kinase II) contains autophosphorylatable subunits of M, 50,000 and 60,000/58,000, constitutes ~0.4% of total brain protein (1–3), and phosphorylates a variety of substrate proteins (4). The subcellular distribution of CaM kinase II within neurons has not been studied in detail. However, the enzyme is present in both soluble and particulate fractions of brain tissue (1), and the M, 50,000 subunit of the kinase is identical to the major postsynaptic density (PSD) protein (5–7). In addition, co-localization of the kinase with its physiological substrates is to be expected. It is of interest, therefore, that synapsin I, which is present in nerve terminals (8), and microtubule-associated protein 2 (MAP 2), which is present in dendrites and somata (9), are phosphorylated by CaM kinase II (1, 4).

The regional distribution of CaM kinase II throughout the brain is also not known in detail. Assay for CaM kinase II activity in various brain regions by phosphorylation of exogenous synapsin I indicates that this enzyme is unevenly distributed in the brain (10, 11). Calcium/calmodulin-dependent phosphorylation of endogenous M, ~50,000 and ~60,000/58,000 polypeptides [recently found to represent subunits of CaM kinase II (unpublished observations in collaboration with S. I. Walaas)] also indicates an uneven distribution of the enzyme in the brain and, in addition, shows that the ratio of the subunits is not constant in all regions (10, 11). Consistent with this conclusion, CaM kinase II purified from rat forebrain and cerebellum has been found to exist as isozymes with different proportions of the M, 50,000 and 60,000/58,000 subunits (unpublished observations in collaboration with Y. Lai).

In the present study, a monoclonal antibody that recognizes each of the subunits of the enzyme has been used to examine the cellular and regional distribution of CaM kinase II in rat brain by light and electron microscopic immunocytochemistry. Brief reports on the immunocytochemical localization of this enzyme have been published (3, 12).

METHODS

Production of Monoclonal Antibody C42.1. CaM kinase II, purified as described (3), was used to produce monoclonal antibodies by modification of the method of Köhler and Milstein (13). Spleen cells from BALB/c × C57 Black mice, which had been immunized with CaM kinase II, were fused with SP2/0 Ag14 myeloma cells using polyethylene glycol. Hybridomas secreting antibodies specific for CaM kinase II were selected by screening culture supernatants for their ability to precipitate 125I-labeled and 32P-labeled M, 50,000 and 60,000/58,000 subunits. Positive cultures were subcloned and then injected intraperitoneally into pristane-primed mice for the production of ascites fluid. Antibodies were partially purified from the ascites fluid by two sequential precipitations with 45% ammonium sulfate. The antibody C42.1 precipitate was redissolved to a protein concentration of 17 mg/ml in phosphate-buffered saline (P/NaCl) (150 mM NaCl/10 mM potassium phosphate, pH 7.4), dialyzed overnight against P/NaCl, and stored in aliquots at –70°C.

Immunoblots. Whole brains were dissected from male Sprague–Dawley rats (100–200 g) and immediately homogenized by hand in 20 vol of boiling 1% NaDodSO4 in a Teflon/glass homogenizer. The homogenate was then boiled for an additional 3 min. Brain homogenate and purified CaM kinase II [purified as described (3)] were subjected to NaDodSO4/10% polyacrylamide gel electrophoresis as described (1) and then transferred to nitrocellulose sheets by modification (14) of the method of Towbin et al. (15). The nitrocellulose sheets were cut longitudinally into 5-mm strips and were stained, either for protein with amido black or for antibody reactivity with an alkaline phosphatase-linked procedure as described (14). All incubations were carried out in an incubation tray (Bio-Rad) with 22.8 × 1 cm wells; a 1:50 dilution of mouse monoclonal antibody C42.1 was used with an incubation time of 2 hr; a 1:500 dilution of goat anti-mouse IgG F(ab')2 fragment conjugated to alkaline phosphatase (Boehringer Mannheim) was used with an incubation time of 2.5 hr. Antibody C42.1 was highly selective for the Mr, 50,000 and 60,000/58,000 kinase subunits in brain homogenates, as shown in Fig. 1. Specificity was further demonstrated by immunoblots showing that the antibody did not cross-react with other calmodulin-binding proteins including.

Abbreviations: CaM kinase II, calcium/calmodulin-dependent protein kinase II; PSD, postsynaptic density.
smooth (gift of A. Nairn) and skeletal (gift of D. Hathaway) muscle myosin light chain kinases, calcineurin (gift of C. Klee), and calcium/calmodulin-dependent synapsin I kinase I (16) (gift of A. Nairn) (data not shown).

**Tissue Preparation and Immunocytochemical Staining.** Male Sprague–Dawley rats (100–200 g) were anesthetized with chloral hydrate (350 mg/kg) and perfused through the aorta with a variety of fixatives for light microscopy. The zinc/formaldehyde fixative of Mignani and Dahl (17) (4% formaldehyde/0.5% zinc salicylate in unbuffered saline) provided the highest retention of immunoreactivity and was therefore used for the light microscopic studies. Vibratome sections 20- to 50-μm thick were collected into 0.1 M Tris saline (pH 7.6) and sequentially incubated with antibody C42.1 directed against CaM kinase II, 1:4000, 48 hr; rabbit anti-mouse IgG, 1:20, 1 hr; and mouse peroxidase/antiperoxidase (Sternberger-Meyer) 1:100, 1 hr. All antibodies were diluted in 0.1 M Tris buffer (pH 7.6) containing non-immune rabbit serum (1:100). Each incubation with antibody was followed by three 10-min washes in Tris saline. The sections

**FIG. 1.** Immunoblot showing specificity of monoclonal antibody C42.1 directed against CaM kinase II. (Left) Protein stain of purified CaM kinase II (PK; 6 μg) and rat brain homogenate (Hom; 150 μg). (Right) Antibody reactivity of purified CaM kinase II (PK; 0.1 μg) and rat brain homogenate (Hom; 150 μg). Arrows point to the M, 50,000 (50), 58,000 (58), and 60,000 (60) polypeptides of CaM kinase II.

**FIG. 2.** Electron micrographs of ultrathin sections through the dentate gyrus. (A) Immunolabeled dendrite (d) giving rise to a spine (arrow). Immunoreactivity is greater in the dendrite than in its spine (as can also be seen for two other dendrites and their spines in the upper right corner of D and E. (B–E) Enhanced immunoreactivity on PSDs (between arrowheads) in sections not counterstained with uranyl acetate. In E, compare immunoreactive PSD (arrowheads) to unlabeled PSD (small arrows).] Immunoreactivity on PSDs is greater in strongly labeled (B and C) than in very lightly labeled (D and E) processes. (F) Immunoreactivity in the cytoplasm (c) of a granule cell. By comparison, the nucleus (n) appears to be unlabeled. The arrow points to a lightly labeled nerve terminal. (G) Immunoreactive dendrites (d) and nerve terminals (arrows). Most of the immunoreactivity in nerve terminals is associated with synaptic vesicles. (Bars = 100 nm.)
FIG. 3. Light micrographs of coronal sections through telencephalic structures. Dorsal is up. (A) Layer V of auditory neocortex. Strong immunoreactivity, evenly distributed throughout the cytoplasm, is observed in pyramidal neurons. Nuclei are unlabeled (also see G). (Bar = 10 μm.) (B) Ectal limb of the dentate gyrus showing immunoreactivity in the cytoplasm and initial axonal segment (arrowheads) of granule cells. (These cells have no basal dendrites.) Immunostaining of the cytoplasm of many of these cells appears non-uniform, with the most intense stain frequently in the region of the cytoplasm (arrows) facing the subcellular zone (asterisk). (Bar = 10 μm.) (C) Layers I-VI of visual neocortex. Immunoreactivity is relatively strong in layer I (in fine dendritic branches) and relatively weak in the lower half of layer IV. Very strong immunoreactivity is present in pyramidal neurons in layers II-III and V. (Bar = 0.5 mm.) (D) Rostral hippocampal formation. Immunoreactivity is strong in the ectal limb (ec) of the dentate gyrus and even stronger in the endal limb (en). Strong immunoreactivity is present in large dendrites in the stratum radiatum (sr) and in fine dendritic branches in the stratum lacunosum moleculare (slm). so, stratum oriens; sp, stratum
were then allowed to react with 3,3'-diaminobenzidine and hydrogen peroxide as described by Sternberger (18). The entire procedure was carried out on a tissue shaker at room temperature.

For electron microscopy, tissue was processed as described by Pickel (19): the fixative was 0.2% glutaraldehyde/4% formaldehyde (freshly depolymerized from paraformaldehyde) in 0.1 M phosphate buffer (pH 7.4); antibody C42.1 was used at a dilution of 1:2000 for 24 hr at room temperature. Ultrathin sections (60–80 nm) were examined before and after counterstaining with uranyl acetate.

Tests for specificity included the substitution of the following for antibodies against CaM kinase II: (i) monoclonal antibodies against dinitrophenol, 1:200; (ii) normal mouse serum, 1:100; (iii) monoclonal antibodies directed against other neuronal antigens of known distributions; and (iv) anti-CaM kinase II antibody preabsorbed batchwise with purified CaM kinase II. In addition, the effects of omission of each of the immunoreagents was examined. All such control experiments indicated specificity of staining.

RESULTS AND DISCUSSION

Subcellular Localization. Immunoreactivity was very strong in neuronal soma and dendrites and absent from nuclei (Figs. 2 and 3). In nerve terminals, immunoreactivity was clearly detectable only with the magnification and resolution afforded by electron microscopy (Fig. 2 F and G). Similarly, moderate immunoreactivity was clearly detectable in spines at the electron microscopic level (Fig. 2 A, D, and E), whereas a background of immunoreactive processes throughout the neuropil made the unequivocal identification of spines difficult at the light microscopic level (Fig. 3A). Weak axonal staining was observed in fiber tracts such as the corpus callosum and internal capsule. In general, immunoreactivity was evenly distributed in the perikaryal and dendritic cytoplasm (Figs. 2 and 3A), but in the granule cells of the dentate gyrus a strong accumulation of immunoreactive material was often present in that portion of the cell body adjacent to the subcellular zone (Fig. 3B). Differences in strength of immunoreactivity in different subcellular compartments (strong immunoreactivity in somata and dendrites; weak immunoreactivity in nerve terminals, spines, and axons) must be assessed in terms of the volume of cytoplasm and the accessibility of immunoreagents to kinase in each compartment.

At the electron microscopic level, immunoreactivity was observed on the inner surface of the plasmalemma, on the outer surfaces of mitochondria and synaptic vesicles, and on microtubules (Fig. 2). A heavy deposit of immunoreaction product, apparent in sections that were not counterstained with uranyl acetate, was present on PSDs in heavily labeled dendrites (Fig. 2 B and C), and a very light deposit was present on PSDs in lightly labeled spines (Fig. 2 D and E). The standard methods employed in the present study are not adequate to distinguish between true immunoreactivity of organelles and artificial staining of organelles due to the diffusion of immunoreaction product from nearby sites. Immunoreactivity of the PSD itself is plausible because the M, 50,000 subunit of CaM kinase II is the major protein present in PSDs isolated by subcellular fractionation (5).

CaM kinase II is a multifunctional enzyme capable of phosphorylating a variety of substrate proteins (4). In neurons, CaM kinase II immunoreactivity is present in spines, dendrites (including PSDs), somata, axons, and terminals, supporting the view that it may be involved in a variety of calcium-dependent physiological processes.

Regional Distribution. At the light microscopic level, immunoreactivity was observed in most, if not all, neurons but was not detected in glial cells. (The enzyme might be present in glial cells at levels below the limits of detectability.) Immunoreactivity within somata and dendrites was much stronger in some brain regions than in others (Figs. 3 and 4). It should be kept in mind that differences in immunoreactivity among various brain areas can be affected by a number of factors, including cell packing density and relative amounts of enzyme in different types of neurons. Nevertheless, dramatic differences in staining intensity were observed among various brain regions, with the strongest immunoreactivity in forebrain neurons and the weakest in hindbrain neurons.

In neocortex, pyramidal and nonpyramidal neurons were labeled. The most prominently labeled cells in the neocortex were the large pyramidal neurons of layer V (Fig. 3A and C), and the least prominently labeled were the small neurons in the lower half of layer IV (Fig. 3C). Strong immunoreactivity was present from layer III to the pial surface, especially in fine dendritic branches in layer I (Fig. 3C). Neurons in piriform cortex (Fig. 4E) and entorhinal cortex (not shown) were highly immunoreactive, with the greatest immunoreactivity in layers II and III. Strong immunoreactivity was also present in neurons of the olfactory tubercle and bulb. All elements of the hippocampal formation were very strongly immunoreactive, with the strongest staining in the anterior hippocampal rudiment (Fig. 3F), induseum griseum (Fig. 3E), dentate gyrus (Figs. 3D and 4B), and fascia cinereum (Fig. 3E).

Neurons in the lateral septum were strongly immunoreactive (Fig. 4A). Within the caudatoputamen, immunoreactivity was greatest in the dorsomedial portion adjacent to the lateral ventricle (Fig. 4A). Grossly, immunoreactivity was much weaker in the globus pallidus than in surrounding nuclei (Fig. 4A), but it should be noted that the globus pallidus has a very low cell density; immunoreactivity in individual perikarya in the globus pallidus was only slightly less than that in individual perikarya in the caudatoputamen. Immunoreactivity was very strong in the entire amygdaloid complex (Fig. 4B).

The most thalamic nuclei contained neurons that were less immunoreactive than those in the areas described above (Fig. 4B). The ventromedial hypothalamus was more heavily immunoreactive than other diencephalic regions (Fig. 4B). In the epithalamus, the habenula was notable for its very weak immunoreactivity (Fig. 4B). In general, neurons in brain regions caudal to the diencephalon were weakly immunoreactive. Within the mesencephalon, neurons in the region of the peripenduncular nucleus were more immunoreactive than those in other nuclei. In the cerebellum, both Purkinje neurons and granule cells were weakly labeled. Neurons in the caudal half of the substantia gelatinosa of the spinal trigeminal nucleus were more heavily immunoreactive than neurons elsewhere in the myelencephalon.

The regional distribution of CaM kinase II as shown by light microscopic immunocytochemistry presumably reflects the total amount of CaM kinase II, since the antibody used recognizes each of the kinase subunits. This distribution is in general agreement with that of the M, 50,000 and 60,000/
58,000 subunits as indicated by biochemical methods (10, 11) and the distribution of the M, 50,000 subunit as shown by immunohistochemical methods (20). Interestingly, the regional distribution of CaM kinase II (present results) and that of sodium-independent glutamate binding sites (21) show a striking correlation (unpublished observations in collaboration with S. Halpain and T. Rainbow).

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