Regional Distribution of Type II Ca^{2+}/Calmodulin-dependent Protein Kinase in Rat Brain[1]

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Abstract

The distribution of type II Ca^{2+}/calmodulin-dependent protein kinase has been mapped in rat brain by immunochemical and immunohistochemical methods using an antibody against its α-subunit. The concentration of the kinase, measured by radioimmunoassay, varies markedly in different brain regions. It is most highly concentrated in the telencephalon where it comprises approximately 2% of the total hippocampal protein, 1.3% of cortical protein, and 0.7% of striatal protein. It is less concentrated in lower brain structures, ranging from about 0.3% of hypothalamic protein to 0.1% of protein in the pons/medulla. The gradient of staining intensity observed in brain sections by immunohistochemistry corroborates this distribution. Neurons and neuropil of the hippocampus are densely stained, whereas little staining is observed in lower brain regions such as the superior colliculus. Within the diencephalon and midbrain, dense staining is observed only in thalamic nuclei and the substantia nigra. The skewed distribution of α-subunit appears to be due in part to the occurrence in the cerebellum and pons/medulla of forms of the kinase with a high ratio of β- to α-subunits. However, most of the variation is due to the extremely high concentration of the kinase in particular neurons, especially those of the hippocampus, cortex and striatum. The unusually high expression of the kinase in these neurons is likely to confer upon them specialized responses to calcium ion that are different from those of neurons in lower brain regions.

Changes in the intracellular concentration of calcium regulate a number of important neuronal processes (Greengard, 1981; Reichardt and Kelly, 1983). In order to understand at the molecular level how calcium ion carries out its regulatory roles, it will be necessary to identify the calcium target proteins in neurons and to understand how these proteins compete for calcium to generate a coordinated physiological response. In order to explain the fact that different classes of neurons respond to calcium ion in different ways, it will also be important to understand how distinct sets of calcium target proteins in different neurons generate specialized responses to calcium ion.

Several calcium target proteins have been identified in the nervous

system (Kennedy, 1983; Reichardt and Kelly, 1983). Among them are three classes of calcium-activated protein kinases: the C-kinase (Takai et al., 1979) and at least two different classes of calmodulin-dependent protein kinases (Yamauchi and Fujisawa, 1980, 1983; Kennedy and Greengard, 1981; Bennett et al., 1983; Goldenring et al., 1983). The type II Ca^{2+}/calmodulin-dependent protein kinases (type II CaM kinases) are a class of closely related enzymes all of which have a similar broad substrate specificity. They are large oligomeric proteins composed of subunits with M_r = 50,000 to 60,000. Their exact subunit composition varies from tissue to tissue (Ahmad et al., 1982; Bennett et al., 1983; Goldenring et al., 1983; McGuinness et al., 1983; Woodgett et al., 1983) and in different brain regions (McGuinness et al., 1985; Miller and Kennedy, 1985).

This class of kinases is far more highly expressed in brain than in other tissues. Estimates of the concentration of brain type II CaM kinase, based on its fold-purification, suggest that it makes up at least 0.3% of total brain protein (Bennett et al., 1983). In contrast, the type II CaM kinases expressed in liver and skeletal muscle are less than 0.02% of the total protein in those tissues (Payne et al., 1983; Woodgett et al., 1983). The most prominent form of the enzyme in brain is purified from the forebrain. It contains an average of nine α-subunits (50 kilodaltons (kd)) and three β/β'-subunits (60/58 kd) (Bennett et al., 1983). The cerebellum contains a variant of the brain enzyme, composed of an average of eight β/β'-subunits and two α-subunits (McGuinness et al., 1985; Miller and Kennedy, 1985).

A report by Walaas et al. (1983) suggested that type II CaM kinase activity in rat brain showed more regional variation than either cAMP-dependent kinase or C-kinase activities. In this study we have examined the distribution of the type II CaM kinase by immunochemical methods. We developed a radioimmunoassay based on a monoclonal antibody that binds on immunoblots with high affinity and high specificity to the α-subunit of the brain kinase. Using this assay, we have confirmed the high concentration of the kinase in brain and have demonstrated a marked variation in its concentration in different brain regions. For example, we estimate that the kinase holoenzyme is about 2% of the total protein in the hippocampus and only about 0.1% of the protein in the pons/medulla. We have also used immunohistochemical methods to examine more precisely the distribution of the kinase in neurons within the brain. These results generally confirm those obtained by immunoassay, revealing, for example, dense staining of all neurons in the hippocampus, and very light or no staining of neurons in the medulla. In addition, the histochemical results reveal that, within the diencephalon and midbrain, the thalamic nuclei and the substantia nigra stain more darkly than do other areas. A preliminary report of this work has appeared (Erondu et al., 1983).

Materials and Methods

Materials. [γ-^{32}P]ATP and carrier-free Na^{125}I were purchased from ICN (Irvine, CA), and nitrocellulose membranes (BA 85, 0.45-μm pore diameter)

were purchased from Schleicher and Schuell Inc. (Keene, NH). ATP, dithio-threitol (DTT), imidazole, EDTA, EGTA, phenylmethylsulfonyl fluoride (PMSF), Trizma buffer, soybean trypsin inhibitor, diaminobenzidine, Triton X-100, hemoglobin (bovine type II), naphthol blue black (amido black), polyinosinic-polycytidylic acid (Poly I, Poly C), and mouse IgG were purchased from Sigma Chemical Co. (St. Louis, MO). Protein A, protein A-Sepharose, and agarose C were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Leupeptin was purchased from Peninsula Laboratories, Inc. (Belmont, CA), and ultrapure sucrose was purchased from Schwartz/Mann (Spring Valley, N. Y.). Horseradish peroxidase-conjugated rabbit anti-mouse IgG was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY). Glutaraldehyde was purchased from Polysciences, Inc. (Warrington, PA). Sprague-Dawley rats (140- to 160-gm males) were purchased from Simonsen Laboratories (Gilroy, CA), New Zealand female rabbits were obtained from Lab Pets (Rosemead, CA), and BALB/c ByJ mice were from Jackson Laboratories (Bar Harbor, ME). Rabbit anti-mouse IgG antiserum was obtained from rabbits immunized by repeated subcutaneous injections with mouse IgG in Freund's adjuvant and was affinity-purified by chromatography on Sepharose 4B coupled with mouse IgG (March et al., 1974). Protein A was iodinated by the chloramine T method essentially as described by Fryxell et al. (1983). The specific activity was 2 to 3 Ci/mmol, and 50 to 60% of it was retained by rabbit anti-mouse IgG coated on plastic microwells.

Preparation of hybridoma 6G9. A 6-week-old BALB/c ByJ female mouse was immunized acording to a schedule adapted from Stähli et al. (1980). For each set of injections, 50% pure type II CaM kinase, purified through the calmodulin-Sepharose step as described by Kennedy et al. (1983a), was mixed with an equal weight of Poly I, Poly C in a volume of 300 μl. About half of the mixture was administered intraperitoneally, the other half was given subcutaneously. The mouse was primed with 150 μg of kinase and boosted with 220 μg 3 weeks later. After an additional week, the mouse was bled from the tail and the titer of anti-kinase antibodies was measured by solid phase radioimmunoassay as previously described (Kennedy et al., 1983a). Six weeks after the boost, 195 μg of antigen were administered each day for 4 days in a row. On the fifth day, spleen cells were fused with NS1/SP2 myeloma cells by standard techniques, and fused hybrids were selected in hypoxanthine/aminopterin/thymidine medium (Köhler and Milstein, 1976; Moore et al., 1982). Of 160 antibody-secreting clones, 80 produced antibody against the crude antigen as measured in a solid phase well-binding assay similar to that described by Moore et al. (1982). One of these, 6G9, bound with high specificity and high affinity to the α-subunit on immunoblots. This hybridoma was subcloned and ascites fluids were prepared as previously described (Kennedy et al., 1983a). Antibody from ascites fluids was partially purified by precipitation with 50% saturated ammonium sulfate and dialyzed against buffer A (50 mM Tris-HCl, pH 7.4/0.9% NaCl/0.1% NaN₃). This preparation (20 mg of protein/ml) was used for immunocytochemistry. For the radioimmunoassay, the dialysate was further purified on a protein A-Sepharose affinity column (Ey et al., 1978). The IgG₁ fraction eluted from the column is subsequently referred to as "Pr. A-purified" 6G9.

Immunoassay of brain regions. Male Sprague-Dawley rats (140 to 160 gm body weight) were stunned and decapitated, and their brains were placed in ice-cold buffer B (40 mM Tris (pH 7.5), 1 mM imidazole, containing 150 mM sodium perchlorate, 250 mM sucrose, 5 mM EDTA, 1 mM EGTA, 2 mM DDT, 0.1 mM PMSF, 2 mg/liter of leupeptin, and 25 mg/liter of soybean trypsin inhibitor). Dissections were performed in an ice-cooled Petri dish according to a modification of the method described by Glowinski and Iversen (1966), as shown in Figure 1. The olfactory bulbs, cerebellum, and pons/medulla were separated from the rest of the brain. Then, a sagittal section was made to divide the brain into two halves. The hypothalamus was dissected along the lines shown in Figure 1, using the anterior commissure as a reference point. The midbrain/thalamus was then gently separated from the rest of the brain, exposing the striatum. The striatum and the hippocampus were removed by blunt dissection, and the rest of the brain was taken as the cortex. With this dissection procedure, the "striatum" consists of the basal ganglia of the telencephalon without the amygdala, whereas the "cortex" corresponds to the telencephalon without the "striatum." Each brain region was weighed. The wet weight varied in different dissections by less than 10%. The brain regions were homogenized in 10 vol of buffer B with 12 up-and-down strokes in a glass/Teflon homogenizer rotating at 1500 rpm.

The different brain regions were assayed for α-subunit using a quantitative immunoblot method. Aliquots of the homogenates were subjeted to polyacrylamide gel electrophoresis (PAGE) in the presence of SDS according to the method of Laemmli (1970). Stacking gels were 3.5% acrylamide/0.09% bisacrylamide. Running gels were 10% acrylamide/0.27% bisacrylamide. Standard amounts of purified kinase, containing 35, 70, 140, 210, and 350 ng of α-subunit were loaded onto each gel. Immediately after electrophoresis, the region of the gel containing proteins of M_r = 30,000 to 70,000 was cut
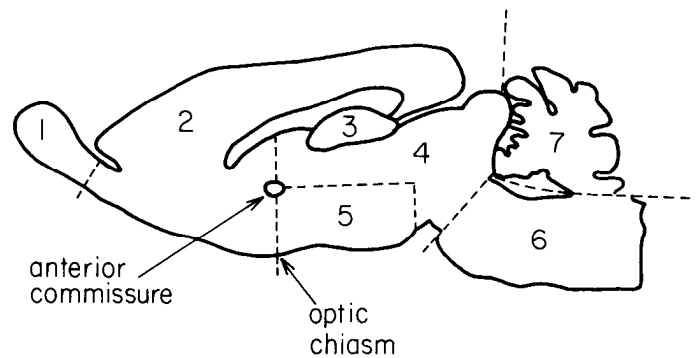

Figure 1. Diagrammatic representation of the procedure used in the dissection of rat brain. *Dashed lines* indicate positions of the sections: *1*, olfactory bulb; *2*, cerebral cortex; *3*, hippocampus; *4*, midbrain/thalamus; *5*, hypothalamus; *6*, pons/medulla; and *7*, cerebellum. Adapted from Glowinski and Iversen (1966).

out, and the separated proteins were transferred onto nitrocellulose paper by a modification of the method of Towbin et al. (1979). The transfer was effected at a constant current of 350 mA for 10 hr in 10 mM sodium borate, pH 9.5. After the transfer, the nitrocellulose sheets were incubated with: (*1*) buffer A containing 5% hemoglobin (2 hr); (*2*) Pr. A-purified 6G9 (3 to 5 μg/ml) diluted into buffer A containing 1% hemoglobin (8 hr); (*3*) wash buffer (buffer A containing 0.5% hemoglobin) (1 hr with three changes); (*4*) rabbit anti-mouse IgG (3 to 5 μg/ml) in wash buffer (2 hr); (*5*) wash buffer (1 hr with three changes); (*6*) ¹²⁵I-labeled protein A (2 to 3 × 10⁵ cpm/ml) in wash buffer (2 hr); and (*7*) wash buffer (1 hr with three changes). The nitrocellulose sheet was dried, and the ¹²⁵I-labeled bands were located by autoradiography, cut out, and counted in a gamma counter. Background counts were obtained by cutting out and counting portions of the nitrocellulose sheet that contained no α-subunit. After subtracting background counts, standard curves were prepared by plotting on a double log scale the counts in labeled bands from lanes containing pure kinase against the amount of α-subunit in each lane. These standard curves were used to compute the amount of α-subunit present in unknown samples of brain region homogenates.

Immunohistochemical staining of α-subunit in brain regions. Male Sprague-Dawley rats (140 to 160 gm body weight) were perfused through the heart for 6 to 8 min with 0.1 M sodium phosphate buffer, pH 7.2, 4% paraformaldehyde, 0.1% glutaraldehyde. After an additional 10 min, brains were removed and placed in the same fixative at 4° C overnight. Fifty-micrometer sections were cut on an Oxford Vibratome under cold 0.1 M phosphate buffer, pH 7.2. The sections were stained by a modification of the method described by Pickel (1981). They were washed briefly in 0.1 M Tris buffer, pH 7.5, 0.9% NaCl (Tris-saline) to inactivate remaining glutaraldehyde (DeCamilli et al., 1983), then transferred for 15 min to Tris-saline containing 0.2% Triton. Some sets of sections (noted in figure legend) were not treated with Triton. The sections were washed twice for 8 to 10 min each in Tris-saline, then incubated for 30 min in 3% non-immune rabbit serum in Tris-saline. They were again washed twice for 8 to 10 min each in Tris-saline and transferred to experimental or control antibody solutions prepared in Tris-saline plus 3% non-immune rabbit serum. Experimental antibody solutions contained ammonium sulfate-purified 6G9 ascites fluid diluted 1/200. Control solutions contained either ammonium sulfate-purified ascites fluid from the NS1/SP2 parent tumor diluted 1/200 or no antibody. Sections were incubated in these solutions at 4°C for 8 to 12 hr, then washed twice for 8 to 10 min each in Tris-saline plus 1% non-immune rabbit serum. They were then transferred to a solution of horseradish peroxidase-conjugated rabbit anti-mouse IgG diluted 1/50 in Tris-saline plus 1% non-immune rabbit serum, incubated for 1 hr at room temperature, and washed twice for 8 to 10 min each in Tris-saline. Reaction product was developed by incubation for 6 to 8 min in 0.0125% diaminobenzidine and 0.003% peroxide in 0.1 M Tris buffer, pH 7.5. The reaction was stopped by two quick rinses in 0.1 M Tris buffer. The mounted sections were examined with a Zeiss Universal microscope and then photographed with a Nikon Multiphot apparatus using the diascopic illuminator.

Agarose slabs containing kinase were prepared by mixing various amounts of pure kinase with a prewarmed solution of 3% agarose in 5 mM phosphate buffer, pH 7.2, to produce solutions containing 1.5% agarose, and 0.5, 0.25, 0.1, 0.05, or 0.02 mg/ml of kinase in a volume of 80 μl. The solutions were quickly formed into 0.17-mm-thick slabs by pipetting them into prewarmed molds as described by DeCamilli et al. (1983). The cooled slabs were fixed

for 30 min at room temperature in 4% paraformaldehyde, 0.1% glutaraldehyde, 0.1 M phosphate buffer, pH 7.2, and then overnight at 4°C in the same solution. The fixed slabs were stained with 6G9 as described above.

Assay for calmodulin-dependent synapsin I kinase activity. Brain regions or other tissues were homogenized in 20 mM Tris (pH 7.5), 1 mM imidazole containing 2 mM DTT, 0.1 mM PMSF, 1.0 mM MgCl$_2$, 25 mg/liter of soybean trypsin inhibitor, and 1 mg/liter of leupeptin. Calmodulin-dependent synapsin I kinase activity was assayed, with minor modifications, as previously described (Bennett et al., 1983; Kennedy et al., 1983b). The reaction mix contained, in a final volume of 100 μl, 50 mM Tris (pH 8.0), 10 mM MgCl$_2$, 5 mM 2-mercaptoethanol, 1 μg of calmodulin, 20 μg of synapsin I, 50 μM[γ-^{32}P]ATP (1 to 2 × 10^3 cpm/pmol), either 0.4 mM EGTA (minus calcium) or 0.4 mM EGTA/0.7 mM CaCl$_2$ (plus calcium), and 2 to 25 μg of crude homogenate protein. Tubes were warmed for 60 sec to 30°C; reactions were initiated by addition of ATP and terminated after 20 sec (to ensure initial rates) by addition of 50 μl of SDS-stop solution. Synapsin I phosphorylation was analyzed by SDS-PAGE as described (Kennedy et al., 1983b). For each brain region or tissue, assays were performed in duplicate with varying amounts of homogenate protein to determine the range in which the rate of phosphorylation was linear with protein. The type II CaM kinase specifically phosphorylates a site on synapsin I that is recovered in a 30-kd fragment after digestion with *Staphyllococcus aureus* V8 protease. Therefore, after assays of brain region homogenates, labeled synapsin I was digested and phosphorylation of this site was determined as described previously (Kennedy and Greengard, 1981). Under the present assay conditions, 75 to 90% of calcium-stimulated phosphorylation of synapsin I occurred on the site recovered in the 30-kd fragment. Specific activities were calculated by dividing the rate of phosphorylation of the site contained in the 30-kd fragment by the amount of homogenate protein in the assay. Protein was measured by the method of Peterson (1977) with bovine serum albumin as standard.

Phosphorylation of the kinase subunits. Incorporation of ^{32}P into the kinase subunits was measured as above with the following modifications. Two hundred nanograms of pure kinase were used per assay, and synapsin I and homogenate protein were omitted. [γ-^{32}P]ATP was used at a higher specific activity (1 to 2 × 10^4 cpm/pmol) and the reaction was terminated after varying lengths of time by the addition of SDS-stop solution. ^{32}P-labeled subunits were localized in gels by autoradiography, cut out, and counted by liquid scintillation spectrometry. For measurement of phosphorylated α-subunit by the immunoblot assay, cold (nonradioactive) ATP was used instead of [γ-^{32}P]ATP.

Results

Radioimmunoassay for the α-subunit of brain Type II CaM kinase. A difficulty with radioimmunoassay of brain type II CaM kinase is that the kinase is found in both soluble and particulate fractions of brain homogenates. Some of the particulate kinase can be solubilized by dilution into a low ionic strength buffer (Kennedy et al., 1983b), but complete solubilization requires a strong detergent. To circumvent this problem, we devised a radioimmunoassay based on quantitative immunoblots. Such an adaptation of the immunoblot technique has been used previously for the quantitation of gap junction proteins (Traub et al., 1983) and P$_0$ glycoprotein of peripheral nerve myelin (Nunn and Mezei, 1984). In this method, the protein sample is completely solubilized in SDS and fractionated by SDS-PAGE before the immunoassay is performed. To develop the assay, we used a monoclonal antibody (6G9) that binds on immunoblots with high specificity and high affinity to the α-subunit of the type II CaM kinase (Kennedy et al., 1983a).

Antibody 6G9 binds specifically to the α-subunit of the kinase in all of the brain regions examined in this study (Fig. 2). In most of the homogenates the antibody reacts with a single band of 50 kd. This band can be removed from homogenates by immunoprecipitation with a different anti-kinase monoclonal antibody, 4A11 (data not shown). In homogenates of the hypothalamus, midbrain/thalamus, and pons/medulla, 6G9 binds to a closely spaced doublet, both bands of which can be immunoprecipitated by 4A11. The second band appears to be an isomeric variant of the α-subunit which is present in these regions, rather than a post-translationally modified form, since *in vitro* translation of whole brain messenger RNA also produces two closely spaced bands, both of which are precipitated by anti-kinase antibodies (M. K. Bennett, unpublished observations).

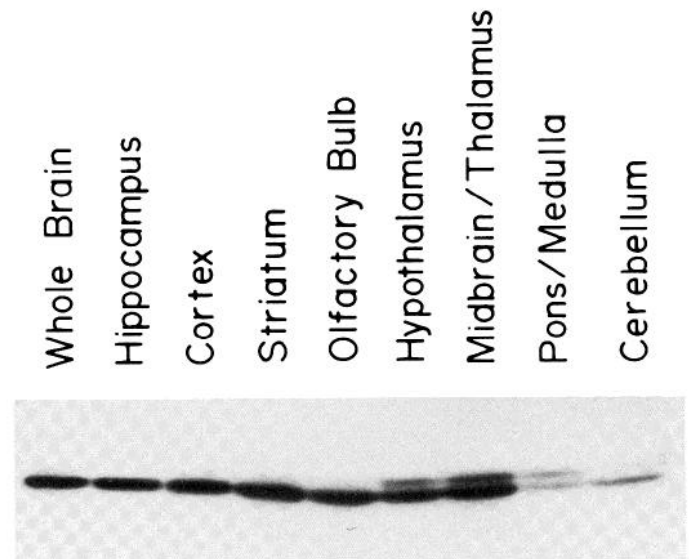Because some antibodies distinguish between "dephospho" and

Figure 2. Autoradiogram illustrating the reaction of antibody 6G9 with crude homogenates from different regions of the rat brain. Aliquots of crude homogenates were subjected to SDS-PAGE and the proteins on the gel were transferred to nitrocellulose paper. The nitrocellulose paper sheet was then reacted with 6G9 by the immunoblot technique as described under "Materials and Methods." Whole brain, 24 μg; hippocampus, 11 μg; cortex, 18 μg; striatum, 29 μg; olfactory bulb, 70 μg; hypothalamus, 57 μg; midbrain/thalamus, 92 μg; pons/medulla, 164 μg; cerebellum, 171 μg.
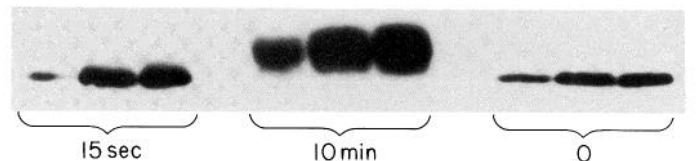

Figure 3. Autoradiogram showing the reaction of antibody 6G9 with "phospho" and "dephospho" forms of the α-subunit. Pure kinase was autophosphorylated for the lengths of time indicated. Aliquots of the reaction mixture containing 35, 70, and 85 ng of the α-subunit were subjected to SDS-PAGE, transferred to nitrocellulose paper and reacted with 6G9 by the immunoblot technique as described under "Materials and Methods."

"phospho" forms of a phosphoprotein (Nairn et al., 1982; Sternberger and Sternberger, 1983), we compared the binding of 6G9 to unphosphorylated and phosphorylated α-subunit (Fig. 3). After 15 sec of autophosphorylation, approximately 1 mol of phosphate was incorporated/mol of subunit and the amount of 6G9 bound was not significantly changed. After 10 min, approximately 3 mol of phosphate were incorporated/mol of subunit, its mobility had shifted considerably (see Bennett et al., 1983), and the amount of 6G9 bound was approximately twice that bound to the same amount of unphosphorylated kinase. Thus, in theory, the amount of α-subunit measured in the various brain regions could depend, within a factor of two, on the proportion of phosphorylated subunit in each region. However, in all of the assays reported in this study, the α-subunit band appeared sharp, rather than diffuse or shifted in mobility. We concluded that the level of phosphorylation in the crude homogenates was not sufficient to affect the results.

The linear range of the immunoassay with purified kinase is 20 to 500 ng of α-subunit (28 to 700 ng of kinase) (Fig. 4). The limit of detection ($p < 0.05$) of α-subunit is 3.5 ng (5 ng of kinase). A standard curve similar to that shown in Figure 4 was used to calibrate each radioimmunoassay as described under "Materials and Methods." The range in which the amount of α-subunit measured in brain homogenates is linear with homogenate protein is shown in Figure 5. The presence of up to 100 μg of homogenate protein does not

affect detection of added standard α-subunit (data not shown); although it does increase the error of detection and decrease the linear range of the assay to approximately 20 to 250 ng of α-subunit.

Distribution of α-subunit in brain regions measured by radioimmunoassay. We measured the concentration of the α-subunit in several brain regions, as described under "Materials and Methods." Two important findings emerge from the results summarized in Table I. First, when measured by radioimmunoassay, the α-subunit is 3 times as abundant in brain homogenates as was originally estimated from purification results (Bennett et al., 1983). It constitutes approximately 0.7% of the total protein in whole brain homogenates. Therefore, the entire type II CaM kinase holoenzyme, which is composed of both α- and β-subunits in approximately a 3 to 1 ratio, makes up nearly 1% of the total brain protein. Second, there is a marked regional variation in the concentration of the α-subunit. It is

TABLE I

Distribution of the α-subunit of type II CaM kinase in rat brain

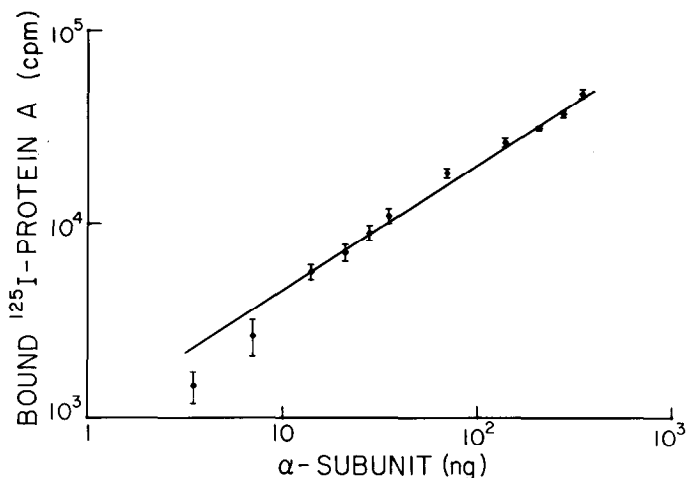| Brain Region	Concentration of α-Subunit		No. of Determinations	No. of Animals
	Nanograms/100 µg of Total Protein[a]	Percentage of Total Protein		
Whole brain	740 ± 42	0.74	15	2
Hippocampus	1420 ± 87	1.4	16	4
Cerebral cortex	860 ± 82	0.86	10	4
Striatum	460 ± 39	0.46	12	4
Olfactory bulb	260 ± 20	0.26	11	4
Hypothalamus	220 ± 13	0.22	8	4
Midbrain/thalamus	160 ± 10	0.16	12	4
Cerebellum	57 ± 6	0.06	16	6
Pons/medulla	50 ± 3	0.05	8	2

[a] Mean ± SEM.

Figure 4. Standard curve. Varying amounts of pure kinase were subjected to SDS-PAGE and transferred to nitrocellulose paper. The paper sheet was then reacted with antibody 6G9 by the immunoblot technique, and labeled bands were cut out and counted as described under "Materials and Methods." The *line* was calculated by weighted linear regression of the data. Each *point* represents the mean ± SD of four determinations.
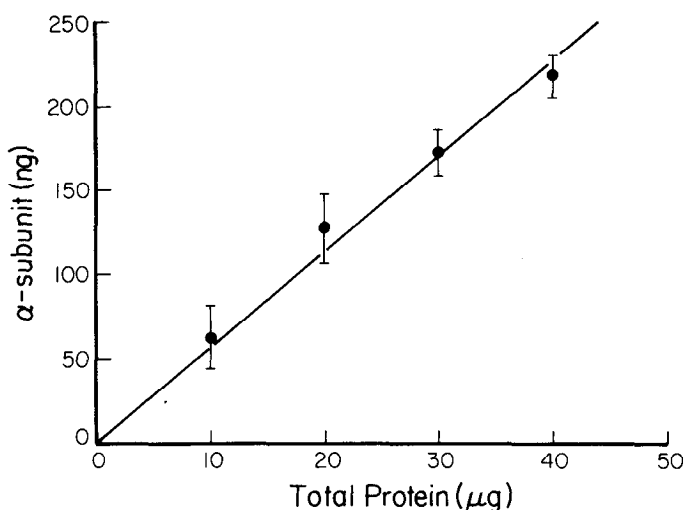

Figure 5. Proportionality between the α-subunit content of whole brain homogenate and total brain protein assayed. Increasing amounts of crude whole brain homogenate were assayed for α-subunit by the immunoblot method as described under "Materials and Methods." The values of α-subunit computed from the standard curve were then plotted against the total protein in the homogenate. The *line* was calculated by linear regression of the data. Each *point* represents the mean ± SD of four determinations.

highly concentrated in the hippocampus where it is 1.4% of total protein. Therefore, the kinase holoenzyme is 2% of the hippocampal protein. It is also highly concentrated in other parts of the telencephalon, whereas its concentration is considerably lower (0.05 to 0.2% of total protein) in the pons/medulla, cerebellum, and midbrain/thalamus. We determined that this difference was not due to enhanced degradation of the α-subunit by measuring purified α-subunit after mixing it with cerebellar homogenates (data not shown).

Immunohistochemical staining for the α-subunit in brain sections. In order to determine which cell types and brain nuclei contain the α-subunit, we used antibody 6G9 and a horseradish peroxidase sandwich method to stain fixed sections of rat brain (Fig. 6). The results generally confirmed the distribution measured by radioimmunoassay. Dark, specific staining was observed in all cortical areas, the septal nuclei, and the caudatoputamen. The most intense staining, however, was in the hippocampus and amygdaloid nuclei. In the diencephalon and midbrain, the most consistently stained structures were the thalamic nuclei and the substantia nigra. Within the same sections, staining of structures such as the superior colliculus and the central gray nucleus was barely perceptible. In the cerebellum, Purkinje cells were stained nonspecifically, and we could not reliably detect specific staining.

In agreement with the results of Ouimet et al. (1984), specific staining in all areas was confined to neuronal cell bodies and/or neuropil (data not shown). We saw no evidence for staining of glia. Staining of neuropil regions was enhanced by treatment of fixed sections with dilute Triton prior to incubation with antibodies (cf. hippocampal staining in Fig. 6, *B* and *C*). Presumably, the brief detergent treatment allowed better access of the antibodies to the interior of small neuropil structures. The Triton-treated sections revealed that neuropil in the hippocampus and amygdala stains more intensely than in the cortex.

Although 6G9 is specific for the α-subunit on immunoblots, it was possible, as with any monoclonal antibody, that tissue fixation had destroyed the 6G9 epitope on the α-subunit and/or exposed a similar epitope in another protein. We used several controls to rule out this possibility. Incubations were performed in which 6G9 ascites fluid was replaced by buffer or by ascites fluid prepared from the parent NS1/SP2 tumor line. All control incubations produced very little staining within brain sections and a band of nonspecific reaction product around the outside (Fig. 6F). These controls indicated that 6G9 was necessary for specific staining. In addition, we tested whether the concentration of kinase measured in brain regions by the radioimmunoassay could account for the range of staining intensity shown in Figure 6. Thin agarose slabs containing kinase concentrations from 0.5 mg/ml to 0.02 mg/ml were prepared as described under "Materials and Methods." They were fixed and stained with 6G9 together with control slabs containing no kinase. Specific staining was observed in the blocks that contained kinase, and the intensity increased with kinase concentration (data not
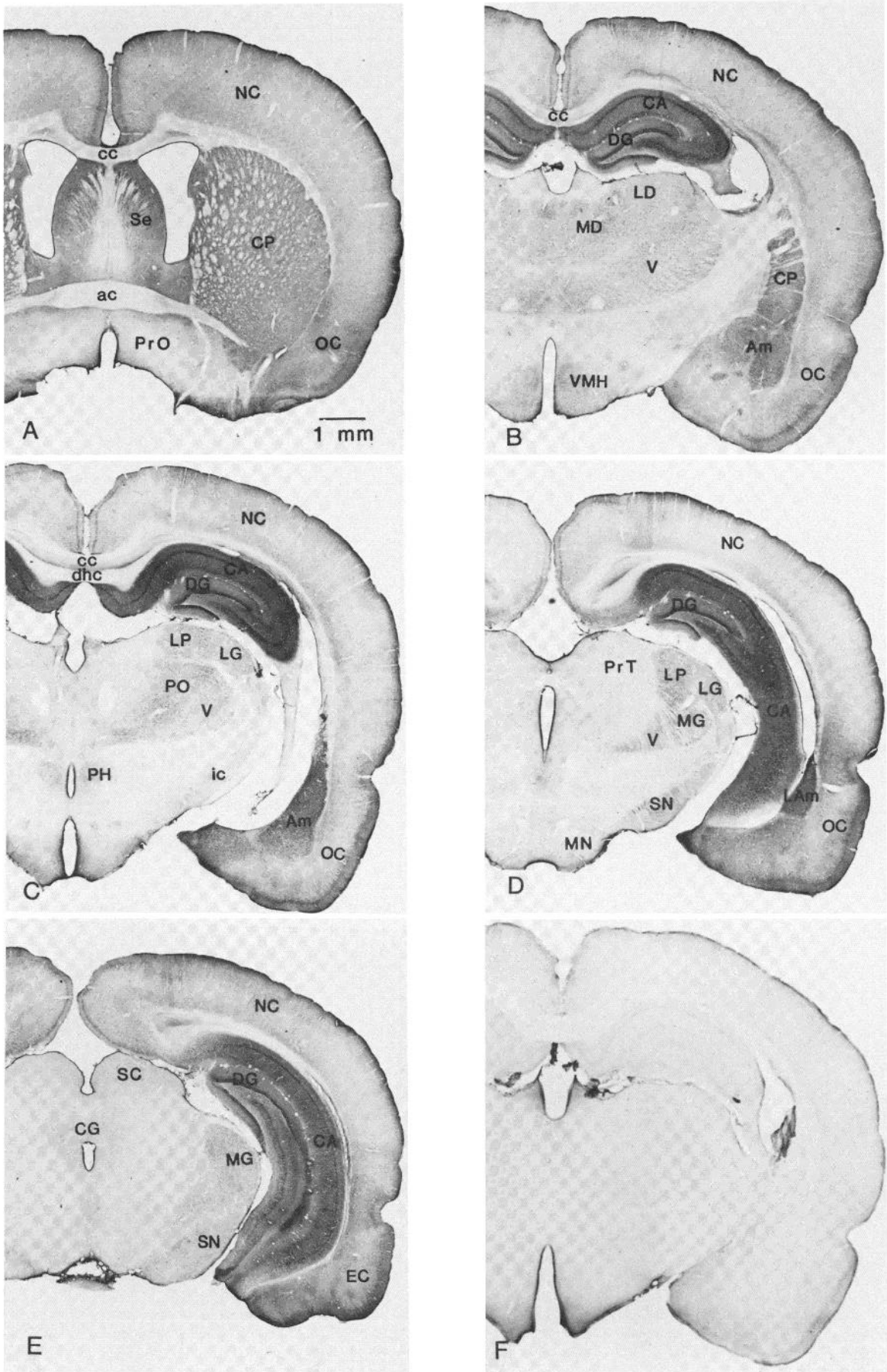
Figure 6

shown). Thus, the presence of the fixed kinase molecule was sufficient to produce the staining intensities observed in brain sections. Finally, we note that the intensity of staining in brain regions varied in parallel with the concentration of α-subunit measured by radioimmunoassay. Taken together, the evidence is quite strong that the staining patterns shown in Figure 6 reflect the variable concentration of α-subunit in different brain regions.

Type II CaM kinase activity in brain regions. The intepretation of these results is complicated by the recent finding that the subunit composition of the type II CaM kinase varies in certain brain regions (McGuiness et al., 1985; Miller and Kennedy, 1985). In particular, the kinase from the cerebellum contains, on average, more β-subunit and less α-subunit than does the forebrain kinase. We measured the specific activity of type II CaM kinase in the different brain regions in order to determine how closely the concentration of the α-subunit parallels the concentration of kinase activity. The results (Table II) indicated a rather close parallel in all brain regions except cerebellum and the pons/medulla. In these two regions, the kinase activity per α-subunit was significantly higher than that of other brain regions. Thus, the average subunit composition of the type II CaM kinase in the pons/medulla may be between that of the forebrain and that of the cerebellum. The results also suggest that the kinase in the olfactory bulb, the hypothalamus, and the midbrain/thalamus may have, on average, a slightly lower proportion of α-subunit than in the hippocampus and cortex.

Measurement of α-subunit in non-neuronal tissues. Because several non-neuronal tissues contain kinases that appear homologous to the brain type II CaM kinase, we examined these tissues to see whether the α-subunit is expressed at low levels. Even in the liver, where the molecular weights of the homologous kinase subunits are near 50,000 (Ahmad et al., 1982; Payne et al., 1983), we could not detect any α-subunit (Table III). Thus, the α-subunit may be specific to neuronal tissue.

Discussion

We have used a quantitative immunochemical technique, together with immunocytochemistry, to map the distribution within the brain of the α-subunit of type II CaM kinase. The most important finding

TABLE II
Ratio of type II synapsin I kinase activity and α subunit in brain regions

Brain Region	Specific Activity[a] (nmol/min/mg of protein)	Activity/α-Subunit[b] (nmol/min/μg of α-subunit)
Whole brain	14.4	1.9
Hippocampus	16.6	1.2
Cortex	12.9	1.5
Striatum	7.4	1.6
Olfactory bulb	4.8	1.8
Cerebellum	4.0	7.0
Hypothalamus	4.3	2.0
Midbrain/thalamus	3.0	1.9
Pons/medulla	1.9	3.8

[a] Measured as described under "Materials and Methods."

[b] Calculated from specific activities and concentrations of α-subunit listed in Table I.

TABLE III
Distribution of type II CaM kinase activity and the α-subunit in non-neuronal tissues

Tissue	Kinase Specific Activity[a] (%)	α Subunit (ng/100 μg of total protein)
Brain	100.0	740 ± 42
Spleen	9.0	<3.5
Testis	3.2	<3.5
Heart	3.0	<3.5
Skeletal muscle	2.4	<3.5
Adrenals	1.4	<3.5
Liver	0.8	<3.5
Kidney	0.5	<3.5

[a] Activity in brain was 16.1 nmol/min/mg. Specific activities were determined as described under "Materials and Methods."

emerging from the study is the extent of differential expression of the type II CaM kinase in different brain regions. The kinase is highly expressed in most telencephalic neurons (hippocampus, cortex, striatum, septum, and amygdala) and in thalamic neurons. Its concentration is highest in the hippocampus where it is about 2% of the total protein. In contrast, the kinase comprises only about 0.1% of the total protein in the pons/medulla. Intermediate concentrations are found in other brain areas. Immunocytochemical staining of brain sections confirms the variation in concentration of the α-subunit measured by radioimmunoassay and reveals its distribution more precisely. In the hippocampus and amygdala, neurons and neuropil appear densely stained. In cortical areas, staining of the neuropil is lighter and more variable. In the diencephalon and midbrain only the thalamic nuclei and the substantia nigra are stained, and in lower brain areas very little specific staining is apparent.

Some of the regional variation in concentration of the α-subunit is due to variation in the subunit composition of the type II CaM kinase. Purified cerebellar kinase has a considerably higher proportion of β-subunit (α-to-β ratio of 1:4; Miller and Kennedy, 1985) than does the forebrain kinase (α-to-β ratio of 3:1; Bennett et al., 1983). This is reflected in the correspondingly high level of kinase activity per α-subunit measured in cerebellar homogenates (Table II). In the pons/medulla, kinase activity per α-subunit is intermediate between that in forebrain and cerebellum, suggesting that the α- and β-subunits may be present there in nearly equal amounts. We recently found that the forebrain kinase isozyme is 10 to 20 times more concentrated in the postsynaptic density fraction from forebrain than is the cerebellar isozyme in cerebellar postsynaptic densities. Thus, the different proportions of α- and β-subunits may affect the subcellular distribution of type II CaM kinase within different cell types (Miller and Kennedy, 1985).

Although the α-subunit has been shown to be identical to the "major postsynaptic density protein" (Kennedy et al., 1983; Goldenring et al., 1984; Kelly et al., 1984) and is probably concentrated in postsynaptic densities *in situ* (Kennedy and Radice, 1984), biochemical studies indicate that a substantial portion of it is free in the cytosol. Immunocytochemical localization of the α-subunit is consistent with a diffuse distribution of the kinase within neurons. Neuronal cell bodies, dendrites, and neuropil regions are stained, as well as

Figure 6. Coronal sections through rat brain stained immunohistochemically for the α-subunit. Sections were fixed, cut, and stained as described under "Materials and Methods." Approximate positions of each section anterior to the interaural line were estimated from those of Paxinos and Watson (1982): *A*, 8.5 mm; *B*, 6.0 mm; *C*, 5.0 mm; *D*, 4.0 mm; *E*, 3.0 mm; *F*, 5.5 mm. All sections except *B* were treated briefly with Triton before incubation with antibody, as described under "Materials and Methods." *ac*, anterior commissure; *Am*, amygdaloid nuclei; *CA*, Ca fields of the hippocampus; *cc*, corpus collosum; *CG*, central gray; *CP*, caudatoputamen; *DG*, dentate gyrus of the hippocampus; *dhc*, dorsal hippocampal commissure; *EC*, entorhinal cortex; *ic*, internal capsule; *LAm*, lateral amygdaloid nucleus; *LD*, laterodorsal thalamic nucleus; *LG*, lateral geniculate thalamic nucleus; *LP*, lateroposterior thalamic nucleus; *MG*, medial geniculate thalamic nucleus; *MN*, mammillary nuclei; *NC*, neocortex; *OC*, olfactory cortex; *PH*, posterior hypothalamic nucleus; *Po*, posterior thalamic nuclei; *PrO*, preoptic area; *PrT*, pretectal area; *SC*, superior colliculus; *Se*, septal nuclei; *SN*, substantia nigra; *V*, ventral thalamic nuclei; *VMH*, ventromedial hypothalamus. Magnification × 8.

some axons in the corpus collosum (Fig. 6C). This agrees with the localization of the type II CaM kinase at the electron microscope level reported by Ouimet et al. (1984). It is consistent with the notion that the kinase regulates several neuronal functions.

At present, the available structural and enzymatic data can only give clues about what these functions may be. In response to calcium, the kinase phosphorylates the synaptic vesicle-associated protein synapsin I at a high rate (Bennett et al., 1983). This phosphorylation reduces the affinity of synapsin I for synaptic vesicles (Huttner et al., 1983). Thus, the kinase may regulate vesicle function in presynaptic terminals (Nestler et al., 1984). The type II CaM kinase also phosphorylates at least two components of the neuronal cytoskeleton *in vitro*; MAP$_2$ at a high rate (Yamauchi and Fujisawa, 1982; Bennett et al., 1983; Schulman, 1984) and tubulin at a lower rate (Goldenring et al., 1983; R. DeLorenzo and M. B. Kennedy, unpublished observations). These findings, the association of the kinase with the postsynaptic density fraction, and its association with the cytoskeleton in *Aplysia* (Saitoh and Schwartz, 1985) suggest that it is involved in regulating the neuronal cytoskeleton. Such regulation could affect neurite outgrowth, receptor clustering, the shape and size of dendrites and spines, and/or transport of materials through neuronal processes. There is as yet no evidence that the kinase phosphorylates ion channels. Better understanding of the various functions of the type II CaM kinase will require more precise structural data, information about additional kinase substrates, and the development of simplified systems in which its role can be studied in intact cells.

The high concentration of type II CaM kinase in telencephalic neurons could specifically affect their functioning in several ways. First, it will allow the kinase to compete more effectively with other calmodulin-binding proteins for calcium-bound calmodulin. As a result, the cascade of functional changes produced by activation of the kinase could begin to dominate the response to calcium in these neurons. Second, the overall rate of phosphorylation by activated kinase will be higher in these neurons, since the velocity of an enzymatic reaction depends on the concentration of enzyme as well as the concentration of substrate. Because the turnover numbers of protein kinases are rather low, 2 to 20/sec, an extremely rapid response by the kinase to a calcium signal may require that its concentration be unusually high. Finally, the kinase may play a structural role in these neurons. Expression of an enzyme at the level of 1 to 2% of total protein is unusual in any cell. Most highly abundant brain proteins, such as tubulin and actin, which are approximately 10% and 7% of total brain protein, respectively (Gordon et al., 1977; Fulton and Simpson, 1979), perform structural roles. The myosin ATPase, which is present at a high concentration in muscle cells, has a structural role in addition to its enzymatic one. By analogy, it is possible that the type II CaM kinase may have both structural and enzymatic roles, especially in neurons in which it is highly expressed. The finding that the kinase is a major component of the postsynaptic density fraction supports this possibility. However, its diffuse distribution within neurons suggests that any structural role may not be confined to postsynaptic densities.

Whatever its precise role, the high concentration of type II CaM kinase in certain sets of neurons undoubtedly confers on them a set of specialized responses to changes in calcium concentration. It is interesting to note that many of the regions that are especially enriched in the α-subunit are also those that have been implicated in memory formation (Thompson et al., 1983) and that display robust long-term potentiation (Swanson et al., 1982; Racine et al., 1983). It is, therefore, possible that the type II CaM kinase and, in particular, its α-subunit, may be important in the cascade of events that lead to long-term, activity-dependent changes in synaptic efficacy.

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