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IDENTIFICATION AND REGIONAL DISTRIBUTION OF A TYPE II CALCIUM/CALMODULIN-DEPENDENT KINASE IN MOUSE BRAIN

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Abstract—A calcium/calmodulin-dependent protein kinase was partially purified from mouse brain cytosol and compared to a type II calcium/calmodulin-dependent protein kinase (CaM kinase II) previously purified from rat brain. The purification (approximately 200-fold) was followed by the ability of the kinase to phosphorylate the high molecular weight microtubule-associated protein, MAP-2. Approximately 40% of the mouse brain kinase was soluble, and it contained two subunits of 50 kD and 58–60 kD. Both subunits bound [^{125}I]calmodulin in a calcium-dependent manner and demonstrated calmodulin-dependent autophosphorylation. The subunits from whole brain were present in a molar ratio of 3/1. The apparent K_m values of the kinase for ATP and calmodulin were 17 μM and 55 nM respectively. The time course, substrate specificity, and subunit phosphopeptide maps were comparable to CaM kinase II from rat brain. Regional distribution studies indicate that the enzyme activity was enriched in hippocampus, cerebral cortex and corpus striatum, whereas activity in cerebellum and pons/medulla was approximately 10-fold lower. All of these characteristics were shared with the rat brain enzyme, indicating that the kinase in mouse brain was a type II calcium/calmodulin-dependent kinase. The mouse may be useful for examining the neuronal localization of CaM kinase II in different brain regions, since this model offers a variety of genetic mutants with well-defined lesions in specific neuronal populations.

CaM kinase II*, a type II calcium/calmodulin-dependent kinase, has been implicated in synaptic neurotransmission [for reviews, see Refs. 1 and 2], regulation of neurotransmitter synthesis [1, 3, 4] and cytoskeletal function [5–7]. The rat brain cytosolic kinase has been purified and biochemically characterized by several laboratories [8–13]. The holoenzyme has a molecular mass of 530–650 kD and contains two calmodulin-binding subunits of 50 kD and 58–60 kD that exhibit calmodulin-dependent autophosphorylation. The primary structures of the 50 kD [14] and 58–60 kD [15] subunits have been determined recently by molecular cloning. Synapsin I and the high molecular weight microtubule-associated protein, MAP-2, are among the best substrates for the kinase. These substrates are also co-localized with CaM kinase II at the synapse [1, 2, 16]. Although CaM kinase II is highly concentrated in neurons, a similar or identical kinase has been characterized in several peripheral tissues including myocardium, lung, spleen [17], and pancreas [18]. Activation of CaM kinase II may provide a rapid biochemical response to an increase in intracellular calcium produced by a variety of cell surface active ligands including neurotransmitters, hormones and pharmacological agents.

Investigators have noted that the distribution of CaM kinase II parallels the distribution of glutamate binding sites [1], suggesting that it is localized in excitatory glutaminergic synapses, but this theory has not been experimentally verified. It may be advantageous to use the mouse as a model for investigating the localization of CaM kinase II in different neurons, since there are a variety of genetic mutants with specific neuronal lesions that have been carefully screened and characterized.

Due to the presence of multiple calcium/calmodulin-dependent kinases in brain [1], it is important to demonstrate the existence and distribution of the kinase of interest. The present study was undertaken to determine whether a type II calcium/calmodulin-dependent kinase is present in mouse brain, as a first step in using this animal model for further study of kinase function.

EXPERIMENTAL PROCEDURES

Materials

Male Swiss–Webster mice (20–30 g) were used for the experiments (Taconic Farms, Germantown, NY). [γ-^{32}P]ATP (5–10 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear, Boston, MA. [^{125}I]Calmodulin (90 mCi/mg) was purchased from Amersham, Arlington Heights, IL. Unlabeled calmodulin was purified from calf brain by chromatography on DEAE-cellulose and Affi-Gel phenothiazine as previously described [19]. Affi-Gel phenothiazine, Affi-Gel calmodulin, and Zeta-Probe blotting membranes were purchased from Bio-Rad Laboratories, Richmond, CA. Molecular weight standards for gel filtration, chromatography and elec-

* Abbreviations: CaM kinase II, type II calcium/calmodulin-dependent protein kinase; MAP-2, microtubule-associated protein-2; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFP, trifluoperazine; and EGTA, ethyleneglycolbis(amino-ethylether)tetra-acetate.

trophoresis, and S-Sepharose resin were obtained from Pharmacia, Piscataway, NJ. Histone (Type VIII-S from calf thymus), phosphorylase *b* (EC 2.4.1.1 from rabbit muscle), casein, and *Staphylococcus aureus* V8 protease (EC 3.4.21.19) were purchased from Sigma, St. Louis, MO. All other chemicals used in the experiments were reagent grade and were obtained from commercial sources.

Methods

CaM kinase II preparation. Mice were decapitated quickly, and whole brain (0.4 g) or different brain regions were rapidly excised and homogenized in 2 ml buffer/brain (50 mM Tris, 10 mM EGTA, 10 mM EDTA, 0.32 mM PMSF) using 3 up-and-down strokes at 1200 rpm in a Teflon/glass homogenizer. Unless otherwise indicated, all solutions were pH 7.2, 4°, and contained 2 mM 2-mercaptoethanol. The crude homogenate was centrifuged at 3000 *g* for 10 min at 4° to obtain a low speed supernatant (S_1). A high speed supernatant (S_2 or cytosol) was prepared by centrifugation of S_1 at 100,000 *g* for 30 min at 4°. Approximately 40% of the calmodulin-dependent kinase activity was recovered in the cytosol, while the remainder of the activity was recovered in the particulate fraction. The cytosol fraction was then applied to S-Sepharose cation-exchange resin equilibrated in 50 mM Tris buffer, and the eluate was collected (column bed size: i.d. 1.2 cm × 15.0 cm; flow rate: 120 ml/hr). Elution of bound protein was achieved by two sequential step gradients of 50 mM Tris buffer containing 150 mM NaCl and 350 mM NaCl respectively. The 150 mM NaCl fractions containing the majority of recovered kinase activity were pooled, and 200 mM NaCl, 2 mM $CaCl_2$ and 10% glycerol (final concentrations) were added. The preparation was applied to calmodulin-affinity resin that had been equilibrated in 50 mM Tris, 200 mM NaCl, 2 mM $CaCl_2$, 10% glycerol (column bed size: i.d. 1.0 cm × 5.5 cm; flow rate: 20 ml/hr). The calmodulin-binding proteins were recovered from the column as a sharp peak by exchanging 2 mM EGTA for calcium in the buffer. As a further purification step, the fractions from the calmodulin-affinity resin containing the majority of recovered kinase activity were pooled, diluted with an equal volume of 50 mM Tris, pH 7.2, and re-applied to S-Sepharose resin equilibrated in 50 mM Tris, 100 mM NaCl (column bed size: 1.5 cm × 2.5 cm; flow rate: 60 ml/hr). Elution of bound protein was achieved with a step gradient of 50 mM Tris, 350 mM NaCl. The putative CaM kinase II from mouse brain was compared to authentic CAM kinase II prepared from rat brain cytosol essentially as described by Goldenring *et al.* [9].

Tubulin and MAP-2 preparation. Sheep brain microtubules were prepared by one cycle of temperature-dependent polymerization/depolymerization [6, 7]. MAP-2 was partially purified from microtubules by heat treatment and clarified by centrifugation as previously described [20]. The heat-stable MAP proteins in the supernatant were subsequently dialyzed overnight against three changes of 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.8, and used in the studies at a concentration of 0.3 mg/ml. Based upon SDS–PAGE, isoelectric focussing and scanning densitometry, the preparation contained primarily MAP-2 and the tau polypeptides. The preparation did not demonstrate calmodulin-dependent kinase activity when tested in the standard phosphorylation assay, indicating that endogenous CaM kinase II was inactivated during heat treatment.

In some studies, tubulin was tested as a substrate for the calmodulin-dependent kinase. Twice-cycled microtubules containing approximately 85% tubulin were prepared from rat brain cytosol as described previously [6, 7]. Tubulin was further purified by chromatography of the microtubule preparation on phosphocellulose resin as described by Weingarten *et al.* [21]. Based upon SDS–PAGE, isoelectric focussing and scanning densitometry, greater than 95% of the phosphocellulose-purified preparation consisted of alpha and beta tubulin.

Measurement of kinase activity and calmodulin-binding proteins. Calmodulin-dependent kinase activity was measured in different samples in the presence and absence of trifluoperazine (TFP), a calmodulin inhibitor, using a phosphorylation system as described previously [7, 9]. MAP-2 was used as an exogenous substrate in these studies under linear rate conditions. In some experiments, incorporation of phosphate into different substrate proteins was measured in the standard phosphorylation assay using 10–50 ng of CaM kinase II and the following final concentrations of substrate protein: tubulin (0.1 mg/ml); histone (0.1 mg/ml); phosphorylase *b* (0.5 mg/ml); and casein (0.5 mg/ml). These concentrations were selected for comparison with previous studies performed with rat brain kinase [9, 10]. The reaction mixture (100 μl final volume) was incubated for 30 sec at 30° prior to termination of the phosphorylation reaction by addition of 50 μl of "SDS-stop" solution as described [7, 9].

SDS–PAGE was performed on 7% or 8% running gels (12 cm) and 4% or 5% stacking gels, respectively (2 cm), with an acrylamide/bis-acrylamide ratio of 30/0.8 as originally formulated by Laemmli [22]. After electrophoresis for 1–3 hr at constant current (50 mA/gel), gels were stained with Coomassie Blue or silver reagent [23], destained, and dried on filter paper, followed by autoradiography on Kodak XAR film. The specific activity and recovery of kinase were assessed by excision of specific proteins from SDS–PAGE and scintillation spectroscopy [9] and/or densitometric scanning of gels and autoradiographs with a Shimadzu CS-930 densitometer. This model densitometer provides automatic integration of the area under the peak.

Calmodulin-binding was assessed by transferring protein from SDS–PAGE to Zeta-Probe blotting membrane, blocking the blot with nonfat dry milk to reduce non-specific binding, and overlaying with [^{125}I]calmodulin (1 μCi/blot), followed by autoradiography. This procedure is essentially as described by Gorelick *et al.* [18] with the modifications of Peluso and Rosenberg [24].

Other procedures. Isoelectric focussing was performed on an Ephortec isoelectric focussing cell (Haake Buchler, Saddle Brook, NJ) using a pH gradient of 3–10 [6, 9]. Protein was determined by

the method of Bradford [25] using bovine serum albumin as a standard. Limited proteolysis and phosphopeptide mapping of kinase subunits from rat and mouse were performed with *S. aureus* V8 protease as described by Cleveland *et al.* [26]. The regional distribution of CaM kinase II was assessed by dissecting different brain regions freehand as described by Walaas *et al.* [27] and Erondu and Kennedy [28].

RESULTS

Purification of calmodulin-dependent kinase activity

S-Sepharose chromatography. Figure 1 depicts the protein elution profile and corresponding calmodulin-dependent kinase activity in fractions that were obtained after chromatography of mouse brain cytosol on cation-exchange resin. The major protein peaks are designated at the top of Fig. 1 in order of elution. They consisted of: an eluate fraction containing two peaks of protein that were not separated completely (E_{1+2}); a third eluate peak (E_3); two peaks that were eluted in the presence of 150 mM NaCl (150_1 and 150_2); and one peak that was eluted in the presence of 350 mM NaCl (350). The specific activity of the calmodulin-dependent kinase was highest in the 150_2 mM NaCl peak, with a 20- to 30-fold enrichment of enzyme activity compared to S_1.

Comparison of endogenous calcium/calmodulin-dependent phosphorylation of proteins from the major peaks is shown in Fig. 2. The E_{1+2} fractions were virtually devoid of calmodulin-dependent kinase activity. The E_3, 150_2, and 350 fractions contained calmodulin-dependent kinase activity that phosphorylated several endogenous proteins, including proteins of 50 kD and 58–60 kD, presumably corresponding to the autophosphorylated subunits of CaM kinase II. The subunit autophosphorylation pattern suggests that three distinct forms of calmodulin-dependent kinase were separated. The apparent ratio of 58–60 kD/50 kD subunit was greatest in the 350 fraction, followed by the 150_2 fraction. The E_3 fraction contained primarily 50 kD subunit.

Calmodulin-affinity chromatography. The 150_2 mM NaCl fractions (No. 115–136) containing the majority of recovered calmodulin-dependent kinase activity were pooled and applied to calmodulin-affinity resin (Fig. 3). The majority of applied protein appeared in the column eluate and contained minimal calmodulin-dependent kinase activity. The calmodulin-binding proteins retained by the resin were subsequently eluted as a sharp peak by replacing calcium in the column elution buffer with EGTA. The majority of calmodulin-dependent kinase activity was recovered in this protein peak, representing a further enzyme enrichment of 5- to 7-fold compared to the 150_2 fractions.

S-Sepharose chromatography. Finally, the peak fractions (No. 45–51) from the calmodulin-affinity column containing the majority of kinase activity were pooled, diluted with an equal volume of 50 mM Tris buffer to reduce the NaCl concentration to 100 mM, and re-applied to S-Sepharose cation exchange resin. Approximately half of the applied protein appeared in the first eluate. The majority of recovered calmodulin-dependent kinase activity was subsequently eluted in the presence of 350 mM NaCl, representing a further 1.5-fold enrichment of enzyme activity. Thus, enzyme prepared in this manner was enriched approximately 200-fold over S_1, with a recovery of 3–5%.

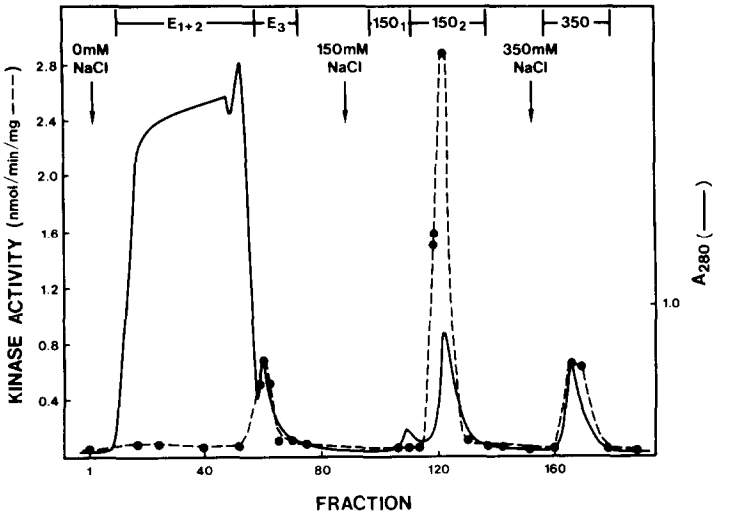

Fig. 1. S-Sepharose elution profile of calcium/calmodulin-dependent kinase activity. Approximately 115 ml of cytosol (6 mg/ml) was chromatographed on S-Sepharose cation-exchange resin under the conditions described in Methods. Protein absorbance was monitored at 280 nM (——), and selected samples were assayed for calcium/calmodulin-dependent kinase activity (- - - -). In the standard assay, samples were incubated at 30° for 30 sec using exogenous MAP-2 as a substrate as described [7, 9]. Protein was resolved on SDS–PAGE (7% resolving gel), and kinase activity was quantitated by excision of MAP-2 from the gel and scintillation spectroscopy. Calmodulin-independent activity was assessed in the presence of 0.1 mM TFP and was subtracted from total activity [7].

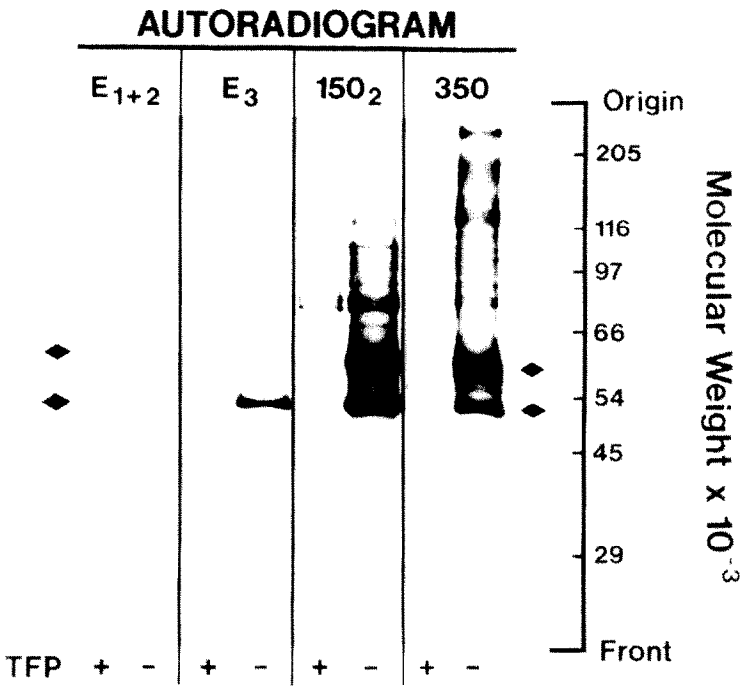

Fig. 2. Comparison of endogenous calcium/calmodulin-dependent phosphorylation of cytosolic protein chromatographed on S-Sepharose. Proteins (30 μg) from the major peaks designated at the top of Fig. 1 were assayed for endogenous calcium/calmodulin-dependent kinase activity in the presence [+] and absence [−] of TFP. Protein (9 μg) was subsequently resolved on SDS–PAGE (8% resolving gel), and phosphoproteins were visualized by autoradiography (exposure time: 2 hr). Symbols (◆) depict the 50 kD and 58–60 kD subunits of the putative CaM kinase II.

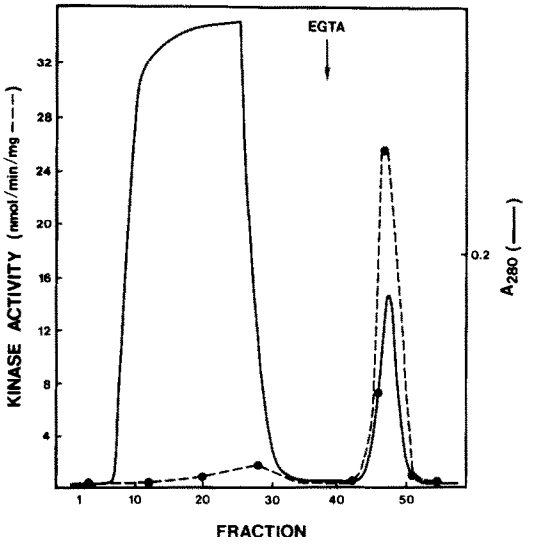

Fig. 3. Calmodulin-affinity elution profile of calcium/calmodulin-dependent kinase activity. The peak fractions (No. 115–136) from the S-Sepharose column were pooled and applied to calmodulin-affinity resin (see Methods). Protein absorbance (——) and kinase activity (- - - -) were quantitated as described in Fig. 1.

Characterization of calmodulin-dependent kinase activity

Protein staining pattern, autophosphorylation, and calmodulin-binding studies. The calmodulin-dependent kinase derived from mouse brain cytosol was compared to authentic CaM kinase II prepared from rat brain cytosol (Fig. 4). The protein composition was assessed by silver-staining a sample of the enzyme preparation after resolution on SDS–PAGE. A major band of 50 kD and a minor doublet of 58–60 kD were observed. Densitometric scans of silver-stained gels were employed to determine the molar ratio of the kinase subunits. The ratio of 50 kD/58–60 kD subunit from whole brain was 3/1, which is consistent with the subunit ratio observed in rat brain [1, 10, 12, 13]. The kinase subunits demonstrated calmodulin-dependent autophosphorylation that was inhibited by TFP. In addition, the kinase subunits were the major calmodulin-binding proteins in the preparation. Both subunits bound [^{125}I]calmodulin in the presence of calcium, but not when the blot was washed in equilibration buffer containing EGTA.

Time course, ATP, and calmodulin dependence. The kinase from mouse brain demonstrated half-maximal autophosphorylation at 17 μM ATP and 55 nM calmodulin respectively. The time course for kinase activation was linear up to 1.5 min and reached a plateau by 10–15 min at 30°.

Substrate specificity studies. The specificity of the kinase from mouse brain for several proteins pre-

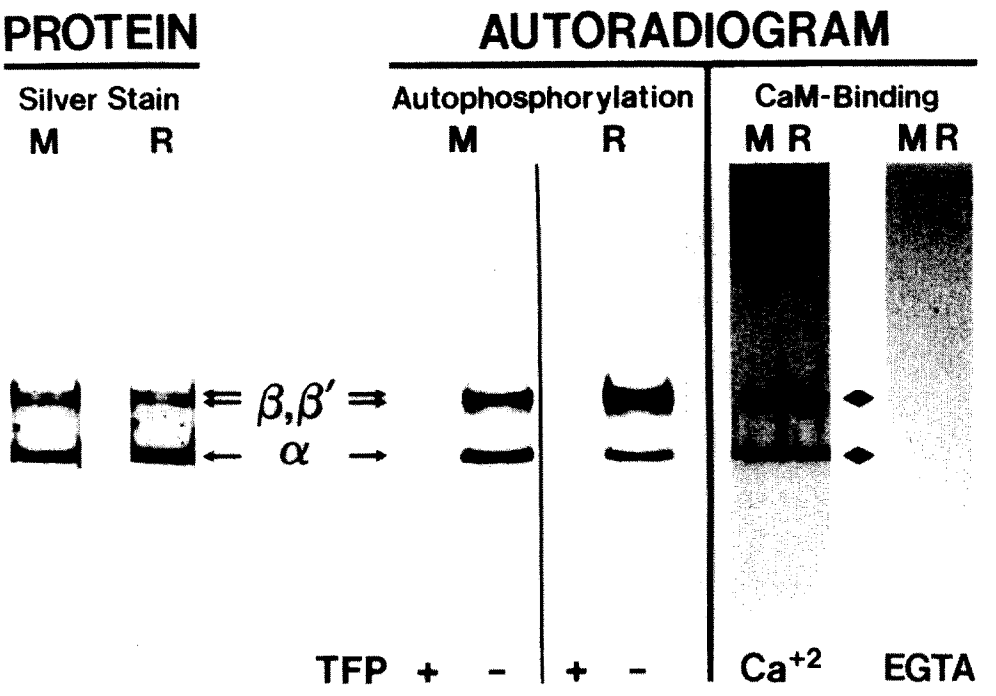

Fig. 4. Comparison of mouse and rat brain kinases by protein staining, endogenous calcium/calmodulin-dependent phosphorylation, and [^{125}I]calmodulin-binding. The purified kinase preparations were resolved on SDS–PAGE (8% resolving gel) and analyzed for protein by silver staining [23]; or assayed for the presence of calcium/calmodulin-dependent kinase activity in the standard phosphorylation assay in the presence [+] and absence [−] of TFP, resolved on SDS-PAGE, and subjected to autoradiography (exposure time: 2 hr); or resolved on SDS–PAGE, transferred to Zeta-Probe blotting membrane and analyzed for [^{125}I]calmodulin-binding proteins by autoradiography (exposure time: 24 hr; symbols (◆) depict 50 kD and 58–60 kD subunits) as described [18]. M = mouse; R = rat; α = 50 kD subunit of CaM kinase II (single arrow); β,β' = 58–60 kD subunit of CaM kinase II (two arrows).

viously used in studies with CaM kinase II derived from rat brain was tested. MAP-2 was the best substrate examined. The specific activity of the kinase for MAP-2 was 31.4 nmol PO_4 transferred/min/mg protein at 30°. This value compares well with the specific activity of 27.6 nmol/min/mg protein previously reported by Schulman for purified rat brain CaM kinase II [13]. The relative rate of phosphate incorporation into tubulin and histone was 5–30% compared to MAP-2 phosphorylation. The enzyme was inactive against phosphorylase *b* and casein (data not shown). The substrate specificity was comparable to CaM kinase II previously purified from rat brain [8–13].

Phosphopeptide mapping studies. The autophosphorylated 50 kD and 58–60 kD subunits from mouse or rat brain were excised from SDS–PAGE and subjected to limited proteolytic digestion using *S. aureus* V8 protease [26]. Although recent evidence suggests that the 58 kD subunit of the kinase may be synthesized on a separate message compared to the 60 kD subunit [15], this doublet was treated as a single protein for mapping studies. Figure 5 depicts the phosphopeptide maps of the rat and mouse 50 kD subunits (α), which were indistinguishable. Similarly, the maps of the 58–60 kD subunits from rat and mouse (β,β') were identical and contained a single major phosphopeptide of approximately 25 kD.

Regional distribution studies. Figure 6A shows the endogenous phosphorylation profile of CaM kinase II activity in different regions of brain. Two major phosphoproteins of 50 kD and 58–60 kD were observed, corresponding to the autophosphorylated subunits of CaM kinase II. The higher molar ratio of 58–60/50 kD subunit in cerebellum compared to cerebrum is in good agreement with previous studies in rat brain where a ratio of 4/1 was observed [29, 30]. The non-kinase phosphoprotein of approximately 55 kD co-migrated with phosphocellulose-purified tubulin on SDS–PAGE and isoelectric focussing. Since tubulin has been identified as an endogenous substrate for CaM kinase II in rat brain cytosol [2], the 55 kD endogenous phosphoprotein in mouse brain is probably tubulin.

The calcium/calmodulin-dependent kinase activity in different brain regions was also measured using exogenous MAP-2 as a substrate in the standard phosphorylation assay. Expressed in picomoles per mg protein per minute, it was most abundant in hippocampus (425.5), cortex (359.4) and striatum (304.9), whereas the activity detected in the cerebellum (42.1) and pons/medulla (44.6) was approximately 10-fold lower.

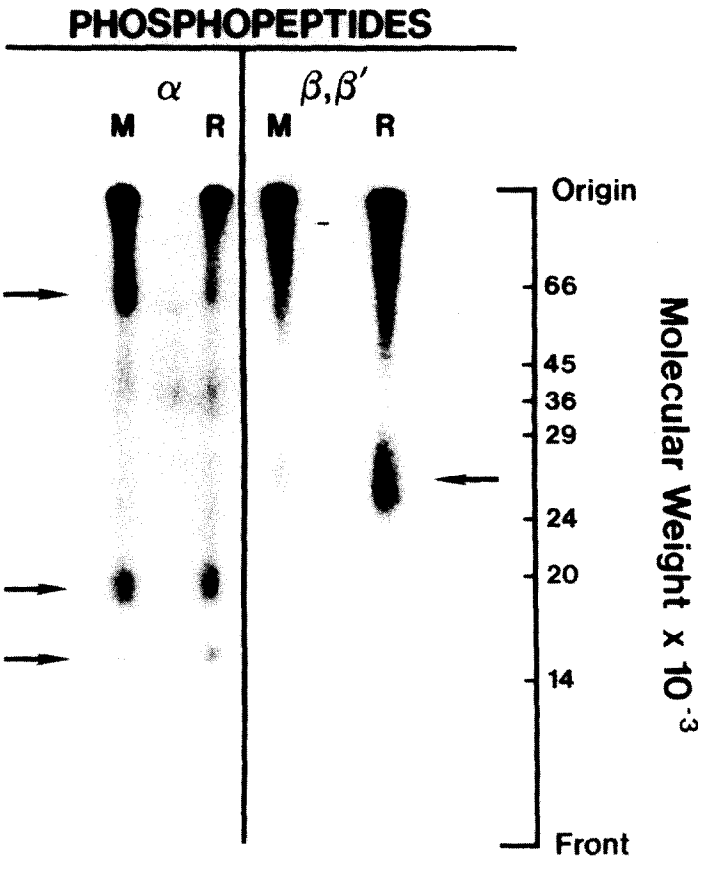

Fig. 5. Comparison of one-dimensional phosphopeptide maps of the 50 kD and 58–60 kD kinase subunits from mouse and rat. The autophosphorylated subunits of purifed kinase from mouse (M) and rat (R) were resolved on SDS–PAGE (8% resolving gel), excised from the gel, and subjected to limited proteolysis for 2 hr in a second gel (15% resolving gel) using 10 μg of *S. aureus* V8 protease [26]. The phosphopeptides were visualized by autoradiography (exposure time: 24 hr). α = 50 kD subunit of CaM kinase II, β,β' = 58–60 kD subunit. Arrows depict positions of major phosphopeptides.

Figure 6B depicts the calmodulin-binding proteins present in different brain regions based upon [^{125}I]calmodulin-overlay studies. As expected, it was very difficult to detect the low amounts of kinase present in crude cytosol by the calmodulin-overlay method. One is also limited by the amount of protein that will transfer with high resolution and quantitatively from SDS–PAGE to blotting membrane. Despite the fact that two times as much protein was transferred from cerebellum and pons/medulla compared to the other regions tested, the calmodulin-binding in these regions was not apparent on the autoradiograph. However, calmodulin-binding to the regions with greater kinase activity is clearly shown. These data are in good agreement with the pattern of autophosphorylation and the enzyme activity measurements, indicating that the levels of CaM kinase II are highest in hippocampus, cerebral cortex and corpus striatum. Moreover, the data confirm previous studies examining the regional distribution of CaM kinase II in rat brain [27, 28].

DISCUSSION

A type II calcium/calmodulin-dependent protein kinase was purified approximately 200-fold from mouse brain cytosol and compared to CaM kinase II from rat brain. The purification was followed by the ability of the kinase to phosphorylate exogenous MAP-2. Based upon subunit molecular weight, calmodulin-binding of subunits, calmodulin-dependent autophosphorylation of subunits, molar ratio of subunits, apparent K_m values for ATP and calmodulin, time course, substrate specificity, subunit phosphopeptide maps, and regional distribution studies, the kinase from mouse brain is identical to CaM kinase II from rat brain. It is distinguished from other calmodulin-dependent kinases in brain based upon its broad substrate specificity and relative abundance [1, 28].

CaM kinase II has been implicated as a mediator of calcium effects in a variety of tissues, especially in synaptic events [1–7]. Evidence suggests that these effects are mediated in part by phosphorylation of synapsin I and MAP-2, which are co-localized with CaM kinase II at the synapse [1, 16]. Investigators have speculated that CaM kinase II may be particularly enriched in excitatory glutaminergic synapses, since its distribution parallels the distribution of sodium-independent glutamate-binding

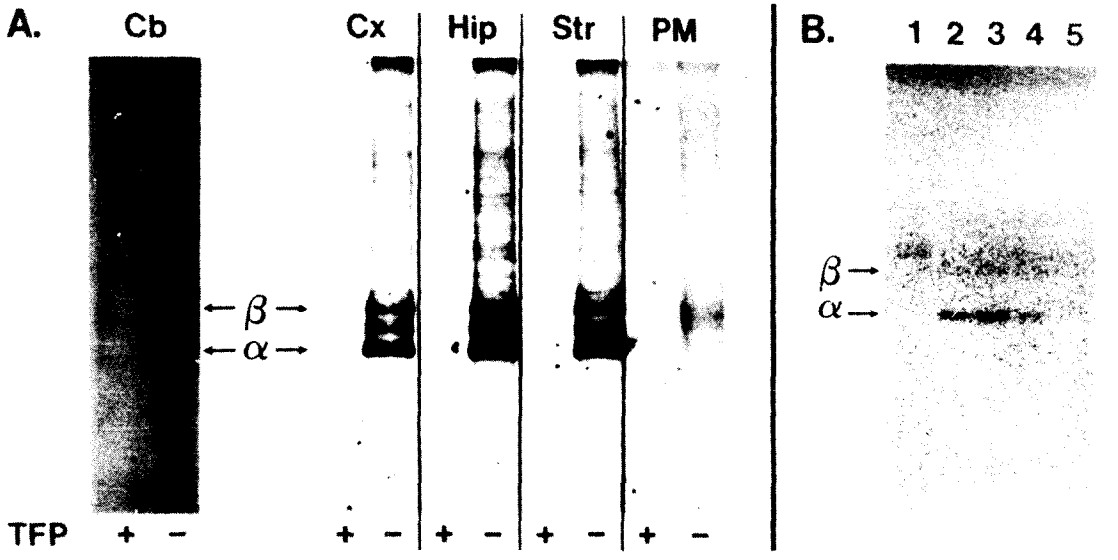

Fig. 6. Regional distribution of CaM kinase II. (A) Comparison of endogenous calcium/calmodulin-dependent phosphorylation of proteins in different brain regions. Aliquots containing 10 μg of cytosolic protein from different brain regions were incubated for 30 sec at 30° under standard assay conditions in the presence [+] and absence [−] of TFP. Protein was subsequently resolved on SDS–PAGE (8% resolving gel) and phosphoproteins were visualized by autoradiography (exposure time: 12 hr). Cb = cerebellum; Cx = cerebral cortex; Hip = hippocampus; Str = striatum; PM = pons/medulla; α = 50 kD subunit of CaM kinase II; β = 58–60 kD subunit of CaM kinase II. The print demonstrating endogenous calmodulin-dependent phosphorylation in cerebellum was exposed two times longer than the print showing phosphorylation in other regions (12 sec vs 6 sec). (B) Comparison of endogenous calmodulin-binding proteins in different brain regions. Aliquots containing 20 μg (lane 1 = Cb, lane 5 = PM) or 10 μg (lane 2 = Cx, lane 3 = Hip, lane 4 = Str) of cytosol were resolved on SDS–PAGE (8% resolving gel), and protein was transferred to Zeta-Probe blotting membrane and assayed for binding of [^{125}I]calmodulin as described [18]. The autoradiograph was exposed for 1 week. Binding of [^{125}I]calmodulin was not observed in the absence of calcium (data not shown).

sites [1]. However, this theory has not been examined carefully. The mouse may provide an interesting model system to study this question, since there are a variety of well-characterized genetic mutants with specific neuronal lesions.

Differences in the regional distribution of CaM kinase II subunits have been demonstrated using biochemical [27, 28, 30] and immunocytochemical [29] approaches, indicating that the enzyme exists as isozymes in different brain regions. An interesting observation that emerged from the present studies is the apparent separation of three distinct isozymes of CaM kinase II after chromatography of cytosol on S-Sepharose cation-exchange resin. The ratio of 58–60 kD/50 kD subunit was greatest in the fraction that was eluted in the presence of 350 mM NaCl, followed by the 150 mM NaCl fraction. The E_3 fraction did not bind to the resin and contained primarily 50 kD subunit. One possible explanation is that the pI of the 58–60 kD subunit is more basic than the 50 kD subunit, thus accounting for binding of the forms that contain a greater ratio of 58–60 kD/50 kD subunit to cation exchange resin. Indeed, Goldenring *et al.* [9] reported a more basic pI for the 58–60 kD subunit of CaM Kinase II. However, the enzyme is poorly soluble on isoelectric focussing so that quantative studies are difficult. At present, one cannot rule out the possibility that other non-kinase proteins in the preparation contribute to differential binding of the enzyme to cation-exchange resin. Studies are in progress to purify and biochemically characterize all three peaks of kinase activity from mouse brain in order to resolve this issue, and to determine whether all three forms of the kinase are active.

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