Distinct Forebrain and Cerebellar Isozymes of Type II
Ca$^{2+}$/Calmodulin-dependent Protein Kinase Associate Differently
with the Postsynaptic Density Fraction*

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Forebrain and cerebellar Type II Ca$^{2+}$/calmodulin-dependent protein kinases have different subunit composi-
tions. The forebrain holoenzyme, characterized in
our laboratory, is a 650-kDa holoenzyme composed of
50-kDa α-subunits and 60-kDa β-subunits assembled
in approximately a 3:1 ratio (Bennett, M. K., Erondu,
12735–12744). The cerebellar isozyme is a 500-kDa
holoenzyme composed of α-subunits and β-subunits as-
sembled in almost the converse ratio, approximately
four β-subunits for each α-subunit. When compared by
trypsin peptide mapping and by immunochemical tech-
niques, the β-subunits from the two brain regions are
indistinguishable and the α-subunits appear closely re-
lated.

The specific activities, substrate specificities, and
catalytic constants of the cerebellar and forebrain iso-
zymes are similar, suggesting that the α- and β-subunits
contain similar catalytic sites. However, two differ-
ces in the properties of the isozymes may result in
functional differences between them in vivo. First, the
apparent affinity of the cerebellar kinase for Ca$^{2+}/$
calmodulin is 2-fold higher than that of the forebrain
kinase. Second, the two isozymes appear to associate
differently with subcellular structures. Approximately
85% of the cerebellar kinase and 50% of the forebrain
kinase remain in the particulate fraction after homog-
enization under standard conditions. However, they
are present in different amounts in postsynaptic den-
sity fractions. Postsynaptic densities prepared from
forebrain contain the forebrain isozyme. Immuno-
chemical measurements show that it comprises ~16%
of their total protein. In contrast, postsynaptic den-
sity preparations from cerebellum contain the cerebellar
isozyme, but it comprises only ~1–2% of their total
protein. Thus, the α-subunit may play a role in anchoring
Type II Ca$^{2+}$/calmodulin-dependent protein kinase
to postsynaptic densities.

Many agents regulate the functions of target cells by alter-
ing the concentration of an intracellular second messenger
such as a cyclic nucleotide, Ca$^{2+}$ ion, or diacglycerol (1–4).
The cyclic nucleotides and diacylglycerol are believed to act
primarily through the activation of specific protein kinases
(5–7). The actions of Ca$^{2+}$ as a second messenger are more
diverse and often require the Ca$^{2+}$/binding protein calmodulin
(8). Among the enzymes regulated by Ca$^{2+}$/calmodulin are a
family of protein kinases. Ca$^{2+}$/calmodulin-dependent protein
kinase activities have been described in mammalian brain (9,
10), pituitary (11), liver (12), pancreas (13, 14), skeletal
and smooth muscle (15–18), avian erythrocytes (19), Torpedo
electric organ (20), and Aplysia ganglia (21, 22). Recent structural
colorization of some of these kinases suggests that there
are at least four and perhaps more distinct Ca$^{2+}$/calmodulin-
dependent protein kinases (15–18, 23–32). The diversity of
these kinases may underlie, in part, the diversity of cellular
responses to Ca$^{2+}$.

We recently reported the purification and characterization
of a Ca$^{2+}$/calmodulin-dependent protein kinase with a broad
substrate specificity that is far more highly expressed in brain
than in other tissues (27). Brain kinases that are structurally
similar and may be identical with this one have been described
by other groups (28, 29, 31, 32). Two of the groups have
referred to the kinase as “Ca$^{2+}$/calmodulin-dependent kinase
II” (29, 31). Because this kinase now appears to occur as a
family of homologous but distinct forms in different tissues
as well as brain regions (29, 33, 34, and this report), we refer
to them as “Type II” Ca$^{2+}$/calmodulin-dependent protein
kinases. These kinases have several features in common. They
are multimeric proteins of high molecular mass (300–700
kDa), composed of structurally related 50–60-kDa subunits.
They exhibit similar substrate specificities. All the kinase
subunits bind calmodulin and undergo a Ca$^{2+}$/calmodulin-
dependent autophosphorylation. The kinases differ in the
exact molecular weights and ratios of their subunits.

The brain Type II CaM kinase previously described by this
lab (27) is a 650-kDa holoenzyme composed of ~9 α (50 kDa)-
and ~3 β/β' (60/58 kDa)-subunits. This isozyme of the kinase
is concentrated in the forebrain (cortex and hippocampus)
and makes up about 75% of the total brain Type II CaM
kinase.1 In this paper we report the purification of a distinct
isozyme of the kinase from cerebellum. It is composed of
subunits similar to those of the forebrain kinase, but they are
assembled in a different ratio. We compare its structural and
enzymatic properties to those of the forebrain kinase.

We and others have shown that the Type II CaM kinase is a
major constituent of brain postsynaptic density fractions
(36–38). The postsynaptic density is a specialization of the
submembranous cytoskeleton that occurs beneath postsyn-
aptic densities.

1 For brevity, we will frequently refer to the brain Type II Ca$^{2+}$/
calmodulin-dependent protein kinases as Type II CaM kinases. They
are not related to the Type II cAMP-dependent protein kinase.

2 N. Erondu, M. Bennett, and M. Kennedy, unpublished observa-
ations.
optic membranes in CNS neurons. We show here that forebrain PSD fractions contain the forebrain isoform as a major component while cerebellar PSD fractions contain the cerebellar isoform, but in greatly reduced amounts. A preliminary report of part of this work has appeared (33). After this manuscript was submitted, another paper describing the physical properties of the cerebellar isoform was published (34).

EXPERIMENTAL PROCEDURES

Materials—Alcohol dehydrogenase, ATP, bovine serum albumin, carbonic anhydrase, casein, chloramine-T, EDTA, EGTA, fast green, fibrinogen, hemoglobin (Type II), histones (Type VIII-S), midezole, ovalbumin, phosvitin, PMSF, polysynthetic-polycytdic acid, and soybean trypsin inhibitor were purchased from Sigma. Albclase, blue dextran 2000, catalase, ferritin, protein A, Superpose 4B, and thyroglobulin were purchased from Pharmacia. DEAE-Bio-Gel A, SDS, acrylamide, and bisacrylamide were purchased from Bio-Rad. Nitrocellulose membranes (BA85, 0.45 μm) were purchased from Schleicher and Schuell, cellulose-coated thin layer chromatography plates from Eastman, trypsin treated with 1-tosylamido-2-phenylethyl chloromethyl ketone (t-2-PACK) from Worthington, ultrapure sucrose from Bethesda Research Laboratories, and Coomassie Brilliant Blue from Searle Diagnostic. Sodium perchlorate was purchased from G. Frederick Smith Chemical Co., dithiothreitol from Boehringer Mannheim, leupeptin from Peninsula Laboratories, Na+-[14C] (carrier-free) from Amer sham, and [γ-32P]ATP from (CN N) Nutritional Biochemicals. Simonsen albino rats (140-160 g, male) were purchased from Simonsen Laboratories. Polyacrylamide, pentadecylamine, phosvitin, PMSF, polyinosinic-polycytidylic acid, and soybean trypsin inhibitor were purchased from Sigma. Aldolase, blue dextran, carbonic anhydrase, casein, chloramine-T, EDTA, EGTA, fast green, histones (Type VII), insulin, N,N,N',N'-tetraacetic acid.

EXPERIMENTAL PROCEDURES

Materials—Alcohol dehydrogenase, ATP, bovine serum albumin, carbonic anhydrase, casein, chloramine-T, EDTA, EGTA, fast green, fibrinogen, hemoglobin (Type II), histones (Type VIII-S), midezole, ovalbumin, phosvitin, PMSF, polysynthetic-polycytdic acid, and soybean trypsin inhibitor were purchased from Sigma. Albclase, blue dextran 2000, catalase, ferritin, protein A, Superpose 4B, and thyroglobulin were purchased from Pharmacia. DEAE-Bio-Gel A, SDS, acrylamide, and bisacrylamide were purchased from Bio-Rad. Nitrocellulose membranes (BA85, 0.45 μm) were purchased from Schleicher and Schuell, cellulose-coated thin layer chromatography plates from Eastman, trypsin treated with 1-tosylamido-2-phenylethyl chloromethyl ketone (t-2-PACK) from Worthington, ultrapure sucrose from Bethesda Research Laboratories, and Coomassie Brilliant Blue from Searle Diagnostic. Sodium perchlorate was purchased from G. Frederick Smith Chemical Co., dithiothreitol from Boehringer Mannheim, leupeptin from Peninsula Laboratories, Na+-[14C] (carrier-free) from Amer sham, and [γ-32P]ATP from (CN N) Nutritional Biochemicals. Simonsen albino rats (140-160 g, male) were purchased from Simonsen Laboratories. Polyacrylamide, pentadecylamine, phosvitin, PMSF, polyinosinic-polycytidylic acid, and soybean trypsin inhibitor were purchased from Sigma. Aldolase, blue dextran, carbonic anhydrase, casein, chloramine-T, EDTA, EGTA, fast green, histones (Type VII), insulin, N,N,N',N'-tetraacetic acid.

Purification of the Cerebellar Type II Ca2+/Calmodulin-dependent Protein Kinase—The purification was carried out as previously described (27) with several modifications. All operations were carried out at 4°C.

Brains were removed from 90 rats after stunning and decapitation. The cerebellar lobes, weighing approximately 0.25 g each, were removed and immediately homogenized by 12 up and down strokes in a Teflon/glass homogenizer driven at 900 rpm in 10 volumes of Buffer A (40 mM Tris (pH 7.5), 1 mM imidazole, 150 mM Na+-perchlorate, 250 mM sucrose, 1 mM EGTA, 5 mM EDTA, 2 mg/dl dithiothreitol, 0.1 mM PMSF, 1 mg/ml of leupeptin, and 25 mg of soybean trypsin inhibitor). The crude homogenate was centrifuged at 2000 × g for 10 min and the resulting supernatant was centrifuged at 170,000 × g for 1 h. The 170,000 × g supernatant was diluted 5-fold into Buffer B (20 mM Tris (pH 7.5), 1 mM imidazole, 2 mM EDTA, 2 mM dithiothreitol, 0.1 mM PMSF, and 0.02% NaN3), mixed with 50 μl of DEAE-agarose previously equilibrated with Buffer B, and stirred slowly for 90 min. The gel slurry was poured into a Buchner funnel and excess supernatant was drained off by gentle suction. The resulting gel slurry was poured into a glass column (1.5 × 30 cm) and packed at 80-100 ml/min. The first column was washed with 100 ml of Buffer B containing 0.1 M NaCl. Enzyme activity was eluted with a linear gradient of 0.1-0.3 M NaCl in Buffer B (total volume of 500 ml) at 80 ml/h while collecting 6-ml fractions. Kinase activity peaked at 0.08 M NaCl. Peak fractions were pooled, adjusted to 0.1 M Tris (pH 7.5), 1 mM dithiothreitol, 0.1 mM PMSF, and brought to 50% saturation by the slow addition of solid ammonium sulfate over 1 h. After 3-12 h, precipitated protein was collected by centrifugation and redissolved in 5 ml of Buffer C (40 mM Tris (pH 7.5), 2 mM EGTA, 2 mM dithiothreitol, 0.1 mM PMSF, and 0.02% NaN3).

Equal volumes of the redissolved ammonium sulfate precipitate were layered onto each of four 37-ml linear gradients of 5-20% sucrose in Buffer C. The gradients were centrifuged for 24 h at 27,000 × g in a swinging-bucket rotor SW 27Ti) rotating at 12,000 rpm. The 1.5 and 2.0 M sucrose steps, which is enriched in Synapsin I, was resuspended in 0.5% Triton X-100, 75 mM KCl and then fractionated by centrifugation through a discontinuous sucrose gradient. The synaposomal fraction obtained from the 1.0-1.3 M sucrose interface was adjusted to 0.5% Triton X-100, 6 mM Tris, pH 8.1, and insoluble material was pelleted by centrifugation. This material was resuspended and centrifuged through a second discontinuous sucrose gradient. The material at the interface of the 1.5 and 2.0 M sucrose steps, which is enriched in PSDs, was resuspended in 0.5% Triton X-100, 75 mM KCl and then pelleted by centrifugation. The final pellet was resuspended in 40 mM Tris (pH 7.5) and other characteristics were maintained. The material at approximately 0.6-0.7 mg/10 g wet tissue from cerebellum and 2-3 mg/10 g wet tissue from forebrain.

Protein Phosphorylation Assays—Kinase activity was assayed as described (27) except that 10 mM dithiothreitol was included in the final reaction mixture. Autophosphorylation of kinase subunits and endogenous phosphorylation of PSDs was performed as described (27) except that [γ-32P]ATP was used at a lower concentration (5-10 μM) and higher specific activity (2.0-5.0 × 10^6 cpm/pmol) and an additional 6 μl of β-mercaptoethanol was added to the stopped reaction just prior to gel electrophoresis.

Determination of Catalytic Constants—Kinetic parameters were measured as described above except that the amount of Synapsin I was increased to 20 μg, except when it was the variable, and the reaction time was decreased to 10-15 s to ensure measurement of initial rates. Each tube contained 20-50 ng of purified enzyme (4-10 pmol subunits). Utilization of substrates (ATP and Synapsin I) was less than 10% in each assay. The apparent K_m values of various substrates and the apparent K_m for Ca^2+/calmodulin were determined by fitting the data to the Michaelis-Menton equation with a weighted nonlinear least squares computer program adapted from Clandel (47). The apparent K_m for Ca^2+/calmodulin was compared in duplicate experiments using the same reagents.

Preparation of Antibodies—Monoclonal antibodies 6G9 and 4A4 were produced and selected as described (35, 36). These antibodies were purified from ascites fluids by precipitation with protein A-Sepharose or by two successive 50% ammonium sulfate precipitations. Polyclonal
rabbit antisera were produced by multiple subcutaneous injections of purified forebrain Type II kinase or of electrophoretically purified subunits in phosphate-buffered saline containing an equal weight of polyinosinic-polyricidylic acid. The antisera were the kind gifts of Ngozi Erondu and Mark Bennett of this laboratory.

Immunoblotting and Radioimmunoassays—Proteins were separated by SDS-PAGE and transferred to nitrocellulose paper as described by Towbin et al. (48). The nitrocellulose sheets containing transferred protein were labeled with antibodies as previously described (38). Radioimmunoassay was carried out by a quantitative immunoblot method, as described by Erondu and Kennedy (55) using either monoclonal antibody 629 for detection of the α-subunit or a rabbit polyclonal antisera for detection of the β-subunit. Briefly, standard amounts of purified forebrain Type II Ca²⁺/calmodulin-dependent protein kinase and unknown samples were separated by SDS-PAGE and transferred to nitrocellulose paper as above. The nitrocellulose sheets were labeled with appropriate antibodies and subjected to autoradiography after drying. The labeled bands were located on the nitrocellulose sheet using the autoradiograph, cut out, and counted in a γ counter. A standard curve was constructed from the counts bound to standard amounts of forebrain Type II Ca²⁺ kinase (after subtracting background). Linear standard curves were obtained on log/log plots for 20–500 ng of α-subunit with Gb9 and for 80–500 ng of β-subunit for the rabbit antisera.

Other Procedures—Protein concentrations were measured by the method of Lowry et al. (49) as modified by Peterson (50). SDS-PAGE was performed by the method of Laemmli (51). Stacking gels (0.5 × 16 cm × 1.5 mm) contained 3.5% acrylamide, 0.09% bisacrylamide, and the running gels (14 × 16 cm × 1.5 mm) contained 10% acrylamide, 0.27% bisacrylamide. Molecular weight standards for SDS-PAGE were: MAP2 (300,000), phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (45,000), alcohol dehydrogenase (41,000), carbonic anhydrase (29,000), chymotrypsinogen (25,000), and soybean trypsin inhibitor (21,500). The sedimentation coefficient (s₂₀,₅₀) of the kinase was determined by sucrose density gradient centrifugation as described by Martin and Ames (52) with ovalbumin (s₂₀,₅₀ = 3.5 S), fibrinogen (s₂₀,₅₀ = 7.9 S), catalase (s₂₀,₅₀ = 11.3 S), and thyroglobulin (s₂₀,₅₀ = 22 S) as standards. The Stokes radii (Å) of the kinase was determined by gel filtration through Sepharose 4B as described by Porath (53), with catalase (52 Å), ferritin (61 Å), thyroglobulin (85 Å), and fibrinogen (107 Å) as standards. Iodinated tryptic maps were prepared by the method of Elder et al. (64).

RESULTS

Purification of Cerebellar Type II Ca²⁺/Calmodulin-dependent Protein Kinase

Purification—The homogenization conditions described in our initial report on the purification of brain Type II Ca²⁺ kinase (27) have been altered to optimize recovery of the cerebellar kinase. When the cerebellum was homogenized in the original homogenization buffer, 80–90% of the Type II Ca²⁺ kinase activity was recovered in the particulate fraction after centrifugation. The addition of the chaotropic salt, sodium perchlorate (0.15 M), to the homogenization buffer released most of the bound kinase resulting in the recovery of 70–80% of it in a soluble form. The cerebellar kinase activity was unstable in the presence of calcium, therefore 0.2 mM CaCl₂ was replaced by 1 mM EGTA, 5 mM EDTA. The solubilized enzyme was adsorbed to DEAE-agarose by a batch process because of the large sample volume after dilution of the 170,000 × g supernatant to an appropriate ionic strength. The purification of the cerebellar kinase is summarized in Table I.

Different salt concentrations were required to elute the forebrain and cerebellar Type II Ca²⁺ kinases from calmodulin-Sepharose. The forebrain isozyme was eluted in EGTA buffers without salt (27), while elution of the cerebellar isozyme required both EGTA and ~0.08 M NaCl. This may result from nonspecific ionic interactions of the cerebellar kinase with the column or from specific low affinity binding of the kinase to calmodulin even in the absence of Ca²⁺. The calmodulin-Sepharose pool was approximately 90% pure as judged by densitometric scans of stained SDS-polyacrylamide gels. It represented a purification of about 400-fold from the crude homogenate with a yield of approximately 5%. The low overall recovery was due largely to low recoveries from DEAE-agarose. The specific activities of the calmodulin-Sepharose pools varied from 1.23 to 2.90 μmol/min/mg under our standard assay conditions and from 3.4 to 8.0 μmol/min/mg at saturating synapsin I concentrations. This compares to an average value for the forebrain kinase of 2.9 μmol/min/mg under standard conditions and 8.0 μmol/min/mg at saturating synapsin I concentrations. The physical properties of the forebrain kinase were not altered by the changes in purification procedure and recoveries were improved, ranging from 6 to 11%.

Subunit Composition—The Type II Ca²⁺ kinases purified from cerebellum and from forebrain both contain two major subunits, α (50 kDa) and β (60 kDa), but they are present in different ratios (Fig. 1). Minor subunits of 56 and 58 kDa (β') in the cerebellar kinase are more prominent than the 58-kDa β'-subunit in the forebrain kinase. As for the forebrain kinase, the cerebellar β- and β'-subunits have identical peptide maps and occur in a constant ratio to the α-subunits from preparation to preparation. All of the subunits of the cerebellar kinase were precipitated with a monoclonal antibody (4A4) that recognizes only the α-subunit on Western blots (data not shown). Thus, they exist together in a holoenzyme complex. In several immunoprecipitation experiments with antibody 4A4, 6–7% of the initial kinase activity remained in the supernatant after immunoprecipitation with maximal amounts of antibody (data not shown). Thus, a small proportion of the cerebellar kinase holoenzyme molecules may not contain α-subunits.

The physical properties of the cerebellar isozyme were determined by the methods used to characterize the forebrain kinase (27). The molar ratio of the subunits was determined from densitometric scans of gels stained with fast green. The β'-doublet was treated as a single band for this analysis. The ratio of β-subunit alone to α-subunit was 3:1 and the ratio of total subunits (α + β'-doublet) to α-subunit was 4:1. The approximate molecular weight of the holoenzyme was calculated from its hydrodynamic properties (Table II). A Stokes radius of 88 Å was measured by gel filtration and a sedimentation coefficient (s₂₀,₅₀) of 14.0 S was measured by sucrose density gradient centrifugation. A molecular weight of 508,000 (±48,000) was calculated from these parameters as described in Table II. The cerebellar isozyme consistently appeared smaller than the forebrain isozyme. This difference was observed even in parallel density gradients run at the same time. The subunit structure of the cerebellar holoenzyme that is most consistent with its molecular weight and the ratio of its subunits is a decamer of ~8 β/β'-subunits and ~2 α-subunits. The molecular weight of such a holoenzyme would be 574,000, slightly higher than the range indicated by its hydrodynamic properties.

Comparison of the Subunits of Forebrain and Cerebellar Isozymes

Iodinated Peptide Maps—we compared maps of iodinated peptides of the α- and β-subunits of the cerebellar kinase to those of the forebrain kinase (Fig. 2). Maps of the β-subunits of the two isozymes were identical. Maps of the α-subunits, however, showed a few reproducible differences. Maps of the β'-subunits from both isozymes (not shown) reveal that they are closely related to the β-subunits. As has been noted before (28, 37), peptide maps of the α- and β-subunits of the forebrain

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they are structurally related. This is also the case for the corresponding subunits in the two holoenzyme forms. kinase contain several peptides in common, indicating that SDS-PAGE on a 12.5% gel, then stained with Coomassie Blue. Positions of molecular weight standards are indicated on the left.

kinase antibodies which recognize specific subunits of the forebrain Type II CaM kinase on immunoblots were used to test the immunological relationship between the subunits of the forebrain and cerebellar isozymes. Two polyclonal rabbit antisera that recognize primarily the β-subunit of the forebrain kinase cross-reacted strongly with the cerebellar β/β'-subunits. A monoclonal antibody, 6G9, which binds to the α-subunit of the forebrain isozyme also bound to the α-subunit of the cerebellar isozyme (data not shown). Thus, the denatured subunits of the two kinases were indistinguishable by these immunological reagents.

**Comparison of Enzymatic Properties of Forebrain and Cerebellar Kinases**—The substrate specificity and catalytic constants of the forebrain and cerebellar isozymes were compared to determine whether their catalytic properties might differ significantly in vivo.

**Substrate Specificity**—The forebrain kinase phosphorylates a number of substrate proteins (27). We measured the ability of the cerebellar kinase to phosphorylate several of these same substrates (Table III). At this level of kinetic resolution, the substrate specificity of the two forms did not differ. Synapsin I and MAP2 were phosphorylated at the highest rate.

**Catalytic Constants**—For each of the two kinases, we determined the apparent $K_m$ values for synapsin I, MAP2, and ATP, as well as the apparent $K_m$ for calmodulin (Table IV). The apparent $K_m$ values for synapsin I and MAP2 were the same, however the $K_m$ for ATP differed by a factor of $\sim 1.8$, the cerebellar kinase having the higher affinity. Since the intracellular ATP concentration is estimated to be about 1–2 mM (55), the difference between the two kinases is not likely to be significant in vivo. However, the cerebellar kinase also

![Fig. 1. Comparison of purified forebrain (FB) and cerebellar (CER) isozymes of rat brain Type II Ca$^{2+}$/calmodulin-dependent protein kinase.](image-url)

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**Table I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity* (µmol/min)</th>
<th>Total protein* (mg)</th>
<th>Specific activity* (µmol/min/mg)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Homogenate</td>
<td>10.5 ± 1.5</td>
<td>2418 ± 450</td>
<td>5</td>
<td>0.006</td>
<td>1.0</td>
</tr>
<tr>
<td>2. 170,000 × g supernatant</td>
<td>10.4 ± 1.9</td>
<td>724 ± 110</td>
<td>5</td>
<td>0.013</td>
<td>2.8</td>
</tr>
<tr>
<td>3. DEAE-agarose pool</td>
<td>1.98 ± 0.3</td>
<td>175 ± 23</td>
<td>5</td>
<td>0.012</td>
<td>2.2</td>
</tr>
<tr>
<td>4. (NH$_4$)$_2$SO$_4$, ppt.</td>
<td>1.74 ± 0.3</td>
<td>83 ± 13</td>
<td>5</td>
<td>0.021</td>
<td>4.1</td>
</tr>
<tr>
<td>5. Sucrose gradient</td>
<td>1.38 ± 0.2</td>
<td>16 ± 4</td>
<td>3</td>
<td>0.091</td>
<td>16.8</td>
</tr>
<tr>
<td>6. Calmodulin-Sepharose pool</td>
<td>0.76 ± 0.2</td>
<td>0.49 ± 0.29</td>
<td>2</td>
<td>2.02</td>
<td>400</td>
</tr>
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</table>

*Values are mean ± S.D. of $n$ experiments.

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**Table II**

<table>
<thead>
<tr>
<th>Property</th>
<th>Forebrain*</th>
<th>Cerebellum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stokes radius (Å)</td>
<td>94.7 ± 1.2</td>
<td>88 ± 1.3</td>
</tr>
<tr>
<td>Sedimentation coefficient (s$_{20w}$)</td>
<td>16.4 ± 0.7</td>
<td>14.0 ± 1.1</td>
</tr>
<tr>
<td>Frictional coefficient ($f$/g)</td>
<td>1.67</td>
<td>1.67$^d$</td>
</tr>
<tr>
<td>Molecular weight (M$_w$)</td>
<td>650,000</td>
<td>508,000 ± 48,000$^d$</td>
</tr>
<tr>
<td>Holoenzyme composition</td>
<td>9 αββ'</td>
<td>2 αββ'</td>
</tr>
<tr>
<td></td>
<td>(654,000 Da)</td>
<td>(574,000 Da)</td>
</tr>
</tbody>
</table>

*Identical values were obtained in three experiments. The range is ±Stokes radii included in one column fraction.

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**Immunochemical Comparison**—Monoclonal and polyclonal antibodies which recognize specific subunits of the forebrain Type II CaM kinase on immunoblots were used to test the immunological relationship between the subunits of the forebrain and cerebellar isozymes. Two polyclonal rabbit antisera that recognize primarily the β-subunit of the forebrain kinase cross-reacted strongly with the cerebellar β/β'-subunits. A monoclonal antibody, 6G9, which binds to the α-subunit of the forebrain isozyme also bound to the α-subunit of the cerebellar isozyme (data not shown). Thus, the denatured subunits of the two kinases were indistinguishable by these immunochemical reagents.
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Fig. 2. Iodinated tryptic peptides of the \( \alpha \)- and \( \beta \)-subunits of Type II CaM kinase isolated from forebrain (FB) and cerebellum (CER). Five \( \mu \)g of pure kinase isolated from each brain region was subjected to SDS-PAGE and stained with Coomassie Blue. The bands corresponding to the \( \alpha \)-, \( \beta \)-, and \( \beta' \)-subunits from each region were cut from the gel. Iodinated tryptic peptide maps were prepared as previously described (54). One \( \mu \)l of the peptide solution was applied to the plate at the lower left. For electrophoresis, the anode was to the left and the cathode to the right for electrophoresis; chromatography was from bottom to top. The spots labeled 1, 2, and 3 were common to both subunits from both brain regions; 4, 5, and 6 were common to the \( \beta \)-subunits (4 was also found in the cerebellar \( \alpha \)-subunit); 7 and 8 were common to the \( \alpha \)-subunits (although differing in intensity); 9 and 10 were found only in the cerebellar \( \alpha \)-subunit; and 11 is found only in the forebrain \( \alpha \)-subunit. The identities of the various spots were confirmed by mapping of mixtures of peptides from the different subunits.

Table III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration in assay</th>
<th>Rate of Ca\textsuperscript{2+}/calmodulin-stimulated phosphorylation</th>
<th>Cerebellum</th>
<th>Forebrain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synapsin I\textsuperscript{a}</td>
<td>0.1 ( \mu )g/ml</td>
<td>100 %</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>MAP\textsubscript{2}\textsuperscript{b}</td>
<td>0.4 ( \mu )g/ml</td>
<td>128 %</td>
<td>141</td>
<td>141</td>
</tr>
<tr>
<td>Histone\textsuperscript{c}</td>
<td>0.2 ( \mu )g/ml</td>
<td>21 %</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>0.4 ( \mu )g/ml</td>
<td>3 %</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Casein</td>
<td>0.4 ( \mu )g/ml</td>
<td>0 %</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phosphorylase ( b )</td>
<td>0.4 ( \mu )g/ml</td>
<td>2 %</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

\( \textsuperscript{a} \) The rate of Ca\textsuperscript{2+}-stimulated phosphorylation of synapsin I was 33–37 pmol/min for the cerebellar kinase and 23–30 pmol/min for the forebrain kinase.

\( \textsuperscript{b} \) The source of MAP\textsubscript{2} was a microtubule protein preparation consisting of approximately 10% MAPs and 90% tubulin.

\( \textsuperscript{c} \) This sample of arginine-rich histones contained approximately 30% histone H3, which was the only protein phosphorylated. Calmodulin concentration in these assays was increased to 0.2 mg/ml.

had a consistently higher apparent affinity for Ca\textsuperscript{2+}/calmodulin. The difference in affinities occurred in a range over which the concentration of the Ca\textsuperscript{2+}/calmodulin complex would be expected to vary in vivo. Thus, this difference may have physiological significance.

Autophosphorylation of Kinase Subunits

The subunits of the Type II CaM kinase undergo rapid and stoichiometrically significant autophosphorylation under conditions in which the kinase is active (27, 28). When crude brain homogenates are phosphorylated under conditions in which the kinase is maximally active, the most prominent endogenous phosphopeptides are the subunits of the kinase itself (27, 56). This probably reflects, at least in part, the relative abundance of the kinase in brain (35). We compared the Ca\textsuperscript{2+}/calmodulin-stimulated endogenous phosphoproteins in forebrain and cerebellar homogenates to the autophosphorylated subunits of the Type II CaM kinase isozymes from each region (Fig. 3). The most prominent endogenous phosphopeptides in both homogenates were the kinase subunits. We confirmed this by comparison of phosphopeptide maps of the endogenous phosphoproteins and the phosphorylated subunits of the pure isozymes (data not shown). The patterns of phosphorylation shown in Fig. 3 were observed in fresh tissue

Table IV

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Apparent ( K_a ) or ( K_m )</th>
<th>Forebrain</th>
<th>Cerebellum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synapsin I\textsuperscript{a}</td>
<td>1.2 ( \mu )M ( \pm 0.1 )</td>
<td>1.1 ( \mu )M ( \pm 0.2 )</td>
<td></td>
</tr>
<tr>
<td>MAP\textsubscript{2}\textsuperscript{b}</td>
<td>130 nM</td>
<td>114 nM</td>
<td></td>
</tr>
<tr>
<td>ATP\textsuperscript{b}</td>
<td>16 ( \mu )M ( \pm 1.3 )</td>
<td>9 ( \mu )M ( \pm 1.1 )</td>
<td></td>
</tr>
<tr>
<td>Calmodulin\textsuperscript{a}</td>
<td>123 nM ( \pm 6 )</td>
<td>69 nM ( \pm 1 )</td>
<td></td>
</tr>
</tbody>
</table>

\( \textsuperscript{a} \) Values are the mean \pm S.D. of at least two experiments.

\( \textsuperscript{b} \) The \( K_m \) of the forebrain kinase for MAP\textsubscript{2} was determined by James Soha of Caltech. The \( K_m \) of the cerebellar kinase was determined in one experiment, using the same preparation of purified MAP\textsubscript{2}.

Conditions in which the kinase is active (27, 28). When crude brain homogenates are phosphorylated under conditions in which the kinase is maximally active, the most prominent endogenous phosphopeptides are the subunits of the kinase itself (27, 56). This probably reflects, at least in part, the relative abundance of the kinase in brain (35). We compared the Ca\textsuperscript{2+}/calmodulin-stimulated endogenous phosphoproteins in forebrain and cerebellar homogenates to the autophosphorylated subunits of the Type II CaM kinase isozymes from each region (Fig. 3). The most prominent endogenous phosphopeptides in both homogenates were the kinase subunits. We confirmed this by comparison of phosphopeptide maps of the endogenous phosphoproteins and the phosphorylated subunits of the pure isozymes (data not shown). The patterns of phosphorylation shown in Fig. 3 were observed in fresh tissue
were incubated under phosphorylating conditions for 10 s with 
pendent phosphorylation of crude brain homogenates and of 
"PIATP as described under "Experimental Procedures." The incu-
bations were carried out in the presence or absence of Ca2+ as 
cerebellum) and pure kinase 
indicated. The samples were then subjected to SDS-PAGE and dried, and labeled bands were detected by autoradiography.

homogenates, indicating that the different proportions of 
kinese subunits were not generated artifactually during puri-

Concentration of Cerebellar and Forebrain Isozymes in 
Postsynaptic Density Fractions

The α-subunit of Type II CaM kinase is a major component of 
postsynaptic density fractions prepared from whole brain (36-38). However, several groups have reported that it is 
reduced in concentration or nearly absent in cerebellar PSDs 
(57-59; in these papers, the α-subunit is referred to as the 
51K PSD protein or the major PSD protein). We wondered 
whether this reduction simply reflected the difference in 
subunit composition of forebrain and cerebellar Type II CaM 
kinese isozymes, so we examined the content of α- and β-
subunits in PSD fractions from the two brain regions.

Endogenous Phosphorylation of PSDs—Freshly isolated 
PSDs from forebrain and cerebellum were labeled with 32P by 
endogenous phosphorylation in the presence of calcium and 
calmodulin (Fig. 4). Under these conditions, the major endog-


protein that is identical with the α-subunit of 
Type II CaM kinase. Protein bands corresponding to the β-

Protein Composition of Cerebellar and Forebrain PSDs—
We also compared Coomassie Blue-stained protein profiles of 
cerebellar and forebrain PSDs to the profiles of purified Type 
II CaM kinases from each region (Fig. 5). As was previously 
reported, the predominant protein in the forebrain PSDs was 
the 50-kDa protein that is identical with the α-subunit of 
Type II CaM kinase. Protein bands corresponding to the β-

DISCUSSION

We have shown that brain “Type II” Ca2+/calmodulin-
dependent protein kinase exists in a different isomeric form 
in cerebellum as compared to forebrain. The cerebellar iso-
zyme contains 50- and 60-KDa α- and β-subunits that are
Cerebellar Type II Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase

**FIG. 5.** Protein composition of PSD and Type II CaM kinase (KIN) isolated from either forebrain (FB) or cerebellum (CER). Six \(\mu\)g of purified kinase and 40 \(\mu\)g of PSD protein from each region were subjected to SDS-PAGE on a 10% gel then stained with Coomassie Blue. Positions of molecular weight standards are indicated on the left.

**TABLE V**

Concentration of \(\alpha\)- and \(\beta\)-subunits of Type II CaM kinase in PSD fractions from forebrain and cerebellum

<table>
<thead>
<tr>
<th>Region</th>
<th>(\alpha)-Subunit/PSD protein(^*)</th>
<th>(\beta)-Subunit/PSD protein(^*)</th>
<th>Molar ratio ((\alpha/\beta))</th>
<th>Kinase holoenzyme as % PSD protein(^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebellum</td>
<td>2.4 ± 0.2 ((8)^b)</td>
<td>10.5 ± 1.1 ((8)^b)</td>
<td>1.0:3.7</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Forebrain</td>
<td>129.6 ± 9.6 ((35)^a)</td>
<td>44.5 ± 2.7 ((12)^a)</td>
<td>3.5:1.0</td>
<td>17.4 ± 1.5</td>
</tr>
</tbody>
</table>

\(^*\) ± S.E.M.

\(^b\) Number of determinations.

closely related to those in the forebrain; however, they are present in a different ratio. The cerebellar \(\alpha:\beta\) ratio is 1:4, whereas the forebrain \(\alpha:\beta\) ratio is 3:1. The resolution of our analytical methods is not sufficient to tell if the kinase holoenzymes from the two brain regions exist as unique oligomers that each contain the same number of \(\alpha\)- and \(\beta\)-subunits. It is possible that we have measured an average molecular weight of heterogeneous oligomers that were assembled randomly from newly synthesized subunits. In the latter case, the difference in average holoenzyme composition between the two regions would simply reflect a difference in the ratio of subunits synthesized. Further studies will be necessary to determine whether intermediate isozyme forms exist in other brain regions (35).

The \(\beta\)-subunits from the two brain regions show extensive immunological cross-reactivity and have identical iodinated tryptic peptide maps. The \(\alpha\)-subunits also show immunological cross-reactivity and their maps differ by only a few peptides. Studies by our laboratory and others have suggested that the \(\alpha\)- and \(\beta\)-subunits are themselves structurally homologous and may both be catalytic (27, 37). The properties of the cerebellar isozyme are consistent with this notion. The specific activity of the purified cerebellar kinase is similar to that of the forebrain kinase, although the ratio of its subunits is dramatically different. In addition, the protein substrate specificities of the two isozymes do not differ, insofar as we have measured them.

What then is the functional significance, if any, of the
different kinase forms? There are several possibilities. In peripheral tissues, closely related isoenzymes sometimes differ in critical kinetic constants or in their association with specific subcellular organelles (60). The cerebellar kinase shows a consistent 2-fold higher apparent affinity for the Ca\(^{2+}\) in comparison to the forebrain kinase. The difference is in a concentration range over which calcium-bound calmodulin is likely to vary in vivo. Thus, the cerebellar kinase may be more sensitive to increases in calcium concentration than the forebrain kinase.

A second possible functional difference between the \(\alpha\)- and \(\beta\)-subunits is in their association with subcellular structures in vivo. Protein isozymes are known to have distinct subcellular locations in peripheral and neural tissue. This specific organization of the cytoplasm may be important for efficient cellular functioning (60). Recently, isoforms of various neuronal cytoskeletal proteins have been shown to have different subcellular locations. In chick cerebellum, \(\beta\)-spectrin is confined to the plasma membrane of the soma, while \(\gamma\)-spectrin (fodrin) is associated with the membranes of both the soma and processes (61). Similarly, MAP2 is located in neuronal dendrites and perikarya, while MAP4 is more evenly distributed throughout the neuron (62, 63). The results presented here suggest that the subcellular locations of the forebrain and cerebellar Type II CaM kinase isoenzymes differ. Although more of the cerebellar isozyme (85%) than of the forebrain isozyme (50%) is recovered in the particulate fraction of brain homogenates, cerebellar PSDs contain only about 8% as much Type II CaM kinase as do forebrain PSDs. This suggests that the \(\alpha\)-subunit is involved in anchoring of the kinase within PSDs, while the \(\beta\)-subunit may have a higher affinity for different subcellular structures. It will be interesting to determine what other particular structures the cerebellar kinase is bound to.

In summary, we have confirmed and extended the observation made by several groups that the major difference in protein composition between cerebellar and forebrain post-synaptic density fractions is their content of a major 51-kDa protein (46, 58, 59). The 50-kDa protein is the \(\alpha\)-subunit of the Type II CaM kinase (56-58). We have shown that PSD fractions from cerebellum contain reduced quantities of this protein because the cerebellar kinase contains less \(\alpha\)-subunit and more \(\beta\)-subunit than the forebrain kinase and has a reduced affinity for the PSD fraction. This quantitative difference in the concentration of the Type II CaM kinase in PSDs from neurons in the two brain regions may produce different responses in these neurons to post-synaptic changes in calcium flux.

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REFERENCES