Purification and Characterization of a Brain-specific Multifunctional Calmodulin-dependent Protein Kinase from Rat Cerebellum*

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A brain-specific multifunctional calmodulin-dependent protein kinase, calmodulin-dependent protein kinase IV, which exhibited characteristic properties quite different from those of calmodulin-dependent protein kinase II, was purified approximately 230-fold from rat cerebellum. The purified preparation gave two protein bands with molecular weights of 63,000 (α) and 66,000 (β) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, both of which showed protein kinase activity as examined by the activity gel method. The molecular weight of the enzyme was estimated as about 67,000 from sedimentation coefficient (3.2 S) and Stokes radius (50 Å), indicating a monomeric structure of the enzyme. The enzyme phosphorylated smooth muscle myosin light chain, synapsin I, microtubule-associated protein 2, tau protein, myelin basic protein, histone H1, and tyrosine hydroxylase in a Ca2+/calmodulin dependent manner, suggesting that the enzyme is a multifunctional calmodulin-dependent protein kinase capable of phosphorylating a large number of substrates. A synthetic peptide, Lys-Ser-Asp-Gly-Gly-Val-Lys-Lys-Arg-Lys-Ser-Ser-Ser-Ser, was found to be a specific substrate for this kinase and, using this peptide as substrate, the distribution of the enzyme activity in various rat tissues was examined. The activity was found in cerebral cortex, brain stem, and cerebellum, most abundantly in cerebellum, but other tissues tested, including liver, spleen, kidney, lung, heart, skeletal muscle, and adrenal gland showed very little activity.

Three multifunctional protein kinases, cAMP-dependent protein kinase, protein kinase C, and calmodulin-dependent protein kinase II (CaM kinase II), are known to be involved in signal transduction in response to their respective second messengers, cAMP, diacylglycerol, and Ca2+*. Since CaM kinase II was first demonstrated to occur abundantly in brain and show a very broad substrate specificity (1), the regulation of the physiological functions by this kinase in the central nervous system has interested a number of laboratories and the possible involvement of CaM kinase II in the regulation of neuronal functions such as neurotransmitter synthesis (2, 3), neurotransmitter release (4), and long term potentiation (5) has so far been demonstrated.

Sikela and his associates (6) isolated a cDNA clone from mouse brain by screening with 125I-labeled calmodulin in 1987, and thereafter identified it as a new brain calmodulin-dependent protein kinase, designated as calmodulin-dependent protein kinase IV (CaM kinase IV) (7). In 1989, Sahyoun and his associates (8) reported a novel neuronal calmodulin-dependent protein kinase, designated as calmodulin-dependent protein kinase Gr, enriched in cerebellar granule cells which catalyzes phosphorylation of synapsin I. Judging from the deduced amino acid sequences (6, 8), both calmodulin-dependent protein kinases appeared to be identical. In the present study, this neuronal calmodulin-dependent protein kinase was purified from rat cerebellum and its properties were examined. Of particular interest, the kinase phosphorylated a number of protein substrates, suggesting the possible involvement in the regulation of a number of neuronal processes. The name of CaM kinase IV was adopted in this paper, because this kinase occurs not only in cerebellar granule cells but also in cerebral cortex and brain stem, CaM kinase II was originally designated by the order of decreasing molecular weights of calmodulin-dependent protein kinases in the rat brain since it eluted from a gel filtration column after phosphorylase kinase (kinase I) but before myosin light chain kinase (kinase III) (1), and the apparent molecular weight of this kinase was lower than that of myosin light chain kinase (kinase III (1)). Thus, the present study suggests that at least two distinct calmodulin-dependent multifunctional protein kinases, CaM kinase II and CaM kinase IV, are involved in the regulation of diverse physiological processes mediated by Ca2+ in the central nervous system.

EXPERIMENTAL PROCEDURES AND RESULTS

Properties of CaM Kinase IV

Molecular Properties—Purified CaM kinase IV gave two protein bands, corresponding to molecular weights of 63,000 (α) and 66,000 (β), on SDS-polyacrylamide gel electrophoresis as shown in Fig. 3A. The molar ratio of α to β was roughly estimated as 4 to 1 from the results of densitometric scanning of silver-stained SDS-polyacrylamide gels. When the enzyme was subjected to sucrose density gradient centrifugation, the enzyme was sedimented as a single and symmetrical peak at

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* Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 1 and 2, and Table 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
that the activities are strongly stimulated in the presence of Ca2+/calmodulin.

**Catalytic Properties**—Phosphorylation of various substrates by CaM kinase IV was examined and compared with that of CaM kinase II as shown in Table III. The activity of CaM kinase IV was measured at pH 6.5 and 8.0, since the reaction of CaM kinase IV for most substrates was optimal at around pH 6.5 and the usual assay for CaM kinase II has been carried out at pH 8.0. Synapsin I, myelin basic protein, MLC, and tau protein were relatively good substrates for both CaM kinase IV and II, although the specific activities of CaM kinase IV toward these substrates were much lower than those of CaM kinase II under the experimental conditions. Tyrosine hydroxylase also served as substrate for both kinases. Casein and tubulin served as substrate for CaM kinase II but not significantly for CaM kinase IV. Histone H1 was, in contrast, a good substrate for CaM kinase IV but was a relatively poor substrate for CaM kinase II. Thus, CaM kinase IV appears to be a multifunctional protein kinase capable of phosphorylating a large number of substrates, although its activity toward each substrate was much lower than that of CaM kinase II. Phosvitin, protamine, and phosphorylase b were not phosphorylated by CaM kinase IV significantly (data not shown). It is of particular interest to examine how many endogenous brain proteins are active as substrate for CaM kinase IV. The crude extract of rat cerebellum whose calmodulin-dependent protein kinases had been inactivated by heat treatment at 55 °C was incubated with CaM kinase IV under the phosphorylating conditions, and incorporation of 32P into proteins was examined by SDS-polyacrylamide gel electrophoresis followed by autoradiography as shown in Fig. 4. CaM kinase IV phosphorylated many endogenous brain cytosol proteins, including 270-, 200-, 190-, 150-, 140-, 125-, 100-, 90-, 80-, 60-, 58-, 54-, 52-, 50-, and 45-kDa proteins, suggesting its involvement in regulating diverse neuronal functions.

Sytntide-2, a synthetic peptide which is often used as substrate for CaM kinase II, was also a very good substrate for CaM kinase IV. However, the specific activity of CaM kinase IV obtained with syntide-2 as substrate was more than 1 order of magnitude lower than that of CaM kinase II. On the other hand, it was inadvertently found that peptide-γ, corresponding to

![Fig. 3. Analysis of purified CaM kinase IV on SDS-polyacrylamide gel electrophoresis. A, purified CaM kinase IV (10 μg) was subjected to SDS-polyacrylamide gel electrophoresis, and stained with Coomassie Brilliant Blue R-250. B, purified CaM kinase IV (0.2 μg) was autophosphorylated with [γ-32P]ATP in the presence (lane 1) and absence (lane 2) of Ca2+/calmodulin as described under "Experimental Procedures," subjected to SDS-polyacrylamide gel electrophoresis, and then analyzed by autoradiography. C, CaM kinase IV (0.2 μg) was electrophoresed on a SDS-polyacrylamide gel containing 0.5 mg/ml MLC, renatured in situ, incubated with [γ-32P]ATP in the presence (lane 1) and absence (lane 2) of Ca2+/calmodulin, and then analyzed by autoradiography.](image)

### Table II

**Molecular properties of CaM kinase IV**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Method of determination</th>
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<tbody>
<tr>
<td>Molecular weight</td>
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<td>Stokes radius and sedimentation coefficient</td>
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<tr>
<td>Subunit weight</td>
<td>66,000</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Stokes radius (Å)</td>
<td>50</td>
<td>Gel filtration</td>
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<tr>
<td>Sedimentation coefficient (ρm)</td>
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<td>Sucrose-density gradient centrifugation</td>
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<tr>
<td>Frictional ratio (f/f0)</td>
<td>1.9</td>
<td>Stokes radius and sedimentation coefficient</td>
</tr>
</tbody>
</table>

* Calculated according to the equation (25): $f/f_0 = a/(3\omega M/4\pi N)^{1/3}$.

* Calculated according to the equation (25): $M_p = 6\pi N\eta a/(1-\eta)$, where $N = $ Avogadro's number, $a = $ Stokes radius, $\eta = $ viscosity of water at 20 °C, $\rho = $ density of water at 20 °C, and $\nu = $ partial specific volume. Partial specific volume was assumed to be 0.725 ml/g (23).

### Table III

**Substrate specificities of CaM kinase IV and CaM kinase II**

Phosphorylation of various protein substrates (0.1 mg/ml) or synthetic peptides (0.4 μM) by CaM kinase IV (0.2 μg) or CaM kinase II (0.019 μg) was measured as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate of phosphorylation</th>
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<tbody>
<tr>
<td></td>
<td>CaM kinase IV</td>
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<tr>
<td>pH 6.5</td>
<td>pH 8.0</td>
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<tr>
<td>MLC</td>
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<tr>
<td>MAP2</td>
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<tr>
<td>Tau protein</td>
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<tr>
<td>Myelin basic protein</td>
<td>58.2</td>
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<tr>
<td>Synapsin I</td>
<td>41.9</td>
</tr>
<tr>
<td>Histone H1</td>
<td>14.0</td>
</tr>
<tr>
<td>Tyrosine hydroxylase</td>
<td>2.2</td>
</tr>
<tr>
<td>Casein</td>
<td>0.1</td>
</tr>
<tr>
<td>Tubulin</td>
<td>0.1</td>
</tr>
<tr>
<td>Syntide-2</td>
<td>173.7</td>
</tr>
<tr>
<td>Peptide-γ</td>
<td>146.1</td>
</tr>
</tbody>
</table>

* Assayed at pH 6.0 (in 40 mM Mes/NaOH buffer).
buffer was added to the reaction mixture, and the mixture was heated in a mini incubator at 55 °C to inactivate endogenous calmodulin-dependent protein kinase IV. Polyacrylamide gel electrophoresis and incorporation of 32P into protein kinases, and 5-μl aliquots (about 7.5 μg of protein) were incubated at 37 °C for 30 min in the standard assay mixture, in a final volume of 50 μl, containing 40 mM Hepes-NaOH (pH 6.5), 5 mM Mg(CH3COO)2, 50 μM γ-[32P]ATP (9520 cpm/pmol), 0.1 mM EGTA, 0.15 mM CaCl2, 0.84 μM calmodulin, and 0.5 μg of CaM kinase IV, with the indicated omissions. After incubation for 30 min at 37 °C, 50 μl of SDS sample buffer was added to the reaction mixture, and the mixture was heated at 100 °C for 1.5 min. An aliquot of 30 μl was subjected to SDS-polyacrylamide gel electrophoresis and incorporation of 32P into proteins was examined by autoradiography. Arrows indicate protein bands markedly phosphorylated by CaM kinase IV. PK IV, CaM kinase IV; and Ext, the crude extract of rat cerebellum.

FIG. 4. Phosphorylation of rat cerebellar proteins by CaM kinase IV. The crude extract of rat cerebellum was heated for 60 min at 55 °C to inactivate endogenous calmodulin-dependent protein kinases, and 5-μl aliquots (about 7.5 μg of protein) were incubated at 37 °C for 30 min in the standard assay mixture, in a final volume of 50 μl, containing 40 mM Hepes-NaOH (pH 6.5), 5 mM Mg(CH3COO)2, 50 μM γ-[32P]ATP (9520 cpm/pmol), 0.1 mM EGTA, 0.15 mM CaCl2, 0.84 μM calmodulin, and 0.5 μg of CaM kinase IV, with the indicated omissions. After incubation for 30 min at 37 °C, 50 μl of SDS sample buffer was added to the reaction mixture, and the mixture was heated at 100 °C for 1.5 min. An aliquot of 30 μl was subjected to SDS-polyacrylamide gel electrophoresis and incorporation of 32P into proteins was examined by autoradiography. Arrows indicate protein bands markedly phosphorylated by CaM kinase IV. PK IV, CaM kinase IV; and Ext, the crude extract of rat cerebellum.

FIG. 5. Elution profile of calmodulin-dependent peptide-γ phosphorylating activity of rat cerebellar crude extract on DEAE-TOYOPEARL. Rat cerebellar crude extract was chromatographed on a DEAE-TOYOPEARL column after ammonium sulfate precipitation, essentially as described in the legend for Fig. 1. ⧫, calmodulin-dependent syntide-2 phosphorylation activity; ▲, calmodulin-dependent peptide-γ phosphorylation activity. The arrow indicates the elution position of CaM kinase IV.

The apparent Kₐ values for syntide-2, peptide-γ, MLC, and MAP2 were obtained from double-reciprocal plots of data. The Kₐ value for ATP and the Kₐ value for calmodulin were measured with syntide-2 as a substrate. The Kₐ value for calmodulin was obtained from the concentration required for half-maximal activation of the enzyme. The molecular weights of syntide-2, peptide-γ, and calmodulin were assumed to be 1,508, 1,451, and 17,000, respectively.

Table IV: Tissue Distribution of CaM Kinase IV

Tissue distribution of CaM kinase IV was examined by the use of its specific substrate, peptide-γ, as shown in Table V. The activity of CaM kinase IV was calculated from the difference between the total peptide-γ phosphorylating activity (a – b, in Table V) and Ca2+/calmodulin-independent peptide-γ phosphorylating activity (c – d, in Table V). The crude extract of cerebellum showed the total activity of 4.48 nmol/min/ml (1.79 nmol/min/mg of protein) and Ca2+/calmodulin-independent activity of 0.61 nmol/min/ml (0.24 nmol/min/mg of protein), and Ca2+/calmodulin-dependent activity was accordingly calculated to be 3.87 nmol/min/ml (1.55 nmol/min/mg of protein). Among a number of tissues

Table V: Tissue Activity of CaM kinase IV

The apparent Kₐ values for syntide-2, peptide-γ, MLC, and MAP2 were obtained from double-reciprocal plots of data. The Kₐ value for ATP and the Kₐ value for calmodulin were measured with syntide-2 as a substrate. The Kₐ value for calmodulin was obtained from the concentration required for half-maximal activation of the enzyme. The molecular weights of syntide-2, peptide-γ, and calmodulin were assumed to be 1,508, 1,451, and 17,000, respectively.

Table IV: Kinetic properties of CaM kinase IV

The kinetic properties of CaM kinase IV are shown in Table IV. The apparent Kₐ value for syntide-2, peptide-γ, MLC, and MAP2 were obtained from double-reciprocal plots of data. The Kₐ value for ATP and the Kₐ value for calmodulin were measured with syntide-2 as a substrate. The Kₐ value for calmodulin was obtained from the concentration required for half-maximal activation of the enzyme. The molecular weights of syntide-2, peptide-γ, and calmodulin were assumed to be 1,508, 1,451, and 17,000, respectively.
examined, cerebellum showed the highest specific activity of CaM kinase IV. Cerebral cortex showed the second highest activity which amounted to about one-half of that of cerebellum, and brain stem showed the activity amounting to one-fourth. In contrast, the tissues other than brain showed little, if any, activity. When tissue distribution of CaM kinase IV was examined by the activity gel method using MLC as substrate, more definitive results were obtained as shown in Fig. 6. Radioactivity corresponding in position to CaM kinase IV in SDS-polyacrylamide gel was observed in cerebellum, cerebral cortex, and brain stem, and the intensity of the radioactivity decreased in this order, in agreement with the results obtained with peptide-γ (Table V). Shorter exposure of the autoradiograph revealed two distinct radioactive bands corresponding to molecular weights of 63,000 and 66,000 in cerebellum, but only one radioactive band corresponding to a molecular weight of 63,000 in cerebral cortex and brain stem. This suggests that both CaM kinase IV-α and -β exists in cerebellum, but that only CaM kinase IV-α exists in cerebral cortex and brain stem. Longer exposure of the autoradiograph revealed a faint radioactive band corresponding to the position of CaM kinase IV in all tissue tested. These results, taken together, suggest the possibility that CaM kinase IV is present abundantly in brain but very sparingly in other tissues. Skeletal muscle gave a clear calmodulin-dependent band corresponding in size to myosin light chain kinase, and all tissues tested gave two distinct calmodulin-independent bands corresponding to molecular weights of 37,000 and 41,000. Casein kinases and the catalytic subunit of cAMP-dependent protein kinase are well known as independent protein kinases possessing such molecular weights.

**DISCUSSION**

CaM kinase IV was first demonstrated in mouse brain by cDNA cloning by Sikela and Hahn (6), and the properties of this protein kinase from rat brain have been extensively studied by Sahyoun and his associates (8, 30, 31). The present study was undertaken to characterize this kinase in further detail to understand its physiological significance.

CaM kinase IV purified from rat cerebellum showed two protein bands, a major band (α) and a minor band (β) corresponding to molecular weights of 63,000 and 66,000, respectively, on SDS-polyacrylamide gel electrophoresis (Fig. 3A), in agreement with the results of Omhstede et al. (8). Both α and β showed calmodulin-dependent protein kinase activity (Fig. 3C) and calmodulin-dependent autophosphorylation activity (Fig. 3B), indicating that both were calmodulin-dependent protein kinases. Although the enzyme was eluted from a gel filtration column at the same elution position as γ-globulin possessing a molecular weight of 150,000 (data not shown), the molecular weight of the native enzyme estimated from the sedimentation coefficient, Stokes radius, and partial specific volume was approximately 67,000. These results suggest that CaM kinase IV is a monomeric enzyme. The frictional ratio was calculated to be 1.9 from the Stokes radius and sedimentation coefficient, indicating that CaM kinase IV was not a typical globular protein.

Among several calmodulin-dependent protein kinases so far reported, only CaM kinase II has been demonstrated to be a multifunctional enzyme, like cAMP-dependent protein kinase and protein kinase C (32). When a number of proteins and peptides were tested for their ability to serve as substrate for CaM kinase IV, myelin basic protein, synapsin I, MLC, MAP2, tau protein, histone H1, tyrosine hydroxylase, syntide-2, and peptide-γ were active as substrates (Table III), indicating the broad substrate specificity of CaM kinase IV. Comparison of the substrate specificities of CaM kinase IV and CaM kinase II indicates that the two protein kinases show different substrate specificities but similar low degrees of the specificities (Table III). An autoradiograph illustrating the incorporation of 32P into endogenous cerebellar cytosol proteins by the action of CaM kinase IV (Fig. 4) indicate that CaM kinase IV can phosphorylate a large number of endogenous brain proteins. Sahyoun et al. (30) has very recently reported that a Ras-related GTP-binding protein, Rap-1b, which is enriched in brain, is selectively phosphorylated by CaM kinase IV but not by CaM kinase II. Thus, CaM kinase IV, like CaM kinase II, may be a multifunctional calmodulin-dependent protein kinase which is involved in the regulation of various neuronal functions.

It was fortunate for us to find that peptide-γ was a specific substrate for CaM-kinase IV. The amino acid sequences of this peptide is Lys1-Ser2-Asp3-Gly4-Gly5-Val6-Lys7-Lys8-Arg9-Lys10-Ser11-Ser12-Ser13-Ser14, corresponding to Lys346 to Ser358 of the predicted amino acid sequences of CaM kinase II-γ (19). This peptide had been synthesized for the purpose of obtaining antibody specific for CaM kinase II-γ. The phosphorylation site of peptide-γ by CaM kinase IV was determined to be Ser11 by analysis by an automated protein/peptide sequencer (Applied Biosystems Model 477A) (data not shown).

The discovery of peptide-γ as a specific substrate for CaM kinase IV enabled us to measure the activity of CaM kinase IV in the crude extract. Among the tissues tested for CaM kinase IV activity using peptide-γ as substrate, only brain showed significant activity and the other tissues, including liver, spleen, kidney, lung, heart, skeletal muscle, and adrenal gland, showed very little, if any, activity (Table V), in good agreement with the earlier results obtained by Western blot analysis (8). An overall purification of CaM kinase IV from cerebellum where the enzyme occurred most abundantly was calculated to be about 230-fold, based on the specific activities of the enzyme of the crude extract and the purified preparation which were measured by the use of peptide-γ as substrate, indicating extreme abundance of CaM kinase IV in cerebellum. The activities of cerebral cortex and brain stem were approximately one-half and one-fourth, respectively, that of cerebellum. Thus, CaM kinase IV appears to occur abundantly specifically in the brain. The activity gel method, developed in this laboratory (22), confirming the results obtained by the use of peptide-γ, revealed that CaM kinase IV-α and IV-β...
were present in cerebellum but only CaM kinase IV-α was present in cerebral cortex and brain stem, and further revealed that occurrence of CaM kinase IV was not completely specific in brain, occurring very sparingly but significantly in other tissues as discussed in the text.

Acknowledgment—We are grateful to Professor M. Namiki (Department of Internal Medicine, Asahikawa Medical College) for support throughout this research.

Addendum.—After submission of this paper, Frangakis et al. (33) reported the molecular properties of the purified enzyme, and Ohmstedt et al. (34) reported the cDNA sequence of the enzyme.

REFERENCES


Supplementary Material to Purification and Characterization of a Brain-specific Multifunctional Calcium-dependent Protein Kinase from Rat Cerebellum

Daisuke Miyano, Toru Kameshita, and Hitoshi Fujisawa

EXPERIMENTAL PROCEDURES

Materials—(1-7)-TTP (1,000 Ci/mmol) was purchased from the Radiochemical Center (Amersham). Ultronic acid (14) was obtained from Sigma. Phosphocellulose 40 and Bio-Gel P-2 were obtained from Bio-Rad Laboratories. Calmodulin and free calcium were obtained from the Sigma Chemical Company. [3H]Phosphocellulose 100,000 (1,000 Ci/mmol) was obtained from Amersham. Dextran blue (1,000,000 mol. wt) was obtained from Pharmacia. Dextran (200,000 mol. wt) was obtained from M. Mallinckrodt. 

Methods—The cDNA sequence of the enzyme was determined by the method of Maniatis (21) with ead 9.7 x 6.6 x 5.1 cm) consisting of 5% (or 10%) acrylamide separation gel and 1% stacking gel.

Activity Determination—Activity gel was carried out essentially as described previously (22), approximately 5 mg/ml each 0.2% NADH, 50 mM Tris-HCl, 50 mM NaCl, and 20% glycerol was added as substrate into the gel. The protein concentration was determined by the method of Bradford (23).

Sucrose Density Gradient Centrifugation—Samples (100 μl) were applied onto a 30% N of a 5% 0.5% linear sucrose density gradient in 45 μl Tris-HE buffer (pH 7.4) containing 1% (v/v) ethylene glycol, 0.5 M NaCl, 1 mM EDTA, and 0.5 mM DTT. Temperature was cooled to 4°C at 47% in 7°C. The concentration coefficient of CaM-kinase IV was determined by the method of Ohmstede et al. (31). The control was purified from rat testes as described previously (31).

The standard buffer consisting of 45 μM Tris-HE (pH 7.4), 1 mM MgCl₂, 50 μM ethylene glycol and 100 μM Tween 40, was used in the purification or other procedures. The buffer was prepared at 2°C.

Assay of Enzyme Activity—CaM-kinase activity was assayed by phosphorylation of substrates to measure enzyme activity. The standard assay mixture contained CaM-kinase IV-α, 30 μCi ATP (1,000 Ci/mmol), 0.1 μg/ml phosphocellulose 100, 20 μM EGTA and 0.5 μM CaCl₂. The total reaction volume (μl) was assumed to be the elution volume of the protein. 200 μl of protein solution was used.

The standard reaction of CaM-kinase IV was determined according to the method of Lauren and Lindberg (24) as described by Krugt and Monty (25).
Preparation of tissue extracts—Rat cerebellum, cerebral cortex, brain stem, liver, spleen, and lung were homogenized by a Teflon-plastic homogenizer and kept at 0°C. The white matter was removed, and the homogenate was then centrifuged at 20,000 g for 20 min. The supernatant was collected and 10 volumes of 50 mM Tris-Cl (pH 7.4) containing 0.5 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml chymostatin, and 10 μg/ml aprolaprin were added. The homogenate was then centrifuged at 100,000 g for 1 h. The resultant crude extracts were used for the assay of the activity of Calmodulin-dependent Protein Kinase IV.

Other procedures—Calmodulin-substituted Sepharose 4B was prepared by coupling calmodulin to CNBr-activated Sepharose 4B according to the procedure described in the Pharmacia manual. The extent of coupling was calculated to be approximately 10 mg of calmodulin per 0.5 ml of Sepharose 4B. The fractions containing Calmodulin-dependent Protein Kinase IV were determined spectrophotometrically, using 5, 7, 4, 277 in 280 and 390 nm, respectively.

RESULTS

Purification

All purification procedures were carried out at 0°C. DEAE-Toyopearl chromatography—The crude extract was applied to a DEAE-Toyopearl 650M column (3.2 × 20 cm) and eluted with a linear NaCl gradient from 0 to 0.5 M NaCl. Fractions containing the enzyme were pooled and dialyzed against 0.5 M Tris-Cl (pH 7.4) and used for the assay of the activity of protein phosphatase 2A.

Calmodulin-Sepharose 4B Affinity Chromatography—The crude extract was applied to a CaM-Sepharose 4B column (4.4 × 25 cm) and eluted with the standard buffer containing 50 mM Tris-Cl and 5 mM CaCl2. Fractions containing the enzyme were pooled and used for the assay of the activity of protein phosphatase 2A.

Collagenase digestion—The crude extract was digested with 1 mg/ml collagenase at 20°C for 1 h. The enzyme activity was measured as described above.

Fig. 1. DEAE-Toyopearl column chromatography in the purification of Calmodulin-dependent Protein Kinase IV. Fraction A was collected using sodium acetate 200 mM (pH 5.0), and fraction B was collected using sodium acetate 300 mM (pH 5.0). The activity was expressed as a percentage of the activity of the crude extract.

Fig. 2. Analysis of Calmodulin-dependent Protein Kinase IV from DEAE-Toyopearl fractions by activity gel method. Two-micrometer tips of the enzyme were applied to the gel according to the procedure described in the Pharmacia manual. The enzyme activity was measured as described above.

Table 1

<table>
<thead>
<tr>
<th>Step</th>
<th>Activity (units/mg)</th>
<th>Specific Purification Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>4</td>
<td>2,500</td>
<td>250</td>
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</table>

The enzyme activity was determined using glycogen as substrate. The specificity of the enzyme was determined using a specific substrate for the enzyme. The enzyme activity was measured as described above.