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Purification and Characterization of Ca^{2+}/Calmodulin-Dependent Protein Kinase Kinase β from Rat Cerebellum[1]

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The existence of isoforms of calmodulin-dependent protein kinase kinase (CaM-kinase kinase) in the rat brain was recently suggested by Northern and Western blot analyses and immunotitration [Okuno, S., Kitani, T., and Fujisawa, H. (1996) *J. Biochem.* 119, 1176-1181]. In the present study, CaM-kinase kinase β, distinct from CaM-kinase kinase α which had been purified and cloned from rat cerebral cortex, was purified approximately 5,000-fold from rat cerebellum and its properties were examined. The purified CaM-kinase kinase β gave a doublet at positions corresponding to molecular weights of 66,000 to 67,000 on SDS-PAGE, and neither protein band reacted with antibody against CaM-kinase kinase α. Both CaM-kinase kinase α and β markedly activated both CaM-kinase I and IV, but CaM-kinase kinase β activated CaM-kinase IV more strongly than did CaM-kinase kinase α. The maximal extents of the activation of CaM-kinase I and IV by CaM-kinase kinase β were almost the same as those by CaM-kinase kinase α, suggesting that the two CaM-kinase kinases activated CaM-kinase I and IV by the same mechanisms.

Key words: brain, Ca^{2+}/calmodulin, CaM-kinase kinase, isoform, protein kinase kinase.

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The calcium ion is known to play important roles in the regulation of a variety of neuronal functions, and most of the diverse actions of Ca^{2+} may be mediated through protein phosphorylation by three multifunctional calmodulin-dependent protein kinases, calmodulin-dependent protein kinase (CaM-kinase) I, II, and IV (see Refs. *1-4* for reviews). These three protein kinases exist relatively widely and abundantly in the central nervous system but are distributed in different regions inside a neuronal cell: CaM-kinase I exists in cytosol, CaM-kinase II exists predominantly in cell membranes such as postsynaptic density and cytosol, and CaM-kinase IV exists in cell nuclei. Each plays different roles in Ca^{2+}-signaling in the brain. In contrast to CaM-kinase II, which is activated through phosphorylation by CaM-kinase II itself (autophosphorylation), CaM-kinase IV and I were reported to be activated through phosphorylation by other Ca^{2+}/calmodulin-dependent protein kinases, CaM-kinase IV kinase, and CaM-kinase I kinase, respectively (*5-8*), and the cDNA for CaM-kinase IV kinase purified from rat cerebral cortex was cloned and sequenced independently in this and other laboratories (*9, 10*). On the other hand, Northern blot, Western blot, and immunotitration analyses suggested that most of the CaM-kinase IV kinase activity of rat cerebellum derived not from the cloned enzyme (CaM-kinase kinase α) but from another CaM-kinase kinase (*10*). In the present study, a new CaM-kinase kinase (CaM-kinase kinase β) was purified from rat cerebellum and its properties were examined.

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Abbreviations: CaM-kinase, calmodulin-dependent protein kinase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TPCK-trypsin, tosylphenylalanyl chloromethyl ketone-treated trypsin.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP (5,000 Ci/mmol) was from Amersham International. Microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin) were from the Peptide Institute (Osaka). DEAE-cellulose (DE52), phosphocellulose paper (P81), and 3MM paper were from Whatman. Fluorotrans, a polyvinylidene difluoride (PVDF) membrane was from Pall BioSupport. Sephacryl S-300 HR, blue-Sepharose CL-6B, and Protein A Sepharose 4 Fast Flow were from Pharmacia-LKB Biotechnology. Peroxidase-conjugated goat anti-rabbit immunoglobulin was from Organon Teknika. TPCK-trypsin was from Worthington Biochemical. Kemptide (LRRASLG) was from Sigma. Syntide-2 (PLARTLSVAGLPGKK) (*11*) was synthesized by the American Peptide Company. Peptide-γ (KSDGGVKKRKSSSS) (*12*) was synthesized by use of a Shimadzu PSSM-8 automated peptide synthesizer. All other reagents were of the highest grade commercially available. Wistar rats were purchased from the Shizuoka Laboratory Animal Center.

Protein Preparations—Calmodulin was purified from *Escherichia coli* transformed with expression vector pET11d carrying a cDNA encoding chicken brain calmodulin (*13*), essentially as described by Gopalakrishna and Anderson (*14*). The cDNA encoding chicken brain calmodulin was kindly donated by A.R. Means (*15*). Recombinant rat brain CaM-kinase IV expressed in Sf9 cells was purified

as described previously (13). A cDNA clone of CaM-kinase I was obtained from a rat cerebral cDNA library using an oligonucleotide corresponding to nucleotides 92-141 of the reported sequence (16) as a probe, and a cDNA fragment containing the entire coding sequence was introduced into a vaculovirus, AcNPV, using the Bac-To-Bac Baculovirus Expression System (Life Technologies). Sf9 cells infected with the recombinant baculovirus were grown as described previously (13), and the harvested cells (0.47 g) were suspended in 5 ml of 20 mM Tris-HCl buffer (pH 7.5 at 4°C) containing 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of microbial protease inhibitors (leupeptin, pepstatin A, antipain, and chymostatin) and disrupted by sonic oscillation. The supernatant obtained on centrifugation was applied to a column of DE52 (5 ml) equilibrated with 40 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM EGTA, 0.5 mM EDTA, 1 mM dithiothreitol, 10% ethylene glycol, and 0.05% Tween 40. The column was washed extensively with the equilibration buffer and the enzyme was eluted with the buffer containing 75 mM NaCl. To the eluate was added solid ammonium sulfate to a final saturation of 60%, and the resulting precipitate, collected by centrifugation, was dissolved in 10 ml of 40 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol, 10% ethylene glycol, 0.05% Tween 40, 10 μg/ml each of the microbial protease inhibitors, and 1.5 mM $CaCl_2$ and applied to a column of calmodulin-Sepharose (2 ml), prepared as described previously (6), equilibrated with the same buffer. The column was washed extensively with 40 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol, 10% ethylene glycol, 0.05% Tween 40, 0.5 mM $CaCl_2$, and 1 M NaCl, and then with the same buffer with NaCl omitted, and the enzyme was eluted with 40 mM Tris-HCl buffer (pH 7.5) containing 1 mM EGTA, 1 mM dithiothreitol, 10% ethylene glycol, 0.05% Tween 40. Approximately 1.4 mg of the purified CaM-kinase I, which gave a single protein band corresponding to an apparent molecular weight of 40,000 on SDS-PAGE, was obtained. Rat brain CaM-kinase kinase α (CaM-kinase IV kinase) was purified as described previously (6). Antibody against CaM-kinase kinase α was prepared and purified by affinity chromatography on immobilized antigen as described previously (10). Antibody-Sepharose used for purification of CaM-kinase kinase β was prepared by linking antibody against CaM-kinase kinase α to Protein A-Sepharose 4 Fast Flow (Pharmacia-LKB). The purified antibody (5 mg) was incubated with 100 μl of the Protein A-Sepharose at 4°C overnight with shaking, and the Sepharose was washed extensively with buffered saline containing 2 M NaCl.

Assay of CaM-Kinase Kinase—For purification of CaM-kinase kinase β, the activity of CaM-kinase kinase β was assayed by measuring the CaM-kinase IV activity generated by incubation with recombinant CaM-kinase IV expressed in *E. coli* under Ca^{2+}/calmodulin-dependent protein phosphorylation conditions, as described previously (6). The CaM-kinase IV activity was determined with peptide-γ as a substrate by the phosphocellulose paper method of Roskoski (17).

Activation of CaM-kinase I and IV by CaM-kinase kinase α or β were carried out at 30°C in a reaction mixture containing 50 mM MOPS-NaOH (pH 7.0 at 30°C), 5 mM magnesium acetate, 0.1 mM ATP, 0.1 mM EGTA, 0.2 mM $CaCl_2$, 1 μM calmodulin, 2 mM dithiothreitol, the indicated amounts of CaM-kinase I or IV, and suitable amounts of CaM-kinase kinase. After incubation for the indicated times, aliquots were withdrawn and the CaM-kinase I or IV activity was determined in a final volume of 50 μl of a reaction mixture containing 50 mM MOPS-NaOH (pH 7.0), 5 mM magnesium acetate, 0.1 mM [γ-^{32}P]ATP (175 cpm/pmol), 0.1 mM EGTA, 0.2 mM $CaCl_2$, 1 μM calmodulin, 2 mM dithiothreitol, and 40 μM syntide-2 for 1 min at 30°C by the phosphocellulose paper method (17).

Other Analytical Procedures—SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (18). Immunoblotting analysis was performed essentially as described by Winston *et al.* (19). *In situ* autophosphorylation of CaM-kinase kinase α and β in a gel was carried out as described previously (20). The protein concentrations of various fractions obtained in the course of purification of CaM-kinase kinase β were determined spectrophotometrically from the absorbance at 280 and 260 nm (21). The concentration of calmodulin was determined spectrophotometrically using an absorption coefficient, A_{280} (1 mg/ml), of 0.21 (22) and the molecular weight of 16,700 (23, 24). Other proteins were determined by the method of Lowry *et al.* (25), as modified by Peterson (26) with bovine serum albumin as a standard.

Purification of CaM-Kinase Kinase β—All purification procedures were carried out at 4°C and centrifugations were done at 27,000 × g for 20 min unless otherwise stated.

Step 1. Extraction: Frozen (−85°C) rat cerebellums (206 g) were homogenized with a Teflon/glass homogenizer in 3 volumes of 10 mM Tris-HCl buffer (pH 7.5 at 4°C) containing 0.1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, and 10 μg/ml each of the microbial protease inhibitors, and the homogenate was centrifuged at 100,000 × g for 1 h.

Step 2. DE52 chromatography: The 100,000 × g supernatant was applied to a column of DE52 (280 ml) equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, and 10% glycerol. The column was washed with 3 bed volumes of the equilibration buffer, then with the equilibration buffer containing 0.05 M NaCl until the A_{280} of the eluate became negligible (about 12 bed volumes). The enzyme was then eluted with 8 volumes of the equilibration buffer containing 0.15 M NaCl.

Step 3. Calmodulin-Sepharose affinity chromatography: To the eluate was added solid ammonium sulfate to a final saturation of 60%. The resulting precipitate, collected by centrifugation, was dissolved in 100 ml of 10 mM Hepes-NaOH buffer (pH 7.2 at 4°C) containing 0.1 mM dithiothreitol, 10% glycerol, 0.05% Tween 80, 1 mM $CaCl_2$, and 10 μg/ml each of the microbial protease inhibitors, and applied to a column of calmodulin-Sepharose (20 ml), prepared as described previously (6), equilibrated with the same buffer as described above. The column was washed sequentially with 10 mM Hepes-NaOH buffer (pH 7.2) containing 0.1 mM dithiothreitol, 10% glycerol, 0.05% Tween 80, 0.2 mM $CaCl_2$, and 2 M NaCl until the A_{280} of the eluate became negligible (about 20 bed volumes), then with 5 bed volumes of the same buffer without NaCl, finally with 7 bed volumes of the same buffer without $CaCl_2$ containing 1 mM EGTA and 20 mM NaCl. The enzyme was then eluted with the same buffer containing 0.2 M NaCl.

Step 4. Sephacryl S-300 gel filtration: To the eluate (28 ml) was added solid ammonium sulfate to a final saturation

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of 60%. The resulting precipitate, collected by centrifugation, was dissolved in a minimum volume of the standard buffer consisting of 10 mM Hepes-NaOH (pH 7.2), 0.1 mM dithiothreitol, 10% glycerol, 0.05% Tween 80, and 0.1 mM EDTA, and dialyzed against the same buffer. The dialyzate was applied to a column (1.6×95 cm, 190 ml) of Sephacryl S-300 HR equilibrated with the same buffer. The column was eluted with the same buffer in fractions of 2 ml and the active fractions were pooled.

Step 5. Blue-Sepharose chromatography: The pooled active solution was applied to a column of blue-Sepharose (0.3 ml) equilibrated with the standard buffer. The column was washed with 30 bed volumes of the standard buffer, then with 20 volumes of the standard buffer containing 0.3 M NaCl, and the enzyme was eluted with 8 volumes of the standard buffer containing 2 M NaCl.

Step 6. Removal of CaM-kinase kinase α by antibody-Sepharose: The eluate was applied to a column of the antibody-Sepharose (100 μl), prepared as described above, to remove CaM-kinase kinase α. To the pass-through fraction was added 40 μl of Protein A Sepharose 4 Fast Flow (Pharmacia-LKB) to remove the antibody released from the antibody-Sepharose. After incubation for 1 h at 4°C with shaking, the mixture was centrifuged to remove the Sepharose.

Step 7. Concentration of the purified enzyme by calmodulin-Sepharose: To the supernatant was added $CaCl_2$ to a final concentration of 1 mM, and the solution was applied to a column of the calmodulin-Sepharose (150 μl). The column was washed with 10 bed volumes of the standard buffer containing 0.2 mM $CaCl_2$ and 2 M NaCl, then with 5 volumes of the same buffer without NaCl, and the enzyme was eluted with 6 volumes of the same buffer containing 1 mM EGTA and 0.2 M NaCl.

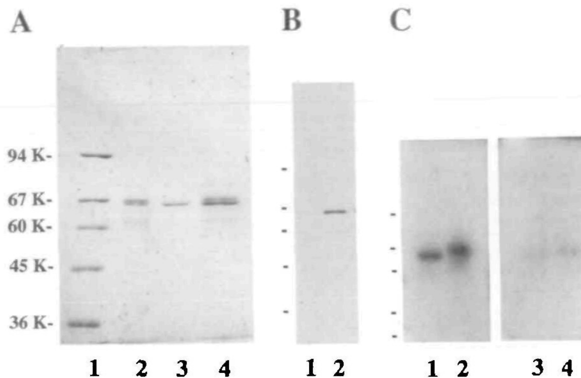

Fig. 1. **Analysis of purified CaM-kinase kinase β on SDS-PAGE.** (A) Approximately 0.4 μg of CaM-kinase kinase β purified from rat cerebellum (lane 2), 0.4 μg of CaM-kinase kinase α (lane 3), both (lane 4), and molecular weight standards (lane 1) were subjected to SDS-PAGE on 7.5% acrylamide gel, and stained with Coomassie Brilliant Blue R-250. The molecular weight standards used were phosphorylase *b* (M_r 94,000), bovine serum albumin (M_r 67,000), catalase (M_r 60,000), ovalbumin (M_r 45,000), and lactate dehydrogenase (M_r 36,000). (B) Approximately 0.16 μg of CaM-kinase kinase β purified from rat cerebellum (lane 1) and 0.01 μg of CaM-kinase kinase α purified from rat cerebral cortex (lane 2) were subjected to SDS-PAGE, then separated proteins were transferred onto a PVDF membrane (Fluorotrans, Pall BioSupport). The membrane was blocked with 5% nonfat milk in phosphate-buffered saline for 30 min at 24°C, then incubated with 0.358 μg/ml antibody to CaM-kinase kinase α in the blocking buffer for 2 h, followed by incubation with 30 μg/ml goat anti-rabbit immunoglobulins (IgA+IgG+IgM) conjugated with peroxidase (Organon Teknika) at 4°C overnight. The positive bands were detected with diaminobenzidine tetrahydrochloride and H_2O_2 in the presence $CoCl_2$. (C) Approximately 0.5 μg each of CaM-kinase kinase β purified from rat cerebellum (lanes 2 and 4) and CaM-kinase kinase α (lanes 1 and 3) was subjected to SDS-PAGE. After electrophoresis, proteins were renatured *in situ* by successive treatments with 20% 2-propanol, 6 M guanidinium hydrochloride, and 0.04% Tween 40, as described previously (*20*). After the gels had been preincubated at 25°C for 30 min with 50 mM MOPS-NaOH (pH 7.0), 5 mM magnesium acetate, 0.1 mM EGTA, and 2 mM dithiothreitol, the autophosphorylation was allowed to proceed by incubation of the gel at 25°C for 30 min with 10 μM [γ-^{32}P]ATP (1.1×10^4 cpm/pmol) in the preincubation mixture in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of 0.2 mM $CaCl_2$ and 1 μM calmodulin. Autophosphorylation of the CaM-kinase kinases in the gel was detected as described previously (*20*).

RESULTS

Table I summarizes a typical purification procedure of CaM-kinase kinase β. Approximately 37 μg of the purified enzyme was obtained from 800 rat cerebellums (about 206 g). The apparent overall purification was about 5,000-fold. The specific activity of CaM-kinase kinase β toward CaM-kinase IV was 2- to 3-fold higher than that of CaM-kinase kinase α (*6*) under our assay conditions. The final purified preparation gave a doublet at positions corresponding to

TABLE II. **Activities of CaM-kinase kinase α and β toward CaM-kinase IV and I.** The activities of CaM-kinase kinase α and β toward CaM-kinase IV and I, respectively, were determined using purified recombinant CaM-kinase IV or I expressed in Sf9 cells at a concentration of 15 μg/ml, under the conditions in which the activities were almost linear with incubation time and the amounts of the CaM-kinase kinases, as described under "EXPERIMENTAL PROCEDURES."

Substrate \ Enzyme	CaM-KKβ (nmol/min/mg)	CaM-KKα (nmol/min/mg)	β/α ratio
CaM-kinase IV	0.57×10^6	0.26×10^6	2.2
CaM-kinase I	3.32×10^6	4.01×10^6	0.83

TABLE I. **Purification of CaM-kinase kinase β from cerebellums (206 g) of 800 rats.**

Purification step	Protein (mg)	Activity [nmol/min (%)]	Specific activity [nmol/ min / mg (fold)]
1. Crude extract	5,707[a]	79,019 (100)	13.8 (1)
2. DE52	1,363	50,892 (64)	37.3 (3)
3. Calmodulin-Seph	3.467	26,611 (34)	7,676 (556)
4. Sephacryl S-300	0.814	14,720 (19)	18,092 (1,311)
5. Blue-Seph	0.120	8,188 (10)	68,120 (4,936)
6. Antibody-Seph	–	3,794 (5)	
7. Calmodulin-Seph	0.037	2,327 (3)	62,386 (4,521)

[a]Determined by the method of Lowry *et al.* (*25*).

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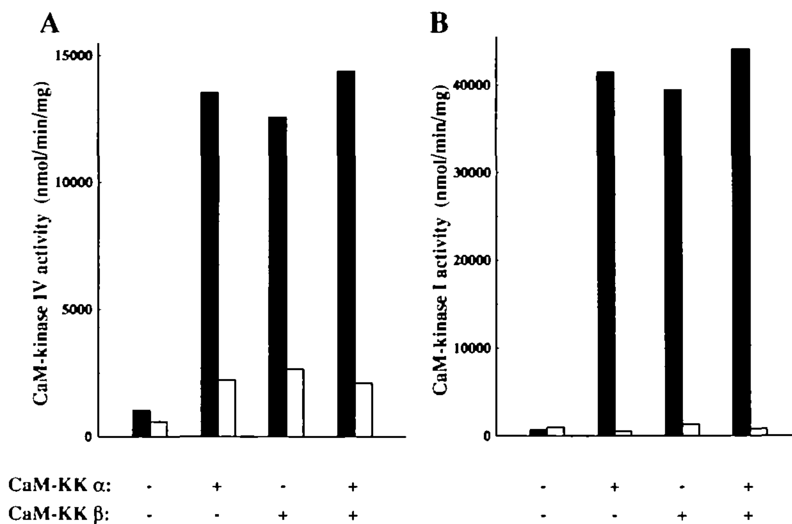

Fig. 2. **Activation of CaM-kinase IV and I by CaM-kinase kinase α and β.** Approximately 15 μg/ml of purified recombinant CaM-kinase IV (A) and I (B) expressed in Sf9 cells were maximally activated by incubation with 0.4 μg/ml of CaM-kinase kinase α, β, or both at 30°C for 10 min, and the activities of CaM-kinase IV (A) and I (B) were determined with syntide-2 as a substrate, in the presence (■) and absence (□) of $CaCl_2$, as described under "EXPERIMENTAL PROCEDURES."

molecular weights of 66,000 to 67,000 on SDS-PAGE, as shown in Fig. 1A. When the final purified preparation was subjected to Western blot analysis with antibody against CaM-kinase kinase α, as much as 157 ng of CaM-kinase kinase β showed no significant band, although 10 ng of CaM-kinase kinase α showed a clear band, as shown in Fig. 1B, indicating that the purified preparation of CaM-kinase kinase β contained no significant amount of CaM-kinase kinase α. The enzyme preparation from step 5 of the purification procedure gave a similar doublet upon SDS-PAGE to that of the final preparation, but Western blot analysis showed that approximately 1 to 2% of the protein of the preparation from step 5 was CaM-kinase kinase α (data not shown). Thus, steps 6 and 7 are necessary to remove a trace amount of CaM-kinase kinase α from the enzyme preparation.

To ascertain whether both bands of the doublet on SDS-PAGE were a protein kinase, the purified preparation was subjected to in-gel autophosphorylation analysis (*20*), as shown in Fig. 1C, since most protein kinases possess autophosphorylation activity. Ca^{2+}/calmodulin-dependent autophosphorylation activity was observed at a position corresponding to the purified protein on SDS-PAGE, but the autoradiography could not distinguish the two bands of the doublet. When approximately 15 μg of purified CaM-kinase kinase β, which was calculated to be about 224 pmol of the enzyme from a molecular weight of 67,000 estimated by SDS-PAGE (Fig. 1A), was digested with TPCK-trypsin, the digested peptides were separated on a C_{18} reverse-phase HPLC column (TSK gel ODS-80Ts, Tosoh) with a 0-100% acetonitrile gradient in 0.06% trifluoroacetic acid, and the amino acid sequence of a purified peptide was determined with a Hewlett Packard G 1005A peptide sequenator, the sequence (in which the numbers in subscript indicate the picomole yield) of $S_{13}F_{80}G_{63}N_{63}P_{39}F_{55}$-$E_{59}G_{47}S_{9.4}R_{34}$ was obtained. The recovery of this peptide, taken together with the initial yield of 51% determined with human serum albumin in the sequenator, as described in the application note from Hewlett Packard, the repetitive yield of about 95% calculated from this analysis, and also the recovery upon the experimental procedures such as the proteolytic digestion and HPLC, indicate that almost all the protein in our purified preparation may be an identical protein, suggesting that both bands of the doublet on SDS-PAGE may be derived from CaM-kinase kinase β.

Comparison of the Activities of CaM-Kinase Kinase α and β—Table II shows a comparison of the activation activities of CaM-kinase kinase β toward CaM-kinase IV and I with those of CaM-kinase kinase α. The activities were determined by measuring the activation of substrate amounts of CaM-kinase IV and I by catalytic amounts of the CaM-kinase kinases, under the conditions in which the activities were almost linear with incubation time and the amounts of the CaM-kinase kinases. Under the experimental conditions, both CaM-kinase kinases activated CaM-kinase I strongly and to almost similar extents. They also activated CaM-kinase IV, though less strongly than they did CaM-kinase I, and CaM-kinase kinase β activated CaM-kinase IV more strongly than did CaM-kinase kinase α.

When CaM-kinase IV and I were almost maximally activated by incubation with excess amounts of CaM-kinase kinase α, β, or both, the enzymes showed similar activities, as shown in Fig. 2. These results suggest that CaM-kinase IV and I were activated through the same mechanism by CaM-kinase kinase α and β. Both the Ca^{2+}/calmodulin-dependent and independent activities of CaM-kinase IV were markedly activated by incubation with CaM-kinase kinases, in agreement with our earlier observations (*6, 27*). In contrast to CaM-kinase IV, the Ca^{2+}/calmodulin-dependent activity of CaM-kinase I was markedly activated by CaM-kinase kinases but the Ca^{2+}/calmodulin-independent activity was not significantly stimulated, in close agreement with the previous observation (*8*) that the Ca^{2+}/calmodulin-dependent activity of CaM-kinase Ia is markedly activated by CaM-kinase Ia activator but the Ca^{2+}/calmodulin-independent activity is not activated.

DISCUSSION

Since our previous reports (*10, 28*) had suggested that an isoform of CaM-kinase kinase (CaM-kinase kinase β) immunologically distinct from the enzyme (CaM-kinase kinase α) cloned from rat cerebral cortex (*9, 10*) occurs in the rat brain, especially abundantly in the cerebellum, we

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tried to purify the β isoform from rat cerebellum in the present study. The purified enzyme gave a doublet of molecular weights of 66,000 to 67,000 on SDS-PAGE (Fig. 1A), but the high recovery of the trypsin-digested peptide of the purified enzyme in the sequence analysis indicated that both protein bands of the doublet were CaM-kinase kinase β. Since the in-gel autophosphorylation analysis (Fig. 1C) indicates that CaM-kinase kinase β possesses Ca^{2+}/calmodulin-dependent autophosphorylation activity, unless there is a contaminating protein kinase comigrating with CaM-kinase kinase β on SDS-PAGE, and a number of protein kinases, such as cAMP-dependent protein kinase (*29*), CaM-kinase II (*30-32*), and CaM-kinase IV (*33*), have been shown to undergo changes in their mobilities on SDS-PAGE upon autophosphorylation, it is conceivable that the doublet observed on SDS-PAGE (Fig. 1A) results from autophosphorylation of CaM-kinase kinase β.

The amino acid sequence of the trypsin-digested peptide of the purified CaM-kinase kinase β, SFGNPFEGSR, was very similar but not identical to the sequence of amino acids 458-468 of CaM-kinase kinase α (*9, 10*), SFGNPFEPQAR (the common amino acids are underlined), suggesting that CaM-kinase kinases α and β might be encoded by related but different genes. A similar amino acid sequence has recently been reported in CaM-kinase I kinase (*34*). CaM-kinase kinases α (*5, 6*) and β (*10*) were discovered by activation of inactive recombinant CaM-kinase IV expressed in *E. coli*, but both can activate CaM-kinase I as well as CaM-kinase IV, activating the former more efficiently as shown in Table II. On the basis of the results that the specific activity of the maximally activated CaM-kinase I was about 3.1-fold higher than that of the CaM-kinase IV (Fig. 2) and that the ratio of activation activities of CaM-kinase kinase α toward CaM-kinase I and IV and that of CaM-kinase kinase β were about 15.4 ($\approx 4.01 \times 10^6/0.25 \times 10^6$) and 5.8 ($\approx 3.32 \times 10^6/0.57 \times 10^6$), respectively (Table II), the activities of CaM-kinase kinases α and β toward CaM-kinase I can be estimated to be approximately 5-fold ($\approx 15.4/3.1$) and 2-fold ($\approx 5.8/3.1$), respectively, higher than those toward CaM-kinase IV. Thus, both CaM-kinase kinases α and β appear to prefer CaM-kinase I to CaM-kinase IV as substrate. On the other hand, CaM-kinase kinase α does not significantly exist in the liver and intestine (*10*) where CaM-kinase I occurs abundantly (*35, 36*), and predominantly exists in cellular nuclei of neurons (*28*) where CaM-kinase IV occurs (*37-39*) but CaM-kinase I does not occur (*35, 36*), suggesting that CaM-kinase kinase α may be involved in the activation of not CaM-kinase I but CaM-kinase IV. The facts that CaM-kinase kinase β appears to occur most abundantly in the cerebellum (*10*) where CaM-kinase IV exists abundantly (*39, 40*), and that the activity of CaM-kinase IV kinase including CaM-kinase kinase β cannot be significantly detected in the liver (*5*) where CaM-kinase I occurs abundantly (*35, 36*) suggest that CaM-kinase kinase β may also be a kinase involved in the activation of not CaM-kinase I but CaM-kinase IV.

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