J. Biochem. 122, 337-343 (1997)

Studies on the Substrate Specificity of Ca^{2+}/Calmodulin-Dependent Protein Kinase Kinase α[1]

Sachiko Okuno, Takako Kitani,[2] and Hitoshi Fujisawa[3]

Department of Biochemistry, Asahikawa Medical College, Asahikawa 078

Received for publication, March 3, 1997

Ca^{2+}/calmodulin-dependent protein kinase (CaM-kinase) kinase α, which is known to activate CaM-kinases IV and I by phosphorylation of Thr^{196} and Thr^{177}, respectively, can only phosphorylate Thr^{196} among many phosphorylation sites of CaM-kinase IV [Kitani, T., Okuno, S., and Fujisawa, H. (1997) *J. Biochem.* 121, 804-810], indicating its high degree of substrate specificity. In the present study, the substrate specificity of CaM-kinase kinase α was examined using various proteins and synthetic peptides as substrates as a means to address its physiological function. Among a number of proteins and synthetic peptides, including several known as good substrates for various protein kinases, only CaM-kinases IV and I and peptides containing the sequence surrounding Thr^{196} of CaM-kinase IV or Thr^{177} of CaM-kinase I were significantly phosphorylated by CaM-kinase kinase α, while the heat-denatured (at 60°C for 5 min) CaM-kinases IV and I were not phosphorylated. Peptides containing the phosphorylation site of CaM-kinase IV or I were far less active as substrates for CaM-kinase kinase α than were native CaM-kinase IV or I. Thus, CaM-kinase kinase α showed a high degree of substrate specificity, recognizing not only specific amino acid sequences but also the native conformation of CaM-kinases IV and I.

Key words: Ca^{2+}/calmodulin, CaM-kinase I, CaM-kinase IV, CaM-kinase kinase, substrate specificity.

Ca^{2+}/calmodulin-dependent protein kinases (CaM-kinases) I, II, and IV are known to be Ca^{2+}-responsive multifunctional protein kinases (see Refs. *1-4* for reviews) which are involved in the regulation of a variety of cellular functions in response to an increase in intracellular Ca^{2+}. Among the three multifunctional CaM-kinases, CaM-kinases IV (*5-7*) and I (*8, 9*) are markedly activated through phosphorylation by upstream Ca^{2+}/calmodulin-dependent protein kinases such as CaM-kinase kinases α (*10, 11*) and β (*11-13*), whereas CaM-kinase II is activated through phosphorylation by itself, that is, autophosphorylation, at Thr^{286} (*14-17*). While CaM-kinase IV possesses many phosphorylation sites (*18, 19*), only Thr^{196} among them can be phosphorylated by the action of CaM-kinase kinase α (*20*), suggesting a strict substrate specificity of CaM-kinase kinase α. On the other hand, CaM-kinase I is activated through phosphorylation at Thr^{177} by a CaM-kinase kinase (*21, 22*). Thus, CaM-kinase kinases appear to possess strict substrate specificities but to be able to phosphorylate both Thr^{196} of CaM-kinase IV and Thr^{177} of CaM-kinase I, which are located in equivalent positions (*23*). The present study was undertaken to examine the substrate specificity of CaM-kinase kinase α, focusing on the phosphorylation sites of CaM-kinases IV and I, to address the physiological functions of CaM-kinase kinases.

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). Phosphocellulose paper (P81) and 3MM paper were from Whatman. All other reagents were of the highest grade commercially available. Wistar rats were purchased from the Shizuoka Laboratory Animal Center.

Preparations of Peptide Substrates—Peptide-γ (KSDGGVKKRKSSSS) (*24*), synapsin I-site 1 peptide (NYLRRRLSDSNF) (*25, 26*), and the following peptide substrates containing the sequence surrounding Thr^{196} of CaM-kinase IV (or Thr^{177} of CaM-kinase I) and four lysyl residues at the amino terminus for binding to phosphocellulose paper in the phosphocellulose protein kinase assay (*27*) were synthesized with a Shimadzu PSSM-8 automated peptide synthesizer. PKIV peptide (KKKKEHQVLMKTVCGTPGY) contains amino acid residues 189-203 of CaM-kinase IV (*28, 29*), and PKIV[T_{200}A] peptide (KKKKEHQVLMKTVCGAPGY) has the same sequence, except for replacement of Thr^{200} with nonphosphorylatable alanine (the substitution site of the oligopeptide is underlined). Short PKIV[T_{200}A] peptide (KKKKLMKTVCGAPGY) contains amino acid residues 193-203 of CaM-kinase IV, but Thr^{200}

[1] This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, the Byotai Taisha Research Foundation, the Uehara Memorial Foundation, the Brain Science Foundation, the Smoking Research Foundation, and the Mitsubishi Foundation.

[2] Affiliated with the Laboratory for Radioactive Isotope Research, Asahikawa Medical College.

[3] To whom correspondence should be addressed.

Abbreviations: CaM-kinase, calmodulin-dependent protein kinase; Mops, 3-(*N*-morpholino)propanesulfonic acid; MAP2, microtubule-associated protein 2.

is replaced with alanine. PKI peptide (KKKKDPGSVLSTACGTPGY) contains amino acid residues 170-184 of CaM-kinase I (*30, 31*), and PKI[$S_{173}A/S_{176}A/T_{181}A$] peptide (KKKKDPGAVLATACGAPGY) has the same sequence, except that Ser[173], Ser[176], and Thr[181] are replaced with alanine residues. Short PKI[$S_{176}A/T_{181}A$] peptide (KKKKVLATACGAPGY) contains amino acid residues 174-184 of CaM-kinase I, but Ser[176] and Thr[181] are replaced with alanine residues. The identities of the synthesized peptides were confirmed by fast atom bombardment-mass spectrometry. Purities were estimated by reverse-phase HPLC on a C_{18} column (TSK gel ODS-80T_M, Tosoh).

Kemptide (LRRASLG) (*32*) was from Sigma. Syntide-2 (PLARTLSVAGLPGKK) (*33*) and autocamtide-2 (KKALRRQETVDAL) (*34*) were synthesized by the American Peptide.

Protein Preparations—Calmodulin was purified from *Escherichia coli* cells transformed with expression vector pET11d carrying a cDNA encoding chicken calmodulin (*35*), essentially as described by Gopalakrishna and Anderson (*36*). The cDNA encoding chicken calmodulin was kindly donated by A.R. Means (*37*). Recombinant rat CaM-kinase kinase α expressed in *E. coli* (*11*) was purified as described previously (*20*). Recombinant rat CaM-kinase IV expressed in Sf9 cells was purified as described previously (*35*). Recombinant rat CaM-kinase IV($K_{71}R$), in which Lys[71] (ATP-binding site) was replaced with arginine, expressed in Sf9 cells was purified as described previously (*20*). Recombinant rat CaM-kinase I expressed in Sf9 cells was purified as described previously (*13*). Synapsin I was purified from bovine cerebral cortex as described by Bennett *et al.* (*38*). Histone H1 was purified from calf thymus (*39, 40*). Microtubule-associated protein 2 (MAP2) was purified from porcine brain microtubule proteins as described by Kim *et al.* (*41*). The porcine brain microtubule proteins were purified by one cycle of assembly according to a modification of the method of Shelanski *et al.* (*42*) as described by Karr *et al.* (*43*). Myosin light chain was prepared from chicken gizzard myosin by the method of Perrie and Perry (*44*). The chicken gizzard myosin was prepared by the method of Ebashi (*45*). Myelin basic protein (bovine brain) and α-casein were from Sigma.

Assaying of CaM-Kinase Kinase—Phosphorylation of protein or peptide substrates by CaM-kinase kinase α was carried out at 30°C in the standard reaction mixture comprising 50 mM Mops-NaOH (pH 7.0 at 30°C), 5 mM $Mg(CH_3COO)_2$, 0.1 mM [γ-^{32}P]ATP (50-300 cpm/pmol), 0.1 mM EGTA, 0.2 mM $CaCl_2$, 1 μM calmodulin, 2 mM dithiothreitol, the indicated amounts of protein or peptide substrates, and suitable amounts of CaM-kinase kinase α. After incubation for 5 min, the incorporation of [^{32}P]phosphate into protein substrates was determined by the 3MM paper method of Corbin and Reimann (*46*), except that the filter papers were washed with ice-cold 10% trichloroacetic acid containing 2 mM ATP. The incorporation of [^{32}P]-phosphate into peptide substrates was determined by the phosphocellulose paper method of Roskoski (*47*).

Other Procedures—SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (*48*). The concentration of calmodulin was determined spectrophotometrically using an absorption coefficient, A_{280} (1 mg/ml), of 0.21 (*49*) and a molecular weight of 16,700 (*50, 51*). Other proteins were determined by the method of Lowry *et al.* (*52*), as modified by Peterson (*53*) with bovine serum albumin as a standard. The amounts of CaM-kinase IV and CaM-kinase IV($K_{71}R$) were corrected for overestimation by a factor of 1.15 by means of Lowry's method (*54*). The molar concentration of CaM-kinase IV($K_{71}R$) was calculated using a molecular weight of 53,161, which was estimated from the molecular wight of 53,133 calculated from the deduced amino acid sequence of the wild-type enzyme (*28, 29*). The molar concentration of CaM-kinase I was calculated using a molecular weight of 41,643, which was obtained from the deduced amino acid sequence of the enzyme (*31*).

RESULTS

CaM-kinase kinase α phosphorylates CaM-kinase IV at Thr[196] to activate it (*20*), and CaM-kinase I kinase phosphorylates CaM-kinase I at Thr[177] to activate it (*21, 22*). CaM-kinase kinase α and its isoform, CaM-kinase kinase β, both phosphorylate to activate both CaM-kinases I and IV (*13*). CaM-kinase I kinase can also phosphorylate to activate not only CaM-kinase I but also CaM-kinase IV (*23*). Thus, these CaM-kinase kinases appear to be very similar enzymes, although it is not yet clear whether CaM-kinase I kinase is the same enzyme as CaM-kinase kinase α or β. The fact that CaM-kinase I kinase can phosphorylate CaM-kinase I only at Thr[177] (*21*), and that CaM-kinase kinase α can phosphorylate CaM-kinase IV only at Thr[196] among its many phosphorylation sites (*20*), indicates the relatively strict substrate specificity of the CaM-kinase kinases. In order to elucidate the physiological function of the CaM-kinase kinase, the substrate specificity of CaM-kinase kinase α was examined, focusing on Thr[196] of CaM-kinase IV and Thr[177] of CaM-kinase I, as shown in Table I. Among a number of proteins examined, including several known to be phosphorylated by various protein kinases, only CaM-kinases IV and I were significantly phosphorylated by CaM-kinase kinase α. It has been demonstrated that CaM-kinase I is phosphorylated only at Thr[177] in the presence of CaM-kinase I kinase under the phosphorylation conditions (*21*), but CaM-kinase IV is phosphorylated at many sites by the action of CaM-kinase IV itself activated by CaM-kinase kinase α in the presence of the kinase kinase, although the phosphorylation site of CaM-kinase IV by the CaM-kinase kinase is only Thr[196] (*20*). Therefore, CaM-kinase IV($K_{71}R$), in which Lys[71] (ATP-binding site) essential for the enzyme activity was replaced with arginine, was used for the experiment. Heat treatment of CaM-kinases IV and I at 60°C for 5 min abolished the phosphorylation by the kinase kinase, suggesting that native conformations of CaM-kinases IV and I are necessary for the phosphorylation by the CaM-kinase kinase. While CaM-kinase IV($K_{71}R$) became inactive as a substrate for the CaM-kinase kinase after heat treatment for 5 min at 50°C, CaM-kinase I retained most of its activity after the heat treatment. When various synthetic peptides, including several known to be phosphorylated by various protein kinases, were tested as substrates for CaM-kinase kinase α, only those containing the sequence surrounding Thr[196] of CaM-kinase IV or Thr[177] of CaM-kinase I were found to be significantly phosphorylated. The enzyme activity toward PKIV peptide containing amino acid residues 189-203 of CaM-kinase IV, which contained two

phosphorylatable amino acid residues (Thr^{196} and Thr^{200}), was almost the same as that toward PKIV[T_{200}A] peptide containing the same sequence, except for replacement of Thr^{200} with nonphosphorylatable alanine, suggesting that only Thr^{196} in PKIV peptide was phosphorylated by CaM-kinase kinase α, in agreement with our earlier observation that only Thr^{196} in CaM-kinase IV is phosphorylated by CaM-kinase kinase α (*20*). The activity toward PKI peptide containing amino acid residues 170-184 of CaM-kinase I, which contained four phosphorylatable amino acid residues (Ser^{173}, Ser^{176}, Thr^{177}, and Thr^{181}), was almost the same as that toward PKI[S_{173}A/S_{176}A/Thr_{181}A] peptide, in which the three phosphorylatable amino acids other than Thr^{177} were replaced with alanine residues, suggesting that only Thr^{177} in PKI peptide was phosphorylated, in accord with the previous observation that only Thr^{177} in CaM-kinase I is phosphorylated in the presence of CaM-kinase I kinase (*21*). Shortening of PKIV peptide and PKI peptide reduced the ability of these peptides to act as substrates for the CaM-kinase kinase. Thus, CaM-kinase kinase α exhibited a high degree of substrate specificity, although the consensus sequence for its substrates remains to be studied.

TABLE I. **Substrate specificity of CaM-kinase kinase α.** The activity of CaM-kinase kinase α for the indicated proteins (0.1 mg/ml) was measured with about 30 ng of the enzyme for 1 min in the reaction mixture containing 5 μM calmodulin, and that for the indicated synthetic peptides (100 μM) was measured with about 100 ng of the enzyme for 5 min in the reaction mixture containing 1 μM calmodulin, in the presence and absence of $CaCl_2$, as described under "EXPERIMENTAL PROCEDURES."

Substrate	Ca^{2+} (+) (nmol/min/mg)	Ca^{2+} (−) (nmol/min/mg)
CaM-kinase IV(K_{71}R)	587	6
CaM-kinase IV(K_{71}R), heated at 50°C[a]	11	
CaM-kinase IV(K_{71}R), heated at 60°C[a]	<10	
CaM-kinase IV, heated at 60°C[a]	<10	
CaM-kinase I	1,290	48
CaM-kinase I, heated at 50°C[a]	1,050	54
CaM-kinase I, heated at 60°C[a]	<10	
Synapsin I	<10	
Histone H1	<10	
α-Casein	<10	
Myelin basic protein	<10	
MAP2	<10	
Myosin light chain	<10	
PKIV peptide[b]	172	5.9
PKIV[T_{200}A] peptide[b]	183	5.9
Short PKIV[T_{200}A] peptide[b]	13	3.8
PKI peptide[b]	63	2.1
PKI[S_{173}A/S_{176}A/T_{181}A] peptide[b]	73	2.6
Short PKI[S_{176}A/T_{181}A] peptide[b]	10	2.3
Syntide-2	<1	
Kemptide	<1	
Peptide-γ	<1	
Autocamtide-2	<1	
Synapsin I-site 1 peptide	<1	

[a]Heated for 5 min. [b]Amino acid sequences of peptides (threonine and serine residues are underlined).

	196
PKIV peptide:	KKKK-EHQVLMKTVCGTPGY
PKIV[T_{200}A] peptide:	KKKK-EHQVLMKTVCGAPGY
Short PKIV[T_{200}A]:	KKKK-LMKTVCGAPGY
	177
PKI peptide:	KKKK-DPGSVLSTACGTPGY
PKI[S_{173}A/S_{176}A/T181A] peptide:	KKKK-DPGAVLATACGAPGY
Short PKI[S_{176}A/T_{181}A]:	KKKK-VLATACGAPGY

The kinetic properties of CaM-kinase kinase α for the putative physiological substrates CaM-kinases IV and I are shown in Figs. 1 and 2, respectively. The experiments were carried out in the presence of 5 μM calmodulin so that the substrates, which were calmodulin-binding proteins, did not exhaust calmodulin in the reaction mixture. The enzyme showed normal kinetic behavior with respect to both CaM-kinases and ATP. The kinetic parameters obtained from the double-reciprocal plots are summarized in Table II. The K_m value for CaM-kinase IV, 0.7 μM (38 μg/ml), was almost the same as that for CaM-kinase I, 0.9 μM (37 μg/ml), but the estimated V_{max} value toward CaM-kinase IV was approximately one-third of that toward CaM-kinase I. For comparison, the kinetic properties of the enzyme for PKIV peptide are shown in Fig. 3. The K_m value for PKIV peptide was two to three orders of magnitude higher than those for CaM-kinases IV and I, although the estimated V_{max} value toward the peptide substrate was similar to those toward the protein substrates, as summarized in Table II. The K_m values for ATP with the protein substrates were approximately one-fifth to one-eighth of

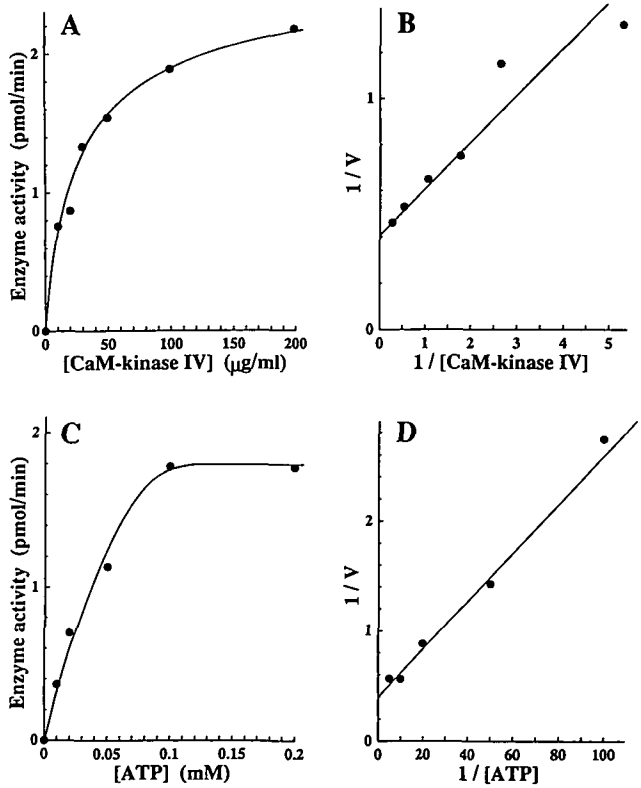

Fig. 1. **Effects of concentrations of CaM-kinase IV(K_{71}R) and ATP on the phosphorylation of CaM-kinase IV(K_{71}R) by CaM-kinase kinase α.** Approximately 2.6 ng of CaM-kinase kinase α was incubated in the reaction mixture containing 0.1 mM [γ-^{32}P]ATP (about 1,000 cpm/pmol) and the indicated concentrations of CaM-kinase IV(K_{71}R) (A), or 50 μg/ml (0.94 μM) CaM-kinase IV(K_{71}R) and the indicated concentrations of [γ-^{32}P]ATP (about 1,000 cpm/pmol) (C) at 30°C for 1 min in the presence of 5 μM calmodulin, and the incorporation of [^{32}P]phosphate into CaM-kinase IV(K_{71}R) was determined by the 3MM paper method (*46*). (B) and (D) represent double reciprocal plots of the activities obtained from the data of (A) and (C), respectively. The data are representative of three independent experiments.

that with the peptide substrate. Thus, Thr[196] was much more susceptible to the phosphorylation by the CaM-kinase kinase in the protein than in the peptide.

To confirm the strict substrate specificity of CaM-kinase kinase α, the phosphorylation of endogenous proteins in the crude extract of rat cerebral cortex by CaM-kinase kinase α was examined as shown in Fig. 4. Since CaM-kinases such as CaM-kinase kinase α, CaM-kinase IV, and CaM-kinase II exist abundantly in the cerebral cortex, the crude extract was incubated at 60°C for 5 min to inactivate the enzymes or passed through a column of calmodulin-Sepharose to remove the enzymes. When the heat-treated crude extract was incubated with [γ-^{32}P]ATP in the presence of Ca^{2+}/calmodulin and CaM-kinase kinase α under the phosphorylation conditions, a single radioactive band was observed on SDS-polyacrylamide gel electrophoresis (Fig. 4B, lane 3), but a similar band was also observed without the crude extract (Fig. 4B, lane 9), suggesting that the radioactive band resulted from the autophosphorylation of CaM-kinase kinase α added into the reaction mixture. Thus, no significant protein phosphorylation by CaM-kinase kinase α in the heat-treated crude cerebral cortex was detected under the experimental conditions. Since heat teatment of CaM-kinases IV and I at 60°C for 5 min abolished their phosphorylation by CaM-kinase kinase α (Table I), the possibility could not be ruled out that the heat treatment resulted in

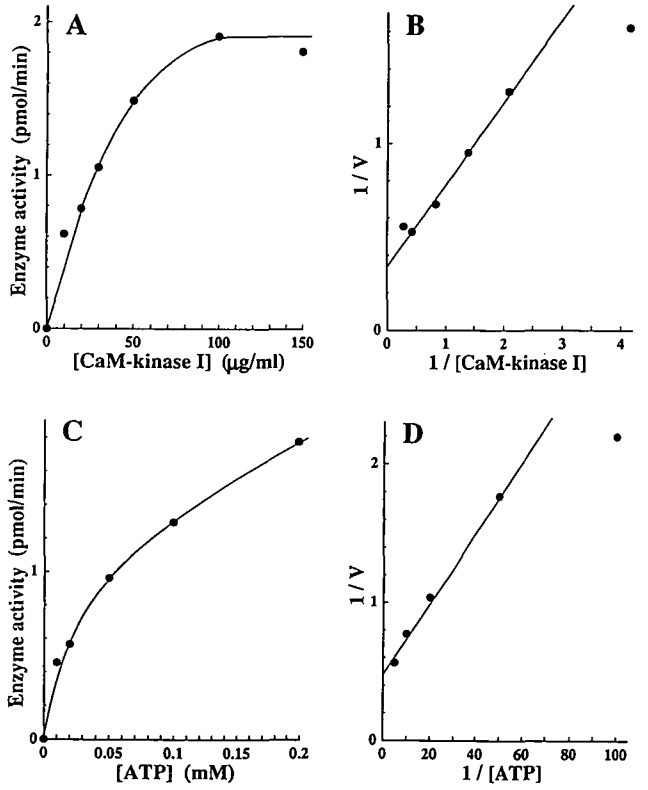

Fig. 2. **Effects of concentrations of CaM-kinase I and ATP on the phosphorylation of CaM-kinase I by CaM-kinase kinase α.** Approximately 0.72 ng of CaM-kinase kinase α was incubated in the reaction mixture containing 0.1 mM [γ-^{32}P]ATP (about 1,000 cpm/pmol) and the indicated concentrations of CaM-kinase I (A), or 50 μg/ml (1.2 μM) CaM-kinase I and the indicated concentrations of [γ-^{32}P]ATP (about 1,000 cpm/pmol) (C) at 30°C for 1 min in the presence of 5 μM calmodulin, and the incorporation of [^{32}P]phosphate into CaM-kinase I was determined by the 3MM paper method (*46*). (B) and (D) represent double reciprocal plots of the activities obtained from the data of (A) and (C), respectively. The data are representative of three independent experiments.

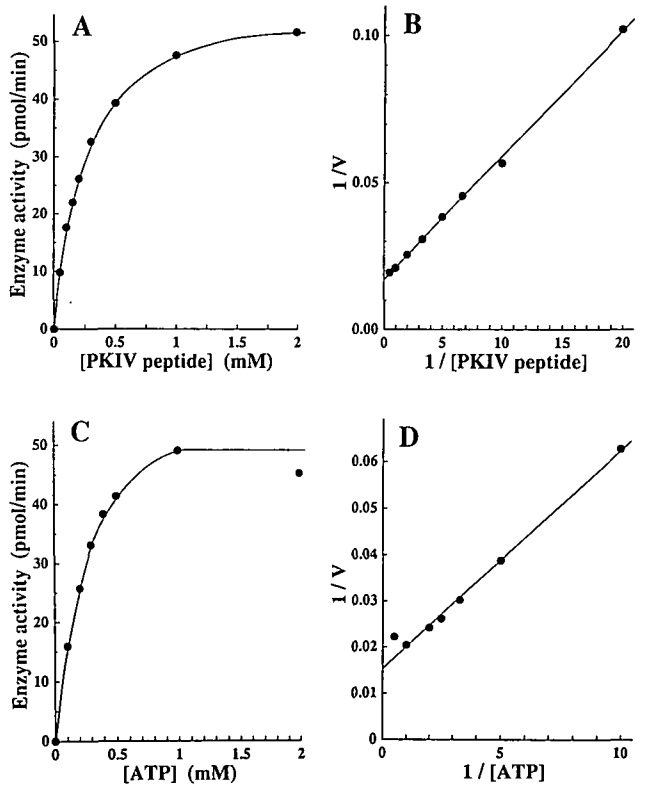

Fig. 3. **Effects of concentrations of PKIV peptide and ATP on the phosphorylation of PKIV peptide by CaM-kinase kinase α.** Approximately 45 ng of CaM-kinase kinase α was incubated in the reaction mixture containing 0.2 mM [γ-^{32}P]ATP (about 50 cpm/pmol) and the indicated concentrations of PKIV peptide (A), or 0.2 mM PKIV peptide and the indicated concentrations of [γ-^{32}P]ATP (about 50 cpm/pmol) (C) at 30°C for 5 min, and the incorporation of [^{32}P]phosphate into PKIV peptide was determined by the phosphocellulose paper method (*47*). (B) and (D) represent double reciprocal plots of the activities obtained from the data of (A) and (C), respectively. The data are representative of three independent experiments.

TABLE II. **Kinetic parameters of CaM-kinase kinase α.** Kinetic parameters were obtained from the double-reciprocal plots presented in Figs. 1-3. The V_{max} values, calculated from apparent V_{max} values on the basis of the Michaelis equation $v = V/(1+K_m/[S])$, are given in parentheses. The results are expressed as the mean and sample standard deviation of three independent experiments.

Substrate	K_m for substrate (μM)	Apparent V_{max} (nmol/min/mg)		K_m for ATP (μM)	Apparent V_{max} (nmol/min/mg)	
PKIV peptide	263±10	1,630±260	(4,290±780)	325±13	1,760±290	(4,090±700)
CaM-kinase IV(K_{71}R)	0.71±0.29	920±160	(1,540±240)	68±10	910±340	(1,670±550)
CaM-kinase I	0.89±0.27	3,830±1,100	(5,430±1,230)	43±13	2,980±280	(5,220±1,120)

complete loss of the susceptibilities to phosphorylation by CaM-kinase kinase α of not only the CaM-kinases but also other protein substrates occurring in the crude cerebral extract. Therefore, the crude extract passed through a column of calmodulin-Sepharose was also examined for phosphorylation by CaM-kinase kinase α (Fig. 4, lanes 5–8). The addition of CaM-kinase kinase α in the presence of Ca^{2+}/calmodulin resulted in an intense broad radioactive band in the 64- to 67-kDa range (Fig. 4, lane 7). However, Western blot analysis revealed that 3 μg of our calmodulin pass-through extract still contained approximately 1 ng of CaM-kinase IV (data not shown), and it is therefore conceivable that the CaM-kinase kinase-induced band was due to the phosphorylation of the endogenous CaM-kinase IV as well as the autophosphorylation of the added CaM-kinase kinase, judging from its migration position on SDS-polyacrylamide gel electrophoresis. Interestingly, the phosphorylation of a protein with somewhat slower mobility than the autophosphorylated CaM-kinase kinase α was strongly stimulated in the absence of Ca^{2+} (Fig. 4, lane 6), while there were many proteins whose phosphorylation was stimulated in the presence of Ca^{2+}.

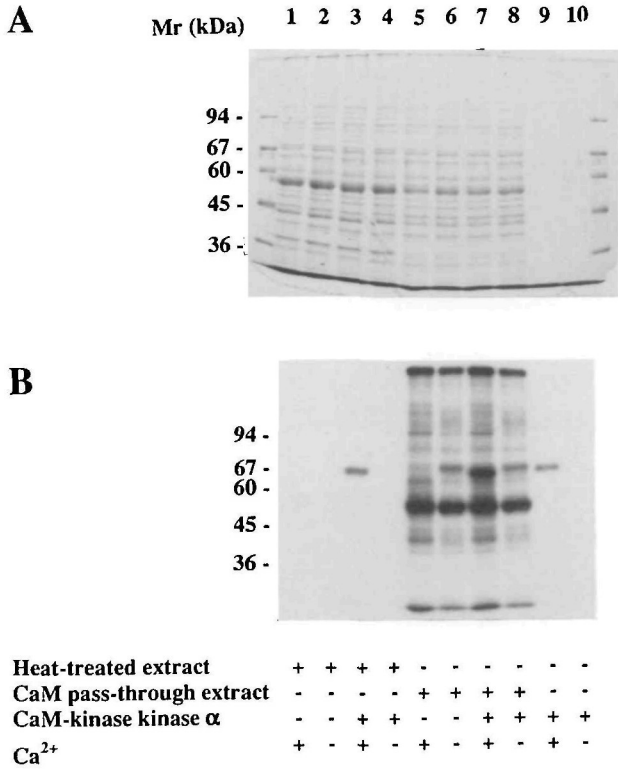

Fig. 4. **Analysis of rat cerebral proteins phosphorylated by CaM-kinase kinase α by SDS-polyacrylamide gel electrophoresis.** Rat cerebral cortex was homogenized with a Potter-Elvehjem homogenizer in 3 volumes of 10 mM Tris-HCl (pH 7.5) containing 0.1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, and 10 μg/ml each of microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin). The residue was removed by centrifugation at 100,000×*g* to generate the crude extract. To obtain the heat-treated extract, the crude extract was diluted with 7 volumes of 10 mM Hepes-NaOH (pH 7.2) containing 10% glycerol, 0.05% Tween 80, 0.1 mM dithiothreitol, and 0.1 mM EDTA, then heated at 60°C for 5 min. To obtain the calmodulin pass-through extract, 1 ml of the crude extract was subjected to ammonium sulfate precipitation between 25 and 75% saturation to remove endogenous calmodulin, and the precipitate dissolved in 1 ml of 10 mM Tris-HCl (pH 7.5) containing 0.1 mM dithiothreitol, 1 mM $CaCl_2$, and 10 μg/ml each of the protease inhibitors was applied to a column of calmodulin-Sepharose (0.5 ml), prepared as described previously (*6*), equilibrated with the same buffer. To the pass-through fraction eluted with 1 ml of 10 mM Tris-HCl (pH 7.5) containing 0.1 mM dithiothreitol, 0.2 mM $CaCl_2$, and 10 μg/ml each of the protease inhibitors, ammonium sulfate was added to 70% saturation, and the precipitate was dialyzed against 10 mM Tris-HCl (pH 7.5) containing 0.1 mM dithiothreitol, 0.1 mM EGTA, and 5 μg/ml each of the protease inhibitors. Approximately 3 μg of the heat-treated extract or calmodulin pass-through (CaM pass-through) extract was incubated in 20 μl of the reaction mixture containing 10 ng of CaM-kinase kinase α and 0.1 mM [γ-^{32}P]ATP (2,500 cpm/pmol) in the presence or absence of $CaCl_2$ at 30°C for 5 min, as indicated at the bottom, then subjected to SDS-polyacrylamide gel electrophoresis on 7.5% gels. (A) Proteins were visualized by staining with Coomassie Brilliant Blue. (B) Incorporation of [^{32}P]phosphate into proteins was examined by autoradiography.

DISCUSSION

It has recently been established that CaM-kinases IV and I are markedly activated upon phosphorylation at Thr^{196} and Thr^{177}, respectively, by upstream Ca^{2+}/calmodulin-dependent protein kinases such as CaM-kinase kinases α and β. CaM-kinases IV and I are known as multifunctional protein kinases catalyzing phosphorylation of a number of protein substrates, but our recent finding (*20*) that CaM-kinase kinase α can only phosphorylate Thr^{196} among many phosphorylation sites of CaM-kinase IV suggests its strict substrate specificity. To determine whether CaM-kinase kinase α functions only to activate CaM-kinases IV and I, the substrate specificity of CaM-kinase kinase α was examined in the present study. Among a number of proteins and synthetic peptides, including several known to be good substrates for various protein kinases, only CaM-kinases IV and I and peptides containing the sequence surrounding Thr^{196} of CaM-kinase IV or Thr^{177} of CaM-kinase I were significantly phosphorylated by CaM-kinase kinase α, and the phosphorylation was not observed with CaM-kinase IV or I that had been denatured at 60°C for 5 min (Table I), suggesting the high degree of substrate specificity of CaM-kinase kinase α, which recognizes the native conformation of CaM-kinases IV and I as substrates. Interestingly, a remarkably similar high degree of substrate specificity has been observed with mitogen-activated protein kinase kinase (*55*). The fact that peptides containing the phosphorylation site of CaM-kinase IV or I were far less active as substrates for CaM-kinase kinase α than were native CaM-kinase IV or I (Table II) also indicates a high degree of substrate specificity.

This high degree of substrate specificity of the CaM-kinase kinase suggests that the physiological function of the CaM-kinase kinase may be only to activate CaM-kinases IV and I. Our attempts to detect protein substrates for CaM-kinase kinase α other than CaM-kinases IV and I in a rat crude cerebral extract which had been heated at 60°C for 5 min to inactivate endogenous CaM-kinases or passed through a column of calmodulin-Sepharose to remove the enzymes were unsuccessful (Fig. 4), suggesting that no protein substrates other than CaM-kinases IV and I are present in the cerebral cortex, although further investigations should be undertaken to substantiate this conclusion. The Ca^{2+}-dependent phosphorylation of the endogenous proteins other than the remaining CaM-kinase IV in the

calmodulin pass-through extract was not significantly stimulated by the addition of CaM-kinase kinase α (Fig. 4, lanes 5 and 7). The Ca^{2+}-dependent phosphorylation may in part reflect the action of the remaining CaM-kinase IV, because the enzyme of the crude brain extract was present in an active form (*56*).

REFERENCES

1. Fujisawa, H. (1990) Calmodulin-dependent protein kinase II. *BioEssays* **12**, 27-29
2. Colbran, R.J. and Soderling, T.R. (1990) Calcium/calmodulin-dependent protein kinase II. *Curr. Top. Cell. Regul.* **31**, 181-221
3. Hanson, P.I. and Schulman, H. (1992) Neuronal Ca^{2+}/calmodulin-dependent protein kinases. *Annu. Rev. Biochem.* **61**, 559-601
4. Nairn, A.C. and Picciotto, M.R. (1994) Calcium/calmodulin-dependent protein kinases. *Sem. Cancer Biol.* **5**, 295-303
5. Okuno, S. and Fujisawa, H. (1993) Requirement of brain extract for the activity of brain calmodulin-dependent protein kinase IV expressed in *Escherichia coli*. *J. Biochem.* **114**, 167-170
6. Okuno, S., Kitani, T., and Fujisawa, H. (1994) Purification and characterization of Ca^{2+}/calmodulin-dependent protein kinase IV kinase from rat brain. *J. Biochem.* **116**, 923-930
7. Tokumitsu, H., Brickey, D.A., Glod, J., Hidaka, H., Sikela, J., and Soderling, T.R. (1994) Activation mechanism for Ca^{2+}/calmodulin-dependent protein kinase IV. Identification of a brain CaM-kinase IV kinase. *J. Biol. Chem.* **269**, 28640-28647
8. Mochizuki, H., Sugita, R., Ito, T., and Hidaka, H. (1993) Phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase V and regulation of its activity. *Biochem. Biophys. Res. Commun.* **197**, 1595-1600
9. Lee, J.C. and Edelman, A.M. (1994) A protein activator of Ca^{2+}-calmodulin-dependent protein kinase Ia. *J. Biol. Chem.* **269**, 2158-2164
10. Tokumitsu, H., Enslen, H., and Soderling, T.R. (1995) Characterization of a Ca^{2+}/calmodulin-dependent protein kinase cascade. Molecular cloning and expression of calcium/calmodulin-dependent protein kinase kinase. *J. Biol. Chem.* **270**, 19320-19324
11. Okuno, S., Kitani, T., and Fujisawa, H. (1996) Evidence for the existence of Ca^{2+}/calmodulin-dependent protein kinase IV kinase isoforms in rat brain. *J. Biochem.* **119**, 1176-1181
12. Edelman, A.M., Mitchelhill, K.I., Selbert, M.A., Anderson, K. A., Hook, S.S., Stapleton, D., Goldstein, E.G., Means, A.R., and Kemp, B.E. (1996) Multiple Ca^{2+}-calmodulin-dependent protein kinase kinases from rat brain. Purification, regulation by Ca^{2+}-calmodulin, and partial amino acid sequence. *J. Biol. Chem.* **271**, 10806-10810
13. Okuno, S., Kitani, T., and Fujisawa, H. (1997) Purification and characterization of Ca^{2+}/calmodulin-dependent protein kinase kinase β from rat cerebellum. *J. Biochem.* **121**, 155-160
14. Kwiatkowski, A.P., Shell, D.J., and King, M.M. (1988) The role of autophosphorylation in activation of the type II calmodulin-dependent protein kinase. *J. Biol. Chem.* **263**, 6484-6486
15. Katoh, T. and Fujisawa, H. (1991) Autoactivation of calmodulin-dependent protein kinase II by autophosphorylation. *J. Biol. Chem.* **266**, 3039-3044
16. Ikeda, A., Okuno, S., and Fujisawa, H. (1991) Studies on the generation of Ca^{2+}/calmodulin-independent activity of calmodulin-dependent protein kinase II by autophosphorylation. *J. Biol. Chem.* **266**, 11582-11588
17. Ishida, A., Kitani, T., and Fujisawa, H. (1996) Evidence that autophosphorylation at Thr-286/Thr-287 is required for full activation of calmodulin-dependent protein kinase II. *Biochim. Biophys. Acta* **1311**, 211-217
18. McDonald, O.B., Merrill, B.M., Bland, M.M., Taylor, L.C.E., and Sahyoun, N. (1993) Site and consequences of the autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase type "Gr." *J. Biol. Chem.* **268**, 10054-10059
19. Okuno, S., Kitani, T., and Fujisawa, H. (1995) Full activation of brain calmodulin-dependent protein kinase IV requires phosphorylation of the amino-terminal serine-rich region by calmodulin-dependent protein kinase IV kinase. *J. Biochem.* **117**, 686-690
20. Kitani, T., Okuno, S., and Fujisawa, H. (1997) Studies on the site of phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase (CaM-kinase) IV by CaM-kinase kinase. *J. Biochem.* **121**, 804-810
21. Sugita, R., Mochizuki, H., Ito, T., Yokokura, H., Kobayashi, R., and Hidaka, H. (1994) Ca^{2+}/calmodulin-dependent protein kinase kinase cascade. *Biochem. Biophys. Res. Commun.* **203**, 694-701
22. Haribabu, B., Hook, S.S., Selbert, M.A., Goldstein, E.G., Tomhave, E.D., Edelman, A.M., Snyderman, R., and Means, A.R. (1995) Human calcium-calmodulin dependent protein kinase I: cDNA cloning, domain structure and activation by phosphorylation at threonine-177 by calcium-calmodulin dependent protein kinase I kinase. *EMBO J.* **14**, 3679-3686
23. Selbert, M.A., Anderson, K.A., Huang, Q., Goldstein, E.G., Means, A.R., and Edelman, A.M. (1995) Phosphorylation and activation of Ca^{2+}-calmodulin-dependent protein kinase IV by Ca^{2+}-calmodulin-dependent protein kinase Ia kinase. Phosphorylation of threonine 196 is essential for activation. *J. Biol. Chem.* **270**, 17616-17621
24. Miyano, O., Kameshita, I., and Fujisawa, H. (1992) Purification and characterization of a brain-specific multifunctional calmodulin-dependent protein kinase from rat cerebellum. *J. Biol. Chem.* **267**, 1198-1203
25. Czernik, A.J., Pang, D.T., and Greengrad, P. (1987) Amino acid sequences surrounding the cAMP-dependent and calcium/calmodulin-dependent phosphorylation sites in rat and bovine synapsin I. *Proc. Natl. Acad. Sci. USA* **84**, 7518-7522
26. DeRemer, M.F., Saeli, R.J., and Edelman, A.M. (1992) Ca^{2+}-calmodulin-dependent protein kinases Ia and Ib from rat brain. I. Identification, purification, and structural comparisons. *J. Biol. Chem.* **267**, 13460-13465
27. Casnellie, J.E. (1991) Assay of protein kinases using peptides with basic residues for phosphocellulose binding. *Methods Enzymol.* **200**, 115-120
28. Means, A.R., Cruzalegui, F., LeMagueresse, B., Needleman, D.S., Slaughter, G.R., and Ono, T. (1991) A novel Ca^{2+}/calmodulin-dependent protein kinase and a male germ cell-specific calmodulin-binding protein are derived from the same gene. *Mol. Cell. Biol.* **11**, 3960-3971
29. Ohmstede, C.-A., Bland, M.M., Merrill, B.M., and Sahyoun, N. (1991) Relationship of genes encoding Ca^{2+}/calmodulin-dependent protein kinase Gr and calspermine: A gene within a gene. *Proc. Natl. Acad. Sci. USA* **88**, 5784-5788
30. Picciotto, M.R., Czernik, A.J., and Nairn, A.C. (1993) Calcium/calmodulin-dependent protein kinase I. cDNA cloning and identification of autophosphorylation site. *J. Biol. Chem.* **268**, 26512-26521
31. Cho, F.S., Phillips, K.S., Bogucki, B., and Weaver, T.E. (1994) Characterization of a rat cDNA encoding calcium/calmodulin-dependent protein kinase I. *Biochim. Biophys. Acta* **1224**, 156-160
32. Kemp, B.E., Graves, D.J., and Benjamini, E. (1976) Synthetic peptide substrates of the cAMP-dependent protein kinase. *Fed. Proc.* **35**, 1384
33. Hashimoto, Y. and Soderling, T.R. (1987) Calcium/calmodulin-dependent protein kinase II and calcium/phospholipid-dependent protein kinase activities in rat tissues assayed with a synthetic peptide. *Arch. Biochem. Biophys.* **252**, 418-425
34. Hanson, P.I., Kapiloff, M.S., Lou, L.L., Rosenfeld, M.G., and Schulman, H. (1989) Expression of a multifunctional Ca^{2+}/calmodulin-dependent protein kinase and mutational analysis of its autoregulation. *Neuron* **3**, 59-70
35. Kitani, T., Okuno, S., and Fujisawa, H. (1995) Inactivation of Ca^{2+}/calmodulin-dependent protein kinase IV by Ca^{2+}/calmodulin and restoration of the activity by Mg^{2+}/EGTA. *J. Biochem.* **117**, 1070-1075
36. Gopalakrishna, R. and Anderson, W.B. (1982) Ca^{2+}-induced hydrophobic site on calmodulin: application for purification of calmodulin by phenyl-Sepharose affinity chromatography. *Bio-*

chem. Biophys. Res. Commun. **104**, 830-836
37. Putkey, J.A., Ts'ui, K.F., Tanaka, T., Lagacé, L., Stein, J.P., Lai, E.C., and Means, A.R. (1983) Chicken calmodulin genes. A species comparison of cDNA sequences and isolation of a genomic clone. *J. Biol. Chem.* **258**, 11864-11870
38. Bennett, V., Baines, A.J., and Davis, J. (1986) Purification of brain analogs of red blood cell membrane skeletal proteins: ankyrin, protein 4.1 (synapsin), spectrin, and spectrin subunits. *Methods Enzymol.* **134**, 55-69
39. Johns, E.W. (1967) The electrophoresis of histones in polyacrylamide gel and their quantitative determination. *Biochem. J.* **104**, 78-82
40. Oliver, D., Sommer, K.R., Panyim, S., Spiker, S., and Chalkley, R. (1972) A modified procedure for fractionating histones. *Biochem. J.* **129**, 349-353
41. Kim, H., Binder, L.I., and Rosenbaum, J.L. (1979) The periodic association of MAP2 with brain microtubules *in vitro*. *J. Cell Biol.* **80**, 266-276
42. Shelanski, M.L., Gaskin, F., and Cantor, C.R. (1973) Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. USA* **70**, 765-768
43. Karr, T.L., White, H.D., and Purich, D.L. (1979) Characterization of brain microtubule proteins prepared by selective removal of mitochondrial and synaptosomal components. *J. Biol. Chem.* **254**, 6107-6111
44. Perrie, W.T. and Perry, S.V. (1970) An electrophoretic study of the low-molecular-weight components of myosin. *Biochem. J.* **119**, 31-38
45. Ebashi, S. (1976) A simple method of preparing actin-free myosin from smooth muscle. *J. Biochem.* **79**, 229-231
46. Corbin, J.D. and Reimann, E.M. (1974) Assay of cyclic AMP-dependent protein kinases. *Methods Enzymol.* **38**, 287-290
47. Roskoski, R., Jr. (1983) Assays of protein kinase. *Methods Enzymol.* **99**, 3-6
48. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
49. Dedman, J.R., Potter, J.D., Jackson, R.L., Johnson, J.D., and Means, A.R. (1977) Physicochemical properties of rat testis Ca^{2+}-dependent regulator protein of cyclic nucleotide phosphodiesterase. Relationship of Ca^{2+}-binding, conformational changes, and phosphodiesterase activity. *J. Biol. Chem.* **252**, 8415-8422
50. Dedman, J.R., Jackson, R.L., Schreiber, W.E., and Means, A.R. (1978) Sequence homology of the Ca^{2+}-dependent regulator of cyclic nucleotide phosphodiesterase from rat testis with other Ca^{2+}-binding proteins. *J. Biol. Chem.* **253**, 343-346
51. Watterson, D.M., Sharief, F., and Vanaman, T.C. (1980) The complete amino acid sequence of the Ca^{2+}-dependent modulator protein (calmodulin) of bovine brain. *J. Biol. Chem.* **255**, 962-975
52. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275
53. Peterson, G.L. (1977) A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.* **83**, 346-356
54. Kameshita, I. and Fujisawa, H. (1995) Preparation and characterization of calmodulin-dependent protein kinase IV (CaM-kinase IV) free of CaM-kinase IV kinase from rat cerebral cortex. *J. Biochem.* **117**, 85-90
55. Seger, R., Ahn, N.G., Posada, J., Munar, E.S., Jensen, A.M., Cooper, J.A., Cobb, M.H., and Krebs, E.G. (1992) Purification and characterization of mitogen-activated protein kinase activator(s) from epidermal growth factor-stimulated A431 cells. *J. Biol. Chem.* **267**, 14373-14381
56. Fujisawa, H., Okuno, S., Kitani, T., Kameshita, I., Ishida, A., and Takeuchi, M. (1994) Effect of nicotine on the regulation of the function of the central nervous system by multifunctional protein kinases in *Smoking Research Foundation Annual Research Report* pp. 180-185, Smoking Research Foundation, Tokyo