J. Biochem. 130, 503–513 (2001)

Regulation of Ca^{2+}/Calmodulin-Dependent Protein Kinase Kinase α by cAMP-Dependent Protein Kinase: I. Biochemical Analysis[1]

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Received June 28, 2001; accepted July 23, 2001

Ca^{2+}/calmodulin-dependent protein kinases (CaM-kinases) I and IV are activated upon phosphorylation of their Thr^{177} and Thr^{196}, respectively, by the upstream Ca^{2+}/calmodulin-dependent protein kinases CaM-kinase kinase α and β, and deactivated upon dephosphorylation by protein phosphatases such as CaM-kinase phosphatase. Recent studies demonstrated that the activity of CaM-kinase kinase α is decreased upon phosphorylation by cAMP-dependent protein kinase (PKA), and the relationship between the inhibition and phosphorylation of CaM-kinase kinase α by PKA has been studied. In the present study, we demonstrate that the activity of CaM-kinase kinase α toward PKIV peptide, which contains the sequence surrounding Thr^{196} of CaM-kinase IV, is increased by incubation with PKA in the presence of Ca^{2+}/calmodulin but decreased in its absence, while the activity toward CaM-kinase IV is decreased by incubation with PKA in both the presence and absence of Ca^{2+}/calmodulin. Six phosphorylation sites on CaM-kinase kinase α, Ser^{24} for autophosphorylation, and Ser^{52}, Ser^{74}, Thr^{108}, Ser^{458}, and Ser^{475} for phosphorylation by PKA, were identified by amino acid sequence analysis of the phosphopeptides purified from the tryptic digest of the phosphorylated enzymes. The presence of Ca^{2+}/calmodulin suppresses phosphorylation on Ser^{52}, Ser^{74}, Thr^{108}, and Ser^{458} by PKA, but accelerates phosphorylation on Ser^{475}. The changes in the activity of the enzyme upon phosphorylation appear to occur as a result of conformational changes induced by phosphorylation on several sites.

Key words: cAMP-dependent protein kinase, Ca^{2+}/calmodulin, CaM-kinase kinase, phosphorylation site, protein phosphorylation.

Calcium ion, cyclic AMP, and diacylglycerol are typical representative intracellular messengers in many eukaryotic signal-transducing pathways, and these messengers function by activating multifunctional protein kinases such as Ca^{2+}/calmodulin-dependent protein kinase (CaM-kinase), PKA, and PKC, respectively. Since these signal-transducing pathways coexist in most cells, cross-talk among these pathways is very important. In 1991, we found that CaM-kinase IV is inactivated by incubation with PKA under phosphorylation conditions, suggesting cross-talk between Ca^{2+}-signaling and cyclic AMP-signaling pathways (*1*). Thereafter, in 1993, we discovered CaM-kinase kinase, which markedly activates CaM-kinase IV through Ca^{2+}/calmodulin-dependent phosphorylation in rat brain (*2*). Since then, it has been well established that CaM-kinases IV (*2–4*) and I (*5, 6*), among the three CaM-kinases I, II, and IV known as multifunctional CaM-kinases, are activated through phosphorylation at Thr^{196} (*7, 8*) and Thr^{177} (*9, 10*), respectively, by upstream Ca^{2+}/calmodulin-dependent protein kinases such as CaM-kinase kinases α (*11, 12*) and β (*12–14*). The present study was undertaken to examine the details of the mechanism of CaM-kinase IV inactivation by PKA. During the course of these studies, Wayman et al. reported that PKA phosphorylates CaM-kinase kinase α, resulting in 50–75% inhibition of the CaM-kinase kinase activity toward CaM-kinase IV due to the phosphorylation of Ser^{458} and Thr^{108} (*15*). On the other hand, Matsushita and Nairn reported that the phosphorylation of Thr^{108} is mainly involved in the decrease in the activity of CaM-kinase kinase α toward CaM-kinase I (*16*). A preliminary report of this work has already been reported (*17*).

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP (5,000 Ci/mmol) was from Amersham Pharmacia Biotech. Microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin) were from the Peptide Institute (Osaka). Phosphocellulose paper (P81) and 3MM paper were from Whatman. PKA inhibitor peptide (TTYADFIASGRTGRRNAIHD) (*18*) and Kemptide (LRRASLG) (*19*) were from Sigma. TPCK-trypsin was from Cooper Biomedical. Syntide-2 (PLARTLSVAGLPGKK) (*20*) was synthesized by the American Peptide Institute. PKIV peptide (KKKKEHQVLMKTVCGTPGY) (*21*), which contains the sequence surrounding Thr^{196} of CaM-kinase IV and four lysyl residues at the amino terminus for binding

[1] This work was supported by Grants-in-Aid #10102002 for Specially Promoted Research on "Signal Transduction Mediated by Multifunctional Ca^{2+}/Calmodulin-Dependent Protein Kinases," the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: CaM-kinase, calmodulin-dependent protein kinase; PKA, cyclic AMP-dependent protein kinase.

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to phosphocellulose paper in the phosphocellulose protein kinase assay (*22*), and PKIV[T_{200}A] peptide (KKKKEHQVLMKTVCGAPGY) (*21*), which has the same sequence except for the replacement of Thr^{200} with nonphosphorylatable alanine, were synthesized with a Shimadzu PSSM-8 automated peptide synthesizer. The PTH-Δ-Threonine standard was from Wako Pure Chemicals (Osaka). All other reagents were of the highest grade commercially available.

Protein Preparations—Calmodulin was purified from *E. coli* cells transformed with expression vector pET11d carrying a cDNA encoding chicken calmodulin (*23*) essentially as described by Gopalakrishna and Anderson (*24*). The cDNA encoding chicken calmodulin was kindly donated by A.R. Means (*25*). Recombinant rat CaM-kinase kinase α expressed in *E. coli* (*12*) was purified as described previously (*8*). Recombinant rat CaM-kinase kinase β expressed in Sf9 cells (*26*) was purified essentially as described for the purification of recombinant CaM-kinase kinase α expressed in *E. coli* (*8*), except for the omission of the streptomycin step. We did not check whether the recombinant CaM-kinase kinases α and β had been phosphorylated in the cells in which the enzymes were expressed. PKA (catalytic subunit of cyclic AMP-dependent protein kinase) was purified from bovine heart as described previously (*27*). Recombinant rat CaM-kinase IV expressed in Sf9 cells was purified as described previously (*23*). 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 (*8*). Recombinant rat CaM-kinase I expressed in Sf9 cells was purified as described previously (*14*).

Phosphorylation Reaction—The phosphorylation of proteins or peptides by protein kinases was carried out at 30°C in a phosphorylation mixture comprising 50 mM Mops-NaOH (pH 7.0 at 30°C), 2 mM dithiothreitol, 5 mM Mg-($CH_3COO)_2$, 0.1 mM nonradioactive or [γ-^{32}P]ATP, 1 μM calmodulin, either 0.1 mM EGTA (minus calcium) or 0.1 mM EGTA/0.2 mM $CaCl_2$ (plus calcium), and the indicated amounts of proteins or peptides. After incubation for the indicated times, the incorporation of [^{32}P]phosphate into the protein substrates was determined by the 3MM paper method of Corbin and Reimann (*28*), except that the filters were washed with ice-cold 10% trichloroacetic acid containing 2 mM ATP. The incorporation of [^{32}P]phosphate into the peptide substrates was determined by the phosphocellulose paper method of Roskoski (*29*).

Analysis of Phosphorylation Sites of CaM-Kinase kinase α—Approximately 100 μg (20 μg/ml) of CaM-kinase kinase α was incubated with or without 2.5 μg (0.5 μg/ml) of PKA in a final volume of 5 ml phosphorylation mixture containing [γ-^{32}P]ATP (200 cpm/pmol) in the presence or absence of Ca^{2+}/calmodulin at 30°C. The reaction was stopped by the addition of 10 mM ATP and 10% ice-cold trichloroacetic acid. The precipitate obtained on centrifugation was washed three times by sonication for 2 min in 1 ml of ice-cold acetone and then three times by sonication for 1 min in 0.3 ml ice-cold acetone, and then air-dried at room temperature. The resulting protein was dissolved in 30 μl of 50 mM Tris-HCl (pH 8.5) containing 8 M urea, then 3 μl of 0.5 M dithiothreitol was added under a stream of argon, and the mixture was incubated at 37°C for 2 h under an argon atmosphere. To the mixture was added 6 μl of 0.4 M iodoacetamide, and then the mixture was incubated at 24°C for 30 min in the dark. The mixture was diluted with 140 μl of 50 mM Tris-HCl (pH 8.0) and, after the addition of 20 μl of 0.1 M $CaCl_2$ and 4.12 μl of 1 mg/ml TPCK-trypsin in 1 mM HCl, the mixture was incubated at 37°C for 24 h and for a further 14 h after the addition of another 4.12 μl of TPCK-trypsin. The mixture was filtered through a 0.22-μm filter, and then loaded onto a C_{18} reverse-phase HPLC column (0.46 × 25 cm, TSK gel ODS-80Ts; Tosoh) equilibrated with buffer A (2% acetonitrile in 20 mM triethylamine phosphate, pH 3). The column was eluted successively at the flow rate of 0.5 ml/min with buffer A for the initial 30 min, then linear gradients of 0–50% (v/v) buffer B (80% acetonitrile in 20 mM triethylamine phosphate, pH 3) for 100 min, and then 50–100% buffer B for 5 min, and finally buffer B for 5 min. The peptide and phosphopeptide peaks were monitored spectrophotometrically at 215 nm using an on-line UV monitor, Tosoh UV-8000, and radiometrically by Cerenkov counting using an on-line detector, Raytest Ramona 90. The fractions corresponding to each radioactive peak were pooled and subjected to a second HPLC on the same column equilibrated with buffer C (2% acetonitrile in 20 mM triethylamine acetate, pH 6.5). The column was eluted with buffer C for the initial 10 min, then linear gradients of 0–50% buffer D (80% acetonitrile in 20 mM triethylamine acetate, pH 6.5) for 100 min and 50–100% buffer D for 10 min, and finally buffer D for 5 min. The amino acid sequences of the radioactive phosphopeptides thus obtained were determined with a Hewlett Packard G1005A peptide sequenator.

Chromatographic identification of phosphoserine or serine, and phosphothreonine or threonine by the peptide sequenator was performed using the ratio of the PTH-amino acid to its dehydroderivative. Phosphorylated amino acids produce more dehydroderivatives, such as dehydroalanine in the case of serine (*30, 31*) and dehydroaminobutyric acid in the case of threonine (*31, 32*), upon Edman degradation than the nonphosphorylated amino acids. When Kemptide phosphorylated with [γ-^{32}P]ATP by PKA, which was purified on a C_{18} reverse-phase HPLC column eluted by a linear gradient of 2 to 50% acetonitrile in 0.1% trifluoroacetic acid, and nonphosphorylated Kemptide were analyzed with the peptide sequenator, the ratios of the peak heights of serine eluted at 7.9 min to those of dehydroalanine eluted at 13.4 min were 0.2 for phosphorylated Kemptide and 2.5 for nonphosphorylated Kemptide, in cycle 5. On the other hand, when PKIV[T_{200}A] peptide phosphorylated with CaM-kinase kinase α, purified as described above, and nonphosphorylated PKIV[T_{200}A] peptide were analyzed, the ratios of the peak heights of threonine eluted at 8.3 min to those of dehydroaminobutyric acid eluted at 13.6 min were 0.4 for phosphorylated PKIV[T_{200}A] peptide and 7.5 for nonphosphorylated PKIV[T_{200}A] peptide in cycle 12. The retention time of PTH-dehydroaminobutyric acid was determined using a PTH-Δ-threonine standard.

Other Procedures—The concentration of calmodulin was determined spectrophotometrically using an absorption coefficient, A_{280} (1 mg/ml), of 0.21 (*33*) and a molecular weight of 16,700 (*34, 35*). Other proteins were determined by the method of Lowry *et al.* (*36*), as modified by Peterson (*37*) 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 (*38*).

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RESULTS

When CaM-kinase kinase α was incubated with PKA in the presence of Ca^{2+}/calmodulin under the phosphorylation conditions, the phosphorylation activity toward PKIV peptide increased progressively with incubation time to twice the initial activity after incubation for 30 min. Incubation without PKA in the presence of Ca^{2+}/calmodulin also resulted in a gradual increase, presumably due to autophosphorylation of the enzyme, as shown in Fig. 1A. In contrast, incubation of the enzyme with PKA in the absence of Ca^{2+}/calmodulin resulted in a rapid decrease in activity, the decrease reaching an almost the maximal level within 1 min, after which no further significant decrease was observed. Phosphate incorporation into the enzyme during incubation was examined in a parallel experiment, as shown in Fig. 1B. The enzyme underwent a gradual autophosphorylation in the presence of Ca^{2+}/calmodulin to about 1.42 mol of phosphate/mol of enzyme after incubation for 40 min, and the phosphate incorporation was accelerated by PKA. The accelerations of the activation and phosphorylation of the enzyme by PKA were both prevented by the addition of a PKA inhibitor, indicating that the acceleration of the enzyme activation in the presence of Ca^{2+}/calmodulin occurs as a result of the phosphorylation by PKA. In the absence of Ca^{2+}/calmodulin, PKA caused a rapid incorporation of phosphate into CaM-kinase kinase α to about 1.28 mol of phosphate/mol of enzyme at 1 min, with a subsequent slow incorporation of phosphate. Since the rapid decrease in the activity of CaM-kinase kinase α produced by PKA in the absence of Ca^{2+}/calmodulin reached a maximal level within 1 min (Fig. 1A), and the inhibition of the following slow phosphorylation by PKA inhibitor did not affect the enzyme activity significantly, only the initial rapid phosphorylation of the enzyme by PKA appears to be involved in the decrease in enzyme activity.

In order to determine the phosphorylation sites responsible for the activation in the presence of Ca^{2+}/calmodulin and the rapid inactivation in their absence, CaM-kinase kinase α phosphorylated with [γ-^{32}P]ATP was digested with trypsin and subjected to reverse-phase HPLC for the purification of radioactive phosphopeptides, as shown in Fig. 2. The enzyme incubated for 5 min in the presence of Ca^{2+}/calmodulin (without PKA) showed one major peak (peak 5) and several minor peaks of radioactive phosphopeptides upon the first HPLC (Fig. 2A). The major peak was separated into two peaks, *a* and *b*, by the second HPLC (Fig. 2D), and the amino acid sequences of phosphopeptides *a* and *b* were VAAIS(PO_4)^3-VA, and VAAIS(PO_4)^3-VAHLEEAEEGPEPAS, respectively, as determined with an automated peptide sequenator (Hewlett Packard G1005A) as described in "EXPERIMENTAL PROCEDURES." The reason why the peptide VAAIS(PO_4)^3-VA (peak α) was produced by our trypsin digestion is not yet understood. The enzyme incubated with PKA for 1 min in the absence of Ca^{2+}/calmodulin showed five major radioactive peaks, *1*, *2*, *3*, *4*, and *5*, and several minor peaks in the first HPLC (Fig. 2C). Among the five major peaks, peaks *1*, *2*, *3*, and *4* were eluted as single peaks upon the second HPLC (data not shown) with amino acid sequences of RPT(PO_4)^3-IESHH, AAS(PO_4)^3-VIPGSASR, KFS(PO_4)^3-LQER, and AAS(PO_4)^3-VIPGSASRPTPVRP, respectively. Peak *5* was separated into two radioactive peaks, *c* and *d*, upon the second HPLC (Fig. 2F), and their amino acid sequences were S(PO_4)^3-FGNPFEPQAR and SMS(PO_4)^3-APGN, respectively. The enzyme incubated with PKA for 5 min in the presence of Ca^{2+}/calmodulin also showed five major peaks, *1*, *2*, *3*, *4*, and *5*, and several minor peaks in the first HPLC (Fig. 2B). Among them, peaks *1*, *2*, *3*, and *4* were eluted as single peaks with elution positions coinciding with those of peaks *1*, *2*, *3*, and *4* in Fig. 2C, respectively, upon the second HPLC (date not shown). Peak *5* was separated into four peaks, *a*, *b*, *c*, and *d*, upon the second HPLC (Fig. 2E), two

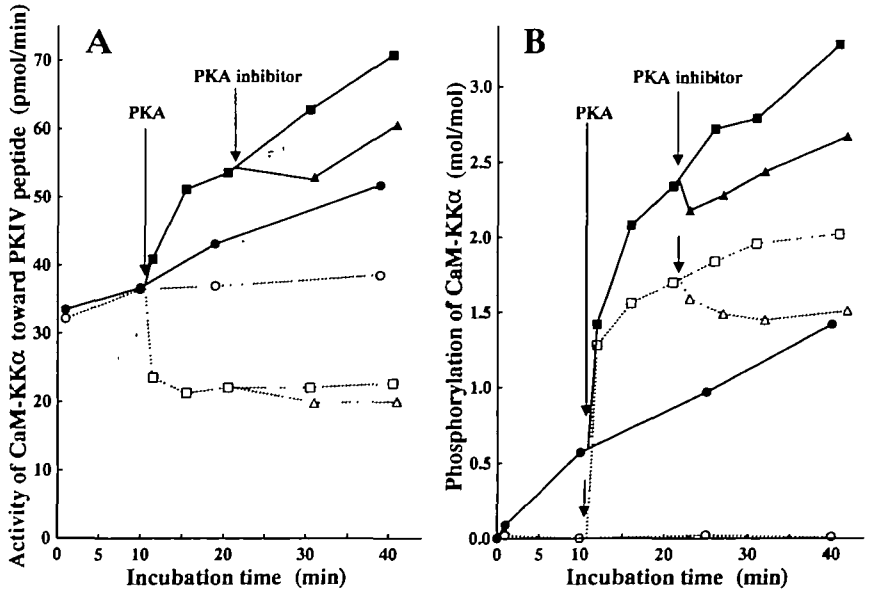

Fig. 1. **Effect of phosphorylation of CaM-kinase kinase α by PKA on the activity toward a peptide substrate.** (A) Approximately 18 μg/ml CaM-kinase kinase α was incubated at 30°C in the presence (●, ■) or absence (○, □) of 0.2 mM $CaCl_2$ and 1 μM calmodulin in a reaction mixture containing 50 mM Mops-NaOH (pH 7.0 at 30°C), 2 mM dithiothreitol, 5 mM $Mg(CH_3COO)_2$, 0.1 mM ATP, and 0.1 mM EGTA. At 10.5 min, 0.5 μg/ml PKA was added (■, □) and at 21 min, 1 μM PKA inhibitor was added (▲, △). At the indicated times, aliquots were withdrawn and the activity of CaM-kinase kinase α was measured for 1 min at 30°C in the standard phosphorylation mixture containing 0.2 mM PKIV peptide as a substrate, as described under "EXPERIMENTAL PROCEDURES." (B) Approximately 18 μg/ml CaM-kinase kinase α was incubated at 30°C in the presence (●, ■) or absence (○, □)) of Ca^{2+}/calmodulin as described above, except that radioactive [γ-^{32}P]ATP (about 2,000 cpm/pmol) was used. At 11 min, 0.5 μg/ml PKA was added (■, □) and at 22 min, 1 μM PKA inhibitor was added (▲, △). At the indicated times, aliquots were withdrawn and the incorporation of [^{32}P]phosphate into the protein was determined by the 3MM paper method.

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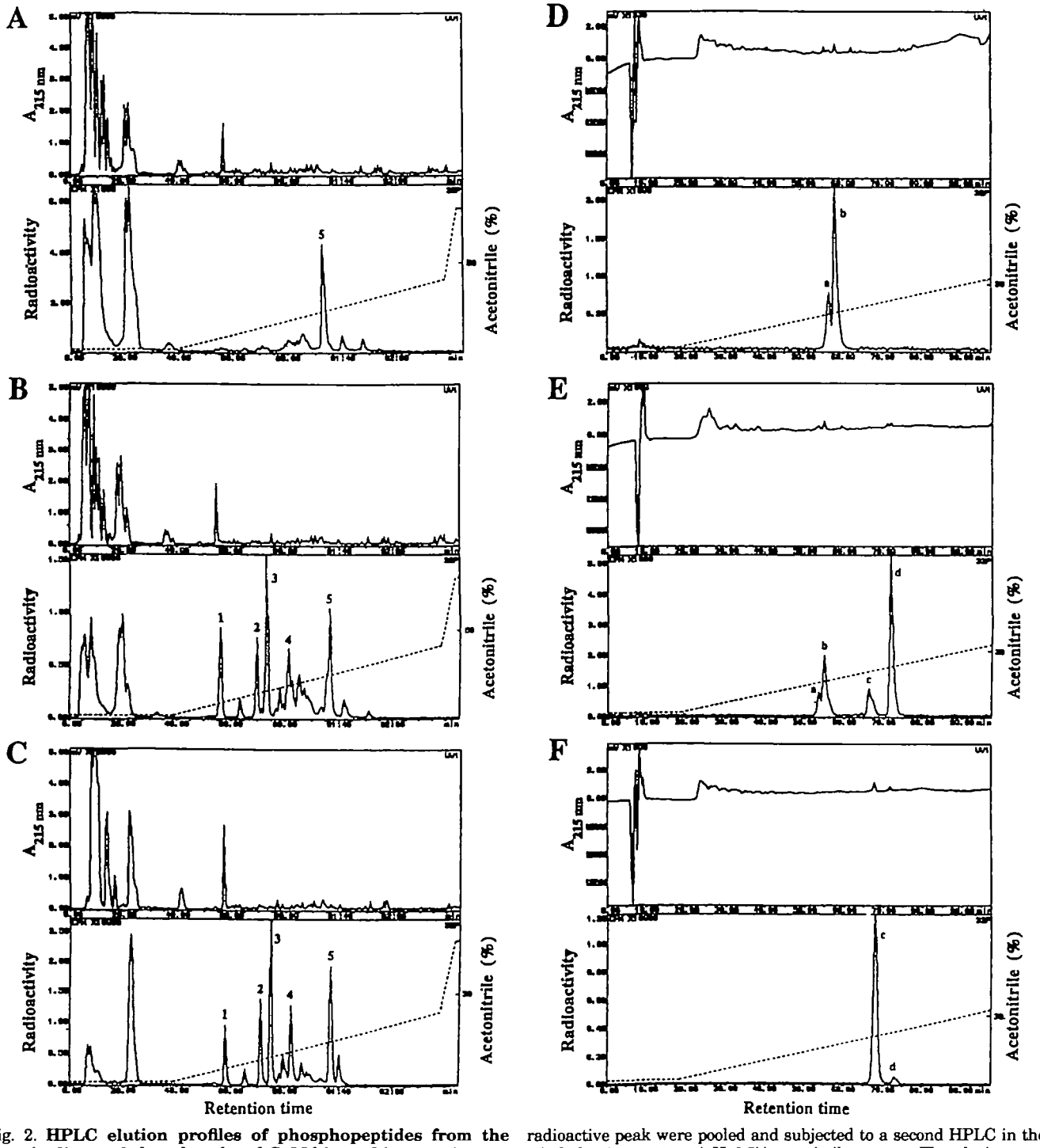

Fig. 2. **HPLC elution profiles of phosphopeptides from the trypsin digest of phosphorylated CaM-kinase kinase α.** Approximately 100 μg (20 μg/ml) of CaM-kinase kinase α was incubated at 30°C for 5 min without (A) or with (B) 2.5 μg (0.5 μg/ml) of PKA in the presence of Ca^{2+}/calmodulin, or for 1 min with PKA in the absence of Ca^{2+}/calmodulin (C) in the standard phosphorylation mixture, as described under "EXPERIMENTAL PROCEDURES." The amounts of phosphate incorporated into protein were about 0.27 mol/mol of enzyme after incubation for 5 min without PKA (plus calcium), 1.45 mol/mol after incubation for 5 min with PKA (plus calcium), and 1.67 mol/mol after incubation for 1 min with PKA (minus calcium), respectively, as measured by the 3MM paper method. The trypsin digests of the radioactive proteins were subjected to HPLC in the triethylamine phosphate (pH 3)/acetonitrile system, as described under "EXPERIMENTAL PROCEDURES." The fractions corresponding to each radioactive peak were pooled and subjected to a second HPLC in the triethylamine acetate (pH 6.5)/acetonitrile system. The elution profiles of peaks 5 in A, B, and C are given in D, E, and F, respectively. Peaks *1*, *2*, *3*, and *4* of B were eluted as single peaks with the same retention times as those of C in the second HPLC (data not shown). The recoveries of radioactivity upon the HPLC of A, B, C, D, E, and F were 90, 90, 92, 88, 88, and 88%, respectively. The amino acid sequences for the peptides from peaks *a* [VAAIS$(PO_4)^3$-VA] and *b* [VAAIS$(PO_4)^3$-VAHLEEAEEGPEPAS] of D, peaks *1* [RPT$(PO_4)^3$-IESHH], *2* [AAS-$(PO_4)^3$-VIPGSASR], *3* [KFS$(PO_4)^3$-LQER], and *4* [AAS$(PO_4)^3$-VIPG-SASRPTPVRP] of B, peak *d* [SMS$(PO_4)^3$-APGN] of E, and peak *c* [S$(PO_4)^3$-FGNPFEPQAR] of F were determined by a peptide sequenator as described under "EXPERIMENTAL PROCEDURES," and the peptides from peaks *1*, *2*, *3*, and *4* of C, peaks *a*, *b*, and *c* of E, and peak *d* of F were identified by their HPLC retention times.

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peaks, *a* and *b*, coinciding with peaks *a* and *b* in Fig. 2D, and the other two peaks, *c* and *d*, coinciding with peaks *c* and *d* in Fig. 2F, respectively. Comparison of the amino acid sequences of the isolated phosphopeptides with the predicted amino acid sequence of CaM-kinase kinase α (*11, 12*) indicates that the phosphorylation sites may be Thr^{108} for phosphopeptide *1*, Ser^{52} for peptides *2* and *4*, Ser^{74} for peptide *3*, Ser^{24} for peptides *a* and *b*, Ser^{458} for peptide *c*, and Ser^{475} for peptide *d*. The amounts of phosphate incorporated into the respective phosphorylation sites were calculated from the total amounts of [^{32}P]phosphate incorporated into the enzyme, the recoveries of radioactivity upon HPLC, and the percentages of radioactivity recovered in the fractions corresponding to the respective peaks, as summarized in Fig. 3. The gradual incorporation of phosphate into CaM-kinase kinase α observed when the enzyme was incubated in the presence of Ca^{2+}/calmodulin appeared to occur as a result of autophosphorylation, since no phosphate incorporation was observed when the enzyme was incubated in the absence of Ca^{2+}. Analysis of the tryptic digest of the autophosphorylated enzyme by HPLC revealed one major phosphorylation site at Ser^{24}, but the amount of phosphate incorporated into Ser^{24} accounted for only 30% of the total amount of phosphate incorporated into the enzyme, suggesting that autophosphorylation might occur at many unidentified sites at much slower rates than that at Ser^{24}. The extent of phosphorylation at Ser^{24} was not affected by PKA, suggesting that Ser^{24} is not a PKA phosphorylation site. Thus, five amino acids in CaM-kinase kinase α, Ser^{52}, Ser^{74}, Thr^{108}, Ser^{458}, and Ser^{475}, were identified as PKA phosphorylation sites. Four of these sites, Ser^{52}, Ser^{74}, Thr^{108}, and Ser^{458}, were more quickly phosphorylated in the absence of Ca^{2+} than in its presence, and only Ser^{475} was much more potently phosphorylated in the presence of Ca^{2+}/calmodulin. It is of particular interest that PKA phosphorylation occurred only slightly on Ser^{458} in the presence of Ca^{2+}/calmodulin and on Ser^{475} in its absence, because the phosphorylation of the enzyme by PKA resulted in an increase in enzyme activity in the presence of Ca^{2+}/calmodulin but a decrease in its absence. Incubation of the enzyme with PKA in the absence of Ca^{2+}/calmodulin caused a rapid decrease in activity, reaching a minimum level within 1 min as shown in Fig. 1A, but the extent of phosphorylation of the five PKA phosphorylation sites, Ser^{52}, Ser^{74}, Thr^{108}, Ser^{458}, and Ser^{475}, were only 0.39, 0.38, 0.13, 0.29, and 0.01 mol/mol, respectively, after incubation for 1 min, as shown in Fig. 3, suggesting that the rapid inactivation can not be accounted for by phosphorylation at only one or two sites. The phosphorylation of the enzyme by PKA in the absence of Ca^{2+}/calmodulin occurred at a very rapid rate for the initial 1 min (1.28 mol phosphate/mol of the enzyme/min), causing a large decrease in enzyme activity, and then at a much slower rate for an additional 30 min (0.74 mol phosphate/mol of the enzyme/30 min), causing no significant decrease in activity (Fig. 1). Thus, the enzyme appears to be converted from an active form to a less active form by a

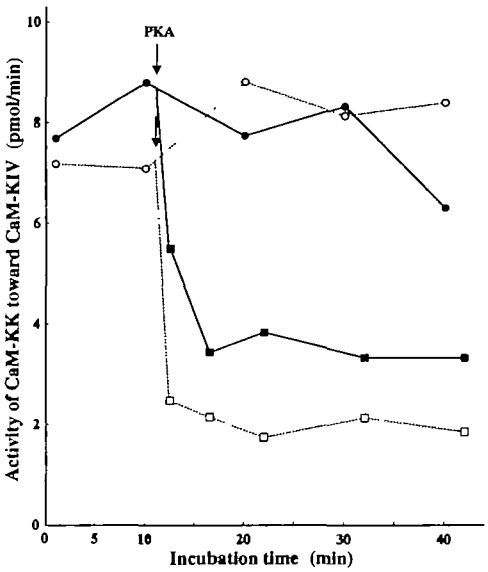

Fig. 4. **Effect of PKA on the activity of CaM-kinase kinase α assayed with CaM-kinase IV.** Approximately 5 μg/ml CaM-kinase kinase α was incubated at 30°C in the presence (●, ■) or absence (○, □) of 0.2 mM $CaCl_2$ and 1 μM calmodulin in reaction mixtures containing 50 mM Mops-NaOH (pH 7.0 at 30°C), 2 mM dithiothreitol, 5 mM $Mg(CH_3COO)_2$, 0.1 mM ATP, and 0.1 mM EGTA. At 11.5 min, 0.5 μg/ml PKA was added (■, □). At the indicated times, aliquots were withdrawn and the activity of CaM-kinase kinase α was measured for 1 min at 30°C in the standard phosphorylation mixture containing 50 μg/ml (about 1 μM) CaM-kinase IV(K_{71}R) as a substrate, as described under "EXPERIMENTAL PROCEDURES," except that the concentration of calmodulin in the reaction mixture was increased to 2 μM.

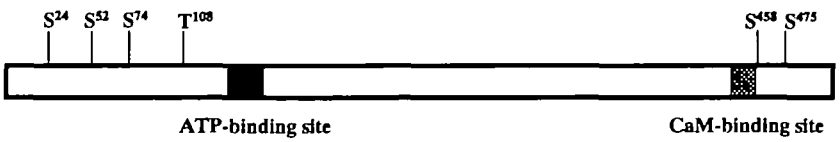

Fig. 3. **Summary of the phosphorylation of CaM-kinase kinase α.** The amounts of phosphate incorporated into CaM-kinase kinase α at each phosphorylation site were calculated from the total amounts of [^{32}P]phosphate incorporated into the enzyme, the recoveries of the radioactivity upon HPLC, and the percentages of radioactivity recovered in the fractions corresponding to each peak.

Incubation	[^{32}P]Phosphate incorporated at (mol Pi / mol CaM-kinase kinase α)						total
	S^{24}	S^{52}	S^{74}	T^{108}	S^{458}	S^{475}	
+Ca^{2+}/-PKA, for 5 min	0.08	-	-	-	-	-	0.27
+Ca^{2+}/+PKA, for 5 min	0.08	0.32	0.24	0.13	0.04	0.13	1.45
-Ca^{2+}/+PKA, for 1 min	-	0.39	0.38	0.13	0.29	0.01	1.67

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conformational change induced upon phosphorylation of any of several phosphorylation sites by PKA, and the conformational change induced by the phosphorylation at one site may interfere with the phosphorylation at other site(s) on the enzyme.

In contrast to the results obtained in assays using PKIV peptide as a substrate, the activity of CaM-kinase kinase α incubated under phosphorylation conditions to phosphorylate CaM-kinase IV(K_{71}R), a mutant in which Lys^{71} involved in ATP-binding was replaced by arginine to prevent autophosphorylation, in both the presence and absence of Ca^{2+}/calmodulin was decreased, as shown in Fig. 4. The decrease in activity caused by incubation with PKA in the absence of Ca^{2+} reached a near maximal level within 1 min (Fig. 4), and the rapid time course of the decrease was similar to that of the activity assayed with PKIV peptide as a substrate (Fig. 1A). In contrast, the time course of the decrease in activity assayed with CaM-kinase IV(K_{71}R) caused by incubation with PKA in the presence of Ca^{2+}/calmodulin (Fig. 4) was much more rapid than the increase in activity assayed with PKIV peptide (Fig. 1A), indicating that the changes in the activities are caused by different mechanisms. Wayman *et al.* (*15*) have reported that the phosphorylation of CaM-kinase kinase α by PKA in the absence of Ca^{2+}/calmodulin results in a 50–75% inhibition of activity, partly owing to the suppression of calmodulin-binding by the phosphorylation of Ser^{458}.

Since CaM-kinase kinase α phosphorylates CaM-kinase IV on Thr^{196} (*7, 8*) and CaM-kinase I on Thr^{177} (*9, 10*), and thereby activates these down-stream protein kinases, the activity of CaM-kinase kinase α can be measured by the activation of the down-stream CaM-kinases. When the activity of CaM-kinase kinase a was measured by the activation of CaM-kinases IV and I, the activity decreased after incubation with PKA for 10 min in both the presence and absence of Ca^{2+}/calmodulin, as shown in Fig. 5 (A and B), in agreement with the results obtained using CaM-kinase IV(K_{71}R) as a substrate (Fig. 4), indicating that the activities of CaM-kinase kinases α toward both CaM-kinases IV and I are regulated in a similar manner by PKA. The question of whether CaM-kinase kinase β, an isoform of CaM-kinase kinase α, is also regulated by PKA was examined, as shown in Fig. 5C. When the activity of CaM-kinase kinase β was measured by the activation of CaM-kinase IV, the activity was not significantly affected by incubation with PKA in the presence of Ca^{2+}/calmodulin, in contrast to CaM-kinase kinase α. When the enzyme was incubated with PKA in the absence of Ca^{2+}/calmodulin for 10 min, the activity was slightly decreased, but the time course of the decrease was much slower than that observed for CaM-kinase kinase α: no significant decrease in the activity of CaM-kinase kinase β was observed after incubation for 1min (data not shown). Thus, CaM-kinase kinases α and β appear to be regulated by PKA in quite different manners. Incubation of CaM-kinase kinase β with PKA in the absence of Ca^{2+} did not cause the rapid incorporation of phosphate as observed with CaM-kinase kinase α, but a gradual incorporation, the extent being only about 0.6 mol of phosphate/mol of enzyme even after incubation for 1 h (data not shown). Incubation in the presence of Ca^{2+}/calmodulin without PKA caused greater incorporation of phosphate than that observed with the α isoform, the extent being about 2.4 mol/mol after incubation for 1 h, and only a slight acceleration in phosphate incorporation by PKA, the increase in the phosphate incorporation by PKA being about only 0.28 mol phosphate/mol enzyme after incubation for 1 h (data not shown). Thus, CaM-kinase kinase β is more rapidly autophosphorylated but far less rapidly phosphorylated by PKA than CaM-kinase kinase α. The tryptic digest of CaM-kinase kinase β autophosphorylated by incubation for 1 h in the presence of Ca^{2+}/calmodulin was subjected to reverse-phase HPLC followed by analysis on an automated

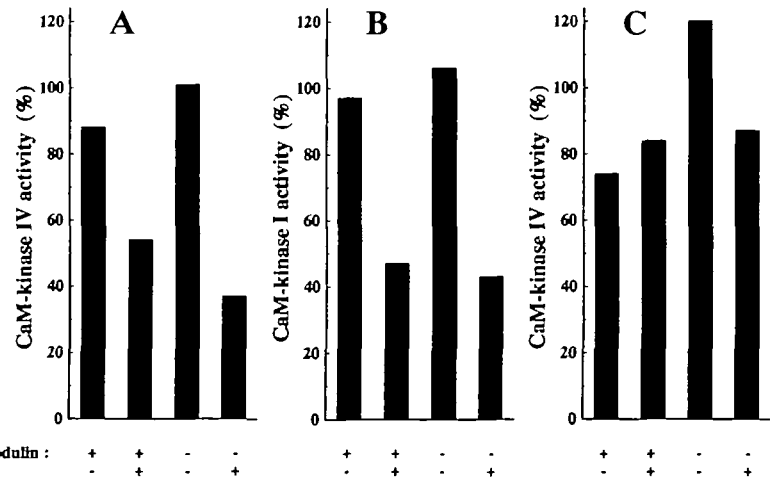

Fig. 5. **Effects of PKA on the activities of CaM-kinase kinases α and β.** (A) Approximately 100 ng of CaM-kinase kinase α (2 μg/ml) was incubated with or without 25 ng of PKA in the presence or absence of 0.2 mM $CaCl_2$ and 1 μM calmodulin, in a final volume of 50 μl of reaction mixture containing 50 mM Mops-NaOH (pH 7.0 at 30°C), 2 mM dithiothreitol, 5 mM $Mg(CH_3COO)_2$, 0.1 mM ATP, and 0.1 mM EGTA. After incubation for 10 min, a 5-μl sample (10 ng of CaM-kinase kinase α) was withdrawn and incubated at 30°C with 250 ng of CaM-kinase IV (10 μg/ml) in a final volume of 25 μl of reaction mixture containing 50 mM Mops-NaOH (pH 7.0 at 30°C), 2 mM dithiothreitol, 5 mM $Mg(CH_3COO)_2$, 0.1 mM ATP, and 1 μM calmodulin. After incubation for 1 min, an aliquot was withdrawn and the CaM-kinase IV activity was measured at 30°C for 1 min as described under "EXPERIMENTAL PROCEDURES," using 40 μM syntide-2 as a substrate. (B) After incubation of CaM-kinase kinase α with or without PKA as described above, a 3-μl sample (6 ng of CaM-kinase kinase α) was withdrawn and incubated with 2 μg of CaM-kinase I (10 μg/ml) in a final volume of 200 μl of reaction mixture containing 50 mM Mops-NaOH (pH 7.0 at 30°C), 2 mM dithiothreitol, 5 mM $Mg(CH_3COO)_2$, 0.1 mM ATP, and 1 μM calmodulin. After incubation for 1 min at 30°C, an aliquot was withdrawn and the CaM-kinase I activity was measured at 30°C for 1 min using 40 μM syntide-2 as a substrate. (C) After incubation of 100 ng of CaM-kinase kinase β (2 μg/ml) with or without 25 ng of PKA as described above, a 5-μl sample (10 ng of CaM-kinase kinase β) was withdrawn and incubated with 250 ng of CaM-kinase IV (10 μg/ml), and then the CaM-kinase IV activity of an aliquot was measured as described in (A). The results are expressed as percentages of the activities of CaM-kinase IV or I after incubation with unincubated CaM-kinase kinase α and β.

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TABLE I. **Summary of the kinetic parameters of CaM-kinase kinase α phosphorylated by PKA.** Kinetic parameters were obtained from the double-reciprocal plots shown in Fig. 6. The V_{max} values were calculated from the apparent V_{max} values on the basis of the Michaelis equation $v = V/(1 + K_m/[S])$.

	K_m for substrate (μM)	K_m for ATP (μM)	V_{max} (nmol/min/mg)
	Assayed with PKIV peptide		
Before incubation	270	290	2,310
After incubation with			
PKA (+Ca^{2+}/calmodulin) for 2 h	220	390	6,270
PKA (–Ca^{2+}/calmodulin) for 10 min	210	500	1,800
	Assayed with CaM-kinase IV(K_{71}R)		
Before incubation	0.45 (24 μg/ml)	29	2,130
After incubation with			
PKA (+Ca^{2+}/calmoduin) for 10 min	1.8 (97 μg/ml)	67	3,120
PKA (–Ca^{2+}/calmodulin) for 10 min	1.7 (89 μg/ml)	87	3,680

peptide sequenator, and Ser^{22}, Ser^{66}, Ser^{94}, Ser^{104}, Thr^{215}, and Thr^{517} were identified as autophosphorylation sites (data not shown).

The kinetic properties of CaM-kinase kinase α incubated under phosphorylation conditions were studied in comparison with those of the original enzyme using PKIV peptide and CaM-kinase IV(K_{71}R) as substrates, as shown in Fig. 6; the kinetic parameters obtained are summarized in Table I. When the enzyme activity was measured with PKIV peptide as a substrate, the enzymes before and after incubation with PKA in the presence and absence of Ca^{2+}/calmodulin showed similar K_m values for the peptide substrate. The enzyme after incubation with PKA in the presence of Ca^{2+}/calmodulin showed a V_{max} about three fold higher than the original enzyme, suggesting that the gradual increase in the PKIV peptide-phosphorylating activity of the enzyme caused by incubation with PKA in the presence of Ca^{2+}/calmo-dulin is due mainly to an increase in the turnover number of the enzyme. After incubation with PKA in the absence of Ca^{2+}/calmodulin, the enzyme showed a K_m about two times higher for ATP and a slightly lower V_{max} than the original enzyme, indicating that the rapid decrease in the PKIV peptide-phosphorylating activity caused by incubation with PKA in the absence of Ca^{2+}/calmodulin is due to both the decreased turnover number and the decreased affinity for ATP. In contrast, when the activity was measured using CaM-kinase IV(K_{71}R) as a substrate, incubation with PKA for 10 min in the presence or absence of Ca^{2+}/calmodulin caused the enzymes to exhibit K_m values about 4 times higher for the protein substrate, 2–3 times higher for ATP, and somewhat higher V_{max} values than the original enzyme, suggesting that the rapid decrease in the activity of the enzyme caused by incubation with PKA in the absence of Ca^{2+} and the somewhat slow decrease caused by incubation with PKA in the presence of Ca^{2+}/calmodulin are both due to the decreased affinities for both the protein substrate and ATP. Thus, the changes in the kinetic properties of the enzyme caused by incubation with PKA depend upon the substrates used for assay, indicating that PKA alters the substrate specificity of CaM-kinase kinase α.

DISCUSSION

The activity of CaM-kinase kinase α is increased upon incubation with PKA in the presence of Ca^{2+}/calmodulin, but decreased upon incubation with PKA in its absence, when assayed using PKIV peptide as the substrate, while the activity assayed using CaM-kinase IV as the substrate decreases upon incubation with PKA irrespective of the presence or absence of Ca^{2+}/calmodulin. To define the detailed mechanism involved in the regulation of CaM-kinase kinase α by PKA, we initially attempted to determine the phosphorylation sites responsible for the decrease and increase in the PKIV peptide-phosphorylating activity of the enzyme. Analysis of the tryptic digest of the phosphorylated enzyme revealed six phosphorylation sites for CaM-kinase kinase α, Ser^{24} as a autophosphorylation site, and Ser^{52}, Ser^{74}, Thr^{108}, Ser^{458}, and Ser^{475} as PKA phosphorylation sites. Decreases in both the PKIV peptide-phosphorylating activity and CaM-kinase IV-phosphorylating activity of the enzyme caused by incubation with PKA in the absence of Ca^{2+} reached a maximal level within 1 min (Figs. 1A and 4), but none of the major five phosphorylation sites for PKA was fully phosphorylated at 1 min as summarized in Fig. 3, suggesting the involvement of phosphorylation at several sites in the decrease in enzyme activity. The rapid phosphorylation of the enzyme by PKA in the absence of Ca^{2+}/calmodulin for the initial 1 min, causing a large decrease in enzyme activity, was followed by a much slower phosphorylation for an additional 30 min, causing no significant change in activity (Fig. 1), suggesting that the phosphorylation at one site of the enzyme might interfere with the phosphorylation at other site(s). The fact that the extents of phosphorylation on Ser^{52}, Ser^{74}, and Ser^{458} are much higher than those on the other two sites (Thr^{108} and Ser^{475}) suggests the possible importance of the three sites in decreasing the enzyme activity. However, Ser^{52} and Ser^{74} are also relatively highly phosphorylated in the enzyme whose PKIV peptide-phosphorylating activity is increased by incubation with PKA in the presence of Ca^{2+}/calmodulin for 5 min, raising doubts concerning the importance of the phosphorylation of Ser^{52} and Ser^{74} in decreasing the PKIV peptide-phosphorylating activity. Incubation with PKA in the presence of Ca^{2+}/calmodulin results in an increase in the PKIV peptide-phosphorylating activity but a decrease in the CaM-kinase IV-phosphorylating activity, and the time course of the increase in PKIV peptide-phosphorylating activity (Fig. 1A) is much slower than that of the decrease in CaM-kinase IV-phosphorylating activity (Fig. 4), indicating that the changes in the two activities caused by incubation with PKA in the presence of Ca^{2+}/calmodulin are not the results of the same event. In contrast, the time course of the decrease in the PKIV peptide-phosphorylating activity caused by incubation with PKA in the absence of Ca2+/calmodulin was the same as that of the decrease in

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the CaM-kinase IV-phosphorylating activity, suggesting that the changes in the two activities caused by incubation with PKA in the absence of Ca^{2+}/calmodulin are due to the same event. Thus, the other phosphorylation site, Ser^{458}, among the three sites well phosphorylated by PKA in the absence of Ca^{2+}/calmodulin, may be partly involved in the decreases in both the PKIV peptide- and CaM-kinase IV-phosphorylating activities. On the other hand, the kinetic analysis using CaM-kinase IV(K_{71}R) as a substrate (Fig. 6 and Table I) revealed that the decrease in the CaM-kinase IV-phosphorylating activity of the enzymes incubated with PKA in both the presence and absence of Ca^{2+}/calmodulin results from 4-fold and 2- to 3-fold increases in the apparent K_m values for CaM-kinase IV and ATP, respectively, although the V_{max} values are slightly higher than that of the original enzyme. The fact that both enzymes show similar kinetic properties suggests that the decreases in the CaM-kinase IV-phosphorylating activity caused by incubation with PKA in both the presence and absence of Ca^{2+}/calmodulin are due to the same event, the phosphorylation of Ser^{52}, Ser^{74}, and/or Thr^{108} (Fig. 3). Overall, the phosphorylation of Ser^{458} in CaM-kinase kinase α may be involved in the decrease in the PKIV peptide-phosphorylating activity, and phosphorylation(s) of Ser^{52}, Ser^{74}, Thr^{108}, and/or Ser^{458} may be involved in the decrease in the CaM-kinase IV-phosphorylating activity.

The phosphorylation of four of the five PKA phosphorylation sites were significantly suppressed by the presence of Ca^{2+}/calmodulin: especially the phosphorylation of Ser^{458} by PKA occurred only slightly in the presence of Ca^{2+}/calmodulin (Fig. 3). Such suppression of phosphorylation through PKA by Ca^{2+}/calmodulin is thought to be due to a conformational change in CaM-kinase kinase α resulting from the binding of calmodulin to the enzyme (not autophosphorylation of the enzyme), because the rate of autophosphorylation was very low compared to the phosphorylation by PKA. Only the remaining site, Ser^{475}, was significantly phosphorylated by PKA in the presence of Ca^{2+}/calmodulin

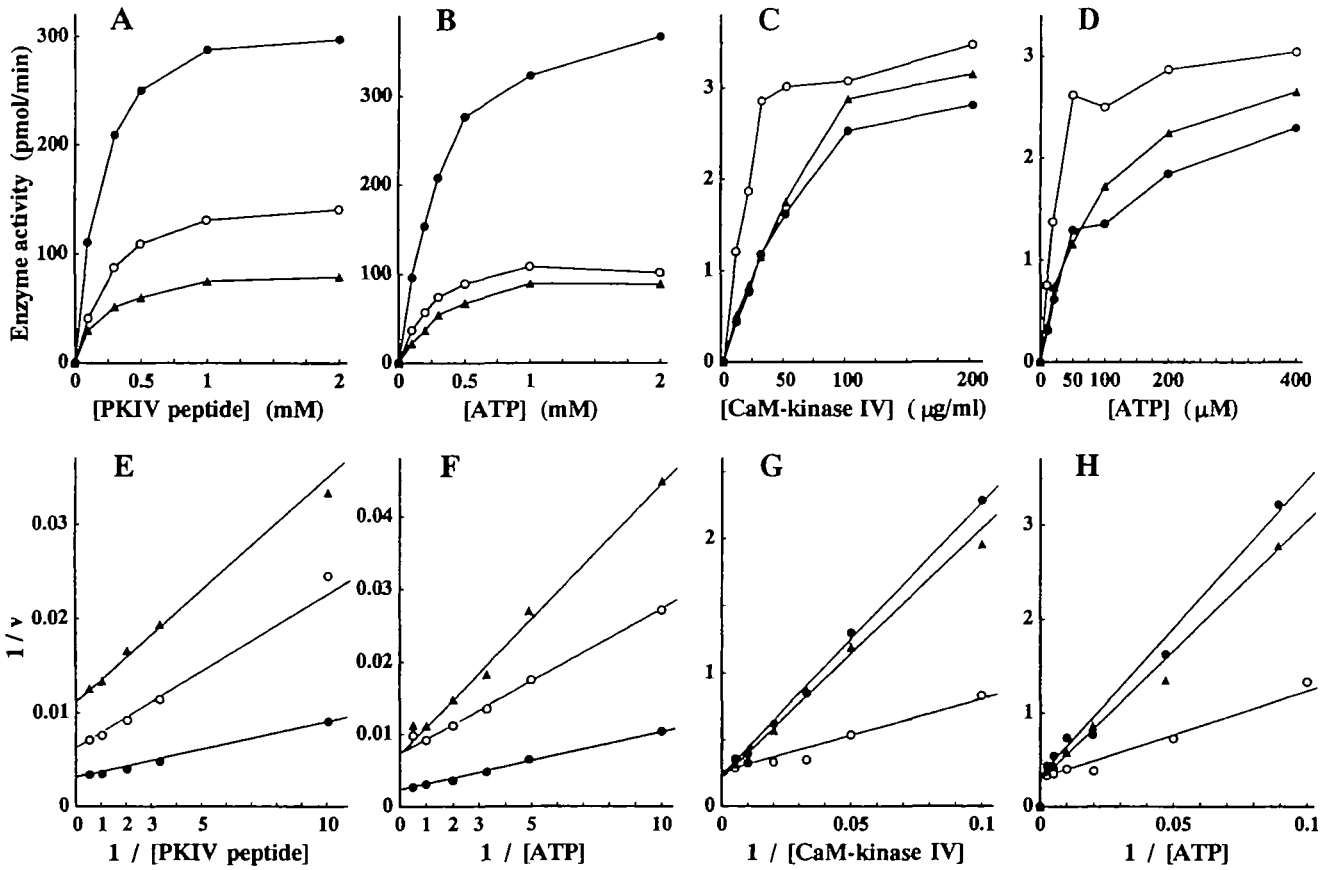

Fig. 6. **Effects of the concentrations the protein or peptide substrate and ATP on the activity of CaM-kinase kinase α phosphorylated by PKA.** (A and B) Approximately 80 μg/ml (about 1.4 μM) CaM-kinase kinase α was incubated at 30°C with or without 0.5 μg/ml PKA in the presence or absence of 0.2 mM $CaCl_2$ and 3 μM calmodulin in a reaction mixture containing 50 mM Mops-NaOH (pH 7.0 at 30°C), 2 mM dithiothreitol, 5 mM $Mg(CH_3COO)_2$, 0.1 mM ATP, and 0.1 mM EGTA. After incubation for 2 h with PKA in the presence of Ca^{2+}/calmodulin (●) or for 10 min with PKA in its absence (▲), or without incubation (○), an aliquot was withdrawn and the kinase activity was measured for 1 min at 30°C in the standard phosphorylation 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) (B). (C and D) Approximately 4 μg/ml CaM-kinase kinase α was incubated at 30°C for 10 min as described above, except that the concentration of calmodulin was 1 μM, and the kinase activity of an aliquot was measured for 1 min at 30°C in the standard phosphorylation 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) (C), or 50 μg/ml (about 1 μM) CaM-kinase IV(K_{71}R) and the indicated concentrations of [γ-^{32}P]ATP (about 1,000 cpm/pmol) (D) in the presence of 5 μM calmodulin. (E), (F), (G), and (H) show double reciprocal plots of the activities obtained from the data in (A), (B), (C), and (D), respectively.

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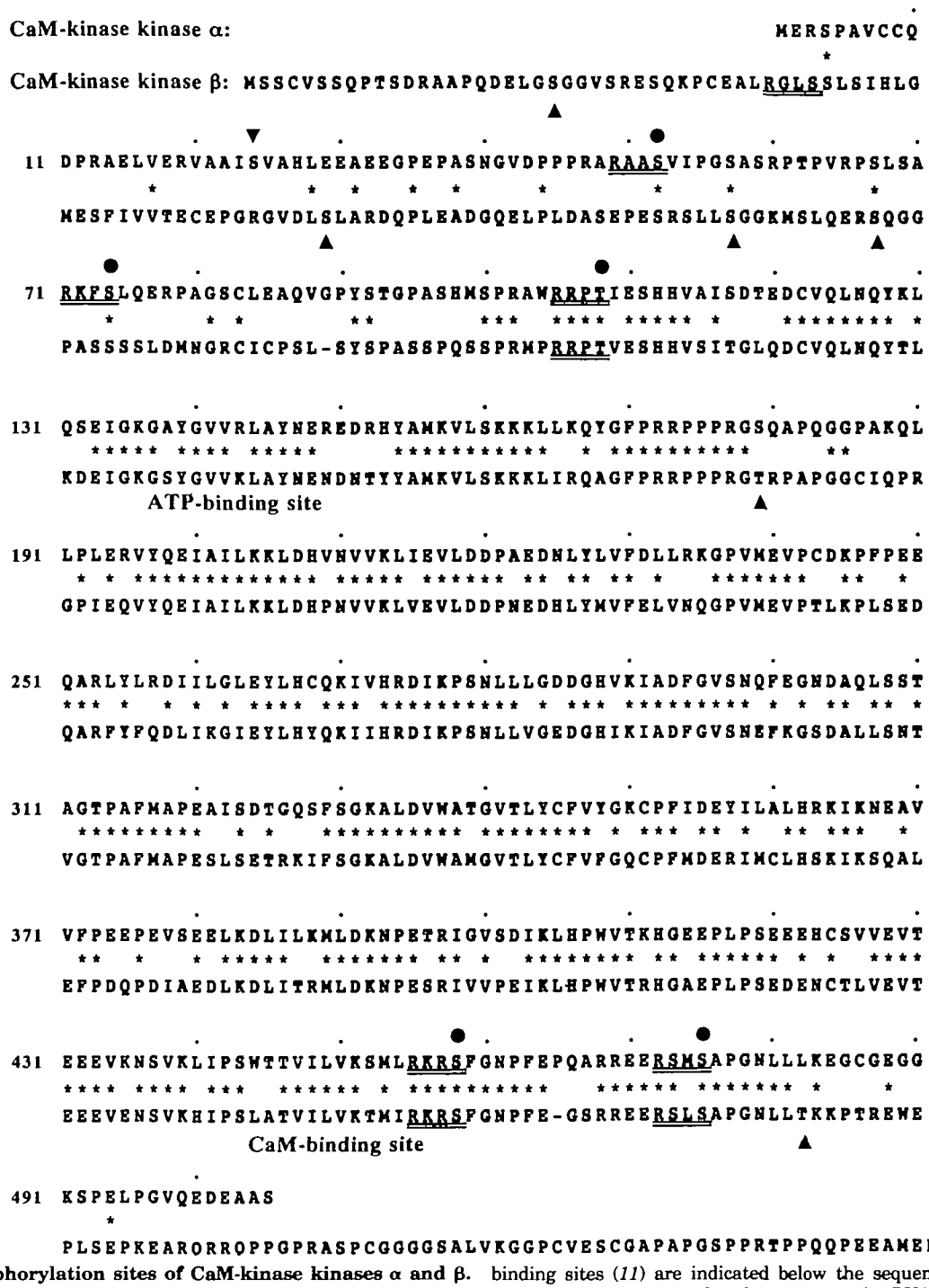

Fig. 7. **Phosphorylation sites of CaM-kinase kinases α and β.** The deduced amino acid sequences of rat CaM-kinase kinases α (*12*) and β (*26*) are aligned for maximal homology; matching amino acids are indicated by asterisks. The putative ATP-binding and calmodulin-binding sites (*11*) are indicated below the sequences. Amino acids phosphorylated by autophosphorylation or by PKA are indicated by the solid triangles or circles, respectively. The consensus sequences for phosphorylation by PKA, R-X-X-S/T, are underlined.

but very little in its absence. Among the six phosphorylation sites on CaM-kinase kinase α, the three sites corresponding to Thr^{108}, Ser^{458}, and Ser^{476} are conserved in CaM-kinase kinase β (Fig. 7), suggesting the importance of these phosphorylation sites. However, the acceleration of the phosphorylation of CaM-kinase kinase β by PKA was very small in both the presence and absence of Ca^{2+}/calmodulin compared to CaM-kinase kinase α, although CaM-kinase kinase β has one putative phosphorylation site for PKA near the amino-terminal end in addition to the above three sites, as shown in Fig. 7. In contrast to the phosphorylation by PKA, the rate of the autophosphorylation of CaM-kinase kinase β was much higher than that of CaM-kinase kinase α, although the major autophosphorylation site of the α isoform, Ser^{24}, is missing from the β isoform.

Thus, the mechanisms involved in changes in CaM-

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kinase kinase α activity caused by phosphorylation are very complex, and the phosphorylation site(s) responsible for the regulation by PKA could not be determined definitely in the present biochemical study. For the further characterization of the phosphorylation sites of the enzyme identified in the present study, mutational analysis of CaM-kinase kinase α was carried out, and the results are described in the following paper (*39*).

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