Inactivation of Calmodulin-dependent Protein Kinase IV by Autophosphorylation of Serine 332 within the Putative Calmodulin-binding Domain*

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When brain calmodulin-dependent protein kinase IV is incubated with calmodulin-dependent protein kinase IV kinase under the phosphorylation conditions in the presence of Ca\(^{2+}\)/calmodulin, rapid initial incorporation of 1 mol of phosphate into 1 mol of the enzyme by the action of the kinase occurs, resulting in marked activation of the enzyme, and the subsequent incorporation of more than 3 mol of phosphate by autophosphorylation occurs, resulting in no significant change in the activity (Okuno, S., Kitani, T., and Fujisawa, H. (1994) J. Biochem. (Tokyo) 116, 923-930; Okuno, S., Kitani, T., and Fujisawa, H. (1995) J. Biochem. (Tokyo) 117, 686-690). After the maximal phosphorylation, the continued incubation in the presence of excess EGTA resulted in additional autophosphorylation of the enzyme, leading to a complete loss of the Ca\(^{2+}\)/calmodulin-dependent activity, while causing no significant change in the Ca\(^{2+}\)/calmodulin-independent activity. The amino acid sequence analysis revealed that the autophosphorylation after removal of Ca\(^{2+}\) occurred on Ser\(^{332}\), Ser\(^{333}\), Ser\(^{337}\), and Ser\(^{342}\). Analysis by site-directed mutagenesis clearly showed that the autophosphorylation site responsible for the inactivation is Ser\(^{332}\). Thus, calmodulin-dependent protein kinase IV activated by the kinase may lose its Ca\(^{2+}\)/calmodulin-dependent activity by autophosphorylation on Ser\(^{332}\) located within the putative calmodulin-binding domain in the absence of Ca\(^{2+}\).

Calmodulin-dependent protein kinase IV (CaM-kinase IV) (also called CaM-kinase Gr) is a Ca\(^{2+}\)/calmodulin-dependent multifunctional protein kinase (1), which is enriched in the brain (2, 3) and T-lymphocytes (4, 5), and is therefore expected to play important roles in controlling a variety of functions in response to an increase in intracellular Ca\(^{2+}\) in the central nervous system and in the immune system. A recent discovery of CaM-kinase IV kinase in the brain (6) suggested the existence of a Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase cascade which is involved in the activation of CaM-kinase IV in the central nervous system. CaM-kinase IV kinase has more recently been purified to apparent homogeneity from rat cerebral cortex (7), and the activation mechanism of rat brain CaM-kinase IV by CaM-kinase IV kinase has been studied (8). The present study demonstrates that CaM-kinase IV activated by CaM-kinase IV kinase lost its Ca\(^{2+}\)/calmodulin-dependent activity by autophosphorylation on Ser\(^{332}\) (9) located within the putative calmodulin-binding domain in the absence of Ca\(^{2+}\). Thus, unlike CaM-kinase II, which is activated upon autophosphorylation on Thr\(^{286}\) in the autoinhibitory domain (9-11), CaM-kinase IV is activated upon phosphorylation by another Ca\(^{2+}\)/calmodulin-dependent protein kinase, CaM-kinase IV kinase, but after activation, both the CaM-kinases are inactivated by autophosphorylation on threonine or serine residue located within their calmodulin-binding domain in the absence of Ca\(^{2+}\) (Thr\(^{305}\) in CaM-kinase IIα (12-16) and Ser\(^{332}\) in CaM-kinase IV).

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ\(^{32}\)P]ATP (6,000 Ci/mmol) was from Amersham International. DEAE-cellulose (DE52), phosphocellulose paper (P81), and 3MP paper were from Whatman. Fluorotrans, a polyvinylidene difluoride membrane, was from Pall BioSupport. Sephacryl S-300 HR, blue-Sepharose CL-6B, phenyl-Sepharose CL-4B, and CNBr-activated Sepharose 4B were from Pharmacia Biotech Inc. A DEAE-Toyopearl 650 h column, a NPR column, and a TSK gel ODS-80Ts column were from Tosoh. NHS-LC-biotin and avidin conjugated with horseradish peroxidase were Pierce. Phosphoserine, phosphothreonine, and phosphotyrosine were from Sigma. Cellulose-precoated thin layer plates (TLC plate) were from Merck. Mitochondrial protease inhibitors (pepsatin A, leupeptin, antipain, and chymostatin) were from the Peptide Institute (Osaka, J. apaan). Lysyl endopeptidase from Actinobacter lyticus was purchased from Wako Chemicals. Syntide-2 (PLARTLSVAGLPG-KK) (17) was synthesized by the American Peptide Co. 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 (18), essentially as described by Gopalakrishna and Anderson (19). Biotinylated calmodulin was prepared from the recombinant chicken calmodulin as described by Mangés and Gnegy (20). Recombinant rat brain CaM-kinase IV \(\alpha\) expressed in E. coli was prepared as described previously (6). Recombinant rat brain CaM-kinase IV \(\alpha\) expressed in Sf9 cells was purified to apparent homogeneity as described previously (18). Rat brain CaM-kinase IV purified from rat cerebral cortex by a modification (7, 8) of the original method (1) was prepared in Sf9 cells was purified to apparent homogeneity as described previously (18). Rat brain CaM-kinase IV purified from rat cerebral cortex by a modification (7, 8) of the original method (1) was purified from rat cerebral cortex as described previously (7).

**Assay of CaM-kinase IV**—The activity of CaM-kinase IV was determined by measuring the phosphorylation of syntide-2 at 30 °C. The standard assay mixture contained, in a final volume of 50 μl, 50 mM Mops-KOH (pH 7.0), 5 mM Mg(CH\(_2\)COO)\(_2\), 2 mM dithiothreitol, either CaCl\(_2\) of a concentration 0.1 mM higher than the total concentrations of EGTA and EDTA or excess EGTA, 1 mM calmodulin, 0.1 mM [γ\(^{32}\)P]ATP (approximately 1 × 10\(^{6}\) cpm/nmol), 40 μM syntide-2, and a suitable amount of CaM-kinase IV. After incubation for 1 or 5 min, the incorporation of [γ\(^{32}\)P]phosphate into syntide-2 was determined by the phosphocellulose paper method of Raskas (21).
Phosphorylation of CaM-kinase IV—CaM-kinase IV was incubated with 0.2 μg/ml CaM-kinase IV kinase in the phosphorylation mixture containing 50 mM Mops-KOH (pH 7.0), 5 mM Mg(CH3COO)2, 2 mM dithiothreitol, 0.14 mM CaCl2, 1 μM calmodulin, and 0.5 mM ATP or \(\gamma\text{-}^{32}\text{P}}\text{ATP (0.4–1.1 × 10^4 cpm/μmol), at 30 °C. Incorporation of \(\gamma\text{-}^{32}\text{P}}\text{phosphate into CaM-kinase IV was determined by the 3MM paper method of Cheng and Reinmann (22), except that the filter papers were washed with ice-cold 10% trichloroacetic acid containing 2 mM ATP.}

Phosphoamino Acid Analysis—CaM-kinase IV phosphorylated with \(\gamma\text{-}^{32}\text{P}}\text{ATP was precipitated by the addition of ice-cold trichloroacetic acid to give a final concentration of 10%. The precipitate obtained on centrifugation was washed twice with ice-cold acetone and hydrolyzed with 10% trichloroacetic acid to give a final concentration of 10%. The precipitate obtained on centrifugation was washed with ice-cold acetone and hydrolyzed with 20% TFA for 2 h at 110 °C. The hydrolysate was electrophoresed on a cellulose-precoated thin layer plate in pH 3.5 buffer consisting of 0.2% pyridine/glacial acetic acid/H2O (10/100/1890), and \(32\text{P}\) radioactivity, and amino acids were detected by autoradiography and staining with ninhydrin, respectively (23).

Lysyl Endopeptidase Digestion of Phosphorylated CaM-kinase IV—Approximately 100 μg (10 μmol) of CaM-kinase IV was incubated with 2 μg (0.2 μg/ml) of CaM-kinase IV kinase, in a final volume of 10 ml, in the phosphorylation mixture containing 0.5 mM ATP or \(\gamma\text{-}^{32}\text{P}}\text{ATP (6.3 × 10^4 cpm/μmol) at 30 °C. After incubation for 60 min, EGTA was added to a final concentration of 0.24 mM, and the incubation was continued for an additional 60 min. The reaction was stopped by the addition of ATP and ice-cold trichloroacetic acid at final concentrations of 2 mM and 10%, respectively. The precipitate obtained on centrifugation was washed by sonication once in 1.6 ml and then seven times in 1.0 ml of ice-cold acetone, and it was air-dried at room temperature. The resulting protein was dissolved in 15 μl of 20 mM Tris-HCl (pH 8.0) containing 8 M urea and after incubation for 30 min at 37 °C, the mixture was diluted with 31 μl of 20 mM Tris-HCl (pH 8.0). After addition of 2.4 μg (7 μl) of lysyl endopeptidase (Achromobacter protease I), the mixture was incubated at 30 °C for 4 h. Another 2.4 μg (7 μl) of lysyl endopeptidase was added and the mixture was incubated for a further 14 h at 30 °C. Then 6 μl of 0.24 mM dithiothreitol (a final concentration of 20 mM) was added under an argon atmosphere. To the mixture was added 5 μl of 0.56 M iodoacetaime (40 mM), and the mixture was incubated for 30 min at room temperature in the dark. Some experiments were carried out on a scale of 1/20.

Reverse-phase HPLC Fractionation of Phosphopeptide—The mixture digested by lysyl endopeptidase as described above was filtered through a 0.22-μm filter and then loaded onto an C18 reverse-phase HPLC column (0.46 × 25 cm, TSK gel ODS-80Ts, Tosoh) equilibrated with 20 mM triethylamine acetate (pH 3). The column was eluted successively, at a flow rate of 0.5 ml/min, with a linear gradient of 0–45% (v/v) acetonitrile in the same buffer for 50 min and then 45–90% acetonitrile for 5 min and with 90% acetonitrile for 5 min and with 90% acetonitrile for 5 min. Peptide and phosphopeptide peaks were monitored spectrophotometrically at A265 and A360 using an on-line UV-monitor, Tosoh UV-8000, and radiochromatographically by Cerenkov counting using an on-line detector, Raytest Ramona 9000, respectively. The collected fractions corresponding to each peak were pooled and subjected to the second HPLC on the same column. The second column was equilibrated with 20 mM triethylamine acetate (pH 6.5) and eluted with a linear gradient of 0–7.2% (v/v) acetonitrile in the equilibration buffer for 5 min, then 7.2–16.2% acetonitrile for 90 min, and then 16.2–90% acetonitrile for 5 min, and with 90% acetonitrile for 5 min, or it was equilibrated with 0.1% trifluoroacetic acid and eluted with a linear gradient of 0–40% (v/v) acetonitrile in 0.1% trifluoroacetic acid for 40 min and then 40–100% acetonitrile for 5 min, and with 100% acetonitrile for 5 min.

Modification of Phosphoserine Residues for Sequence Analysis—The phosphopeptide purified as described above was dried in a 1.5-ml Eppendorf tube by centrifugation under vacuum and solubilized in 50 μl of the modification mixture, consisting of 60 μl of ethanethiol, 200 μl of 0.24 M dithiothreitol (a final concentration of 20 mM) and after incubation for 30 min at 50 °C, the mixture was centrifuged to get rid of the modification mixture, consisting of 60 μl of ethanethiol, 200 μl of 0.24 M dithiothreitol (a final concentration of 20 mM) and then 100 μl of 1 M HI site of the expression vector (6). The corresponding cDNA was inserted in the single-stranded DNA, which was used as a template for site-directed mutagenesis. The sequence of the mutagenic antisense oligonucleotides used were: 5'-TTGGTGGCGCATTCCTGGC-3' (S332A); 5'-TTGGCTCTGTATCGCGTG-3' (S339D); 5'-TGCGGCTCCTGGCTG-3' (S333A); 5'-GGCTGGAGATGCACCA-3' (S337D); 5'-GGCTGGAGATGCACCA3' (S337A); 5'-CCAGCGCTACACAC-3' (S341D); 5'-CCAGCGCTACACAC-3' (S341A). Site-directed mutagenesis was performed using the mutan-K kit (TAKARA), essentially according to the method of Kunkel et al. (26, 27). The mutations were confirmed by dideoxynucleotide sequencing (28). The mutant cDNAs were inserted into the Smal-PstI site of the wild-type CaM-kinase IV cDNA in pET11a expression vector, and the constructs were transformed into E. coli strain BL21(DE3) (29). The bacteria were grown to an \(A_{600}\) of 0.6 to 1.0 at 30 °C in 50 ml of M9SB medium containing 200 μg/ml ampicillin, and then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. After 4 h, the bacteria were harvested by centrifugation, suspended in 1 ml of 20 M Tris-HCl buffer (pH 7.5 at 4 °C) containing 1 mM phenylmethylsulfonyl fluoride, and 10 μM each of microbial protease inhibitors (leupeptin, pepstatin, antipain A, and chymostatin), and then disrupted by sonic oscillation. The residue was removed by centrifugation to generate crude extracts.

Other Analytical Procedures—SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (30). Gel overlay assay by biotinylated calmodulin was carried out as described by Kin- caid et al. (31). The concentration of proteins was determined by the method of Lowry et al. (32), as modified by Peterson (33) with bovine serum albumin as a standard.
RESULTS

Activation of CaM-kinase IV by CaM-kinase IV Kinase followed by Inactivation upon Autophosphorylation in the Absence of Ca\(^{2+}\)—When recombinant rat brain CaM-kinase IV was expressed in insect Sf9 cells and incubated with CaM-kinase IV kinase purified from rat cerebral cortex under the phosphorylation conditions in the presence of Ca\(^{2+}\)/calmodulin, rapid marked activation and rather slow phosphorylation of the enzyme occurred as shown in Fig. 1, in agreement with our earlier observation (8). After the phosphorylation had reached a maximum level, the extent of the phosphorylation being approximately 4.2 mol of phosphate/mol of the enzyme, the addition of excess EGTA to remove free Ca\(^{2+}\) resulted in an additional fairly slow incorporation of phosphate into the enzyme, the extent finally reaching approximately 7.2 mol of phosphate/mol of the enzyme (Fig. 1B), indicating that 3 mol of phosphate was incorporated into 1 mol of the enzyme in the presence of EGTA. The phosphorylation after removal of Ca\(^{2+}\) was accompanied by loss of the Ca\(^{2+}\)/calmodulin-dependent activity but by no significant loss of the Ca\(^{2+}\)/calmodulin-independent activity of the enzyme (Fig. 1A) and after incubation for 5 h, the enzyme became an almost completely Ca\(^{2+}\)/calmodulin-independent form whose activity was 15 times higher than that of the original enzyme. When excess EDTA was added to the incubation mixture to chelate free Mg\(^{2+}\) at 5 min after the addition of EGTA, both the phosphorylation and inactivation of the enzyme in the presence of EGTA were completely blocked instantaneously. These results, taken together, suggest that the phosphorylation of CaM-kinase IV in the presence of EGTA resulted in the almost total loss of the Ca\(^{2+}\)/calmodulin-dependent activity, although not affecting the Ca\(^{2+}\)/calmodulin-independent activity. Similar results were obtained with CaM-kinase IV purified from rat brain (data not shown).

To characterize the mechanism of the phosphorylation causing the inactivation of the enzyme, effect of varying the concentration of the enzyme on the phosphorylation rate was investigated as shown in Fig. 2. The plot of the logarithm of the phosphorylation rate versus the logarithm of the enzyme concentration (van’t Hoff plot) (34, 35) gave a straight line with a slope of 1.2 (approximately 1), suggesting that the phosphorylation of CaM-kinase IV in the absence of Ca\(^{2+}\) causing the enzyme inactivation occurs through an intramolecular autophosphorylation mechanism. The fact that the rate of the phosphorylation after removal of Ca\(^{2+}\) was not affected by increasing the concentration of CaM-kinase IV kinase (data not shown) provides support for the autophosphorylation mechanism.

Identification of Autophosphorylation Sites in the Absence of Ca\(^{2+}\)—Analysis of the phosphorylated amino acids in CaM-kinase IV which had been phosphorylated by incubation with CaM-kinase IV kinase in the presence of Ca\(^{2+}\)/calmodulin or phosphorylated after the addition of EGTA, as shown in Fig. 3, indicated that CaM-kinase IV was much more strongly phos-
phorylated on serine residues than on other phosphorylatable amino acids in either case. In order to identify the phosphorylation sites of CaM-kinase IV after removal of Ca$^{2+}$, phosphorylated CaM-kinase IV was digested with lysyl endopeptidase and subjected to reverse-phase HPLC (Fig. 4), and the purified phosphopeptides were analyzed by a protein sequenator (Fig. 5). The HPLC analysis (in triethylamine phosphate, pH 3) of the enzyme, which had been phosphorylated with radioactive ATP for 60 min in the presence of CaM-kinase IV kinase and Ca$^{2+}$/calmodulin and then for another 60 min in the presence of excess EGTA, revealed four radioactive peaks, a big broad peak at about 48 min in B were subjected to the second HPLC in the triethylamine phosphate/acetonitrile solvent system. E and F, the radioactive fractions eluted as a big peak at about 48 min in B were subjected to the second HPLC in the trifluoroacetic acid/acetonitrile solvent system. G, the radioactive fractions eluted as a big peak at about 48 min in D were subjected to the second HPLC in the trifluoroacetic acid/acetonitrile solvent system. H, the radioactive fractions corresponding Peak E2 in B were subjected to the second HPLC in the triethylamine acetate/acetonitrile solvent system. Peptide (A, E, and inset in H) and phosphopeptide (B–D and F–H) peaks were monitored spectrophotometrically and radiometrically, respectively, as described under "Experimental Procedures." The broken lines indicate the concentrations of acetonitrile. The recoveries of radioactivity upon HPLC were 80–97%.

FIG. 4. Reverse-phase HPLC elution profiles of phosphopeptides from lysyl endopeptidase digest of phosphorylated CaM-kinase IV. A and B, brain CaM-kinase IV was incubated with CaM-kinase IV kinase in the phosphorylation mixture containing radioactive ATP for 60 min and then for another 60 min in the presence of excess EGTA. C, brain CaM-kinase IV was incubated with CaM-kinase IV kinase in the mixture containing radioactive ATP for 60 min and then for another 60 min in the mixture containing nonradioactive ATP in the presence of excess EGTA. After phosphorylation, the protein was digested with lysyl endopeptidase, and the resulting phosphopeptides were fractionated by HPLC in the triethylamine phosphate/acetonitrile solvent system, as described under "Experimental Procedures." E and F, the radioactive fractions eluted as a big peak at about 48 min in B were subjected to the second HPLC in the trifluoroacetic acid/acetonitrile solvent system. G, the radioactive fractions eluted as a big peak at about 48 min in D were subjected to the second HPLC in the trifluoroacetic acid/acetonitrile solvent system. H, the radioactive fractions corresponding Peak E2 in B were subjected to the second HPLC in the triethylamine acetate/acetonitrile solvent system. Peptide (A, E, and inset in H) and phosphopeptide (B–D and F–H) peaks were monitored spectrophotometrically and radiometrically, respectively, as described under "Experimental Procedures." The broken lines indicate the concentrations of acetonitrile. The recoveries of radioactivity upon HPLC were 80–97%.

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vation of the enzyme, were subjected to HPLC in triethylamine acetate, pH 6.5, six contiguous radioactive peaks, E2-1, E2-2, E2-3, E2-4, E2-5, and E2-6, were obtained (Fig. 4). Similar results were obtained with recombinant CaM-kinase IV expressed in Sf9 cells, except that a small radioactive peak, C2 (Fig. 4, B and C), was not observed with the recombinant enzyme, suggesting that C2 was derived from the amino-terminal serine-rich segment (MSCAGNDQAAASGSSSGGI-FRSPAAK) of CaM-kinase IVβ (36), because only this segment of 28 amino acids is deleted in CaM-kinase IVα (37, 38), respectively (Fig. 8). Since C1 contains only one serine residue, the phosphorylation site in C1 may be Ser437, consistent with our earlier observation (39). To determine the precise serine residues phosphorylated in phosphopeptides C3 (Fig. 5A), C3 (Fig. B), E1 (Fig. C), and E2-4 (Fig. D) and E2-6 (Fig. E) were subjected to amino acid sequence analysis after treatment with alkali ethanethiol, as described under “Experimental Procedures.” Recoveries of PTH-derivatives (open circles) and the relative amounts of S-ethylcysteine determined from the ratio toward the internal standard (closed circles) at each cycle are presented.

**Fig. 5.** Sequence analysis of the phosphopeptides. A, peptide C1 was subjected to amino acid sequence analysis. B–E, peptides C3 (B), E1 (C), E2-4 (D), and E2-6 (E) were subjected to amino acid sequence analysis after treatment with alkali ethanethiol, as described under “Experimental Procedures.” Recoveries of PTH-derivatives (open circles) and the relative amounts of S-ethylcysteine determined from the ratio toward the internal standard (closed circles) at each cycle are presented.

Analysis by Site-directed Mutagenesis—In order to establish which serine residue of Ser332, Ser333, Ser337, and Ser341 is responsible for the enzyme inactivation upon autophosphorylation in the absence of Ca2+, these four serine residues were replaced with alanine to eliminate their phosphorylation and with aspartic acid to mimic their phosphorylation. Fig. 6 shows the time course of changes in the enzyme activity of the recombinant wild-type and mutant enzymes during the incubation with CaM-kinase IV kinase in the presence of Ca2+/calmodulin followed by the incubation in the presence of excess EGTA. The reactions were carried out using the same amounts (2.8 μl) of the E. coli extracts. The activity of the wild-type enzyme was markedly increased with incubation under the phosphorylation conditions in the presence of CaM-kinase IV kinase and Ca2+/calmodulin, and the subsequent addition of excess EGTA re-
sulted in a time-dependent loss of Ca\(^{2+}\)/calmodulin-dependent activity without affecting the Ca\(^{2+}\)/calmodulin-independent activity (Fig. 6A), in accord with the result obtained with the recombinant Sf9 enzyme (Fig. 1A). In contrast, the mutant enzyme S332A, in which Ser\(^{332}\) was replaced with alanine, was markedly activated by incubation with CaM-kinase IV kinase under the phosphorylation conditions but the subsequent addition of EGTA caused no significant decrease in the enzyme activity (Fig. 6B), indicating the involvement of the phosphorylation of Ser\(^{332}\) in the inactivation of the enzyme after removal of Ca\(^{2+}\). The incubation of the mutant enzyme S332D, in which Ser\(^{332}\) was replaced with aspartic acid, with CaM-kinase IV kinase under the phosphorylation conditions resulted in a similar activation of Ca\(^{2+}\)/calmodulin-independent activity to that of the wild-type enzyme, but caused no marked activation of Ca\(^{2+}\)/calmodulin-dependent activity (Fig. 6C). These results suggest that Asp\(^{332}\) causes the enzyme to become insensitive to stimulation by Ca\(^{2+}\)/calmodulin by mimicking phospho-Ser\(^{332}\), confirming the importance of Ser\(^{332}\) in the enzyme inactivation. Replacements of Ser\(^{333}\), the amino acid residue next to Ser\(^{332}\), with alanine (Fig. 6D) or aspartic acid (Fig. 6E), and Ser\(^{337}\) and Ser\(^{341}\) with alanine or aspartic acid (data not shown), did not affect the time-dependent changes in the enzyme activity essentially. Thus, the phosphorylation of Ser\(^{332}\) appears to cause loss of the Ca\(^{2+}\)/calmodulin-dependent activity without affecting the Ca\(^{2+}\)/calmodulin-independent activity.

Since Ser\(^{332}\) is located within the putative calmodulin-binding domain of CaM-kinase IV (40, 41) (Fig. 8), the loss of the Ca\(^{2+}\)/calmodulin-dependent activity of the wild-type enzyme autophosphorylated in the presence of EGTA and the mutant S332D was thought to be due to loss of Ca\(^{2+}\)/calmodulin binding. Calmodulin overlay analysis (Fig. 7) showed that the wild-type enzyme, unphosphorylated or phosphorylated in the presence of Ca\(^{2+}\)/calmodulin, and the mutant enzyme S333D bound calmodulin in the presence of Ca\(^{2+}\), but the wild-type enzyme phosphorylated after addition of EGTA and the mutant S332D did not significantly bind calmodulin even in the presence of Ca\(^{2+}\). Thus, the autophosphorylation of CaM-kinase IV on Ser\(^{332}\) occurring only in the absence of Ca\(^{2+}\) appears to cause loss of the ability of the enzyme to bind calmodulin, thereby leading to loss of its Ca\(^{2+}\)/calmodulin-dependent activity. The shift in mobility on SDS-polyacrylamide gel electrophoresis of the enzyme upon phosphorylation in the presence of Ca\(^{2+}\)/calmodulin observed in Fig. 7A (lane 2) has been reported previously (42).

DISCUSSION

CaM-kinase IV is thought to play important roles in the functioning of Ca\(^{2+}\) in the central nervous system, along with another Ca\(^{2+}\)-responsive multifunctional protein kinase, CaM-
CaM-kinase II is activated upon Ca\(^{2+}\) and therefore the regulation of its activity is very important. Discovery of CaM-kinase IV kinase in the brain (6) provided insight into the mechanism by which CaM-kinase IV activity is regulated, and phosphorylation of CaM-kinase IV by CaM-kinase IV kinase has recently been demonstrated to cause a marked activation of the enzyme (8). Unlike CaM-kinase IV kinase, CaM-kinase IV kinase has recently been demonstrated to cause CaM-kinase IV loss of Ca\(^{2+}\)/calmodulin-dependent activity by a large number of studies occurring abundantly in the brain and activated upon phosphorylation by two contrasting mechanisms. After activation by Ca\(^{2+}\)/calmodulin-dependent autophosphorylation at Thr\(^{286}\) (9–11), the two Ca\(^{2+}\)/calmodulin-responsive multifunctional protein kinases occurring abundantly in the brain are activated upon phosphorylation, by two contrasting mechanisms. After activation by Ca\(^{2+}\)/calmodulin-dependent autophosphorylation at Thr\(^{286}\), CaM-kinase IV undergoes autophosphorylation at Thr\(^{305}\) in the absence of Ca\(^{2+}\), resulting in decrease in the Ca\(^{2+}\)/calmodulin-dependent activity without decrease in the Ca\(^{2+}\)/calmodulin-independent activity (12–16). The present study demonstrates that CaM-kinase IV loses its Ca\(^{2+}\)/calmodulin-dependent activity by a similar autophosphorylation mechanism.

When CaM-kinase IV was incubated with CaM-kinase IV kinase under the phosphorylation conditions in the presence of Ca\(^{2+}\)/calmodulin until the phosphorylation reached a maximum level (4.2 mol of phosphate/mol of enzyme), both the Ca\(^{2+}\)/calmodulin-dependent and independent activities were rapidly activated (Fig. 1) and phosphorylation of several serine residues (Ser\(^{8}\), Ser\(^{11}\), Ser\(^{15}\), etc.) in the segment of Val\(^{4}\)/Lys\(^{34}\), Ser\(^{437}\), and Ser\(^{353}\) was observed (Figs. 4 and 5). Although threonine residues (Thr\(^{196}\) and Thr\(^{286}\)) also have been reported to be phosphorylated in the presence of Ca\(^{2+}\)/calmodulin (42, 43), our phosphoamino acid analysis (Fig. 3) could not detect phosphothreonine, probably owing to a low ratio of phosphothreonine to phosphoserine in the phosphorylated enzyme preparation. When the incubation was continued after the addition of EGTA to remove free Ca\(^{2+}\) until the additional phosphorylation reached a maximum (additionally 3 mol of phosphate/mol of enzyme), the Ca\(^{2+}\)/calmodulin-dependent activity was decreased and finally completely lost (Fig. 1) and phosphorylation of Ser\(^{332}\), Ser\(^{333}\), Ser\(^{337}\), Ser\(^{341}\), and Ser\(^{353}\) was observed (Figs. 4 and 5). Thus, among many phosphorylation sites, four serine residues, Ser\(^{332}\), Ser\(^{333}\), Ser\(^{337}\), and Ser\(^{341}\), were phosphorylated only after removal of Ca\(^{2+}\).

Replacement of Ser\(^{332}\) with alanine by site-directed mutagenesis completely blocked the inactivation by the incubation after removal of Ca\(^{2+}\) (Fig. 6B), probably by elimination of the phosphorylation site responsive for the inactivation. Replacement of Ser\(^{332}\) with aspartic acid made the enzyme possess only very low activity of the Ca\(^{2+}\)/calmodulin-dependent activity even after activation by CaM-kinase IV kinase (Fig. 6C), probably by the action of Asp\(^{332}\) mimicking phospho-Ser\(^{332}\). Replacement of Ser\(^{333}\), Ser\(^{337}\), and even Ser\(^{337}\) next to Ser\(^{332}\) with alanine or aspartic acid had essentially no effect on the time course of the enzyme activity (Figs. 6, D and E). Thus, among four serine residues which were phosphorylated in the presence of EGTA, the phosphorylation of only Ser\(^{332}\) appears to cause the inactivation of Ca\(^{2+}\)/calmodulin-dependent activity, although the possibility of a little involvement of other phosphorylation sites in the inactivation cannot be excluded, because the mutant enzyme S332D activated by CaM-kinase IV kinase exhibited very little but significant Ca\(^{2+}\)/calmodulin-dependent activity, which was lost on incubation in the presence of EGTA (Fig. 6C). The rate of the inactivation appears to occur more slowly than that of the phosphorylation, judging from the result of Fig. 1, suggesting that the phosphorylation of Ser\(^{332}\) occurs relatively slowly. The finding that only Ser\(^{332}\) of the four serine residues which become phosphorylatable after removal of Ca\(^{2+}\) was not phosphorylated in phosphopeptide E2-4 (Fig. 5E), although all the four serine residues were phosphorylated in E2-6 (Fig. 5D), also suggests the slow phosphorylation of Ser\(^{332}\). The inactivation rate of the mutant enzyme S333A was much higher than that of the wild-type enzyme, and that of S333D was also significantly higher than that of the wild-type enzyme, as shown in Fig. 6. It is indicated that the adjacent amino acids affect the phosphorylation rate of Ser\(^{332}\).

As shown in Fig. 8, alignment of the amino acid sequences of CaM-kinase IV and CaM-kinase II showed that Ser\(^{332}\) may be located within or near the calmodulin-binding domain of CaM-kinase IV. The fact that replacement of Ser\(^{332}\) with aspartic acid strongly blocked the calmodulin binding indicates that Ser\(^{332}\) is located within the calmodulin-binding domain. In contrast, replacement of Ser\(^{333}\), the residue next to Ser\(^{332}\), with aspartic acid did not affect the calmodulin binding, indicating that this amino acid residue is not involved in the calmodulin binding.
A similar regulatory mechanism by which phosphorylation of serine or threonine residue located within a calmodulin-binding domain abolishes calmodulin binding, thereby leading to a loss of the Ca$$^{2+}$$/calmodulin-dependent activity, is also observed with CaM-kinase II; autophosphorylation of Thr$^{305}$ within the calmodulin-binding domain in CaM-kinase II leads to a loss of the Ca$$^{2+}$$/calmodulin-dependent activity (12–16). This together with the fact that Ser$^{332}$ is conserved in rat (36, 38), mouse (41), and human (44–46) CaM-kinase IV suggests that the inactivation of Ca$$^{2+}$$/calmodulin-dependent activity by autophosphorylation at a serine or threonine residue located within a calmodulin-binding domain in the absence of Ca$$^{2+}$$ may be a rather common and physiologically important regulatory mechanism for Ca$$^{2+}$$/calmodulin-dependent protein kinases.

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