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Ca^{2+}/Calmodulin-Dependent Protein Kinase Kinase Cascade

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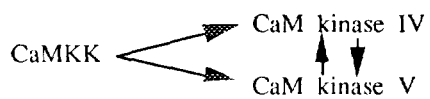
SUMMARY Recently we obtained evidence for the existence of Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK), which was partially purified in the process of the purification step of Ca^{2+}/calmodulin-dependent protein kinase V (CaM kinase V). This enzyme promoted phosphorylation of the autophosphorylation site on CaM kinase V and activated the activity (Mochizuki, H. et al. (1993) Biochem. Biophys. Res. Commun. 197, 1595-1600). Present study revealed that CaMKK also phosphorylated CaM kinase IV associated with the activation of its activity. Phosphorylation of CaM kinase IV by CaMKK occurred on multiple sites. Furthermore, CaM kinase IV and CaM kinase V phosphorylated each other which resulted in their activation. The phosphorylation site of CaM kinase V by CaM kinase IV was the same as the autophosphorylation site. Our study suggests the existence of a heretofore CaM kinases cascade consisting of a sequential activation of each CaM kinase.



Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases) play important roles as neurotransmitter release, long term potentiation, and gene expression (1-6). CaM kinase II is the most abundant and has to date been best characterized in rat brain (7, 8). CaM kinase II has a unique autophosphorylation manner. After autophosphorylation, CaM kinase II develops activity that no longer depends on Ca^{2+}/calmodulin. Its autonomous activities range from 20% to 80% of the full activity in the presence of Ca^{2+}/calmodulin (8, 9, 10). CaM kinase IV (or Gr) which is abundant in the brain (11) and thymus (12), is also multifunctional (13). After autophosphorylation, it also develops Ca^{2+}/calmodulin-independent activity (14, 15).

We reported the purification and characterization of CaM kinase V from rat cerebrum

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Abbreviations: CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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(16), which was widely distributed (17). The localization of CaM kinase V in rat brain was different from CaM kinase II more or less. Functions of CaM kinase V have not been identified yet. But CaM kinase V is able to phosphorylate a number of endogenous proteins of rat cerebral cortex, suggesting that CaM kinase V is a multifunctional type of protein kinase like CaM kinase II and IV and associated with neuronal functions (16).

Recently we reported CaM kinase kinase (CaMKK) which was copurified with CaM kinase V. CaMKK phosphorylated CaM kinase V and activated it (18). Gomez, N. et al. (19) reported protein kinase which phosphorylated mitogen activated protein kinase (MAP kinase) and suggested MAP kinases cascade. In the present work we discovered a new CaM kinases cascade, relationship among CaMKK, CaM kinase IV, and CaM kinase V.

MATERIALS AND METHODS

Materials Syntide-2 was purchased from Peptide Institute Inc. [γ-^{32}P]ATP was from ICN Biomedicals. ATP and bovine serum albumin were from Sigma. HEPES, Tween 20, and dithiothreitol were from Wako Pure Chemicals. Protein assay reagent was from Pierce and P-81 phosphocellulose filter was from Whatman. *Staphylococcus aureus* V8 protease was from Boehringer Mannheim. Recombinant CaM kinase IV expressed in insect cells infected with Baculovirus was a gift from Dr. Soderling, T. R. and Dr. Brickey, D. A. All other chemicals were of the highest grade available.

Protein Purifications CaM kinase V was purified from the rat cerebrum by the method of Mochizuki et al. (16). CaMKK was purified by the method of Mochizuki et al (18). CaM kinase IV was purified from the rat cerebellum by the method of Miyano et al. (13). Calmodulin was purified from bovine brain by the method of Yazawa et al. (20).

Kinase Assay The standard assay mixture contained, in a final volume of 100 µl, 35 mM HEPES, pH 8.0, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.005% Tween 20, 1.5 µM calmodulin, 0.2 mM [γ-^{32}P]ATP (10cpm/pmol), 50 µM syntide-2, and various amounts of enzymes. Reaction was carried out at 37 °C for 10 min. Incorporation of ^{32}P into syntide-2 was measured essentially according to the method of Roskoski (21). After the incubation, 50-µl aliquots of reaction mixture were spotted onto phosphocellulose paper and immediately placed in 75 mM phosphoric acid. The phosphocellulose paper was washed three times for 5 min each in 75 mM phosphoric acid and air dried, and its radio activity was determined by Cerenkov counting in a liquid scintillation spectrophotometer.

Phosphorylation of CaM Kinases The reaction was carried out at 37 °C in the standard assay mixture contained various amounts of enzymes in a final volume of 30 µl, except that syntide-2 was omitted and [γ-^{32}P] ATP at higher specific activity was used. After incubation for an indicated time, the reaction was stopped by adding 6 µl of SDS-PAGE sample buffer (200 mM Tris-HCl, pH 6.8, 30% glycerol, 2% SDS, 5 mM EGTA, 6% 2-mercaptoethanol, and 0.001% bromophenol blue) and immediately boiled for 2 min. Then SDS-PAGE was performed and ^{32}P incorporation was visualized and calculated using the Fujix BAS 2000 system. For the assay of phosphorylation of CaM kinase IV by CaMKK, CaMKK was preliminarily autophosphorylated in the standard assay mixture minus syntide-2 with non-radioactive ATP at 37 °C for 30 minutes. Then the autophosphorylated enzyme was added to the assay mixture as a substrate.

Peptide Sequencing of Phosphorylated CaM Kinase V Approximately 140 µg of CaM kinase V was phosphorylated by CaMKK (0.1 µg) for 30 min at 37 °C. phosphorylated CaM kinase V was then digested with 10 µg/ml of *Staphylococcus aureus* V8 protease for 16 hours at 37 °C. The resulting peptides were separated by reverse phase HPLC on a Nucleosil C_{18} column using a linear gradient of 0-80 % acetonitrile (over 100 min) in 0.1 % trifluoroacetic acid. ^{32}P-Radioactivity was determined by liquid scintillation spectrophotometer and phosphopeptide

peaks were chosen for peptide sequencing. The amino acid sequence of peptides was determined using an Applied Biosystems 473A pulse-liquid protein sequencer equipped with on-line PTH analyzer. Analysis of phosphorylated residues was performed by the method of Wang, Y. et al. (22).

Phosphorylation of CaMKinase V by Recombinant CaM Kinase IV CaM kinase V(4 μg) was phosphorylated by recombinant CaM kinase IV (16 μg/ml) at 37 °C for 60 min in a final volume of 30 μl of the standard assay mixture. CaM kinase V was preliminarily boiled at 60 °C for 60 min in order to exclude the effect of the autophosphorylation. Other procedures were same as "Phosphorylation of CaM kinases."

PhosphoaminoAcid Analysis of CaM Kinase V 0.3 μg CaM kinase V was autophosphorylated at 37 °C for 20 min. Phosphoamino acid analysis was carried out as described by Hunter, T. et al.(23).

Other Procedures SDS-PAGE was done according to the method of Laemmli (24), using a 7.5% acrylamide gel. Protein concentrations were measured by the method of Smith et al. (25), with bovine serum albumin as a standard. All experiments were repeated twice or more times.

RESULTS AND DISCUSSION

Previously we obtained evidence for the existence of a CaM kinase kinase (CaMKK) which was partially copurified in the process of a purification step of CaM kinase V. We screened the fractions of gel filtration column chromatography, which was a final purification step of CaM kinase V, for activities able to activate CaM kinase V. These experiments revealed that addition of first peak fraction of 280 nm absorbance to the assay mixture of CaM kinase V increased phosphorylation of syntide-2. Autoradiogram revealed that a new Ca^{2+}/calmodulin-dependent protein kinase(CaMKK) phosphorylated CaM kinase V and approximate molecular mass of the protein was 64 kDa (18).

Incubation of CaM kinase V with CaMKK led to a rapid increase in phosphorylation of CaM kinase V (Fig. 1A). It reached a maximal level for only 1 min of incubation.

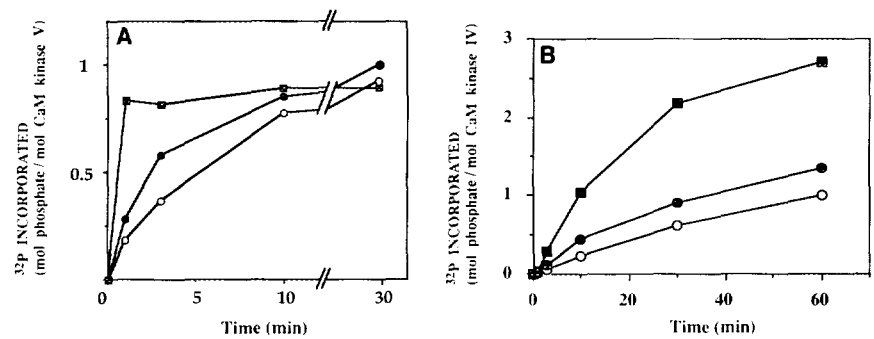


Figure 1. Time course of phosphorylation of CaM kinases.
Enzyme phosphorylations were measured as described under Materials and Methods. (A) Time course of phosphorylation of CaM kinase V in the presence of CaMKK(■), CaM kinase IV (●), or in the absence of other kinases (○). (B) Time course of phosphorylation of CaM kinase IV in the presence of CaMKK(■), CaM kinase V(●) or in the absence of other kinases(○). 0.15 μg/ml of CaM kinase V, 2.5 μg/ml of CaM kinase IV and 1.0 μg/ml of CaMKK were used.

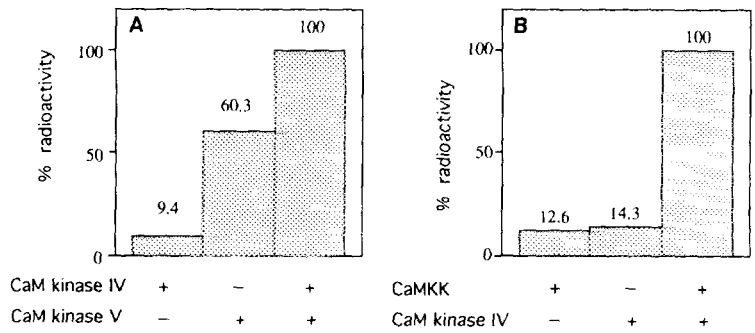


Figure 2. Phosphorylation of syntide-2.
Syntide-2 kinase activity was shown as a % of maximal activity. (A) 0.1 μg/ml of CaM kinase V and 1.0 μg/ml of CaM kinase IV were used. (B) 0.3 μg/ml of CaM kinase IV and 1.0 μg/ml of CaMKK were used. All assays were carried out at 37 °C for 10 min in 100 μl of standard assay mixture as described under Materials and Methods.

Similarly it led to an increase in activity of CaM kinase V toward syntide-2 (18). However, after 30 min of incubation, difference between in the presence of CaMKK and its absence was not observed because activation by autophosphorylation of CaM kinase V reached a maximal level (data not shown).

Next, we examined a relationship between CaM kinase IV and CaM kinase V. Incubation of CaM kinase V in the presence of CaM kinase IV increased phosphorylation of CaM kinase V (Fig. 1A). In addition, incubation of CaM kinase IV in the presence of CaM kinase V increased phosphorylation of CaM kinase IV (Fig. 1B). And it led to an increase in activity toward syntide-2 (Fig. 2A). These results suggested that CaM kinase IV and CaM kinase V phosphorylated each other and resulted in their activation.

However CaM kinase V was not phosphorylated by other protein kinases, such as cyclic AMP-dependent protein kinase, protein kinase C, and CaM kinase II (data not shown).

We also examined a relationship between CaMKK and CaM kinase IV. Activity toward syntide-2 of both CaMKK and CaM kinase IV was very low in this assay condition. However, incubation of CaM kinase IV with CaMKK led to a dramatic increase in activity toward syntide-2 (Fig. 2B). Autoradiogram revealed that phosphorylation of CaM kinase IV in the presence of CaMKK increased three times as much as in its absence (Fig. 1B). On the other hand phoshorylation of CaMKK didn't increase in the presence of CaM kinase IV (data not shown). These results suggested that CaMKK also phosphorylated CaM kinase IV and resulted in its activation. However, full activation required over 60 min of incubation.

CaM kinase IV undergoes autophosphorylation which regulates its autonomous activity (14, 15). The autophosphorylation sites of CaM kinase IV were controversial. Frangakis, M. V. et al. (14) reported that the stoichiometry of CaM kinase IV ranges from 0.6 to

1.7 mol phosphate/mol enzyme according to concentrations of the enzyme and a Ser residue was the autophosphorylation site. McDonald, O. B. et al. (26) reported that the autophosphorylation sites of CaM kinase IV were potentially Ser^{8}, Ser^{11}, Ser^{12}, Ser^{15}, Thr^{196}, or Thr^{200}. Kameshita, I. et al. (15) reported that Ser^{437} was the autophosphorylation site controlling the activity of CaM kinase IV. In our experiments, the stoichiometry of the autophosphorylation of CaM kinase IV was approximately 1 mol phosphate/mol enzyme. On the other hand, the stoichiometry of phosphorylation of CaM kinase IV by CaMKK was 2.7 mol phosphate/mol enzyme and the stoichiometry by CaM kinase V was 1.3 mol phosphate/mol enzyme (Fig. 1B). We have not determined yet whether phosphorylation sites of CaM kinase IV by CaMKK and CaM kinase V correspond to these autophosphorylation sites of CaM kinase IV or not.

CaM kinase V also underwent autophosphorylation, but required Ca^{2+} and calmodulin for its activity even after autophosphorylation (18). The stoichiometry of autophosphorylation of CaM kinase V was approximately 1 mol phosphate/mol enzyme (Fig. 1A). In addition, the stoichiometry of phosphorylation of CaM kinase V in the presence of CaMKK or CaM kinase IV was also approximately 1 mol phosphate/mol CaM kinase V (Fig. 1A). Phosphoamino acid analysis revealed that a Thr residue was an autophosphorylation site (Fig. 3). CaMKK couldn't phosphorylate CaM kinase V under conditions where CaM kinase V had been autophosphorylated in advance (1). Similarly, autophosphorylated CaM kinase V was not phosphorylated by CaM kinase IV(Fig.4A lane3). These results suggested that both CaMKK and CaM kinase IV phosphorylated a Thr residue of CaM kinase V which was same as the autophosphorylation site. CaM kinase V was phosphorylated using [γ-^{32}P] ATP by CaMKK and then digested with *Staphylococcus aureus* V_8 protease. The resulting peptides were separated by reversed-phase HPLC (Fig. 5). When the 53.0 min fraction which was major peak of radioactivity was analyzed by aminoacid sequence, the sequence was identified as follows: DSKIMISDFGLSKMEDPGSVLSTAXGTPGYVAP. Candidate threonines were

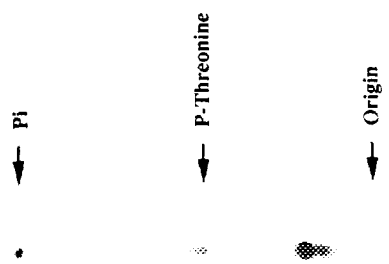


Figure 3. Phosphoamino acid analysis of CaM kinase V.
CaM kinase V (3 μg/ml) was autophosphorylated at 37 °C for 20 min and hydrolyzed with 6 N HCl at 110 °C for 2 h. The hydrolyzed sample was electrophoresed on a cellulose-coated thin plate and the radioactive spots were detected by autoradiography.

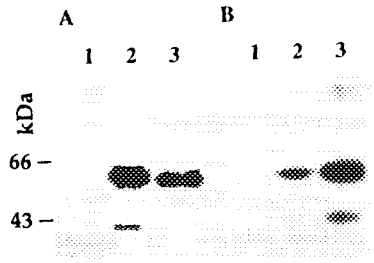


Figure 4. Phosphorylation of CaM kinase V by CaM kinase IV.
(A) Phosphorylation of CaM kinase V by rat cerebellar CaM kinase IV. In lane 1, CaM kinase V (0.1 μg/ml) was incubated for 1 min at 37 °C, as described under Materials and Methods. The reaction was terminated by adding SDS-PAGE sample buffer, then subjected to SDS-PAGE, and radioactivity was visualized as described. In lane 2, CaM kinase V was incubated in the presence of CaM kinase IV (6 μg/ml) for 1 min at 37 °C. In lane 3, CaM kinase V was preliminarily autophosphorylated with non-radioactive ATP for 30 min at 37 °C, then the reaction was initiated by adding CaM kinase IV and [γ-^{32}P]ATP. After 1 min, the reaction was terminated. The band at M_r=41 kDa in lane 2 represents phosphorylated CaM kinase V by CaM kinase IV. The band at M_r=63-65 kDa in lanes 2 and 3 represents autophosphorylated CaM kinase IV. In lane 1, autophosphorylated CaM kinase V can't be observed because of a low concentration of CaM kinase V used and a short reaction time.
(B) Phosphorylation of heat-treated CaM kinase V by recombinant CaM kinase IV. CaM kinase V was preliminarily boiled at 60 °C for 60 min. Heat-treated CaM kinase V (4 μg) was incubated at 37 °C for 60 min in the absence (lane 1) or in the presence (lane 3) of recombinant CaM kinase IV(16 μg/ml) as described under Materials and Methods. In lane 2 recombinant CaM kinase IV was incubated in the absence of heat-treated CaM kinase V at 37 °C for 60 min. The reactions were terminated by adding SDS-PAGE sample buffer then subjected to SDS-PAGE and radioactivity visualized as described. The band at Mr=41 kDa in lane 3 represents phosphorylated CaM kinase V. The band at Mr=63 kDa in lanes 2 and 3 represents autophosphorylated CaM kinase IV.

presented at residues 23 and 27. We used the technique described by Wang, Y. et al. (22) in order to assign which Thr residue is phosphorylated. The Thr at cycle 23 is clearly the predominant phosphorylation site. When the 54.3 min fraction which was

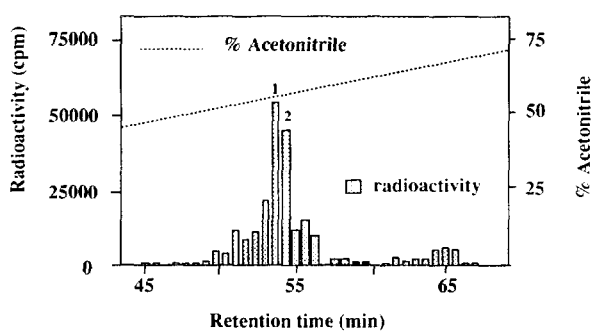
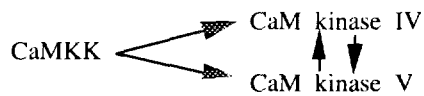


Figure 5. Analysis of digested peptides of CaM kinase V by HPLC.
Radiolabeled peptides were detected by Cherencov radiation given in cpm. The 53.0 min fraction (peak 1) which was major peak of radioactivity and the 54.3 min fraction (peak 2) which was a minor peak of radioactivity were analyzed with protein sequencer as described under Materials and Methods.

minor peak of radioactivity was analyzed by aminoacid sequence, the sequence was identified as : QIKKNFAKSKWKQAFNRTAVV, and the Thr at cycle 18 was the predominant phosphorylation site.

It was reported that CaM kinase IV expressed in *Escherichia coli* had lower activity than that from rat cerebellum (27). The existence of CaMKK in rat brain raises several possibilities. One possibility is that CaM kinase IV from rat cerebellum has already been phosphorylated by CaMKK, at least partially. The second possibility is that CaM kinase IV preparation from rat cerebellum contains CaMKK as a contaminant. If this is a case, it raises the other possibility that CaM kinase IV dose not phosphorylate CaM kinase V and a contaminating CaMKK in the CaM kinase IV preparation phosphorylates CaM kinase V. Then recombinant CaM kinase IV expressed in insect cells infected with Baculovirus was examined with heat-treated CaM kinase V as a substrate. As shown in Fig. 4B, recombinant CaM kinase IV clearly phosphorylated CaM kinase V. Our results suggested a hypothetical CaM kinase cascade summarized as follows:



After only 1 min of incubation, activation of CaM kinase V by CaMKK reached a maximal level. On the other hand, activation of CaM kinase V by CaM kinase IV was slower than by CaMKK. In addition, incubation of CaM kinase IV in the presence of CaMKK did not generate a rapid increase in phosphorylation of CaM kinase IV. Our results suggested that activation of CaM kinase V by CaMKK was principal and others were auxiliary mechanisms for keeping CaM kinase V as a phosphorylated form. Hanson, P. I. et al. (28) reported that the autophosphorylated CaM kinase II retained trapped calmodulin during the interspike interval of Ca^{2+} and CaM kinase II consequently became highly active when the next Ca^{2+} spike arrived. In case of CaM kinase V our results suggested that multiple phosphorylation mechanisms enabled CaM kinase V to become highly active.

Examination of the partial amino acid sequence of several peptides derived from CaM kinase V (17) revealed that the kinase is likely identical to CaM kinase I (29). The major phosphorylation site of CaM kinase V is located at a position equivalent to that of Thr^{177} autophosphorylated in CaM kinase I. The minor phosphorylation site is equivalent to Thr^{310} which is located at putative calmodulin binding domain in CaM kinase I.

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