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Regulation of Ca^{2+}/Calmodulin-Dependent Protein Kinase IV (CaM-Kinase IV) by Changing Its Susceptibility to Phosphorylation by CaM-Kinase Kinases[1]

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Calmodulin-dependent protein kinase IV (CaM-kinase IV) is markedly activated on the phosphorylation of Thr^{196} by an upstream protein kinase, CaM-kinase kinase. The phosphorylation of CaM-kinase IV by CaM-kinase kinase is strongly suppressed on incubation with calmodulin in the presence of Ca^{2+}, which results in a decrease in the enzyme activity, and completely restored on subsequent incubation with Mg^{2+} in the absence of Ca^{2+}, which results in an increase in the activity. That a downstream protein kinase regulates its activity through reversible changes in its susceptibility to phosphorylation by an upstream protein kinase is a new regulatory mechanism.

Key words: Ca^{2+}/calmodulin-dependent protein kinase, CaM-kinase IV, CaM-kinase kinase, protein phosphorylation, regulation of enzyme activity.

Some signal transduction pathways include a regulatory system in which a protein kinase regulates the activity of a downstream protein kinase through phosphorylation, presumably because chain reactions of proteins which have progressed evolutionally are necessary to make up a highly sophisticated regulatory device. For example, MAP kinase kinase kinase activates MAP kinase kinase, which activates MAP kinase (*1*), and calmodulin-dependent protein kinase (CaM-kinase) kinase activates CaM-kinase through phosphorylation (*2*). The present paper reports that the activity of CaM-kinase IV, which is thought to play an important role in the functioning of Ca^{2+} as a Ca^{2+}/calmodulin-dependent multifunctional protein kinase in the central nervous and immune systems, is regulated through reversible changes in its susceptibility to phosphorylation by CaM-kinase kinase (*3, 4*).

Calmodulin was purified from *Escherichia coli* cells transformed with an expression vector, pET11d, carrying cDNA encoding chicken brain calmodulin, and CaM-kinase IV was purified from Sf9 cells transfected with a recombinant vaculovirus, AcNPV, carrying cDNA encoding rat brain CaM-kinase IV as described (*5*). CaM-kinase IV-($K_{71}R$), in which Lys^{71} was replaced with Arg, was purified from Sf9 cells transfected with a vaculovirus carrying cDNA prepared from CaM-kinase IV cDNA by site-directed mutagenesis as described (*4*). CaM-kinase I was purified from Sf9 cells transfected with a vaculovirus carrying cDNA for rat brain CaM-kinase I (*6*). CaM-kinase kinase α was purified from *E. coli* cells transfected with vector pET11d carrying cDNA for rat brain CaM-kinase kinase α (CaM-kinase IV kinase) (*7*). CaM-kinase kinase β was purified from Sf9 cells transfected with a vaculovirus carrying cDNA for rat brain CaM-kinase kinase β (*8*). The incubation of CaM-kinases with calmodulin was carried out at 30°C in an inactivation mixture comprising 50 mM Mops-NaOH (pH 7.0 at 30°C), 2 mM dithiothreitol, 10% ethylene glycol, 0.05% Tween 40, 0.1 mM $CaCl_2$, and 2 μM calmodulin, essentially as described previously (*5*). The phosphorylation of CaM-kinases with CaM-kinase 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 [γ-^{32}P]ATP (1,000-2,000 cpm/pmol), 0.1 mM EGTA, 0.2 mM $CaCl_2$, 2 μM calmodulin, and the indicated amounts of proteins, and the incorporation of [^{32}P]phosphate into the proteins was determined, essentially as described previously (*9*). The activity of CaM-kinase IV was determined in an assay mixture comprising, in a final volume of 50 μl, 50 mM Mops-NaOH (pH 7.0 at 30°C), 2 mM dithiothreitol, 5 mM $Mg(CH_3COO)_2$, 0.1 mM [γ-^{32}P]ATP (200 cpm/pmol), 0.1 mM $CaCl_2$, 1 μM calmodulin, 40 μM syntide-2, and the indicated amounts of proteins, essentially as described previously (*5*).

Our recent findings that CaM-kinase IV is inactivated on incubation with calmodulin in the presence of Ca^{2+} and that the activity is completely restored on incubation with Mg^{2+} in the absence of Ca^{2+} (*5*) led us to examine the possibility that the inactivation of the enzyme is due to a decrease in its susceptibility to phosphorylation at Thr^{196} by an upstream CaM-kinase kinase, because CaM-kinase IV is markedly activated on phosphorylation at Thr^{196} by CaM-kinase kinase (*3, 4*). As CaM-kinase IV autophosphorylates many sites after being activated through phosphorylation

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

by CaM-kinase kinase, the phosphorylation of CaM-kinase IV by CaM-kinase kinase was measured with CaM-kinase IV($K_{71}R$) in which Lys^{71} (ATP-binding site) was replaced with Arg to block autophosphorylation. When CaM-kinase IV($K_{71}R$) was incubated with calmodulin in the presence of Ca^{2+}, its susceptibility to phosphorylation by CaM-kinase kinase α decreased progressively, the minimum level being reached within a few minutes under the experimental conditions used, as shown in Fig. 1A. Since the phosphorylation of CaM-kinase IV($K_{71}R$) by CaM-kinase kinase α only occurs at Thr^{196}, which is involved in the marked activation of the enzyme (*4*), the result indicates that incubation with calmodulin in the presence of Ca^{2+} makes the enzyme less susceptible to phosphorylation at Thr^{196}. When the enzyme was then incubated with Mg^{2+} in the presence of EGTA added to remove Ca^{2+}, the susceptibility to phosphorylation at Thr^{196} by CaM-kinase kinase α increased progressively, almost the original level being recovered within a few minutes. The susceptibility decreased again on the addition of Ca^{2+}, indicating that the lowering and recovery of the susceptibility of CaM-kinase IV to phosphorylation by CaM-kinase kinase α are reversible processes. It was confirmed by SDS-PAGE that the phosphorylation occurred on CaM-kinase IV, as shown in Fig. 1C. Changes in the enzyme activity were examined by a parallel experiment involving wild-type CaM-kinase IV, as shown in Fig. 1B. The incubation of CaM-kinase IV with calmodulin in the presence of Ca^{2+} resulted in a progressive decrease in the enzyme activity detected in the presence of CaM-kinase kinase α, the minimum level being reached within a few minutes. Subsequent incubation with Mg^{2+} in the absence of Ca^{2+} resulted in a progressive increase in the enzyme activity, and the activity decreased again on the addition of Ca^{2+}. These results indicate that Ca^{2+}/calmodulin lowers the susceptibility of CaM-kinase IV to phosphor-

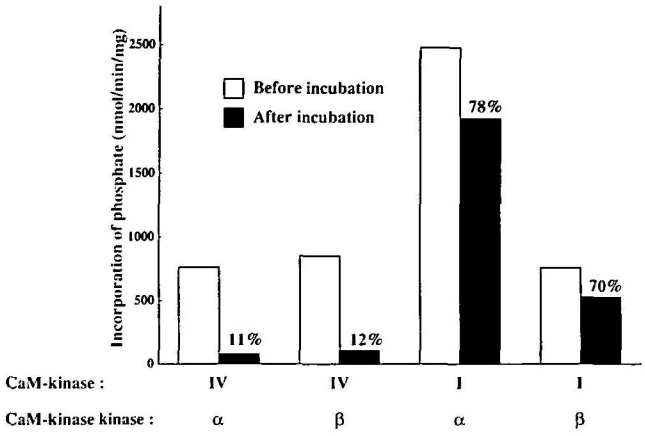

Fig. 2. Effects of Ca^{2+}/calmodulin on the phosphorylation of CaM-kinases IV and I by CaM-kinase kinases α and β. The phosphorylation of CaM-kinase IV($K_{71}R$) and CaM-kinase I by CaM-kinase kinase α or CaM-kinase kinase β was determined for 1 min at 30°C before and after incubation of 50 μg/ml CaM-kinase IV($K_{71}R$) or CaM-kinase I with 2 μM calmodulin in the presence of 0.1 mM $CaCl_2$ for 5 min at 30°C, essentially as described in the legend to Fig. 1A. The phosphorylation of CaM-kinase IV (2 μg) was carried out with 5 ng of CaM-kinase kinase α or 2.5 ng of CaM-kinase kinase β, and that of CaM-kinase I (2 μg) was carried out with 2.5 ng of CaM-kinase kinase α or β in 50 μl of the phosphorylation mixture. The activity after incubation with Ca^{2+}/calmodulin is expressed as a percentage of the activity before incubation.

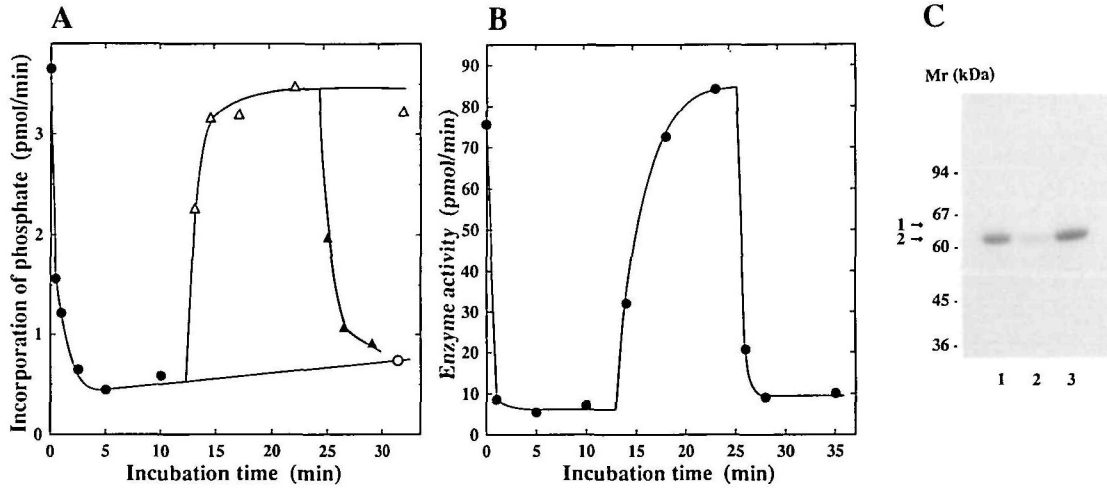

Fig. 1. Decrease in the phosphorylation of CaM-kinase IV by CaM-kinase kinase α and a concomitant decrease in CaM-kinase IV activity caused by incubation with Ca^{2+}/calmodulin. (A) Approximately 50 μg/ml CaM-kinase IV($K_{71}R$) was incubated at 30°C with 2 μM calmodulin in the presence of 0.1 mM $CaCl_2$ in the inactivation mixture (●). At 10.5 min, 0.2 mM EGTA was added to remove Ca^{2+} (○); at 12 min, 5 mM $Mg(CH_3COO)_2$ was added (△); and at 24 min, 0.2 mM $CaCl_2$ was added (▲). At the indicated times, 40-μl aliquots were incubated with 5 ng of CaM-kinase kinase α for 1 min at 30°C, and then the phosphorylation of CaM-kinase IV by CaM-kinase kinase was determined. (B) Approximately 10 μg/ml wild type CaM-kinase IV was incubated with calmodulin in the presence of $CaCl_2$ at 30°C. At 12, 13, and 25 min, 0.2 mM EGTA, 5 mM $Mg(CH_3COO)_2$, and 0.2 mM $CaCl_2$ were successively added, respectively. At the indicated times, 10-μl aliquots were assayed for kinase activity with 40 μM syntide-2 as a substrate for 1 min at 30°C in the presence of 1 ng of CaM-kinase kinase α. (C) Approximately 50 μg/ml CaM-kinase IV($K_{71}R$) (lane 1), after incubation with calmodulin in the presence of $CaCl_2$ at 30°C for 10 min (lane 2), or after incubation with Ca^{2+}/calmodulin for 10 min followed by incubation with EGTA/$Mg(CH_3COO)_2$ for another 10 min (lane 3), was incubated with CaM-kinase kinase α for 1 min as described in (A), and then a 10-μl aliquot was analyzed by SDS-PAGE followed by autoradiography. The arrows, 1 and 2, indicate the positions of CaM-kinase kinase α and CaM-kinase IV, respectively.

ylation at Thr[196] by CaM-kinase kinase α, thereby suppressing the activation of CaM-kinase IV by CaM-kinase kinase α, which consequently results in a decrease in the enzyme activity, and that Mg^{2+} restores the susceptibility in the absence of Ca^{2+}, consequently restoring the enzyme activity. Preliminary kinetic analysis suggested that the lowering of the susceptibility to phosphorylation was not due to an increase in the K_m value of CaM-kinase kinase α for CaM-kinase IV or ATP, but to a decrease in the V_{max} value (data not shown). The rate of the decrease in susceptibility was strongly decreased at 4°C (data not shown). The chromatographic behavior of CaM-kinase IV(K_{71}R) on gel filtration on Superdex 200 HR 10/30 (Pharmacia LKB Biotechnology) did not significantly change on incubation with calmodulin in the presence of Ca^{2+} (data not shown), indicating that incubation with calmodulin did not result in a change in the quaternary structure of the enzyme (such as dimerization).

Since CaM-kinase IV is activated upon phosphorylation not only by CaM-kinase kinase α but also by CaM-kinase kinase β (*6*), an isoform of CaM-kinase kinase α (*8*), and CaM-kinase I is also activated by both CaM-kinase kinases α and β (*6*), the susceptibilities of CaM-kinases IV and I to phosphorylation by CaM-kinase kinases α and β were examined after incubation with calmodulin in the presence of Ca^{2+}, as shown in Fig. 2. As CaM-kinase I, in contrast to CaM-kinase IV, is only phosphorylated at Thr[177] (*10*), which is involved in the activation of the enzyme (*11*), in the presence of a CaM-kinase kinase, the phosphorylation of CaM-kinase I by CaM-kinase kinase α or β was measured with wild-type CaM-kinase I. When CaM-kinase IV(K_{71}R) was incubated with calmodulin in the presence of Ca^{2+} at 30°C for 5 min, the susceptibility of the enzyme to phosphorylation by CaM-kinase kinase β was decreased to the same extent as that to the phosphorylation by CaM-kinase kinase α. In contrast, the susceptibility of CaM-kinase I to phosphorylation by CaM-kinase kinase α or β was little decreased on incubation with calmodulin in the presence of Ca^{2+}, but the extent was much lower than that in the case of CaM-kinase IV. Since CaM-kinase IV is directly inactivated on incubation with Ca^{2+}/calmodulin and the activity of the inactivated enzyme is restored on subsequent incubation with EGTA/Mg^{2+} (*5*), in order to clarify the relationship between the previously observed direct inactivation and the decrease in the susceptibility to phosphorylation by CaM-kinase kinases described in the present paper, the effects of Ca^{2+}/calmodulin and EGTA/Mg^{2+} on the activity of CaM-kinase IV or I, and on the activation of the CaM-kinase by CaM-kinase kinase α were compared, as shown in Table I. The extents of the inactivation of CaM-kinase IV by Ca^{2+}/calmodulin were almost the same before and after activation by CaM-kinase kinase α, indicating that the binding of Ca^{2+}/calmodulin to CaM-kinase IV induces a conformational change of the enzyme under the experimental conditions used, leading not only to a decrease in the activity of the enzyme, but also a decrease in its susceptibility to phosphorylation by CaM-kinase kinase α. In contrast to CaM-kinase IV, the activity of CaM-kinase I was not significantly affected by incubation with Ca^{2+}/calmodulin under the present experimental conditions. It is interesting to note that the binding of Ca^{2+}/calmodulin to CaM-kinase IV has a quite opposite effect on this CaM-kinase cascade, because it was recently reported that the binding of Ca^{2+}/calmodulin to CaM-kinase IV is necessary for the activation by CaM-kinase kinase (*12*). Thus, in the present paper, a novel regulatory mechanism in which a downstream protein kinase shows change in its susceptibility to activation by an upstream protein kinase is reported, although the details of the mechanism underlying the interconversion of CaM-kinase IV between the sensitive and insensitive forms remain unknown.

It has been well established that CaM-kinase IV is activated upon phosphorylation at Thr[196] through the action of CaM-kinase kinase in the presence of Ca^{2+}/calmodulin (*3, 4*), and that the activated CaM-kinase IV is inactivated upon phosphorylation at Ser[332] by itself in the absence of Ca^{2+} (*13*). In addition to Ser[332], CaM-kinase IV possesses many autophosphorylation sites (*13-15*), whose regulatory roles are currently unknown. Thus, CaM-kinase IV appears to be controlled by an intricate regulatory mechanism including a number of self-regulatory processes.

TABLE I. **Effects of Ca^{2+}/calmodulin on the activities of CaM-kinases IV and I.** Approximately 50 μg/ml CaM-kinase IV or I was incubated for 5 min at 30°C with 2 μM calmodulin in the presence of 0.1 mM $CaCl_2$ in the inactivation mixture (60 μl), and then a 5-μl aliquot was assayed for kinase activity with 100 μM syntide-2 as a substrate. Another 5-μl aliquot was incubated for 1 min at 30°C with CaM-kinase kinase α (25 ng for CaM-kinase IV and 1.25 ng for CaM-kinase I) in the phosphorylation mixture (25 μl), and then an aliquot was assayed for kinase activity. To the remaining 50 μl of the enzyme treated with Ca^{2+}/calmodulin, 1 μl each of 11 mM EGTA and 260 mM $Mg(CH_3COO)_2$ were added, and then the mixture was incubated for 5 min at 30°C. A 5-μl aliquot was assayed for kinase activity, and another 5-μl aliquot was incubated for 1 min at 30°C with CaM-kinase kinase α (25 ng for CaM-kinase IV and 1.25 ng for CaM-kinase I) in the phosphorylation mixture (25 μl), and then an aliquot was assayed for kinase activity.

	Enzyme activity (nmol/min/mg) before and after incubation with CaM-kinase kinase α	
CaM-kinase IV		
Untreated	642 (100%)	7,960 (100%)
Treated with Ca^{2+}/calmodulin	85 (13)	1,438 (18)
Treated with EGTA/Mg^{2+}	781 (122)	6,833 (86)
CaM-kinase I		
Untreated	828 (100%)	14,372 (100%)
Treated with Ca^{2+}/calmodulin	959 (116)	16,708 (116)
Treated with EGTA/Mg^{2+}	748 (90)	15,473 (108)

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