*J. Biochem.* **129**, 193–199 (2001)

# Regulation of the Activities of Multifunctional $Ca^{2+}$/Calmodulin-Dependent Protein Kinases

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Received November 29, 2000; accepted December 8, 2000

**$Ca^{2+}$/calmodulin-dependent protein kinases (CaM-kinases) II, IV, and I play important roles as $Ca^{2+}$-responsive multifunctional protein kinases in controlling a variety of cellular functions in response to an increase in intracellular $Ca^{2+}$, and hence regulation of their activities is very important. CaM-kinase II is activated through autophosphorylation of threonine-286 (in the case of α isoform), and CaM-kinases IV and I are activated through phosphorylation of threonine-196 and 177, respectively, by CaM-kinase kinase. After activation, CaM-kinases II and IV lose their $Ca^{2+}$/calmodulin-dependent activity upon autophosphorylation of threonine-305 and serine-332, respectively, in the absence of $Ca^{2+}$, becoming $Ca^{2+}$/calmodulin-independent forms. The activated CaM-kinases II, IV, and I are deactivated upon dephosphorylation of phosphothreonine-286, 196, and 177, respectively, by CaM-kinase phosphatase or other multifunctional protein phosphatases and restored to the original ground states. Thus, the activities of the three multifunctional CaM-kinases are regulated by phosphorylation and dephosphorylation.**

**Key words: calcium ion, calmodulin-dependent protein kinase, protein kinase kinase, protein phosphatase, signal transduction.**

Mammalian cells contain a number of $Ca^{2+}$/calmodulin-dependent protein kinases (CaM-kinases). Among them, CaM-kinases II, IV, and I have broad substrate specificities, and these $Ca^{2+}$-responsive protein kinases, along with cAMP-dependent protein kinase (PKA) and diacylglycerol-responsive protein kinase C (PKC), play crucial roles as second messenger–responsive multifunctional protein kinases in signal transduction in the cell. When the concentration of $Ca^{2+}$ in the cytosol is raised by the opening of $Ca^{2+}$ channels in the plasma membrane or in the membrane surrounding an internal $Ca^{2+}$ store, a number of $Ca^{2+}$-binding proteins are activated. $Ca^{2+}$/calmodulin, a small protein (17 kDa) which is the most ubiquitous $Ca^{2+}$-binding protein in eukaryotic cells, binds to activate a number of proteins, such as CaM-kinases II, IV, and I, which phosphorylate many target proteins to regulate their activities. The present review describes the regulatory mechanisms of the three multifunctional CaM-kinases, CaM-kinases II, IV, and I, by $Ca^{2+}$/calmodulin.

## Regulation of CaM-kinase II by phosphorylation

CaM-kinase II is known to undergo rapid autophosphorylation at $Thr^{286}$ within the autoinhibitory domain in the presence of $Ca^{2+}$/calmodulin (*1–3*), and subsequent dissociation of calmodulin (in the presence of $Ca^{2+}$ chelator such as EGTA) results in autophosphorylation at $Thr^{305}$ located in the calmodulin-binding site (*4*, *5*) (Fig. 1). The observations that CaM-kinase II activity is apparently maximal in the presence of $Ca^{2+}$/calmodulin under the conventional assay conditions but is not expressed in the absence of $Ca^{2+}$ without preincubation under the phosphorylation conditions in the presence of $Ca^{2+}$/calmodulin led to the popular misconception that CaM-kinase II is converted from a $Ca^{2+}$/calmodulin-dependent form to a $Ca^{2+}$/calmodulin-independent form by autophosphorylation at $Thr^{286}$. However, Kwiatkowski *et al.* (*6*) investigated the effect of autophosphorylation of CaM-kinase II on the enzyme activity by using short assay times under the limiting phosphorylation conditions with low concentrations of ATP, and obtained results suggesting that autophosphorylation of CaM-kinase II in the presence of $Ca^{2+}$/calmodulin is an obligatory step for full activation of the enzyme. This report led us to think that the velocity of autophosphorylation of $Thr^{286}$ of CaM-kinase II is too fast to observe the activation process of the enzyme by the autophosphorylation under the conventional assay conditions in the presence of $Ca^{2+}$/calmodulin; that is, the enzyme is instantaneously activated upon the autophosphorylation in the conventional assay mixture and therefore it apparently shows a high activity without preincubation for the autophosphorylation. Indeed, as shown in Fig. 2, when CaM-kinase II is incubated for various periods with ATP (Fig. 2A) or ATPγS (Fig. 2B) under the (thio)-phosphorylation conditions in the presence of $Ca^{2+}$/calmodulin, the enzyme activity subsequently measured under the limiting (thio)phosphorylation conditions, at low ATP (2 μM) and high syntide-2 (200 μM) concentrations (Fig. 2C) (*7*) or with ATPγS in place of ATP at a low temperature (5°C) (Fig. 2D) (*8*), increases both in the absence of $Ca^{2+}$/calmodulin and in its presence as incubation time for auto-(thio)phosphorylation increases, finally reaching a maximum level. The time course of the increase in the enzyme activity in both the presence and absence of $Ca^{2+}$/calmodulin is similar to the time course of the phosphorylation (Fig. 2, A and C) or thiophosphorylation (Fig. 2, B and D) of the enzyme. The amount of phosphate or thiophosphate incorporated into the enzyme is approximately 8 mol/mol of

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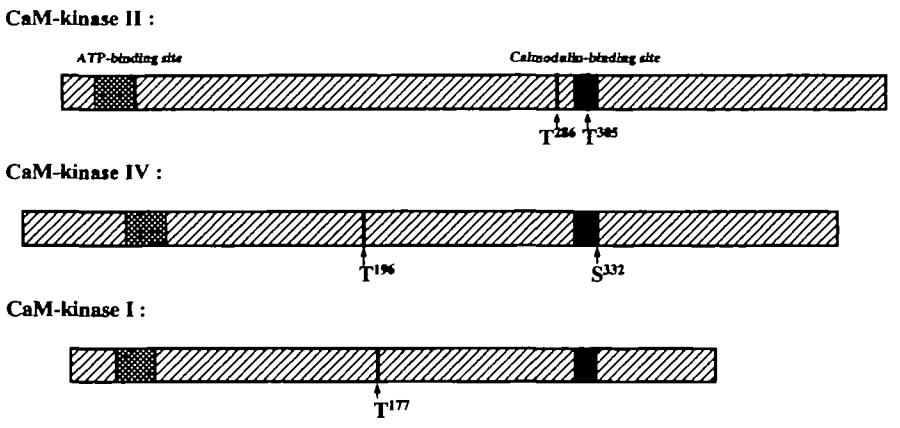


Fig. 1. **Schematic representation of phosphorylation sites involved in controlling the activities of multifunctional CaM-kinases.** CaM-kinases II, IV, and I are aligned so that the calmodulin-binding sites are in a line.

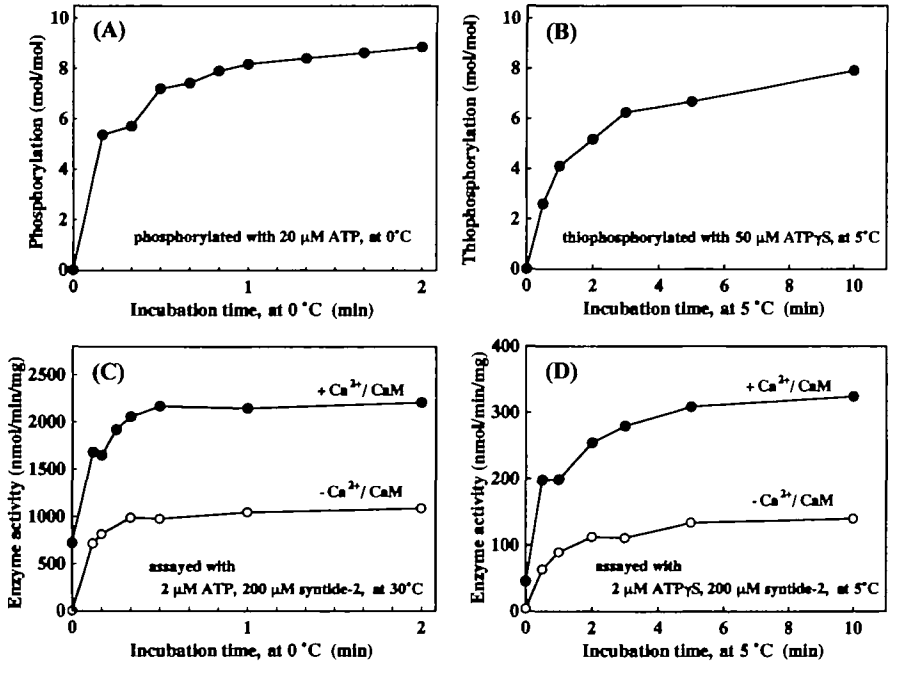


Fig. 2. **Time course of autophosphorylation of CaM-kinase II (A and B) and concomitant changes in its activities in the presence (●) and absence (○) of $Ca^{2+}$/calmodulin (C and D).** (A and C) CaM-kinase II was incubated with 20 μM [γ-$^{32}$P]ATP (A) or non-radioactive ATP (C) at 0°C in the presence of $Ca^{2+}$/calmodulin under the phosphorylation conditions. After incubation for the indicated periods, aliquots were taken for the determination of the amounts of [$^{32}$P]phosphate incorporated into CaM-kinase II (A) and for the measurement of the enzyme activity in the presence (●) and absence (○) of $Ca^{2+}$ using 2 μM [γ-$^{32}$P]ATP and 200 μM syntide-2 as substrate (C). For details, see Ref. 7. (B and D) CaM-kinase II was incubated with 50 μM [γ-$^{35}$S]ATPγS (B) or non-radioactive ATPγS (D) at 5°C in the presence of $Ca^{2+}$/calmodulin. After incubation for the indicated periods, aliquots were taken for the determination of the amounts of [$^{35}$S]thiophosphate incorporated into CaM-kinase II (B) and for the measurement of the enzyme activity in the presence (●) and absence of (○) of $Ca^{2+}$ using 2 μM ATPγS and 200 μM syntide-2 as substrate (D). For details, see Ref. 8.

holoenzyme, which corresponds to 1 mol/mol of subunit. Thr$^{286}$ is identified as a rapid $Ca^{2+}$/calmodulin-dependent autophosphorylation site (*1–3*) and the thiophosphorylation with ATPγS at 5°C occurs exclusively at Thr$^{286}$ (*9*). These results together indicate that CaM-kinase II activity in the presence of $Ca^{2+}$/calmodulin, as well as in its absence, is fully activated upon autophosphorylation at Thr$^{286}$. CaM-kinase II autophosphorylated at Thr$^{286}$ shows the same $V_{max}$ values in the presence and absence of $Ca^{2+}$/calmodulin, while the $K_m$ values in the presence of $Ca^{2+}$/calmodulin are two- to fivefold lower than those in its absence, depending upon the substrates used (*9*); for example, the $K_m$ values for syntide-2 are 5.4 μM in the presence of $Ca^{2+}$/calmodulin and 28 μM in its absence, but the $V_{max}$ is approximately 40,000 nmol/min/mg, irrespective of the presence or absence of $Ca^{2+}$/calmodulin. This means that the apparent $Ca^{2+}$-independence of CaM-kinase II may vary depending on the substrates and their concentrations. Autophosphorylation of Thr$^{286}$ is an intramolecular (*10, 11*) and intersubunit-catalyzed reaction (*12*). Since nonautophosphorylated CaM-kinase II possesses a basal low level of $Ca^{2+}$/calmodulin-dependent activity (*8*), subunits of the enzyme may phosphorylate Thr$^{286}$ of one another within an enzyme molecule in the presence of $Ca^{2+}$/calmodulin, all the subunits being phosphorylated at Thr$^{286}$ and thereby fully activated. Thus, the increase in $Ca^{2+}$/calmodulin-independent activity of CaM-kinase II upon autophosphorylation at Thr$^{286}$ is a consequence of the activation of the enzyme, but not due to the conversion of the enzyme from a $Ca^{2+}$/calmodulin-dependent form to a $Ca^{2+}$/calmodulin-independent form.

After autophosphorylation of CaM-kinase II in the presence of $Ca^{2+}$/calmodulin, addition of EGTA enhanced the autophosphorylation of the enzyme, causing a decrease in the $Ca^{2+}$/calmodulin-dependent activity (*13*). The phosphorylation site in the presence of EGTA has been identified as Thr$^{305}$ in the calmodulin-binding site of the enzyme (*5, 6*), and it has more recently been reported that autophosphorylation at either Thr$^{305}$ or Thr$^{306}$ blocks both binding and

activation of the enzyme by $Ca^{2+}$/calmodulin (*14*). The autophosphorylation of $Thr^{305}$ occurs only after removal of $Ca^{2+}$ (*5*), suggesting that this phosphorylation site located within the calmodulin-binding site becomes accessible for autophosphorylation upon removal of calmodulin. Thus, CaM-kinase II is activated upon autophosphorylation of $Thr^{286}$ in the presence of $Ca^{2+}$/calmodulin, and subsequent removal of $Ca^{2+}$ causes further autophosphorylation of $Thr^{305}$ within the calmodulin-binding site, making the enzyme unable to bind calmodulin and thereby leading to complete loss of the $Ca^{2+}$/calmodulin-dependent activity. Thus, the conversion of CaM-kinase II into a $Ca^{2+}$/calmodulin-independent form is not caused by autophosphorylation of $Thr^{286}$ in the presence of $Ca^{2+}$/calmodulin, but it is caused by autophosphorylation of $Thr^{305}$ in the absence of $Ca^{2+}$/calmodulin. A similar loss of $Ca^{2+}$/calmodulin-dependent activity by autophosphorylation of a calmodulin-binding site is also observed with another multifunctional CaM-kinase, CaM-kinase IV, as described in the next section.

## Regulation of CaM-kinases IV and I by phosphorylation

In contrast to the high activity of CaM-kinase II in the presence of $Ca^{2+}$/calmodulin, rat brain CaM-kinase IV expressed in *Escherichia coli* showed little activity in the presence of $Ca^{2+}$/calmodulin. The inactive CaM-kinase IV was phosphorylated and became highly active on incubation with a rat brain extract in the presence of $Ca^{2+}$/calmodulin under the phosphorylation conditions, and this finding led to the discovery that CaM-kinase IV is activated upon phosphorylation by an upstream $Ca^{2+}$/calmodulin-dependent protein kinase, CaM-kinase kinase (*15*, *16*). Thereafter, CaM-kinase I was also reported to be activated upon phosphorylation by an upstream $Ca^{2+}$/calmodulin-dependent protein kinase (*17*). There are two isoforms of CaM-kinase kinase in the brain (*18*), called CaM-kinase kinases α (*18*, *19*) and β (*20*, *21*), and both of them phosphorylate and activate both CaM-kinases IV and I (*19–22*). CaM-kinase kinase α is localized in the nucleus of cells, where CaM-kinase IV exists, and CaM-kinase kinase β is primarily distributed in the cytoplasm, where CaM-kinase I exists, as described later. The phosphorylation site involved in the activation of CaM-kinase I by CaM-kinase kinase has been identified as $Thr^{177}$ by amino acid sequence analysis (*23*), this being supported by site-directed mutational analysis (*24*). The corresponding phosphorylation site in CaM-kinase IV was suggested by site-directed mutational analysis to be $Thr^{196}$, which is located at an equivalent position to $Thr^{177}$ in CaM-kinase I (*22*, *25*), and this was confirmed by amino acid sequence analysis of the phosphorylated enzyme (*26*). CaM-kinase kinase phosphorylates only $Thr^{196}$ of CaM-kinase IV (*26*), though the activated CaM-kinase IV autophosphorylates several sites. For the phosphorylation of $Thr^{196}$ of CaM-kinase IV by CaM-kinase kinase, $Ca^{2+}$/calmodulin must bind not only to CaM-kinase kinase but also to CaM-kinase IV (*27*). The phosphorylation sites of CaM-kinases I and IV by CaM-kinase kinase, $Thr^{177}$ and $Thr^{196}$, respectively, are equivalent to the activating phosphorylation sites (in the activation loop) of a number of signal transducing protein kinases, such as PKA, cyclin-dependent protein kinase 2, and MAP kinases (*28*, *29*), though the autophosphorylation site responsible for the activation of CaM-kinase II, $Thr^{286}$, is not an equivalent.

After activation of CaM-kinase IV upon phosphorylation of $Thr^{196}$ by CaM-kinase kinase in the presence of $Ca^{2+}$/calmodulin, addition of EGTA to remove $Ca^{2+}$ enhances the phosphorylation of the enzyme, causing a decrease in the $Ca^{2+}$/calmodulin-dependent activity (*30*), as observed with CaM-kinase II (as described in the previous section). As shown in Fig. 3, incubation of CaM-kinase IV with CaM-kinase kinase under the phosphorylation conditions in the presence of $Ca^{2+}$/calmodulin results in rapid, marked activation and rather slow phosphorylation of the enzyme. After the phosphorylation has reached a maximum level, the addition of excess EGTA to remove $Ca^{2+}$ results in an additional phosphorylation of the enzyme and a decrease in the enzyme activity measured in the presence of $Ca^{2+}$/calmodulin without affecting the activity measured in the presence of excess EGTA, and both the activities in the presence and absence of $Ca^{2+}$/calmodulin finally become identical, indicating that CaM-kinase IV is in a completely $Ca^{2+}$/calmodulin-independent form. Serine residues $Ser^{332}$,

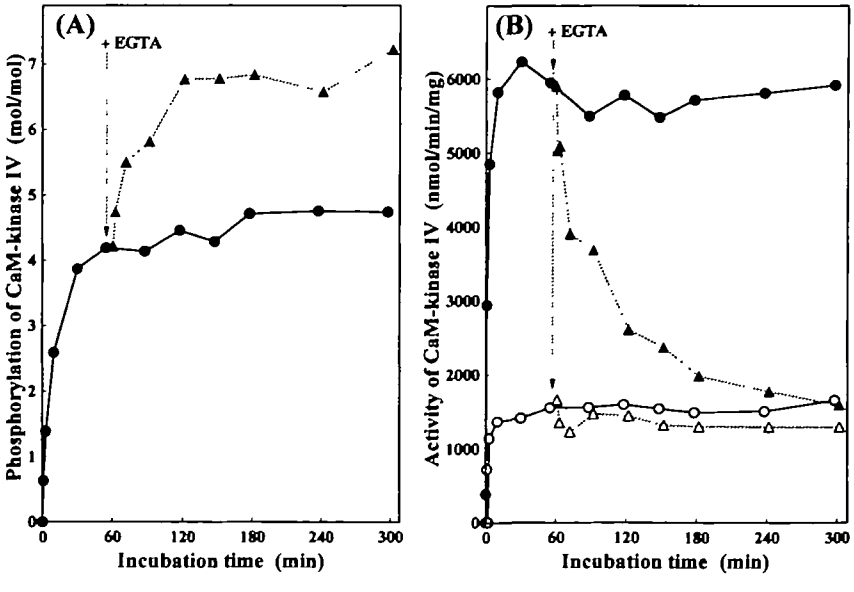


Fig. 3. **Time course of phosphorylation of CaM-kinase IV (A) and concomitant changes in its activity (B).** (A) CaM-kinase IV was incubated with CaM-kinase kinase in the presence of $Ca^{2+}$/calmodulin in the phosphorylation mixture containing [γ-$^{32}$P]ATP at 30°C (●). At 60 min, EGTA was added to remove $Ca^{2+}$ (▲). After incubation for the indicated periods, aliquots were taken for the determination of the amounts of [$^{32}$P]phosphate incorporated into protein. (B) CaM-kinase IV was incubated with non-radioactive ATP under the same conditions as described above, except that aliquots were taken for the measurement of the activity of CaM-kinase IV in the presence of $Ca^{2+}$ (●, ▲) or EGTA (○, △). For details, see Ref. *30*.

$Ser^{333}$, $Ser^{337}$, and $Ser^{341}$ of CaM-kinase IV have been identified as phosphorylation sites in the presence of EGTA by amino acid sequence analysis, among which $Ser^{332}$ has been identified as the phosphorylation site responsible for the inactivation of the $Ca^{2+}$/calmodulin-dependent activity by site-directed mutational analysis (*30*). Phosphorylation in the absence of $Ca^{2+}$ causing the loss of the $Ca^{2+}$/calmodulin-dependent activity occurs primarily through an intramolecular autophosphorylation mechanism (*30*), that is, a process occurring within a polypeptide, because CaM-kinase IV consists of a single polypeptide chain. Calmodulin overlay analysis of mutant enzymes has suggested that CaM-kinase IV phosphorylated at $Ser^{332}$ does not significantly bind calmodulin, but the enzyme phosphorylated at $Ser^{333}$ does bind calmodulin to the same extent as does the non-phosphorylated enzyme (*30*). It has been predicted that the region of amino acid residues 317–334 of CaM-kinase IV is the calmodulin-binding domain (*31*). Thus, CaM-kinase IV is activated upon phosphorylation of $Thr^{196}$ by CaM-kinase kinase in the presence of $Ca^{2+}$/calmodulin, and subsequent removal of $Ca^{2+}$ causes autophosphorylation of $Ser^{332}$ located in the calmodulin-binding domain, which makes the enzyme unable to bind calmodulin, thereby resulting in complete loss of the $Ca^{2+}$/calmodulin-dependent activity. Thus, the activities of CaM-kinases II and IV are regulated by very similar mechanisms. In the case of CaM-kinase I, such a loss of the $Ca^{2+}$/calmodulin-dependent activity by autophosphorylation in the absence of $Ca^{2+}$ has not been observed.

## Deactivation of CaM-kinases II, IV, and I by dephosphorylation

Since CaM-kinases II, IV, and I are markedly activated upon phosphorylation at $Thr^{286}$, $Thr^{196}$, and $Thr^{177}$, respectively, protein phosphatases dephosphorylating these phosphorylated threonine residues are thought to play important roles in the regulation of the activities of these multifunctional CaM-kinases. Protein phosphatases 1 (PPase 1) (*5*, *32*), 2A (PPase 2A) (*3*, *11*, *33*), and 2C (PPase 2C) (*34*) have been reported to dephosphorylate phosphorylated $Thr^{286}$ and also $Thr^{305/306}$ of CaM-kinase II, suggesting the possibility that these well-known multifunctional protein phosphatases are involved in restoring stimulated CaM-kinase II to its original state. It has recently been reported that dephosphorylation of soluble and postsynaptic density-associated CaM-kinase II in the forebrain is catalyzed predominantly by PPase 2A and PPase 1, respectively (*35*). Like CaM-kinase II, activated CaM-kinase IV has also been reported to be deactivated by PPase 2C (*36*), PPase 2A (*37*), PPase 1 (*38*), and calcineurin (*38*), although there are conflicting results concerning PPase 1 (*37*). Interestingly, CaM-kinase IV has been demonstrated to be associated with PPase 2A in Jurkat T cell and rat brain extracts (*39*). Activated CaM-kinase I has also been reported to be deactivated by PPase 2A (*40*). Thus, most of the well-known multifunctional protein phosphatases appear capable of dephosphorylating the phosphorylated residues involved in the regulation of the activities of CaM-kinases II, IV, and I.

On the other hand, a protein phosphatase (CaM-kinase phosphatase) that was first detected by an in-gel protein phosphatase assay with a phosphorylated synthetic peptide corresponding to the region around $Thr^{286}$ of CaM-kinase II as a substrate (*41*), has been demonstrated to fairly specifically dephosphorylate the phosphorylated $Thr^{286}$, $Thr^{196}$, and $Thr^{177}$ of CaM-kinases II, IV, and I, respectively (*42*, *43*), thereby deactivating these activated CaM-kinases. As shown in Fig. 4A, CaM-kinase IV is activated by incubation with CaM-kinase kinase under the phosphorylation conditions in the presence of $Ca^{2+}$/calmodulin. When the activated CaM-kinase IV is gel-filtered to remove of low-molecular weight substances such as ATP and calcium ion and then incubated with CaM-kinase phosphatase, its activity decreases progressively. CaM-kinase I activated by CaM-kinase kinase is also deactivated by incubation with CaM-kinase phosphatase, as shown in Fig. 4B. The recovery of the activity of CaM-kinase I after gel filtration is much better than that of CaM-kinase IV, presumably reflecting the higher stability of CaM-kinase I. Since CaM-kinase II be-

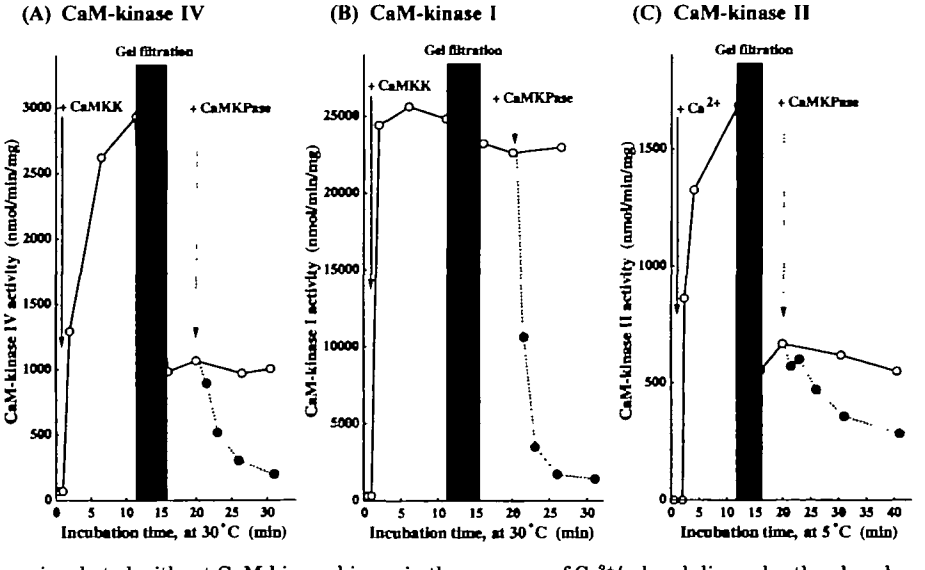


Fig. 4. **Deactivation of activated CaM-kinases IV (A), I (B), and II (C) by CaM-kinase phosphatase.** (A) CaM-kinase IV (20 μg/ml) was incubated with CaM-kinase kinase α (1 μg/ml) in the presence of $Ca^{2+}$/calmodulin under the phosphorylation conditions at 30°C, then the mixture was subjected to gel filtration on Sephadex G-50 (superfine) to remove ATP and $Ca^{2+}$, and the incubation was continued (○). At the indicated point, the mixture was divided into two portions, and CaM-kinase phosphatase was added to one portion (●). Aliquots were withdrawn at the indicated points and the enzyme activity was determined at 30°C using syntide-2 (40 μM) as a substrate in the presence of $Ca^{2+}$/calmodulin. (B) CaM-kinase I (10 μg/ml) was incubated with CaM-kinase kinase α (0.2 μg/ml), and the experiment was performed as described above. (C) CaM-kinase II (40 μg/ml) was incubated without CaM-kinase kinase in the presence of $Ca^{2+}$/calmodulin under the phosphorylation conditions at 5°C, and the experiment was performed as described above, except that the incubation was carried out at 5°C and the enzyme activity was measured at 30°C in the presence of EGTA added to remove $Ca^{2+}$.

comes very unstable upon autophosphorylation, the incubation is carried out at 5°C rather than 30°C (Fig. 4C). To avoid rapid autophosphorylation of the dephosphorylated CaM-kinase II during assay, as described in the first section in this review, the activity of CaM-kinase II is measured in the absence of $Ca^{2+}$. Studies of phosphorylated proteins including phosphorylase kinase, histones, myelin basic protein, and casein that had been phosphorylated by PKA, and phosphorylase *a* that had been phosphorylated by phosphorylase kinase showed that, unlike CaM-kinases I, II, and IV, none was significantly dephosphorylated by CaM-kinase phosphatase (*42*, *43*), and that no other proteins than CaM-kinase II in rat brain extract that had been phosphorylated by CaM-kinase II were dephosphorylated (*42*). Thus, CaM-kinase phosphatase, unlike such protein phosphatases as PPase 1, PPase 2A, PPase 2C, and calcineurin, which have a low degree of substrate specificity, is much more specific for the phosphorylation sites involved in the marked activation of CaM-kinases I, II, and IV. The activities of multifunctional CaM-kinases are thus regulated by phosphorylation and dephosphorylation catalyzed by a specific protein kinase and phosphatase.

**Tissue and subcellular distributions**

CaM-kinase II has four isoforms encoded by different genes, α, β, γ, and δ (*44*). CaM-kinase IIα is much more concentrated in brain than in other tissues, constituting almost 1% of total rat cerebral cortex protein. From 20 to 35% of of the enzume exists in soluble form in adult rat forebrain, and the remaining 65 to 80% is particulate and concentrated in the postsynaptic density (see Ref. *45* for a review). The other three isoforms of CaM-kinase II (β, γ, and δ) are all present not only in brain but also in other tissues in varying concentrations (*44*).

CaM-kinase I is widely distributed in the cytosol (*46*) of various neuronal and non-neuronal tissues (*47*, *48*), occurring at the highest level in the cerebral cortex (*47*). In contrast, CaM-kinase IV is predominantly distributed in the nuclei at highest levels in cerebellum, forebrain, and thymus, and at lower levels in spleen and testis, and was undetectable in several other tissues (see Ref. *45* for a review). Crude extracts of rat cerebral cortex, brain stem, and cerebellum showed high activity of CaM-kinase IV but a crude extract of thymus showed very little activity (*15*), suggesting that CaM-kinase IV exists mostly in an inactive form in the thymus, presumably reflecting an insufficiency of CaM-kinase kinase α or β in the thymus (*18*, *21*). CaM-kinase kinase α occurs at the highest levels in the cerebral cortex and brain stem and at lower levels in the cerebellum and retina, and was undetectable in the other tissues tested (*18*). CaM-kinase kinase β significantly exists only in the brain (*21*).

In their intracellular localization, CaM-kinase kinase α is present in cellular nuclei of virtually all neurons in the brain (*49*), though there is a conflicting report (*50*) suggesting the possibility that CaM-kinase kinase α is an upstream enzyme of CaM-kinase IV in the brain, because CaM-kinase IV occurs exclusively in the nuclei of neurons in the brain (*51*). In contrast, CaM-kinase kinase β is distributed in the cytoplasm (*50*) or in both the cytoplasm and nucleus (*52*), suggesting that the possibility that CaM-kinase kinase β is an upstream enzyme of CaM-kinase I.

Subcellular localization of CaM-kinase phosphatase, which dephosphorylates the phosphorylation sites involved in the activation of CaM-kinases I, II, and IV, is of particular interest, because CaM-kinases I and IV are almost exclusively distributed in the cytoplasm and cellular nucleus, respectively. Western blot analysis shows the ubiquitous tissue distribution of CaM-kinase phosphatase (*53*), and immunocytochemical analysis shows that the phosphatase is localized only in the cytoplasm and is never observed in the nucleus (*53*, *54*), indicating that CaM-kinase I can be a target for CaM-kinase phosphatase but that CaM-kinase IV cannot. Recently, we have found a new protein phosphatase (*55*), called CaM-kinase phosphatase N, which shows very similar catalytic properties including substrate specificity to those of CaM-kinase phosphatase. CaM-kinase phosphatase N, in contrast to CaM-kinase phosphatase, is particularly abundant in the brain and exclusively localized in the cellular nucleus, indicating the possibility that CaM-kinase IV is a target for CaM-kinase phosphatase N.

**Epilogue**

The present review has two objectives: one is to correct a widespread misconception concerning the regulation of CaM-kinase II activity, and the other is to provide an insight into the relationships between CaM-kinases I and IV and their upstream protein kinases and phosphatases.

The misconception that CaM-kinase II is converted to a $Ca^{2+}$/calmodulin-independent form upon autophosphorylation of its $Thr^{286}$ derived from the observation that CaM-kinase II exhibited maximal activity in the presence of $Ca^{2+}$/calmodulin but required preincubation under the phosphorylation conditions in the presence of $Ca^{2+}$/calmodulin in orders to exhibit the maximal activity in the absence of $Ca^{2+}$/calmodulin. This conclusion was apparently been supported by experiments with recombinant site-directed mutated enzymes in which $Thr^{286}$ was replaced with other amino acid residues. However, it is very difficult to keep the natural conformation of CaM-kinase II in the recombinant enzymes, whether expressed in *E. coli* or in eukaryotic cells such as Sf-9 cells; for example, most of the mutated enzyme in which $Thr^{286}$ is replaced with alanine or aspartic acid is not recovered in the soluble fraction after purification (unpublished observation). The specific activity of even the wild-type enzyme expressed in Sf-9 cells and purified to apparent homogeneity using calmodulin affinity chromatography is sometimes much lower than that of the enzyme purified from the brain. Experiments with the enzyme having a high specific activity using the limiting phosphorylation conditions clearly show that both $Ca^{2+}$/calmodulin-dependent and independent activities of CaM-kinase II increase with the concomitant phosphorylation of $Thr^{286}$, as described above (Fig. 2). Furthermore, the enzyme autophosphorylated at $Thr^{286}$ shows th same $V_{max}$ values for various substrates in the presence and absence of $Ca^{2+}$/calmodulin, indicating that activities in the presence and absence of $Ca^{2+}$/calmodulin are generated through the same mechanism and accordingly that the increase in the activity in the absence of $Ca^{2+}$/calmodulin results from the increase in the total activity. Thus, CaM-kinase II is not converted to a $Ca^{2+}$/calmodulin-independent form but activated upon autophosphorylation at $Thr^{286}$ in the presence of $Ca^{2+}$/calmodulin. The conversion to a $Ca^{2+}$/calmodulin-independent form of CaM-kinase II is a consequence of loss of

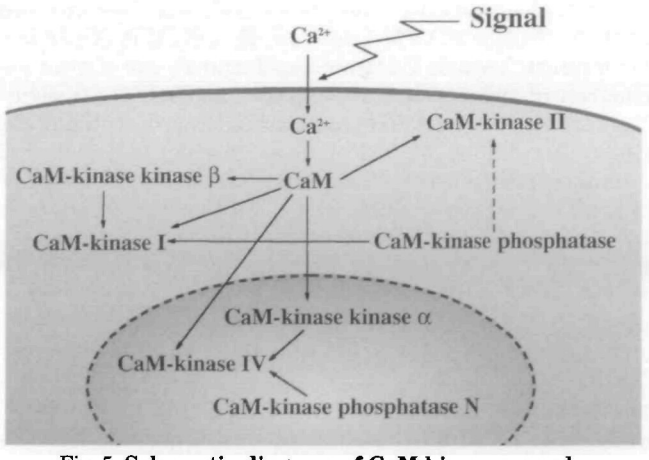


Fig. 5. **Schematic diagram of CaM-kinase cascade.**

the calmodulin-binding activity caused by the autophosphorylation of $Thr^{306}$ located within the calmodulin-binding site. A similar mechanism is also observed with CaM-kinase IV as described above.

The possible relationships between CaM-kinases I and IV and their regulatory system of CaM-kinase kinases and phosphatases are schematically summarized in Fig. 5. The $Ca^{2+}$ signaling systems consisting of CaM-kinase kinase α, CaM-kinase IV, and CaM-kinase phosphatase N in the cellular nuclei, and of CaM-kinase kinase β, CaM-kinase I, and CaM-kinase phosphatase in the cytoplasm are suggested by subcellular distribution studies of the respective enzymes.

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