Characterization of the Mechanism of Regulation of Ca\(^{2+}\)/
Calmodulin-dependent Protein Kinase I by Calmodulin and by Ca\(^{2+}\)/
Calmodulin-dependent Protein Kinase Kinase*

(Received for publication, March 9, 1998, and in revised form, June 4, 1998)

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Ca\(^{2+}\)/calmodulin-dependent protein kinase I (CaMKI) is maintained in an autoinhibited state by the interaction of a COOH-terminal helix-loop-helix (Ile286-Met316) regulatory domain with the catalytic core. Activation of the enzyme by calmodulin (CaM) also allows CaMKI to be phosphorylated and activated by a second enzyme, CaM kinase (CaMKK). To more thoroughly characterize the regulation of CaMKI by CaM and its interrelationship with phosphorylation by CaMKK, we have carried out a detailed structure-function analysis using recombinant wild-type (WT) and mutant forms of CaMKI and CaMKK. CaMKI-WT, in the absence of CaM, or CaMKI-299 and CaMKI-298 were autoinhibited and could not be phosphorylated by CaMKK-453 (a truncated constitutively active form of CaMKK). Removal of Phe298 (CaMK-297) generated a constitutively active form of CaMKI that was also phosphorylated by CaMKK-433. CaMKI-WT was essentially inactive in the absence of CaM (K_m for activation by CaM ~30 nM). Mutation of Ile294 and Phe298 to alanine (CaMKI-2A) resulted in measurable basal enzyme activity. Additional mutation of Ile296 and Val290 to alanine (CaMKI-4A) increased this basal activity. Mutation of Trp303 (CaMKI-W303S) resulted in a large increase in the K_m for CaM (~100 \muM), supporting a role for this residue as an initial target for CaM. Mutation of Phe297 (CaMKI-F307A) resulted in increased basal enzyme activity, supporting a role for this residue in autoinhibition of CaMKI. Together these studies demonstrate the critical role of specific amino acids in the autoinhibition of CaMKI and also in its activation by CaM and phosphorylation by CaMKK.

Many of the intracellular actions of Ca\(^{2+}\) in eukaryotic cells are mediated by activation of the family of Ca\(^{2+}\)/calmodulin-dependent protein kinases (CaMKs),\(^1\) that include the monofunctional enzymes, myosin light chain kinase (MLCK) and phosphorylase kinase, and the multifunctional enzymes, CaMKI, CaMKII, and CaMKIV (1–6). New members of the family continue to be characterized, including CaMKK that phosphorylate and activate CaMKI and CaMKIV as part of a kinase cascade (7–15). A number of novel CaM kinases of unknown function also have been described recently (16, 17). Elongation factor 2 kinase (EF2 kinase), originally thought to represent a member of this family, now appears to be related to a distinct class of protein kinases (18–21).

The CaMKs share many common structural and regulatory features. They each have a catalytic domain that is relatively highly conserved (~40–50% amino acid identity) and a COOH-terminal regulatory domain that is not as highly conserved among family members (3–6). Like cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), the CaMKs are maintained in an inactive state by an intrasteric autoinhibitory mechanism (6, 22–28), a central feature of which is that amino acid residues within the regulatory domain bind to the active site of the kinases and sterically block the access of substrates. The CaMKs are activated by a high affinity interaction of the Ca\(^{2+}\)/CaM complex with the regulatory domain which removes the autoinhibitory sequence from the active site.

Many details concerning the regulation of the CaMK family have come from mutagenesis studies of MLCK, CaMKI, CaMKII, and CaMKIV (23, 24, 26, 27, 29–31) and from the recent determination of the crystal structure of CaMKI in its autoinhibited form (28). CaMKI is a monomeric enzyme of ~42 kDa that has widespread tissue distribution and that can phosphorylate a number of substrates including synapsin I, synapsin II (32, 33), and CREB, the cAMP-response element-binding protein (34). In the crystal structure of a truncated autoinhibited fragment of CaMKI, the regulatory domain (residues Ile286-Met316) binds to the catalytic domain via an αR1-helix-loop-αR2-helix structure not observed in other protein kinases (28). Hydrophobic residues in the αR1 helix interact with a hydrophobic channel on the surface of the COOH-terminal lobe of the catalytic domain. Following αR1, the regulatory domain does not enter the active site, but instead turns away to form a protuberant loop (residues 300–304) that includes Thr303. The αR2 helix (residues 305–316) then unexpectedly interacts with the outside of the ATP-binding loop in the NH2-terminal lobe of the catalytic domain, resulting in a striking distortion of this loop. Thus the regulatory domain of CaMKI forms multiple interactions with the catalytic domain that appear likely to interfere with both peptide substrate and MgATP binding.

In addition to their regulation by CaM, CaMKI and CaMKIV are activated by phosphorylation by a highly specific CaMKK at an equivalent threonine (Thr177 in CaMKI) in the so-called “activation-“ or “T-loop”, present near the active site of both kinases (7–15). The phosphorylation of CaMKI by CaMKK is dependent on CaM in two distinct ways. CaMKK must bind to CaM to be active. In addition, CaMKK cannot phosphorylate Thr177 of CaMKI unless CaM is bound to the substrate enzyme (10, 35). Notably, in the structure of the autoinhibited form of

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1 The abbreviations used are: CaMK, Ca\(^{2+}\)/calmodulin-dependent protein kinase; CaM, calmodulin; CaMKI, Ca\(^{2+}\)/CaM-dependent protein kinase I; CaMKIV, Ca\(^{2+}\)/CaM-dependent protein kinase IV; CaMKK, Ca\(^{2+}\)/CaM-dependent protein kinase kinase; PKA, cAMP-dependent protein kinase; GST, glutathione S-transferase; MAP kinase, mitogen-activated protein kinase; MLCK, myosin light chain kinase; RT-PCR, reverse-transcription polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; WT, wild type.
CaMKI, the activation loop, although disordered, appears to be accessible (28).

The crystal structure of CaMKI has suggested a general model of autoinhibition of enzyme activity. To address the function played by specific amino acids in the autoinhibition of CaMKI, and also in its activation by CaM and phosphorylation by CaMKK, we have carried out a detailed structure-function analysis of the regulation of CaMKI, using recombinant wild-type and mutants forms of CaMKI and CaMKK. The results obtained from these enzymatic studies of full-length CaMKI, together with the crystal structure of the autoinhibited enzyme, provide a detailed model for the regulation of this enzyme by CaM and phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—ATP, Nonidet P-40, dithiothreitol, 2-mercaptoethanol, EDTA, EGTA, Tris, Coomassie Brilliant Blue R-250, SDS, and bovine serum albumin were from Sigma. HEPES, phenylmethylsulfonyl fluoride were from Calbiochem. γ-32P-ATP was from NEN Life Science Products. Thin layer cellulose sheets were from Eastman Kodak. Phosphocellulose P-81 paper was from Whatman. CaM was purified from rabbit brain as described (36). Recombinant CaMKIV expressed in Sf9 cells was provided by Dr. Anthony Means.

Synthesis of Peptides—The peptide YLRRRLSDSNF-amide (corresponding to residues 10–29 of CaMKK) and the peptide encompassing residues 10–29 of CaMKII (QDPRAELVVERAIAVHLE) (15) were synthesized by the Keck Foundation Biopolymer Facility at Yale University. The peptides were purified by preparative reversed-phase high performance liquid chromatography, were >95% pure as analyzed by high performance liquid chromatography, and had the expected amino acid composition and mass spectra.

Cloning of CaMKKα cDNA—PC12 mRNA was reverse-transcribed with 10 μM of random mix primers using an RT-PCR kit (Stratagene). The following oligonucleotides (Operon) were synthesized. Oligo KI-1S corresponded to cDNA encoding rat CaMKKβ and introduced a BamHI site. Oligo KI-505AS corresponded to the complement of rat CaMKKβ and introduced an EcoRI site. PCR was performed using: KI-1S (5′-CCCGGATCCATGCCAGGGGCAGTGGAAGGCCCC-3′) and KI-505AS (5′-CCCAATTGTCCAGGATGCAGCCTCATCTTC-3′) using the following conditions: 30 cycles 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min. The amplified product was purified using an agarose electrophoresis gel purification kit (Qiagen) and cloned into the TA vector (Invitrogen) and all other deletion or substitution constructs were sequenced by the Rockefeller University DNA sequence Facility.

Construction of COOH-terminal Truncated Mutants of CaMKKα—The following oligonucleotides (Operon) were synthesized: KK-433AS (5′-GGCGATCCATGCAGCAGGGCCAGTGGAAGGCCCC-3′) and KK-444AS (5′-GGCGATCCATGCAGCAGGGCCAGTGGAAGGCCCC-3′). PCR was performed under the conditions described above except that an EcoRI site was added to the 5′ end of the template, and the following primers were used: KI-4A-S (5′-AGGCGATCCATGCAGCAGGGCCAGTGGAAGGCCCC-3′) and KI-F307A-AS (5′-AGGCGATCCATGCAGCAGGGCCAGTGGAAGGCCCC-3′). PCR was performed under the following conditions: 16 cycles at 95°C for 1 min, 56°C for 1 min and 68°C for 12 min. The reaction mixtures were digested with Dpn1 for 1 h, then 1 μl of digested samples was transfected into XL1 blue Escherichia coli.

Expression and Purification of Recombinant Proteins—BL21 (DE3) cells containing each expression plasmid were grown overnight at 37 °C in 100 ml of LB plus 100 μg/ml ampicillin. 1 liter of LB plus 100 μg/ml ampicillin was inoculated with 100 ml of the overnight culture and incubated at 30°C to an A600 of 0.6. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration 0.3 mM, and each culture was incubated for 3 h. Cells were harvested by centrifugation at 4,000 × g for 10 min and resuspended in 100 ml of lysis buffer containing 50 mM Tris (pH 7.5), 2 mM MgCl2, and 100 μM phenylmethylsulfonyl fluoride. The cell suspension was sonicated and eluted with 50 mM Tris-HCl (pH 8.8) containing 5 mM glutathione. Fractions were collected, and protein was analyzed by SDS-polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue. Fractions containing expressed protein were pooled and dialyzed against equilibration buffer. Protein concentrations were determined using a BCA kit (Pierce). Glyceraldehyde was added to 20% (v/v), and aliquots were stored at −80°C.

Antibody Preparation—An antibody was prepared against a synthetic peptide encompassing residues 10–29 of CaMKKα (QDPRAELVVERAIAVHLE) (15). Purified peptide was coupled to bovine thyroglobulin using glutaraldehyde. Antiserum CC135 was prepared in New Zealand White rabbits by Cocalico Biologicals, Inc. The synthetic peptide was coupled to activated CH-Sepharose beads and used to affinity-purify the sera essentially as described (37). For immunoblotting, proteins were transferred to nitrocellulose filters as described (38). Immune complexes were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Phosphorylation Assays—The standard reaction mixture (100 μl) contained 50 mM HEPES (pH 7.5), 1 mM magnesium acetate, 1 mM EGTA, 5 mM dithiothreitol, 100 μM γ-32P-ATP (specific activity, 2 × 10⁶ cpm/pmol), 1 μM CaM, and 0.5 μg CaMKI. For studies of the phosphorylation of CaMKIIα and CaMKIIβ, the concentrations of CaM were 1 μg/ml. The concentration of CaMKIIα was 10 μg/ml, and CaMKIIβ, 5 μg/ml. After 2 min of preincubation at 30°C, reactions were initiated by the addition of ATP. Reactions were terminated by the addition of 100 μl of SDS sample buffer (1% SDS, 60 mM Tris-HCl (pH 6.8), 5% (v/v) glycerol, 0.2 mM β-mercaptoethanol). Samples were analyzed by SDS-PAGE (10% polyacrylamide). Gels were stained with Coomassie Brilliant Blue, destained, dried, and subjected to autoradiography or analysis using a PhosphorImager (Molecular Dynamics). For studies of the effect of phosphorylation on enzyme activity, recombinant CaMKI and CaMKIV (10 μg/ml) were preincubated in the absence or presence of CaMKK (2 μg/ml) for the indicated times at 30°C under the conditions described above except that nonradioactive ATP was used. A 1/10 volume of each preincubation mixture was added to a second phosphorylation assay using synapsin I site 1 peptide (100 μM) as substrate. Reactions were stopped by addition of acetic acid to a final concentration of 10%, and 32P-incorporation into the peptide substrate was determined using a standard P-81 filter paper assay. For analysis of the CaM dependence of CaMKI and CaMKIV, enzyme activity was measured using the synapsin I site 1 peptide (100 μM) as substrate, and various concentrations of CaM as indicated.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as described (32). Gel pieces containing 32P-labeled proteins were excised from dried SDS-polyacrylamide gels and digested with trypsin. Phosphoamino acid analysis was performed as described (32). Gel pieces containing 32P-labeled proteins were excised from dried SDS-polyacrylamide gels and digested with trypsin.
Phosphorylation and Activation of CaMKI and CaMKIV by CaMKK—The purpose of the present study was to investigate the regulation of CaMKI by CaM and phosphorylation by CaMKK. For these studies, rat CaMKKα was cloned from PC12 cells by RT-PCR, and recombinant protein was expressed as a GST-fusion protein. CaMKKα was initially identified as an enzyme that could phosphorylate and activate CaMKIV (15, 39). In addition, CaMKKα was found to phosphorylate CaMKI (15, 40), but information regarding the relative efficiency of phosphorylation of the two substrates was not available. We compared the ability of recombinant CaMKKα to phosphorylate and activate CaMKI and CaMKIV (Figs. 1 and 2). CaMKI was phosphorylated essentially only on threonine, and CaMKIV was phosphorylated on both serine and threonine (Fig. 1B). Notably, the total incorporation of 32P into CaMKIV was much greater than that into CaMKI, but this higher phosphorylation resulted from increased serine phosphorylation, presumably as a result of autophosphorylation of activated CaMKIV (41, 42). Phosphorylation of either CaMKI or CaMKIV by CaMKKα was associated with large increases in enzyme activity that paralleled the phosphorylation of threonine in both enzymes (Fig. 2). The rate of increase in threonine phosphorylation, as well as the fold-increase in kinase activity, was higher for CaMKI than for CaMKIV, suggesting that CaMKKα slightly prefers CaMKI as a substrate compared with CaMKIV.

CaM Dependence of Phosphorylation of CaMKI by CaMKKα—CaMKKα, like other CaM kinases, contains putative autoinhibitory and CaM-binding domains following its catalytic domain (15) (Fig. 3A). It was necessary to define the positions of these regulatory subdomains for use in the studies described below. CaMKK-WT and several COOH-terminal truncations were expressed as recombinant proteins (Fig. 3, A and B). Enzyme activity was measured using CaMKI-293 or CaMKI-WT as a substrate in the absence or presence of Ca2+/CaM (Fig. 3C and Fig. 4). CaMKI-293 is constitutively active following removal of the autoinhibitory and CaM-binding domains of CaMKI (27). Consistent with previous studies using a rat brain preparation (10), recombinant CaMKK-WT phosphorylated CaMKI-293 in a Ca2+/CaM-dependent manner (Fig. 3C). In contrast, CaMKK-433 was basally active in the absence of Ca2+/CaM and was slightly inhibited by their addition. Moreover, CaMKK-433 could only phosphorylate CaMKI-WT in the presence of Ca2+/CaM. CaMKK-457, like CaMKK-WT, phosphorylated CaMKI-293 in a Ca2+/CaM-dependent manner; however, CaMKK-444 was basally inactive and could not be activated by addition of Ca2+/CaM. Taken together, these studies indicate that the CaM-binding domain of CaMKK is contained within residues 444–457, and the autoinhibitory domain is contained within residues 433–444. Similar conclusions have recently been made based on studies of slightly different CaMKK mutants (43). The CaM dependence of CaMKK was measured at different concentrations of CaM, and the results were compared with that of CaMKI and CaMKIV (Fig. 4). Using synapsin I site 1 peptide as substrate, the concentration of CaM required for half-maximal activation (K0.5) of both CaMKI and CaMKIV was ~30 mM. Using CaMKI-293 as substrate, the K0.5 of CaMKK was ~5 nM.

Relationship between the Regulation of CaMKI by CaM and Phosphorylation by CaMKK—To further investigate the structural basis for the interrelationship between activation by Ca2+/CaM and phosphorylation by CaMKK, a number of truncation mutants of CaMKI were prepared (Fig. 5A), and their phosphorylation by CaMKK-433 was compared with their regulation by CaM. CaMKI-299, which was inactive in either the absence or presence of Ca2+/CaM (Fig. 5B), was not phosphorylated by CaMKK-433 (Fig. 5C). Removal of A299 to produce CaMKI-298, maintained the enzyme in its autoinhibited form. Moreover, CaMKI-298 was not phosphorylated by CaMKK-433. However, removal of Phe298 to produce CaMKI-297, generated an enzyme that was not only constitutively active but was also phosphorylated by CaMKK-433. Notably, the activity of CaMKI-297 and the level of phosphorylation of this mutant by CaMKK-433 was slightly lower than that of CaMKI-293 (Fig. 5 and data not shown). CaMKI-294 exhibited essentially the same properties as CaMKI-293 (data not shown), suggest-

![Figure 1](https://example.com/figure1.png)
sulted in a large increase in the K\textsubscript{m} for MgATP for CaMKI-W307A (measured in the absence of Ca\textsuperscript{2+}/CaM) compared with CaMKI-WT (measured in the presence of Ca\textsuperscript{2+}/CaM) (data not shown).

The results described in Fig. 5 indicate the critical role that Phe\textsuperscript{298} plays in the autoinhibition of CaMKI and the ability of the enzyme to be phosphorylated by CaMKK. Phe\textsuperscript{298} is situated at the COOH terminus of the \alpha1 helix in CaMKI that interacts with the catalytic domain via three additional hydrophobic amino acids, Ile\textsuperscript{286}, Val\textsuperscript{290}, and Ile\textsuperscript{294}. Replacement of Ile\textsuperscript{294} and Phe\textsuperscript{298} with alanine (CaMKI-2A) resulted in an increase in the basal activity of the mutant (Fig. 6B). Additional mutation of Ile\textsuperscript{286} and Val\textsuperscript{290} (CaMKI-4A) resulted in a further increase in the basal activity of enzyme activity. For both mutants, there was no significant change in the K\textsubscript{0.5} for CaM, although like CaMKI-F307A, small leftward shifts in the CaM activation curves were consistently observed. In addition, there were no significant changes in the apparent K\textsubscript{m} for peptide substrate for CaMKI-2A or -4A (measured in the absence of Ca\textsuperscript{2+}/CaM) compared with CaMKI-WT (measured in the presence of Ca\textsuperscript{2+}/CaM) (data not shown). However, notably, CaMKI-2A and CaMKI-4A, but not CaMKI-F307A (Fig. 6C) or CaMKI-W303S (data not shown), were phosphorylated at Thr\textsuperscript{177} by CaMKK-433.

**DISCUSSION**

The results obtained from the present enzymatic studies using full-length CaMKI provide strong support for the mechanism of autoinhibition and activation by CaM that was suggested by the crystal structure of the truncated kinase. The results also provide insight into the interrelationship between the autoinhibition of CaMKI and the phosphorylation of the T-loop by CaMKK.

An important observation made in this study is that, in the truncated form of the kinase, Phe\textsuperscript{298} alone is sufficient to maintain CaMKI in an inactive, autoinhibited state. This result extends previous deletion mutagenesis studies that had indicated a role for residues 294–299 in autoinhibition of CaMKI (10, 27). The consensus amino acid sequence found in substrates for CaMKI is Hyd-B-Arg-B-X-Ser/Thr-X-X-Hyd (where X is any amino acid, B is preferably a basic residue, Hyd is a hydrophobic residue, and Ser/Thr represents the residue phosphorylated at the P0 position) (47). In the crystal structure of autoinhibited CaMKI, Lys\textsuperscript{300} interacts with Ghu\textsuperscript{152}, and we have suggested that Lys\textsuperscript{300} mimics the P-3 basic amino acid in the peptide substrate (28), a suggestion supported by the observation that mutation of Ghu\textsuperscript{152} alters the P-3 specificity of the enzyme.\textsuperscript{2} In the autoinhibited form of CaMKI, Phe\textsuperscript{298} interacts with a hydrophobic pocket formed by Ile\textsuperscript{286}, Pro\textsuperscript{216}, and Ile\textsuperscript{294}. Replacement of Ile\textsuperscript{294} with alanine (CaMKI-2A) resulted in an increase in the basal activity of the enzyme to be phosphorylated by CaMKK. Phe\textsuperscript{298} plays an important role in the autoinhibition of CaMKI and the ability of the enzyme to be phosphorylated by CaMKK. Phe\textsuperscript{298} is situated at the COOH terminus of the \alpha1 helix in CaMKI that interacts with the catalytic domain via three additional hydrophobic amino acids, Ile\textsuperscript{286}, Val\textsuperscript{290}, and Ile\textsuperscript{294}. Replacement of Ile\textsuperscript{294} and Phe\textsuperscript{298} with alanine (CaMKI-2A) resulted in an increase in the basal activity of the mutant (Fig. 6B). Additional mutation of Ile\textsuperscript{286} and Val\textsuperscript{290} (CaMKI-4A) resulted in a further increase in the basal activity of enzyme activity. For both mutants, there was no significant change in the K\textsubscript{0.5} for CaM, although like CaMKI-F307A, small leftward shifts in the CaM activation curves were consistently observed. In addition, there were no significant changes in the apparent K\textsubscript{m} for peptide substrate for CaMKI-2A or -4A (measured in the absence of Ca\textsuperscript{2+}/CaM) compared with CaMKI-WT (measured in the presence of Ca\textsuperscript{2+}/CaM) (data not shown). However, notably, CaMKI-2A and CaMKI-4A, but not CaMKI-F307A (Fig. 6C) or CaMKI-W303S (data not shown), were phosphorylated at Thr\textsuperscript{177} by CaMKK-433.

The interaction of CaMKK with CaM is bipartite; the NH\textsubscript{2}-terminal hydrophobic residue interacts primarily with the COOH-terminal domain of CaM, whereas the COOH-terminal hydrophobic residue interacts with the NH\textsubscript{2}-terminal domain of CaM. In several cases, the NH\textsubscript{2}-terminal hydrophobic amino acid in CaM-binding pep-
tides is a tryptophan. Mutation of Trp303 of CaMKI resulted in a large decrease in the $K_{0.5}$ for CaM. This result, together with the fact that the side-chain of Trp 303 points outward from the catalytic core in the crystal structure of CaMKI, is consistent with this residue being the initial target for the COOH-terminal domain of CaM and that this interaction is important for the normal activation of the enzyme. Based on the structures of CaM in complexes with different CaM-binding peptides (44–46) and truncation mutagenesis studies of CaMKI (10, 27), residues Trp303 and Val312 (or Val313) most likely represent the important anchoring hydrophobic residues. However, based on the crystal structure of the CaMKII peptide/CaM complex (46), it is probable that the interaction of the COOH-terminal domain of CaM with CaMKI includes residues Phe298 to Trp303.

**Fig. 3.** Construction and expression of a series of COOH-terminal truncated mutants of CaMKK as GST fusion proteins. A, schematic representation of wild type CaMKK and mutant enzymes. A series of DNAs were amplified using PCR from a cDNA encoding rat CaMKKα as described under “Experimental Procedures.” The DNA fragments were subcloned into pGEX-2T and expressed in E. coli as GST fusion proteins. B, recombinant CaMKK mutants (0.1 μg) were separated by SDS-PAGE (10% polyacrylamide) and analyzed by immunoblotting using an antibody that recognizes the NH₂ terminus of CaMKKα. C, CaMKI-293 (10 μg/ml) and CaMKI-WT (10 μg/ml) were incubated with CaMKKα mutants (5 μg/ml) as indicated, for 60 min, at 30 °C in the absence or presence of 1.5 mM CaCl₂, 1 μM CaM as described under “Experimental Procedures.” Proteins were separated by SDS-PAGE (10% polyacrylamide), and autoradiography was performed. The low level of incorporation of ³²P into CaMKI-293 in the CaMKK-WT and CaMKK-457 samples (absence of CaCl₂/CaM) and CaMKK-444 sample (absence and presence of CaCl₂/CaM) reflects a low level of autophosphorylation of CaMKI-293.

**Fig. 4.** CaM dependence of CaMKI, CaMKIV, and CaMKK. CaMKI (0.1 μg/ml), CaMKIV (0.1 μg/ml) and CaMKK-WT (5 μg/ml) were assayed in the presence of 1 mM CaCl₂ with the indicated concentrations of CaM. For CaMKI and CaMKIV, synapsin I site 1 peptide was used as substrate. For CaMKK-WT, CaMKI-293 (50 μg/ml) was used as substrate. ³²P-incorporation into peptide was determined as described under “Experimental Procedures.” ³²P-incorporation into CaMKI-293 was determined by PhosphorImager analysis with basal phosphorylation of CaMKI-293 being subtracted.
The phosphorylation of CaMKI by CaMKK.

A. activities of CaMKI-WT and CaMKI mutants (0.1 

Lys300, from the catalytic core of the enzyme. 

pseudosubstrate interactions in autoinhibition, these results 

reflects a low level of autophosphorylation of these mutants. 

The low level of incorporation of32P into CaMKI-298 and CaMKI-299 

described under “Experimental Procedures.” Samples were analyzed by 

described under “Experimental Procedures.” Results are the average of 

Km for ATP for CaMKI. This dependence of the 

Km for ATP on alteration of the NH2-terminal domain of CaM is consistent 

with the idea that interaction of residues 307–316 of CaM, Phe307, Ala 309, Ala 311, Val 312, Val 313, and Met 316 in the 

Phe307, Ala 309, Ala 311, Val 312, Val 313, and Met 316 in the 

CHK and CaMKII have demonstrated that Met124, in 

the COOH terminal domain of the bound target 

peptides. In CaMKI, this would include Trp303, and perhaps Phe298 (see above). Mutation of several hydrophobic amino 

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ing to the enzyme, and to contribute to an additional level of 

autoinhibition.

The results obtained in the present study indicate that the mechanisms involved in autoinhibition of CaMKII are also im-

portant for regulating the phosphorylation of the enzyme by 

CaMKK. Thus, removal of a single amino acid, Phe298, (in the 

truncated form of CaMKI) resulted in a constitutively active 

enzyme, or in CaMKI-WT in the presence of CaM, once the 

phorylatable; see below) state.

The crystal structure of autoinhibited CaMKI revealed an 

unexpected interaction of the CaM-binding domain of the en-

zyme with the outer surface of the ATP-binding domain. Ex-

tensive hydrophobic interactions are mediated by Ala306, 
Phe307, Ala309, Ala311, Val312, Val313, and Met316 in the aR2 

helix, with Leu26, Gly27, Phe31, Leu36, Leu45, Leu79, Ser99, and 

Gly101 in the ATP-binding domain. These interactions lead to a 

large displacement of the ATP-binding loop relative to that 

observed in a substrate-bound PKA structure (28, 50, 51). Such 

a displacement of the ATP-binding domain would likely lead 

to inhibition of binding of MgATP to the enzyme. Mutation of 
Phe307 (CaMKI-F307A) led to the generation of basal CaM-

independent kinase activity that was ~30% of the maximal 

CaM-dependent activity. This result, that was obtained in 

CaMKI-WT in solution, is consistent with a role for Phe307 in 

the autoinhibition of the kinase. Notably, the effect of mutation of Phe307 occurred in full-length CaMKI in which Ile286, Val290, 

Ile294, and Phe298 would still be expected to play a significant 

role in blocking peptide substrate binding to the enzyme. This 
suggests that multiple interactions between residues in the 
aR1 and aR2 helices contribute to the autoinhibition of CaMKI. 

However, following truncation of the COOH terminus of the 

enzyme, including the activation of CaMKI by CaM mutants (52). Mutation of 

M124, located in the COOH-terminal domain of CaM (M124Q), 

resulted in an ~60-fold increase in the Ka5 for CaM. Solution 

and crystal structures of CaM bound to CaM-binding peptides 

from MLCK and CaMKII have demonstrated that Met124, in 

the COOH terminus of CaM, plays an important role in the 

interaction with the NH2-terminal domain of the bound target 

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CaMKK. Thus, removal of a single amino acid, Phe298, (in the 

truncated form of CaMKI) resulted in a constitutively active 

enzyme and generated a substrate for CaMKK-433. In the 

crystal structure of autoinhibited CaMKI, the T-loop that con-

tains the threonine residue phosphorylated by CaMKK (Thr177) 

is disordered, although apparently exposed to solvent. Phe298 is 
situated at the COOH-terminal end of aR1, some distance from 

Thr177, and it does not appear possible that Phe298 can influ-

ence the organization of the T-loop via any direct interaction. 

CaMKK must therefore recognize structural elements of fully 

active CaMKI that are only present in the truncated active 

enzyme, or in CaMKI-WT in the presence of CaM, once the 

Therefore, in the full-length enzyme, binding of the COOH-

terminal domain of CaM to CaMKI would be sufficient to re-

move the pseudosubstrate domain, including Phe298 and 

Lys300, from the catalytic core of the enzyme.

The interaction of aR1 with the catalytic domain is mediated by 

Ile286, Val290, Ile294, and Phe298 (28). The involvement of 

hydrophobic amino acids in the interaction of the regulatory domain with the catalytic domain was unexpected because several previous studies of CaM regulation had focused on potential roles for basic amino acids in mediating charge-based 
pseudosubstrate interactions (23, 24, 49). Mutation of Ile294 

and Phe298 to alanine resulted in the generation of a modest 

level of CaM-independent enzyme activity. Additional mutation of Ile286 and Val290 resulted in a significant increase in this 

CaM-independent activity. Taken together with the role of 
pseudosubstrate interactions in autoinhibition, these results 
support the idea that, although Ile286, Val290, and Ile294 may 

not be absolutely critical for autoinhibition, they serve to an-

chor the aR1 helix in a position such that Phe298 plays a major 

role in maintaining the enzyme in an inhibited (and nonphos-
inhibitory influence of Phe\textsuperscript{298} is removed. Movement of Phe\textsuperscript{298} away from the hydrophobic pocket of CaMKI may expose a specific binding site for CaMKK, or more likely result in changes in the overall structure of CaMKI that allows CaMKK to bind and phosphorylate Thr\textsuperscript{177}. Notably, acidic residues in domains V and VI (Glu\textsuperscript{102} and Glu\textsuperscript{145} of CaMKI), that are important for recognition of basic amino acids within substrate recognition motifs for CaMKI, CaMKII, CaMKIV, and PKA, are not conserved in CaMKK (15). This supports the idea that CaMKK must utilize other distinct interactions for recognition of its substrates, CaMKI and CaMKIV. This would be consistent with studies of enzymes in the various MAP kinase pathways that have revealed that protein-protein interactions away from the active site of the upstream kinase, and away from the T-loop of the downstream substrate, are important for directing the specificity of a kinase cascade (53–55).

The discovery that CaMKI and CaMKIV are regulated by T-loop phosphorylation is not surprising, given that this is a mechanism that is common to many protein kinases. However, the physiological significance of the coupling of two protein kinases that are both tightly regulated by CaM is not clear at this time. In many respects the regulation of CaMKK and CaMKI by CaM is analogous to that of 5\textsuperscript{-}AMP activated protein kinase kinase (AMPKK) which phosphorylates and activates 5\textsuperscript{-}AMP activated protein kinase (AMPK) (56), or 3-phosphoinositide-dependent protein kinase (PDK1) which phosphorylates and activates protein kinase B (PKB) (57, 58). Studies of the protein kinase cascades involved in the regulation of glycogen metabolism and the MAP kinases suggest that one function of a cascade is signal amplification (59–61). Theoretical analysis of the MAP kinase cascade also suggests that cooperative relationships between enzymes leads to a more

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**Fig. 6. CaM dependence of CaMKI-WT and CaMKI regulatory domain mutants.**

A, comparison of the amino acid sequences of CaMKI-WT and CaMKI mutants. B, CaMKI (0.1 \( \mu \)g/ml) and CaMKI regulatory domain mutants (0.1 \( \mu \)g/ml) were assayed in the presence of 1 mM CaCl\textsubscript{2} with the indicated concentrations of CaM using synapsin I site 1 peptide as substrate. Activity is normalized to that of fully active CaMKI-WT. Except for CaMKI-W303S, the maximal activity of CaMKI-WT and the other mutants was identical. C, CaMKI mutants (1 \( \mu \)g/ml) were incubated with CaMKK-433 (0.5 \( \mu \)g/ml) in the absence of Ca\textsuperscript{2+}/CaM as described under “Experimental Procedures.” Samples were analyzed by SDS-PAGE (10\% polyacrylamide), and autoradiography was performed. The low level of incorporation of \(^{32}\)P into CaMKI-WT and CaMKI-F307A reflects a low level of autophosphorylation of these mutants.
switch-like response at the final point of the cascade (60, 61). The present study suggests that CaMKK has an intrinsically higher apparent affinity for CaM than dephospho-CaMKI (summarized in Fig. 7), raising the possibility that it would be activated at lower Ca\(^{2+}\) concentrations within cells (62). However, CaM must bind to CaMKI before CaMKK can phosphorylate Thr\(^{177}\) thereby placing a constraint on the ability of active CaMKI to interact with its substrate. Studies of CaMKII have shown that autophosphorylation of Thr\(^{286}\) leads to a significant increase in the affinity of that enzyme for CaM and that this may be responsible for the ability of CaMKII to decode oscillatory Ca\(^{2+}\) signals (63, 64). However, studies of CaMKI\(^3\) and CaMKIV (35) indicate that phosphorylation of either enzyme by CaMKK does not affect their affinity for CaM. Kinetic analysis of the activities of dephospho- and phospho-CaMKI indicate that phosphorylation by CaMKK is accompanied by a large increase in catalytic efficiency that is associated with a reduced \(K_m\) and an increased \(k_{cat}\) (Fig. 7).\(^2\) Therefore it is likely that the physiologically relevant form of CaMKI is the fully phosphorylated and CaM-bound form of the enzyme. The differential activation of CaMKI and CaMKIV by CaM may therefore ensure that CaMKI is only activated at relatively high concentrations of Ca\(^{2+}\). Alternatively, activation of CaMKI may occur only as a consequence of repeated increases in intracellular Ca\(^{2+}\), as would occur, for example, during tonic stimulation in neurons.

In conclusion, the results from the present study as well as those of the recent study of CaM mutants (52) support a model in which the COOH-terminal region of CaM initially binds to the exposed Trp\(^{265}\) of autoinhibited CaMKI. This initial interaction with CaM is likely to be sufficient to remove the region of \(\alpha R\), including Phe\(^{280}\), that makes pseudosubstrate contacts with the P-5 and P-3 amino acid binding sites. This may allow peptide substrate to begin to bind to the enzyme. However, additional interaction of the NH\(_2\)-terminal region of CaM with \(\alpha R\)2 is necessary to release the ATP-binding region from its constrained position. Although kinetic analyses of PKA have indicated that random binding of peptide or MgATP is possible, thermodynamic and structural considerations suggest that MgATP is likely to bind prior to peptide (50, 51, 65). The reorientation of the ATP-binding site is likely to be associated with rotation of the two lobes of CaMKI that will result in a closed “active” conformation. Removal of Phe\(^{265}\) from the P-5 binding site alone, or in combination with the reorientation of the ATP-binding domain, is also associated with conformational changes that allow the T-loop to be phosphorylated. In turn, as revealed by recent studies of phosphorylase kinase and the insulin receptor tyrosine kinase (66, 67), the phosphorylated T-loop is likely to form an antiparallel \(\beta\)-sheet structure that fully defines the binding sites for P+1 to P+4 amino acids in the peptide substrate. Future structural studies will hopefully reveal additional details of this mechanism of regulation of CaMKI and address the general application of the mechanism to the regulation of other CaMks.

Acknowledgments—We thank Dr. Anthony Means for the generous gift of CaM-KIV, Gloria Bertuzzi for technical assistance, and Dr. Marina Picciotto for helpful discussions.

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Characterization of the Mechanism of Regulation of Ca$^{2+}$/ Calmodulin-dependent Protein Kinase I by Calmodulin and by Ca$^{2+}$/Calmodulin-dependent Protein Kinase
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doi: 10.1074/jbc.273.34.21473

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