The Regulatory Region of Calcium/Calmodulin-dependent Protein Kinase I Contains Closely Associated Autoinhibitory and Calmodulin-binding Domains*

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The mechanism for the regulation of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase I (CaM kinase I) was investigated using a series of COOH-terminal truncated mutants. These mutants were expressed in bacteria as fusion proteins with glutathione S-transferase and purified by affinity chromatography using glutathione Sepharose 4B. A mutant (residues 1-332) showed complete Ca\textsuperscript{2+}/CaM-dependent activity. Truncation mutants (residues 1-321, 1-314, and 1-309) exhibited decreasing affinities for Ca\textsuperscript{2+}/CaM and also exhibited decreasing Ca\textsuperscript{2+}/CaM-dependent activities. Truncation mutants (residues 1-305 or 1-299) were unable to bind Ca\textsuperscript{2+}/CaM and were inactive. In contrast, truncation mutants (residues 1-293 or 1-277) were constitutively active at a slightly higher level (2-fold) than fully active CaM kinase I. These results indicate the location of the Ca\textsuperscript{2+}/CaM-binding domain on CaM kinase I (residues 294-321) and predict the existence of an autoinhibitory domain near, or overlapping, the Ca\textsuperscript{2+}/CaM-binding domain. These conclusions were supported by studies which showed that a synthetic peptide (CaM kinase I (294-321)) corresponding to residues 294-321 of CaM kinase I inhibited the fully active kinase in a manner that was competitive with Ca\textsuperscript{2+}/CaM and also inhibited the constitutively active mutant (residues 1-293) in a manner that was competitive with Syntide-2, a peptide substrate, (Ki = 1.2 \mu M) but was non-competitive with ATP. Thus, these results suggest that CaM kinase I is regulated through an intrasteric mechanism common to other members of the family of Ca\textsuperscript{2+}/CaM-dependent protein kinases.

Calcium (Ca\textsuperscript{2+}) is widely recognized as an essential intracellular second messenger in eukaryotic systems regulating processes such as muscle contraction, neurotransmitter release, gene expression, and cell proliferation (for reviews, see Campbell, 1983; Davis, 1992). In a number of cases the effects of Ca\textsuperscript{2+} are believed to be mediated by the ubiquitously distributed Ca\textsuperscript{2+} receptor, calmodulin (CaM). For reviews, see Manalan and Klee, 1984). Strong evidence, in turn, indicates that the effects of Ca\textsuperscript{2+}/CaM are often achieved through the regulation of protein phosphorylation (for reviews, see Nairn et al., 1985; Hanson and Schulman, 1992; Nairn and Picciotto, 1994). A family of Ca\textsuperscript{2+}/CaM-dependent protein kinases has been identified: phosphorylase kinase, myosin light chain kinase, and EF-2 kinase (CaM kinase III) that are highly specific enzymes, while CaM kinases II and IV are multifunctional enzymes. CaM kinase I was first purified from bovine brain based on its ability to phosphorylate site 1 of the neuronal protein synapsin I (Nairn and Greengard, 1987). Since then the enzyme has been found to be expressed in both neuronal and non-neuronal tissues (Ito et al., 1994a; Picciotto et al., 1995), thus necessitating a re-evaluation of the kinase as a potential multifunctional kinase. In this respect, two other good substrates for CaM kinase I have been identified in vitro: CREB, a cAMP-response element-binding protein (Sheng et al., 1991), and CF-2, a portion of the R-domain of the cystic fibrosis transmembrane conductance regulator (Picciotto et al., 1992).

CaM kinase I is active as a monomer (Nairn and Greengard, 1987), although various molecular masses of 37–43 kDa have been assigned to the enzyme. A preparation from bovine brain, purified using synapsin I as a substrate, was found to consist of two major polypeptides of M\textsubscript{r} 37,000 and 39,000 and a minor polypeptide of M\textsubscript{r} 42,000 (Nairn and Greengard, 1987). Two other Ca\textsuperscript{2+}/CaM-dependent protein kinases, termed CaM kinases Ia (M\textsubscript{r} 43,000) and Ib (M\textsubscript{r} 39,000), have been purified from rat brain using a synthetic peptide based on site 1 of synapsin I as a substrate (DeRemer et al., 1992a, 1992b). Another preparation, termed CaM kinase V (M\textsubscript{r} 41,000), has been purified from rat brain using the synthetic peptide, Syntide-2, as a substrate and its partial amino acid sequences determined (Mochizuki et al., 1993). The full-length cDNA for rat CaM kinase I has been cloned and a M\textsubscript{r} of 41,643 deduced from the amino acid sequence (Picciotto et al., 1993; Cho et al., 1994). Based on a high identity of amino acid sequences, CaM kinase V appears to be closely related to, though distinct from...
CaM kinase I (Picciotto et al., 1993). These results, together with recent immunological studies of CaM kinase I and V (Ito et al., 1994a, 1994b; Picciotto et al., 1995), suggest the possibility that CaM kinase I, Ia, Ib, and V form a family of related isoforms. Comparison of the deduced amino acid sequence of CaM kinase I with that of the α subunit of rat brain CaM kinase II revealed a high identity (approximately 42%) throughout the catalytic domain (Picciotto et al., 1993). In addition, a second domain was identified near the COOH terminus of CaM kinase I that showed limited identity (approximately 32%) to the Ca$^{2+}$/CaM-binding domain of the CaM kinase II subunit. In the present study, the structural basis for the Ca$^{2+}$/CaM-dependent regulation of the enzyme has been established. The results obtained indicate that CaM kinase I is regulated through an intrasegment mechanism common to other members of the family of Ca$^{2+}$/CaM-dependent protein kinases.

**EXPERIMENTAL PROCEDURES**

Materials—Phenylnethylsulfanyl fluoride, dithiothreitol, bovine serum albumin (BSA), Tween-20, Tween-80, dimethyl sulfoxide, and ATP were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). [3H]- or [125I]-labeled CaM was purified from bovine brain as described (Endo et al., 1985). CaM kinase I from bovine brain was purified with recent immunological studies of CaM kinase I and V (Ito et al., 1994a, 1994b; Picciotto et al., 1995). CaM was purified from bovine brain as described (Endo et al., 1981). Smooth muscle myosin light chain kinase (smMLCK) was purified from rat smooth muscle. CaM kinase I(294–321) was purified by preparative reversed-phase HPLC (C-18). Aliquots of CaM kinase I (294–321) were synthesized with a model 431A peptide synthesizer (Applied Biosystems Inc.). CaM kinase I(294–321) was purified by preparative reversed-phase HPLC (C-18). Aliquots of CaM kinase I(294–321) were subjected to analytical reversed-phase HPLC (C-18) and Pico-tag amino acid analysis (Waters Associates). The peptide was further characterized by amino acid sequencing with a model 473A protein sequence (Applied Biosystems Inc.) (data not shown).

Protein Kinase Inhibitors, H-89 and H-7, and Ca$^{2+}$/CaM Antagonist, W-2, H-10, and W-7 with a Ca$^{2+}$/CaM Antagonist, W-7 were synthesized by the method of Hidaka et al. (1984).

Construction and Expression of a Series of COOH-terminal Truncated Mutants of CaM Kinase I as GST Fusion Proteins—The following oligonucleotides were synthesized with a model 381A DNA synthesizer (Applied Biosystems Inc.) and purified by OLIGO-PAK EX (Millipore) according to the manufacturer’s instructions. The peptide, CaM kinase I(294–321) was purified by preparative reversed-phase HPLC (C-18). Aliquots of CaM kinase I(294–321) were subjected to analytical reversed-phase HPLC (C-18) and Pico-tag amino acid analysis (Waters Associates). The peptide was further characterized by amino acid sequencing with a model 473A protein sequence (Applied Biosystems Inc.) (data not shown).

Radiodination of CaM and 125I-CaM Gel Overlay—CaM (200 μg) was treated with 125I-Bolton-Hunter reagent (18.5 Mbq) in 10 μl of 0.1 mM sodium borate (pH 8.5) for 60 min at room temperature. The reaction was stopped by addition of 500 μM of 0.1 mM sodium borate (pH 8.5), and 0.2 μM glycine, and placed on ice for 10 min. 125I-CaM was separated from unreacted 125I by passing through a column of Sephadex G-25M (Pharmacia LKB Biotechnology Inc.) pre-equilibrated with wash buffer containing 20 mM Tris (pH 7.5), 2 mM MgCl$\text{2}$, 1 mM β-mercaptoethanol, and 100 μM phenylmethylsulfonyl fluoride. The column was washed extensively with wash buffer and eluted with elution buffer containing 0.1 M sodium borate (pH 8.5) and 0.5 mM glutathione. Aliquots of CaM kinase I(294–321) were collected in tubes containing 1 ml of 200 mM Tris (pH 7.5) for pH neutralization. Protein concentrations were determined by the Bradford assay (1976) and fractions containing peak protein values were pooled (approximately 12 ml in all cases), dialyzed against wash buffer, adjusted with glycerol to a final concentration of 20% and stored in aliquots at −70 °C. CaM kinase I fusion proteins were essentially homogeneous as judged by staining of SDS-polyacrylamide gels with Coomassie Brilliant Blue and the protein concentration of the pure protein was used to determine the specific activity (see below).

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washed with the corresponding EGTA- and CaCl$_2$-containing buffer at 4 °C for 2 days and exposed, without drying, to x-ray film with an intensifying screen at −70 °C overnight. The film was developed with a FPM800A film processor (Fuji medical systems Ltd., Tokyo, Japan).

Protein Kinase Assays—Analysis of the deduced amino acid sequence of CaM kinase I has suggested that the catalytic domain is included within residues 1–270 (Picciotto et al., 1993). In addition, a second domain showing moderate identity to the Ca$^{2+}$/CaM-binding domains of other Ca$^{2+}$/CaM-binding proteins was found near the COOH terminus of CaM kinase I (for example, ~32% identity to CaM kinase II). To directly identify the location of the regulatory region containing the Ca$^{2+}$/CaM-binding domain, a series of COOH-terminal truncated mutants were generated. The sense oligonucleotide, Oligo-1S, corresponding to the 5′ end of the coding region and one of several antisense oligonucleotides corresponding to different parts of the 3′ end of the coding region were used to amplify DNA using PCR. The DNAs encoding a series of COOH-terminal truncated mutants were subcloned into pGex-2T. The clones were expressed in E. coli as GST-fusion proteins (Fig. 1A). The resulting products were purified by affinity chromatography on a column of glutathione Sepharose 4B. Transformants with plasmids carrying the mutants 1–293 or 1–277 displayed inhibited growth following induction with IPTG (data not shown). Thus, the yield of mutants 1–293 and 1–277 were significantly decreased.

All preparations were purified to apparent homogeneity as demonstrated by staining of SDS-polyacrylamide gels using BSA as a carrier protein. The mixtures were centrifuged at 3,000 revolutions/min for 10 min. The pellets were washed with 5% trichloroacetic acid, the centrifugation repeated, and the pellet dissolved in 1 ml of 1 N NaOH prior to quantification of Cerenkov radiation.

Other Procedures—SDS-PAGE was performed according to the method of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue (0.25% in 45% methanol, and 10% acetic acid) followed by destaining in 10% methanol and 10% acetic acid. Gels were dried onto cellulophane paper in vacuo. Apparent molecular weights on SDS-PAGE were determined using SDS-PAGE molecular standards (Bio-Rad). Protein was determined by the Bradford assay (1976) using BSA as a standard.

RESULTS

Construction and Expression of a Series of COOH-terminal Truncated Mutants—Analysis of the deduced amino acid sequence of CaM kinase I has suggested that the catalytic domain is included within residues 1–270 (Picciotto et al., 1993). In addition, a second domain showing moderate identity to the Ca$^{2+}$/CaM-binding domains of other Ca$^{2+}$/CaM-binding proteins was found near the COOH terminus of CaM kinase I (for example, ~32% identity to CaM kinase II). To directly identify the location of the regulatory region containing the Ca$^{2+}$/CaM-binding domain, a series of COOH-terminal truncated mutants were generated. The sense oligonucleotide, Oligo-1S, corresponding to the 5′ end of the coding region and one of several antisense oligonucleotides corresponding to different parts of the 3′ end of the coding region were used to amplify DNA using PCR. The DNAs encoding a series of COOH-terminal truncated mutants were subcloned into pGex-2T. The clones were induced by addition of IPTG and expressed in Escherichia coli as GST-fusion proteins (Fig. 1A). The resulting products were purified by affinity chromatography on a column of glutathione Sepharose 4B. Transformants with plasmids carrying the mutants 1–293 or 1–277 displayed inhibited growth following induction with IPTG (data not shown). Thus, the yield of mutants 1–293 and 1–277 were significantly decreased.

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Coomassie Brilliant Blue (Fig. 1B). Mutant 1–332 exhibited a molecular mass of 62 kDa, consistent with the predicted value of 37 kDa of rat CaM kinase I plus 26 kDa of GST. A proportional increase in mobility on SDS-PAGE was observed with removal of increasing amounts of the COOH-terminal region (i.e., mutants 1–321, 1–314, 1–309, 1–305, and 1–299, Fig. 1B). In contrast, mutant 1–293 migrated slower than mutant 1–299 although residues 294–299 had been deleted. In addition, mutant 1–277, which was expected to migrate with the fastest mobility, co-migrated with mutant 1–299. The basis for the anomalous migrations of mutant 1–293 and 1–277 is not known. Previous studies of mitogen-activated protein kinase (Matsuda et al., 1992) and cdc25-C (Hoffmann et al., 1993) have indicated that phosphorylation by their respective activating kinases resulted in decreased mobility on SDS-PAGE. Recent studies have indicated that CaM kinase I is also phosphorylated at an active site Thr residue by one or more kinases (Sugita et al., 1994), and that this results in a slower mobility on SDS-PAGE.² However, there is no evidence that any of the recombinant proteins were phosphorylated during preparation.

² H. Yokokura, M. R. Picciotto, A. C. Nairn, and H. Hidaka, unpublished results.

Ca²⁺-dependent CaM Binding and Ca²⁺/CaM Independent and Dependent Activities of Recombinant Kinases—

Fig. 2. Ca²⁺-dependent CaM binding and Ca²⁺/CaM independent and dependent activities of recombinant kinases. A, aliquots (0.9–1.7 μg of protein/lane) of recombinant kinases were subjected to SDS-PAGE (10% acrylamide). The gels were incubated with ¹²⁵I-CaM (5 x 10⁴ cpm/ml) in a buffer containing 1 mM CaCl₂ (upper panel) or 1 mM EGTA (lower panel), washed in the corresponding buffer, and exposed, without drying, to x-ray film prior to development as described under “Experimental Procedures.” B, aliquots (0.3–0.39 mg) of mutants 1–332, 1–314, 1–309, and 1–293 were adjusted to 1 mM CaCl₂, applied onto a column of CaM-coupled Sepharose HP (indicated by Apply), washed extensively, and proteins eluted with 1 mM EGTA (indicated by EGTA) as described under “Experimental Procedures.” Protein in the eluate was monitored by absorbance at 280 nm. C, protein kinase activities of mutants were measured in the presence of 1 mM EGTA (solid bars), or 0.5 mM CaCl₂ plus 1.5 μM CaM (open bars) as described under “Experimental Procedures.” The enzymes were added to a final concentration of 5.5–12 μg/ml. Mean values and standard errors were determined from three independent experiments, each performed in duplicate.

Wild-type recombinant CaM kinase I was completely dependent on Ca²⁺/CaM (Fig. 2C), as previously demonstrated (Picciotto et al., 1993). Using Syntide-2 as a substrate, its specific activity was 73.8 ± 7.6 nmol/min/mg. The activity of recombinant CaM kinase I is significantly lower than the fully active form of the enzyme that requires phosphorylation by an activating kinase (Sugita et al., 1994). However, recent studies
have indicated that the substrate specificity and CaM dependence of recombinant CaM kinase I are the same as the fully activated enzyme. Mutants 1–332 and 1–321 displayed similar properties to wild-type kinase (Fig. 2C). However, the Ca\(^2\+\)/CaM-dependent activities of mutants 1–314 and 1–309 were significantly decreased, and mutants 1–305 and 1–299 were not activated at all by Ca\(^2\+)/CaM. Mutants 1–293 and 1–277 were fully active in the absence of Ca\(^2\+)/CaM. Notably, they were 2-fold more active than the Ca\(^2\+)/CaM-activated mutant 1–323. In fact, these two mutants were slightly inhibited by Ca\(^2\+) in the absence or presence of CaM (data not shown).

Taken together, these results suggest that residues T\(^{310}\)AVVR\(^{314}\) play a pivotal role in the binding of CaM kinase I to Ca\(^2\+)/CaM. The Ca\(^2\+)/CaM-binding domain of the enzyme contains at least residues 310–314 and may extend further toward the NH\(_2\) terminus of the enzyme. Mutant 1–293 acquired Ca\(^2\+)/CaM-independent activity when residues I\(^{294}\)KKNA\(^{299}\) were deleted, suggesting the existence of an autoinhibitory domain, which may extend toward the COOH terminus from residues 294–299.

Effect of CaM Kinase I(294–321) on CaM kinase I and smMLCK—The peptide, CaM kinase I(294–321), was expected from the studies described above to contain both the Ca\(^2\+)/CaM-binding domain and the autoinhibitory domain of CaM kinase I. Mutant 1–332 was activated by Ca\(^2\+)/CaM in a dose-dependent manner with its activation curve showing a sigmoidal relationship (Fig. 3A). The concentration of CaM producing 50% of the maximal activity (K\(_{act.}\)) was 51.0 ± 4.5 nM (mean ± S.E.), comparable to that of the wild-type enzyme (K\(_{act.}\) of 42.8 ± 3.3 nM, data not shown). The enzyme was inhibited by CaM kinase I(294–321) in a dose-dependent manner as illustrated by the right shift in the K\(_{act.}\) for Ca\(^2\+)/CaM. However, maximal activity was measured upon addition of excess Ca\(^2\+)/CaM, suggesting that peptide inhibition is competitive with respect to Ca\(^2\+)/CaM. We further examined the effect of CaM kinase I(294–321) on smMLCK, another Ca\(^2\+)/CaM-dependent protein kinase (Fig. 3B). smMLCK was activated by Ca\(^2\+)/CaM in a dose-dependent manner with a K\(_{act.}\) of 5.4 nM. The enzyme was also inhibited by CaM kinase I(294–321) competitively with respect to Ca\(^2\+)/CaM. These results suggest that CaM kinase I(294–321) directly binds Ca\(^2\+)/CaM, preventing it from activating the two kinases.

Effects of CaM Kinase I(294–321) or Other Kinase Inhibitors on Ca\(^2\+)/CaM-independent Protein Kinases—The ability of CaM kinase I(294–321) to inhibit the constitutively active mutant 1–293 as well as other Ca\(^2\+)/CaM-independent protein kinases was examined (Table I). Mutant 1–293 was inhibited by CaM kinase I(294–321) with an IC\(_{50}\) value of 0.62 nM. H-89 and H-7 are established as specific inhibitors of PKA (Chijiwa et al., 1990) and a relatively selective inhibitor of protein kinase C (Hidaka et al., 1984), respectively. Mutant 1–293 was not inhibited by either H-89 or H-7 with concentrations of at least 100 μM and was not inhibited by the Ca\(^2\+)/CaM antagonist, W-7 (Hidaka and Tanaka, 1983). PKA was inhibited by H-89 with an IC\(_{50}\) value of 0.33 μM whereas it was not inhibited by CaM kinase I(294–321) at all. Protein kinase C was inhibited by H-89 with an IC\(_{50}\) value of 24 μM, though not inhibited by CaM kinase I(294–324). Thus, CaM kinase I(294–321) inhibited only mutant 1–293 among the Ca\(^2\+)/CaM-independent protein kinases tested, suggesting that the peptide inhibits the catalytic mechanism of CaM kinase I in a manner relatively specific to that enzyme.

Kinetic Analyses of the Inhibition of Mutant 1–293 by CaM Kinase I(294–321)—Kinetic analyses were carried out to fur-

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\(^{2}\)M. R. Picciotto and A. C. Nairn, manuscript in preparation.
distinct from that previously reported (Picciotto et al., 1993). Analysis of the two cDNAs suggested that the original DNA sequence was incorrect. We have carried out further DNA sequencing of our original cDNA and have found that indeed there are a number of sequencing errors (Picciotto et al., 1995). Notably, Arg³⁰⁹ is replaced by Ala³⁰⁹ and a frameshift occurred sequenc ing of our original cDNA (Fig. 5A). Wild-type kinase (residues 1–374) and a truncation mutant corresponding to that predicted by the original cDNA (mutant 1–332) were expressed, digested with thrombin, and compared by SDS-PAGE (Fig. 5B). Wild-type kinase migrated with a mobility of $M_r$ of 41,643 predicted from the corrected sequence. A truncated mutant corresponding to that predicted by the original cDNA (10.4 $\mu$g/ml) was incubated (30°C, 10 min) with either 0 $\mu$M (○), 0.5 $\mu$M (△), or 1.0 $\mu$M (□) CaM kinase I(294–321) using 25–1000 $\mu$M Syntide-2. B, the enzyme (5.5 $\mu$g/ml) was incubated (30°C, 10 min) with either 0 $\mu$M ([△△△]), 0.5 $\mu$M (△), or 1.0 $\mu$M (□) CaM kinase I(294–321) using 25–1000 $\mu$M ($^3$2P)ATP. $^3$2P incorporation into Syntide-2 was determined as described under "Experimental Procedures." The results are representative of three independent experiments, each performed in duplicate, and presented as double-reciprocal plots. Kinetic constants were estimated by fitting the data to the Michaelis-Menten equation using the method of least-squares. Inset, the apparent $K_m/V_{max}$ is plotted as a function of CaM kinase I(294–321) concentration.

DISCUSSION

In the present study, we have directly analyzed the structural basis for the regulation of CaM kinase I by analyzing the activity and Ca$^{2+}$/CaM-binding of a series of COOH-terminal truncated mutants. Analysis of the binding of Ca$^{2+}$/CaM to the mutants indicated that the Ca$^{2+}$/CaM-binding domain of the kinase is located between residues 294–321. This conclusion was supported by the demonstration that a synthetic peptide encompassing these residues competed with Ca$^{2+}$/CaM in the activation of either CaM kinase I or smMLCK. Deletion of residues 322–332 reduced the binding of Ca$^{2+}$/CaM as measured by $^{125}$I-CaM overlay, but had little effect on binding of Ca$^{2+}$/CaM as determined by affinity chromatography on CaM-Sepharose or activity measurements. Truncation of residues $T^{310}$AVVR$^{314}$ had the greatest effect on binding of Ca$^{2+}$/CaM, particularly as measured by CaM-Sepharose chromatography. Further truncation of residues $K^{305}$SWK$^{305}$ completely abolished binding of Ca$^{2+}$/CaM. Notably, analysis of binding of Ca$^{2+}$/CaM by CaM overlay, CaM-Sepharose chromatography and activation of kinase activity indicated that there is not an absolute correlation between all measurements of binding of Ca$^{2+}$/CaM and stimulation of kinase activity.

The Ca$^{2+}$/CaM-binding domains of numerous proteins have been shown to have a propensity for the formation of amphipathic $\alpha$-helices (O'Neil and DeGrado, 1990). Helical wheel analysis of residues 299–316 indicated that these residues of CaM kinase I are predicted to contain a hydrophobic domain and a basic, hydrophilic domain (Fig. 6B). In addition, analysis of residues 303–316 by the method of Chou and Fasman (1978) predicted an $\alpha$-helical conformation (data not shown). Direct structural analysis of the binding of Ca$^{2+}$/CaM to synthetic peptides encompassing Ca$^{2+}$/CaM-binding domains have indi-
CaM kinase I (residues 294–321) and other Ca2+/CaM-binding domains were aligned with these key positions. Amino acid sequences known to be directly involved in binding Ca2+/CaM with structural studies (Meador et al., 1992, 1993; Ikura et al., 1992) are underlined. Ca2+/CaM-binding domains: skMLCK (Ikura et al., 1992), smMLCK (Meador et al., 1988), CaM kinase II (Meador et al., 1993), CaM kinase IV (Sikera et al., 1987), calcineurin (Kincaid et al., 1988), plasma membrane Ca2+ pump (Kataoka et al., 1991), and constitutive nitric oxide synthase (cNOS) (Zhang et al., 1994). B, the amino acid residues 299–316 of CaM kinase I were plotted as an α-helical wheel. The diagram represents an end-on view of this region being in a right-handed α-helical conformation viewed from the NH2 end. A basic, hydrophilic face and a hydrophobic face are underlined with a corresponding note.

Comparison of several common features that are critical for high affinity binding (Ikura et al., 1992; Meador et al., 1992, 1993) (Fig. 6A). Upon binding of Ca2+ to the four binding sites in CaM, the two lobes of the protein fold around the helical structure making contact between the NH2-terminal domain of Ca2+/CaM and the COOH-terminal part of the peptide, and between the COOH-terminal domain of Ca2+/CaM and the NH2-terminal part of the peptide. Two hydrophobic amino acids, 8–12 residues apart (Trp300 and Phe303 in skMLCK), are necessary to bind to hydrophobic pockets in Ca2+/CaM, although extensive contact between many other hydrophobic residues (Phe290 and Val297 in skMLCK) are apparent. In addition, specific basic residues (Arg311 in smMLCK) are believed to act as "fulcrums" to initiate the interaction between the central helix of Ca2+/CaM and the target enzymes (Meador et al., 1992). The variability in the exact location of the principal hydrophobic amino acids in the different structure is tolerated by the fact that the central helix of Ca2+/CaM is highly flexible and extends to accommodate the particular binding peptide (Meador et al., 1993).

Comparison of the proposed Ca2+/CaM-binding domain of CaM kinase I with that of other Ca2+/CaM-binding proteins indicated several conserved features (Fig. 6A). Residues T310AV313 of CaM kinase I are likely to represent the COOH-terminal end of the proposed Ca2+/CaM-binding domain. These residues are essential for binding to Ca2+/CaM and are predicted from the method of Kyte and Doolittle (1982) to be the most hydrophobic region of the proposed Ca2+/CaM-binding domain. Residues K305KWKQ305 are also critical for binding Ca2+/CaM and likely represent the NH2-terminal end of the Ca2+/CaM-binding domain. Notably, Trp303 of CaM kinase I aligns with conserved tryptophan residues in several other Ca2+/CaM-binding proteins (Fig. 6A). Thus, the results from the present study, together with the results obtained from the crystallographic analyses (Meador et al., 1993), suggest that the Ca2+/CaM-binding domain of CaM kinase I contains at least residues 302–314. However, the results from the CaM binding and activity studies (Fig. 2, A and C) suggest that the presence of some part or all of residues 315–321 (HMRKLQL) contribute to binding of CaM, possibly by stabilization of the structure of the CaM-binding domain. In addition, the precise NH2-terminal limit of the CaM-binding domain cannot be assigned based on the present experimental results.

Full-length wild-type CaM kinase I is completely dependent on Ca2+/CaM for activity. Deletion of residues 294–332 resulted in a fully active enzyme. In contrast, a slightly longer CaM kinase I mutant (resides 1–299) was basally inactive and could not be activated by addition of Ca2+/CaM. In addition, the constitutively active kinase was inhibited by a synthetic peptide corresponding to residues 294–321 in a manner competitive with respect to peptide substrate. Together, these results indicate that an autoinhibitory domain exists within residues 294–321 and that the presence of residues 294–299 is sufficient to inactivate CaM kinase I catalytic activity.

The elucidation of the molecular basis for the regulation of protein kinases has been the subject of a number of recent studies (Knighton et al., 1991, 1992; Cruzalegui et al., 1992, 1993; Ito et al., 1991; Fitzsimons et al., 1992). The structure of the catalytically subunit of PKA in a complex with the high affinity pseudosubstrate inhibitor (PKI (5–24)) has been determined by x-ray crystallography (Knighton et al., 1991, 1992), Arg18 in PKI (the P(−3) position) interacts with several carboxyl side chains of the enzyme in a manner that places a non-phosphorylatable alanine residue in the place of the normally phosphorylated serine or threonine residue at the P(0) position (Fig. 7). Furthermore, a phenylalanine residue (Phe20) that interacts with a hydrophobic pocket formed by residues Y239PPHH239 of PKA is critical for the high affinity of the inhibitor.

The best characterized substrates for CaM kinase I are the members of the synapsin family. Synapsin I and II are both phosphorylated at NH2-terminal serine residues (Fig. 7) that are also phosphorylated by PKA (Czernik et al., 1987). The substrate specificity of CaM kinase I has recently been investigated and compared with that of PKA using a series of peptide substrates based on the sequence of site 1 of synapsin I (Lee et al., 1994). These studies indicate that the second of three consecutive arginine residues corresponds to the basic amino acid residue found in substrates for PKA at the P(−3) position (Fig. 7). In addition, the studies by Lee et al. indicated that hydrophobic amino acids are required at the P(−5) and
Comparison of the amino acid sequence of residues 294–321 of CaM kinase I with that of PKI (5–24) and the phosphorylation sites of synapsin I, syntide-2, and CREB (Fig. 7) revealed several common features that support the possibility that the autokinase domain of CaM kinase I interacts with the catalytic domain through a pseudosubstrate mechanism. Three alignments of the autokinase domain that support a pseudosubstrate mechanism were possible. In the first (which required insertion of two gaps in the sequence), Lys304 of CaM kinase I at the P(−3) position would place Trp303 and Ala311 at the P(−5) and P(+4) positions, respectively. These two positions would correspond to the critical hydrophobic amino acids present in each of the known substrates for the enzyme; however, alanine is not normally considered a hydrophobic residue. In this alignment, Phe307 would replace the phosphorylatable residue at P(0). Notably, in this alignment Phe298 is placed at the P(−11) position in an analogous position to that of the critical phenylalanine found in PKI (5–24). In PKI, Phe10 interacts with the hydrophobic domain Y235PPFY239 in PKA catalytic subunit (Knighton et al., 1991). By analogy, Phe298 may interact with the corresponding hydrophobic domain Y215PPFY219 in CaM kinase I. However, this model is weakened by the fact that the alignment required insertion of gaps, the presence of an alanine residue at the P(+4) position, and the fact that the pseudosubstrate domain would correspond to W303KQAFNATA311, which is not supported by the observation that residues I294KKNAF299 are sufficient to maintain the enzyme inactive form. An alternative alignment that maintains the necessary features of the pseudosubstrate places residues I294KKNAFKSKW303 in the P(−5) to P(+4) positions. Important features of this alignment are the lack of insertions of gaps in the sequence, the presence of alanine instead of phenylalanine at the P(0) position, and the excellent alignment of the hydrophobic amino acids at P(−5) and P(+4) positions. Finally, a third alignment that also maintains all the necessary features of a pseudosubstrate places Phe298 and Phe307 in the P(−5) and P(+4) positions. In either alignment two or three elements of the CaM-binding domain overlap the proposed pseudosubstrate domains, suggesting that while distinct regions of the regulatory domain of CaM kinase I are primarily responsible for mediating autoinhibition and CaM-binding, the two domains are closely associated and probably overlap.

Comparison of the results obtained in this study with that of other CaM kinases reveals a number of similarities, as well as a number of differences. There is general agreement concerning the position of the Ca2+/CaM-binding domain of the various enzymes (Fig. 6A), although the exact features of the mechanism of autoinhibition vary. A number of studies of MLCK, including detailed molecular modeling (Ito et al., 1991; Knighton et al., 1992), have suggested that this enzyme may be regulated by a strict pseudosubstrate mechanism. However, other studies using site-directed mutagenesis and/or preparations of truncated MLCK mutants have suggested that the original pseudosubstrate model requires modification (Fitzsimons et al., 1992; Yano et al., 1993). Recently, the crystal structure of a fragment of twitchin kinase, which shares a high level of identity with smMLCK, has been elucidated (Hu et al., 1994). While the structure clearly establishes the regulation of twitchin kinase by an intrasteric autoinhibitory mechanism, the lack of amino acid sequence identity of the regulatory domain of twitchin kinase with MLCK and the absence of information from site-directed and deletion mutagenesis make it difficult to evaluate the existence of a strict pseudosubstrate mechanism. Studies of CaM kinase II (Cruzalegui et al., 1992) and CaM kinase IV (Cruzalegui et al., 1993) have also suggested regulation by an autoinhibitory mechanism. In the case of CaM kinase II, the presence of residues K290KFN294 (Fig. 6A) was, like CaM kinase I, sufficient to maintain the enzyme in an inactive state. However, replacement of KKFKN by AAAL had little effect on Ca2+/CaM-dependent activation of the enzyme, although deletion of the amino acids generated a partially Ca2+/CaM-independent kinase. Based on these results, it was therefore concluded that autoinhibition of CaM kinase II required determinants in addition to residues 291–294. In the case of CaM kinase IV, truncation at Leu313 (K311K Fig. 6A) generated a fully active kinase that did not require Ca2+/CaM. Notably, truncation of CaM kinase I, II, and IV at an analogous hydrophobic residue (Fig. 6A) resulted in an active enzyme; however, the position of the truncation placed a pair of lysine residues either as part (CaM kinase I and II) of the autokinase domain or not (CaM kinase IV). These results, together with the observations that the substrate specificity of CaM kinase I depends on hydrophobic amino acids, suggest that at least in the case of CaM kinase I, the mechanism of autoinhibition is highly dependent on hydrophobic amino acids.

As discussed above, there was not an absolute correlation between binding of Ca2+/CaM and stimulation of kinase activity. These results suggest, therefore, that although Ca2+/CaM may bind efficiently to a limited domain in the regulatory domain of the kinase, the complete regulatory domain is necessary (including COOH-terminal amino acid residues) to allow the removal of the autoinhibitory domain from the active site of the enzyme upon binding of Ca2+/CaM. The models presented above for the position of the proposed pseudosubstrate domain also raise additional questions concerning the exact role of the binding of Ca2+/CaM in the activation of the kinase. In the case of the second or third models, Ca2+/CaM would be predicted to bind directly to the pseudosubstrate domain and release it from the active site of the kinase. In contrast, in the first model binding of Ca2+/CaM would be accompanied by both removal of the pseudosubstrate domain from the active site and an alteration in the interaction of residues 294–299 with the catalytic domain of the kinase.

In summary, these various studies suggest that the CaM kinases are not all regulated by exactly the same structural mechanism. CaM kinase I appears to be the smallest of the autoinhibited kinases, shows an overlapping substrate specificity with PKA, and appears to be regulated by a pseudosubstrate mechanism that shares more features with PKA than the other CaM kinases. In contrast, the autoinhibitory mechanism used by MLCK and CaM kinase II may have evolved from the pseudosubstrate mechanism, perhaps as a reflection of the changes in substrate specificity of these enzymes. The present studies, together with future elucidation of the structure of CaM kinase I and other CaM kinases, should help to resolve these issues.

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