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Active site-directed inhibition of Ca^{2+}/calmodulin-dependent protein kinase type II by a bifunctional calmodulin-binding peptide

(autoregulation/phosphorylation/synthetic peptides)

PAUL T. KELLY*†, RON P. WEINBERGER*, AND M. NEAL WAXHAM‡

Departments of *Neurobiology and Anatomy, and ‡Neurology, University of Texas Health Science Center, Houston, TX 77225

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ABSTRACT **The activation of Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaM-KII) by Ca^{2+}/CaM results in autophosphorylation and the generation of Ca^{2+}/CaM-independent enzyme activity. We postulated that CaM binding and subsequent autophosphorylation alters the conformation of CaM-KII and exposes its substrate-binding and catalytic site(s). Previous peptide mapping studies on CaM-KII demonstrated the close proximity of CaM-binding and autophosphorylation domains. Analyses of the deduced amino acid sequences encoding CaM-KII have allowed the identification of its CaM-binding domain and have revealed two consensus phosphorylation sites that flank this regulatory domain. We report herein the distinct properties of two synthetic peptides modeled after the CaM-binding domain of CaM-KII. The first peptide binds CaM in a Ca^{2+}-dependent manner and is an antagonist of CaM-KII activation ($IC_{50} \approx 75$ nM). It does not, however, inhibit CaM-KII activity. A second peptide containing the same CaM-binding domain plus a putative autophosphorylation sequence at its N terminus displayed bifunctional regulatory properties. In addition to being a CaM antagonist, the latter was a potent inhibitor of Ca^{2+}/CaM-independent kinase activity ($IC_{50} \approx 2$ μM). We suggest that this bifunctional peptide represents an active site-directed inhibitory element of CaM-KII. The separation of CaM antagonist and active site-directed inhibitory properties of this peptide distinguishes CaM-KII from other CaM-dependent enzymes in which bifunctional regulatory properties appear to reside in the same peptide domain. These results indicate that the definition of site-directed inhibitory peptides should, in some cases, be expanded to include bona fide phosphorylation sites.**

Ca^{2+}/calmodulin (CaM)-dependent protein kinase type II (CaM-KII) is an abundant protein kinase in mammalian brain tissues (1–3). CaM-KII is concentrated at postsynaptic densities of asymmetric synapses (4–6), whereas a second form of the kinase is present in brain cytosol (1–3). CaM-KII from rodent forebrain is composed of two distinct but related protein subunits of 50 and 60 kDa in a ratio of ≈12:3, respectively (1–3, 7). Both subunits contain regulatory (CaM-binding) and catalytic domains and undergo autophosphorylation in a Ca^{2+}/CaM-dependent manner (8–10). Numerous reports have documented that autophosphorylation of CaM-KII results in the generation of catalytic activity that is no longer dependent on Ca^{2+}/CaM (11–17).

Although the precise mechanisms through which CaM-dependent activation and autophosphorylation regulate CaM-KII activity are unknown, the recent cloning of both subunits (18–20) and identification of the CaM-binding domain in the 50-kDa subunit of CaM-KII (18) have revealed potential autophosphorylation sites in close proximity to this regulatory domain. We have proposed that in the absence of Ca^{2+}/CaM, CaM-KII subunits exist in conformations that allow intramolecular interaction between catalytic and CaM-binding domains, thus prohibiting ATP binding and subsequent substrate phosphorylation and/or autophosphorylation (13, 18). The experiments herein are a logical extension of recent studies on myosin light chain kinase (MLCK) from smooth or skeletal muscle in which synthetic peptides containing their respective CaM-binding domains were shown to display pseudosubstrate or active site-directed inhibitory properties (21, 22). An important distinction between MLCK and CaM-KII, however, is that the latter's activity is regulated by autophosphorylation (12–17).

Pearson *et al.* (23) have recently defined the "minimum" substrate phosphorylation sequence (Arg-Xaa-Xaa-Ser/Thr) for the CaM-KII from skeletal muscle. Only two phosphorylation sites conforming to this minimum consensus sequence are found in the 50-kDa subunit of CaM-KII, and they flank the amino acid core of its CaM-binding domain [Arg-Gln-Glu-Thr (RQET) and Arg-Asn-Phe-Ser (RNFS) at its N and C terminus, respectively; see Fig. 1 and refs. 18 and 20]. These sequences also flank the CaM-binding domain of the 60-kDa subunit (19). We and others have proposed that the key location of one or both of these potential autophosphorylation sites may underlie the autoregulatory properties of CaM-KII and/or the generation of Ca^{2+}/CaM-*independent* activity (13, 17, 18, 20). In addition, results from this laboratory (13) and those of Lai *et al.* (17) indicated that the autophosphorylation of a threonine residue in either the 50- or 60-kDa subunit of CaM-KII accompanies the generation of Ca^{2+}/CaM-independent activity. To elucidate the potential interrelationships between the regulatory and autophosphorylation properties of CaM-KII, we synthesized a *C*aM-*b*inding *p*eptide (CBP) modeled after the CaM-binding domain of the 50-kDa subunit. CBP contains the N-terminal phosphorylation sequence Arg-Gln-Glu-Thr (see Fig. 1) and was examined for its interactions with CaM and CaM-KII. To evaluate the role of this potential phosphorylation site, a second CaM-binding peptide that lacked the complete phosphorylation sequence present in CBP was also examined. Our results demonstrate that the synthetic peptide CBP is an active site-directed inhibitor of the Ca^{2+}/CaM-independent CaM-KII. Moreover, the inhibitory activity of CBP requires the intact phosphorylation recognition sequence Arg-Gln-Glu-Thr at its N terminus.

METHODS

Synthesis and Purification of Synthetic Peptides. Synthetic peptides (CBP and CBP_{-3}; see Fig. 1) were prepared on an Applied Biosystems 430A solid-phase peptide synthesizer and were further purified essentially as described by Kemp *et*

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Abbreviations: CaM, calmodulin; CaM-KII, Ca^{2+}/CaM-dependent protein kinase type II; CBP, CaM-binding peptide; MLCK, myosin light chain kinase.

†To whom reprint requests should be addressed.

al. (21). Each peptide was purified by preparative HPLC. Analysis of purified peptides by analytical HPLC showed that each was at least 95% pure. The amino acid composition and molar concentration of each purified peptide were verified by quantitative amino acid analysis using norleucine as an internal standard. Molar peptide concentrations were determined through calculations of the number of nanomoles of glycine, aspartic acid, or glutamic acid in a known volume of material. The synthetic peptide Pro-Leu-Arg-Arg-Thr-Leu-Ser-Val-Ala-Ala, modeled after the 10 N-terminal residues of skeletal muscle glycogen synthase (23), was kindly provided by Bruce Kemp (University of Melbourne, Australia).

Phosphorylation Assays. Cytosolic CaM-KII was purified from rat forebrain as described elsewhere and was ≈95% pure (7). Synapsin I was prepared from bovine brain and was a generous gift from T. McGuinness and P. Greengard (Rockefeller University, New York). Assays of the autophosphorylation of CaM-KII and the phosphorylation of synapsin I were carried out as described by Kelly *et al.* (5).

The Ca^{2+}/CaM-independent form of CaM-KII was prepared by autophosphorylation of kinase (5–10 μg) in the presence of $CaCl_2$ (0.5 mM) plus CaM (700 nM) in 10 mM Hepes (pH 7.0) containing $MgCl_2$ (0.1 mM) and unlabeled ATP (15 μM). After autophosphorylation for 1 min at 30°C, the kinase was diluted 1:5 in 10 mM Hepes containing EGTA (0.5 mM). $MgCl_2$ and ATP were then added to final concentrations of 0.5 mM and 15 μM, respectively, and additional Ca^{2+}/CaM-independent autophosphorylation was carried out at 30°C for 1 min. Autophosphorylation under these conditions resulted in 2–4 mol of P_i per mol of holoenzyme; phosphorylated CaM-KII was 85–95% independent of Ca^{2+}/CaM for further enzymatic activity when compared to its nonphosphorylated counterpart. The Ca^{2+}/CaM-independent CaM-KII prepared by this procedure displayed no loss of catalytic activity compared to the nonphosphorylated kinase activated by Ca^{2+}/CaM. Ca^{2+}/CaM-independent CaM-KII was used to measure the effects of synthetic peptides on synapsin I phosphorylation or continued Ca^{2+}/CaM-independent autophosphorylation of CaM-KII. Assays were performed in a final volume of 50 μl containing Hepes (10 mM), EGTA (0.5 mM), $MgCl_2$ (5 mM), and [^{32}P]ATP (15 μM, 3.5 μCi per reaction; 1 Ci = 37 GBq) with or without synapsin I (4 μg). Reactions were terminated by the addition of 4-fold concentrated $NaDodSO_4$ sample buffer; individual polypeptides were resolved on polyacrylamide gels as described previously (24). ^{32}P incorporation into CaM-KII subunits, synapsin I, or synthetic peptides was determined by liquid scintillation counting of the appropriate gel band. Assays that examined the phosphorylation of the glycogen synthase-derived synthetic peptide were carried out by using the phosphocellulose filter method described by Kemp *et al.* (21). Electrophoresis of Ca^{2+}/CaM/peptide mixtures under nondenaturing conditions was performed as previously described (18).

RESULTS

Synthetic Peptides. Two peptides were synthesized whose sequences were modeled after the CaM-binding domain of CaM-KII (Fig. 1; ref. 18). CBP is a 29-amino acid peptide, whereas CBP_{-3} is identical to CBP with the exception that the three N-terminal amino acids of CBP are absent from CBP_{-3} (Fig. 1). Also shown in Fig. 1 is CBP_{-9}, which was previously used to define the CaM-binding domain of CaM-KII (18), as well as the analogous CaM-binding region of the 60-kDa subunit of CaM-KII (19). These peptides reside between amino acids 281 and 314 in the 50-kDa subunit.

CaM-Binding Properties of Synthetic Peptides. CBP and CBP_{-3} were tested for their ability to bind to and retard the electrophoretic migration of CaM in a Ca^{2+}-dependent manner on nondenaturing gels (results not shown). By using this gel system (18), peptides rich in basic amino acids, such as CBP and CBP_{-3}, migrate toward the cathode and in the opposite direction from CaM. At molar ratios of 1:1 (peptide to CaM), CBP and CBP_{-3} retarded the migration of virtually all the CaM in a Ca^{2+}-dependent manner. In the absence of Ca^{2+} (i.e., excess EGTA), neither peptide affected the migration of CaM. These results demonstrate that in the presence of Ca^{2+}, ternary Ca^{2+}/CaM/peptide complexes are formed, which decrease the electrophoretic mobility of CaM.

As a second test of their CaM-binding properties, CBP and CBP_{-3} were examined in a competition assay together with CaM and CaM-KII. These experiments determined the degree of Ca^{2+}/CaM-stimulated CaM-KII autophosphorylation at increasing concentrations of peptides while using a CaM concentration that half-maximally activated CaM-KII. As the concentration of either peptide increased from 10 to 200 nM, >90% inhibition of CaM-KII autophosphorylation was observed (Fig. 2). The degree of inhibition of CaM-KII autophosphorylation was essentially the same for CBP and CBP_{-3} at a given peptide concentration. Autophosphorylation of the 50-kDa subunit was inhibited at slightly lower peptide

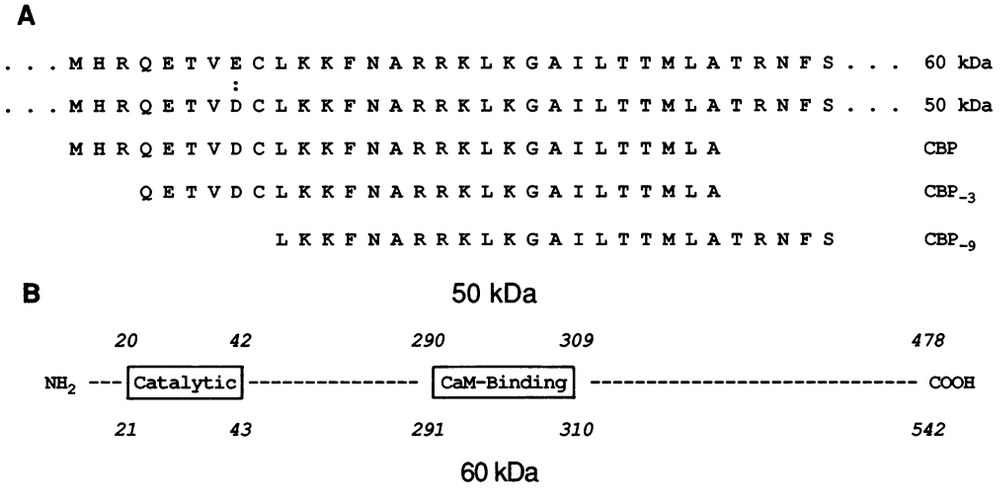

FIG. 1. (*A*) Amino acid sequences encompassing the CaM-binding domains of the 60- (19) and 50- (18) kDa subunits of CaM-KII and the synthetic peptides designated CBP (residues 281–309), CBP_{-3} (residues 284–309), and CBP_{-9} (residues 290–314). Sequences are given in the standard one-letter amino acid code. (*B*) Linear model of CaM-KII structure indicating catalytic and CaM-binding domains of the 50- and 60-kDa subunits. The numbers above and below the linear map designate amino acids that define each domain and the C termini of each kinase subunit.

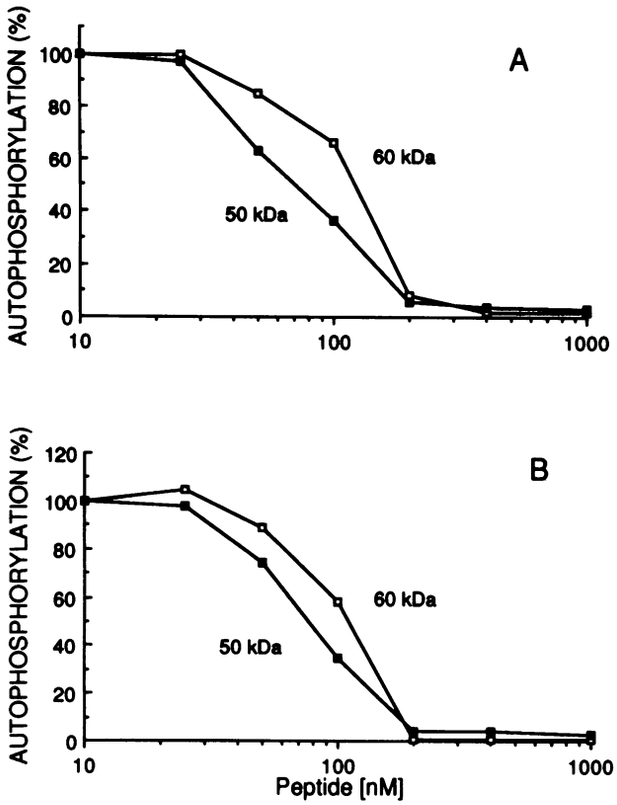

FIG. 2. Inhibitory effects of peptide analogs on Ca^{2+}/CaM-dependent autophosphorylation of the 50- and 60-kDa subunits of CaM-KII. Maximal levels of autophosphorylation were obtained in the absence of peptides and were set at 100% (*y* axis). CaM-KII (150 ng) plus CaM (600 nM final concentration) were incubated in the presence or absence of peptides at 4°C for 20 min; autophosphorylation was initiated by the addition of Mg[^{32}P]ATP (3.5 μCi per reaction) and incubation at 30°C for 30 sec. (*A*) Inhibitory effects of CBP at increasing peptide concentration. (*B*) Inhibitory effects of CBP_{-3} at increasing peptide concentration. Quantitation of ^{32}P incorporation into the 50- and 60-kDa subunits was performed by liquid scintillation counting on excised gel bands. Values represent the average of duplicate reactions from two separate experiments; individual determinations varied less than 5% from each other under the same assay conditions.

concentrations ($IC_{50} \approx 75$ nM) compared to the 60-kDa subunit ($IC_{50} \approx 120$ nM). These results demonstrated that both peptides possessed an affinity for CaM that was equal to or greater than the affinity between CaM and CaM-KII. Since CBP could not be distinguished from CBP_{-3} on the basis of relative affinities for CaM, the three amino acids absent from the N terminus of CBP_{-3} are not involved in the CaM-binding domain of CBP.

Analyses of interactions between peptides and CaM were extended by examining whether CBP and CBP_{-3} could inhibit the stimulatory activity of Ca^{2+}/CaM already bound to CaM-KII. CaM-KII (150 ng) was preincubated in Ca^{2+} plus CaM (1 mM and 600 nM, respectively) for 30 min at 4°C. CBP or CBP_{-3} were then added (10 μM final concentration), and autophosphorylation was initiated 10 sec later by the addition of Mg[^{32}P]ATP and the incubation of reactions at 30°C for 15 sec. Either synthetic peptide inhibited >90% of 50- and 60-kDa autophosphorylation in this assay. These results demonstrated that both peptides rapidly inhibited the Ca^{2+}/CaM-dependent autophosphorylation of CaM-KII in preformed kinase/Ca^{2+}/CaM ternary complexes.

Effects of Synthetic Peptides on Ca^{2+}/CaM-Independent CaM-KII Activity. To determine whether CBP or CBP_{-3} possessed inhibitory activities independent of their CaM-antagonist properties, a form of the autophosphorylated CaM-KII that is largely independent of Ca^{2+}/CaM for activity was used (see *Methods*). The truncated peptide CBP_{-3} showed little, if any, inhibition of autophosphorylation of either the 50-kDa or 60-kDa subunit of CaM-KII at peptide concentrations up to 250 μM (Fig. 3 *A* and *B*). In sharp contrast, CBP displayed potent inhibition of CaM-KII autophosphorylation. At 25 μM, CBP inhibited >90% of both 50- and 60-kDa subunit autophosphorylation (Fig. 3). The concentration of CBP necessary to inhibit 50% (IC_{50}) of 50- or 60-kDa autophosphorylation was $\approx$2 μM.

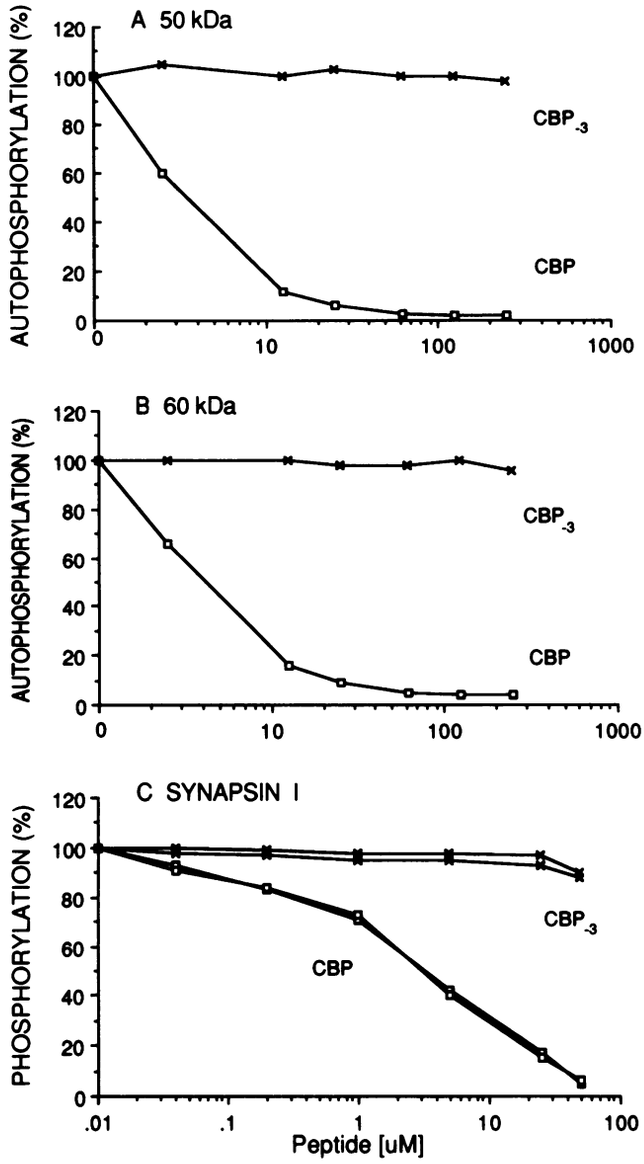

FIG. 3. Effects of peptide analogs on continued autophosphorylation (*A* and *B*) and synapsin I phosphorylation (*C*) by Ca^{2+}/CaM-independent CaM-KII. Maximal levels of autophosphorylation and substrate phosphorylation were obtained in the absence of peptides and were set at 100% (*y* axis). Mixtures of Ca^{2+}/CaM-independent kinase (200 ng per reaction), 0.5 mM EGTA, 10 mM Hepes (pH 7.0), and the designated amount of synthetic peptide (final concentrations indicated on *x* axis) were constituted at 4°C. The reactions in *C* also contained synapsin I (4 μg). Phosphorylation was initiated by addition of Mg[^{32}P]ATP (5 mM $MgCl_2$ and 25 μM ATP; 3.5 μCi/reaction) and incubation at 30°C for 30 sec. ^{32}P incorporation into individual polypeptides was quantitated as described in Fig. 2. Values represent the average of duplicate reactions from two separate experiments; individual determinations varied less than 7% from each other under the same assay conditions. The pairs of lines in *C* represent each of two separate experiments.

To determine if either peptide would inhibit the phosphorylation of exogenous substrate by the Ca^{2+}/CaM-independent CaM-KII, we examined the phosphorylation of synapsin I. Peptide CBP_{-3} at concentrations ≤ 25 μM did not inhibit synapsin I phosphorylation. Slight inhibition (5–10%) was seen at 50 μM CBP_{-3}, which suggested that the low affinity of CBP_{-3} for CaM-KII could be partially overcome by a large excess of peptide. Conversely, CBP was a potent inhibitor of synapsin I phosphorylation and displayed an IC_{50} of 2.5 μM. These results demonstrate that CBP can inhibit the interaction of CaM-KII with a natural substrate. More importantly, the observation that CBP_{-3} did not inhibit synapsin I phosphorylation demonstrated that the important determinants for active site-directed inhibition reside in the first several N-terminal residues of CBP.

Finally, we examined the active site-directed inhibitory properties of CBP in the presence of Ca^{2+}/CaM. These experiments also used the Ca^{2+}/CaM-independent form of CaM-KII (see *Methods*) and increasing amounts of CBP plus CaM at molar ratios of 1:1 (CBP to CaM). These results demonstrated that the ternary complex CBP/CaM/Ca^{2+} displayed an IC_{50} of 19 ± 3 μM (mean ± SEM) toward the inhibition of kinase autophosphorylation. This result demonstrated that CBP was approximately 10 times less potent in inhibiting CaM-KII activity in the presence of Ca^{2+}/CaM as compared to CBP in the absence of Ca^{2+}/CaM (see above).

Phosphorylation of Synthetic Peptides by Ca^{2+}/CaM-Independent CaM-KII. The ability of CBP to inhibit both autophosphorylation and exogenous substrate phosphorylation indicated its high affinity for the substrate binding site of CaM-KII. CBP has the N-terminal residues Met-His-**Arg-Gln-Glu-Thr**, which contain a minimum consensus phosphorylation sequence for skeletal muscle CaM-KII (23). CBP_{-3} does not contain the residues Met-His-Arg at its N terminus. CBP and CBP_{-3} were examined as substrates in phosphorylation assays using the Ca^{2+}/CaM-independent form of CaM-KII (Table 1). CBP displayed an apparent K_m of 6.1 μM with a low but measurable V_{max} of 0.001 μmol·min^{-1}·mg^{-1}. At concentrations between 2.5 and 25 μM, CBP was phosphorylated to the extent of approximately 0.001 mol of P_i per mol of peptide. Only [^{32}P]threonine was detected in CBP when it was analyzed for phospho amino acid composition following phosphorylation at peptide concentrations ranging from 2.5 to 250 μM (results not shown). CBP_{-3} was a very poor substrate for CaM-KII; its V_{max} was approximately 100 times less than that for CBP, and its K_m was approximately 500 μM (Table 1).

The above results with CBP indicated that although it displayed a rather high affinity for CaM-KII as indicated by its K_m in the low micromolar concentration range, its V_{max} was extremely low. These properties are consistent with CBP being an active site-directed inhibitor of Ca^{2+}/CaM-independent CaM-KII activity. To determine if these inhibitory properties were specific to CBP or were simply a general property of synthetic peptide substrates, we compared the characteristics of CBP to another peptide substrate—namely, a synthetic peptide (designated syntide) modeled after the 10 N-terminal residues of glycogen synthase. Syntide has been shown to be a good *in vitro* substrate for the CaM-KII from skeletal muscle (23). Experiments with syntide and the Ca^{2+}/CaM-independent form of CaM-KII revealed a K_m similar to that observed for CBP (9 μM); however, the V_{max} (0.33 μmol·min^{-1}·mg^{-1}) for syntide was approximately 300-fold greater than that of CBP (Table 1). Further analyses demonstrated that syntide was a modest inhibitor of continued autophosphorylation of the Ca^{2+}/CaM-independent CaM-KII with an IC_{50} of 28 μM.

Table 1. Phosphorylation kinetics of synthetic peptides

Peptide	K_m, μM	V_{max}, μmol·min^{-1}·mg^{-1}
CBP	6.1 ± 4	0.001 ± 0.0004
CBP_{-3}	500 ± 25	Negligible*
Syntide	9.0 ± 1	0.33 ± 0.1

Peptide phosphorylation was determined using peptide concentrations between 1 and 250 μM and the Ca^{2+}/CaM-independent CaM-KII. Kinetic constants (mean ± SEM) were determined by fitting data to the Michaelis–Menten equation using double reciprocal plots.

*Less than 0.01 nmol·min^{-1}·mg^{-1} at a peptide concentration of 50 μM.

DISCUSSION

Our results demonstrated that CBP and CBP_{-3} were indistinguishable in terms of Ca^{2+}-dependent CaM antagonist properties and inhibited Ca^{2+}/CaM-dependent autophosphorylation of CaM-KII with similar IC_{50} values (75–120 nM). Using nondenaturing gel electrophoresis, CBP and CBP_{-3} bound CaM in a Ca^{2+}-dependent manner at molar ratios of 1:1 (peptide to CaM). Furthermore, both peptides rapidly inhibited the CaM-dependent autophosphorylation of preformed Ca^{2+}/CaM/CaM-KII complexes. The potent CaM antagonist properties of CBP and CBP_{-3} are comparable to the IC_{50} of 46 nM for synthetic peptide analogs of the CaM-binding domains of smooth or skeletal muscle MLCK (21, 22). The focus of the studies herein was not the identification of the minimum CaM-binding sequence of CaM-KII. However, our results with CBP_{-3} together with previous findings with CBP_{-9} (18) indicate that the sequence encompassing residues 290–309 of the 50-kDa subunit contains the core CaM-binding domain (Fig. 1). In addition, experiments examining the CaM antagonist properties of CBP and CBP_{-3} indicated that the 60-kDa subunit displayed a consistently higher affinity for CaM as compared to the 50-kDa subunit.

Examination of the potencies of CBP and CBP_{-3} in inhibiting Ca^{2+}/CaM-independent CaM-KII activity revealed striking differences between the two peptides. The Ca^{2+}/CaM-independent form of CaM-KII was ideally suited to these studies since it no longer requires CaM for activity (11–17) and interactions between kinase and peptide can be directly assessed. Comparisons between CBP and CBP_{-3} revealed that the N terminus, which contains the complete consensus phosphorylation sequence Arg-Gln-Glu-Thr was critical to active site-directed inhibitory activity. CBP inhibited autophosphorylation of Ca^{2+}/CaM-independent CaM-KII and phosphorylation of synapsin I with an IC_{50} of approximately 2 μM, whereas CBP_{-3} displayed little, if any, inhibitory activity. Additional support for the high-affinity interaction of CBP with CaM-KII came from kinetic analyses in which CBP was shown to possess a K_m of approximately 6 μM.

It is noteworthy that the specific activity of Ca^{2+}/CaM-independent CaM-KII toward CBP was at least 300 times lower than its specific activity toward synapsin I or syntide. There are precedents for this correlation since the properties that make a peptide an effective active site-directed inhibitor are not always coincident with those that make it a good substrate (22). Kennelly *et al.* demonstrated that serine-substituted homologues of the CaM-binding autoinhibitory domain of MLCK were very poor substrates for this enzyme (22). In addition, a serine-substituted peptide modeled after the heat-stable inhibitor of cAMP-dependent protein kinase was an extremely poor substrate for this kinase (25). On the other hand, strategies to create potent inhibitors of cAMP-dependent protein kinase through modifications of the sequence of the extremely good peptide substrate kemptide have yielded little success (26, 27).

The results herein indicate that the N-terminal phosphorylation sequence adjacent to the CaM-binding domain of CaM-KII functions as an active site-directed inhibitory element. First, the inhibitory activity of CBP is dependent on the intact consensus phosphorylation sequence at its N terminus. Second, the IC_{50} for CBP inhibition of Ca^{2+}/CaM-independent kinase activity (2 μM) is only slightly higher than the reported K_m of CaM-KII for its native substrate synapsin I (K_m = 0.4–1.2 μM; refs. 2 and 15). Third, the active site-directed inhibitory activity of CBP appears specific and not a property of protein substrates in general. For example, high concentrations of synapsin I (5–15 μM) resulted in no detectable inhibition of Ca^{2+}/CaM-independent autophosphorylation of CaM-KII (data not shown). In addition, the synthetic peptide modeled after the phosphorylation site of glycogen synthase was not an inhibitor of CaM-KII catalytic activity, although it did display apparent competitive inhibition of autophosphorylation of the Ca^{2+}/CaM-independent form of CaM-KII with an IC_{50} of 28 μM. Further support for active site-directed inhibitory mechanisms comes from studies on MLCK from skeletal (22) or smooth muscle (21). Synthetic peptides modeled after the CaM-binding domains of either MLCK display active site-directed inhibitory capabilities of their parent enzyme with IC_{50} or K_i values in the 1–3 μM range.

An important conclusion from our results is the finding that the CaM-binding and active site-directed inhibitory domains contained in CBP appear to reside in nonoverlapping elements of a contiguous amino acid sequence. In contrast to CaM-KII, autoinhibitory and CaM-binding domains of MLCK from either smooth or skeletal muscle appear to reside in identical or highly overlapping domains (21, 22). One major difference between autoinhibitory peptides derived from CaM-KII versus MLCK is that peptides inhibiting the latter lack bona fide phosphorylation sequences that are similar to those that are present in the natural substrate myosin light chain (21, 22). Both CaM-KII and MLCK undergo Ca^{2+}/CaM-dependent autophosphorylation (7). Unlike MLCK, autophosphorylation of CaM-KII has a profound effect on its Ca^{2+}/CaM dependence, such that autophosphorylation results in the generation of Ca^{2+}/CaM-independent kinase activity (11–17). Thus, the strategic location of a potential autophosphorylation sequence adjacent to the CaM-binding domain of CaM-KII suggests that it may play a critical role in the autoregulatory properties of this kinase.

Our results suggest that the region of the 50-kDa subunit represented by CBP contains a bifunctional regulatory domain. In the absence of Ca^{2+}/CaM, we propose that the substrate binding site of each CaM-KII subunit is occupied with the phosphorylation sequence near the N terminus of the CaM-binding domain. During the Ca^{2+}/CaM-dependent activation of CaM-KII, we envision that the CaM-binding domain first undergoes a conformational change that generates an active catalytic domain and allows ATP binding prior to catalysis. This model is consistent with earlier results showing that the photoaffinity labeling of CaM-KII with 8-azido-[α-^{32}P]ATP is a Ca^{2+}/CaM-dependent process (8). After activation by Ca^{2+}/CaM and ATP binding, we postulate that autophosphorylation of the sequence Arg-Gln-Glu-Thr may be a prerequisite to relaxing active site-directed inhibition by this regulatory element. Thus, our model would predict that CBP would become a less potent active site-directed inhibitor after binding CaM. Consistent with this notion is our observation that CBP is a 10 times less potent inhibitor of kinase autophosphorylation when present in a ternary complex with Ca^{2+}/CaM. Autophosphorylation may promote additional conformational alterations in CaM-KII that would allow substrate proteins easy access to substrate-binding and catalytic domains. Finally, it is tempting to speculate that the phosphorylation of the N-terminal sequence in CBP is responsible for the generation of Ca^{2+}/CaM-independent activity. Although our results do not address the latter point, they provide fundamental information on which to base future site-directed mutagenesis studies that may ultimately define the autoregulatory domains of this major neuronal protein kinase.

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