Inhibition of the Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase I Cascade by cAMP-dependent Protein Kinase*

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Several recent studies have shown that Ca\(^{2+}\)/calmodulin-dependent protein kinase I (CaMKI) is phosphorylated and activated by a protein kinase (CaMKK) that is itself subject to regulation by Ca\(^{2+}\)/calmodulin. In the present study, we demonstrate that this enzyme cascade is regulated by cAMP-mediated activation of cAMP-dependent protein kinase (PKA). In vitro, CaMKK is phosphorylated by PKA and this is associated with inhibition of enzyme activity. The major site of phosphorylation is threonine 108, although additional sites are phosphorylated with lower efficiency. In vitro, CaMKK is also phosphorylated by CaMKI at the same sites as PKA, suggesting that this regulatory phosphorylation might play a role as a negative-feedback mechanism. In intact PC12 cells, activation of PKA with forskolin resulted in a rapid inhibition of both CaMKK and CaMKI activity. In hippocampal slices CaMKK was phosphorylated under basal conditions, and activation of PKA led to an increase in phosphorylation. Two-dimensional phosphopeptide mapping indicated that activation of PKA led to increased phosphorylation of multiple sites including threonine 108. These results indicate that in vitro and in intact cells the CaMKK/CaMKI cascade is subject to inhibition by PKA-mediated phosphorylation of CaMKK. The phosphorylation and inhibition of CaMKK by PKA is likely to be involved in modulating the balance between cAMP- and Ca\(^{2+}\)-dependent signal transduction pathways.

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 specific enzymes: myosin light chain kinase, phosphorylase kinase, and the multifunctional enzymes, CaMKI, CaMKII, and CaMKIV (1–7). Elongation factor 2 kinase, originally thought to represent a member of this family now appears to be related to a distinct class of protein kinases (8–10). CaMKI was initially identified in brain, but subsequently the enzyme was found to have widespread tissue and cellular distribution (11, 12). More recently several cDNAs have been isolated that appear to be isoforms of CaMKI derived from distinct genes (13). CaMKI was originally purified from brain based on its ability to phosphorylate the synaptic vesicle protein synapsin I at site 1 (14), and physiological substrates for CaMKI include synapsin I and synapsin II (14, 15). In vitro, CaMKI has also been found to phosphorylate cAMP response element-binding protein (16), and CF-2, a portion of the R-domain of the cystic fibrosis transmembrane conductance regulator (17). A potential role for CaMKI in the phosphorylation and regulation of the transcription factor, ATF-1, has also been suggested (18). Notably, for all of the well characterized substrates, CaMKI and cAMP-dependent protein kinase (PKA) phosphorylate the same residue, although CaMKI phosphorylates only a subset of PKA substrates, indicating differences in the recognition of substrates by the two enzymes.

CaMKI, myosin light chain kinase, CaMKII, and CaMKIV are activated upon binding of Ca\(^{2+}\)/CaM to a COOH-terminal regulatory domain of the enzyme, which in turn leads to removal of a short pseudosubstrate-like autoinhibitory region from the active site of the enzyme (7, 19–26). In addition, several recent studies have indicated that in a manner analogous to the MAP kinases and cyclin-dependent protein kinases, CaMKI and CaMKIV activities are strongly dependent on phosphorylation by a highly specific CaMKI kinase (CaMKK) at an equivalent threonine (Thr\(^{177}\) in CaMKI) in the activation loops of both kinases (26–36). Two isoforms of CaMKK have been identified that appear to recognize both CaMKI and CaMKIV (36, 37). However, unlike autophosphorylated CaM kinase II (4), phosphorylated CaMKI remains fully dependent on Ca\(^{2+}\)/CaM for activity. In contrast to CaMKII and CaMKIV, CaMKII, myosin light chain kinase, and phosphorylase kinase are not regulated by phosphorylation within the activation loop.

The precise physiological consequences of linking two CaMKs as part of an enzyme cascade remains to be clarified. However, this is likely to contribute to amplification of the Ca\(^{2+}\) signal since CaMKK appears to be more sensitive to activation by Ca\(^{2+}\)/CaM than CaMKI (26). In addition, by analogy to kinase cascades upstream of the MAP kinases, the CaMK cascade may serve to integrate information from other signal transduction pathways. In the present study, we report that both in vitro and in intact cells the CaMKK/CaMKI cascade is subject to inhibition by PKA-mediated phosphorylation of CaMKK. The phosphorylation and inhibition of CaMKK by PKA suggests a novel mechanism to modulate the balance between cAMP- and Ca\(^{2+}\)-dependent signal transduction pathways in eukaryotic cells. CaMKK is also phosphorylated by CaMKI, reflecting the overlapping substrate specificities of CaMKI and PKA, and suggesting the possibility of a classical negative feedback mechanism.

EXPERIMENTAL PROCEDURES

Materials—ATP, Nonidet P-40, diethiothreitol, 2-mercaptoethanol, EDTA, EGTA, Tris, Coomassie Brilliant Blue R-250, SDS, and bovine...
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serum albumin were from Sigma. HEPES and phenylmethylsulfonyl fluoride were obtained from Calbiochem. Nerve growth factor (NGF) was from Life Technologies, Inc. Forskolin, H89, and PDBu were obtained from Alexis Laboratories. [γ-32P]ATP was from NEN Life Science Products Inc. TPCCK-trypsin was from Worthington. Phosphocellulose (P-81 paper) was from Whatman. Nifedipine (1 μM final concentration) was from Schleicher & Schuell. X-ray film was from DuPont. Cellulose thin layer chromatographic sheets were from Eastman Kodak. Leupeptin was from Chemicon. 125I-CaM was from Amersham. CaM was purified from rabbit brain as described (38). The catalytic subunit of PKA was purified from bovine heart as described (39). Recombinant GST tagged CaMKK-WT, GST-CaMKK-S98A, -CaMKK-299, CaMKI-WT, and CaMKI-S98A were produced in Escherichia coli and purified as described previously (26). Serum from nonimmunized rabbits (nonimmune serum) was obtained from Pierce. Protein A-Sepharose was purchased from Pharmacia Biotech Inc. Rats were purchased from Charles River Laboratory.

Synthesis of Peptides—Peptide YLRRLSDSNF-amide (corresponding to residues 3–13 of synapsin I peptide) and PKI fragment (5–24) were synthesized by the Keck Foundation Biopolymer Facility at Yale University. 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.

Site-directed Mutagenesis of CaMKK and Preparation of Truncation Mutants—CaMKKα cDNA was prepared as described (26). The following oligonucleotides were synthesized by OPERON: KR-108M-S (5' -CCCCAGTCTATGAGATCAGCTCAGCTGCGG3'), KR-108M-A (5' -GTGGGACTCGATCCCACTCCCTGATCGTCG3'), and KR-433-AS (5' -CCCCATTTCACACCACCTTCCTCCTCTGATCGTCG3'). Oligo KR-108M-S corresponded to the coding strand and introduced a BamHI site. Oligo KR-433-AS corresponded to the complement of the coding strand and introduced an EcoRI site. The truncation mutants were amplified using appropriate primers from 10 ng of CaMKKα cDNA using pfu polymerase (Stratagene). The amplified DNA was purified and digested with BamHI and EcoRI. The fragments were subcloned into the BamHI and EcoRI site of pGEX-2T (Pharmacia) using a ligation kit (Takara).

Site-directed mutagenesis of full-length CaMKKα cDNA was carried out with a Quick Change Site-directed Mutagenesis Kit (Stratagene) according to the manufacturers protocol using pGEX-2T/CaMKKα WT as a template and the following primers: KR-T108A-S (5' -GGGGACTCGATCCCACTCCCTCCTCTGATCGTCG3') and KR-433-AS (5' -CCCCATTTCACACCACCTTCCTCCTCCTGATCGTCG3'). Oligo KR-108M-S corresponded to the coding strand and introduced a BamHI site. Oligo KR-433-AS corresponded to the complement of the coding strand and introduced an EcoRI site. The truncation mutants were amplified using appropriate primers from 10 ng of CaMKKα cDNA using pfu polymerase (Stratagene). The amplified DNA was purified and digested with BamHI and EcoRI. The fragments were subcloned into the BamHI and EcoRI site of pGEX-2T (Pharmacia) using a ligation kit (Takara).

Cell Culture—PC12 cells were incubated in Dulbecco's modified Eagle's medium plus 1% horse serum 18–24 h prior to drug or KCl treatment. Replicate cultures (1 × 105 cells) were treated with agonists for the indicated times and at the concentrations indicated in the figure legends. Forskolin (10 μM) and H89 (1 μM) were dissolved in MeSO. Depolarization of intact cells was accomplished by raising extracellular K+ to 40 mM by addition of an appropriate volume of 150 mM KCl.

Immunoblotting and 125I-CaM Overlay—Proteins (100 μg) were separated by SDS-PAGE and transferred to nitrocellulose filters as described (26). Immune complexes were incubated with HRP-conjugated protein G-Sepharose, washed, and analyzed by chemiluminescence kit (Amersham). For the 125I-CaM overlay, nitrocellulose filters were incubated with a blocking buffer containing: 3% (v/v) glycerol, 0.2 M NaCl, 0.5% β-mercaptoethanol, 50 mM Tris-HCl (pH 7.6) and purified as described previously (38). Serum from nonimmunized rabbits (nonimmune serum) was obtained from Pierce. Protein A-Sepharose was purchased from Pharmacia Biotech Inc. Rats were purchased from Charles River Laboratory.

Immunoprecipitation—PC12 cells were rinsed with phosphate-buffered saline, and lysates were prepared as described previously (41). CaMKI and CaMKKα were immunoprecipitated with CC76 or CC135, 150 μl of lysates were coimmunoprecipitated with 1 μl of IP buffer (1 Triton X-100, 150 μM NaCl, 50 mM Tris-HCl, pH 8.0, 2.5 mM sodium fluoride, 5 mM EDTA, 5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, aprotnin (60 kallikrein inactivating units/ml), 100 μM okadaic acid, and 1 mM sodium orthovanadate). Lysates were allowed to stand on ice for 10 min. After preclearing the lysates with 6 μg of protein A-Sepharose, 1 μg of lysate protein was incubated for 1 h with 1 μl of CC76, 10 μl of CC135 or nonimmune serum. Immunoprecipitates were washed twice with 1 ml of IP buffer, and then twice with 1 ml of 50 mM Tris-HCl (pH 7.6). Immunocomplexes were immediately resuspended in an assay mixture consisting of: 50 mM Tris (pH 7.6), 0.5 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, 1 mM CaCl2, 1 μM CaM, 10 mM MgCl2, 200 μM [γ-32P]ATP (100 cpm/μmol). For CaMKI activity, 50 μl synapsin site 1 peptide was used as substrate. For CaMKK activity, GST fragment or GST-CaMKI was used as substrate. For CaMKI, the assay mixtures were incubated at 30 °C for 5 min, at which time 20-μl aliquots were removed and 32P incorporation into synapsin site 1 peptide was quantified by adsorption of the peptide to phosphocellulose P81 papers and scintillation counting. For CaMKK, the assay mixtures were incubated at 30 °C for 20 min. Reactions were stopped by adding SDS sample buffer, samples were analyzed by SDS-PAGE, and 32P incorporation into GST-CaMKI was quantified by PhosphorImager analysis. Assays were linear with time and protein concentration.

32P Labeling of Brain Slices—A Mcllwain tissue chamber was used to prepare 400-μm thick slices from freshly dissected rat hippocampus. Dissection, cutting, and preincubation (for 30 min) were carried out in a modified Krebs-bicarbonate buffer (phosphate free) of the following composition: 124 mM NaCl, 4 mM KCl, 25 mM NaHCO3, 1.5 mM MgSO4, 20 mM glucose, and 20 μM t-glucose, pH 7.4. Slices were then incubated at 30 °C for 30 min, each incubation was separated by freezing in liquid nitrogen. Slices were homogenized using IP buffer and immunoprecipitation was performed as described above. Proteins were analyzed by SDS-PAGE (10% polyacrylamide), and gel pieces containing CaMKKα were identified by preincubation with the radiogram and excised. After measuring 32P incorporation by scintillation counting (Cerenkov), gel pieces from triplicate samples were pooled and processed for two-dimensional phosphopeptide mapping.

Two-dimensional Phosphopeptide Mapping and Phosphoamino Acid Analysis—Gel pieces containing 32P-labeled CaMKKα were excised from dried SDS-polyacrylamide gels, and two-dimensional phosphopeptide mapping and phosphoamino acid analysis were performed as described (14).

RESULTS

Phosphorylation of CaMKK by CaMKI and PKA—When incubated together, CaMKKα WT phosphorylated CaMKI-WT in a Ca2+/CaM-dependent manner (Fig. 1A). A low but significant level of phosphorylation of CaMKK-WT was also observed in the presence of Ca2+/CaM (Fig. 1A). In contrast, CaMKK-WT, when incubated alone, was not phosphorylated under any condition (Fig. 1B). This raised the possibility that the phosphorylation of CaMKK-WT was catalyzed by activated CaMKI-
WT. To test this idea further, CaMKI-293, a constitutively active form of CaMKI, and CaMKI-299, an autoinhibited form of CaMKI (23, 30), were incubated with CaMKK-WT (Fig. 1A). With CaMKI-293, phosphorylation of CaMKK-WT was observed in both the absence and presence of Ca\(^{2+}/\)CaM. However, with CaMKI-299 no phosphorylation of CaMKK-WT or CaMKI-299 was detected. The failure to observe phosphorylation of CaMKI-299 was expected since CaMKI has to be in an active form to act as a substrate for CaMKK (26, 30). Moreover, the failure to observe phosphorylation of CaMKK when incubated alone or in the presence of CaMKI-299 indicated that active forms of CaMKI catalyzed phosphorylation of CaMKK.

Notably, all substrates for CaMKI identified to date are also phosphorylated by PKA or CaMKI, and of CaMKK mutants (see Fig. 8) suggested that peptides 1, 2, and 3 may be derived from alternative tryptic digestion of residues surrounding a single phosphorylation site.

Fig. 1. Phosphorylation of CaMKK by CaMKI and PKA. A, CaMKK (1 \(\mu\)g/ml) was incubated with CaMKI-WT, -CaMKI-293, or -CaMKI-299 (10 \(\mu\)g/ml) in the absence or presence of Ca\(^{2+}/\)CaM for 10 min at 30 °C as described under “Experimental Procedures.” B, CaMKK-WT (1 \(\mu\)g/ml) was incubated in the absence or presence of PKA (0.2 \(\mu\)g/ml), in the absence or presence of Ca\(^{2+}/\)CaM for 20 min. For both A and B, samples were analyzed by SDS-PAGE and autoradiograms of the dried gels are shown.

Fig. 2. Peptide mapping and phosphoamino acid analysis of CaMKK phosphorylated by CaMKI or PKA. CaMKK was phosphorylated with PKA or CaMKI as described in the legend to Fig. 1. CaMKK was digested with TPCK-trypsin and digests were separated on thin layer cellulose plates by electrophoresis in the first dimension and chromatography in the second dimension. O, origin in lower left. Upper panel, tryptic digest of CaMKK phosphorylated by CaMKI. Lower panel, tryptic digest of CaMKK phosphorylated by PKA. Right panels, aliquots of tryptic digests were hydrolyzed with HCl and phosphoamino acids were separated by electrophoresis. \(^{32}\)P-Phosphoamino acids were visualized by autoradiography. The position of phosphotyrosine (pY), phosphothreonine (pT), and phosphoserine (pS) standards are indicated. Analysis of additional peptide maps of CaMKK phosphorylated by PKA or CaMKI, and of CaMKK mutants (see Fig. 8) suggested that peptides 1, 2, and 3 may be derived from alternative tryptic digestion of residues surrounding a single phosphorylation site.

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Thr\(^{108}\) Is the Major Site in CaMKK Phosphorylated by PKA in Vitro—Analysis of the amino acid sequence of CaMKK indicated the presence of three good consensuses phosphorylation sites for PKA, at Ser\(^{433}\) (RKPSL), Thr\(^{108}\) (RRPTI), and Ser\(^{458}\) (RKRSF). Removal of the COOH terminus of CaMKK at amino acid 433 (CaMKK-433) did not affect significantly phosphorylation by PKA, or the inhibition of enzyme activity as measured by phosphorylation of CaMKK-WT (Fig. 4A). This suggested that Ser\(^{458}\) was not a major site of phosphorylation, and that phosphorylation did not contribute to inhibition of CaMKK activity, under the conditions used in this assay. Mutation of Thr\(^{108}\) to alanine (T108A) resulted in a large decrease in phosphorylation by PKA (Fig. 4B). Additional mutation of Ser\(^{433}\) to alanine (S74A) resulted in a further small reduction in phosphorylation. These results indicated that Thr\(^{108}\) was the major site of CaMKK phosphorylated by PKA in vitro. However, a low
PKA as described in CaMKK activity. CaMKK was incubated in the absence or presence of 100 μM CaM (pH 7.5), 10 mM magnesium acetate, 1 mM EGTA, 5 mM dithiothreitol, striatum and nucleus accumbens, and low in cerebellum. Very low expression was highest in cortex and hippocampus, intermediate in forebrain regions or PC12 cells, expressing barely detectable amounts (Fig. 5B). Based on these results, forebrain regions or PC12 cells appeared good preparations for further analysis.

As shown in part in a previous study in PC12 cells (41), membrane depolarization, but not treatment with NGF or PDBu, led to activation of CaMKI (Fig. 6). Membrane depolarization, NGF, and PDBu did not affect CaMKK activity. However, treatment with forskolin resulted in a decrease in both CaMKK and CaMKI activities to approximately the same magnitude. The forskolin-dependent inhibition of both CaMKK and CaMKI activities occurred rapidly, but transiently, reaching a peak within 5 min, after which both activities returned to an intermediate level (Fig. 7). Pretreatment with H89, a relatively specific inhibitor of PKA, significantly attenuated the effect of forskolin on both CaMKK and CaMKI activities.

**Phosphorylation of CaMKK in Hippocampal Slices**—The low level of CaMKK in PC12 cells precluded an analysis of the phosphorylation of CaMKK by PKA in situ. Therefore, we carried out studies in rat hippocampus, the brain region that expresses the highest level of CaMKK (see Fig. 5 above). Hippocampal slices were prelabeled with [32P] proteoliposomes treated without or with forskolin, and CaMKK was immunoprecipitated (Fig. 8). A significant level of phosphorylation of CaMKK was measured under basal conditions and this was increased 2.0 ± 0.3-fold.
CaMKI-293 for the samples was 100, 90, 98, 102, 47, 115, and 113% (%

**FIG. 5. Distribution of CaMKK in rat tissues and cell lines.** 100 µg of each sample was subjected to immunoblotting. Molecular weights (in kDa) are shown on the left of each panel. A, rat tissues; Striat, striatum; Nuc Ac, nucleus accumbens; Panc, pancreas; Salivery G, salivery gland. B, cell lines. CHO, Chinese hamster ovary. The autoradiogram shown in panel B was exposed ~5 times longer than that shown in panel A. Based on these and other results, we estimate that PC12 cells contained ~20% of the amount of CaMKK found in hippocampus.

**FIG. 6. Regulation of CaMKK and CaMKI activity in PC12 cells.** PC12 cells were treated with control solution, NGF (50 ng/ml), PDBu (2 µM), forskolin (5 µM), a depolarizing concentration of KCl (40 mM), KCl plus NGF, or KCl plus PDBu for 5 min. CaMKI and CaMKK activities were measured following immunoprecipitation, using synapsin I peptide or CaMKI-293, respectively. Upper panel, CaMKI activity results are representative of three experiments, and standard deviations are shown. Lower panel, CaMKK assay samples were separated by SDS-PAGE and analyzed using a PhosphorImager. 32P incorporation into CaMKK-293 for the samples was 100, 90, 98, 102, 47, 115, and 113% (%

In addition, following deletion of the NH2-terminal 108 amino acids, only peptide 1’ was phosphorylated by PKA. These results suggested that peptides 1–3 were derived by alternative tryptic cleavage of amino acids flanking Thr108 in CaMKK, peptides 2’, 4, and 5 were derived from the NH2-terminal 108 amino acids of CaMKK, and peptide 1’ was derived from the COOH-terminal 397 amino acids of CaMKK. In hippocampal slices incubated under basal conditions, CaMKK was phosphorylated at multiple sites, several of which appeared to be the same as the minor sites phosphorylated by PKA in vitro (for example, peptides 1’, 4, and 5). Incubation with forskolin not only increased phosphorylation of sites found in the control condition, but significantly increased phosphorylation of peptide 1 and either peptides 2 or 2’. In general, the pattern obtained from CaMKK-WT phosphorylated in vitro with PKA, and that obtained from CaMKK phosphorylated in hippocampal slices following incubation with forskolin, were the same. Based on these results, we conclude that in situ, stimulation of PKA leads to an increase in the phosphorylation of several sites in CaMKK, one of which is Thr108.

**DISCUSSION**

In the present study, we have shown that the CaMKK/ CaMKI cascade is subject to regulation via phosphorylation and inactivation of CaMKK by PKA. In in vitro studies, PKA was found to efficiently phosphorylate Thr108 of CaMKKα, a consensus PKA site (RRPTI) located at the NH2 terminus of the catalytic domain of the enzyme. Phosphorylation of Thr108 was sufficient to mediate the inhibition of enzyme activity. Under conditions where Thr108 is the major site phosphorylated by PKA, CaMKK activity was significantly inhibited. In addition, CaMKK-433, a constitutively active form of the enzyme that does not contain the autoinhibitory or CaM-binding domains, was also phosphorylated by PKA and inhibited. However, direct assessment of the role of Thr108 phosphorylation was precluded by the fact that CaMKK-T108A was inactive when expressed in bacteria (data not shown). In vitro, phosphorylation on serine was also found within residues 109–505 of CaMKK, and a consensus PKA site at Ser458 (RKRSF) may be a potential candidate. Ser458 is present within the CaM-binding domain of CaMKK (28, 36, 44), and autophosphorylation of CaMKII and CaMKIV, within or close to the CaM-binding domains, inhibits enzyme activity by influencing the binding of Ca2+/CaM (4, 45). Under our standard assay conditions, we did not detect any effect of Ca2+/CaM on the phosphorylation of CaMKK by PKA, or of phosphorylation of CaMKK on binding of Ca2+/CaM. However, phosphorylation with high concentrations of PKA resulted in a reduction in binding of Ca2+/CaM, presumably as

(u)(average of three experiments) by forskolin. Under the same conditions used for analysis of phosphorylation of CaMKK, forskolin treatment of hippocampal slices resulted in ~30% decrease in CaMKK activity (data not shown).

Two-dimensional peptide mapping was used to compare the site(s) phosphorylated in CaMKK in vitro and in situ (Fig. 8). In vitro, CaMKK-WT was phosphorylated by PKA at multiple sites (see also Fig. 2). As revealed by site-directed mutagenesis, two major peptides and a minor peptide (numbered 1, 2, and 3) were not detected in the peptide map obtained from CaMKK-T108A. In addition, following deletion of the NH2-terminal 108 amino acids, only peptide 1’ was phosphorylated by PKA. These results suggested that peptides 1–3 were derived by alternative tryptic cleavage of amino acids flanking Thr108 in CaMKK, peptides 2’, 4, and 5 were derived from the NH2-terminal 108 amino acids of CaMKK, and peptide 1’ was derived from the COOH-terminal 397 amino acids of CaMKK. In hippocampal slices incubated under basal conditions, CaMKK was phosphorylated at multiple sites, several of which appeared to be the same as the minor sites phosphorylated by PKA in vitro (for example, peptides 1’, 4, and 5). Incubation with forskolin not only increased phosphorylation of sites found in the control condition, but significantly increased phosphorylation of peptide 1 and either peptides 2 or 2’. In general, the pattern obtained from CaMKK-WT phosphorylated in vitro with PKA, and that obtained from CaMKK phosphorylated in hippocampal slices following incubation with forskolin, were the same. Based on these results, we conclude that in situ, stimulation of PKA leads to an increase in the phosphorylation of several sites in CaMKK, one of which is Thr108.

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In intact cells, CaMKK was basally phosphorylated, and stimulation of PKA activity was associated with increased phosphorylation. Two-dimensional peptide mapping suggested that Thr108 was one of several sites phosphorylated by PKA. Therefore, the present studies support the conclusion that phosphorylation of Thr108 leads to inhibition of CaMKK in vitro and that this is likely to contribute to the inhibition observed in intact cells.

During the completion of this work, Wayman et al. (46) published results from studies of the effect of PKA on the regulation of the CaMKK/CaMKIV cascade. Several of the general conclusions were similar to those obtained in the present study. However, it was concluded that phosphorylation of both Thr108 and Ser458 by PKA contributed equally to inhibition of CaMKK, and that as a consequence of phosphorylation of Ser458, suppression of Ca2+/CaM binding contributed to inhibition of enzyme activity. The reasons for the differences in the conclusions are not immediately clear, but notably Wayman et al. (46) demonstrated at least in some of their studies that phosphorylation of Ser458 occurred only following prolonged incubation of CaMKK with PKA. Thus, while phosphorylation
of Ser<sup>458</sup> or other serine(s) by PKA may contribute to the inhibition observed in vitro and in intact cells, our results suggest that Thr<sup>108</sup> is likely to be more efficiently phosphorylated than Ser<sup>458</sup>, and that phosphorylation of Thr<sup>108</sup> represents the major mechanism of regulation of the enzyme.

There are a number of potential mechanisms by which phosphorylation of Thr<sup>108</sup> may lead to inhibition of CaMKK. Recent studies of several protein kinases have indicated that the NH<sub>2</sub>-terminus is important for regulation of kinase activity (47–51), and phosphorylation of Thr<sup>108</sup> may confer an autoinhibitory function. In the catalytic subunit of PKA, the catalytic core of the enzyme is preceded by a 39-residue NH<sub>2</sub>-terminal amphipathic α-helical domain (the A helix) that is important for the stability of the enzyme and for maintaining conformation at the active site (52, 53). Thr<sup>108</sup> would be predicted to be at the junction of the NH<sub>2</sub>-terminal and catalytic domains of CaMKK.

Phosphorylation by PKA may therefore modulate a stabilizing influence of the NH<sub>2</sub>-terminal domain. Studies of the enzymes within the various MAP kinase cascades have revealed that protein-protein interactions away from the active site of the upstream kinase, and away from the activation loop of the downstream kinase, are important for directing the specificity of a given cascade (54), and for mediating high affinity interactions (49, 50). These interactions have been found to be close to the NH<sub>2</sub>-terminus of the catalytic domain (54), or within NH<sub>2</sub>-terminal extensions of the particular kinases (49, 50). Thus rather than directly inhibiting CaMKK activity, phosphorylation of Thr<sup>108</sup> of CaMKK by PKA may interfere with its ability to interact with and/or to phosphorylate CaMKI or CaMKIV.

The results obtained using PC12 cells indicate that activation of CaMKK is primarily dependent on increases in intracellular Ca<sup>2+</sup>. In contrast, and in agreement with previous studies (41), phosphorylation of CaMKI by CaMKK leads to a stable activated form of the enzyme that can be assayed following immunoprecipitation in the presence of protein phosphatase inhibitors. Notably, forskolin treatment led to a significant decrease in CaMKI activity, that presumably reflects a decrease in the level of basally activated, phosphorylated CaMKI. Moreover, the effect of forskolin on either CaMKK or CaMKI activity was somewhat transient, most likely resulting from dephosphorylation of CaMKK and CaMKI by cellular protein phosphatases. Our preliminary studies suggest that phosphorylation of CaMKI is a good substrate in vitro for protein phosphatase 2A, but the identity of the phosphatase(s) that dephosphorylate(s) CaMKK or CaMKI in intact cells is not known.

A large number of studies have indicated that there is an intricate inter-relationship between Ca<sup>2+</sup>/CaM-dependent and cAMP-dependent signaling pathways in cells. For example, CaM-dependent adenyl cyclases, as well as CaM-dependent cAMP phosphodiesterases play a critical role in the generation and degradation of cAMP (55, 56). The CaM-dependent protein phosphatase, calcineurin (protein phosphatase 2B), antagonizes the actions of cAMP through the dephosphorylation of various substrates for PKA (57, 58). Interestingly, recent studies have shown that PKA and calcineurin can exist in a multienzyme complex through their interaction with the PKA-anchoring protein, AKAP-79 (59). PKA is also able to phosphorylate and activate phospholipase kinase (60) and to phosphorylate elongation factor-2 kinase, leading to the generation of a CaM-independent enzyme activity (61, 62). Therefore, phosphorylation of CaMKKs by PKA represents a new mechanism that adds to our understanding of the interaction of Ca<sup>2+</sup> and cAMP signaling mechanisms.

CaMKI phosphorylates a subset of proteins that are also phosphorylated on a common site by PKA. For example, CaMKI and PKA phosphorylate Ser<sup>1</sup> of the neurotransmitter vesicle-associated proteins, synapsin I and II, and CaMKIV and PKA phosphorylate the transcription factor cAMP response element-binding protein at Ser<sup>133</sup> (16, 63–65). Given that PKA and either CaMKI or CaMKIV phosphorylate common sites in these substrates, the precise physiological consequences of regulation of either CaMKI or CaMKIV by PKA remains to be elucidated. On first examination, this regulatory mechanism appears somewhat paradoxical, however, any changes in the phosphorylation of downstream targets for CaMKI or CaMKIV as a result of phosphorylation of CaMKK by PKA would reflect the relative subcellular distribution of the enzymes, and more importantly reflect the integration of the activities of protein phosphatases toward the various phosphorylated species involved. Regulation of CaMKK by PKA phosphorylation may also be more relevant to other downstream targets. For example, recent studies have indicated that CaMKIV can stimulate various MAP kinase cascades, although the exact mechanism is not yet established (66). Phosphorylation and inhibition of CaMKK by PKA could therefore contribute to the down-regulation of MAP kinase observed in several cell types (67).

These studies of the regulation of CaMKK by PKA resulted from an initial observation that CaMKK was phosphorylated by CaMKI. Peptide mapping studies and phosphoamino acid analysis indicated that Thr<sup>108</sup> was the major site phosphorylated by both PKA and CaMKI. Other studies have also suggested that CaMKK is phosphorylated by CaMKIV in vitro (26). These results raise the possibility that phosphorylation of CaMKK by CaMKI, CaMKIV, or both, may represent a classical feedback mechanism to control the CaMKK cascade. In this respect, the situation would be analogous to that observed for the analog of MEK, STE7, found in Saccharomyces cerevisiae (43, 68). Our present studies did not address this possibility experimentally. However, preliminary studies have shown in hippocampal slices that depolarization, or incubation with the Ca<sup>2+</sup> ionophore, ionomycin, increased threonine phosphorylation of CaMKK. Future studies will hopefully establish whether phosphorylation and inhibition of CaMKK subserves such a feedback mechanism.

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