Inhibitory Cross-talk by cAMP Kinase on the Calmodulin-dependent Protein Kinase Cascade*

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The calmodulin-dependent kinase (CaM-K) cascade, a Ca2+-triggered system involving phosphorylation and activation of CaM-KI and CaM-KIV by CaM kinase kinase (CaM-KK), regulates translocation through direct phosphorylation of transcription factors such as cAMP response element-binding protein. We have shown previously that activated CaM-KIV can activate the mitogen-activated protein kinases (Enslen, H., Tokumitsu, H., Stork, P. J. S., Davis, R. J., and Soderling, T. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10803–10808), and the present paper describes a novel regulatory cross-talk between cAMP kinase (PKA) and CaM-KK. PKA gave rapid phosphorylation in vitro and in cells of recombinant CaM-KK, resulting in 50–75% inhibition of CaM-KK activity, part of which was due to suppression of CaM-binding by phosphorylation of Ser458 in the CaM-binding domain. However, the Ser458→Ala mutant, or a truncation mutant in which the CaM-binding and auto-inhibitory domains were deleted, was still partially suppressed by PKA-mediated phosphorylation. The second inhibitory site was identified as Thr108 by site-specific mutagenesis. Treatments of COS-7, PC12, hippocampal, or Jurkat cells with the PKA activators forskolin or isoproterenol gave 30–90% inhibition of either endogenous or transfected CaM-KK and/or CaM-KIV activities. These results demonstrate that the CaM kinase cascade is negatively regulated in cells by the cAMP/PKA pathway.

Calcium ion is one of the most ubiquitous cellular signaling molecules, especially in neural tissues where many of its physiological responses are mediated by the Ca2+-binding protein calmodulin (CaM). The Ca2+/CaM complex allosterically activates numerous proteins including a diverse family of Ca2+/CaM-dependent protein kinases (CaM-Ks), including CaM-KI and CaM-KIV (reviewed in Refs. 1 and 2). Several groups identified a factor(s) in brain extracts that activates CaM-KI and/or CaM-KIV (3–5), and this led to the cloning of a CaM-KK (6). Recombinant CaM-KK phosphorylates CaM-KI and CaM-KIV, resulting in 10–20-fold increases in their activities. Operation of this CaM-K cascade has been demonstrated for CaM-KIV in Jurkat cells upon stimulation of their CD3 receptor (7, 8) and for CaM-KI in PC12 cells upon depolarization with KCl (9).

One of the physiological systems regulated by this CaM-K cascade is gene transcription mediated through phosphorylation of CREB. CaM-KIV only phosphorylates the transactivating serine (Ser133) in CREB, and activation by CaM-KK of CaM-KIV increases its Vmax 10-fold for CREB phosphorylation (10). Using transfected COS-7 cells expressing GAL4/CREB and a GAL4/luciferase reporter gene, co-transfection with CaM-KK plus CaM-KIV gave a 14-fold increase in luciferase expression over transfection with either kinase alone (6). Furthermore, CREB-mediated gene expression has been implicated in forms of learning and memory such as late phase long term potentiation (11), and a recent study in cultured hippocampal neurons strongly implicates CaM-KIV as the catalyst for CREB phosphorylation in this system (12).

Because of the complexity of the Ca2+-modulated cellular responses, we have begun to explore cross-talk between this CaM-K cascade and other signaling pathways. We recently demonstrated that the CaM-K cascade can also indirectly regulate gene transcription through the ability of activated CaM-KK to activate the extracellular signal-regulated kinase, Jun-NH2-terminal kinase, and p38 (13). We have now explored cross-talk between the cAMP kinase (PKA) system and the CaM-K system, and we demonstrate that PKA rapidly phosphorylated CaM-KK and resulted in its partial inhibition both in vitro and in multiple cultured cells.

**MATERIALS AND METHODS**

Recombinant CaM-KIV from SF9 cells was purified on CaM-Sepharose (5, 6). CaM-KK was either expressed in Escherichia coli and purified on CaM-Sepharose or co-transfected in COS cells as a FLAG-tagged construct and purified by immunoprecipitation with the FLAG antibody. Standard in vitro CaM-KK phosphorylation reactions contained 5–25 mM PKA, the indicated concentrations of CaM-KK (or CaM-KIV), either 1 mM CaCl2, 5 μM CaM or 1 mM EGTA, and the other standard protein kinase assay constituents (5). For assays of CaM-KK activity, the reactions contained either 40 μg/ml His-CaMKIV transfected with CaM-KIV (15, 16) or 5 μM CaM-KIV (0.88 μM was indicated) and 5 μM CaM (1 μM in Fig. 2C). Reactions were terminated by either addition of SDS sample buffer and resolution on SDS-PAGE (for His CaM-KIV transfected with CaM-KIV (17) or by dilution of reaction aliquots, which were then assayed for CaM-KIV activity using syntide-2 as substrate in the presence of EGTA (5, 6). In vitro phosphatase treatments of CaM-KK were performed as described (8). FP1C was a kind gift from Dr. Anna DePaoli-Roach (Indiana University), and human red blood cell PP2A catalytic subunit was from Upstate Biotechnology, Inc. Site-directed mutants of CaM-KK were made using a site-specific plasmid DNA mutagenesis kit (5 Prime→3 Prime, Inc., Boulder, CO). 32P incorporation and CaM binding to CaM-KK were quantitated by densitometry using a computer program “Image” (National Institutes of Health, Research Service Branch).

For studies in intact cells, COS-7 cells were transfected with plasmid (Mock) or CaM-KK (6, 14), and PC12 cells (10), primary cultures of hippocampal neurons (15), or Jurkat cells (8) were transfected and/or cultured as referenced. Cells were lysed in buffer A (14) which contains the protein phosphatase inhibitors pyrophosphate (10 μM), NaF (50

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The abbreviations used are: CaM, calmodulin; CaM-K, Ca2+/CaM-dependent protein kinase; PAGE, polyacrylamide gel electrophoresis; CREB, cAMP response element-binding protein; PKA, cAMP kinase; cBimp, 5,6-dichloro-1-b-D-ribofuranosylbenzimidazole-3'-5'-cyclic monophosphorothioate, Sp-isomer.
Inhibition of CaM kinase Cascade by cAMP kinase

RESULTS AND DISCUSSION

We first determined whether PKA could phosphorylate in vitro recombinant CaM-KIV or CaM-KK and found that CaM-KK was rapidly phosphorylated by PKA in the absence (Fig. 1A, lanes 7 versus 5) or presence (lane 8 versus 6) of Ca\(^{2+}\)/CaM (Fig. 1A, A and B) (see “Note Added in Proof”). In the presence of Ca\(^{2+}\)/CaM, CaM-KK also exhibited strong auto-phosphorylation (Fig. 1A, lane 6 versus 5), which appeared to be additive with PKA-mediated phosphorylation of CaM-KK (Fig. 1, A and B). Tryptic peptide mapping revealed several 32P-labeled peptides, suggesting that PKA phosphorylated several sites in CaM-KK (not shown). In contrast to a previous report using purified rat cerebellar CaM-KIV (16), we were unable to obtain phosphorylation of recombinant mouse CaM-KIV by PKA (Fig. 1A, lanes 1–4). This is analogous to the previous claim that the C-terminal CaM-KIV could be activated by “auto-phosphorylation” (17), whereas the recombinant CaM-KIV could not (18–20). Subsequent experiments showed that recombinant CaM-KIV is activated by recombinant CaM-KK (6). Thus, it is possible in the previous report (16) that PKA phosphorylated the contaminating CaM-KK in the rat brain CaM-KIV, and this suppressed the ability of this CaM-KK to subsequently activate the CaM-KIV (see below). Such an effect could easily be interpreted as PKA-mediated inhibition of CaM-KIV.

To investigate whether the PKA-mediated phosphorylation of CaM-KK was regulatory, we determined its effect on CaM-KK activity using two different assays. CaM-KK was phosphorylated by PKA for the indicated times, and aliquots were subjected to SDS-PAGE and either autoradiography (upper inset) to quantitate 32P incorporation (filled circles) or to gel overlay with biotinylated-CaM (lower inset) to assess CaM binding (open circles). PKA phosphorylation of 32P suppressed CaM binding. Partially purified wild-type and Ser\(^{52}-\)→Ala mutant of CaM-KK were phosphorylated for 20 min without (-) or with (+) PKA (6.7 ng/ml) as described above. Aliquots of the reaction mixtures were then subjected to SDS-PAGE followed by the biotinylated CaM overlay. C and D, identification of regulatory PKA phosphorylation sites in CaM-KK. Wild-type CaM-KK mutants and mutants S458A. T108G, or the double mutant T108G/ S458A were expressed as FLAG-tagged constructs in COS cells, immunoprecipitated with FLAG antibody, and treated for 20 min with (+) or without (-) PKA (25 nM). The CaM-KK was then assayed by the standard CaM-KIV activation reaction using 0.08 μM CaM-KIV and 1 μM CaM (C). The results show mean ± S.E. of triplicate assays for Ca\(^{2+}\)-independent CaM-KIV. In D the PKA phosphorylation used [γ-\(^{32}\)P]ATP, the reactions were resolved by SDS-PAGE, and the CaM-KK protein was quantitated by Coomassie staining. The CaM-KK bands were then excised, and 32P incorporation was determined by liquid scintillation and normalized for protein. Phosphorylation of wild type CaM-KK is defined as 100%, and results are shown as means ± S.E. of triplicate phosphorylations.

The biochemical mechanisms involved in PKA-mediated inhibition of CaM-KK are complex and not easily characterized kinetically. Phosphorylation of CaM-KK by PKA in the absence of Ca\(^{2+}\)/CaM (see “Note Added in Proof”) strongly suppressed binding of Ca\(^{2+}\)/CaM to CaM-KK (Fig. 2A), and this effect was lost when Ser\(^{52}\), a consensus PKA phosphorylation site in the CaM-binding domain, was mutated to Ala (Fig. 2B). It should...
be noted that the biotinylated CaM-overlay technique used in Fig. 2, A and B, is only qualitative, and PKA phosphorylation of CaM-KK does not completely block its activation by Ca\(^{2+}\)/CaM.

For example, in the experiment of Fig. 1D where 5 μM CaM was used, PKA gave a 45% inhibition of CaM-KK activity, whereas in Fig. 2C inhibition was about 80% with 1 μM CaM in the assay. It is well known that the activities of several enzymes can be inhibited due to phosphorylation of their CaM-binding domains, but this mechanism cannot account for all of the CaM-KK inhibition, since PKA still gave significant inhibition (Fig. 2D), where isoproterenol produced a rapid 5.4% inhibition, suggesting a K\(_{\text{m}}\) effect.

We next identified this second site of inhibitory regulation. CaM-KK has an extended NH\(_{2}\) terminus containing several PKA consensus phosphorylation sites (e.g. Ser\(^{52}\) and Ser\(^{74}\)), but when we expressed CaM-KKK\(_{434}\), its activity was still inhibited 30–35% by PKA (not shown), indicating that the second site was in the catalytic domain. We therefore individually mutated the two remaining candidate PKA phosphorylation sites, Thr\(^{108}\) and Ser\(^{179}\), in CaM-KK\(_{434}\). Although the Ser\(^{179}\) 

→ Ala mutant was still inhibited by PKA, the Thr\(^{108}\) → Gly mutant was not inhibited (not shown). These two mutations were then made singularly or as the double mutant (T108G/S458A) in the full-length CaM-KK, and the effects of PKA on their inhibition (Fig. 2C) and phosphorylation (Fig. 2D) were consistent with the conclusion that PKA-mediated inhibition of CaM-KK due to the combined phosphorylations of Thr\(^{108}\) in the catalytic domain and Ser\(^{458}\) in the CaM-binding domain. PKA can apparently phosphorylate additional sites, since the double mutant still exhibited some \(^{32}\)P incorporation (Fig. 2D), but this residual phosphorylation of the double mutant had no apparent effect on CaM-KK activity (Fig. 2C).

We next wanted to ascertain whether activation of PKA in cultured cells would result in inhibition of CaM-KK. COS-7 cells were transfected with wild-type CaM-KK or the double mutant, and the effect of PKA activation was determined upon forskolin treatment for 20 min. Cells were then lysed in the presence of phosphatase inhibitors, and CaM-KK activity in the lysate was assayed for its ability to increase the Ca\(^{2+}\)-independent activity of recombinant CaM-KIV (Fig. 3A, left panel). Extracts from transfected cells exhibited robust CaM-KK activity, which was decreased by forskolin treatment by about 45% for wild-type CaM-KK but not for the double mutant. When cells were \(^{32}\)P-labeled, forskolin treatment strongly enhanced phosphorylation of wild-type CaM-KK, but forskolin-induced labeling of the double mutant CaM-KK was strongly suppressed compared with wild-type (Fig. 3A, right panel). Similar activation of CaM-KK was obtained on the endogenous CaM-KK in PC12 cells where the inhibitory effect of forskolin (41%) was mimicked by the CaM-KK analog eBmps (35%) but not by the inactive forskolin analog 1,9-dideoxyforskolin (Fig. 3B). Strong suppression of endogenous CaM-KK activity was also observed for forskolin treatment of cultured hippocampal neurons (65.9 ± 10.3%, Fig. 3C) and Jurkat cells (52.0 ± 5.4%, Fig. 3D), where isoproterenol produced a rapid (t\(_{1/2}\) = 30 s), partial suppression (36.0 ± 2.4%) of CaM-KK activity (Fig. 3E). These results clearly demonstrate in a variety of cells that either transfected CaM-KK or endogenous
CaM-KK activities could be partially inhibited by agonists known to activate PKA. The selective PKA inhibitor H89 blocked the inhibition of CaM-KK by forskolin treatment in PC12 and Jurkat cells (not shown).

To confirm that the PKA suppression of CaM-KK activity produced a decrease in activation of its downstream target, CaM-KIV, we determined the effect of forskolin treatment on the Ca\(^{2+}\)-dependent activation of either transfected or endogenous CaM-KIV. We have shown previously that COS-7 cells transfected with CaM-KK and CaM-KIV exhibit a rapid Ca\(^{2+}\)-dependent activation of the CaM-KIV upon treatment with ionomycin (14). This activation is due to phosphorylation of CaM-KIV by CaM-KK, because transfections with either the Thr\(^{196}\) → Ala mutant of CaM-KIV plus CaM-KK or by CaM-KIV alone do not exhibit Ca\(^{2+}\)-dependent activation. We used ionomycin in the present study because agonist-dependent mobilization of Ca\(^{2+}\) can be modulated by activated PKA, and this would complicate interpretation of results. Fig. 4A shows that the Ca\(^{2+}\)-dependent activation of expressed CaM-KIV in COS-7 cells was blocked about 40% by pretreatment with forskolin (left bars), whereas forskolin had no effect when the CaM-KK double mutant was the catalyst (right bars). With PC12 cells we used the endogenous CaM-KK, but transfected with His-tagged CaM-KIV, because PC12 cells do not contain detectable CaM-KIV. Forskolin pretreatment of the transfected PC12 cells gave a 62.4 ± 0.7% inhibition of the ionomycin-stimulated activation of CaM-KIV (Fig. 4B). Last, we used the same treatment protocol on Jurkat cells as they contain both endogenous CaM-KK and CaM-KIV, which can be rapidly activated by stimulation of the CD3 receptor or by ionomycin (8). Ionomycin treatment alone gave a 5-fold activation of CaM-KIV, and this activation was largely blocked by forskolin pretreatment (Fig. 4C).

Based on the results reported herein, we conclude that activation of PKA partially suppresses the activity of CaM-KK, and this inhibition of CaM-KK is transmitted to its downstream target CaM-KIV. These results are consistent with a pattern of extensive cross-talk between the cAMP/PKA and calcium intracellular signaling pathways. For example, both the synthesis and degradation of cAMP are highly regulated by Ca\(^{2+}\) as several adenylate cyclases can be either activated or inhibited by Ca\(^{2+}\) or Ca\(^{2+}\)/CaM (22), and type I phosphodiesterases are stimulated by Ca\(^{2+}\)/CaM (23). Furthermore, CaM-KII can inhibit type III adenylate cyclases (24) and type Ib phosphodiesterase (25), whereas CaM-KIV can inhibit type I adenylate cyclases (26). Conversely, PKA can phosphorylate several CaM-dependent enzymes, such as type 1A phosphodiesterase (27) and myosin light chain kinase (28), thereby inhibiting their CaM-binding and enzyme activations, and this same mechanism appears to account in part for the inhibition of CaM-KK by PKA reported here. Thus, it is clear that these major signaling systems in cells exhibit extensive positive and inhibitory interactions at numerous sites along their pathways. Because PKA and several of the CaM kinases can phosphorylate numerous substrates, this cross-talk allows fine-tune controls for these complex physiological responses.

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Note Added in Proof—Recent experiments indicate that phosphorylation of Ser\(^{458}\) by PKA is blocked when Ca\(^{2+}\)/CaM is bound to CaM-KK (H. Tokumitsu, G. A. Wayman, and T. R. Soderling, manuscript in preparation).

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