Differential activation of CREB by Ca^{2+}/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity

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The cAMP response element-binding protein (CREB) has been shown to mediate transcriptional activation of genes in response to both cAMP and calcium influx signal transduction pathways. The roles of two multifunctional calcium/calmodulin-dependent protein kinases, CaMKIV and CaMKII, were examined in transient transfection studies that utilized either the full-length or the constitutively active forms of these kinases. The results indicate that CaMKIV is much more potent than CaMKII in activating CREB in three different cell lines. It was also found in these studies that Ser^{133} of CREB is essential for its activation by CaMKIV. Because both CaMKII and CaMKIV can phosphorylate CREB, we pursued further the mechanism by which CaMKII and CaMKIV differentially regulate CREB activity. Mutagenesis studies and phosphopeptide mapping analysis demonstrated that in vitro, CaMKIV phosphorylates CREB at Ser^{133} only, whereas CaMKII phosphorylates CREB at Ser^{133} and a second site, Ser^{142}. Transient transfection studies revealed that phosphorylation of Ser^{142} by CaMKII blocks the activation of CREB that would otherwise occur when Ser^{133} is phosphorylated. When Ser^{142} was mutated to alanine, CREB was activated by CaMKII, as well as by CaMKIV. Furthermore, mutation of Ser^{142} to alanine enhanced the ability of Ca^{2+} influx to activate CREB, suggesting a physiological role for the phosphorylation of Ser^{142} in modulation of CREB activity. These data provide evidence for a new mechanism for regulation of CREB activity involving phosphorylation of a negative regulatory site in the transcriptional activation domain. The studies also provide new insights into possible interactions between the cAMP and Ca^{2+} signaling pathways in the regulation of transcription. In particular, changes in intracellular Ca^{2+} have the potential to either inhibit or augment the ability of cAMP to stimulate transcription, depending on the presence of specific forms of Ca^{2+}/calmodulin-dependent protein kinases.

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Several recent studies have provided evidence for a convergence of the cAMP and Ca^{2+} signaling pathways in the regulation of transcription (Sheng et al. 1990, 1991; Dash et al. 1991). These studies suggest that both cAMP and Ca^{2+}-dependent pathways can lead to phosphorylation and activation of the cAMP response element-binding protein (CREB). CREB was originally characterized as a transcription factor mediating responses to cAMP (Montminy and Bilezikjian 1987; Hoeffler et al. 1988; Yamamoto et al. 1988; Gonzalez et al. 1989). CREB binds to a conserved cAMP response element that is present in the 5′-flanking region of many cAMP-responsive genes (Montminy et al. 1986; Silver et al. 1987; Roesler et al. 1988). The possibility that CREB might also mediate transcriptional responses to changes in intracellular Ca^{2+} concentrations was initially suggested by studies of the c-*fos* promoter (Sheng et al. 1990). These studies demonstrated that the Ca^{2+}-responsive DNA elements of the c-*fos* promoter mapped to a previously identified cAMP-responsive DNA element. In addition, Ca^{2+} influx was found to lead to phosphorylation of Ser^{133} of CREB. Ser^{133} of CREB is phosphorylated by the cAMP-dependent protein kinase (PKA), and phosphorylation of this residue is essential for PKA-mediated activation of CREB (Gonzalez and Montminy 1989; Lee et al. 1990;

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Gonzalez et al. 1991). Thus, these studies suggested that Ca^{2+} influx could lead to phosphorylation and activation of CREB. In subsequent studies the ability of Ca^{2+} influx to alter the activity of CREB has been confirmed (Sheng et al. 1991). In addition, in vitro studies have shown that the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) can phosphorylate Ser^{133} of CREB (Dash et al. 1991; Sheng et al. 1991). Overall, these studies suggest that CREB may permit transcriptional regulation in response to both the Ca^{2+} and cAMP pathways and that the effects of Ca^{2+} are likely mediated by activation of CaMKII. However, recent transfection studies suggest that CaMKII may not be a very efficient activator of CREB (Enslen et al. 1994; Matthews et al. 1994). In contrast, a different Ca^{2+}/calmodulin-dependent protein kinase, CaMKIV, may be the enzyme that mediates Ca^{2+}-stimulated activation of CREB.

In the present study we have compared the ability of CaMKII and CaMKIV to activate CREB. We confirm that CaMKII is not very effective in activating CREB in vivo. However, an expression vector for a constitutively active form of CaMKIV was quite effective in activating CREB in several different cell lines. We further explored the possible mechanisms for this differential regulation, by use of a combination of phosphopeptide mapping analysis and mutagenesis studies. The results suggest that both CaMKII and CaMKIV can phosphorylate Ser^{133} of CREB. However, CaMKII also phosphorylates a second site within the transcriptional activation domain of CREB. Phosphorylation at this site blocks the activation that would otherwise occur following phosphorylation of Ser^{133}. Phosphorylation of CREB by CaMKII can block the ability of PKA to activate CREB. This finding demonstrates that phosphorylation of this negative regulatory site can lead to antagonism between the Ca^{2+} and cAMP signaling pathways for activation of CREB. Thus, the findings provide new insight into the possible interactions between the Ca^{2+} and cAMP signaling pathways.

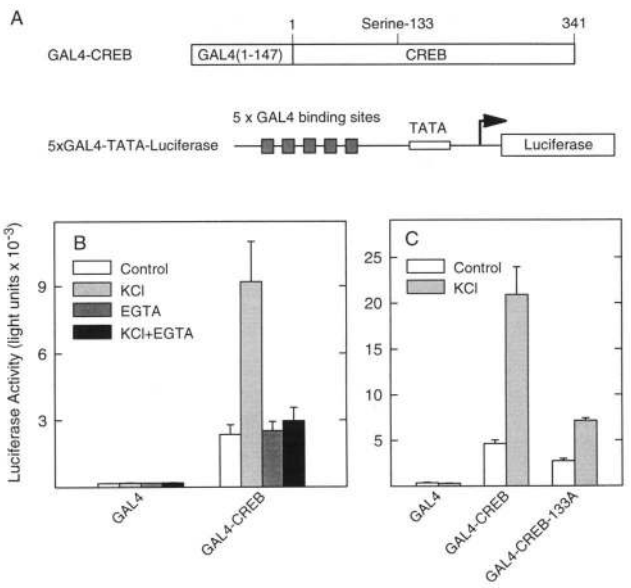

Figure 1. Activation of CREB by membrane depolarization. (*A*) Schematic diagram of a GAL4–CREB fusion protein and the GAL4-responsive luciferase reporter gene. The GAL4–CREB fusion protein contains the DNA-binding domain of GAL4 fused to the complete coding sequence of rat CREB. The reporter gene contains multiple GAL4 DNA-binding sites (indicated by shading) upstream of a TATA box from the E1B promoter and the luciferase-coding sequence. The arrow represents the transcription initiation site of the luciferase reporter gene. (*B*) KCl-induced membrane depolarization activates CREB in a Ca^{2+}-dependent manner. GH_3 cells were transfected by electroporation with 4 μg of an expression vector for GAL4(1–147) or a GAL4–CREB fusion protein and 10 μg of the 5×GAL4–TATA–luciferase reporter gene and 14 μg of BSSK(−). After transfection the cells were divided into four 60-mm dishes. Cells were treated by addition of 80 mM KCl or 3.75 mM EGTA to the medium ~18 hr after transfection. EGTA was added to the medium 0.5 hr before the KCl treatment. Cells were assayed for luciferase activity 6 hr after treatment with KCl. Values are the mean±S.E.M. for three separate transfections. (*C*) Ser^{133} of CREB is essential for activation by membrane depolarization. GH_3 cells were transfected with 2 μg of an expression vector for either GAL4(1–147) or the GAL4–CREB fusion protein and 5 μg of 5×GAL4–TATA–luciferase reporter gene and 7 μg of BSSK(−). After transfection, the cells were divided into two 60-mm dishes and half of the cultures were treated by the addition of 80 mM KCl to the medium 18 hr after transfection. Luciferase activity was determined 6 hr after KCl treatment. Values are the mean±S.E.M. for three separate transfections.

Results

An expression vector encoding a constitutively active form of CaMKIV is much more effective than a constitutively active form of CaMKII in activating CREB

We have used transient transfection assays to study the role of specific Ca^{2+}/calmodulin protein kinases in activating CREB. To specifically study activation of CREB in the presence of other endogenous proteins that bind to CREs, a GAL4–CREB fusion protein was used (Berkowitz and Gilman 1990; Lee et al. 1990; Sheng et al. 1991; Sun et al. 1992). The fusion protein contains the complete coding sequence of CREB fused in-frame to the DNA-binding and dimerization domain of the yeast transcription factor, GAL4 (Fig. 1A). To assess activation of the GAL4–CREB protein, cells were cotransfected with a luciferase indicator gene (5×GAL4-TATA-luciferase) that contains five copies of a GAL4-binding site upstream of a TATA box from the E1B promoter. GH_3 pituitary tumor cells, which express voltage-sensitive Ca^{2+} channels (Matteson and Armstrong 1986; Gammon et al. 1989; Enyeart et al. 1990; Li et al. 1992), were used as recipients in transfection assays to examine activation of the GAL4–CREB fusion protein. Stimulation of Ca^{2+} influx by KCl-induced depolarization led to increased expression of the GAL4-dependent indicator gene when cotransfected with the GAL4–CREB expression vector but not when cotransfected with an expres-

sion vector for the DNA-binding domain of GAL4 (Fig. 1B). Addition of EGTA to the medium greatly reduced the ability of depolarization to increase reporter gene expression in the presence of the GAL4–CREB expression vector. Thus, KCl-induced activation of the GAL4–CREB protein appears to be Ca^{2+}-dependent. We also tested the effect of mutating the CREB-coding sequence so that Ser^{133} is changed to alanine (Fig. 1C). As was seen previously in PC12 cells (Sheng et al. 1991), mutation of Ser^{133} substantially reduced the ability of GAL4–CREB to mediate transcriptional activation in response to KCl-induced depolarization, consistent with the view that phosphorylation of this residue is important for mediating transcriptional activation of CREB (Gonzalez and Montminy 1989; Lee et al. 1990).

We used GH_3 cells to test the ability of two different multifunctional Ca^{2+}/calmodulin-dependent protein kinases to activate CREB. Although there are a number of Ca^{2+}/calmodulin-dependent protein kinases, most appear to have rather restricted substrate specificity. Until recently, it was thought that only CaMKII was a multifunctional enzyme that played a general and widespread role in mediating responses to Ca^{2+} signaling (Colbran et al. 1989). CaMKII is a multisubunit protein in which each of the subunits contains both catalytic and regulatory domains. Multiple isoforms of CaMKII are present in many different tissues (Tobimatsu and Fujisawa 1989). In the present studies we have tested the role of only the α isoform of CaMKII. Recently a second multifunctional Ca^{2+}/calmodulin-dependent protein kinase, CaMKIV, has been identified (Jones et al. 1991; Means et al. 1991; Miyano et al. 1992; Ohmstede et al. 1991). CaMKIV, which is also known as CaMK Gr, shares ~40% sequence identity with CaMKII isoforms. As a substantial amount of CaMKIV has been reported to be found in the nucleus (Jensen et al. 1991), it is a good candidate as a protein kinase involved in the regulation of transcription. To study the role of CaMKII and CaMKIV in the activation of CREB, we have prepared expression vectors encoding constitutively active forms of the enzymes. The functional domains of both CaMKII and CaMKIV are arranged so that an amino-terminal protein kinase domain is followed by an autoinhibitory-regulatory domain that contains a calmodulin-binding site (Fig. 2A). CaMKII also contains a protein association domain that is involved in forming the multisubunit complex. Removal of the carboxy-terminal region, including the autoinhibitory-regulatory domain of either CaMKII or CaMKIV, has been shown to result in a constitutively active form of enzyme that no longer requires Ca^{2+}/calmodulin for activation (Kapiloff et al. 1991; Cruzalegui and Means 1993). The expression vectors for the truncated forms of CaMKII and CaMKIV were then tested, by use of a transient transfection assay, for the ability of the enzymes to alter the activity of the GAL4–CREB fusion protein (Fig. 2B). As reported recently (Enslen et al. 1994; Matthews et al. 1994), the $CaMKII_{1–290}$ expression vector had little effect on the ability of GAL4–CREB to increase expression of the reporter gene. In contrast, the expression vector for the constitutively active form of CaMKIV substantially increased the ability of GAL4–CREB to stimulate expression of the reporter gene. In this experiment CaMKIV stimulated expression of the reporter gene to about half of the activity

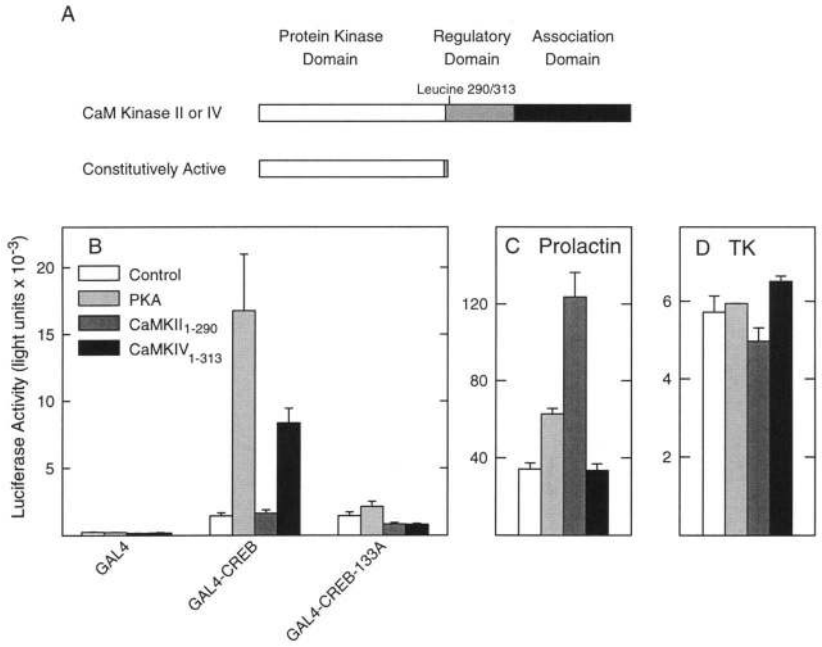

Figure 2. Effects of expression vectors encoding constitutively active forms of CaMKII and CaMKIV on activation of a CREB-dependent promoter, the prolactin promoter, and the thymidine kinase promoter. (*A*) Schematic maps indicating the functional domains of CaMKII and CaMKIV. The truncations used to create the constitutively active forms of CaMKII and CaMKIV are also indicated. (*B*) $CaMKIV_{1–313}$ and PKA, but not $CaMKII_{1–290}$, activate CREB, and Ser^{133} of CREB is essential for activation by both PKA and $CaMKIV_{1–313}$. GH_3 cells were transfected with 2 μg of an expression vector for either GAL4(1–147) or the GAL4–CREB fusion protein, 5 μg of 5×GAL4–TATA–luciferase reporter gene and either 5 μg of BSSK(−) as a control or 5 μg of an expression plasmid for PKA, $CaMKII_{1–290}$ or $CaMKIV_{1–313}$. Luciferase activity was determined 24 hr after transfection. Values are means±S.E.M for three separate transfections. (*C*,*D*) The constitutively active forms of CaMKII and CaMKIV differentially regulate the expression of the prolactin promoter and thymidine kinase promoter. GH_3 cells were transfected with 5 μg of either a reporter gene containing 2.5 kb of the 5′-flanking region and promoter of the rat prolactin gene or the thymidine kinase promoter (TK) linked to luciferase and either 5 μg of BSSK(−) as control or 5 μg of the expression plasmids for PKA, $CaMKII_{1–290}$, or $CaMKIV_{1–313}$. Luciferase activity was determined 24 hr after transfection. Values are means±S.E.M. for three separate transfections.

obtained with PKA. In some experiments the ability of CaMKIV to activate GAL4–CREB approached the activity obtained with PKA. CaMKII and CaMKIV have been found to phosphorylate a number of the same substrates (Miyano et al. 1992), and analysis of the ability of CaMKIV to phosphorylate a series of peptide substrates suggests a consensus phosphorylation site containing the sequence Arg-X-X-Ser/Thr-X (Cruzalegui and Means 1993). As this is the same consensus site that is recognized by CaMKII (Kennelly and Krebs 1991), it is clear that CaMKII and CaMKIV share at least some structural requirements for phosphorylation. The region surrounding Ser^{133} of CREB contains the sequence, ^{130}Arg-Arg-Pro-Ser-Tyr^{134}, which would fit the consensus sequence for phosphorylation by either CaMKII, CaMKIV, or PKA. Mutation of Ser^{133} of CREB to alanine substantially reduced the ability of GAL4–CREB to mediate transcriptional activation in response to either PKA or CaMKIV (Fig. 2B). Thus, these findings suggest that both PKA and CaMKIV can activate CREB by phosphorylation of Ser^{133}. Furthermore, although CaMKII is also able to phosphorylate Ser^{133} (Dash et al. 1991; Sheng et al. 1991), CaMKII is not efficient in activating CREB.

The $CaMKII_{1-290}$ or $CaMKIV_{1-313}$ expression vectors were also tested for their effects on the prolactin and thymidine kinase promoters (Fig. 2C,D). The prolactin promoter showed the opposite pattern for responsiveness to CaMKII and CaMKIV as was obtained for activation of CREB. The prolactin promoter is responsive to CaMKII but has little or no response to CaMKIV. We do not understand the reason that the prolactin promoter has different responses from those observed for activation of CREB. There is evidence that Ca^{2+} effects on prolactin transcription may be mediated by a mechanism that does not involve CREB (Yan and Bancroft 1991; Liang et al. 1992). In any case, these findings demonstrate that the CaMKII expression vector produces a functional enzyme in GH_3 cells. Thus, the differences that were observed between CaMKII and CaMKIV in the activation of GAL4–CREB likely reflect different biochemical properties of the two enzymes. We found that none of the kinase expression vectors had major effects on the thymidine kinase promoter (Fig. 2D), demonstrating further that the effects of the kinases are promoter specific.

To compare further the activation of GAL4–CREB by CaMKII and CaMKIV, titration experiments were performed with two different cell lines. In these experiments increasing amounts of the $CaMKII_{1-290}$ or $CaMKIV_{1-313}$ expression plasmids were cotransfected with the GAL4–CREB expression vector and the indicator gene (Fig. 3). At all tested concentrations of the kinase expression vectors, $CaMKIV_{1-313}$ was much more effective than $CaMKII_{1-290}$ in stimulating the ability of GAL4–CREB to activate the reporter gene in both PC12 and JEG-3 cells. Similar results were obtained in kinase titration studies in GH_3 cells (data not shown). These studies demonstrate that CaMKIV is more effective than CaMKII in activating CREB in several different cell types, and the findings also suggest that the failure of CaMKII to activate is probably not attributable to production of supraphysiological concentrations of protein kinase in the transient transfection assays. Although it remains remotely possible that the failure of CaMKII to activate CREB is attributable to overproduction of kinase, the use of varying concentrations of kinase expression vectors provided no evidence for conditions in which CaMKII was as efficient as CaMKIV in activating CREB.

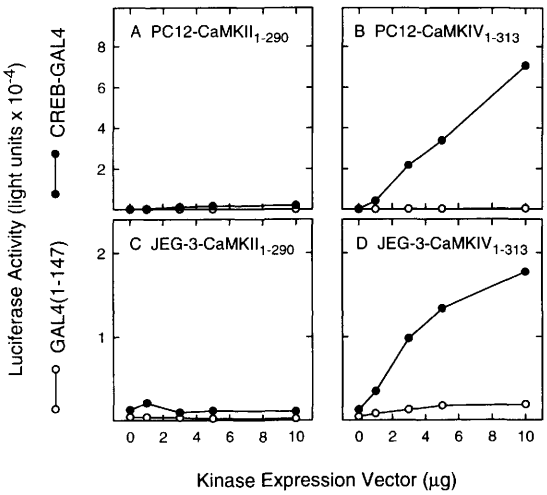

Figure 3. Comparison of the ability of $CaMKII_{1-290}$ and $CaMKIV_{1-313}$ to activate CREB in PC12 and JEG-3 cells. PC12 (*A,B*) or JEG-3 (*C,D*) cells were transfected with 2 μg of an expression vector for GAL4(1–147) (○) or GAL4–CREB fusion protein (●) and 5 μg of the 5×GAL4–TATA–luciferase reporter gene and increasing amounts (0, 1, 3, 5, or 10 μg) of the expression plasmid for $CaMKII_{1-290}$ (*A,C*) or $CaMKIV_{1-313}$ (*B,D*). The total amount of DNA for each transfection was equalized using BSSK(−). Luciferase activity was determined 24 hr after transfection. Values are the mean for two separate transfections.

The constitutively active forms of CaMKII and CaMKIV are expressed at similar levels in transfected cells

The failure of the constitutively active form of CaMKII to activate the GAL4-CREB fusion protein might be attributable to relatively poor expression of the protein or production of an inactive enzyme. The preceding experiments showed that $CaMKII_{1-290}$ can activate the prolactin promoter, demonstrating the production of a functional enzyme. To examine the possibility that CaMKII and CaMKIV may be expressed at different levels after transfection of expression vectors, we compared the protein levels of $CaMKII_{1-290}$ and $CaMKIV_{1-313}$ in transfected cells. To facilitate the comparison, we prepared expression vectors encoding epitope-tagged versions of the constitutively active forms of CaMKII and CaMKIV. A 9-amino-acid sequence from the influenza virus hemagglutinin that contains the minimal recognition sequence for the 12CA5 monoclonal antibody (Wilson et al. 1984) was fused in-frame to the carboxy-terminal coding sequence of $CaMKII_{1-290}$ and $CaMKIV_{1-313}$. The

epitope-tagged kinases were then cloned into a mammalian expression vector and tested in transient transfection assays. The expression vectors for the epitope-tagged kinases demonstrated abilities to regulate transcription similar to those obtained with the expression vectors for the nontagged kinases (data not shown). Immunoblotting analysis with mAb 12CA5 revealed that both $CaMKII_{1-290}$ and $CaMKIV_{1-313}$ were expressed in COS-1 cells (Fig. 4), and $CaMKII_{1-290}$ was expressed at somewhat higher levels than $CaMKIV_{1-313}$. Similar observations were made in GH_3 and PC12 cells (data not shown). Thus, the failure of the truncated form of CaMKII to activate the GAL4–CREB fusion protein is apparently not the result of relatively low levels of expression.

Full-length CaMKIV, but not CaMKII, potentiates the activation of CREB by Ca^{2+} influx in vivo

Although the constitutively active form of CaMKIV was found to be much more potent than the constitutively active form of CaMKII in activating GAL4–CREB, a question remains concerning the ability of the native, full-length kinases to participate in Ca^{2+}-regulated signal transduction. It is possible that the truncated, constitutively active forms of the kinases have properties that are not shared by the full-length enzyme. For instance, the process of truncation to produce the constitutively active enzyme leads to a smaller enzyme that might have enhanced access to the nucleus. This is particularly true for CaMKII in which the native enzyme forms a rather large, multisubunit complex. To explore this question, we prepared expression vectors encoding the full-length enzymes. The expression vectors for full-length CaMKII and CaMKIV were transfected into GH_3 cells by transient transfection (Fig. 5). It was found that the expression vector for full-length CaMKIV could augment the ability of membrane depolarization to activate GAL4–CREB (Fig. 5B). On the other hand, the expression vector for full-length CaMKII had little or no effect on activation of GAL4–CREB. Both CaMKII and CaMKIV had little, if any, effects on transcriptional activation when tested with the GAL4 DNA-binding domain alone (Fig. 5A). Thus, full-length CaMKIV can augment the ability of depolarization to activate CREB, suggesting a possible role for this enzyme in Ca^{2+}-dependent transcriptional activation.

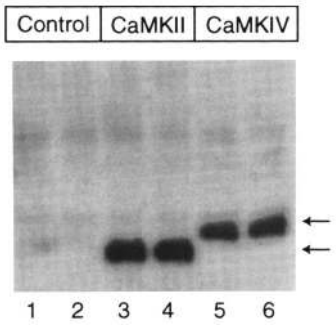

Figure 4. Western blot analysis of the expression of $CaMKII_{1-290}$ and $CaMKIV_{1-313}$ in transfected cells. COS-1 cells were transfected with 9 μg of BSSK(−) (lanes *1,2*) or expression vectors encoding epitope-tagged $CaMKII_{1-290}$ (lanes *3,4*) or $CaMKIV_{1-313}$ (lanes *5,6*) per 60-mm dish. Two separate transfections were performed for each construct. Cells were collected 24 hr after transfection and lysed by sonication, and 70 μg of protein from each sample was separated on an SDS–polyacrylamide gel. The proteins were transferred to a PVDF membrane and the epitope-tagged kinase visualized by immunostaining. The arrows indicate the migration of bands corresponding to $CaMKII_{1-290}$ and $CaMKIV_{1-313}$.

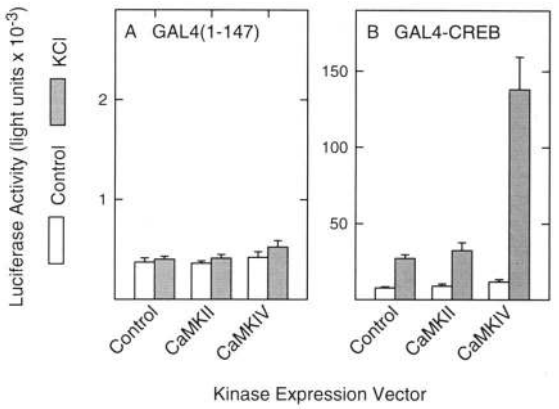

Figure 5. Expression vectors encoding full-length CaMKIV, but not CaMKII, augment activation of CREB in response to membrane depolarization. GH_3 cells were transfected with 2 μg of an expression vector for either GAL4(1–147) (*A*) or the GAL4–CREB fusion protein (*B*) and 5 μg of the 5×GAL4–TATA–luciferase reporter gene, 3 μg of BSSK(−), and 5 μg of an expression plasmid for globin or full-length CaMKII or CaMKIV. After transfection the cells were divided into two 60-mm dishes, one of which (shaded bars) was treated by addition of 80 mM KCl to the medium 18 hr after transfection. Luciferase activity was determined 6 hr after KCl treatment. Values are means±S.E.M. for three separate transfections.

CaMKII and CaMKIV differentially phosphorylate Ser^{142} of CREB

One possible explanation that might account for differences in the ability of CaMKII and CaMKIV to activate CREB would involve differential phosphorylation of CREB. Phosphorylation of Ser^{133} is known to be crucial for activation of CREB (Gonzalez and Montminy 1989; Lee et al. 1990). Although CaMKII is known to be able to phosphorylate Ser^{133}, it is possible that CaMKII and CaMKIV might quantitatively differ in their ability to phosphorylate this site. On the other hand, the two enzymes might have different abilities to phosphorylate CREB at other sites. Previous studies have demonstrated that CaMKII can phosphorylate CREB on Ser^{133} (Sheng et al. 1991), and there is also evidence that CaMKII may be able to phosphorylate a second site in CREB (Dash et al. 1991). More recently it has been shown that CaMKII

stoichiometrically phosphorylates CREB at two sites (Enslen et al. 1994).

We have used phosphopeptide mapping to determine the sites in CREB that are phosphorylated (Fig. 6). As expected, phosphorylation by PKA generated a single, major phosphopeptide, and a very similar pattern of phosphorylation was obtained with CaMKIV. The finding that PKA results in a single, major site of phosphorylation is consistent with several previous studies (Yamamoto et al. 1988; Gonzalez and Montminy 1989; Gonzalez et al. 1989, 1991; Lee et al. 1990). In contrast to PKA and CaMKIV, CaMKII was found to phosphorylate CREB at two major sites. The single site phosphorylated by both PKA and CaMKIV and one of the two sites phosphorylated by CaMKII has been confirmed as Ser^{133} by analysis of a mutant CREB in which Ser^{133} was changed to alanine (data not shown).

As a starting point to mapping the second site that is phosphorylated by CaMKII, the amino acid sequence of CREB was examined for consensus phosphorylation sites. An excellent candidate was found in the sequence ^{95}Arg-Leu-Phe-Ser-Gly^{99}, which fits the CaMKII consensus phosphorylation sequence of Arg-X-X-Ser/Thr-X (Kennelly and Krebs 1991). However, the second site that is phosphorylated by CaMKII is not Ser^{98}, suggesting that CaMKII phosphorylates a site within CREB that does not fit the CaMKII consensus recognition sequence. The ability of CaMKII to phosphorylate nonconsensus sites has been reported for other proteins (Kennelly and Krebs 1991). In an effort to map the second site that is phosphorylated by CaMKII, a series of mutant CREB proteins containing amino-terminal deletions were examined. The results of these studies suggested that the second site is located between amino acids 126 and 160 (data not shown). We then mutated each of the serine and threonine residues within this region to alanines. The mutated residues include Ser^{129}, Ser^{142}, Ser^{143}, Ser^{156}, Thr^{160}, and Ser^{161}. The mutant CREBs were first tested in transient transfection assays (see below). On the basis of the transfection studies, we examined the phosphorylation of one of the mutant CREB proteins in vitro. We found that mutation of Ser^{142} to alanine eliminated the phosphorylation of the second site by CaMKII but had no effect on the phosphorylation of CREB by PKA or CaMKIV. These results identify the second site that is phosphorylated by CaMKII as Ser^{142}.

Although the preceding phosphopeptide mapping studies demonstrate that CaMKII can phosphorylate CREB at two sites in vitro, further studies were required to determine whether this can occur in intact cells. For these studies we transfected GH_3 cells with expression vectors for constitutively active forms of CaMKII and CaMKIV, as well as an expression vector for GAL4–CREB under the same conditions used to test for transcriptional activation of CREB. The cells were then incubated with [^{32}P]orthophosphate, and GAL4–CREB was isolated and subjected to phosphopeptide mapping (Fig. 6B). The peptide maps are more complicated than obtained after in vitro labeling with the appearance of several additional radiolabeled peptides. However, both CaMKIV and CaMKII appear to phosphorylate a major peptide, which corresponds to Ser^{133}. In addition, CaMKII phosphorylates an additional peptide not found in the CaMKIV phosphopeptide map, and this peptide

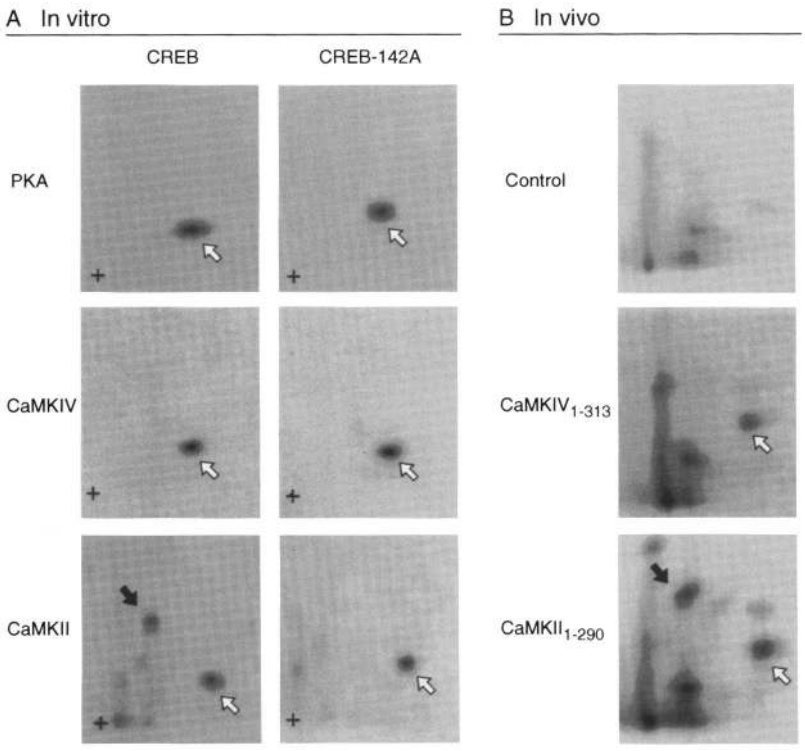

Figure 6. Two-dimensional phosphopeptide mapping of the phosphorylation of CREB by PKA, CaMKIV, and CaMKII. (*A*) In vitro phosphorylation of CREB. Recombinant poly(His)–CREB proteins (wild type or mutants as indicated) were phosphorylated in vitro by PKA, CaMKIV, or CaMKII as indicated in the presence of [γ-^{32}P]ATP. The phosphorylation products were purified by SDS-PAGE, digested with trypsin, and oxidized. Peptides were separated by high voltage electrophoresis in the first dimension and by thin-layer chromatography in the second dimension. The phosphopeptides were visualized by autoradiography. The plus indicates the origin of the two-dimensional separation, the open arrows indicate the phosphopeptide that contains Ser^{133}, and the closed arrows indicate the phosphopeptide that contains Ser^{142}. (*B*) In vivo phosphorylation of CREB by CaMKII and CaMKIV. GH_3 cells were transfected with expression vectors for GAL4–CREB and either $CaMKII_{1-290}$ or $CaMKIV_{1-313}$. The cells were then labeled for 4 hr with [^{32}P]orthophosphate, incubated for 15 min with okadaic acid, and nuclear extracts were prepared and radiolabeled GAL4–CREB protein was isolated by immunoprecipitation and SDS–gel electrophoresis. Phosphopeptides were prepared and analyzed as described above.

appears to correspond to Ser^{142}. Other in vivo phosphorylation experiments with GAL4–CREB in which Ser^{142} was mutated to alanine confirmed that this additional phosphopeptide represents phosphorylation of Ser^{142} (data not shown). Thus, the phosphopeptide mapping experiments demonstrate that CaMKII, but not CaMKIV, can phosphorylate Ser^{142} both in vitro and in vivo.

Phosphorylation of CREB by CaMKII blocks the ability of PKA to activate CREB

The finding that CaMKII can phosphorylate CREB at both Ser^{133} and Ser^{142} suggested a possible mechanism that might account for the failure of CaMKII to activate GAL4–CREB. Phosphorylation at Ser^{142} might block the activation of CREB. Phosphorylation at this site might block essential conformational changes in CREB that occur after phosphorylation of Ser^{133} (Gonzalez et al. 1991). Alternatively, phosphorylation at the second site might interfere with the interaction of CREB with an adapter protein important for transcriptional activation (Chrivia et al. 1993). It is also possible, although perhaps unlikely, that phosphorylation at a second site might alter DNA binding by CREB and/or GAL4–CREB. If phosphorylation of Ser^{142} by CaMKII is sufficient to block the activation of CREB that occurs upon phosphorylation of Ser^{133}, then CaMKII should be able to block the activation of CREB that occurs following phosphorylation by PKA. We tested this prediction by transfecting PC12 cells with expression vectors for PKA plus increasing amounts of an expression vector for $CaMKII_{1-290}$ (Fig. 7). By itself, the PKA vector increased the ability of the GAL4–CREB fusion protein to activate transcription. Addition of increasing amounts of the $CaMKII_{1-290}$ expression vector reduced the transcriptional activation that was observed with PKA alone. In contrast, cotransfection of the $CaMKIV_{1-313}$ vector plus the PKA vector did not inhibit activation of CREB, and similar results were found in GH_3 cells. As would be expected, the $CaMKII_{1-290}$ expression vector was also able to reduce the ability of the $CaMKIV_{1-313}$ vector to activate CREB (data not shown). The ability of $CaMKII_{1-290}$ to block PKA-induced activation of GAL4–CREB supports the view that CaMKII can phosphorylate a second site within CREB and that phosphorylation at this second site blocks the activation of CREB that would otherwise occur after phosphorylation of Ser^{133} by PKA, CaMKII, or CaMKIV. When Ser^{142} of CREB was mutated to alanine, CaMKII was no longer able to block the effects of PKA to activate CREB (data not shown), again consistent with a negative role for phosphorylation of Ser^{142}.

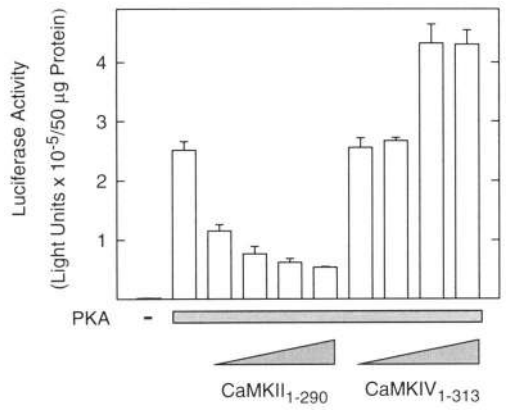

Figure 7. An expression vector for $CaMKII_{1-290}$ blocks activation of CREB by PKA in vivo. PC12 cells were transfected with 2 µg of an expression vector for GAL4–CREB, 5 µg of 5×GAL4–TATA–luciferase reporter gene, 5 µg of BSSK(−) or the expression plasmid for PKA, and increasing amounts (0, 1, 3, 5, or 10 µg) of the expression plasmid for $CaMKII_{1-290}$ or $CaMKIV_{1-131}$. The total amount of DNA for each transfection was equalized with BSSK(−). Luciferase activity was determined 24 hr after transfection. Values are means±S.E.M. for three separate transfections.

Mutation of Ser^{142} of CREB permits CaMKII to activate CREB and enhances activation of CREB in response to Ca^{2+} influx

The preceding experiments are consistent with a model in which CaMKII phosphorylates both Ser^{133} and Ser^{142} and that CaMKII fails to activate CREB because of the phosphorylation of Ser^{142}. If this is correct, then mutation of Ser^{142} to a residue that cannot be phosphorylated should permit CaMKII to activate CREB.

This prediction was tested through the use of expression vectors for wild-type and mutant GAL4–CREB fusion proteins (Fig. 8). As observed in the previous experiments, transfection of expression vectors encoding the constitutively active form of CaMKII and wild-type GAL4–CREB did not stimulate expression of the reporter gene. However, when Ser^{142} of CREB was mutated to alanine, $CaMKII_{1-290}$ was able to stimulate expression of the reporter gene (Fig. 8A). With the Ser^{142} mutant, both $CaMKII_{1-290}$ and $CaMKIV_{1-313}$ had similar abilities to stimulate transcription. The Ser^{142} mutation did not change the response of CREB to PKA activation (data not shown). Replacement of Ser^{129}, Ser^{143}, Ser^{156}, or Thr^{160} plus Ser^{161} with alanine did not permit $CaMKII_{1-290}$ to activate CREB. In some cells the effects of CaMKII on the activation of CREB may be more complicated, as mutation of Ser^{142} to alanine did not permit CaMKII to activate GAL4–CREB in JEG-3 cells (data not shown). Although we do not understand the reason that CaMKII cannot activate the mutant CREB in JEG-3 cells, it should be emphasized that the Ser^{142} to alanine mutation has consistently permitted activation of CREB by CaMKII in GH_3 cells. Mutation of Ser^{142} to alanine also enhanced the response to KCl-induced Ca^{2+} influx (Fig. 8B). The mutation had a slight effect in the enhancement of basal expression as well as a somewhat stronger effect in the enhancement of KCl-stimulated activation of the GAL4–CREB fusion protein. The wild-type GAL4–CREB protein permitted a 36-fold activation by KCl, whereas the GAL4–CREB-S142A protein permitted a 66-fold activation by KCl. The finding that a mutation that blocks phosphorylation of Ser^{142} enhances the response to Ca^{2+}

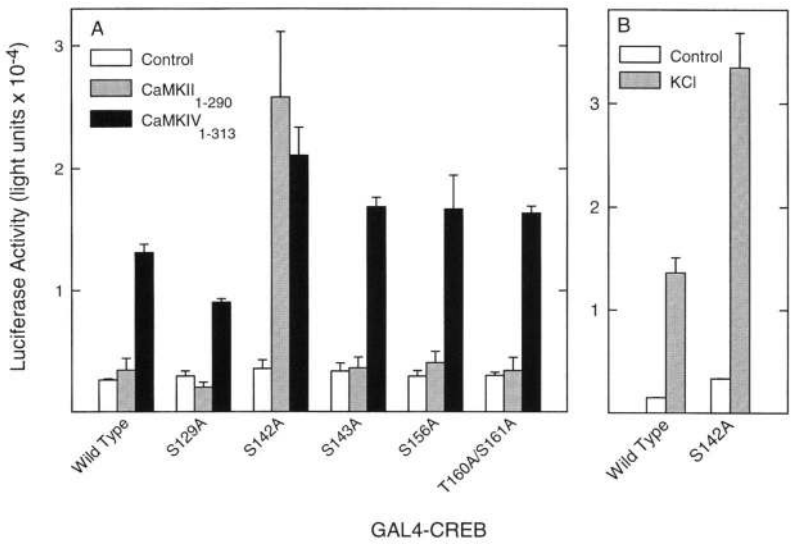

Figure 8. Mutation of Ser^{142} of CREB to alanine permits activation of CREB by both $CaMKII_{1-290}$ and $CaMKIV_{1-313}$ in GH_3 cells and enhances activation of CREB in response to KCl. (*A*) GH_3 cells were transfected with 2 μg of an expression vector for the GAL4–CREB fusion protein (wild-type or mutants as indicated), 5 μg of 5×GAL4–TATA–luciferase reporter gene, and 5 μg of BSSK(−) or the expression vector for $CaMKII_{1-290}$ or $CaMKIV_{1-313}$. Luciferase activity was determined 24 hr after transfection. Values are means±S.E.M. for three separate transfections. (*B*) GH_3 cells were transfected with 2 μg of the indicated GAL4–CREB fusion protein and 5 μg of the 5×GAL4–TATA–luciferase reporter gene. After transfection, the cells were divided in two, and half of the cultures were treated by the addition of 80 mM KCl to the medium 18 hr after transfection. Luciferase activity was determined 6 hr after KCl treatment. Values are the mean±S.E.M. for three separate transfections.

influx suggests a physiological role for phosphorylation at this site.

Discussion

We have explored the ability of two different multifunctional Ca^{2+}/calmodulin-dependent protein kinases, CaMKII and CaMKIV, to activate CREB. We find that CaMKIV is much more potent than CaMKII in activating CREB. Not only is CaMKII not very effective in activating CREB, but expression of CaMKII acts to oppose the activation of CREB that occurs after phosphorylation of CREB by either PKA or CaMKIV. These studies provide new insights into the role of specific protein kinases in mediating transcriptional responses to Ca^{2+} as well as the mechanisms that regulate the activity of CREB.

Previous studies have shown that CREB can be activated by Ca^{2+} influx (Sheng et al. 1990, 1991) and that in vitro, CREB can be phosphorylated by CaMKII (Dash et al. 1991; Sheng et al. 1991). Furthermore, KN-62, a specific inhibitor of CaMKII (Tokumitsu et al. 1990), was found to reduce the ability of Ca^{2+} influx to stimulate transcription mediated by a cAMP response element (Schwaninger et al. 1993). These findings led to the view that the multifunctional enzyme CaMKII likely mediates or at least contributes to the ability of Ca^{2+} influx to activate CREB. Recently, it has been shown that CaMKIV can phosphorylate CREB, raising the possibility that this enzyme may participate in mediating Ca^{2+} effects on the activation of CREB (Cruzalegui and Means 1993). The present transient transfection studies confirm recent studies (Enslen et al. 1994; Matthews et al. 1994), which found that CaMKIV is more likely to be the enzyme that mediates the ability of Ca^{2+} influx to activate CREB. In our studies the greater activation by CaMKIV occurred despite the fact that the expression vectors led to higher protein levels for CaMKII than CaMKIV. Furthermore, an expression vector encoding full-length CaMKIV, but not CaMKII, potentiated the ability of membrane depolarization to activate CREB. The present finding that CaMKII is rather ineffective in activating CREB is quite different from the conclusions reached in studies that examined the ability of CaMKII to activate CREB by use of an in vitro transcription assay (Dash et al. 1991). At least a part of this discrepancy may be attributable to the use of different assays. Analysis of phosphorylation-induced activation of CREB by use of in vitro transcription assays is difficult because of rapid dephosphorylation of CREB in these systems (Hagiwara et al. 1992; Sun et al. 1992; Wadzinski et al. 1993). It is possible that differences between in vivo and in vitro dephosphorylation of specific sites on CREB account for the differences in the ability of CaMKII to activate transcription in the two different assays. In any case, the present studies demonstrate that in intact cells, CaMKII is not very efficient at activating CREB, whereas CaMKIV is quite capable of activating CREB. Overall, the present studies suggest that CaMKIV is an excellent candidate as an enzyme mediating the ability of Ca^{2+} influx to activate CREB. This view is also consistent with the finding that CaMKIV has been reported to be localized to the nucleus (Jensen et al. 1991).

The inability of CaMKII to efficiently activate CREB appears to involve phosphorylation of CREB on Ser^{142}. Initially it was somewhat surprising to find that CaMKII was a poor activator of CREB in view of the data showning that CaMKII can phosphorylate CREB on Ser^{133} in vitro (Dash et al. 1991; Sheng et al. 1991). Both CaMKII and PKA demonstrate similar kinetics for phosphorylation of CREB in vitro (Enslen et al. 1994). Phosphopeptide and mutagenesis studies resolved this apparent contradiction and demonstrated that CaMKII phosphorylates CREB at two major sites, whereas PKA and CaMKIV phosphorylate CREB at only a single major site. All three enzymes phosphorylate CREB on Ser^{133}, a site that has been shown to be important for activation of

CREB (Gonzalez and Montminy 1989; Lee et al. 1990; Gonzalez et al. 1991). In addition to Ser^{133}, CaMKII also phosphorylates CREB on Ser^{142}. Two different findings suggest that phosphorylation of CREB at Ser^{142} blocks transcriptional activation. First, an expression vector for a constitutively active form of CaMKII inhibits the ability of PKA to activate CREB, presumably through phosphorylation of Ser^{142}. Second, mutation of Ser^{142} to alanine permitted CaMKII to activate CREB. Thus, these findings are consistent with a model in which CaMKII and CaMKIV have opposite effects in the regulation of the activity of CREB by differential phosphorylation at Ser^{142}.

Although both the in vivo and in vitro data clearly suggest that Ser^{142} is phosphorylated by CaMKII, the amino acid sequence of this region, ^{139}Asn-Asp-Leu-Ser^{*}-Ser-Asp^{144}, does not fit the CaMKII consensus phosphorylation site, Arg-X-X-Ser/Thr (Kennelly and Krebs 1991). This is not an unprecedented observation, as nine different CaMKII phosphorylation sites have been identified in smooth muscle caldesmon, and seven of these sites do not match the CaMKII consensus site (Ikebe and Reardon 1990). Similarly, a nonconsensus site has also been detected in vimentin (Ando et al. 1991). Analysis of peptide substrates based on the nonconsensus CaMKII site of vimentin suggests that the sequence Ser-X-Asp may also serve as a recognition site for phosphorylation by CaMKII (Ando et al. 1991). The sequence surrounding Ser^{142} of CREB would fit this alternative consensus sequence, and the present findings offer additional support for the recognition of the Ser-X-Asp motif by CaMKII.

There are interesting parallels and differences between the present findings concerning phosphorylation-mediated inhibition of CREB and previous studies of phosphorylation-mediated negative regulation of c-Jun activity. Several phosphorylation sites have been identified that inhibit the ability of c-Jun to stimulate transcription (Boyle et al. 1991a; Lin et al. 1992). These sites are located adjacent to the DNA-binding domain of c-Jun, and phosphorylation of these sites negatively regulates AP-1 activity by inhibiting DNA binding. Activation of AP-1 involves dephosphorylation of these inhibitory sites. The present study demonstrates that the activity of CREB can also be inhibited by phosphorylation, but the inhibitory phosphorylation site is within the transcriptional activation domain rather than adjacent to the DNA-binding domain. Phosphorylation of CREB by CaMKII does not have a detectable effect on DNA binding (P. Sun, unpubl.). Thus, phosphorylation of Ser^{142} almost certainly inhibits transcriptional activation by mechanisms other than the inhibition of DNA binding. Phosphorylation of Ser^{142} may prevent conformational changes (Gonzalez et al. 1991) that are important for CREB activation. Alternatively, phosphorylation of Ser^{142} may interfere with the interaction between CREB and coactivators such as the CREB-binding protein (Chrivia et al. 1993) or with the general transcription apparatus. Observations have demonstrated that the region of CREB surrounding Ser^{142} is important for transcriptional activation. Deletion of residues 140–144 has been found to produce a CREB mutant that has greatly reduced basal and cAMP-stimulated transcriptional activity (Gonzalez et al. 1991). Although the Δ140–144 CREB mutant has very limited basal and inducible activity, the protein is well expressed, targeted to the nucleus, and can be phosphorylated by PKA. The finding that the 140- to 144-residue region of CREB is quite important for transcriptional activation suggests a role for this region in interacting with other components of the transcriptional apparatus. In this regard it is quite interesting that deletion of residues 140–160 of CREB abolishes interactions of phospho-CREB with the coactivator CBP (Chrivia et al. 1993). It should be interesting to determine whether phosphorylation of Ser^{142} of CREB interferes with binding to CBP.

The finding that mutation of Ser^{142} to alanine augments the ability of Ca^{2+} influx to activate CREB in GH_3 cells suggests that phosphorylation at this site is physiologically important for modulating CREB activity. On the other hand, it is also clear that Ca^{2+} influx in GH_3 cells activates CREB, despite the fact that GH_3 cells contain a substantial amount of CaMKII (Jefferson et al. 1991). If phosphorylation of CREB by CaMKII blocks activation by PKA or CaMKIV, why doesn't the presence of CaMKII in GH_3 cells block the ability of Ca^{2+} influx to activate CREB? It is likely that the CaMKII holoenzyme (m.w. ~500,000) does not have ready access to the nucleus in GH_3 cells and therefore cannot effectively phosphorylate CREB. Although this might suggest that CaMKII is not a physiological regulator of CREB, there are at least two mechanisms that could generate a form of CaMKII that could phosphorylate CREB. Recently, an alternatively spliced form of CaMKII that contains a nuclear localization signal has been identified. This nuclear localization signal is sufficient to localize the CaMKII holoenzyme to the nucleus (H. Schulman, pers. comm.). Tissue-specific expression of a nuclear form of CaMKII would permit the kinase to effectively phosphorylate Ser^{142} of CREB. A second mechanism that could generate a form of CaMKII that could phosphorylate Ser^{142} of CREB would involve proteolysis. Proteolytic cleavage of CaMKII can produce a constitutively active catalytic domain of CaMKII (Colbran et al. 1988) that is similar to the active form produced by the expression vector used in this study. As noted above, the observation that mutation of Ser^{142} to alanine enhanced the transcriptional response to Ca^{2+} influx argues that phosphorylation at this site physiologically modulates CREB activity.

A possible role for phosphorylation of Ser^{142} of CREB by a nuclear form of CaMKII adds a new perspective to the possible regulation of CREB activity in cells. Differences in the ratio of the nuclear form of CaMKII to CaMKIV in cells could likely lead to considerable differences in the interaction between the cAMP and Ca^{2+}-signaling pathways. A predominance of nuclear CaMKII activity would likely lead to Ca^{2+} having an inhibitory effect on cAMP-induced activation of CREB. Alternatively, a predominance of CaMKIV activity should produce positive interactions between the cAMP and Ca^{2+} pathways. It should be noted that CaMKIV is expressed

in a number of tissues, but it is not ubiquitous (Jones et al. 1991; Means et al. 1991). There may be other Ca^{2+}/calmodulin-dependent enzymes that are able to activate CREB. For instance, CaMKI has been found to phosphorylate CREB on Ser^{133} (Sheng et al. 1991). There are also other mechanisms that may alter the interaction between the Ca^{2+} and cAMP pathways. Ca^{2+} can alter the activity of Ca^{2+}/calmodulin-stimulated adenylyl cyclase (Choi et al. 1993), phosphodiesterases (Bentley and Beavo 1992), or protein phosphatases (Cohen 1989), permitting differential interactions between the Ca^{2+} and cAMP signaling systems at multiple levels. The present studies suggest that developmental or tissue-specific changes in the nuclear concentrations of CaMKII could lead to an altered role of Ca^{2+} in regulating the activity of cAMP-responsive genes.

Materials and Methods

Plasmid constructions

The indicator plasmid 5×GAL4–TATA–luciferase contains five copies of a GAL4-binding site upstream of the TATA box from the viral E1B promoter, which is linked to the coding sequence of firefly luciferase. This plasmid was prepared by insertion of a *Hin*dIII–*Bam*HI DNA fragment containing the GAL4-binding sites and the E1B TATA box from G_5CAT (Carey et al. 1990) into a plasmid containing the luciferase-coding sequence (d'Emden et al. 1992; de Wet et al. 1987). An indicator gene containing the rat prolactin promoter, 2.5PRL–luciferase, has been described previously (Maurer 1989).

To construct expression vectors for the GAL4–CREB fusion protein, the full-length coding sequence for wild-type or mutant CREB was fused in-frame to the 3′ end of the coding sequence for the DNA-binding domain of GAL4 (amino acids 1–147) in plasmid pSG424 (Sadowski and Ptashne 1989). The GAL4–CREB fusion fragment was then isolated and subcloned downstream of the viral CMV promoter in plasmid pcDNAI–amp (Invitrogen). Specific mutations within the CREB-coding sequence were prepared by oligonucleotide directed mutagenesis (Hutchison et al. 1978), and the complete coding sequence was confirmed by nucleotide sequence analysis (Sanger et al. 1977).

Expression vectors for several different protein kinases were used in these studies. The expression vectors for constitutively active CaMKII and CaMKIV were constructed by polymerase chain amplification of appropriate portions of the coding sequence with single-stranded cDNA as a starting point. The coding sequence for the amino-terminal 290 amino acids of rat CaMKII were isolated by polymerase chain amplification with cDNA synthesized from GH_3 pituitary tumor cells. The coding sequence for the amino-terminal 313 amino acids of mouse CaMKIV was isolated in a similar manner using single-stranded cDNA from αT3-1 cells. The correct sequences of the amplified and cloned coding sequences were confirmed by nucleotide sequence analysis (Sanger et al. 1977) and comparison with the published sequences (Lin et al. 1987; Jones et al. 1991). The truncated CaMKII- and CaMKIV-coding sequences were then used to replace the globin coding sequence in the RSV-β-globin expression vector (Gorman et al. 1983). The full-length CaMKII- and CaMKIV-coding sequences were also cloned into the same vector. To prepare expression vectors encoding epitope-tagged variants of CaMKII and CaMKIV, the 3′ terminus of $CaMKII_{1-290}$ or $CaMKIV_{1-313}$ was fused in-frame to an oligonucleotide encoding a 9-amino-acid peptide (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) from influenza hemagglutinin, which is the minimal recognition site for the mAb 12CA5 (Wilson et al. 1984). The epitope-tagged $CaMKII_{1-290}$ and $CaMKIV_{1-313}$ were then cloned into pcDNAI–amp. The preparation of an expression vector for the catalytic subunit of PKA has been described previously (Maurer 1989).

Cell culture and transfections

PC12 cells were maintained in RPMI 1640 medium supplemented with 10% heat treated equine serum and 5% fetal bovine serum (FBS). JEG-3 and COS-1 cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS. GH_3 cells were maintained in DMEM with 15% equine serum and 2.5% FBS.

PC12 cells were transfected by calcium phosphate precipitation. One day before transfection, PC12 cells were subcultured into DMEM containing 10% equine serum and 5% FBS in 60-mm dishes that were treated previously with an aqueous solution containing 100 μg/ml of poly(L-lysine). The next day DNAs were transfected by the calcium phosphate method, by use of reagents from GIBCO–BRL and following the directions of the supplier. Both JEG-3 and GH_3 cells were transfected by electroporation with a single pulse at 220 V and 960 μF. Typically, cells received 5 μg of the 5×GAL4–TATA–luciferase indicator DNA, 2 μg of the GAL4–CREB expression vector and 5 μg of kinase expression vector. For experiments involving EGTA treatments, after electroporation, the GH_3 cells were plated on dishes treated with poly(L-lysine).

In some experiments, cells were treated with KCl at the indicated concentrations, 18 hr after transfection. EGTA treatments were performed 30 min before the KCl treatment. Cells were collected and lysates prepared 24 hr post-transfection or 6 hr after KCl treatments. The protein concentration of the lysates was determined (Bradford 1976), and luciferase activities were measured with a constant amount of protein (de Wet et al. 1987). Each experiment included three separate transfections for each experimental group, and the experiments have been repeated three to five times.

Western blot analysis of the expression of constitutively active forms of CaMKII and CaMKIV

COS-1 cells were transfected with 9 μg of expression vectors encoding epitope-tagged, $CaMKII_{1-290}$ or $CaMKIV_{1-313}$ per 60-mm dish by use of LipofectACE reagent (GIBCO–BRL) according to instructions from the supplier. The cells were collected 24 hr after transfection. Cell lysates were prepared by sonication on ice with three 10-sec bursts at 10-sec intervals in the buffer containing 0.4 M NaCl, 10 mM HEPES (pH 7.6), 1 mM EGTA, 1 mM EDTA, 0.1 mM PMSF, and 5 mg/liter of leupeptin. Protein concentrations of cell lysates were determined (Bradford 1976), and 70 μg of total protein from each sample was separated on a 12% polyacrylamide, SDS-containing gel. After electroblotting to a PVDF membrane, the filters were blocked and incubated with a 1:6667 dilution of the mAb 12CA5 (BAbCO, Inc.), and immune complexes were visualized by use of the ImmunoSelect Photoblot Chemiluminescent System (GIBCO–BRL) following the manufacturer's protocol.

Expression and purification of recombinant poly(His)–CREB proteins

To facilitate isolation of recombinant CREB protein, poly-histidine (His)-tagged CREB was prepared. The 5′ terminus of the CREB-coding sequence was mutated by polymerase chain am-

plification to insert a poly(His) tag followed by the Factor Xa cleavage site (Met-Ala-His-His-His-His-His-His-Ile-Glu-Gly-Arg). After confirmation of the CREB-coding sequence, the poly(His)-tagged CREB sequence was subcloned in the pET3c bacterial expression vector (Studier and Moffatt 1986). Recombinant poly(His)–CREB proteins were expressed in *Escherichia coli*, and a crude extract prepared as described previously (Pei et al. 1991). The crude extract from each liter of culture was diluted with 1 volume of 8 M urea and then mixed with 1 ml of Ni^+–NTA–agarose (Qiagen) in the presence of 0.8 mM imidazole. The mixture was rotated at 4°C for 1 hr before being packed into a column. The column was washed sequentially with 0.8 mM, 8 mM, and 40 mM imidazole in 20 mM Tris-HCl (pH 7.4), 6 mM DTT, and 4 M urea. The poly(His)–CREB was eluted with 100 mM imidazole in the same buffer. The purified protein was dialyzed against 20 mM HEPES (pH 7.9), 50 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, and 10% glycerol and stored at −80°C until use.

Phosphorylation of CREB and phosphopeptide mapping

Recombinant CREB protein (1 μg) was phosphorylated in vitro with recombinant CaMKIIα (Brickey et al. 1990), CaMKIV or the catalytic subunit of PKA in the presence of [γ-^{32}P]ATP (1000–5000 cpm/pmole). Recombinant CaMKIIα and CaMKIV were prepared by use of the baculovirus expression system (Brickey et al. 1990). For phosphorylation by CaMKII and CaMKIV, the reaction mixtures contained 400 μM [γ-^{32}P]ATP (5000 cpm/pmole), 50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 50 nM of kinase, 0.5 mM $CaCl_2$, and 1 μM calmodulin. Reactions with PKA contained 400 μM [γ-^{32}P]ATP, 50 nM PKA, 1 mM of EGTA, and 50 mM potassium phosphate (pH 6.8). Reactions were initiated by addition of protein kinase and incubated for 45 min at 30°C. For two-dimensional phosphopeptide mapping, CREB was fractionated by electrophoresis on a 10% polyacrylamide, SDS-containing gel, and phospho-CREB was identified by autoradiography of the dried gel. The phospho-CREB bands were cut from the gel, and two-dimensional tryptic phosphopeptide maps were prepared on the extracted proteins as described (Boyle et al. 1991b). Briefly, the gel pieces were rehydrated in 50 mM ammonium bicarbonate and digested with 10 μg of trypsin in a 0.2-ml reaction. After oxidation of the tryptic peptides, approximately the same amount of radioactivity for each sample was loaded on a cellulose plate and fractionated by high voltage electrophoresis for 25 min at 1 kV by use of the Hunter Thin Layer Electrophoresis system (HTLE-7000, CBS Scientific Co.). Samples were separated in the second dimension by thin-layer chromatography with *n*-butanol/pyridine/acetic acid/water in volume ratios of 0.375, 0.25, 0.075, and 0.30. The phosphopeptides were visualized by autoradiography. For analysis of phosphopeptides after in vivo labeling, GH_3 cells were transfected with 10 μg of an expression vector for GAL4–CREB fusion protein and 10 μg of expression vectors for the constitutively active forms of CaMKII or CaMKIV. After transfection, cells were plated into 100-mm dishes and incubated at 37°C for 20 hr in normal growth medium. The cells were labeled for 4 hr in the presence of 1 mCi/ml of [^{32}P]orthophosphate in 4 ml of phosphate-free DMEM containing 1% FBS. To enhance detection of phosphorylated CREB, okadaic acid was added to the culture medium to a final concentration of 1 μM 15 min before collection. After rinsing with buffered saline, the cells were collected and homogenized in a buffer containing 10 mM HEPES (pH 7.4), 5 mM DTT, 5 mM EDTA, 1 mM benzamidine, 0.2 mM PMSF, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, and 10 mM β-glycerophosphate. Nuclei were collected by centrifugation and resuspended in 250 μl of homogenization buffer containing 0.4 M KCl. After incubation for 30 min on ice, the nuclei were removed by centrifugation. The supernatants were then mixed with 15 μl of a slurry of protein A–agarose beads (Sigma, St Louis, MO) and incubated for 15 mins. After centrifugation, the supernatants were combined with 15 μl of an antiserum to CREB (Sun et al. 1992) and incubated on ice for 2 hr. Immune complexes were isolated by addition of 100 μl of a slurry of protein A–agarose beads. After rotation at 4°C for 1 hr, the protein A–agarose beads were washed five times with 1 ml of 1% Triton X-100, 1% sodium deoxycholate, 0.5% SDS, 10 mM Tris (pH 7.4), and 150 mM NaCl. The proteins bound to beads were eluted and separated on an SDS–polyacrylamide gel. The radioactive band corresponding to the GAL4–CREB fusion protein was visualized by autoradiography, excised from the gel, and subjected to two-dimensional phosphopeptide mapping analysis as described above.

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