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Calcium/Calmodulin-Dependent Protein Kinase Types II and IV Differentially Regulate CREB-Dependent Gene Expression

RANDOLPH P. MATTHEWS,[1] CHRIS R. GUTHRIE,[1] LAUREN M. WAILES,[1] XINYU ZHAO,[1] ANTHONY R. MEANS,[2] AND G. STANLEY McKNIGHT[1]*

Department of Pharmacology, University of Washington, Seattle, Washington 98195,[1] and Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710[2]

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Phosphorylation of CREB (cyclic AMP [cAMP]- response element [CRE]-binding protein) by cAMP-dependent protein kinase (PKA) leads to the activation of many promoters containing CREs. In neurons and other cell types, CREB phosphorylation and activation of CRE-containing promoters can occur in response to elevated intracellular Ca^{2+}. In cultured cells that normally lack this Ca^{2+} responsiveness, we confer Ca^{2+}-mediated activation of a CRE-containing promoter by introducing an expression vector for Ca^{2+}/calmodulin-dependent protein kinase type IV (CaMKIV). Activation could also be mediated directly by a constitutively active form of CaMKIV which is Ca^{2+} independent. The CaMKIV-mediated gene induction requires the activity of CREB/ATF family members but is independent of PKA activity. In contrast, transient expression of either a constitutively active or wild-type Ca^{2+}/calmodulin-dependent protein kinase type II (CaMKII) fails to mediate the transactivation of the same CRE-containing reporter gene. Examination of the subcellular distribution of transiently expressed CaMKIV and CaMKII reveals that only CaMKIV enters the nucleus. Our results demonstrate that CaMKIV, which is expressed in neuronal, reproductive, and lymphoid tissues, may act as a mediator of Ca^{2+}-dependent gene induction.

A variety of hormonal and electrophysiological stimuli can induce changes in the intracellular levels of the second messengers, Ca^{2+} and cyclic AMP (cAMP). Many of the downstream effects of these second messengers depend on the activation of specific protein kinases and the subsequent phosphorylation of regulatory proteins such as transcription factors. cAMP directly leads to the dissociation of protein kinase A (PKA) holoenzyme and release of active catalytic (C) subunit. Elevation of Ca^{2+} may be caused by influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels or by release of Ca^{2+} from intracellular stores in response to elevated inositol trisphosphate. Some of the effects of Ca^{2+} are mediated through a family of protein kinases stimulated by the Ca^{2+}/calmodulin complex. These include dedicated protein kinases such as myosin light-chain kinase and phosphorylase kinase and the multifunctional Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases) (43).

The type II CaM kinase (CaMKII) isoforms are highly expressed in the central nervous system but are also distributed in other tissues (20). CaMKIIα self-associates into an 8- to 10-mer (25), and in vitro it phosphorylates a wide variety of substrates, including the transcription factors CREB (cAMP response element [CRE]-binding protein) (10, 47) and C/EBPβ (CAAT enhancer-binding protein β) (55) and a wide variety of proteins important in neuronal function (20). CaMKII has been implicated in many physiological phenomena, including regulation of the cell cycle, smooth muscle contraction, secretion, and growth factor desensitization (43). Hippocampal slices from mice made deficient in CaMKIIα by gene-targeted homologous recombination show a defect in long-term potentiation, and the mutant mice are also deficient in certain tasks designed to test spatial memory (48, 49).

* Corresponding author. Mailing address: Department of Pharmacology SJ-30, University of Washington, Seattle, WA 98195. Phone: (206) 543-7485. Fax: (206) 685-3822.

The type IV CaM kinase (CaMKIV) is a more recently discovered member of the CaM kinases. CaMKIV exists as a monomer and is not distributed as widely as is the type II isoform; CaMKIV mRNA levels are highest in the cerebellum, forebrain, testis, spleen, and thymus (14, 22, 29, 33). The deduced primary sequence of CaMKIV shares only 32% identity to the CaMKIIα sequence, but the structural organization of CaMKIV is similar to that of CaMKII, including an amino-terminal catalytic domain and a central calmodulin binding regulatory domain. The carboxy-terminal domain in CaMKIV is rich in acidic residues and differs from the corresponding carboxy-terminal association domain of CaMKII (14, 22, 29). CaMKIV protein can be localized in the nucleus of cerebellar granule cells (21), which would allow the kinase ready access to transcription factors when activated by Ca^{2+}. The substrate specificity of CaMKIV appears to differ from that of CaMKII; sequences from ribosomal protein S6 (9), CaMKIIγ (31), and Rap-1b protein (41) are phosphorylated more effectively by CaMKIV than by CaMKIIα. CaMKIV phosphorylates the transcription factors CREB and serum response factor in vitro (9). Phosphorylation of substrates in vitro by CaMKIV, however, occurs at a much reduced rate compared with phosphorylation by CaMKIIα (9, 13). This may be due to the requirement for a protein activator; CaMKIV appears to require phosphorylation for activity, but the site of phosphorylation and the identity of the activating kinase remain controversial (9, 15, 24, 27, 28, 34). CaMKIV is also phosphorylated by the C subunit of PKA in vitro, which leads to decreased activity (23).

There are several sites of potential interaction between the Ca^{2+} and cAMP signaling systems, including the Ca^{2+}/calmodulin-stimulated adenylyl cyclases (4), calmodulin-dependent phosphodiesterases (2), and Ca^{2+}-activated phosphatases (6). The activity of voltage-sensitive Ca^{2+} channels can be directly regulated by PKA phosphorylation (45). One other possible point of convergence between the Ca^{2+} and cAMP signaling systems is at the phosphorylation and subsequent

activation of CREB. This bZIP transcription factor has been shown to mediate transactivation of CRE-containing promoters after phosphorylation of serine 133 by PKA (17). Investigators have also shown that a CRE in the *fos* promoter can mediate the Ca^{2+}-dependent induction of *fos* after depolarization of PC12 cells (46). Depolarization activates CaMKII (18), suggesting the possibility that CaMKII mediates the induction of *fos* expression in response to depolarization of PC12 cells (46). The subsequent finding that CREB is phosphorylated in vitro by CaMKII (10, 47) supports a role for CaMKII in gene induction. In addition to being phosphorylated by CaMKII and PKA, CREB is also phosphorylated in vitro by type I (47) and type IV (9) CaM kinase, suggesting that there are other possible Ca^{2+}-responsive kinases that could mediate transcriptional regulation of CRE-driven promoters.

Here we demonstrate the transactivation of a CRE-containing reporter gene by CaMKIV in transient expression assays and show that CaMKIV acts through CREB/ATF family members to activate transcription. We also demonstrate that CaMKII is not capable of activating this CRE-containing reporter, although CaMKII does stimulate transcription from the *fos* promoter. Our results suggest a possible mechanism to explain the convergent activation of CRE-containing promoters by both cAMP and Ca^{2+} in cells that express CaMKIV.

MATERIALS AND METHODS

CaM kinase expression vectors. A 1.2-kb *Nco*I-*Apa*I fragment was prepared from a 15-cycle PCR using rat CaMKIV cDNA as the template, a 5′ oligonucleotide primer (CGGC GACCATGGTCAAAGTCACGGTGC) that introduces an *Nco*I site at the initiator net and changes the next amino acid from Leu to Val, and a 3′ oligonucleotide primer (GGCCT GGGGCCCTAAAGGAAG). CaMKIV cDNA templates included both a full-length form (CaMKIVwt) and a mutated form (CaMKIV313) in which the codon for Gln-314 has been mutated to a stop codon, thus terminating the protein at Leu-313 (9) and introducing an *Xba*I site. The 1.2-kb PCR product was substituted into the CEV expression vector originally constructed to produce the Cα subunit of PKA (35). Expression vectors consisting of full-length and truncated constitutive CaMKIIα (9) driven by the metallothionein promoter (CaMKIIwt and CaMKII290) were constructed by similar methods, using CaMKIIα cDNA as the template, a 5′ oligonucleotide primer (GTGCCACCATGGCTACCATCAC CTGC) that introduces an *Nco*I site at the initiator Met and conserves the Ala at position 2, and a 3′ oligonucleotide primer (GAATTCGGGCCCTCAATGGGGCAGGACGGAG).

The CaMKIIα-IHG expression vector was constructed by using a 15-cycle PCR of CaMKIIα cDNA, the 5′ primer described above, and a 3′ oligonucleotide primer (GAATTCG GGCCCTCAGGCGTAATCAGGGACGTCGTAAGGGTA ATGGGCAGGACGGAGGGCGC) that encodes a short peptide epitope (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) from hemagglutinin (IHG) recognized by monoclonal antibody 12CA5 (Berkeley Antibody); the 1.4-kb *Nco*I-*Apa*I fragment was then substituted into the CEV expression vector (35). For the Cα-TAG construct, a 1.2-kb *Nco*I-*Apa*I fragment was prepared by using PCR of the Cα expression vector as a template, a 5′ primer previously described (35), and a 3′ oligonucleotide primer (GAATTCGGGCCCCTAGTTCAGA TCCTCCTCAGAAATAAGCTTCTGCTCCATAAACTCA GTAAACTCCTTGCCACACTT) that contains a sequence encoding a peptide epitope recognized by monoclonal antibody AB-1 (Oncogene Science). The PCR product was then substituted into the CEV expression vector (35). The epitope-tagged C subunit displays the same ability to activate gene expression in transfected cells as the wild-type C subunit. All of these expression vectors are driven by the Zn^{2+}-dependent mouse metallothionein-1 promoter and terminated with a polyadenylation signal from the human growth hormone gene.

Cell culture and transient transfections. JEG-3 cells were grown in Dulbecco modified Eagle medium plus 10% fetal bovine serum (Life Technologies) as described previously (7) in 24-well plates until the cells were ~60% confluent, at which point the amount of medium was reduced to 250 μl per well. Three to six hours later, a mixture of calcium phosphate and DNA, including 2.5 ng of α168-luciferase (30), 50 ng of the internal control plasmid RSV-*lacZ* (12), 30 ng of CaMK expression vector, or 1 ng of Cα expression vector, plus enough pBluescript KS(+) (Stratagene) to produce a final DNA amount of 250 ng per well, was added to the medium on the cells as previously described (36). For those experiments examining *fos-lacZ* expression, 75 ng of *fos-lacZ* (42) and 5 ng of promoter driven Rous sarcoma virus (RSV)-luciferase (11) were added in the mixture in place of α168-luciferase and RSV-*lacZ*. RSV-KCREB (54), and MT-R_{AB} (7) expression vectors were added at various concentrations as described in the legends to Fig. 3 and 4. To control for promoter competition, we kept the amount of metallothionein promoter constant by adding the appropriate amount of ZEM3 (26), an empty vector containing the metallothionein promoter, to those conditions not including the CaMK, Cα, or R_{AB} expression vector. We also controlled for promoter competition in the RSV-KCREB experiments by adding the appropriate amounts of RSV-*neo*. DNA was prepared by the Qiagen procedure (Diagen) or standard CsCl methods. After 24 h of DNA exposure in 3% CO_2, the medium was replaced with medium containing 2.5% fetal bovine serum and 80 μM $ZnSO_4$ in Dulbecco modified Eagle medium, and the cells were returned to incubation at 10% CO_2 for 12 to 18 h.

For those cells to be treated with ionomycin or thapsigargin, medium containing 2.5% fetal bovine serum and 80 μM $ZnSO_4$ remained on the cells for only 10 to 14 h, at which point ionomycin (CalBiochem) or thapsigargin (Sigma) was added directly to the medium on the cells for 5 to 6 h. The concentrations of ionomycin and thapsigargin are indicated in Fig. 2 to 4. Stock solutions of ionomycin (6 mM) and thapsigargin (1.54 mM) were prepared in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide remained constant for all drug treatments. The final concentration of $CaCl_2$ on the cells was 1.5 mM: 1.0 mM from Dulbecco modified Eagle medium and 0.5 mM added with the drug treatment.

Following incubation with drugs or $ZnSO_4$-containing medium, cells were washed once with ice-cold phosphate-buffered saline, harvested, and assayed for luciferase activity as described previously (36). β-Galactosidase activity was determined by using a modification of the Galacto-Light system (Tropix). Substrate buffer consisting of 100 μl of 35 μM AMPGD {3-(4-methoxyspiro[1,2-dioxetane-3,2′-tricyclo [3.3.1.$1^{3,7}$]decan]-yl) phenyl β-D-galactopyranoside}, 10 mM $MgCl_2$, and 100 mM sodium phosphate (pH 8.0), was added to cell lysates (5 μl) from each well in 10-s intervals; these reactions proceeded at room temperature for 60 min. Emerald chemiluminescence amplifier (100 μl) was injected into the tubes containing the cell lysates by a Berthold luminometer, which determined the amount of luminescence produced during a 5-s period. Luciferase activity was divided by this β-galactosidase activity to normalize for differences in transfection efficiency, except for those experiments examining *fos-lacZ* expression, in which case the reciprocal ratio was used.

All transfection experiments presented were performed at least three times with consistently reproducible results.

In vitro phosphorylation of CREB. We generated CREB S133A by using a CREB expression vector as the template and standard in vitro mutagenesis techniques. Phosphorylation reactions with CREB (17) or CREB S133A were performed with purified recombinant CaMKIV313 described previously (9). Each reaction mixture contained 1.75 μM CREB or CREB S133A in a final volume of 50 μl of 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0)–0.5 mM dithiothreitol–10 mM magnesium acetate–100 μM ATP–1 mM $CaCl_2$ or 5 mM EGTA–60 ng of CaMKIV313. The specific activity of [γ-^{32}P]ATP was 4,200 cpm/pmol. The reactions were initiated by addition of kinase, the mixtures were incubated at 30°C for the time specified in the legend to Fig. 5, and the reactions were terminated by the addition of 4× sample buffer and analyzed on 12.5% polyacrylamide gels.

Immunocytochemistry. Cells for immunocytochemical staining were maintained and transfected as described above. The RIIα expression vector has been described previously (37). Following incubation with 80 μM Zn^{2+}, cells were washed once with cold methanol and then fixed for 15 min at −20°C in methanol. Following fixation, cells were washed in phosphate-buffered saline and incubated overnight at 4°C with blocking buffer (5% bovine serum albumin, 10% horse or goat serum). After removal of blocking buffer, diluted primary antibody was incubated with the cells for 1 h at room temperature in blocking buffer. Monoclonal antibody AB-1 (Oncogene Science) was incubated on the cells at a dilution of 1:100, 12CA5 ascites fluid (Berkeley Antibody) was used at a dilution of 1:400, or anti-CaMKIV antiserum (28) was used at a dilution of 1:25. Following primary antibody incubation, cells were washed with phosphate-buffered saline. For cells treated with antibody AB-1 or 12CA5, biotinylated horse anti-mouse affinity-purified immunoglobulin G (Vector Laboratories) was then added at a dilution of 1:100 in blocking buffer for 1 h. For cells treated with anti-CaMKIV antiserum, biotinylated goat anti-rabbit affinity-purified immunoglobulin G (Vector Laboratories) was added at a dilution of 1:100 in blocking buffer. Following another set of washes, cells were incubated with Avidin D-FITC (Vector Laboratories) diluted 1:4,000 in HEPES-buffered saline (pH 8.2) for 30 min at room temperature. Cells were washed three times as before, then stained with 5 μM propidium iodide (Molecular Probes) in deionized water for 20 min, and washed once with deionized water for 1 min. Cells were then mounted under coverslips with Vecta-Shield (Vector Laboratories), wells were punched out with a 1-cm diameter cork borer, and the cells were mounted on slides. Stained cells were then examined in a Bio-Rad MRC 600 confocal microscope with a focal plane thickness of 1 μm. Images were prepared and labeled with Adobe Photoshop version 2.5.

RESULTS

CaMKIV transactivates a CRE-containing reporter gene. We designed expression vectors containing CaMKII or CaM KIV cDNA, downstream of the metallothionein promoter, as described in Materials and Methods. These constructs contain cDNA clones that produce either a full-length (CaMKIVwt) or a truncated (CaMKIV313) version of CaMKIV (Fig. 1A); the CaMKII constructs that produce full-length (CaMKIIwt) and truncated (CaMKII290) forms of CaMKII are similar (not shown). Truncation of CaMKIV at leucine 313 (9) and of CaMKII at leucine 290 (8) has been shown to produce calcium-independent activity of these enzymes. Figure 1B displays the transactivation of two different reporter constructs, α168-luciferase and *fos-lacZ*, by the CaMK expression vectors in transient transfections of JEG-3 cells. The α168-luciferase construct consists of the 168-bp promoter of the glycoprotein hormone α subunit, containing two tandem consensus CREs upstream of firefly luciferase cDNA (30). The *fos-lacZ* reporter construct contains the 611-bp *fos* promoter, including the Ca/CRE site, serum response element, and *sis*-inducible element, upstream of a fusion gene that encodes the 315 amino-terminal amino acids from Fos and 1,015 carboxy-terminal amino acids from β-galactosidase (42). CaMKIV313 is the only CaMK expression vector that transactivates α168-luciferase, while *fos-lacZ* is transactivated by either CaMKII290 or, to a lesser extent, CaMKIV313 (Fig. 1B). Expression of the full-length form of CaMKII or

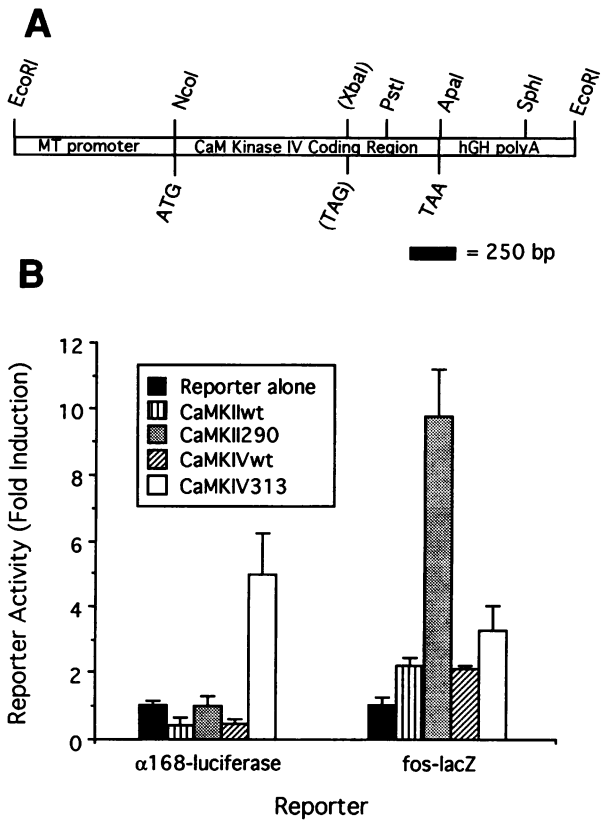

FIG. 1. Abilities of various CaM kinases to induce α168-luciferase and *fos-lacZ* in JEG-3 cells. (A) Coding regions for CaMKIVwt and CaMKIV313 are downstream of the metallothionein (MT) promoter and are followed by the human growth hormone (hGH) polyadenylation signal. The termination codon and *Xba*I site unique to the CaMKIV313 construct are noted in parentheses. (B) JEG-3 cells were transiently transfected with, per well, either (i) 30 ng of CaMKIIwt (vertically hatched columns), CaMKII290 (speckled columns), CaM KIVwt (diagonally hatched columns), or CaMKIV313 (open columns) expression vector and 2.5 ng of α168-luciferase reporter or (ii) 30 ng of CaMK expression vector and 75 ng of *fos-lacZ* reporter. After exposure to DNA precipitates for 24 h, medium was replaced and cells were treated with medium containing 80 μM Zn^{2+} for 16 h before harvesting. Extracts were analyzed for luciferase and β-galactosidase activity as described in Materials and Methods. The activity of the inducible construct was divided by the activity from the constitutive construct, and the activity ratio for reporter alone (closed columns) was normalized to 1. The transfections were performed in triplicate, and the mean ± standard deviation is shown for each datum point.

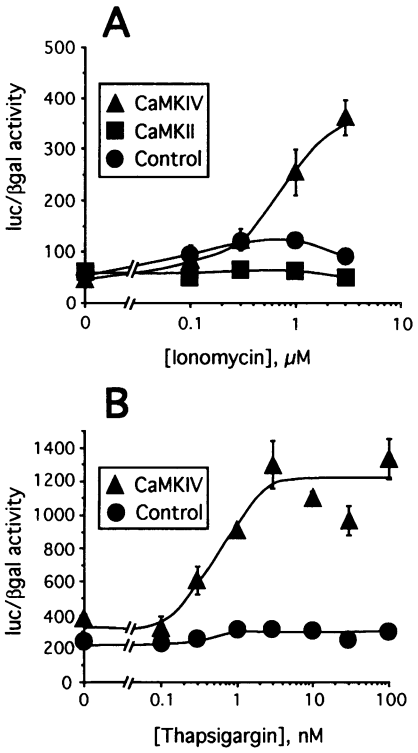

FIG. 2. Ionomycin and thapsigargin induce a CRE-containing reporter in CaMKIVwt-transfected JEG-3 cells. Cells were transfected with, per well, 30 ng of CaMKIVwt expression vector and 2.5 ng α168-luciferase (triangles), 30 ng of CaMKIIwt expression vector and 2.5 ng of α168-luciferase (squares in panel A), or 2.5 ng of α168-luciferase alone (circles) and then treated with medium containing 80 μM Zn^{2+} for 14 h. (A) Following Zn^{2+} treatment, cells were incubated with the indicated concentrations of ionomycin and 1.5 mM $CaCl_2$ for 5 h. (B) Following Zn^{2+} treatment, cells were incubated with the indicated concentrations of thapsigargin and 1.5 mM $CaCl_2$ for 5 h, when cells were harvested and assayed as for Fig. 1. Both sets of transfections were performed in triplicate, and the mean ± standard deviation is shown for each datum point. Datum points are presented as a ratio of luciferase (luc) activity to β-galactosidase (βgal) activity, resulting from expression of the internal control plasmid, RSV-*lacZ*.

CaMKIV does not transactivate expression of α168-luciferase in the absence of agents that increase intracellular calcium, although we see a small increase in *fos-lacZ* expression in response to the wild-type CaMKs (Fig. 1B).

CaMKIV confers Ca^{2+} sensitivity to JEG-3 cells. When JEG-3 cells are transiently transfected with CaMKIVwt, subsequent treatment with the calcium ionophore ionomycin results in transactivation of α168-luciferase expression (Fig. 2A). At 3 μM ionomycin, CaMKIVwt stimulation approaches the level of stimulation seen with the constitutive CaMKIV313 (Fig. 2A). Ionomycin has very little effect on α168-luciferase activity in control cells not transfected with a CaMK expression vector. There is also no change in α168-luciferase expression when cells are cotransfected with CaMKIIwt and treated with ionomycin (Fig. 2A).

Thapsigargin inhibits reuptake of Ca^{2+} into intracellular stores and leads to an elevation of intracellular calcium (52). In JEG-3 cells that have been transiently transfected with CaMKIVwt, thapsigargin (3 nM) produces transactivation of α168-luciferase activity to approximately the same fold induction as seen with the constitutive CaMKIV313 (Fig. 2B). Thapsigargin did not induce α168-luciferase expression in the absence of CaMKIVwt (Fig. 2B). Analogous cotransfection experiments with the CaMKIIwt expression vector produce no increase in α168-luciferase expression when cells are treated with thapsigargin (data not shown).

CaMKIV-induced CRE transactivation does not require PKA. Since the effects of CaMKIV on gene expression could be directly or indirectly influenced by PKA, we used a dominant negative PKA mutant (R_{AB}) in cotransfection experiments to examine the role of PKA in the CaMKIV response. Incorporation of this mutant form of the PKA RIα subunit into the PKA tetramer inactivates the C subunit and prevents the dissociation of C and R subunits in the presence of cAMP (5, 7). Increasing amounts of R_{AB} decrease the basal expression of α168-luciferase but do not reduce the induction by CaMKIV313 (Fig. 3A). In contrast, Fig. 3B demonstrates that transactivation of α168-luciferase expression by cotransfected PKA Cα subunit is completely inhibited as the amount of R_{AB} expression vector increases. In Fig. 3C, the data from Fig. 3A and B are depicted after elimination of background α168-luciferase expression so that the α168-luciferase induction produced by Cα and CaMKIV313 may be directly compared. Although we observe a small decrease in CaMKIV313-mediated α168-luciferase expression by R_{AB}, the same amount of R_{AB} results in the complete inhibition of Cα-mediated α168-luciferase expression. These results indicate that the induction of α168-luciferase by CaMKIV313 does not require PKA activity.

To test whether the Ca^{2+} response observed in cells cotransfected with CaMKIVwt is also independent of PKA, we performed experiments similar to those described above, using an amount of R_{AB} vector (25 ng) sufficient to completely inactivate endogenous PKA activity. JEG-3 cells that have been cotransfected with R_{AB} and CaMKIVwt respond to ionomycin treatment with a fivefold stimulation of α168-luciferase expression (Fig. 3D). There is no stimulation of α168-luciferase expression by ionomycin in cells that have not been transfected with the CaMKIV expression vector. Thus, we continue to see a Ca^{2+}-stimulated response in CaMKIV-transfected cells in which PKA has been inactivated.

Inhibition of CaMKIV-induced transactivation by a dominant negative CREB mutant. We cotransfected JEG-3 cells with an expression vector for the dominant negative CREB mutant KCREB (3, 54) to examine the dependence of the transactivation of α168-luciferase by CaMKIV on endogenous CREB. KCREB contains a mutation (Arg-287→Leu) in the DNA-binding domain of CREB, which allows KCREB to heterodimerize with CREB and other members of the CREB/ATF family that heterodimerize with wild-type CREB (such as ATF-1 or CREM) and reduces the ability of these transcription factors to interact with DNA (54). CaMKIV313-mediated α168-luciferase expression is completely inhibited by as little as 1 ng of KCREB expression vector per well (Fig. 4A). As expected, PKA-mediated α168-luciferase expression is also inhibited by KCREB expression (Fig. 4B), which supports previous studies demonstrating the importance of CREB in the activation of gene transcription by PKA (17). The stimulation of α168-luciferase expression produced by 1 μM ionomycin treatment of CaMKIVwt-transfected JEG-3 cells is attenuated by KCREB coexpression (Fig. 4C) to a similar degree as is CaMKIV313-mediated transactivation (Fig. 4A). The effect of KCREB on CaMKIV- and Cα-stimulated activity may be directly compared in Fig. 4D, which displays the data from Fig. 4A to C as a percentage of maximal induction. As shown in Fig. 4D, both CaMKIV- and Cα-mediated inductions of α168-luciferase are inhibited in parallel by KCREB. Neither

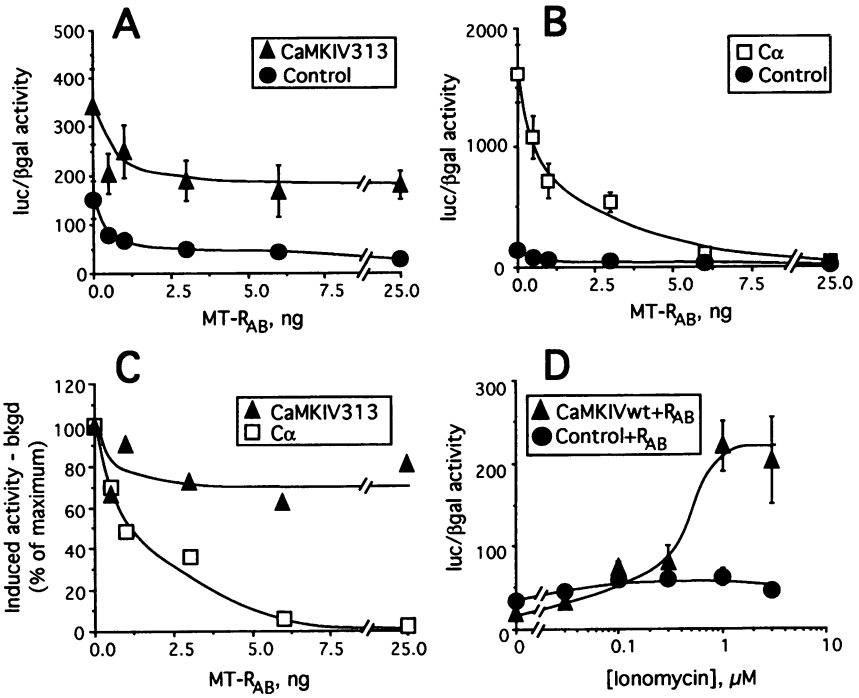

FIG. 3. CaMKIV-induced α168-luciferase expression does not require active PKA. (A) JEG-3 cells were transfected with the indicated amounts of R_{AB} plus, per well, 30 ng of CaMKIV313 and 2.5 ng of α168-luciferase (triangles) or 2.5 ng of α168-luciferase alone (filled circles). After 24 h of DNA exposure, cells were treated with medium containing 80 μM Zn^{2+} for 16 h before assay. (B) JEG-3 cells were transfected with the indicated amounts of R_{AB} plus, per well, 1 ng of Cα and 2.5 ng of α168-luciferase (open squares) or 2.5 ng of α168-luciferase alone (filled circles). After 24 h of DNA exposure, cells were treated with medium containing 80 μM Zn^{2+} for 16 h, lysed, and assayed. (C) The background (DKgd) control activity was subtracted from each point in panels A (triangles) and B (open squares), and the absolute activity at 0 ng of R_{AB} per well was set at 100%. (D) JEG-3 cells were transfected with, per well, 25 ng of R_{AB} plus 30 ng of CaMKIVwt and 2.5 ng of α168-luciferase (triangles) or 2.5 ng of α168-luciferase alone (circles). After 24 h of DNA exposure, cells were treated with medium containing 80 μM Zn^{2+} for 14 h, at which point cells were incubated with medium containing the indicated concentrations of ionomycin and 1.5 mM $CaCl_2$ for 5 h. Cells were lysed and assayed for luciferase and β-galactosidase activity. The transfections were performed in triplicate, and the mean ± standard deviation is shown for each datum point. Datum points in panels A, B, and D are presented as a ratio of luciferase (luc) activity to β-galactosidase (βgal) activity, resulting from expression of the internal control plasmid, RSV-*lacZ*. MT, metallothionein-1 promoter.

KCREB nor R_{AB} coexpression significantly decreased the activity of the constitutively active construct, RSV-*lacZ*, which is cotransfected with α168-luciferase in all of our studies to control for transfection efficiency.

In all of these experiments, the basal expression of α168-luciferase is also decreased by KCREB (Fig. 4), suggesting that this basal activity is CREB dependent. Given the similar reduction seen with R_{AB} cotransfection (Fig. 3), we conclude that endogenous PKA activity accounts for much of the basal transcription. The difference in the effects of R_{AB} and KCREB inhibition is most clearly demonstrated by comparing Fig. 3C and 4D, which display the data so that the effect of background expression is eliminated. KCREB and R_{AB} overexpression effectively inhibit Cα-mediated transactivation (Figs. 3C and 4D), while only KCREB overexpression inhibits CaMKIV-mediated transactivation of α168-luciferase (Fig. 4D). This finding suggests that the activity of CREB/ATF family members, but not PKA activity, is necessary for the CaMKIV-mediated stimulation of α168-luciferase.

CaMKIV phosphorylates CREB exclusively at serine 133 in vitro. The site of CREB phosphorylation by CaMKIV in vitro was examined by using recombinant CaMKIV313 to phosphorylate wild-type CREB and CREB S133A. As depicted in Fig. 5, purified CaMKIV313 phosphorylates wild-type CREB to a maximum level of one incorporated phosphate per CREB molecule, reaching this maximum after 6 to 8 min. CREB S133A is not appreciably phosphorylated by CaMKIV (Fig. 5). The inset displays an autoradiograph depicting the phosphorylation of wild-type CREB by recombinant CaMKIV313 after an 8-min incubation at 30°C with or without Ca^{2+}, demonstrating that the constitutive CaMKIV is active in the absence of Ca^{2+} and that CaMKIV313 does not transfer detectable phosphate to CREB S133A. We conclude from these data that CaMKIV stoichiometrically phosphorylates Ser-133 on CREB and does not recognize additional sites.

Subcellular localization of transiently expressed CaMKII, CaMKIV, and Cα. We constructed expression vectors encoding CaMKIIα-IHG and Cα-TAG, which contain sequences encoding peptide epitopes, as described in Materials and Methods. We investigated the subcellular localization of CaMKIIα-IHG, CaMKIV, and Cα-TAG in transient transfections of JEG-3 cells to determine whether the transiently expressed kinases were able to gain access to the nucleus. The localization of transiently expressed CaMKIV was determined by using an antibody that recognizes the central regulatory domain (29); nuclei were visualized by using propidium iodide as described in Materials and Methods. Confocal images that optically section through the upper half of CaMKIV-transfected cells detect CaMKIV in the nucleus, although apparently excluded from nucleoli (Fig. 6A and B), while those through the bottom of the same cells display staining for CaMKIV that appears to be either primarily cytoplasmic (Fig.

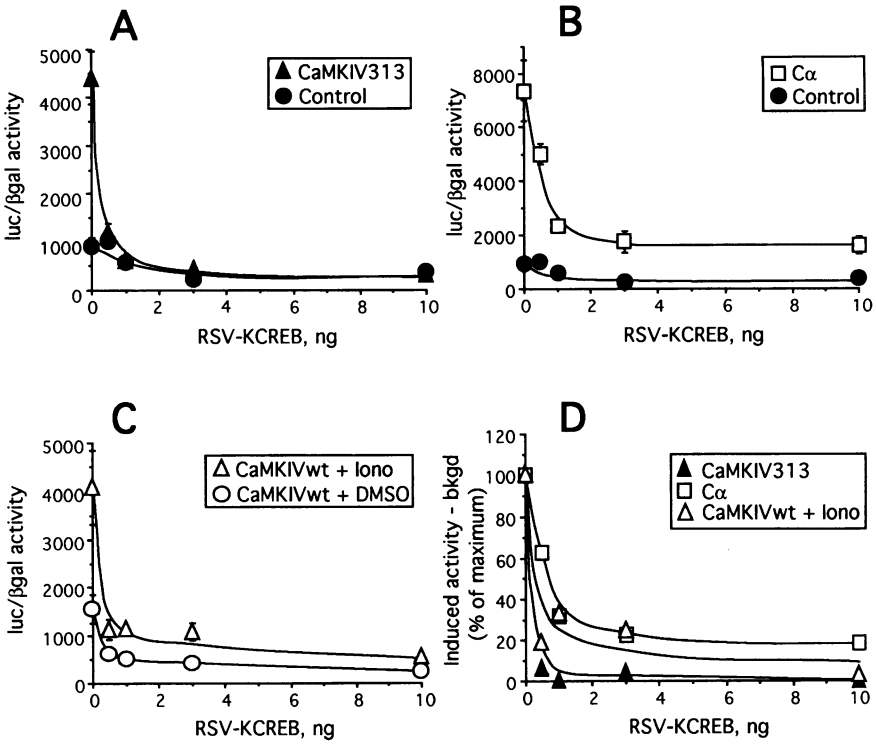

FIG. 4. CaMKIV-induced α168-luciferase expression is inhibited by a dominant negative CREB mutant. (A) JEG-3 cells were transfected with the indicated amounts of KCREB plus, per well, 30 ng of CaMKIV313 and 2.5 ng of α168-luciferase (filled triangles) or 2.5 ng of α168-luciferase alone (filled circles). After 24 h of DNA exposure, cells were treated with medium containing 80 μM Zn^{2+} for 16 h, at which point cells were lysed and assayed. (B) JEG-3 cells were transfected with the indicated amounts of KCREB plus, per well, 1 ng of Cα and 2.5 ng of α168-luciferase (open squares) or 2.5 ng of α168-luciferase alone (filled circles) and were then treated and assayed, as for panel A. (C) JEG-3 cells were transfected with the indicated amounts of KCREB plus 30 ng of CaMKIVwt and 2.5 ng of α168-luciferase per well. After 24 h of DNA exposure, cells were treated with medium containing 80 μM Zn^{2+} for 14 h, at which point cells were incubated with medium containing 1 μM ionomycin (Iono) and 1.5 mM $CaCl_2$ for 5 h (open triangles) or with the same amount of dimethyl sulfoxide (DMSO) (open circles). The transfections were performed in triplicate, and the mean ± standard deviation is shown for each datum point. In all experiments, the amount of RSV promoter was kept constant by the addition of RSV-*neo* so that all conditions included 10 ng of RSV per well in addition to 50 ng of RSV-*lacZ* per well. Datum points are presented as a ratio of luciferase (luc) activity to β-galactosidase (βgal) activity, resulting from expression of the internal control plasmid, RSV-*lacZ*. (D) The background (bkgd) control activity was subtracted from each point in panels A (filled triangles), B (open squares), and C (open triangles), and the absolute activity at 0 ng of KCREB per well was set at 100%.

6C) or in both nuclear and cytoplasmic compartments (Fig. 6D). Figures 6E and F depict JEG-3 cells transiently transfected with the CaMKIIα-IHG expression vector and stained with anti-IHG antibody and propidium iodide as described in Materials and Methods. In contrast to the cells depicted in Figs. 6A to D, the CaMKIIα-IHG-transfected cells display CaMKIIα-IHG staining exclusively in the cytoplasm (Fig. 6E and F), throughout all cellular focal planes (not shown). By comparison, Cα-TAG is localized entirely in the cytoplasm when RIIα is coexpressed (Fig. 6G), forming the inactive holoenzyme. When Cα-TAG is expressed without RIIα, the staining for Cα-TAG is found in both the nucleus and the cytoplasm (Fig. 6H). These results indicate that transiently expressed CaMKIV and Cα-TAG may be localized to the nucleus in conditions that correlate with the activation of α168-luciferase expression described above. Transiently expressed full-length CaMKIIα-IHG is excluded from the nucleus.

DISCUSSION

The transient expression of CaMKIV in JEG-3 cells confers on these cells the ability to induce a CRE-containing reporter, α168-luciferase. This transactivation is elicited directly by a truncated, constitutively active form of CaMKIV or by wild-type CaMKIV if cells have also been treated with ionomycin or thapsigargin to elevate intracellular calcium. The stimulation of α168-luciferase by CaMKIV appears to be independent of endogenous PKA activity, since we have shown that transient coexpression of a dominant inhibitory R subunit (R_{AB}) does not block transactivation by CaMKIV. The induction of α168-luciferase by CaMKIV does appear to be dependent on CREB or other members of the CREB/ATF family such as ATF-1 or CREM, however, since coexpression of a dominant negative CREB inhibits CaMKIV stimulation of α168-luciferase. Furthermore, using purified proteins, we have shown that CaMKIV stoichiometrically phosphorylates a single residue on CREB, serine 133, which corresponds to the residue previously shown to be necessary for CREB activation by PKA in vivo (17). These results demonstrate that CaMKIV is capable of mediating a Ca^{2+}-dependent activation of CRE-containing promoters.

In contrast to CaMKIV, transient expression of CaMKIIα does not mediate the activation of α168-luciferase in JEG-3 cells. We have shown that neither the constitutively active form of CaMKIIα nor the wild-type form, in the presence of agents

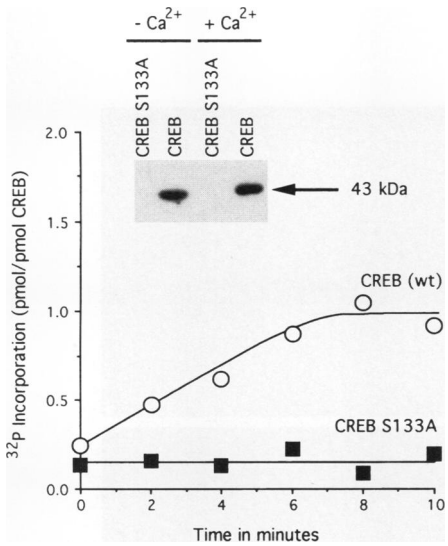

FIG. 5. CaMKIV phosphorylates CREB at serine 133 in vitro. Recombinant CaMKIV313 was incubated with wild-type (wt) CREB or CREB S133A at 30°C for the times indicated as described in Materials and Methods. Data are depicted as a stoichiometric ratio of phosphate incorporated per CREB molecule. The inset displays an autoradiogram of the polyacrylamide gel electrophoresis analysis of CaMKIV313 phosphorylation reactions with CREB or CREB S133A with or without Ca^{2+} after an 8-min incubation at 30°C.

that increase intracellular calcium, activates α168-luciferase. The cells are expressing CaMKIIα protein, as demonstrated by immunocytochemistry, and the constitutive CaMKII290 is biologically active and capable of activating a *fos-lacZ* reporter gene, presumably through a nuclear event. This activation of *fos-lacZ* by CaMKII290 is not dependent on either PKA or CREB activity (19), implying that CaMKII targets a transcription factor other than CREB in the activation of the *fos* promoter. Other reports have suggested that CaMKII may be responsible for the calcium-mediated activation of CRE-modulated genes in PC12 cells and in hippocampal neurons (10, 47). Our results suggest that CaMKIV may be a more likely mediator of Ca^{2+}-dependent gene activation involving CRE-containing promoters.

The activation of CRE-containing promoters by phosphorylation of CREB requires that the kinase have access to CREB, which is localized in the nucleus (53). CaMKIV has been shown previously to be localized to the nucleus by electron microscopy (21); our results indicate that in JEG-3 cells, expressed CaMKIV is detectable not only in the nucleus but in the cytoplasm as well. This distribution favoring the cytoplasm may be cell type specific or influenced by the relatively high expression levels seen in transient transfections. The nonuniform distribution of CaMKIV within the nucleus as visualized at different focal planes may indicate the presence of specific nuclear binding sites. Our results clearly indicate, however, that CaMKIV is able to reach the nuclear compartment where phosphorylation of CREB is thought to occur. A comparison of the apparent ratio of nuclear to cytosolic CaMKIV with that of the relative distribution of the PKA Cα subunit between nucleus and cytosol indicates that the relative amount of CaMKIV in the nucleus is less than that of the C subunit, suggesting that most of the transfected CaMKIV remains in the cytosol. This difference in localization may limit access of CaMKIV to CREB and therefore may partially explain the reduced effectiveness of CaMKIV in gene induction relative to PKA.

In contrast to CaMKIV, staining of cells transfected with IHG-tagged full-length CaMKIIα indicates that this protein is found only in the cytoplasm, in agreement with previous studies examining the localization of CaMKII in neurons (38). Recently, however, alternatively spliced isoforms of the CaMKII family that contain nuclear translocation signals have been discovered (44). In our transactivation studies, we used the truncated CaMKII290, because this protein is both constitutively active and unlikely to be excluded from the nucleus. Overexpression of CaMKII290 has been shown previously to cause arrest of the cell cycle in G_2 in C127 cells (40). Slightly higher molecular weight truncations (positions 1 to 326) of CaMKII are able to enter the nucleus, as determined by kinase assays of nuclear preparations (44). Our results for CaMKII290 suggest that even if the full-length CaMKIIα were able to translocate into the nucleus, it would not induce transcription of CRE-driven promoters when activated by Ca^{2+}/calmodulin.

The inability of the constitutively active CaMKII290 to transactivate a CREB-dependent reporter such as α168-luciferase was surprising, especially since CaMKIV313 is an active inducer. Both kinases phosphorylate CREB on Ser-133 in vitro, but CaMKII has also been shown to phosphorylate other unidentified sites on CREB as well (10, 47). Recent work by Sun et al. (51) demonstrates that CaMKII phosphorylates CREB on Ser-142 as well as Ser-133 and that phosphorylation of CREB at Ser-142 inhibits the ability of CREB to activate transcription. These investigators and others also demonstrate that CaMKIV is an effective inducer of CREB-dependent transcription in GH_3 pituitary cells, as determined by CaMKIV-mediated stimulation of GAL4 constructs by GAL4-CREB (13, 51), which supports our results for JEG-3 cells.

The magnitude of α168-luciferase stimulation by PKA is much higher than that with CaMKIV under similar conditions. The induction of α168-luciferase by CaMKIV ranges from 3- to 5-fold, while the maximal C-induced stimulation is as high as 50-fold. The expression vectors used are identical except for the coding region, but we cannot directly compare the levels of expressed protein in our immunocytochemistry studies since different antibodies were used for detection. Recently, in vitro analysis of CREB phosphorylation by CaMKIV and PKA has revealed that although the K_ms of CaMKIV and PKA for CREB are similar, the V_{max} of CREB phosphorylation by PKA is 30-fold greater than the CREB phosphorylation V_{max} for CaMKIV (13). Certainly such a difference in the kinetics of in vivo phosphorylation in our experiments on JEG-3 cells may be responsible for the different degrees of stimulation by CaMKIV and PKA. Partial exclusion of CaMKIV from the nucleus, due to limited diffusion of CaMKIV into the nucleus or a rate-limiting step in nuclear transport, may also explain the reduced activity of CaMKIV as a transcriptional activator. Others have suggested that CaMKIV must be phosphorylated for maximal activity and that this phosphorylation requires Ca^{2+}/calmodulin and a protein activator that may also be a protein kinase (34). This phosphorylation appears to be on sites near the amino terminus (27) and would therefore be present in both CaMKIVwt and CaMKIV313. If JEG-3 cells do not contain the putative CaMKIV activator, this could explain the lower apparent activity of CaMKIV relative to Cα in our transfection assays.

Our results help clarify several issues regarding the ability of Ca^{2+} to stimulate gene transcription. We have demonstrated a remarkable specificity in the ability of the type II and type IV

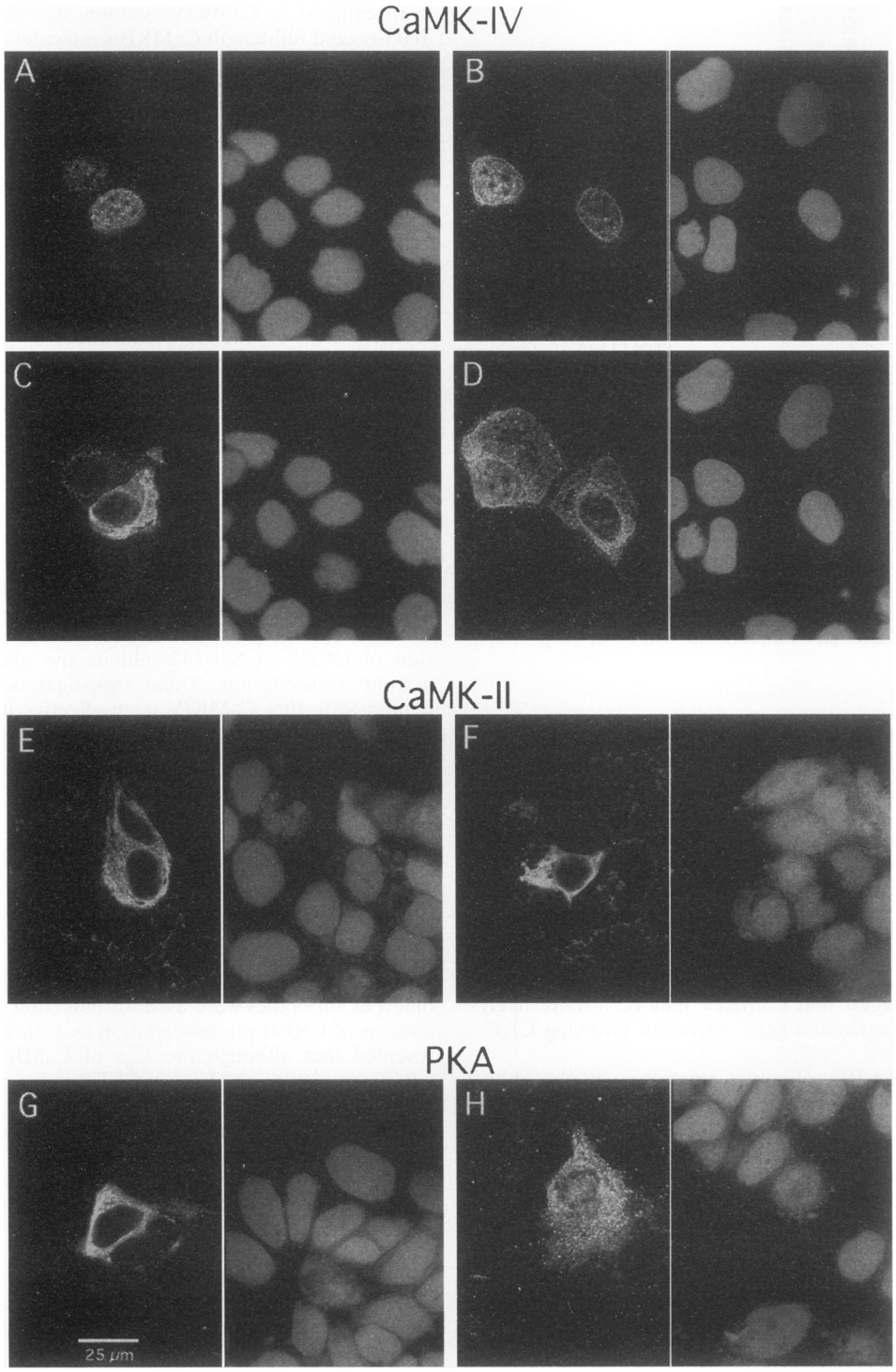

FIG. 6. Subcellular localization of expressed CaMKIV, CaMKII, and the PKA Cα subunit. JEG-3 cells were transiently transfected with, per well, 30 ng of CaMKIVwt expression vector (A to D), 30 ng of CaMKIIα-IHG expression vector (E and F), 2.5 ng of Cα-TAG and 25 ng of RIIα expression vectors (G), or 2.5 ng of Cα-TAG expression vector alone (H) and then subjected to Zn^{2+} treatment and fluorescent staining as described in Materials and Methods. Cells were stained with anti-CaMKIV antiserum (A to D, left panels), anti-IHG antibody 12CA5 (E and F, left panels), anti-TAG antibody AB1 (G and H, left panels), and propidium iodide (A to H, right panels). Panels A and B display 1-μm confocal optical slices though the upper half of cells, while panel C and D represent 1-μm optical slices through the lower half of the same cells. Panels E to H are 1-μm optical slices taken through the lower half of cells.

CaM kinases to induce CRE-driven gene expression in transient transfection assays and conclude that only the type IV kinase has this ability. Although we have yet to determine whether CaMKIV stimulates Ca^{2+}-mediated CRE-driven gene expression in actual physiological processes, we believe that the ability of CaMKIV to elicit Ca^{2+}-mediated gene activation in our transfection studies strongly suggests that CaMKIV is a likely player in Ca^{2+}-mediated gene activation in vivo. Other potential multifunctional Ca^{2+}/calmodulin-dependent protein kinases, such as CaMKI (32, 39) and other isoforms of CaMKII (43), may activate CRE-mediated transcription through Ca^{2+}, although their ability to do so remains to be examined. The overall extent of CRE-dependent gene induction in response to elevated Ca^{2+} may depend on which types of CaM kinases are expressed in specific cell types.

We have also shown that the more complex *fos* promoter responds to both CaMKII290 and CaMKIV313. Preliminary evidence suggests that the CaMKII-mediated induction of the *fos* promoter does not depend on CREB but does require the upstream serum response element (19). The response of the *fos* promoter to CaMKIV may be regulated through the several CREs that are contained within the promoter, including the one also termed the calcium response element at nucleotide −60 (46). *fos* promoter induction has been studied in hippocampal neurons in response to Ca^{2+} entry through either glutamate receptors or L-type Ca^{2+} channels. Constructs containing the CRE were induced by Ca^{2+} entry through L-type Ca^{2+} channels but not by glutamate receptor activation, whereas similar constructs containing the serum response element regained the ability to respond to glutamate receptor activation (1). The hippocampal neurons used in these studies may express isoforms of CaMKII as well as CaMKIV; our results would suggest that they could play distinct roles in the induction pathway.

We must also consider the potential role of PKA in the response to intracellular Ca^{2+}, since many neurons express Ca^{2+}/calmodulin-sensitive adenylate cyclases (4) which have been shown to become activated in neurons after depolarization (50). Interestingly, the induction of *fos* in response to depolarization is strongly inhibited if PC12 cells are deficient in PKA activity (16). Thus, the PKA system may also become activated by elevated Ca^{2+} in certain cell types, which would then lead to an increase in CRE-driven gene expression similar to the increase produced by CaMKIV activation. Although our experiments demonstrate that Ca^{2+}, acting through CaMKIV, can induce a CRE-driven promoter in the absence of functional PKA, it seems very likely that both kinase systems interact with each other in the regulation of gene expression under physiological conditions.

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REFERENCES

1. **Bading, H., D. D. Ginty, and M. E. Greenberg.** 1993. Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. Science **260:**181–186.
2. **Bentley, J. K., and J. A. Beavo.** 1992. Regulation and function of cyclic nucleotides. Curr. Opin. Cell Biol. **4:**233–240.
3. **Boutillier, A.-L., F. Barthel, J. L. Roberts, and J.-P. Loeffler.** 1992. β-Adrenergic stimulation of cFOS by protein kinase A is mediated by cAMP regulatory element binding protein (CREB)-dependent and tissue-specific CREB-independent mechanisms in corticotrope cells. J. Biol. Chem. **267:**23520–23526.
4. **Choi, E. J., Z. Xia, E. C. Villacres, and D. R. Storm.** 1993. The regulatory diversity of mammalian adenylyl cyclases. Curr. Opin. Cell Biol. **5:**269–273.
5. **Clegg, C. H., L. A. Correll, G. C. Cadd, and G. S. McKnight.** 1987. Inhibition of intracellular cAMP-dependent protein kinase using mutant genes of the regulatory type I subunit. J. Biol. Chem. **262:**13111–13119.
6. **Cohen, P.** 1989. The structure and regulation of protein phosphatases. Annu. Rev. Biochem. **58:**453–508.
7. **Correll, L. A., T. A. Woodford, J. D. Corbin, P. L. Mellon, and G. S. McKnight.** 1989. Functional characterization of cAMP-binding mutations in type I protein kinase. J. Biol. Chem. **264:**16672–16678.
8. **Cruzalegui, F. H., M. S. Kapiloff, J. P. Morfin, B. E. Kemp, M. G. Rosenfeld, and A. R. Means.** 1992. Regulation of intrasteric inhibition of the multifunctional calcium/calmodulin-dependent protein kinase. Proc. Natl. Acad. Sci. USA **89:**12127–12131.
9. **Cruzalegui, F. H., and A. R. Means.** 1993. Biochemical characterization of the multifunctional Ca^{2+}/CaM-dependent protein kinase type IV expressed in insect cells. J. Biol. Chem. **268:**26171–26178.
10. **Dash, P. K., K. A. Karl, M. A. Colicos, R. Prywes, and E. R. Kandel.** 1991. cAMP response element-binding protein is activated by Ca^{2+}/calmodulin- as well as cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA **88:**5061–5065.
11. **de Wet, J. R., K. V. Wood, M. deLuca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7:**725–737.
12. **Edlund, T., M. D. Walker, P. J. Barr, and W. J. Rutter.** 1985. Cell-specific expression of the rat insulin gene: evidence for role of two distinct 5′ flanking elements. Science **230:**912–916.
13. **Enslen, H., P. Sun, D. Brickey, S. H. Soderling, E. Klamo, and T. R. Soderling.** 1994. Characterization of Ca^{2+}/calmodulin-dependent protein kinase IV: role in transcriptional regulation. J. Biol. Chem. **269:**15520–15527.
14. **Frangakis, M. V., T. Chatila, E. R. Wood, and N. Sahyoun.** 1991. Expression of a neuronal Ca^{2+}/calmodulin-dependent protein kinase, CaM kinase-Gr, in rat thymus. J. Biol. Chem. **266:**17592–17596.
15. **Frangakis, M. V., C.-A. Ohmstede, and N. Sahyoun.** 1991. A brain-specific Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase-Gr) is regulated by autophosphorylation. J. Biol. Chem. **266:**11309–11316.
16. **Ginty, D. D., D. Glowacka, D. S. Bader, H. Hidaka, and J. A. Wagner.** 1991. Induction of immediate early genes by Ca^{2+} influx requires cAMP-dependent protein kinase in PC12 cells. J. Biol. Chem. **266:**17454–17458.
17. **Gonzalez, G. A., and M. R. Montminy.** 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell **59:**675–680.
18. **Griffith, L. C., and H. Schulman.** 1988. The multi-functional calcium/calmodulin-dependent protein kinase mediates calcium-dependent phosphorylation of tyrosine hydroxylase. J. Biol. Chem. **263:**9542–9549.
19. **Guthrie, C. R., and G. S. McKnight.** Unpublished data.
20. **Hanson, P. I., and H. Schulman.** 1992. Neuronal Ca^{2+}/calmodulin-dependent protein kinases. Annu. Rev. Biochem. **61:**559–601.
21. **Jensen, K. F., C.-A. Ohmstede, R. S. Fisher, and N. Sahyoun.** 1991. Nuclear and axonal localization of Ca^{2+}/calmodulin-dependent protein kinase type Gr in rat cerebellar cortex. Proc. Natl. Acad. Sci. USA **88:**2850–2853.
22. **Jones, D. A., J. Glod, D. Wilson-Shaw, W. E. Hahn, and J. M.**

Sikela. 1991. cDNA sequence and differential expression of the mouse Ca^{2+}/calmodulin-dependent protein kinase IV gene. FEBS Lett. **289:**105–109.

23. **Kameshita, I., and H. Fujisawa.** 1991. Phosphorylation and functional modification of calmodulin-dependent protein kinase IV by cAMP-dependent protein kinase. Biochem. Biophys. Res. Commun. **180:**191–196.
24. **Kameshita, I., and H. Fujisawa.** 1993. Autophosphorylation of calmodulin-dependent protein kinase IV from rat cerebral cortex. J. Biochem. (Tokyo) **113:**583–590.
25. **Kaneseki, T., Y. Ikeuchi, H. Sugiura, and T. Yamauchi.** 1991. Structural features of Ca^{2+}/calmodulin-dependent protein kinase II revealed by electron microscopy. J. Cell Biol. **115:**1049–1060.
26. **Low, M. J., R. E. Hammer, R. H. Goodman, J. F. Habener, R. D. Palmiter, and R. L. Brinster.** 1985. Tissue-specific posttranslational processing pre-prosomatostatin encoded by a metallothionein-somatostatin fusion gene in transgenic mice. Cell **41:**211–219.
27. **McDonald, O. B., B. M. Merrill, M. M. Bland, L. C. E. Taylor, and N. Sahyoun.** 1993. Site and consequences of the autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase type "Gr." J. Biol. Chem. **268:**10054–10059.
28. **Means, A. R.** Unpublished data.
29. **Means, A. R., F. Cruzalegui, B. LeMagueresse, D. S. Needleman, G. R. Slaughter, and T. Ono.** 1991. A novel Ca^{2+}/calmodulin-dependent protein kinase and a male germ cell-specific calmodulin-binding protein are derived from the same gene. Mol. Cell. Biol. **11:**3960–3971.
30. **Mellon, P., C. H. Clegg, L. A. Correll, and G. S. McKnight.** 1989. Regulation of transcription by cyclic AMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA **86:**4887–4891.
31. **Miyano, O., I. Kameshita, and H. Fujisawa.** 1992. Purification and characterization of a brain-specific multifunctional calmodulin-dependent protein kinase from rat cerebellum. J. Biol. Chem. **267:**1198–1203.
32. **Mochizuki, H., T. Ito, and H. Hidaka.** 1993. Purification and characterization of Ca^{2+}/calmodulin-dependent protein kinase V from rat cerebrum. J. Biol. Chem. **268:**9143–9147.
33. **Ohmstede, C.-A., K. F. Jensen, and N. Sahyoun.** 1989. Ca^{2+}/calmodulin-dependent protein kinase enriched in cerebellar granule cells. Identification of a novel neuronal calmodulin-dependent protein kinase. J. Biol. Chem. **264:**5866–5875.
34. **Okuno, S., and H. Fujisawa.** 1993. Requirement of brain extract for the activity of brain calmodulin-dependent protein kinase IV expressed in *Escherichia coli*. J. Biochem. (Tokyo) **114:**167–170.
35. **Orellana, S. A., and G. S. McKnight.** 1990. The S49 kin- cell line transcribes and translates a functional mRNA coding for the catalytic subunit of cAMP-dependent protein kinase. J. Biol. Chem. **265:**3048–3053.
36. **Orellana, S. A., and G. S. McKnight.** 1992. Mutations in the catalytic subunit of cAMP-dependent protein kinase result in unregulated biological activity. Proc. Natl. Acad. Sci. USA **89:** 4726–4730.
37. **Otten, A. D., and G. S. McKnight.** 1989. Overexpression of the type II regulatory subunit of the cAMP-dependent protein kinase eliminates the type I holoenzyme in mouse cells. J. Biol. Chem. **264:**20255–20260.
38. **Ouimet, C. C., T. L. McGuinness, and P. Greengard.** 1984. Immunocytochemical localization of calcium/calmodulin-dependent protein kinase II in rat brain. Proc. Natl. Acad. Sci. USA **81:** 5604–5608.
39. **Picciotto, M. R., A. J. Czernik, and A. C. Nairn.** 1993. Calcium/calmodulin-dependent protein kinase I. J. Biol. Chem. **268:**26512–26521.
40. **Planas-Silva, M. D., and A. R. Means.** 1992. Expression of a constitutive form of calcium/calmodulin dependent protein kinase II leads to arrest of the cell cycle in G2. EMBO J. **11:**507–517.
41. **Sahyoun, N., O. B. McDonald, F. Farrell, and E. G. Lapetina.** 1991. Phosphorylation of a ras-related GTP-binding protein, rap-1b, by a neuronal Ca^{2+}/calmodulin-dependent protein kinase, CaM kinase Gr. Proc. Natl. Acad. Sci. USA **88:**2643–2647.
42. **Schilling, K., D. Luk, J. I. Morgan, and T. Curran.** 1991. Regulation of a fos-lacZ fusion gene: a paradigm for quantitative analysis of stimulus-transcription coupling. Proc. Natl. Acad. Sci. USA **88:**5665–5669.
43. **Schulman, H.** 1993. The multifunctional Ca^{2+}/calmodulin-dependent protein kinases. Curr. Opin. Cell. Biol. **5:**247–253.
44. **Schulman, H. (Stanford University).** Personal communication.
45. **Sculptoreanu, A., T. Scheuer, and W. A. Catterall.** 1993. Voltage-dependent potentiation of L-type Ca^{2+} channels due to phophorylation by cAMP-dependent protein kinase. Nature (London) **364:**240–243.
46. **Sheng, M., G. McFadden, and M. E. Greenberg.** 1990. Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. Neuron **4:**571–582.
47. **Sheng, M., M. A. Thompson, and M. E. Greenberg.** 1991. CREB: a Ca^{2+}-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science **252:**1427–1430.
48. **Silva, A. J., R. Paylor, J. M. Wehner, and S. Tonegawa.** 1992. Impaired spatial learning in α-calcium-calmodulin kinase II mutant mice. Science **257:**206–211.
49. **Silva, A. J., C. F. Stevens, S. Tonegawa, and Y. Wang.** 1992. Deficient hippocampal long-term potentiation in α-calcium-calmodulin kinase II mutant mice. Science **257:**201–206.
50. **Suidan, H. S., R. D. Murrell, and A. M. Tolkovsky.** 1991. Carbachol and bradykinin elevate cyclic AMP and rapidly deplete ATP in cultured rat sympathetic neurons. Cell Regul. **2:**13–25.
51. **Sun, P., H. Enslen, P. S. Myung, and R. A. Maurer.** Submitted for publication.
52. **Thastrup, O., P. J. Cullen, B. K. Drøbak, M. R. Hanley, and A. P. Dawson.** 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc. Natl. Acad. Sci. USA **87:**2466–2470.
53. **Waeber, G., and J. F. Habener.** 1991. Nuclear translocation and DNA recognition signals colocalize within the bZIP domain of cyclic adenosine 3′,5′-monophosphate response element-binding protein CREB. Mol. Endocrinol. **5:**1431–1438.
54. **Walton, K. M., R. P. Rehfuss, J. C. Chrivia, J. E. Lochner, and R. H. Goodman.** 1992. A dominant repressor of cyclic adenosine 3′,5′-monophosphate (cAMP)-regulated enhancer binding protein activity inhibits the cAMP-mediated induction of the somatostatin promoter in vivo. Mol. Endocrinol. **6:**647–655.
55. **Wegner, M., Z. Cao, and M. G. Rosenfeld.** 1992. Calcium-regulated phosphorylation within the leucine zipper of C/EBPβ. Science **256:**370–373.