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Regulation of mitogen-activated protein kinases by a calcium/calmodulin-dependent protein kinase cascade

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ABSTRACT **Membrane depolarization of NG108 cells gives rapid (<5 min) activation of Ca^{2+}/calmodulin-dependent protein kinase IV (CaM-KIV), as well as activation of c-Jun N-terminal kinase (JNK). To investigate whether the Ca^{2+}-dependent activation of mitogen-activated protein kinases (ERK, JNK, and p38) might be mediated by the CaM kinase cascade, we have transfected PC12 cells, which lack CaM-KIV, with constitutively active mutants of CaM kinase kinase and/or CaM-KIV (CaM-KK_c and CaM-KIV_c, respectively). In the absence of depolarization, CaM-KK_c transfection had no effect on Elk-dependent transcription of a luciferase reporter gene, whereas CaM-KIV_c alone or in combination with CaM-KK_c gave 7- to 10-fold and 60- to 80-fold stimulations, respectively, which were blocked by mitogen-activated protein (MAP) kinase phosphatase cotransfection. When epitope-tagged constructs of MAP kinases were cotransfected with CaM-KK_c plus CaM-KIV_c, the immunoprecipitated MAP kinases were activated 2-fold (ERK-2) and 7- to 10-fold (JNK-1 and p38). The JNK and p38 pathways were further investigated using specific c-Jun or ATF2-dependent transcriptional assays. We found that c-Jun/ATF2-dependent transcriptions were enhanced 7- to 10-fold by CaM-KIV_c and 20- to 30-fold by CaM-KK_c plus CaM-KIV_c. In the case of the Jun-dependent transcription, this effect was not due to direct phosphorylation of c-Jun by activated CaM-KIV, since transcription was blocked by a dominant-negative JNK and by two MAP kinase phosphatases. Mutation of the phosphorylation site (Thr^{196}) in CaM-KIV, which mediates its activation by CaM-KIV kinase, prevented activation of Elk-1, c-Jun, and ATF2 by the CaM kinase cascade. These results establish a new Ca^{2+}-dependent mechanism for regulating MAP kinase pathways and resultant transcription.**

In many cell types, extracellular stimuli, such as growth factors, neurotransmitters, and lymphokines, regulate critical cellular events, such as growth, differentiation, and apoptosis, through activation of protein kinase cascades. Although many of these stimuli trigger Ser/Thr kinase cascades through initial activation of their receptor-associated tyrosine kinases, recent studies indicate that elevated intracellular Ca^{2+} can also activate some cascades, including the mitogen-activated protein (MAP) kinase pathways (1–7). The activated MAP kinases regulate the transcription of mitogen- (8, 9) or stress-activated (10) genes through the phosphorylation of transcription factors, such as TCF, c-Jun, and ATF2 (11, 12). In the case of the extracellular regulated kinase (ERK) subgroup of MAP kinases, it is clear that activation of voltage-dependent Ca^{2+} channels results in a Src/Ras-dependent activation of ERK in PC12 cells (2, 7). Calcium has also been reported to regulate the 42- and 44-kDa isoforms of MAP kinases (ERK-1 and ERK-2) in several different cell types (1, 2, 4, 6) and to activate another member of the MAP kinase family, the c-Jun N-terminal kinase (JNK; ref. 13) cascade (3–5). The JNK family generally promotes cell growth inhibition (14–17) and apoptosis (18) in response to stress signals. Thus, while it is clear that Ca^{2+} can modulate the MAP kinase pathways, the detailed mechanisms are not established.

One of the most common mechanisms by which elevated intracellular Ca^{2+} regulates cellular events is through its association with calmodulin (CaM). The Ca^{2+}/CaM complex binds to and modulates the functions of multiple key regulatory proteins, including a family of CaM kinases (CaM-K; ref. 19). A role for transcriptional regulation by CaM-K is suggested by the observation that the Ca^{2+}-dependent transcription of three immediate early genes (c-*fos*, NGFI-A, and NGFI-B) was blocked 80% by the CaM-K inhibitor KN-62 (20). In the past 2 years, a CaM-K cascade has been identified (21, 22) in which CaM-K kinase (CaM-KK; ref. 23) phosphorylates and activates *in vitro* CaM-KI and CaM-KIV (23–25). CaM-KIV, which has significant nuclear localization (26), phosphorylates transcription factors, such as cAMP-responsive element binding protein (CREB) and serum response factor (SRF; refs. 27–29). Several studies have shown that CaM-KIV can mediate transcriptional stimulation through CREB phosphorylation (28, 30, 31). More recently we have demonstrated that cotransfection of CaM-KIV with CaM-KK stimulates CREB-mediated transcription 14-fold relative to either kinase alone (23). This is consistent with the *in vitro* observation that the V_{max}/K_m ratio of CREB phosphorylation was increased 30-fold by activation of CaM-KIV by CaM-KK (32).

In this report we have investigated the potential interactions between the CaM-K cascade and members of the MAP kinase signaling pathways. We find that the CaM-K cascade can activate the MAP kinases (ERK-2, JNK-1, and p38) and stimulate transcription through the phosphorylation of Elk-1, c-Jun, and ATF2. The results of our study suggest that CaM-KIV may direct transcriptional activation of mitogen- and stress-activated genes through this mechanism, and that the CaM-kinase cascade may mediate some of the effects of intracellular Ca^{2+} on cellular growth and stress responses.

MATERIALS AND METHODS

Plasmids. The pMe18S vector and the full-length, constitutively active form ($HMDT^{308}$ to $DEDT^{308}$) of CaM-KIV (CaM-KIV_c) cloned into the *Xba*I–*Not*I restriction sites of pME18S have been described (22, 23). CaM-KIV_cA was made by digesting the cDNA encoding CaM-KIV_c with *Bst*BI and *Bst*XI, and insertion of the *Bst*BI–*Bst*XI fragment (502 bp) of

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Abbreviations: MAP, mitogen-activated protein; ERK, extracellular regulated kinase(s); JNK, c-Jun N-terminal kinase(s); CaM, calmodulin; CaM-K, CaM kinase(s); CaM-KK, CaM-K kinase(s); CaM-KIV_c, constitutively active form of CaM-KIV; CaM-KK_c, constitutively active form of CaM-KK; MKP, MAP kinase phosphatase; DnJNK, dominant-negative JNK; CREB, cAMP-responsive element binding protein; SRE, serum response element; SRF, serum response factor.

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the CaM-KIV cDNA wild-type, in which Thr^{196} had been mutated to Ala (33). The constitutively active, truncated form of CaM-KK (residues 1–413; CaM-KK_c) was made as follows. The PCR fragment of the 3′-truncated end of CaM-KK, amplified using 5′-GGG GCG GCC GCT CAT CCA TGC TTG GTC ACC CAA GGG-3′ and 5′-AAA GAC CTG ATC CTG AA-3′ as primers and wild-type CaM-KK cDNA as template, was digested with *Eco*RV and *Not*I and inserted into *Eco*RV–*Not*I-digested pME-CaM-KK wild-type plasmid. The sequence of the inserted portion of this plasmid was confirmed by conventional nucleotide sequencing. The plasmids 5G4E1B-Luc, Gal4-Elk-1, Gal4-Elk-1S383A, Gal4-Jun, Gal4-JunS63/73A, MAP kinase phosphatase (MKP)-1, MKP-2, dominant-negative JNK (DnJNK), constitutively-active Raf-1 (BxB-raf), the human oncogene H-*ras* (EJ-*ras*), MKK3 (MKK3 glu), JNK-1-flag, p38-flag, and ERK2-HA have been described (12, 13, 23, 34–41).

Cell Culture and Transient Transfection Assays. NG108 cells were maintained in Dulbecco's modified Eagle's serum containing 10% fetal calf serum supplemented with hypoxanthine, aminopterin, and thymidine at 37°C in 5% CO_2. PC12 cells were maintained in Dulbecco's modified Eagle's serum containing 5% horse serum and 5% fetal calf serum at 37°C in 5% CO_2. Transfections were done (42) in 10-cm plates (Primaria; GIBCO/BRL) with 10–15 μg of the reporter plasmid 5Gal4E1B-luc, 8 μg of either the Gal4-Elk-1, Gal4-Elk-1S383A, Gal4-Jun, or Gal4-JunS63/73A plasmids, 10–15 μg of the KIV_c and/or KK_c or KII_c plasmids, and 15–20 μg of MKP-1, MKP-2, or DnJNK. For the kinase assay, 5–15 μg of plasmid encoding ERK2 tagged with the hemagglutinin epitope (generously provided by P. Lenormand, Institut National de la Santé et de la Recherche Médicale, Université de Nice, France) or JNK and p38 tagged with the flag epitope were used.

Transcriptional Activation. The cells were rinsed three times with ice-cold PBS, pelleted, and frozen. The frozen pellets were thawed and resuspended with the Luciferase Cell Culture Lysis Reagent from Promega (catalog no. E1531) according to the manufacturer's instructions and assayed for luciferase activity. The results are presented as light units per μg of total protein.

Immunoprecipitation and Kinase Assay. Endogenous CaM-KK activity present in NG108 cells or transfected CaM-KK_c activity present in the cell extract of the transfected COS7 cells was tested on Sf9 cell-expressed and purified CaM-KIV and/or CaM-KIV_c (22) as described (23). Immunoprecipitation and kinase assays of epitope-tagged ERK-2, JNK-1, and p38 have been described (13, 40, 41). Immunoprecipitation and kinase assays of endogenous CaM-KIV and endogenous JNK-1 were done following the same protocol and recombinant His-tagged CREB (32) was used as a substrate for the CaM-KIV assay. The results were quantitated by PhosphoImager (Molecular Dynamics) analysis. The anti-flag epitope antibody was purchased from Kodak, the anti-hemagglutinin antibody was generously provided by P. Lenormand, and the anti-JNK-1 (C-17, catalog no. sc-474) and CaM-KIV (catalog no. C28420) antibodies were purchased from Santa Cruz Biotechnology and Transduction Laboratories (Lexington, KY), respectively.

RESULTS

Membrane Depolarization Activates CaM-KIV and JNK-1 in NG108 Cells. To determine which cell lines contain the components of the CaM-K cascade, we screened several cell lines by Western blot using an antibody against human CaM-KIV. Fig. 1*A* shows that CaM-KIV protein could be detected in Jurkat cells (lane 1) and NG108 cells (lane 3) but not in PC12 cells (lane 2). The inability to detect CaM-KIV in PC12 cells was not due to a species difference, as rat brain gave a strongly reacting 60-kDa CaM-KIV band (data not shown). We have previously purified CaM-KK from Jurkat cells (43). The presence of CaM-KK in NG108 cells was determined by its ability to activate recombinant wild-type CaM-KIV (Fig. 1*B*). Elevation of intracellular Ca^{2+} by treatment of Jurkat cells with anti-CD3 receptor or with ionomycin (43) or NG108 cells with 80 mM KCl plus 10 mM Ca^{2+} (Fig. 1*C*) results in activation of CaM-KIV within 1–5 min. We next tested the effect of an increased concentration of intracellular Ca^{2+} on the activation of a member of the MAP kinase family, JNK, in NG108 cells. Fig. 1*D* shows that, within 5 min of depolarization, there was a very strong activation of JNK-1 (lane 2), which was maintained for up to 2 hr (lane 5). These results demonstrate that, in a cell line containing the components of the CaM-kinase cascade (i.e., CaM-KIV and CaM-KK), an increased intracellular concentration of Ca^{2+} rapidly activated both CaM-KIV and JNK-1.

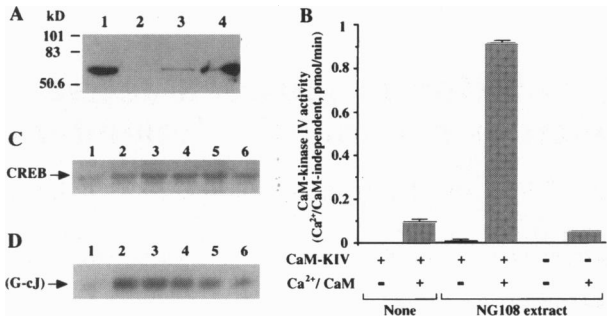

FIG. 1. Ca^{2+}-dependent activation of the CaM-K cascade and JNK in NG108 cells. (*A*) Western analyses for CaM-KIV. Twenty micrograms of total cell extracts from Jurkat (lane 1), PC12 (lane 2), and NG108 (lane 3) cells, as well as 10 ng of recombinant mouse CaM-kinase IV (lane 4), were analyzed using anti-CaM-KIV as described. (*B*) CaM-KK activity in NG108 cells. NG108 cell extract (2 μg), prepared as described, or the extraction buffer alone was incubated with 5.4 μM recombinant CaM-KIV (or kinase dilution buffer alone) in a kinase reaction in the presence or absence of 1 mM $CaCl_2$/10 μM CaM for 20 min at 30°C. After terminating the reaction, Ca^{2+}/CaM-independent activity of CaM-KIV was measured in the presence of 2 mM EGTA, 10 mM $Mg(Ac)_2$ and 0.4 mM [γ-^{32}P]ATP using 40 μM syntide-2 as a substrate. (*C*) Ca^{2+}-dependent activation of CaM-KIV in NG108 cells. NG108 cells were treated for 0, 0.5, 1, 3, 5, and 10 min (lanes 1–6, respectively) with 80 mM KCl and 10 mM $CaCl_2$. After lysing the cells, the ability of immunoprecipitated CaM-KIV to phosphorylate recombinant His-tagged CREB (CREB) was tested. (*D*) Ca^{2+}-dependent activation of JNK in NG108 cells. NG108 cells were treated for 0, 5, 20, 45, and 120 min (lanes 1–5, respectively) with 80 mM KCl and 10 mM $CaCl_2$ or for 45 min to UV irradiation (lane 6). Cells were lysed, and the ability of immunoprecipitated JNK to phosphorylate a recombinant glutathione *S*-transferase–c-Jun fusion protein (G-cJ) was tested.

Activation of CaM-KIV_c by Constitutively Active Truncated CaM-KK_{1-413}. We wanted to test the involvement of the CaM-kinase cascade in the regulation of the MAP kinases in cells lacking CaM-KIV and in the absence of elevated intracellular calcium by transfecting with CaM-KIV_c and CaM-KK_c. We have previously shown that a charge-insertion mutation ($HMDT^{308}$ to $DEDD^{308}$) in the autoinhibitory domain of CaM-KIV is active without Ca^{2+}/CaM (22) and that this mutant (CaM-KIV_c) can be further activated 10-fold by CaM-KK (23). To generate CaM-KK_c, we truncated at residue 413 to remove the regulatory domain (33). Fig. 2*A* shows that a cell extract from COS7 cells transfected with CaM-KK_c potentiates, in the absence of Ca^{2+}/CaM, by 10- to 15-fold the activity of recombinant CaM-KIV_c *in vitro*. This data demonstrate that CaM-KK_c is constitutively active (i.e., does not require Ca^{2+}/CaM) and potentiates CaM-KIV_c activity through the phosphorylation of Thr^{196} (25, 33).

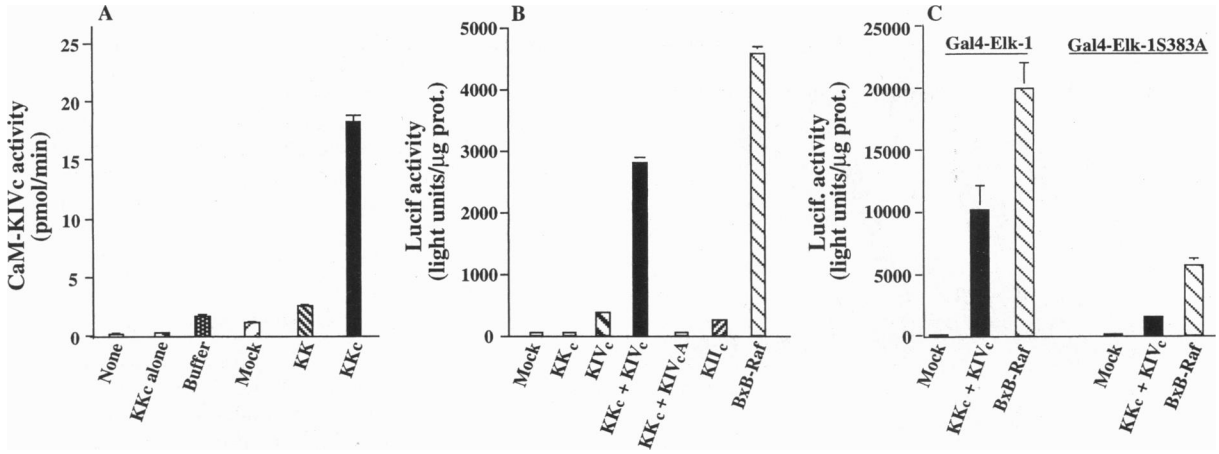

FIG. 2. The CaM-kinase cascade increases gene expression through the phosphorylation of Elk-1. (*A*) Activation of recombinant CaM-KIV$_c$ by CaM-KK$_c$. Recombinant CaM-KIV$_c$ was incubated at 30°C for 30 min with either the lysate buffer (Buffer), lysate from COS-7 cells (3 μg) transfected with plasmid (Mock), plasmid expressing CaM-KK wild type (KK), or CaM-KK$_c$ (KK$_c$) in a kinase activation reaction with 1 mM EGTA. After terminating the activation, CaM-KIV$_c$ activity was measured using 40 μM syntide-2 in the presence of EGTA under standard assay conditions (23). Kinase activity toward syntide-2 of the assay reaction mix (None) or from the COS-7 lysate of cells transfected with CaM-KK$_c$ (KK$_c$ alone) were also measured in the absence of exogenous CaM-KIV$_c$ under the same conditions, but they were negligible. The mean ± SEM of three experiments using three independent transfections is shown. (*B*) PC12 cells were transfected with vectors encoding a Gal4-luciferase reporter gene, a Gal4–Elk-1 fusion protein and CaM-KIV$_c$ and/or CaM-KK$_c$ as indicated. Luciferase activity was measured as detailed, and data from a single experiment (transfections in triplicate) are reported as mean ± SE and are representative of at least three different experiments. The effect of the constitutively active members of the CaM-kinase cascade (KK$_c$ and KIV$_c$) were compared with a constitutively-active Raf-1 kinase (BxB-Raf). The ability of a mutated form of KIV$_c$ (Thr^{196} → Ala, KIV$_c$A) that cannot be activated by CaM-KK and a truncated, constitutively active form of CaM-KII (KII$_c$) to transactivate Elk-1 were determined. (*C*) The effect of mutation of the activating Elk-1 phosphorylation site Ser^{383} → Ala was examined as in *B*.

The CaM-Kinase Cascade Activates Transcription Through Phosphorylation of Elk-1. Both ERK and JNK have been reported to be regulated by Ca^{2+} and to activate transcription through the phosphorylation of the transcription factor Elk-1 (34, 39, 44–46). To investigate whether the CaM-K cascade can modulate ERK or JNK, we assessed the ability of transfected CaM-Kinases to stimulate transcription mediated by Elk-1. The use of CaM-KK$_c$ and CaM-KIV$_c$ allowed us to bypass the requirement of the CaM-K cascade for Ca^{2+}/CaM, thereby avoiding the complex effects of increased intracellular Ca^{2+} on endogenous members of the MAP kinases (2, 5, 7) and phosphatases. CaM-KK$_c$ and CaM-KIV$_c$ were cotransfected, individually or in combination, with an expression vector that encodes the Elk-1 activation domain (residues 307–428) fused to the GAL4 DNA-binding domain. Transcription was quantitated using a GAL4/luciferase reporter gene construct.

Transfection with CaM-KK$_c$ had no effect on Elk-1-mediated transcription, whereas transfection with CaM-KIV$_c$ gave a 7- to 10-fold increase in transcription, which was enhanced a further 6- to 10-fold by cotransfection of both kinases (Fig. 2*B*). Since CaM-KK activates CaM-KIV through phosphorylation on Thr^{196} (25, 33), we determined the effectiveness of the Thr^{196} → Ala mutant of CaM-KIV$_c$ (CaM-KIV$_c$A). Cotransfection of CaM-KK$_c$ with the mutant CaM-KIV$_c$A did not activate Elk-1-dependent transcription, supporting the conclusion that the CaM-K cascade mediates the increased transcriptional activity (Fig. 2*B*). The 60- to 80-fold transcriptional induction by CaM-KK$_c$ plus CaM-KIV$_c$, which was blocked (70–90%) by cotransfection with a MPK expression vector (data not shown), was similar in magnitude to the induction by a constitutively active form of Raf-1 (BxB-Raf; Fig. 2 *B* and *C*). The transcriptional activation by CaM-KK$_c$ plus CaM-KIV$_c$ was due to phosphorylation of Elk-1, since mutation of the regulatory phosphorylation site Ser^{383} in Elk-1 to Ala (34) inhibited the effect of CaM-KK$_c$ and CaM-KIV$_c$ (Fig. 2*C*). A constitutively active form of CaM-KII truncated at residue 290 very weakly activated Elk-1-dependent transcription (Fig. 2*B*), but CaM-KII is not activated by CaM-KK (23). Taken together, these data demonstrate that the CaM-K cascade (i.e., CaM-KIV plus CaM-KK) can potently enhance transcription through the phosphorylation of Elk-1.

The CaM-Kinase Cascade Activates MAP Kinases ERK, JNK, and p38. Transcriptional activation by CaM-KIV through Elk-1 phosphorylation could be due to direct phosphorylation of Elk-1 or through activation of ERK, JNK, or p38 MAP kinases, which all phosphorylate Elk-1 (34, 39, 44–46). To determine if CaM-KIV can activate ERK, we cotransfected epitope-tagged ERK-2 with CaM-KK$_c$ and CaM-KIV$_c$. The activity of the transfected ERK-2 was determined *in vitro* after immunoprecipitation using myelin basic protein as substrate. Fig. 3*A* shows that transfected CaM-KK$_c$ plus CaM-KIV$_c$ gave a 2-fold activation of the epitope-tagged ERK-2 and that this activation was blocked by cotransfection with a vector encoding MKP-2. This 2-fold activation of ERK-2 by the CaM-kinase cascade was small compared with the 5-fold activation of ERK-2 produced by nerve growth factor treatment (Fig. 3*A*).

Since JNK and p38 can also phosphorylate Elk-1, it is possible that much of the Elk-1-mediated transcription could result from activation of JNK and/or p38 by the CaM-kinase cascade. We therefore determined the ability of CaM-KIV to activate cotransfected, epitope-tagged JNK-1 or p38. CaM-KK$_c$ plus CaM-KIV$_c$ gave 7-to 10-fold activations of JNK-1 and p38, which were blocked by cotransfected MKP-2 (Fig. 3 *B* and *C*). Expression vectors encoding constitutively active form of Ras and MKK3 were used as positive controls for JNK and p38, respectively.

CaM-KIV Activates Transcription Through the Phosphorylation of c-Jun and ATF-2. The CaM-kinase cascade activates JNK and p38 more strongly than ERK (Fig. 3). Therefore, we tested the ability of the CaM-K cascade to direct transcription through JNK and p38 using vectors that encode the activation domain of c-Jun (residues 1–223) or ATF2 (residues 1–109) fused to the GAL4 DNA-binding motif. Fig. 4 *A* and *B* illustrate that CaM-KIV$_c$ enhanced ATF2 and Jun-dependent transcription by 7- to 10-fold, and this increase was potentiated to 20- to 30-fold by cotransfection of CaM-KK$_c$ plus CaM-KIV$_c$. The CaM-K-dependent activation of transcription through the phosphorylation of ATF2 was totally abolished in the Thr^{69}/Thr^{71} → Ala/Ala

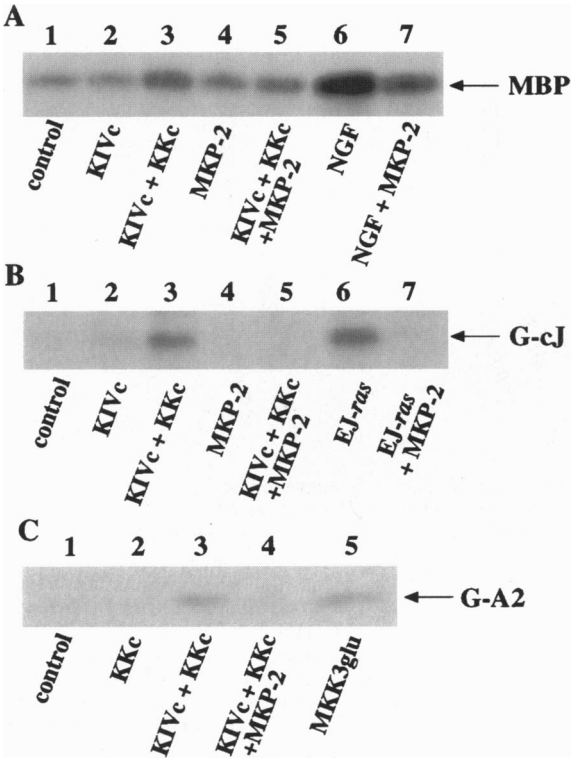

FIG. 3. The CaM-kinase cascade activates members of the MAP kinase family. (*A*) PC12 cells were transfected with vectors encoding epitope-tagged ERK-2 alone (lanes 1 and 6) or cotransfected with KIV_c, KK_c, and/or MKP-2 as indicated. In lanes 6 and 7, the cells were stimulated with 50 ng/ml of nerve growth factor for 5 min. (*B* and *C*) Similar experiments were performed on cells transfected with epitope-tagged JNK-1 (*B*) or p38 (*C*). Oncogenic Ras (EJ-*ras*), lanes 6 and 7 of *B*, or activated MKK3 (MKK3 glu), lane 5 of *C*, were used as positive controls. After immunoprecipitation of the epitope-tagged kinase, the protein kinase activity was determined using myelin basic protein (MBP, *A*), recombinant glutathione *S*-transferase–c-Jun (G-cJ, *B*), or recombinant glutathione *S*-transferase–ATF-2 (G-A2, *C*) as substrates. The samples were resolved by SDS/PAGE, and the autoradiographs shown are representative of at least three experiments.

mutant (Fig. 4*A*), while the $Ser^{63}/Ser^{73} \rightarrow Ala/Ala$ mutant of c-Jun decreased by 60–80% transcriptional activation (Fig. 4*B*). The residual activity in the Ala mutant of c-Jun is due to the phosphorylation of Thr residues close to positions 63 and 73 which become alternative substrates in the absence of Ser at positions 63/73 (47). Phosphorylation of these residues (Thr^{91} and Thr^{93} or Thr^{95}) induces small transcription activity. The 20- to 30-fold induction of transcription by the CaM-K cascade is much stronger than the 3-fold induction by nerve growth factor (Fig. 4*C*). Constitutively active CaM-KII exhibited very little stimulation of ATF2 and Jun-dependent transcription (Fig. 4 *A* and *B*). Mutation of Thr^{196} to Ala in $CaM\text{-}KIV_c$ ($CaM\text{-}KIV_cA$) blocked both ATF2 and c-Jun activation by the CaM-K cascade (Fig. 4 *A* and *B*).

CaM-KIV-Stimulated Transcription Through the Phosphorylation of c-Jun Is Mediated by the JNK Pathway. To further establish the interaction between the CaM-kinase cascade and the JNK cascade, we used DnJNK in which residues Thr^{183} and Tyr^{185} were mutated to Ala and Phe, respectively (12). This mutant blocked 50–60% of c-Jun induction by the MAP kinase kinase kinase/JNK kinase kinase MEKK-1 (48, 49) in the same experiment (data not shown). Fig. 4*C* confirms that DnJNK, MKP-1, and MKP-2 blocked the $CaM\text{-}KK_c$ plus $CaM\text{-}KIV_c$ stimulation of Jun-dependent transcription by 75–95%. Western blot analyses of the transfected CaM-KIV (Fig. 4*C Inset*) demonstrated that cotransfection of the different enzymes did not affect the level of expression of CaM-KIV. The DnJNK, however, had little effect on the stimulation of Elk-1-mediated transcription by $CaM\text{-}KIV_c$ and $CaM\text{-}KK_c$ (data not shown), indicating a role for p38 (39) and ERK (44–46) MAP kinases in Elk-1 regulation by the CaM-K cascade. These data clearly establish that the simulation of Jun-dependent transcription was mediated by the JNK pathway and was not due to direct phosphorylation of c-Jun by CaM-KIV. Thus, the CaM-K cascade positively regulates c-Jun-mediated transcription through the activation of JNK.

DISCUSSION

The results presented in this paper establish that the CaM-K cascade, CaM-KK plus CaM-KIV, can activate MAP kinase pathways, particularly JNK and p38. Transfected $CaM\text{-}KK_c$ plus $CaM\text{-}KIV_c$ caused a 7- to 10-fold activation of JNK-1 (Fig. 3*B*) and p38 (Fig. 3*C*) and also increased Jun-dependent and ATF2-dependent transcription by 25- to 30-fold (Fig. 4). This enhanced transcription was blocked by expression of DnJNK or MKP-1 and MKP-2 (Fig. 4*C*), which inhibit JNK and p38 (Fig. 3), confirming that the effects of the CaM-kinase cascade on c-Jun-dependent transcription was mediated by the JNK pathway. Involvement of the CaM-kinase cascade is strongly supported by the observation that the mutant of $CaM\text{-}KIV_c$, in which the phosphorylation site (Thr^{196}) responsible for its activation by $CaM\text{-}KK_c$ (25, 33) was mutated to Ala, was ineffective as an activator of Elk-1, ATF2, or c-Jun (Figs. 2*B* and 4 *A* and *B*). These data demonstrate that phosphorylation of Thr^{196} is critical for the transmission of the signal through the CaM-kinase cascade to the MAP kinase pathways.

Intracellular Ca^{2+} is known to play a modulatory role in the regulation of growth (50) and stress responses (51). For example, in Rat1A fibroblasts transfected with either the m1 or m2 muscarinic receptor, carbachol treatment induces a Ca^{2+}-dependent activation of JNK (5). Activation of voltage-gated Ca^{2+} channels in PC12 cells induces a Src/Ras-dependent activation of ERK (2, 7). Ca^{2+}-dependent activation of the ERK has also been reported in fibroblasts, human epidermal carcinoma cells, and the glucose-responsive β-cell line INS-1 (1, 2, 4, 6), as well as in other cell types. In Jurkat cells, where we have previously demonstrated activation of the CaM-kinase cascade in response to CD3-receptor stimulation (43), full activation of JNK requires Ca^{2+} (3). Here we report that CaM-KIV can direct the activation of p38 kinase, demonstrating for the first time that this subgroup of MAP kinases can also be regulated by Ca^{2+}.

It is likely that other kinases and/or phosphatases that can be modulated by Ca^{2+} may also regulate members of the MAP kinase pathways. For example, the Src/Ras-dependent activation of ERK by Ca^{2+} influx in PC12 cells (2, 7) may be mediated by the recently cloned PYK2 tyrosine kinase, which is activated in response to elevated intracellular Ca^{2+} and by PKC activation (52). Unlike PC12 cells, all the necessary components of the CaM-kinase cascade are present in NG108 cells (Fig. 1), and we demonstrated here that an increase in intracellular Ca^{2+} results in CaM-KIV activation that slightly precedes activation of JNK. We are now in the process of engineering dominant-negative mutants of CaM-KIV and CaM-KK to directly test the involvement of the CaM-K cascade in the activation of MAP kinases by calcium in various cell types.

CaM-Ks have been demonstrated to be involved in the Ca^{2+}-dependent regulation of immediate early genes (20), especially the protooncogene c-*fos*. Ca^{2+} regulation of c-*fos* can be mediated by the cAMP-responsive element (28, 30, 31, 53) and/or the serum response element (SRE) present in the c-*fos* promoter (54). It has been previously demonstrated that CaM-KIV, but not CaM-KII, can activate transcription through the direct phosphorylation of CREB bound to the

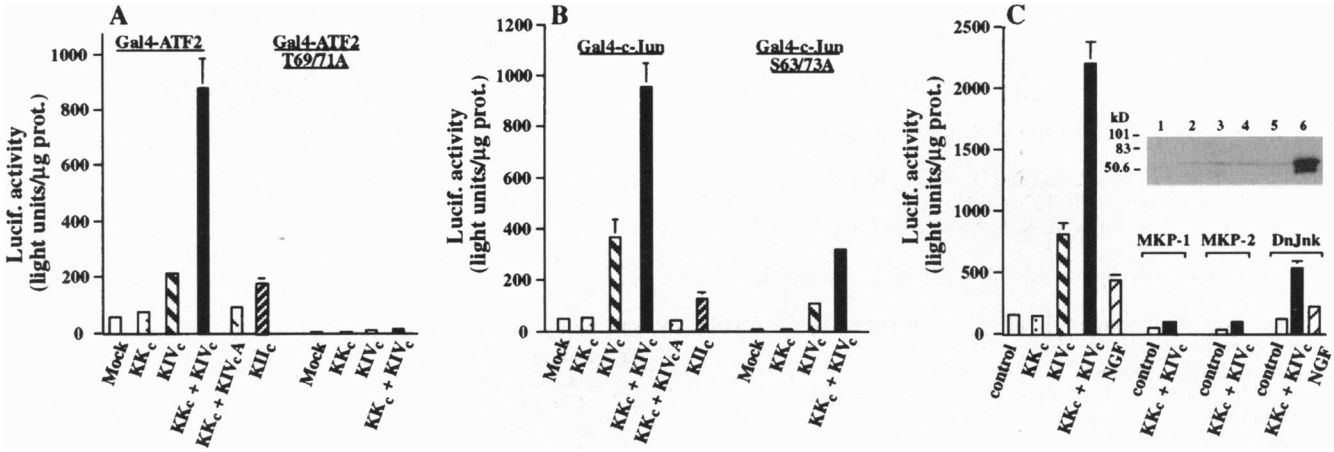

FIG. 4. The CaM-kinase cascade increases gene expression through the phosphorylation of ATF2 and c-Jun. Data from a single experiment (transfection in triplicate) are reported as mean ± SEM and are representative of at least three experiments. (*A*) PC12 cells were transfected as in Fig. 2 except that Gal4-Elk-1 was replaced with Gal4-ATF2 or Gal4-ATF2-S69/71A. (*B*) PC12 cells were transfected as described in the legend of *A* except that Gal4-ATF2 was replaced with Gal4-c-Jun or Gal4-c-Jun-S63/73A. (*C*) Transactivation by the CaM-kinase cascade through c-Jun is mediated by JNK. PC12 cells were transfected (as in *A* and *B*) or with additional cotransfection of MKP-1, MKP-2, or DnJNK. Where indicated, cells were stimulated with 50 mg/ml of nerve growth factor for 5 hr. (*Inset*) Western analysis (50 μg of extract) of CaM-KIV expression in cells transfected with plasmid (Mock, lane 1), CaM-KK_c plus CaM-KIV_c alone (lane 2), or plus DnJNK (lane 3), MKP-1 (lane 4), or MKP-2 (lane 5). Lane 6 is recombinant CaM-KIV purified from Sf9 cells.

cAMP-reponsive element (28, 30, 31). We report here that CaM-KIV can also stimulate transcriptional activity through the phosphorylation of Elk-1 by MAP kinases (ERK, JNK, and p38). These data provide a mechanism by which Ca^{2+} can regulate SRE-mediated transcription. SRF and Elk-1, although directly interacting with each other, bind to different sequences of the SRE (55). CaM-KIV phosphorylates SRF *in vitro* (27, 29). Miranti *et al.* (54) have reported using PC12 cells that Ca^{2+}-regulated, Ras-independent, SRE-dependent activation of c-*fos* requires the SRF but not Elk-1. A transfected, constitutively active, truncated form of CaM-KIV-stimulated, SRF-dependent transcription, although weaker than the Ca^{2+} stimulation in the absence of transfected CaM-KIV (54). Although the interaction of the SRF with the SRE may be required for all SRE functions (56), recent reports suggest that SRF binding alone is not sufficient for some of these functions (57, 58). The absence of a requirement for Elk-1 in the Ca^{2+} induction of c-*fos* reported by Miranti *et al.* (54) is consistent with the finding that PC12 cells do not express CaM-KIV. Furthermore, the CaM-KIV induction through the SRF appears to be weak (2.8-fold ± 1.2-fold; ref. 54). The relative contribution of SRF and Elk-1 phosphorylation to transcriptional induction by CaM-KIV remains to be determined. However, it is possible that the phosphorylation of both SRF and Elk-1 by the CaM-KIV pathway causes synergistic activation of SRE-dependent gene expression.

Activation of c-Jun by CaM-KIV, through JNK activation, also provides a mechanism by which Ca^{2+} can regulate the activity of both preexisting and newly synthesized AP-1 proteins. Phosphorylation of c-Jun causes activation of AP-1, which also results from increased expression of Fos and Jun proteins (11). Activation of JNK and p38 also results in the phosphorylation and activation of ATF-2 (12, 39). ATF-2 can heterodimerize with c-Jun and bind to the TRE-like site present in the c-*jun* promoter (59). The effect of Ca^{2+} to increase AP-1 transcriptional activity is thus mediated by increased phosphorylation of c-Jun and ATF-2 by JNK and/or p38, and by increased expression of c-Fos and c-Jun proteins. Our data suggest that CaM-KIV, which can activate JNK and p38, represents one mediator of Ca^{2+}-regulated cell growth and stress responses that lead to increased AP-1 and SRE-dependent gene expression.

We propose a working model in which Ca^{2+}, through CaM-KIV, regulates transcription through different signal transduction pathways: (*i*) the CRE integrates the Ca^{2+} signal through direct phosphorylation of CREB by CaM-KIV; (*ii*) the TRE mediates the effects of JNK activation by CaM-KIV on both preexisting and newly synthesized ATF-2 and AP-1 proteins; and (*iii*) the SRE appears to integrate the Ca^{2+} signal through activation of the ERK signal transduction pathway, which also mediates the effects of protein kinase C and receptor tyrosine kinases, and the JNK/p38 signal transduction pathway, which also mediates the effects of proinflammatory cytokines and environmental stress.

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