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Cross Talk between ERK and PKA Is Required for Ca^{2+} Stimulation of CREB-Dependent Transcription and ERK Nuclear Translocation

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Summary

Although Ca^{2+}-stimulated cAMP response element binding protein– (CREB-) dependent transcription has been implicated in growth, differentiation, and neuroplasticity, mechanisms for Ca^{2+}-activated transcription have not been defined. Here, we report that extracellular signal–related protein kinase (ERK) signaling is obligatory for Ca^{2+}-stimulated transcription in PC12 cells and hippocampal neurons. The sequential activation of ERK and Rsk2 by Ca^{2+} leads to the phosphorylation and transactivation of CREB. Interestingly, the Ca^{2+}-induced nuclear translocation of ERK and Rsk2 to the nucleus requires protein kinase A (PKA) activation. This may explain why PKA activity is required for Ca^{2+}-stimulated CREB-dependent transcription. Furthermore, the full expression of the late phase of long-term potentiation (L-LTP) and L-LTP–associated CRE-mediated transcription requires ERK activation, suggesting that the activation of CREB by ERK plays a critical role in the formation of long lasting neuronal plasticity.

Introduction

Activity-dependent increases in cytosolic Ca^{2+} trigger the expression of hundreds or perhaps thousands of genes. Activation of gene expression by Ca^{2+} regulates fundamental biological processes, including synaptic plasticity, and may be important for some forms of learning and memory (Ghosh et al., 1994; Ginty, 1997). Since the induction of late-response genes is often mechanistically complex, many studies have focused on immediate-early genes (IEGs). The analysis of IEG promoters led to the identification of the cAMP response element (CRE) as a major Ca^{2+}-responsive transcriptional element (Ginty, 1997). The CRE is also regulated by cAMP and integrates coincident increases in Ca^{2+} and cAMP via synergistic increases in transcription (Deutsch et al., 1987; Sheng et al., 1990; Impey et al., 1994).

Since the CRE can integrate Ca^{2+} and cAMP signaling, it has been proposed that the CRE binding protein (CREB) transcription factor family couples temporally overlapping signals to long-term changes in synaptic strength (Sheng et al., 1990). In support of this idea, late phase long-term potentiation– (L-LTP–) associated increases in CRE-mediated gene transcription in mice depend on both cAMP and Ca^{2+} signaling (Impey et al., 1996). In addition, CREB-like proteins have been implicated in the formation of long-term memory and long-term synaptic plasticity in *Aplysia*, *Drosophila*, and mice (Bourtchuladze et al., 1994; Yin et al., 1994; Bartsch et al., 1995; Martin et al., 1997).

Ca^{2+} is thought to transactivate the CRE via phosphorylation of CREB (Sheng et al., 1990). Phosphorylated CREB then binds to its coactivator, CREB-binding protein (CBP), which facilitates the unwinding of DNA and interacts with the basal transcriptional machinery (Chrivia et al., 1993; Kwok et al., 1994). However, phosphorylation of CREB at Ser-133 is necessary but not sufficient for the activation of transcription (Ginty et al., 1994; Brindle et al., 1995; Thompson et al., 1995; Impey et al., 1996).

The prevailing model posits that Ca^{2+}-stimulated CRE-mediated transcription occurs via phosphorylation of CREB at Ser-133 by a Ca^{2+}/CaM-dependent kinase (CaMK) (Sheng et al., 1990; Bito et al., 1996). Overexpression of CaMKI or CaMKIV can induce CRE-mediated gene expression in cell lines (Enslen et al., 1994; Matthews et al., 1994; Sun et al., 1996). Recent work also shows that antisense inhibition of CaMKIV expression partially reduces early CREB phosphorylation (Bito et al., 1996). However, since only prolonged CREB phosphorylation correlates with CREB-dependent gene expression (Bito et al., 1996; Impey et al., 1996; Liu and Graybiel, 1996), it has not been demonstrated that CaMKs are required for Ca^{2+}-stimulated CRE-mediated gene expression. Moreover, CREB (or activating transcription factor 1 [ATF1]) can be phosphorylated by multiple Ca^{2+}-activated kinases including protein kinase A (PKA), Rsk1, Rsk2, mitogen-activated protein kinase–activated protein (MAPKAP) kinase 2, p70 S6 kinase, and protein kinase C (PKC) (de Groot et al., 1993; Ginty et al., 1994; Brindle et al., 1995; Tan et al., 1996; Xing et al., 1998). It is also clear that other signaling events are necessary, because Ca^{2+}-stimulated transcription depends on cAMP-dependent PKA activity in PC12 cells and primary neuron cultures (Ginty et al., 1991; Impey et al., 1994; Thompson et al., 1995). Thus, it remains to be determined which signaling cascades lead to the activation of CRE-mediated gene expression by Ca^{2+}.

Recently, it was discovered that Ca^{2+} activates the extracellular signal–related protein kinase/mitogen-activated protein (ERK/MAP) kinase pathway in PC12 cells and primary neuron cultures (Bading and Greenberg, 1991; Rosen et al., 1994). Ca^{2+} influx also increases intracellular cAMP in PC12 cells and neurons (Sheng et al., 1990; Mark et al., 1995; Wu et al., 1995). Work with a c-fos promoter fragment also suggests a partial involvement of ERK-dependent signaling for Ca^{2+}-stimulated CRE-mediated transcription (Johnson et al., 1997). Therefore, the objectives of this study were to define the roles of the ERK/MAP kinase and PKA pathways in the activation of CRE-mediated transcription by Ca^{2+}.

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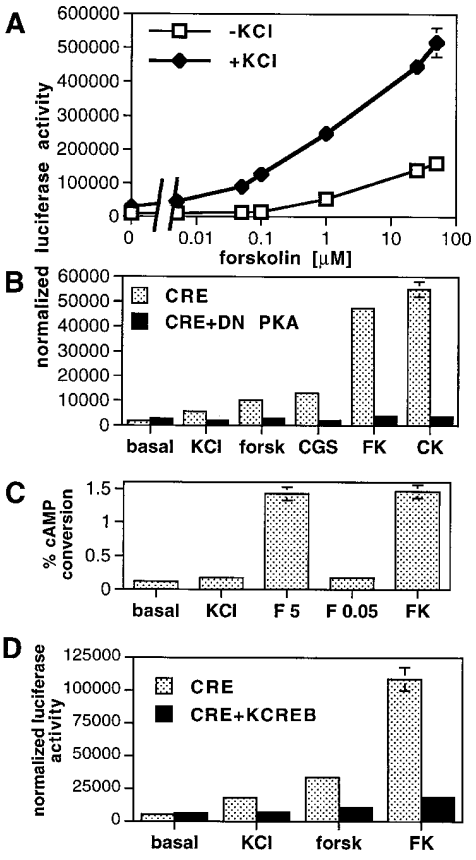

Figure 1. Induction of CRE-Mediated Transcription by Ca^{2+} Requires PKA Activity but Does Not Correlate with Intracellular cAMP in PC12 Cells

(A) PC12 cells were transfected with a CRE–luciferase construct and a 3-fold excess of control DNA. Luciferase expression was measured as a function of forskolin concentration in the absence or presence of KCl (60 mM).

(B) PC12 cells were transfected with a CRE–luciferase construct and a 10-fold excess of control DNA (CRE) or dominant negative PKA DNA (CRE + DN PKA). FK denotes the pairing of KCl with forskolin. KCl was 60 mM, forskolin (forsk) 5 μM.

(C) Intracellular cAMP was monitored after treatment with 60 mM KCl, forskolin, or the combination of 5 μM forskolin and 60 mM KCl (FK). F 5 and F 0.05 denote forskolin at 5 μM and 50 nM, respectively.

(D) PC12 cells were transfected with a CRE–luciferase construct and a 10-fold excess of control or dominant negative CREB (KCREB) DNAs. The cells were treated as described in (B).

Experiments are averages of triplicate determinations. Error is expressed as the standard deviation of the mean.

Results

Ca^{2+}-Stimulation of CRE-Mediated Transcription Requires PKA Activity

The regulation of CRE-mediated transcription was examined by transfecting PC12 cells with a CRE-regulated luciferase reporter construct. Depolarization increased CRE-mediated transcription 3-fold (Figures 1A and 1B), presumably through the activation of voltage-gated Ca^{2+} channels (Sheng et al., 1990; Mark et al., 1995). Furthermore, the pairing of KCl depolarization with activators of adenylyl cyclase synergistically activated transcription, and coexpression of a dominant negative regulatory subunit of PKA (DN PKA) blocked Ca^{2+}-stimulated transcription (Figures 1A and 1B). Activation of serum response element– (SRE-) mediated gene expression by Ca^{2+} was not affected by coexpression of DN PKA, thus ruling out a nonspecific effect on transcription (data not shown). It should also be noted that the inhibitors and dominant negatives used in this study did not affect depolarization-stimulated Ca^{2+} influx (data not shown).

Because Ca^{2+} can activate gene expression by stimulating CaM-regulated adenylyl cyclases (Impey et al., 1994), we measured the effect of depolarization on intracellular cAMP in PC12 cells. Consistent with earlier studies (Sheng et al., 1990; Mark et al., 1995), depolarization caused a modest (40%–80%) but significant ($p < 0.05$, $n = 3$) increase in intracellular cAMP (Figure 1C). However, this increase cannot mediate Ca^{2+}-stimulated gene expression, because 50 nM forskolin generated comparable cAMP increases but did not activate CRE-mediated gene expression (Figures 1A and 1C). Evidently, the depolarization-mediated increase in intracellular cAMP serves to enhance or synergize with other depolarization-activated signaling events.

To determine whether CREB or homologous transcription factors are required for Ca^{2+}-stimulated gene expression, we used a dominant negative CREB mutant (KCREB) in which the DNA binding domain was inactivated (Walton et al., 1992). Coexpression of KCREB attenuated forskolin- and KCl depolarization–stimulated transcription (Figure 1D). Thus, CREB or one of its dimerization partners is required for transactivation of the CRE by Ca^{2+} in PC12 cells.

ERK/MAP Kinase Signaling Is Required for Ca^{2+}-Stimulated Transcription in PC12 Cells

Because Ca^{2+} activates ERK signaling in PC12 cells (Rosen et al., 1994), the role of this kinase cascade in Ca^{2+}-stimulated gene expression was evaluated. Coexpression of dominant negative Ras (Ras TN) or MAP kinase kinase (MEK) (MEK SA) markedly attenuated Ca^{2+}-stimulated gene expression (Figure 2A). The potentiation of KCl-stimulated transcription by cAMP was also inhibited by expression of Ras TN or MEK SA. In addition, treatment with a selective inhibitor of MEK, PD98059, blocked Ca^{2+}-stimulated gene expression (Figure 2A). Because several members of the ATF family bind to the CRE, direct transactivation of CREB using a Gal4 two-hybrid transcriptional assay was examined. Coexpression of MEK SA also inhibited the transactivation of CREB by Ca^{2+} (Figure 2B). These data indicate that ERK/MAP kinase signaling is required for the regulation of CRE-regulated transcription by Ca^{2+} in PC12 cells.

We also explored whether direct activation of the ERK signaling cascade can activate CRE-regulated transcription. Cotransfection of constitutively activated Ras (Ras QL) or MEK (MEK SE) caused a large increase in CRE-mediated transcription that was enhanced by forskolin (Figure 2C). Cotransfection of KCREB attenuated the transcriptional responses to both Ras QL and

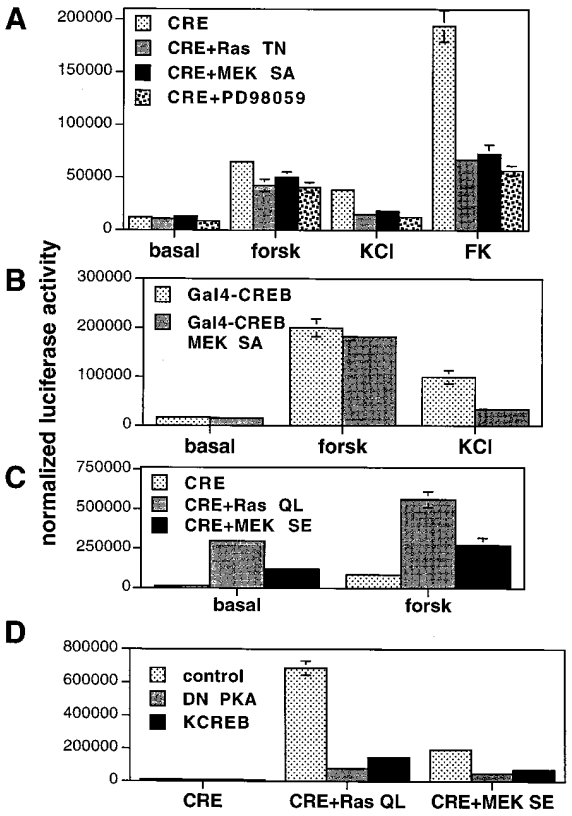

Figure 2. Activation of CRE-Mediated Gene Expression by Ca^{2+} Is Dependent on the ERK/MAP Kinase Cascade

(A) PC12 cells were cotransfected with a CRE–luciferase construct and a 6-fold excess of control (CRE), dominant negative Ras (CRE + Ras TN), or dominant negative MEK (CRE + MEK SA) DNAs. The cells were treated with 5 μM forskolin (forsk), 60 mM KCl, or the pairing of forskolin with KCl (FK) and assayed for luciferase activity. PD98059 was used at 50 μM.

(B) PC12 cells were transfected with a Gal4–CREB fusion protein construct, a Gal4–luciferase construct, and a 6-fold excess of control or dominant negative MEK (MEK SA) DNAs. Treatments were as in (A).

(C) PC12 cells were cotransfected with a CRE–luciferase construct and a 2-fold excess of constitutively active Ras (Ras QL), MEK (MEK SE), or control DNAs. Twenty-four hours following transfection, PC12 cells were treated with 5 μM forskolin (forsk) for 5 hr and assayed for luciferase activity.

(D) Coexpression of control (CRE), constitutively active Ras (CRE + Ras QL), or constitutively active MEK (CRE + MEK SE) DNAs with a CRE–luciferase construct with and without dominant negative PKA (DN PKA) or dominant negative CREB (KCREB) constructs. The constructs (CRE–luciferase–vector or constitutive–vector or DN) were used at a ratio of 2:1:6 (w/w).

Experiments are averages of triplicate determinations. Error is expressed as the standard deviation of the mean.

MEK SE, suggesting that Ras and MEK acted specifically on CREB-dependent transcription (Figure 2D). Interestingly, the induction of gene expression by activators of ERK was also inhibited by DN PKA (Figure 2D).

Ca^{2+}-Stimulated Transcription in Neurons Requires ERK Activity

To define the relationship between the regulation of CRE-mediated transcription and synaptic plasticity, we examined the regulation of CRE-mediated transcription in hippocampal neuron cultures from CRE-LacZ transgenic mice (Impey et al., 1996). Forskolin or KCl depolarization both increased LacZ expression, and the pairing of the two was synergistic (Figure 3A). The L-type Ca^{2+} channel antagonist isradipine blocked the induction of LacZ by depolarization, indicating that depolarization-induced transcription was dependent on Ca^{2+} influx (Figure 3A). Moreover, treatment with the MEK inhibitor PD98059 inhibited depolarization-stimulated but not forskolin-stimulated gene expression (Figure 3A).

To confirm a role for ERK, we coexpressed dominant negative MEK SA with a CRE-luciferase construct and found that it markedly inhibited depolarization-mediated transcription in hippocampal cultures (Figure 3B; 45% of control). Although MEK SA slightly attenuated forskolin-stimulated gene expression (Figure 3B; 76% of control), this was not unexpected, because forskolin activates ERK in hippocampal neurons (Figure 6A). Coexpression of MEK SA inhibited the transactivation of CREB in hippocampal neurons (Figure 3C). Conversely, expression of constitutively active MEK SE or Ras QL stimulated CRE-mediated transcription and potentiated forskolin-stimulated transcription in hippocampal neurons (Figure 3D). The selective inhibitor of PKA, Rp-8-Br-MB-cAMPS, decreased both depolarization and forskolin-stimulated transcription (Figure 3E), indicating that PKA activity is also required for CRE-mediated transcription in hippocampal neurons. Thus, the regulation of CRE-mediated gene expression by Ca^{2+} in PC12 cells and hippocampal neurons shares a common mechanism requiring both ERK and PKA signaling.

ERK Activity Is Synergistically Activated by cAMP and Ca^{2+}

The cAMP/PKA pathway can activate and potentiate ERK signaling in PC12 cells (Mark et al., 1995; Yao et al., 1995) through stimulation of Rap1b (Vossler et al., 1997). Consequently, the effects of forskolin, KCl depolarization, and combinations of the two on ERK activity were examined. ERK activity was synergistically activated by the pairing of depolarization with forskolin in PC12 cells (Figure 4A) and in hippocampal neurons (Figure 6A). ERK activity was also monitored with an antibody that specifically recognizes dually phosphorylated ERK. The phosphorylation state of ERK mirrored its kinase activity in PC12 cells (Figure 4A) and hippocampal neurons (Figure 6A). As expected, a selective MEK inhibitor, PD98059, attenuated ERK phosphorylation in PC12 cells (Figure 4A) and hippocampal neurons (Figure 6A).

Rsk2 Is a Ca^{2+}-Stimulated CREB Kinase in PC12 Cells

Depolarization-stimulated CREB-kinase activity was completely blocked by PD98059 in PC12 cells (Figure 4C). Depolarization-stimulated CREB-kinase activity assayed in the presence of Ca^{2+}/CaM was also attenuated by PD98059 (data not shown). Although ERK/MAP kinase signaling is necessary for CREB transactivation in PC12 cells, CREB lacks consensus ERK phosphorylation sites. Nevertheless, the ERK-activated kinases Rsk1, Rsk2, and Rsk3 can phosphorylate CREB in response to growth factors (Xing et al., 1998). The activation of Rsk2 was

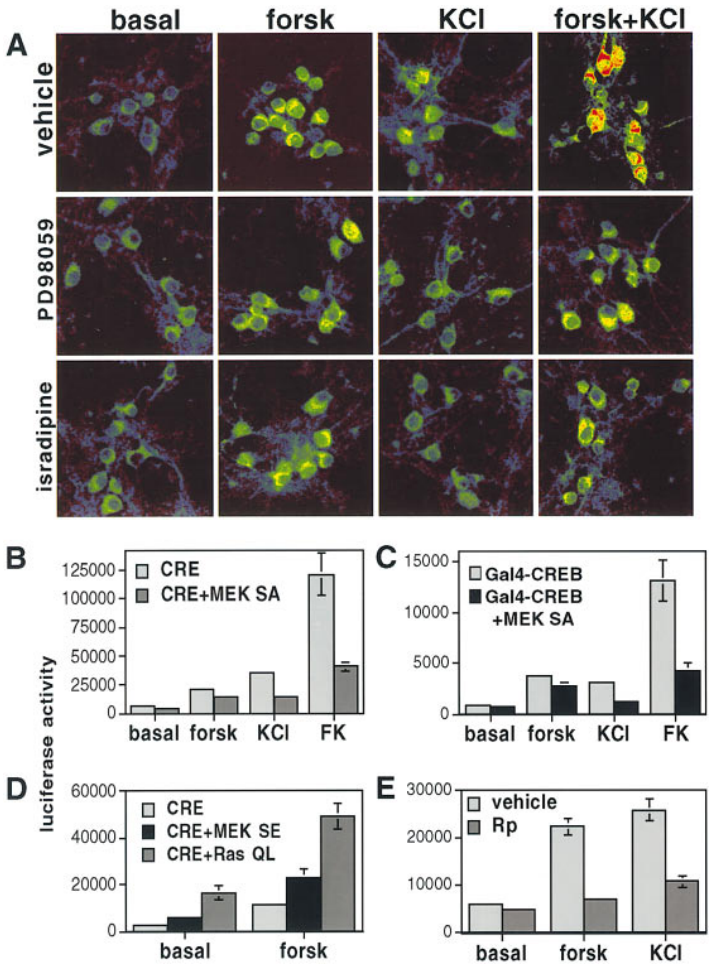

Figure 3. Ca^{2+}-Stimulated Gene Expression in Primary Hippocampal Neurons Requires ERK Activity

(A) Primary hippocampal neurons were cultured from CRE-LacZ transgenic mice. The neurons were pretreated with 50 μM PD98059, 5 μM isradipine, or vehicle. Day 8 in vitro neurons were treated with 5 μM forskolin or 60 mM KCl for 30 min and allowed to recover in conditioned medium for 6 hr. The neurons were fixed and stained immunocytochemically for β-galactosidase.

(B) Hippocampal neurons were transfected with a CRE-regulated reporter construct and a 5-fold excess of control DNA or dominant negative MEK SA DNA. At day 7 in vitro, the neurons were treated with 5 μM forskolin or 30 mM KCl for 6 hr and assayed for luciferase activity. FK denotes the pairing of forskolin and KCl.

(C) Hippocampal neurons were transfected with Gal4–CREB, Gal4–luciferase, and a 4-fold excess of control or dominant negative MEK SA DNAs. The neurons were treated as in (B).

(D) Hippocampal neurons were cotransfected with a 2-fold excess of constitutively active Ras (Ras QL), MEK (MEK SE), or a control DNA with a CRE–luciferase construct. The neurons were treated with 5 μM forskolin 24 hr posttransfection (day 6 in vitro) and assayed for luciferase activity.

(E) Hippocampal neurons were transfected with a CRE–luciferase construct and a 3-fold excess of control DNA. Day 7 in vitro neurons were pretreated with Rp-8-Br-MB-cAMPS (Rp 500 μM). The cells were treated as described in (B) and assayed for luciferase activity.

Experiments are averages of quadruplicate determinations. Error is expressed as SEM. Similar results were obtained in at least three independent experiments.

monitored by measuring the phosphorylation of CREBtide. Rsk2 was activated by depolarization, nerve growth factor (NGF), and forskolin, suggesting that Rsk2 or other Rsks may be Ca^{2+}-activated CREB kinase (Figure 4B).

To characterize the kinase that phosphorylates CREB in response to Ca^{2+}, extracts from untreated KCl- or NGF-stimulated PC12 cells were fractionated by Mono Q anion exchange chromatography and assayed for phosphotransferase activity against CREBtide. Consistent with Xing et al. (1996), NGF treatment generated a peak of CREB kinase activity that copurified with Rsk2. KCl depolarization evoked a peak of activity that overlapped with the NGF-induced peak and also copurified with Rsk2 (Figure 4D). To confirm that Rsk2 is a major Ca^{2+}-stimulated CREB kinase and to determine if other Rsk isoforms are CREB kinases, we immunoprecipitated Rsk1, Rsk2, and Rsk3 from the Mono Q column fractions. Only Rsk2 had significant KCl-stimulated CREB kinase activity in the fractions corresponding to the peak of KCl depolarization–stimulated CREB kinase activity (Figure 4E). In agreement with Xing et al. (1996), we found that Rsk1 was present in flow through fractions and that Rsk3 was eluted by high salt (fractions 27–31; 0.35–0.4 M NaCl) (Figure 4E). Interestingly, we found significant depolarization-stimulated Rsk1-mediated CREB kinase activity in flow through fractions (Figure 4E). Evidently, Rsk1 is also a depolarization-stimulated CREB kinase in PC12 cells.

Rsk2 Translocates to the Nucleus and Is a Major Nuclear CREB Kinase in PC12 Cells

A putative CREB kinase must either translocate to the nucleus or be activated in the nuclear compartment. Therefore, we isolated nuclear extracts from depolarization-stimulated PC12 cells and assayed Rsk-mediated CREB kinase activity. Using immune complex kinase assays, we found that only Rsk2 had detectable depolarization-stimulated nuclear CREB kinase activity (Figure 5A). Both Rsk1 (3 ± 0.5-fold) and Rsk2 (3 ± 0.2-fold) were activated by K^+ depolarization in the cytosolic fractions. Immunodepletion of Rsk2 decreased KCl-stimulated nuclear CREB kinase activity by 60% and confirmed that Rsk2 is a major nuclear CREB kinase in PC12 cells (Figure 5B). It is possible that the disparate regulation of nuclear Rsk2 and Rsk1 activity is a consequence of a differential ability to translocate. Accordingly, we found that Rsk2 was translocated to the nuclear fraction to a far greater extent than Rsk1 (Figure 5C). Interestingly, Rsk2 but not Rsk1 interacts with ERK in vivo (Zhao et al., 1996). We propose that the nuclear

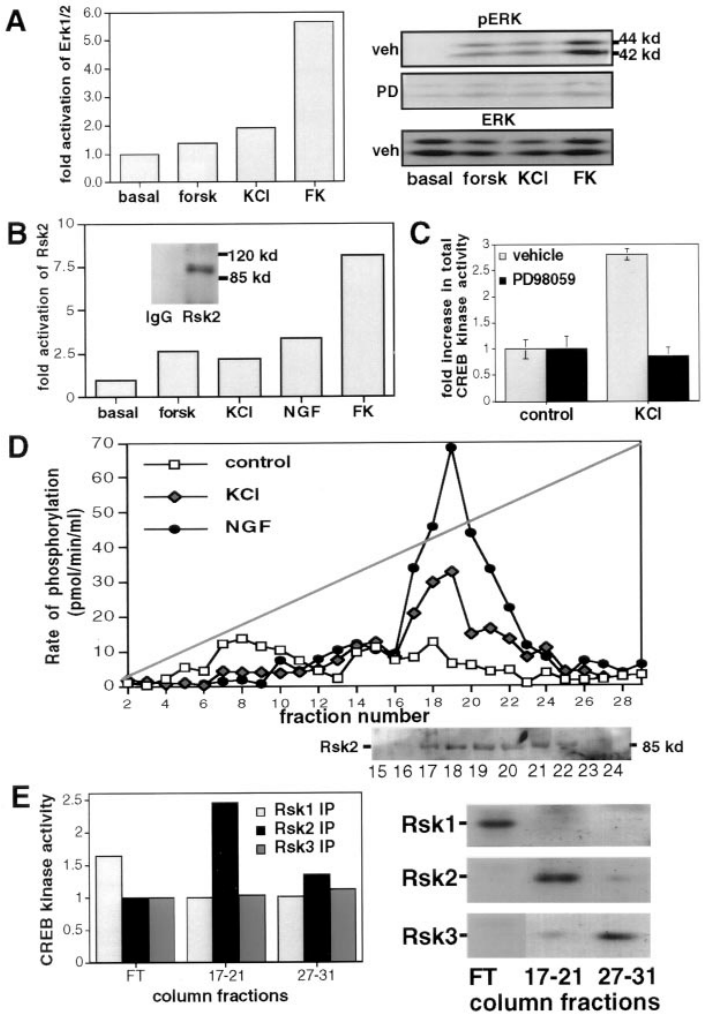

Figure 4. Rsk Is a Ca^{2+}-Stimulated CREB Kinase in PC12 Cells

(A) PC12 cells were treated with agonists for 5 min. ERK was immunoprecipitated, and kinase activity was determined. Forsk denotes 5 μM forskolin, FK denotes the pairing of forskolin and KCl, and NGF was 100 ng/ml. KCl was 60 mM. Extracts were also Western blotted with anti-Tyr and anti-Thr–phosphorylated ERK.

(B) PC12 cells were treated with agonists for 5 min as described in (A). Rsk2 was immunoprecipitated and assayed for kinase activity. The inset depicts a Western blot of immunoprecipitated Rsk2 and an IgG control.

(C) PC12 cells were treated as indicated in (A) for 5 min. Cell extracts were isolated, and total kinase activity was determined. PD98059 was used at 50 μM. Error is expressed as SEM ($n = 3$).

(D) PC12 cells were treated as indicated in (A), and extracts were subjected to Mono Q anion exchange chromatography over a linear 0.4 M NaCl gradient. The fractions were assayed for CREB kinase activity. The gel depicted below (D) is a Western blot of immunoprecipitated Rsk2 from fractions 15–24 of KCl-stimulated PC12 cells.

(E) Rsk1, Rsk2, and Rsk3 were immunoprecipitated from the Mono Q column fractions indicated for control and KCl-treated extracts. The immunoprecipitates were assayed for CREB-kinase activity. The Rsk1, Rsk2, and Rsk3 immunoprecipitates from the KCl-treated column fractions were also Western blotted with Rsk1, Rsk2, and Rsk3 antibodies, respectively.

translocation of Rsk2 depends on the ability of Rsk2 to bind to ERK, which is robustly translocated to the nucleus (Traverse et al., 1992).

Ca^{2+}-Stimulated CREB Kinases in Hippocampal Neurons

Our work in PC12 cells suggests that Rsk2 may function as a Ca^{2+}-stimulated CREB kinase in hippocampal neurons. Accordingly, depolarization markedly stimulated Rsk2 activity in an immune complex CREB kinase assay in hippocampal neurons (Figure 6B). Depolarization-stimulated Rsk2 activity was also attenuated by treatment with the MEK inhibitor PD98059. Interestingly, PD98059 attenuated Rsk2 activation (Figure 6B) to the same degree (~60%) as CREB phosphorylation (Figure 7D). We examined Ca^{2+}-stimulated CREB kinase activities by Mono Q chromatography in cortical neurons and found multiple peaks of activity (data not shown). Since Mono Q chromatography is limited to correlating protein elution to kinase activity, we examined the Ca^{2+}-activated CREB kinase activity of known CREB kinases directly by immune complex kinase assay. In hippocampal neurons, depolarization robustly increased the nuclear CREB kinase activity of Rsk2 and to a lesser extent, of Rsk1, Rsk3, and CaMKIV (Figure 6C). The robust activation of Rsk2 in hippocampal neurons makes Rsk2 a good candidate for a Ca^{2+}-activated CREB kinase that mediates the prolonged phosphorylation of CREB.

Ca^{2+}-Mediated CREB Phosphorylation Is Attenuated by PD98059

Using an antibody that recognizes CREB and ATF1 phosphorylated at Ser-133 (Tan et al., 1996), we found that CREB phosphorylation paralleled the transcriptional response in PC12 cells (Figure 7A) and hippocampal neurons (Figure 7C). PD98059 attenuated KCl-induced CREB phosphorylation in PC12 cells (Figure 7A), whereas the expression of constitutively activated MEK SE promoted CREB phosphorylation (Figure 7B). Although Bito et al. (1996) found that CaMKIV activity is required for depolarization-mediated CREB phosphorylation at 1 min, other potential CREB kinases, such as Rsk and PKA, have more delayed kinetics (Chen et al., 1991; Hagiwara et al., 1993). Furthermore, only long lasting CREB phosphorylation correlates with the induction of gene expression in neurons (Bito et al., 1996; Liu and Graybiel, 1996; Impey et al., 1996). Interestingly,

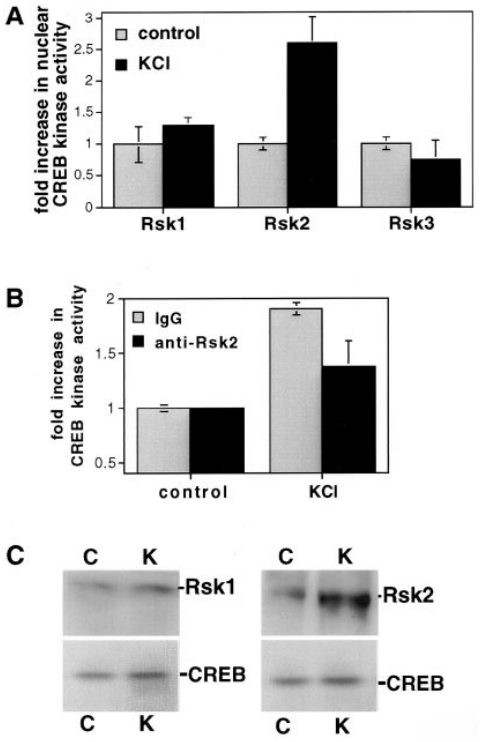

Figure 5. Rsk2 Is a Major Nuclear Ca^{2+}-Stimulated CREB Kinase in PC12 Cells

(A) PC12 cells were treated with 60 mM KCl for 5 min, and nuclear extracts were prepared. Rsk1, Rsk2, and Rsk3 were immunoprecipitated from the nuclear fraction and assayed for CREB kinase activity. Error is SEM (n = 3).

(B) PC12 cell nuclear extacts were prepared as in (A) and were immunodepleted with anti-Rsk2 or control IgG antibodies. The depleted extract was assayed for CREB kinase activity. Error is SEM (n = 3).

(C) PC12 cell nuclear extracts were prepared as in (A) and Western blotted for Rsk1 or Rsk2. The extracts were Western blotted for CREB to demonstrate equal protein loading.

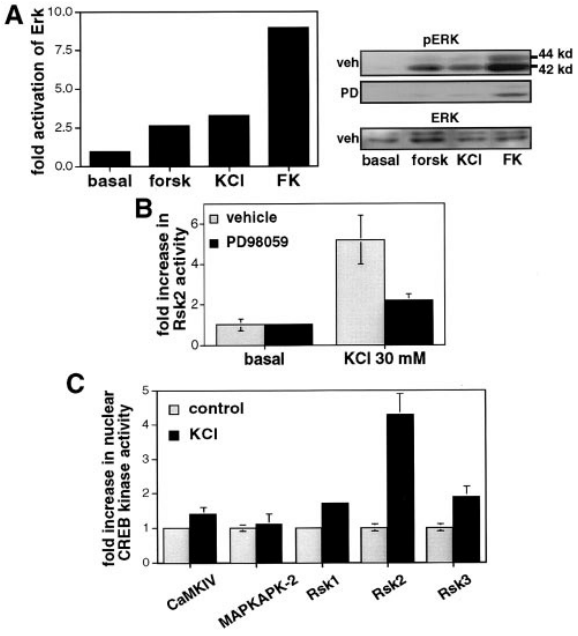

Figure 6. Hippocampal Ca^{2+}-Stimulated CREB Kinases

(A) Hippocampal neurons were treated with agonists for 5 min. ERK was immunoprecipitated and assayed for kinase activity. Forsk denotes 5 μM forskolin, FK denotes the pairing of forskolin and KCl, and KCl was 30 mM. Extracts were also Western blotted with anti–phospho-ERK.

(B) Hippocampal neurons were treated with 30 mM KCl for 10 min in the presence and absence of 50 μM PD98059. Rsk2 immunoprecipitates were assayed for kinase activity by using CREBtide as substrate. Error is SEM (n = 4).

(C) Hippocampal neurons were treated with 60 mM KCl for 15 min, and nuclear extracts were prepared. The indicated kinases were immunoprecipitated from equal amounts of nuclear extract and assayed for CREB kinase activity. Error is SEM. Similar results were obtained in at least two independent experiments.

PD98059 significantly inhibited depolarization-stimulated CREB-phosphorylation at 15 min (Figures 7C and 7D) but not at 1 min (Figure 7D). This suggests a role for ERK signaling in the prolongation of CREB phosphorylation in neurons.

As demonstrated previously (Impey et al., 1996), tetani that generated LTP in area CA1 also induced CREB phosphorylation (Figures 7E and 7F). LTP-associated CREB phosphorylation was dependent on the influx of extracellular Ca^{2+}, because perfusion with the NMDA receptor antagonist amino-phosphono-valeric acid (APV) and the L-type Ca^{2+} channel blocker isradipine blocked tetanus-induced CREB phosphorylation (data not shown). Because our data implicate ERK signaling in Ca^{2+}-stimulated CREB phosphorylation, we also examined the role of the ERK/MAP kinase cascade. Perfusion of the MEK inhibitor PD98059 significantly decreased tetanus-induced CREB phosphorylation measured by Western analysis (Figure 7E; 92% inhibition by densitometry) and by immunohistochemistry (Figure 7F; n = 5).

Since tetanization could increase CREB phosphorylation independent of postsynaptic Ca^{2+} influx, we also examined the effect of K^+ depolarization on CREB phosphorylation in hippocampal slices. We blocked neurotransmission by treatment with APV, 6,7-dinitroquinoxaline-2,3-dione (DNQX), and tetrodotoxin (TTX) to enable us to study CREB-phosphorylation triggered specifically by Ca^{2+} influx. K^+ depolarization markedly increased CREB phosphorylation throughout area CA1 (Figure 7G). Just as with tetanus-induced CREB phosphorylation, PD98059 treatment blocked depolarization-induced CREB phosphorylation (Figures 7G and 7H; $p < 0.01$; control, n = 6; PD98059, n = 5). In addition, the ERK inhibitor olomoucine also attenuated depolarization-induced CREB phosphorylation in CA1 pyramidal neurons (Figure 7H; $p < 0.01$; control, n = 6; olomoucine, n = 3). Both PD98509 and olomoucine also blocked depolarization-induced Erk phosphorylation in hippocampal slices (data not shown). Furthermore, the time course of depolarization-induced CREB phosphorylation closely paralleled Erk phosphorylation in hippocampal area CA1 (Figure 7I).

CaMKIV Activity Is Dispensable for Ca^{2+}-Mediated CREB-Dependent Transcription

CREB can be phosphorylated and activated by multiple Ca^{2+}-activated kinases other than Rsk2, including

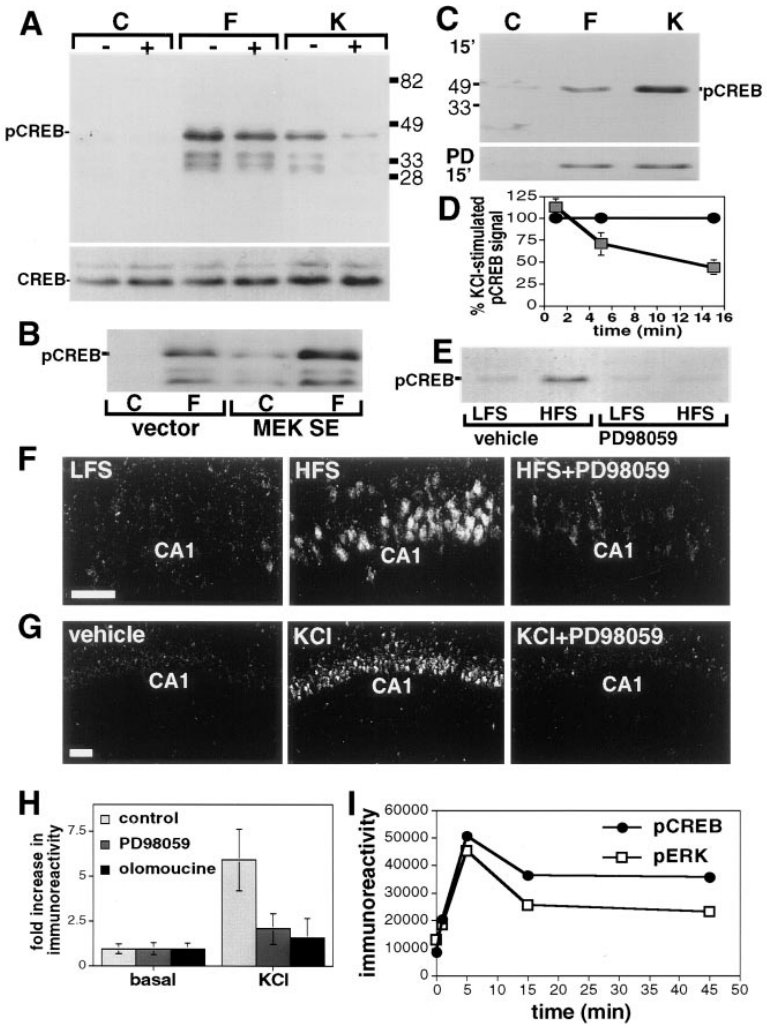

Figure 7. CREB Phosphorylation at Ser-133 Depends on ERK/MAP Kinase Signaling

(A) PC12 cells were treated with the indicated agonists for 5 min in the absence (−) or presence (+) of 50 μM PD98059 and Western blotted for phospho-CREB. C denotes untreated cells, F denotes forskolin (5 μM), K denotes KCl (60 mM), and FK denotes the pairing of forskolin and KCl. The smaller molecular weight doublet comigrates with ATF1.

(B) PC12 cells were transfected with control or constitutively active MEK SE DNAs and treated as described in (A).

(C) Hippocampal neurons were treated with 5 μM forskolin or 30 mM KCl for 15 min in the absence (veh) or presence of PD98059 (PD) and Western blotted.

(D) Densitometry was conducted on K^+-stimulated hippocampal phospho-CREB blots. The black circles show K^+-stimulated pCREB signal in the presence of vehicle, and the gray squares show PD98069-treated pCREB signal. For each time point, PD98059-treated pCREB signal was expressed as a percentage of vehicle. At the 15′ time point, PD98059-treated KCl-stimulated pCREB signal was 44% ± 8% of vehicle ($p < 0.01$; vehicle, $n = 9$; PD98059, $n = 9$).

(E) Slices of hippocampal area CA1 were perfused with the indicated drugs for at least 30 min prior to tetanization (HFS; 100 Hz, 1 s, 0.2 ms pulse). Fifteen minutes following tetanization, slices were frozen and stored at −80C. The pooled tissue was homogenized, and equivalent amounts of protein were Western blotted for phospho-CREB. LFS denotes slices stimulated at 0.05 Hz.

(F) Representative examples of hippocampal slices treated as described in (E) and stained for phospho-CREB. The scale bar is 50 μm.

(G) Hippocampal slices were treated with the indicated drugs at least 60 min prior to depolarization with 40 mM KCl. Fifteen minutes following depolarization, the slices were stained for phospho-CREB. PD denotes PD98059. The scale bar is 75 μM.

(H) Immunoreactivity for phospho-CREB in area CA1 from slices treated as in (G) was quantitated. Error is expressed as the SEM.

(I) Transverse slices of hippocampal area CA1 were treated as in (G). The slices were fixed and processed immunohistochemically for phospho-CREB and phospho-ERK. The graph depicts the averaged immunoreactivity in area CA1 for phospho-CREB and phospho-ERK ($n = 5$–6).

In the above experiments, PD98059 was 50 μM, and olomoucine was 100 μM.

MAPKAP kinase 2, p70 S6 kinase, and CaMKIV (Enslen et al., 1994; Matthews et al., 1994; Tan et al., 1996; Xing et al., 1996). However, neither KN62, a potent inhibitor of CaMKs, SB203580, a selective inhibitor of p38 MAP kinase (an activator of MAPKAP kinase 2), nor rapamycin, a selective inhibitor of a p70 S6 kinase activator, had a significant effect on depolarization-stimulated gene expression in PC12 cells (Figure 8A). Since CaMKIV but not CaMKI is localized to the nuclear compartment, CaMKIV is thought to be the major Ca^{2+}/CaM-stimulated CREB kinase (Picciotto et al., 1995; Bito et al., 1996). However, PC12 cells do not express measurable CaMKIV, as detected by Western blot (Figure 8B; Enslen et al., 1996; Finkbeiner et al., 1997). Thus, it is not surprising that expression of a dominant negative CaMKIV (DN CaMKIV) construct does not inhibit Ca^{2+}-stimulated CREB-dependent transcription (Figure 8C). Conversely, expression of DN CaMKIV blocked the activity of transfected CaMKIV in PC12 cells (Figure 8D). Interestingly, expression of a 5-fold excess of DN CaMKIV also failed to inhibit K^+ depolarization–stimulated gene expression in hippocampal neurons (Figure 8E). Owing to the low efficiency of transfection in hippocampal neurons, we could not assess the effect of DN CaMKIV on CaMKIV activity. However, hippocampal neurons transfected with a Flag-tagged DN CaMKIV were immunostained for the Flag epitope and clearly show a high degree of DN CaMKIV expression (Figure 8F). Furthermore, DN CaMKIV is functional in hippocampal neurons, because DN CaMKIV expression inhibited K^+-stimulated SRE-mediated gene expression (data not shown).

We further explored the role of CaMKIV in Ca^{2+}-regulated transcription by examining CaMK activity under conditions that produce CRE-mediated transcription. Although depolarization with 30 mM KCl markedly activated CREB-dependent transcription, it did not significantly activate CaMKIV activity (Figures 8G and 8H). On the other hand, 60 mM KCl markedly activated CaMKIV activity but did not lead to additional increases in CRE-mediated gene expression or CREB phosphorylation

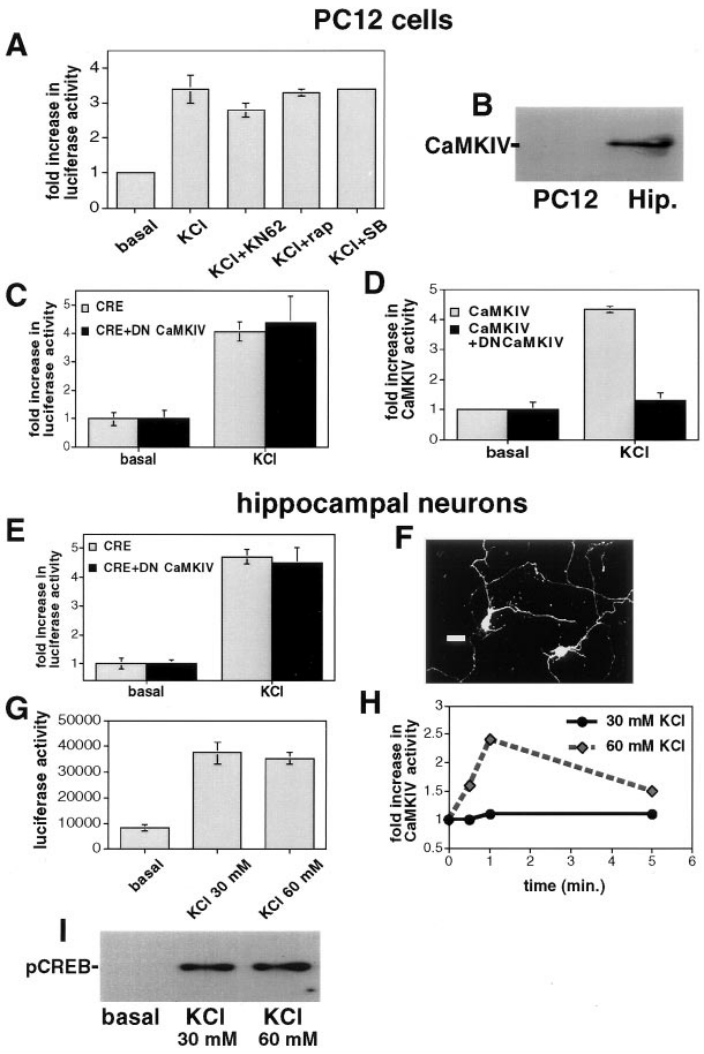

Figure 8. CaMKIV Activity Is Dispensable for Ca^{2+}-Stimulated CREB-Dependent Gene Expression

(A) PC12 cells were transfected with a CRE–luciferase reporter contruct, treated with 60 mM KCl for 6 hr, and asayed for luciferase activity. KN62 was 10 μM, SB203580 was 5 μM, and rapamycin was 100 nM.

(B) PC12 cell nuclear extracts (20 μg) and total extracts from hippocampal neurons (10 μg) were Western blotted for CaMKIV.

(C) PC12 cells were transfected with a CRE–luciferase reporter and a 5-fold excess of control DNA (CRE) or dominant negative CaMK IV DNA (CRE + DN CaMKIV), treated with KCl for 5 hr, and assayed for luciferase activity.

(D) PC12 cells were transfected with wild-type Flag-tagged CaMKIV and a 4-fold excess of DN CaMKIV DNA. Transfected CaMKIV was immunoprecipitated and assayed for kinase activity. Experiments are averages of duplicate determinations. Error is standard deviation.

(E) Hippocampal neurons were transfected with a CRE–luciferase construct and a 5-fold excess of DN CaMKIV DNA, treated with 30 mM KCl for 4 hr, and assayed for luciferase activity. Experiments are averages of quadruplicate determinations. Error is SEM. Similar results were seen in five independent experiments.

(F) Hippocampal neurons were transfected with Flag-tagged DN CaMKIV and stained for the Flag epitope.

(G) Hippocampal neurons were transfected with CRE–luciferase DNA and a 3-fold excess of control DNA and treated with the indicated concentrations of KCl. Error is SEM.

(H) Hippocampal neurons were treated with 30 mM or 60 mM, and CaMKIV was assayed for kinase activity. Experiments are averages of duplicate determinations.

(I) Hippocampal neurons were treated with 30 mM or 60 mM KCl for 15 min, and extracts were Western blotted for phospho-CREB.

(Figures 8H and 8I). Consistent with our observation that a MEK-activated kinase contributes to Ca^{2+}-stimulated CREB phosphorylation, we found that 30 mM KCl robustly stimulated the CREB-kinase activity of Rsk2 in hippocampal neurons (Figure 6B). These results indicate that CaMKIV activity is dispensable for Ca^{2+}-stimulated gene expression in PC12 cells. Furthermore, CaMKIV activation is apparently not required for Ca^{2+}-stimulated CRE-mediated transcription in hippocampal neurons.

PKA Activity Is Required for CREB Phosphorylation and ERK Nuclear Translocation

Our results and those of Thompson et al. (1998) indicate that the activation of CRE-regulated transcription by Ca^{2+} requires PKA activity. However, Ca^{2+}-stimulated CREB phosphorylation was not attenuated in PC12 cells deficient for PKA activity (Thompson et al., 1995). Because this clonal line retains 15%–20% of wild-type PKA-stimulated transcription (data not shown), we explored the role of PKA for Ca^{2+}-stimulated CREB phosphorylation further. Interestingly, Ca^{2+}-mediated CREB phosphorylation in wild-type PC12 cells was decreased by the selective PKA inhibitor Rp-8-Br-MB-cAMPS (Figure 9A). With a high efficiency transfection protocol (>80% transfected; data not shown), expression of DN PKA also attenuated Ca^{2+}-stimulated CREB phosphorylation (Figure 9A). In addition, treatment with the PKA inhibitors Rp-8-Br-MB-cAMPS and Rp-8-CPT-cAMPS also inhibited depolarization-mediated CREB phosphorylation in primary hippocampal neurons (Figure 9B). These results suggest that in PC12 cells and hippocampal neurons, PKA activity is required for Ca^{2+}-induced CREB phosphorylation.

Because the nuclear translocation of ERK may be necessary for ERK-activated transcription (Blenis, 1993), and PKA is required for Ca^{2+} stimulation of CREB phosphorylation, we monitored nuclear translocation of ERK when PKA is inhibited. To efficiently induce the nuclear translocation of ERK by Ca^{2+}, PC12 cells were treated with KCl and BAYK 8644 (a direct activator of L-type Ca^{2+} channels). Depolarization induced the phosphorylation of ERK and its translocation to the nucleus in both

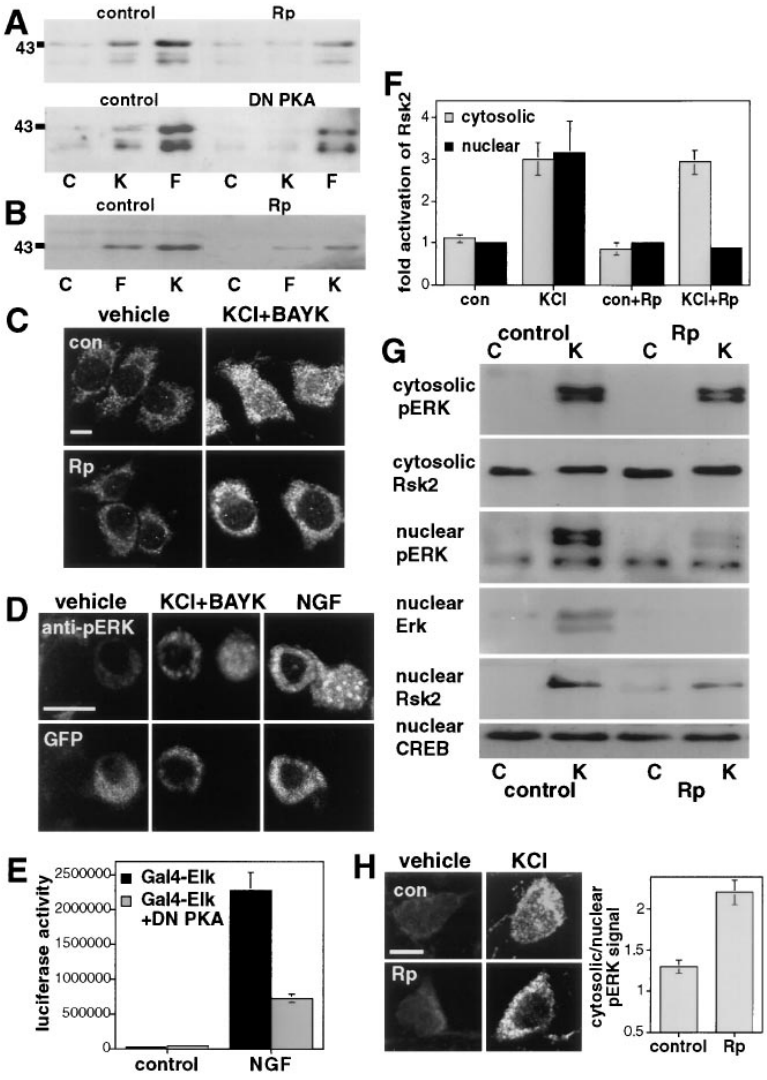

Figure 9. PKA Activity Is Required for the Nuclear Translocation of ERK

(A) PC12 cells were treated with 60 mM KCl (K) or 5 μM forskolin (F) for 5 min. Cell extracts were Western blotted for phospho-CREB. Rp denotes PC12 cells treated with Rp-8-Br-MB-cAMPS (500 μM). DN PKA denotes PC12 cells in which a dominant negative PKA construct was transiently transfected by using lipofectamine plus.

(B) Hippocampal neurons were treated with 30 mM KCl (K) or 5 μM forskolin (F) for 5 min in the absence and presence of 300 μMRp-8-Br-MB-cAMPS/Rp-8-CPT-cAMPS (Rp). Extracts were Western blotted for phospho-CREB.

(C) PC12 cells were treated with 60 mM KCl and 20 μM BAYK 8644 (KCl + BAYK) for 6 min, fixed, and stained immunocytochemically for phospho-ERK. Rp denotes cells treated with 400 μM Rp-8-Br-MB-cAMPS and con denotes cells that were not treated with inhibitors. Scale bar is 25 μm.

(D) PC12 cells were transfected with a GFP–dominant negative PKA fusion protein and treated as described in (C). Anti-pERK shows immunocytochemical staining for phospho-ERK and GFP depicts GFP-DN PKA fusion protein fluorescence in the same field. Scale bar is 25 μm.

(E) PC12 cells were cotransfected with Gal4-Elk, Gal4-luciferase, and a 4-fold excess of control or dominant negative PKA DNAs (DN PKA). Forty-eight hours following transfection, the cells were treated with 100 ng/ml NGF for 5 hr and assayed for luciferase activity. Experiments are averages of triplicate determinations. Error is standard deviation.

(F) PC12 cells were treated with 60 mM KCl for 10 min in the presence and absence of 300 μM Rp-8-Br-MB-cAMPS/Rp-8-CPT-cAMPS. Rsk2 was immunoprecipitated from cytosolic and nuclear extracts, and kinase activity was assayed using CREBtide as a substrate. The results are the average of three experiments. Error is SEM.

(G) PC12 cells were treated as in (F). Nuclear or cytosolic extracts were immunoblotted for the indicated epitopes.

(H) Hippocampal neurons treated with 40 mM KCl for 10 min, fixed, and stained immunocytochemically for phospho-ERK. Rp denotes cells treated with 400 μM Rp-8-Br-MB-cAMPS and con denotes untreated cells. Scale bar is 25 μm. The cytosolic-to-nuclear phospho-ERK ratio was quantitated by confocal microscopy for control and Rp-8-Br-MB-cAMPS-treated neurons (n = 22-28 cells). Error is SEM. Each of the above experiments was repeated at least three times with similar results.

PC12 cells (Figures 9C, 9D, and 9G) and hippocampal neurons (Figures 6C and 9H). The specific PKA inhibitors Rp-8-Br-MB-cAMPS and Rp-8-CPT-cAMPS inhibited the nuclear translocation of Erk in PC12 cells (Figure 9C) and hippocampal neurons (Figure 9H). Furthermore, Western blotting of cytosolic fractions shows that the inhibition of Erk translocation by treatment with Rp-8-Br-MB-cAMPS and Rp-8-CPT-cAMPS was not the result of an effect on Erk activation (Figure 9G). To verify that PKA is required for the nuclear translocation of ERK, the cytosolic-to-nuclear ratio of phospho-ERK in KCl-stimulated hippocampal neurons was also quantitated. The PKA inhibitor Rp-8-Br-MB-cAMPS significantly inhibited the translocation of ERK to the nucleus (Figure 9H). The importance of PKA activity for ERK nuclear translocation was confirmed by transiently transfecting PC12 cells with a dominant negative PKA fused to green fluorescent protein (DN PKA–GFP). Only cells that expressed DN PKA–GFP showed impaired nuclear translocation of phospho-ERK (Figure 9D).

These results suggest that PKA is required for the phosphorylation and transactivation of CREB by Ca^{2+}, because PKA is required for the nuclear translocation of ERK. However, since Rsk2 is a major Ca^{2+}-activated CREB kinase in PC12 cells, inhibition of Erk translocation should also block the activation of nuclear but not cytosolic Rsk2. Accordingly, inhibition of PKA blocked the activation of Rsk2 in the nuclear fraction but not in the cytosolic fraction (Figure 9G). In addition, Rp-8-Br-MB-cAMPS/Rp-8-CPT-cAMPS treatment attenuated the nuclear translocation of Rsk2. This is not surprising, because it is known that both ERK and Rsk2 are tightly associated in vivo and cotranslocate to the nucleus (Hsiao et al., 1994; Zhao et al., 1996). Collectively, these

data indicate that PKA may be necessary for the phosphorylation and transactivation of CREB by Ca^{2+}, because PKA is required for the nuclear translocation of ERK and subsequent nuclear activation of the CREB kinase Rsk2.

Inhibition of PKA also significantly impaired the translocation of ERK to the nucleus in response to NGF (Figure 9D). Interestingly, coexpression of DN PKA attenuated NGF-stimulated Elk1 transcriptional activation (Figure 9E). Evidently, the modulation of ERK translocation by PKA activity plays a general role in the activation of transcription by mitogens and neurotrophic factors. In support of this, Yao et al. (1998) also found a reduced ERK translocation after inhibition of PKA. NGF does not detectably elevate intracellular cAMP (data not shown), suggesting that basal PKA activity is sufficient for neurotrophic factors and other strong ERK activators to induce nuclear translocation of ERK. Nevertheless, in the case of depolarization, which activates ERK to a lesser degree, the concomitant depolarization-mediated increase in cAMP levels enhances ERK translocation (data not shown).

PD98059 Inhibits L-LTP and L-LTP–Associated Increases in CRE-Regulated Transcription

Stimuli that induce protein synthesis–dependent L-LTP also generate a Ca^{2+}- and PKA-dependent increase in CRE-mediated gene expression (Impey et al., 1996). Therefore, we evaluated the role of the ERK/MAP kinase cascade in L-LTP and L-LTP–associated CRE-regulated gene expression. Transcription in CRE-LacZ transgenic mice was monitored by immunohistochemistry for β-galactosidase (Impey et al., 1996). L-LTP in area CA1 was consistently generated (eight of eight slices) by three 100 Hz, 1 s tetani at 5 min intervals (Figure 10A). Slices that expressed L-LTP also evinced a significant increase in LacZ expression in area CA1 (Figures 10C and 10D; $p < 0.001$; low frequency stimulation (LFS), $n = 6$; L-LTP, $n = 8$). However LFS did not generate LTP or increase transcription relative to control slices (Figures 10A and 10C; $p > 0.3$; control, $n = 4$; LFS, $n = 6$). Treatment with the MEK inhibitor PD98059 blocked L-LTP (Figure 10A) and significantly decreased L-LTP–associated LacZ expression (Figures 10C and 10D; $p < 0.001$; high frequency stimulation (HFS), $n = 8$; HFS + PD, $n = 7$). PD98059 treatment also attenuated but did not abolish (146% at 60 min) the early phase of LTP (Figure 10A). Moreover, PD98059 specifically inhibited the induction of LTP, because it did not affect paired-pulse facilitation (data not shown), basal synaptic transmission, or posttetanic potentiation (Figure 10A). PD98059 also failed to block previously established L-LTP, indicating that the ERK/MAP kinase cascade is not involved in the maintenance of L-LTP (Figure 10B).

Interestingly, HFS but not LFS markedly increased immunohistochemical staining for activated ERK in CA1 pyramidal cell bodies and dendrites (Figure 10E). As expected, PD98059 treatment blocked tetanus-induced phospho-ERK staining (data not shown). Thus, activated ERK may regulate synaptic efficacy at the postsynaptic membrane and possibly play a role in targeting long-term changes to activated synapses.

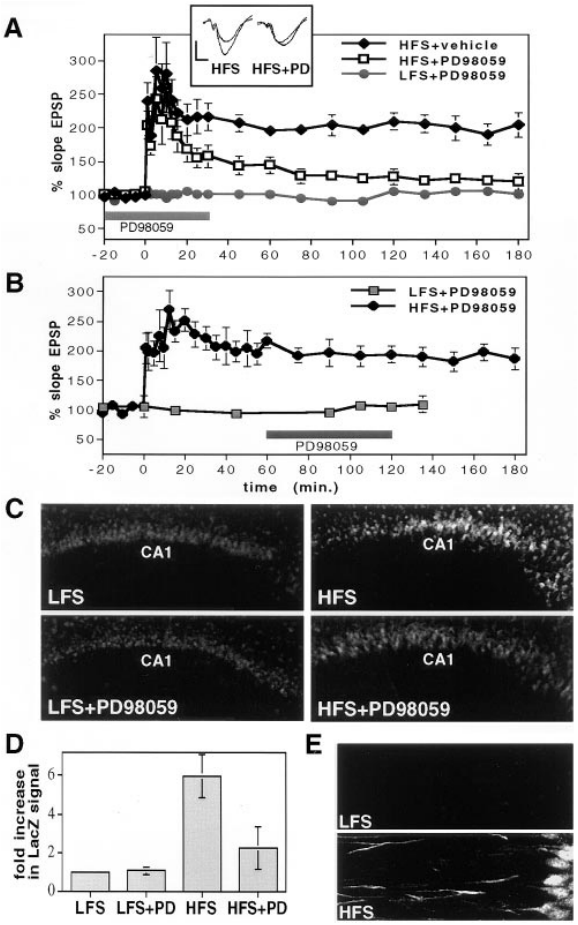

Figure 10. Inhibition of L-LTP and L-LTP–Associated Increases in CRE-LacZ Expression by PD98059

(A) L-LTP was induced by a high frequency tetanus (HFS) (3× 100 Hz, 1 s tetani at 5 min intervals) of the Schaffer collateral–CA1 pyramidal cell synapse. PD98059 (50 μM) was perfused at least 30 min prior to the tetanus. LFS was at 0.05 Hz. The inset depicts fEPSPs from HFS and HFS + PD98059 experiments before and after potentiation. Calibration, 1 mV; 3 ms.

(B) Exposure to 50 μM PD98059 did not inhibit established L-LTP.

(C) Representative examples of immunohistochemistry for LacZ in hippocampal slices from (A). Slices were fixed 5 hr after tetanization.

(D) Fold increase in LacZ signal in the CA1 molecular layer in slices from (A). PD denotes PD98059. Error is expressed as SEM.

(E) Staining for phospho-ERK in slices fixed 15 min after LFS and HFS.

Collectively, our data implicate the ERK/MAP kinase cascade in the induction of gene expression–dependent neuronal plasticity in the mouse hippocampus. Because L-LTP requires the coincident activation of both the PKA and Ca^{2+} signaling pathways (Impey et al., 1996), it is possible that these pathways converge on ERK to facilitate the formation of L-LTP. The coactivation of ERK and PKA may optimally stimulate the expression CREB-regulated genes that facilitate the formation of L-LTP. In support of this hypothesis, L-LTP–associated CRE-mediated gene expression depends on ERK signaling (Figures 10C and 10D) and PKA activity (Impey et al., 1996).

Discussion

In this study, we explored the mechanisms by which Ca^{2+} stimulates CRE-mediated gene expression in PC12 cells and primary hippocampal neurons. Our findings indicate that the induction of CRE-mediated transcription by Ca^{2+} requires both PKA and ERK activities. We propose that PKA is required for Ca^{2+}-stimulated CREB-mediated transcription, because PKA activity is necessary for the nuclear translocation of ERK and subsequent nuclear activation of Rsk2. Furthermore, we demonstrate that the persistent activation of ERK increases CRE-mediated gene expression. Our results suggest that Ca^{2+}-stimulated gene expression is mediated at least in part by an ERK-mediated phosphorylation of CREB and that the regulation of CRE-mediated gene expression by the ERK/MAP kinase cascade plays a critical role in the formation of long lasting neuroplasticity in the hippocampus.

Ca^{2+}-Stimulated Gene Expression and the ERK/Rsk2 MAP Kinase Cascade

Earlier work in PC12 cells and cortical neurons showed that Ca^{2+} promotes the activation of ERK by enhancing the GTPase activity of Ras (Rosen et al., 1994; Rusanescu et al., 1995). These observations led us to explore whether activation of ERK promotes Ca^{2+}-stimulated gene expression. Expression of dominant negative CREB blocked Ca^{2+}-stimulated transcription, indicating that CREB or its dimerization partners mediates transactivation of the CRE by Ca^{2+} in vivo. We found that activation of CRE-mediated gene expression also requires functional MEK in PC12 cells, cultured hippocampal neurons, and in the adult hippocampal slice preparation. This suggests that ERK may play a general role in the activation of CRE-mediated transcription by Ca^{2+} in neurons of the CNS and possibly in other tissues.

Our data support the hypothesis that the persistent activation of ERK leads to the activation of Rsk2, which directly phosphorylates and transactivates CREB. Ca^{2+}-induced CREB phosphorylation was inhibited by the selective MEK inhibitor PD98059 in PC12 cells, cultured hippocampal neurons, and the adult hippocampal slice preparation. Furthermore, Ca^{2+}-stimulated CREB kinase activity in PC12 cells was blocked by PD98059. Because immunodepletion of Rsk2 markedly attenuated depolarization-stimulated nuclear CREB kinase activity, we conclude that Rsk2 is a major Ca^{2+}-stimulated CREB kinase in PC12 cells. By immune complex kinase assay, we identify Rsk2, and to lesser degree Rsk1 and Rsk3, as major Ca^{2+}-stimulated CREB kinases in cultured hippocampal neurons. The inhibition of tetanus- and depolarization-induced CREB phosphorylation by PD98059 in hippocampal slices suggests that a Rsk family member may also function as a CREB kinase in the intact hippocampus.

In support of our hypothesis that the ERK–Rsk signaling cascade plays an important role in activity-dependent gene expression in the CNS, Finkbeiner et al. (1997) recently reported that brain-derived neurotrophic factor– (BDNF-) induced CREB phosphorylation and CRE-mediated transcription depend on a MEK-dependent CREB kinase. In an interesting parallel to our research, they find that inhibition of MEK markedly attenuates late ($>$10 min) CREB phosphorylation, while inhibition of CaMKs attenuates early (5 min) CREB phosphorylation.

Although the work of Bito et al. (1996) suggests that CaMKIV contributes to early ($<$2 min) Ca^{2+} stimulation of CREB phosphorylation, it is not known if CaMKIV activity is required for prolonged Ca^{2+}-stimulated CREB phosphorylation. Indeed, preliminary work suggests that CaMKIV activity is required for early depolarization–mediated CREB phosphorylation but is not required for delayed CREB phosphorylation (S. I. and D. S., unpublished data). CaMKIV may also lead to CREB phosphorylation via the activation of downstream kinases. For example, CaMKIV can activate MAP kinases (Enslen et al., 1996). Recently, Shieh et al. (1998) and Finkbeiner et al. (1997) also implicate CaMKIV in the activation of CREB-dependent gene expression in cortical neuron cultures. Although we found that CaMKIV activity was dispensable for Ca^{2+}-stimulated CRE-mediated gene expression, we used hippocampal cultures, which have a homogenous population of excitatory pyramidal neurons. Furthermore, Shieh et al. (1998) examined Ca^{2+}-stimulated transcription in the context of the BDNF exon III promoter, which requires a cooperative interaction between CREB and other inducible transcription factors. Nevertheless, our work does not exclude the possibility that CaMKIV and ERK-dependent signaling interact cooperatively to induce Ca^{2+}-stimulated CRE-mediated gene expression.

The Role of PKA in Ca^{2+}-Stimulated Gene Expression and ERK Translocation

Why is PKA obligatory for the activation of CREB- and CRE-mediated transcription? The nuclear translocation of ERK, which occurs in response to mitogens, neurotrophic factors, and Ca^{2+}, is thought to be a prerequisite for the activation of transcription by the ERK/MAP kinase cascade. Our data indicate that Ca^{2+}- and growth factor–stimulated ERK nuclear translocation depend on PKA activity. Yao et al. (1998) also report reduced ERK nuclear localization after PKA inhibition. We also show that the inhibition of ERK nuclear translocation attenuates the nuclear translocation and nuclear activation of Rsk2. It is interesting to note that ERK and Rsk are tightly associated in vivo and cotranslocate to the nucleus (Hsiao et al., 1994; Zhao et al., 1996). Optimal activation of nuclear Rsk may involve the translocation of a signaling complex consisting of MEK, ERK, and Rsk. Recent work suggests that dimerization of ERK is required for its prolonged translocation (Khokhlatchev et al., 1998). However, because ERK is not a substrate for PKA (Graves et al., 1993), it is not likely that PKA directly modulates ERK dimerization. Nevertheless, it is possible that PKA indirectly modulates the dimerization of ERK or the machinery that facilitates the nuclear transport of ERK dimers. The discovery that PKA activity is required for the nuclear translocation of ERK and Rsk2 provides important new insights concerning the role of PKA in Ca^{2+}- and growth-factor–stimulated transcription.

The synergism between PKA and Ca^{2+} signaling is more robust at the level of transcription than at the level of CREB phosphorylation in PC12 cells. This suggests that PKA may regulate CRE-mediated gene expression at additional sites, such as the association of CREB with its coactivator CBP (Brindle et al., 1995). We also found that PKA activation enhanced the association of CREB with CBP in PC12 cells (S. I. and D. S., unpublished data). Thus, PKA may contribute to Ca^{2+}-stimulated transcription by multiple mechanisms, including stimulation of the ERK pathway, facilitation of ERK nuclear translocation, and the association of CREB with its transcriptional coactivators.

Is the Activation of CREB by ERK Important for Neuronal Plasticity and Learning?

Although a large body of evidence suggests that CREB-mediated transcription plays a fundamental role in neuronal plasticity and learning, it is not known which signaling cascades mediate plasticity-associated increases in gene expression. Both the Ca^{2+} and cAMP-mediated signaling pathways have been implicated in neuronal plasticity and learning. Coactivation of Ca^{2+} and cAMP signaling may generate optimal levels of CREB-dependent gene expression. For example, L-LTP and L-LTP–associated increases in CRE-regulated gene expression require the coactivation of both cAMP and Ca^{2+} signaling (Impey et al., 1996). Here, we show that the ERK/MAP kinase pathway potentiates the activation of CRE-mediated transcription by PKA.

ERK is robustly activated by Ca^{2+} or synaptic activity in the CNS. For example, NMDA receptor stimulation leads to the activation of ERK in neuron cultures (Bading and Greenberg, 1991). Furthermore, tetanic stimulation generates both LTP and ERK activation (English and Sweatt, 1996), and the ERK cascade may be important for the induction of LTP (English and Sweatt, 1997). Interestingly, our data indicate that Erk-dependent signaling is required for activity-induced CREB phosphorylation in hippocampal area CA1 and for the formation of L-LTP and L-LTP–associated CRE-mediated gene expression.

Two recent studies implicate the ERK/MAP kinase pathway in the formation of long-term facilitation (LTF) in *Aplysia.* ERK activation is both associated with and necessary for LTF (Martin et al., 1997) and is required for the plasticity-associated downregulation of apCAM (Bailey et al., 1997). This is intriguing, because downregulation of the *Drosophila* apCAM homolog Fascicilin II, in concert with CREB activation, promotes functional plasticity at the neuromuscular junction (Davis et al., 1996). Other studies also provide indirect evidence that ERK/MAP kinase signaling may be important for learning and memory. The 14–3–3 protein, Leonardo, which is a positive regulator of Ras1-mediated signaling, is required for olfactory learning in *Drosophila* (Skoulakis and Davis, 1996). Furthermore, mice lacking the Ras guanine nucleotide exchange factor Ras-GRF show impaired learning (Brambilla et al., 1997). Finally, mutations of Rsk2 cause Coffin-Lowry Syndrome, which is characterized by mental retardation and cranial dysmorphism (Trivier et al., 1996).

Our observations that ERK signaling is necessary for Ca^{2+}-stimulated transcription suggest that ERK may modulate neuronal plasticity. In support of this hypothesis, stimuli that induce CREB-dependent L-LTP also promote the activation of ERK, the phosphorylation of CREB, and the stimulation of CRE-regulated gene expression (English and Sweatt, 1996; Impey et al., 1996). Moreover, our work indicates that activation of ERK/MAP kinase signaling is critical for the induction of L-LTP and L-LTP–associated CRE-regulated transcription. In this study, we delineate for the first time a clear link between two processes strongly implicated in neuronal plasticity and learning: the ERK/MAP kinase cascade and CREB-regulated gene expression.

Experimental Procedures

Pharmacology and Drug Treatments

Neurons or PC12 cells were pretreated with inhibitors for at least 90 min. Exposure of PD98059 and isradipine to light was minimized. The sources and handling of inhibitors are provided upon request.

Plasmids

The following plasmids have been described previously: dominant negative and constitutively active MEK (Seger et al., 1994), dominant negative Ras T17N (Coso et al., 1995), constitutively active Ras Q61L (Johnson and Nathanson, 1994), KCREB (Walton et al., 1992), Gal4–CREB (Hagiwara et al., 1992), CRE(α168)-luciferase (Matthews et al., 1994), and dominant negative CaMK (Gringhuis et al., 1997). Details on the subcloning and purification of plasmids is provided on request.

Cell Culture

Early passage PC12 cells (p19–30) were cultured as described in Mark et al. (1995). Neurons were cultured as described in Chan et al. (1998).

Determination of cAMP Accumulation

Intracellular cAMP levels were measured as described by Impey et al. (1994).

Protein Kinase Assays

For immune complex kinase assays, cells were washed twice in cold Hank's balanced salt solution (HBSS) and collected at 4°C in 0.5–1 ml of nondenaturing PBS-RIPA (PBS, pH 7.4, 1% NP-40, 1 mM EDTA, 2 mM Na_3VO_4, 10 nM Calyculin A, 1 mM benzamidine, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethysulfonyl fluoride [PMSF], and 10 mM NaF). The lysates were triturated repeatedly through a 25.5 gauge needle and spun 10 min at 14,000 $\times$ g. Lysates were precleared with 20 μl protein A or G (Pierce). Five to twenty micrograms of goat anti-Rsk1, goat anti-Rsk2, or goat anti-Rsk3 antibody (Santa Cruz), 5 μg of mouse anti-ERK antibody (Zymed), 5 μg of goat anti-rabbit p70 S6 kinase antibody (Santa Cruz), or 2.5 μg of sheep anti-rabbit MAPKAP kinase 2 antibody (UBI) was incubated with the supernatant for 4–6 hr. Thirty microliters protein A or G agarose (Pierce) was added for an additional 4–16 hr. The protein A or G agarose was washed 5 times in RIPA wash buffer (PBS-RIPA with 0.25% NP-40). The protein A or G agarose was resuspended in 20 μl buffer H, and 5 μl was assayed for kinase activity as described by Ahn et al. (1991). Myelin basic protein (MBP), S6 peptide, MAPKAP kinase 2 assay peptide, or CREBtide were used as substrates. The CaMKIV immune complex kinase assay was performed as described in Park and Soderling (1995).

Western Blotting

Agonist-treated cells were lysed in 2.5$\times$ boiling sample buffer and boiled for 10 min. CA1 mini-slices were sonicated in 50 μl buffer H (with 1 mM dithiothreitol [DTT]), centrifuged at 13,000 g, and lysed

in 1× boiling sample buffer. Protein assays (Pierce-BCA) were conducted on the CA1 slice sonicates. The samples were centrifuged (5′ at 13,000 g) and electrophoresed by standard procedures. Gels were transferred to Immobilon-P membranes (Millipore) and blocked with 10% powdered milk in PBS. The following primary antibodies were used overnight at 4°C in PBST (0.1% Triton X-100) with 5% bovine serum albumin (BSA): rabbit anti–phospho-CREB, 1:2000 (NEB); mouse anti-CREB, 1:1000 (Santa Cruz); mouse anti-MAP kinase, 1:500 (Zymed); rabbit anti–phospho-MAP kinase, 1:10000 (Promega); rabbit anti-ATF1, 1:500 (Santa Cruz); and goat anti-Rsk1, -Rsk2, and -Rsk3 (Santa Cruz), 1:1000–2000. The secondary antibodies (affinity-purified AP-conjugated anti-IgG, 1:2500; Cappel) were used at 4°C for 6–16 hr in 5% milk PBST. Immunoblots were developed by using the alkaline phosphatase chemiluminescence (Tropix).

Mono Q Anion Exchange Chromatography

PC12 cells or neurons (10^7 cells) were treated as indicated, washed twice in ice-cold HBSS, lysed in 1 ml of buffer H (Ahn et al., 1991) by dounce homogenization (20 times with pestle B), and spun at 14,000 × g for 15 min. The supernatant was applied to a Mono Q anion exchange column (Pharmacia) that had been preequilibrated with buffer H. The column was washed with 10 ml buffer H, and proteins were eluted with a 0–0.4 M linear NaCl gradient in buffer H. Thirty-one 1 ml fractions were collected at a flow rate of 1 ml/min.

Nuclear Fractionation

Nuclear fractionation was conducted using standard techniques (Schreiber et al., 1989), except that two extractions were performed.

Immunochemistry

Cultured cells were fixed with 90% methanol, 10% glacial acetic acid (1 min at −20°C). Hippocampal slices were fixed in 6% formaldehyde in HBSS (pH 7.4, 50 mM HEPES). Tissue was cryoprotected in 30% sucrose PBS. Cells and tissue were blocked (4 hr at 25°C) in 5% BSA in PBS with 0.05% Triton X-100 and 10 mM NaF (PBST). Labeling was conducted in 2.5% BSA PBST. The rabbit anti-LacZ antibody was used at 1:1000 (Cappel) and was visualized with 3 μg/ml LRSC goat anti-rabbit IgG (Jackson). The rabbit anti–phospho-ERK (NEB), mouse anti-ERK (Zymed), rabbit anti–phospho-CREB (NEB), and anti-Flag epitope (Kodak) antibodies were used at 1:250–500. The ERK, phospho-CREB, and phospho-ERK antibodies were labeled with 3–6 μg/ml biotin-labeled anti-IgG (Jackson) and LRSC or Cy5-labeled streptavidin (Jackson) at 4 μg/ml. For double label experiments, the following antibodies were used: 2 μg/ml FITC anti-rabbit (Jackson), 4 μg/ml Alexa-594 anti-mouse (Molecular Probes), and 4 μg/ml Alexa-488 anti-FITC (Molecular Probes).

Quantitation of Immunohistochemistry

Quantitation was carried out as described in Impey et al. (1996), except that signal was normalized to staining in the stratum lacunosum.

Transfection

PC12 cells were transfected with lipofectamine (Gibco-BRL) or lipofectamine plus (Gibco-BRL) as described by the manufacturer. Hippocampal neurons were transfected with DOSPER (Boehringer Mannheim) as described by the manufacturer. Briefly, 1–2× 10^5 neurons (per well of a 24-well plate) at day 5–7 in vitro were transfected with a complex of 0.6 μg of DNA and 6 μl of DOSPER in 250 μl of Neurobasal (Gibco-BRL).

Reporter Assays

Reporter assays were conducted 48 hr posttransfection unless otherwise noted. Luciferase activity was measured as described in Matthews et al. (1994) and normalized to β-galactosidase activity (Impey et al., 1996).

Electrophysiology

Experiments were conducted as described by Impey et al. (1996), with the following changes. Slices were perfused at 30°C, and afferents were stimulated with pulses consisting of 0.2 ms square waves.

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References

Ahn, N.G., Seger, R., Bratlien, R.L., Diltz, C.D., Tonks, N.K., and Krebs, E.G. (1991). Multiple components in an epidermal growth factor–stimulated protein kinase cascade. In vitro activation of a myelin basic protein/microtubule–associated protein 2 kinase. J. Biol. Chem. *266*, 4220–4227.

Bading, H., and Greenberg, M.E. (1991). Stimulation of protein tyrosine phosphorylation by NMDA receptor activation. Science *253*, 912–914.

Bailey, C.H., Kaang, B., Chen, M., Martin, K.C., Lim, C., Casadio, A., and Kandel, E.R. (1997). Mutation in the phosphorylation sites of MAP kinase blocks learning-related internalization of apCAM in *Aplysia* sensory neurons. Neuron *18*, 913–924.

Bartsch, D., Ghirardi, M., Skehel, P.A., Karl, K.A., Herder, S.P., Chen, M., Bailey, C.H., and Kandel, E.R. (1995). Aplysia CREB2 represses long-term facilitation: relief of repression converts transient facilitation into long-term functional and structural change. Cell *83*, 979–992.

Bito, H., Deisseroth, K., and Tsien, R.W. (1996). CREB phosphorylation and dephosphorylation: a Ca^{2+}- and stimulus duration–dependent switch for hippocampal gene expression. Cell *87*, 1203–1214.

Blenis, J. (1993). Signal transduction via the MAP kinases: proceed at your own rsk. Proc. Natl. Acad. Sci. USA *90*, 5889–5892.

Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., and Silva, A.J. (1994). Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. Cell *79*, 59–68.

Brambilla, R., Gnesutta, N., Minichiello, L., White, G., Roylance, A.J., Herron, C.E., Ramsey, M., Wolfer, D.P., Cestari, V., Rossi-Arnaud, C., et al. (1997). A role for the Ras signaling pathway in synaptic transmission and long-term memory. Nature *390*, 281–286.

Brindle, P., Nakajima, T., and Montminy, M. (1995). Multiple protein kinase A–regulated events are required for transcriptional induction by cAMP. Proc. Natl. Acad. Sci. USA *92*, 10521–10525.

Chan, G.C., Hinds, T.R., Impey, S., Storm, D.R. (1998). Hippocampal neurotoxicity of Delta9-tetrahydrocannabinol. J. Neurosci. *18*, 5322–5332.

Chen, R.H., Chung, J., and Blenis, J. (1991). Regulation of pp90rsk phosphorylation and S6 phosphotransferase activity in Swiss 3T3 cells by growth factor–, phorbol ester–, and cyclic AMP–mediated signal transduction. Mol. Cell. Biol. *11*, 1861–1867.

Chrivia, J.C., Kwok, R.P.S., Lamb, N., Hagiwara, M., Montminy, M.R., and Goodman, R. (1993). Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature *365*, 855–859.

Coso, O.A., Chiariello, M., Yu, J.C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J.S. (1995). The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell *81*, 1137–1146.

Davis, G.W., Schuster, C.M., and Goodman, C.S. (1996). Genetic dissection of structural and functional components of synaptic plasticity. III. CREB is necessary for presynaptic functional plasticity. Neuron *17*, 669–679.

de Groot, R.P., den Hertog, J., Vandenheede, J.R., Goris, J., and Sassone-Corsi, P. (1993). Multiple and cooperative phosphorylation

events regulate the CREM activator function. EMBO J. *12*, 3903–3911.

Deutsch, P.J., Jameson, J.L., and Habener, J.F. (1987). Cyclic AMP responsiveness of human gonadotropin-alpha gene transcription is directed by a repeated 18-base pair enhancer. Alpha-promoter receptivity to the enhancer confers cell-preferential expression. J. Biol. Chem. *262*, 12169–12174.

English, J.D., and Sweatt, J.D. (1996). Activation of p42 mitogen-activated protein kinase in hippocampal long term potentiation. J. Biol. Chem. *271*, 24329–24332.

English, J.D., and Sweatt, J.D. (1997). A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. J. Biol. Chem. *272*, 19103–19106.

Enslen, H., Sun, P., Brickey, D., Soderling, S.H., Klamo, E., and Soderling, T.R. (1994). Characterization of Ca^{2+}/calmodulin-dependent protein kinase IV. Role in transcriptional regulation. J. Biol. Chem. *269*, 15520–15527.

Enslen, H., Tokumitsu, H., Stork, P.J.S., Davis, R.J., and Soderling, T.R. (1996). Regulation of mitogen-activated protein kinases by a calcium/calmodulin-dependent protein kinase cascade. Proc. Natl. Acad. Sci. USA *93*, 10803–10808.

Finkbeiner, S., Tavazoie, S.F., Maloratsky, A., Jacobs, K.M., Harris, K.M., and Greenberg, M.E. (1997). CREB: a major mediator of neuronal neurotrophin responses. Neuron *19*, 1031–1047.

Ghosh, A., Ginty, D.D., Bading, H., and Greenberg, M.E. (1994). Calcium regulation of gene expression in neuronal cells. J. Neurobiol. *25*, 294–303.

Ginty, D.D. (1997). Calcium regulation of gene expression: isn't that spatial? Neuron *18*, 183–186.

Ginty, D.D., Glowacka, D., Bader, D.S., Hidaka, H., and Wagner, J.A. (1991). Induction of immediate early genes by Ca^{2+} influx requires cAMP-dependent protein kinase in PC12 cells. J. Biol. Chem. *266*, 17454–17458.

Ginty, D.D., Bonni, A., and Greenberg, M.E. (1994). NGF activates a novel Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. Cell *77*, 713–725.

Graves, L.M., Bornfeldt, K.E., Raines, E.W., Potts, B.C., Macdonald, S.G., Ross, R., and Krebs, E.G. (1993). Protein kinase A antagonizes platelet-derived growth factor–induced signaling by mitogen-activated protein kinase in human arterial smooth muscle cells. Proc. Natl. Acad. Sci. USA *90*, 10300–10304.

Gringhuis, S.I., de Leij, L.F., Wayman, G.A., Tokumitsu, H., and Vellenga, E. (1997). The Ca^{2+}/calmodulin-dependent kinase type IV is involved in the CD5-mediated signaling pathway in human T lymphocytes. J. Biol. Chem. *272*, 31809–31820.

Hagiwara, M., Alberts, A., Brindle, P., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S., and Montminy, M. (1992). Transcriptional attenuation following cAMP induction requires PP-1–mediated dephosphorylation of CREB. Cell *70*, 105–113.

Hagiwara, M., Brindle, P., Harootunian, A., Armstrong, R., Rivier, J., Vale, J., Tsien, R., and Montminy, M.R. (1993). Coupling of hormonal stimulation and transcription via the cAMP responsive factor CREB is rate limited by nuclear entry of protein kinase A. Mol. Cell. Biol. *13*, 4852–4859.

Hsiao, K.M., Chou, S.Y., Shih, S.J., and Ferrell, J.E., Jr. (1994). Evidence that inactive p42 mitogen-activated protein kinase and inactive Rsk exist as a heterodimer in vivo. Proc. Natl. Acad. Sci. USA *91*, 5480–5484.

Impey, S., Wayman, G., Wu, Z., and Storm, D.R. (1994). Type I adenylyl cyclase functions as a coincidence detector for control of cyclic AMP response element–mediated transcription: synergistic regulation of transcription by Ca^{2+} and isoproterenol. Mol. Cell. Biol. *14*, 8272–8281.

Impey, S., Mark, M., Villacres, E.C., Poser, S., Chavkin, C., and Storm, D.R. (1996). Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. Neuron *16*, 973–982.

Johnson, C.M., Hill, C.S., Chawla, S., Treisman, R., and Bading, H. (1997). Calcium controls gene expression via three distinct pathways that can function independently of the Ras/mitogen-activated protein kinases (ERKs) signaling cascade. J. Neurosci. *17*, 6189–6202.

Johnson, J.A., and Nathanson, N.M. (1994). Differential requirements for p21ras and protein kinase C in the regulation of neuronal gene expression by nerve growth factor and neurokines. J. Biol. Chem. *269*, 18856–18863.

Khokhlatchev, A.V., Canagarajah, B., Wilsbacher, J., Robinson, M., Atkinson, M., Goldsmith, E., and Cobb, M.H. (1998). Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. Cell *93*, 605–615.

Kwok, R.P.S., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G.E., Green, M.R., and Goodman, R.H. (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature *370*, 223–226.

Liu, F.C., and Graybiel, A.M. (1996). Spatiotemporal dynamics of CREB phosphorylation: transient versus sustained phosphorylation in the developing striatum. Neuron *17*, 1133–1144.

Mark, M.D., Liu, Y., Wong, S.T., Hinds, T.R., and Storm, D.R. (1995). Stimulation of neurite outgrowth in PC12 cells by EGF and KCl depolarization: a Ca^{2+}-independent phenomenon. J. Cell Biol. *130*, 701–710.

Martin, K.C., Michael, D., Rose, J.C., Barad, M., Casadio, A., Zhu, H., and Kandel, E.R. (1997). MAP kinase translocates into the nucleus of the presynaptic cell and is required for long-term facilitation in *Aplysia*. Neuron *18*, 899–912.

Matthews, R.P., Guthrie, C.R., Wailes, L.M., Zhao, X., Means, A.R., and McKnight, G.S. (1994). Calcium and calmodulin dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. Mol. Cell. Biol. *14*, 6107–6116.

Park, I.K., and Soderling, T.R. (1995). Activation of Ca^{2+}/calmodulin-dependent protein kinase (CaM-kinase) IV by CaM-kinase kinase in Jurkat T lymphocytes. J. Biol. Chem. *270*, 30464–30469.

Picciotto, M.R., Zoli, M., Bertuzzi, G., and Nairn, A.C. (1995). Immunochemical localization of calcium/calmodulin-dependent protein kinase I. Synapse *20*, 75–84.

Rosen, L.B., Ginty, D.D., Weber, M.J., and Greenberg, M.E. (1994). Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of ras. Neuron *12*, 1207–1221.

Rusanescu, G., Qi, H., Thomas, S.M., Brugge, J.S., and Halegoua, S. (1995). Calcium influx induces neurite growth through a Src–Ras signaling cassette. Neuron *15*, 1415–1425.

Schreiber, E., Matthias, P., Muller, M.M., and Schaffner, W. (1989). Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res. *17*, 6419.

Seger, R., Seger, D., Reszka, A.A., Munar, E.S., Eldar-Finkelman, H., Dobrowolska, G., Jensen, A.M., Campbell, J.S., Fischer, E.H., and Krebs, E.G. (1994). Overexpression of mitogen-activated protein kinase kinase (MAPKK) and its mutants in NIH 3T3 cells. J. Biol. Chem. *269*, 25699–25709.

Sheng, M., McFadden, G., and Greenberg, M.E. (1990). Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. Neuron *4*, 571–582.

Skoulakis, E.M., and Davis, R.L. (1996). Olfactory learning deficits in mutants for leonardo, a Drosophila gene encoding a 14–3–3 protein. Neuron *17*, 931–944.

Sun, P., Lou, L., and Maurer, R.A. (1996). Regulation of activating transcription factor-1 and the cAMP response element–binding protein by Ca^{2+}/calmodulin-dependent protein kinases type I, II, and IV. J. Biol. Chem. *271*, 3066–3073.

Tan, Y., Rouse, J., Zhang, A., Cariati, S., Cohen, P., and Comb, M.J. (1996). FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. EMBO J. *15*, 4629–4642.

Thompson, M.A., Ginty, D.D., Bonni, A., and Greenberg, M.E. (1995). L-type voltage-sensitive Ca^{2+} channel activation regulates c-fos transcription at multiple levels. J. Biol. Chem. *270*, 4224–4435.

Traverse, S., Gomez, N., Paterson, H., Marshall, C., and Cohen, P. (1992). Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of NGF and EGF. Biochem. J. *288*, 351–355.

Trivier, E., De Cesare, D., Jacquot, S., Pannetier, S., Zackai, E.,

Young, I., Mandel, J.L., Sassone-Corsi, P., and Hanauer, A. (1996). Mutations in the kinase Rsk-2 associated with Coffin-Lowry syndrome. Nature *384*, 567–570.

Vossler, M.R., Yao, H., York, R.D., Pan, M.G., Rim, C.S., and Stork, P.J. (1997). cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. Cell *89*, 73–82.

Walton, K.M., Rehfuss, R.P., Chrivia, J.C., Lochner, J.E., and Goodman, R.H. (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.

Wu, Z.L., Thomas, S.A., Villacres, E.C., Xia, Z., Simmons, M.L., Chavkin, C., Palmiter, R.D., and Storm, D.R. (1995). Altered behavior and long-term potentiation in type I adenylyl cyclase mutant mice. Proc. Natl. Acad. Sci. USA *92*, 220–224.

Xing, J., Ginty, D.D., and Greenberg, M.E. (1996). Coupling of the RAS–MAPK pathway to gene activation by RSK2, a growth factor–regulated CREB kinase. Science *273*, 959–963.

Xing, J., Kornhauser, J.M., Xia, Z., Thiele, E.A., and Greenberg, M.E. (1998). Nerve growth factor activates extracellular signal–regulated kinase and p38 mitogen activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. Mol. Cell. Biol. *18*, 1946–1955.

Yao, H., Labudda, K., Rim, C., Capodieci, P., Loda, M., and Stork, P.J. (1995). Cyclic adenosine monophosphate can convert epidermal growth factor into a differentiating factor in neuronal cells. J. Biol. Chem. *270*, 20748–20753.

Yao, H., York, R.D., Misra-Press, A., Carr, D.W., and Stork, P.J. (1998). The cyclic adenosine monophosphate–dependent protein kinase (PKA) is required for the sustained activation of mitogen-activated kinases and gene expression by nerve growth factor. J. Biol. Chem. *273*, 8240–8247.

Yin, J.C., Wallach, J.S., Del Vecchio, M., Wilder, E.L., Zhou, H., Quinn, W.G., and Tully, T. (1994). Induction of a dominant negative CREB transgene specifically blocks long-term memory in Drosophila. Cell *79*, 49–58.

Zhao, Y., Bjorbaek, C., and Moller, D.E. (1996). Regulation and interaction of pp90(rsk) isoforms with mitogen-activated protein kinases. J. Biol. Chem. *271*, 29773–29779.