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CREB Transcriptional Activity in Neurons Is Regulated by Multiple, Calcium-Specific Phosphorylation Events

Jon M. Kornhauser,[1] Christopher W. Cowan,[1] Adam J. Shaywitz,[1,2] Ricardo E. Dolmetsch,[1] Eric C. Griffith,[1] Linda S. Hu,[1] Chia Haddad,[1] Zhengui Xia,[1,4] and Michael E. Greenberg[1,3]
[1]Division of Neuroscience
Children's Hospital and
Department of Neurobiology
Harvard Medical School
[2]Program in Biological and Biomedical Sciences
Harvard Medical School
300 Longwood Avenue
Boston, Massachusetts 02115

Summary

The transcription factor CREB mediates diverse responses in the nervous system. It is not known how CREB induces specific patterns of gene expression in response to different extracellular stimuli. We find that Ca^{2+} influx into neurons induces CREB phosphorylation at Ser133 and two additional sites, Ser142 and Ser143. While CREB Ser133 phosphorylation is induced by many stimuli, phosphorylation at Ser142 and Ser143 is selectively activated by Ca^{2+} influx. The triple phosphorylation of CREB is required for effective Ca^{2+} stimulation of CREB-dependent transcription, but the phosphorylation of Ser142 and Ser143, in addition to Ser133, disrupts the interaction of CREB with its cofactor CBP. These results suggest that Ca^{2+} influx triggers a specific program of gene expression in neurons by selectively regulating CREB phosphorylation.

Introduction

The transcription factor CREB (cAMP/Ca^{2+} response element binding protein) is activated in neurons in response to *trans*-synaptic signaling and regulates the expression of genes that are important for adaptive neuronal responses, such as behavioral adaptation to changes in the environment (Ginty et al., 1993), as well as more complex neural functions, such as learning and memory (reviewed in Frank and Greenberg, 1994). CREB target genes include immediate-early genes, such as *c-fos* (Sheng et al., 1990), and molecules important for synaptic function, such as brain derived neurotrophic factor (BDNF) (Tao et al., 1998; Shieh et al., 1998) and neuronal nitric oxide synthase (nNOS) (Sasaki et al., 2000). In addition to its functions in mature neurons, CREB regulates cell proliferation, differentiation, and survival responses in a range of cell types in developing vertebrates (Bonni et al., 1999; Riccio et al., 1999; Walton et al., 1999).

CREB is a bZIP transcription factor that forms both homo- and heterodimers with itself or other members of the CREB family, including ATF1 and CREM. These dimers interact with a specific DNA element having the consensus sequence TGACGTCA that is present within the regulatory regions of CREB target genes. CREB is inactive as a transcription factor until a cell is exposed to any one of a range of extracellular stimuli that trigger the phosphorylation of CREB at a specific site, Ser133, within its kinase-inducible domain (KID) (reviewed in Shaywitz and Greenberg, 1999). Phosphorylation of the KID at Ser133 promotes the association of CREB with a coadaptor protein, the CREB binding protein (CBP), via the CBP KIX domain (Chrivia et al., 1993). The recruitment of CBP by CREB to the promoter of a CREB target gene then induces the assembly of an active polymerase II transcription complex, thus leading to target gene activation (Kwok et al., 1994).

In neurons, *trans*-synaptic release of glutamate triggers Ca^{2+} influx through the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors and L type voltage-sensitive Ca^{2+} channels (L-VSCCs). The resulting elevated levels of cytoplasmic and nuclear Ca^{2+} promote the activation of the Ca^{2+}/calmodulin-dependent kinase IV (CaMKIV), which then phosphorylates CREB at Ser133 within the nucleus (Matthews et al., 1994; Bito et al., 1996). In addition, Ca^{2+} activates the Ras/MAP kinase pathway which leads to the activation of Rsk protein kinases that phosphorylate CREB at Ser133 (Rosen et al., 1994; Xing et al., 1996). While CaMKIV mediates the early phase of Ser133 phosphorylation in membrane-depolarized neurons, the MAPK pathway is responsible for prolonging Ser133 phosphorylation (Wu et al., 2001).

An unresolved issue is how Ca^{2+} induction of Ser133 phosphorylation selectively activates the transcription of particular genes in neurons, since a number of extracellular stimuli that induce CREB Ser133 phosphorylation fail to activate Ca^{2+}-specific, CREB-dependent transcriptional programs. Although CREB Ser133 phosphorylation is crucial for Ca^{2+}-induction of CREB-dependent transcription, Ser133 phosphorylation alone is not sufficient for target gene activation under some circumstances (Bonni et al., 1995; Tao et al., 1998). In neurons, Ca^{2+} influx through L-VSCCs triggers CREB Ser133 phosphorylation within minutes, but the transcriptional induction of a number of CREB-regulated genes does not occur until later (Tao et al., 1998; West et al., 2001). In addition, CREB Ser133 phosphorylation is maintained past the time when the transcription of some CREB-dependent genes has shut off. These findings suggest that CREB Ser133 phosphorylation alone may not trigger CREB-dependent transcription. One possibility is that there are other sites on CREB, CBP, or an as yet unidentified factor that must be phosphorylated at the same time as CREB Ser133 for Ca^{2+} to effectively activate CREB-dependent transcription (Chawla et al., 1998; Hardingham et al., 1999; Hu et al., 1999).

Previous studies have identified additional sites on CREB that can be phosphorylated in vitro or by overex-

[3]Correspondence: michael.greenberg@tch.harvard.edu
[4]Present address: Departments of Environmental Health and Pharmacology, University of Washington, Box 357234, Seattle, Washington, 98195.

pression of protein kinases in cells. These examples include Ser129 phosphorylation by glycogen synthase kinase III (Fiol et al., 1994), Ser142 phosphorylation by CaM kinase II (CaMKII) (Enslen et al., 1994; Sun et al., 1994; Matthews et al., 1994), and phosphorylation of either Ser142 or Ser143 by casein kinase II (CKII) (Parker et al., 1998). In cotransfection studies, CaMKII-catalyzed phosphorylation of both Ser133 and Ser142 failed to stimulate CREB-dependent reporter gene transcription (Sun et al., 1994). In contrast, when CREB Ser142 was converted to an alanine so that only Ser133 could be phosphorylated by CaMKII, CREB-dependent transcription was enhanced. On the basis of these studies, it was suggested that the phosphorylation of CREB at Ser142 inhibits CREB function. Nevertheless, it was not known whether extracellular stimuli induce the phosphorylation of endogenous CREB at Ser142 or any site other than Ser133. It was also not known how phosphorylation at sites in addition to Ser133 regulates CREB function in neurons under conditions of physiologic stimulation.

In this study, we have investigated the role of CREB phosphorylation at Ser142 and Ser143 in CREB-dependent transcription in neurons. We find that Ca^{2+} influx through L-VSCCs or NMDA receptors selectively induces the phosphorylation of CREB at Ser142 and Ser143, in addition to Ser133. The kinetics of CREB Ser142 and Ser143 phosphorylation suggest that when both of these phosphorylation events occur together with Ser133 phosphorylation they promote CREB activation. Mutation of Ser142 and Ser143 to alanines reduces Ca^{2+}-induced CREB-dependent transcription, indicating that phosphorylation of these sites contributes to CREB activation. However, in vitro experiments indicate that the phosphorylation of CREB at Ser142 and Ser143 inhibits the interaction of CREB with the KIX domain of CBP, raising the possibility that Ca^{2+} influx into neurons may activate CREB by a CBP KIX-independent mechanism.

Results

Characterization of Antibodies Specific for CREB Phosphorylated at Ser142 and Ser143

CREB Ser142 was a candidate site for regulation by Ca^{2+} influx into neurons, given evidence that Ser142 phosphorylation is catalyzed by CaMKII in vitro and modulates CREB function. In addition, since the residue immediately C-terminal to Ser142, Ser143, has been identified as a potential phosphorylation site (de Groot et al., 1993; Parker et al., 1998), we also investigated whether Ser143 phosphorylation is regulated by Ca^{2+} influx. By in vitro phosphotryptic mapping (Figure 1A) and peptide phosphorylation (data not shown), we found that CKII phosphorylates CREB at Ser143 and, to a lesser extent, Ser142, while CaMKII phosphorylates Ser142, but not Ser143.

We were unable to detect CREB phosphopeptides containing phosphorylated Ser142 and Ser143 by ^{32}P-labeling of cultured neurons and phosphotryptic mapping; we later determined that incubation of neurons with ^{32}P inhibits depolarization-induced Ser142 phosphorylation. Therefore, we generated a series of phosphospecific antibodies that recognize CREB phosphorylated at Ser142 and/or Ser143. The phospho-Ser142-specific CREB antibody (anti-P142) was specific for Ser142-phosphorylated CREB when tested by the following criteria. First, anti-P142 recognized the immunizing peptide that was phosphorylated at Ser142 (P142) but did not cross-react with peptides that were unphosphorylated (un-P), phosphorylated at the adjacent Ser143 (P143), or dually phosphorylated at both serines (P142/143) (Figure 1B). Second, wild-type (wt) CREB protein that was phosphorylated in vitro by CaMKII, which phosphorylates CREB at Ser142, but not at Ser143 (Figure 1A), was recognized by Western blotting using anti-P142 (Figure 1C). Replacement of Ser142 with an alanine (S142A) abolished the ability of anti-P142 to recognize in vitro phosphorylated CREB (Figure 1C), while replacement of Ser143 by alanine did not affect the ability of the antibody to detect CREB (data not shown). Third, wild-type CREB overexpressed in COS cells, together with a constitutively active CaMKII, was detected by anti-P142, while the mutation of Ser142 to an alanine abolished detection by this antibody (Figure 1D). Anti-P142 also did not recognize wild-type CREB overexpressed in COS cells in the absence of CaMKII (data not shown). Finally, mutation of Ser133 to alanine (S133A) had no effect upon the ability of anti-P142 to recognize CREB, although an antibody specific for Ser133-phosphorylated CREB (anti-P133) no longer recognized CREB S133A. Taken together, these results indicate that anti-P142 is specific for CREB that is phosphorylated at Ser142.

We also produced phosphospecific antibodies that recognize either Ser143-phosphorylated CREB (anti-P143) or CREB phosphorylated at both Ser142 and Ser143 (anti-P142/143). We established the specificity of these antibodies against CREB protein phosphorylated with CKII in vitro (Figure 1E) and synthetic phosphopeptides (data not shown) using criteria similar to those described above for the anti-P142 antibody. These experiments indicate that anti-P143 specifically recognizes the Ser143-phosphorylated form of CREB and does not bind to CREB when it is phosphorylated at Ser142 alone or phosphorylated at both Ser142 and Ser143. Anti-P142/143, by contrast, recognizes CREB only when it is phosphorylated at both Ser142 and Ser143 and not when it is phosphorylated at either Ser142 or Ser143 alone.

Stimuli that Evoke Neuronal Ca^{2+} Influx Induce the Coordinated Phosphorylation of CREB at Ser142 and Ser143

We used the phosphospecific anti-CREB antibodies to determine whether extracellular stimuli that induce CREB Ser133 phosphorylation in neurons also regulate the phosphorylation of Ser142 and Ser143. Stimulation of cultured cortical neurons using membrane-depolarizing concentrations of KCl to activate L-VSCCs or glutamate treatment to activate NMDA receptors induced CREB phosphorylation at Ser142, as well as at Ser133 (Figure 2A). By contrast, exposure of cortical neurons to BDNF or a cell-permeable cyclic AMP analog (CPT-cAMP) failed to induce Ser142 phosphorylation, although both agents stimulated the phosphorylation of CREB at Ser133.

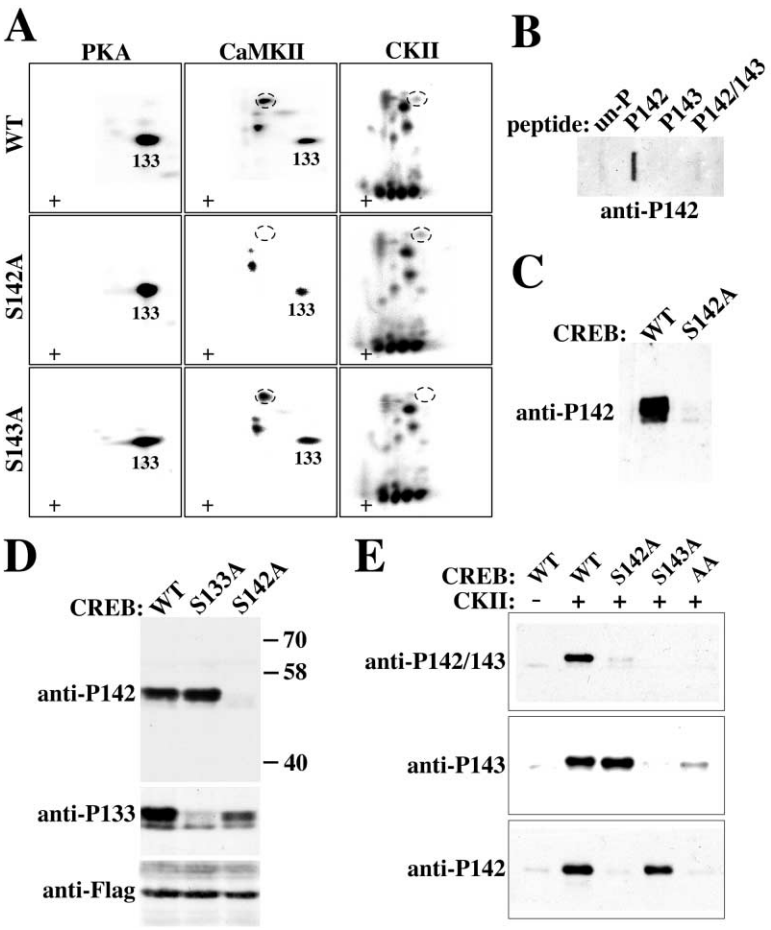

Figure 1. Characterization of Antibodies Specific for CREB Phosphorylated at Ser142, Ser143, or Both

(A) Phosphorylation of CREB in vitro at Ser142 and Ser143. Bacterially expressed CREB protein was phosphorylated in vitro using PKA, CaMKII, or CKII in the presence of [^{32}P-γ]ATP. Tryptic digestion was performed followed by two-dimensional chromatography to resolve tryptic fragments. The position of the single peptide phosphorylated by PKA is shown (133) (Yamamoto et al., 1988). CaMKII phosphorylates the same Ser133-containing peptide as PKA and an additional site (dashed circle) that is abolished by mutation of Ser142 (S142A), but not Ser143 (S143A). CKII catalyzes phosphate incorporation into a number of peptides, one of which (dashed circle) is abolished by an alanine mutation of Ser143, but not Ser142. This tryptic peptide comigrates with the peptide phosphorylated at Ser142 by CaMKII, as well as with Ser142- or Ser143-phosphorylated synthetic peptides corresponding to the predicted tryptic fragment that were detected by ninhydrin staining (not shown). The tryptic fragment representing phosphorylation of both Ser142 and Ser143 was not detected in this experiment. The sample origin is indicated by a "+."

(B–E) Rabbit antisera against phosphopeptides in which Ser142, Ser143, or both sites were phosphorylated were affinity purified and tested for specificity. (B) The immunizing peptide that was phosphorylated at Ser142 (P142) and identical peptides that were either unphosphorylated (un-P), phosphorylated at the adjacent Ser143 (P143), or phosphorylated at both serines (P142/143) were slot-blotted in equal amounts onto nitrocellulose membranes and probed with anti-P142. (C) Recombinant CREB protein was phosphorylated in vitro with CaMKII, and Western blots were performed using anti-P142. The antibody recognized wild-type phosphorylated CREB, while S142A mutant CREB was not recognized. Mutation of the adjacent Ser143 (S143A) did not affect recognition of Ser142-phosphorylated CREB by anti-P142 (not shown). Blotting with a total CREB and anti-P133 CREB antibodies was done to confirm equal loading and the activity of CaMKII upon Ser133 (not shown). (D) Cos cells were transfected with expression constructs for a Flag-tagged CREB, either wild-type, S142A, or S133A, together with a constitutively active CaMKII. Cell lysates were analyzed by Western blotting with anti-P142, anti-P133, or an anti-Flag antibody. (E) Characterization of antibodies specific for CREB phosphorylated at Ser143 (anti-P143) or phosphorylated at both Ser142 and Ser143 (anti-P142/143). CREB protein, either wild-type or mutant, was phosphorylated in vitro with CKII or was left unphosphorylated. Western blots were performed using anti-P143, anti-P142/143, or anti-P142. Anti-P142/143 recognized wild-type phosphorylated CREB, while S142A, S143A, or S142A/S143A mutants were not recognized. Anti-P143 recognized wild-type phosphorylated CREB, while S143A CREB was not recognized; the S142A mutation did not affect recognition of Ser143-phosphorylated CREB by anti-P143. Anti-P142 recognized phosphorylated CREB that was wild-type or S143A mutant, but the mutation of Ser142 abolished recognition. Parallel blots were probed using a total CREB antibody to confirm equal loading (data not shown).

The depolarization induction of CREB Ser142 phosphorylation in cortical neurons requires an influx of extracellular Ca^{2+} through L-VSCCs, as KCl induction of CREB Ser142 phosphorylation was blocked when cells were pretreated with the L-VSCC blocker nimodipine (Figure 2B). Likewise, glutamate stimulation of CREB Ser142 phosphorylation was blocked by the NMDA antagonist APV (data not shown), suggesting that Ca^{2+} influx through NMDA receptors is required for glutamate induction of CREB Ser142 phosphorylation. These findings indicate that CREB becomes newly phosphorylated at Ser142 selectively in response to stimuli that evoke Ca^{2+} influx into neurons.

Using the anti-P143 antibody, we were not able to detect CREB that was phosphorylated at Ser143, but not Ser142, either in unstimulated neurons or in neurons that were depolarized or exposed to cAMP or BDNF for various times (minutes to hours; data not shown). Therefore, it appears that membrane depolarization fails to induce the phosphorylation of CREB at Ser143 in the absence of Ser142 phosphorylation. Alternatively, it is possible that anti-P143 is not sensitive enough to detect endogenous Ser143-phosphorylated CREB.

Using anti-P142/143, we found that membrane depolarization of cortical neurons led to an increase in the amount of CREB that was phosphorylated at both Ser142 and Ser143 (Figure 2C). The coordinated phosphorylation of CREB Ser143 together with Ser142 was selectively induced by agents that trigger Ca^{2+} influx into neurons. No increase in the amount of CREB that was phosphorylated at both Ser142 and Ser143 was detected in cortical neurons exposed to BDNF (Figure

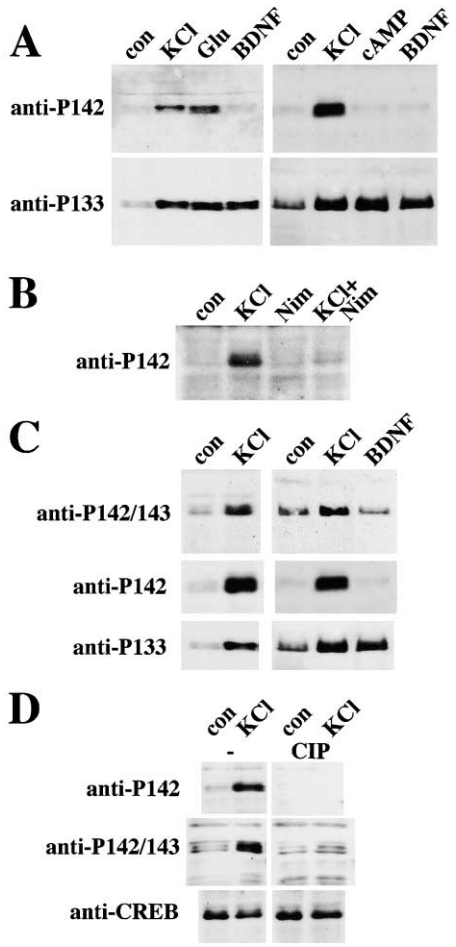

Figure 2. CREB Becomes Phosphorylated at Both Ser142 and Ser143, Specifically in Response to Neuronal Ca^{2+} Influx

(A) Cortical neurons were either untreated or stimulated with a depolarizing concentration of KCl, glutamate (Glu), BDNF, or the cAMP analog, CPT-cAMP (cAMP). Cells were treated with stimulating agents for 10 min (for anti-P133) or 20 min (for anti-P142) and then lysed and analyzed by Western blotting using the anti-P142 or anti-P133 CREB antibodies.

(B) Cortical neurons were treated with the VSCC inhibitor nimodipine (Nim) for 1 hr or left untreated and were then stimulated by depolarization (KCl) for 20 min or left untreated. Cells were then lysed and analyzed by Western blotting with anti-P142.

(C) Membrane depolarization of neurons induces CREB phosphorylation at both Ser142 and Ser143. Primary cultures of cortical neurons were either untreated (con) or stimulated with KCl or BDNF. Stimulating agents were added for 10 min (for determination of Ser133 phosphorylation) or 30 min (for measurement of Ser142 and Ser143 phosphorylation). Cell extracts were analyzed by Western blotting using the anti-P142/143, anti-P142, or anti-P133 CREB antibodies.

(D) Cortical cultures were untreated or stimulated with KCl for 20 or 30 min and then lysed. Western blots of cell extracts were incubated in the presence (CIP) or absence (−) of calf intestinal alkaline phosphatase, washed, and then probed with anti-P142, anti-P142/143, or a total anti-CREB antibody. Because a minor portion of the binding of anti-P142/143 to CREB is resistant to phosphatase treatment and is seen in both the untreated and KCl-stimulated samples, there appears to be some cross-reactive binding of anti-P142/143 to unphosphorylated CREB.

2C) or a cAMP analog (data not shown), although these agents stimulated Ser133 phosphorylation.

Membrane depolarization of the pheochromocytoma cell line PC12 also induced the phosphorylation of CREB at Ser142 and at Ser142 together with Ser143, as detected by Western blot analysis of extracts from PC12 cells (data not shown). Exposure of PC12 cells to a cAMP analog or to nerve growth factor failed to induce a detectable increase in CREB Ser142 and Ser143 phosphorylation, although these stimuli effectively induced CREB Ser133 phosphorylation. In addition, stimulation of COS or HEK 293 cells with agents that activated CREB Ser133 phosphorylation, including cAMP analogs, epidermal growth factor (EGF), and phorbol esters, failed to induce Ser142 or Ser142/Ser143 phosphorylation (data not shown).

We performed an additional control experiment to verify that the depolarization-stimulated increases in the CREB species detected in cortical neurons by anti-P142 and anti-P142/143 represented inducible phosphorylations of CREB. Western blots of lysates from unstimulated or depolarized cortical cultures were treated with a nonspecific phosphatase, calf intestinal alkaline phosphatase (CIP), or incubated in the same buffer without phosphatase (−) prior to antibody detection (Figure 2D). The depolarization-induced increases in the species detected by both anti-P142 and anti-P142/143 were abolished by phosphatase treatment, confirming that these antibodies recognize inducibly-phosphorylated species of CREB in cortical neuronal lysates. Anti-P142/143 was able to bind to CREB to a limited extent in the absence of depolarization (Figure 2C). However, this binding to CREB in extracts of unstimulated cells most likely reflects the presence of antibodies that recognize an unphosphorylated form of CREB, since this binding was not altered when Western blots were treated with phosphatase (Figure 2D).

Immunostaining of cortical neurons using the anti-P142, anti-P142/143, and anti-P133 antibodies revealed that membrane depolarization induced the phosphorylation of CREB at Ser142, at Ser142 together with Ser143, as well as at Ser133 within the nuclei of neurons (Figure 3A). The specificity of the antibodies used for immunostaining was confirmed by the ability of appropriately phosphorylated CREB peptides to abolish staining, whereas the identical unphosphorylated peptides did not block staining (Figure 3A). Thus, in addition to inducing CREB Ser133 phosphorylation within the nucleus, membrane depolarization induces CREB phosphorylation at both Ser142 and Ser143 within the nucleus. Moreover, these coupled phosphorylation events occur specifically in response to stimuli that lead to Ca^{2+} influx into neurons and are not activated by BDNF or cAMP (data not shown).

The observation that elevated Ca^{2+} induces the phosphorylation of CREB Ser142 and Ser143 in cultured neurons suggested that these phosphorylation events might also be regulated in the brain by stimuli that trigger Ca^{2+} influx. To test this possibility, we induced seizure activity in rats using the convulsant drug Metrazol (pentylenetetrazol), a stimulus known to induce Ca^{2+} influx into neurons leading to both CREB Ser133 phosphorylation and gene transcription (Morgan et al., 1987; Moore et al., 1996). Metrazol treatment caused an increase in the

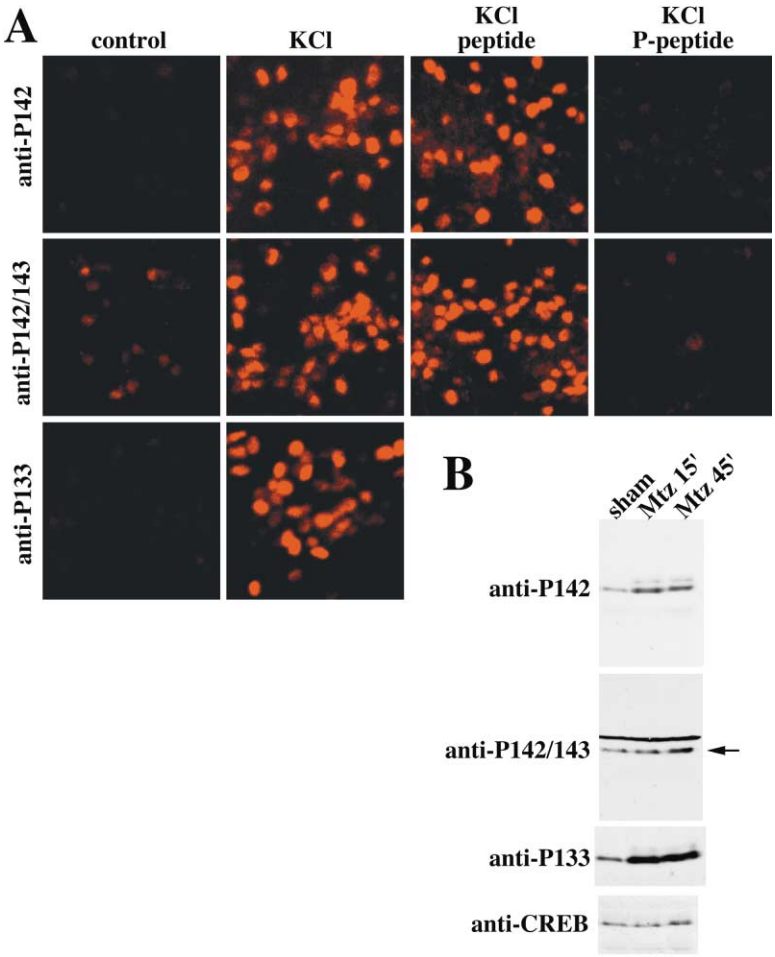

Figure 3. CREB Ser142 and Ser143 Phosphorylation Is Induced in Nuclei of Cortical Neurons by Membrane Depolarization and in the Cerebral Cortex by Synaptic Stimulation

(A) Cortical neuron cultures were untreated (con) or stimulated by depolarization (KCl) and then stained by immunocytochemistry using anti-P142, anti-P142/143, or anti-P133. No nuclear staining was observed using preimmune antisera instead of primary antibodies (data not shown). For competition controls, antibodies were incubated with a CREB peptide that was unphosphorylated (peptide) or phosphorylated (P-peptide) at Ser142 (for anti-P142) or Ser142 and Ser143 (for anti-P142/143).

(B) Adult rats were given intraperitoneal injections of Metrazol (Mtz) or of saline vehicle (sham). Animals were sacrificed 15 min or 45 min after the onset of seizure activity; vehicle-injected controls were sacrificed at a comparable time after injection as the "Mtz 15 min" animals. Equal amounts of total protein were loaded per lane. Three independent experiments were performed with similar results.

levels of CREB Ser133 and Ser142 phosphorylation and a modest increase in Ser142/Ser143 phosphorylation that occurred later (Figure 3B). This suggests that increased synaptic activity in the brain leads to the induction of the coordinated phosphorylation of CREB at Ser133, Ser142, and Ser143.

To investigate the function of CREB Ser142 and Ser143 phosphorylation, we determined the time courses of Ca^{2+}-stimulated Ser142 and Ser143 phosphorylation and dephosphorylation. Membrane depolarization of cortical neurons induced the rapid phosphorylation of CREB at Ser133 within 1–2 min, which was sustained at a high level for at least 60 min (see also Tao et al., 1998). By contrast, the depolarization-induced phosphorylation of CREB at Ser142 occurred more slowly, was not detectable until 2–5 min, and reached its highest levels approximately 15 min after depolarization (Figure 4). CREB that was phosphorylated at both Ser142 and Ser143 became detectable slightly later and accumulated more gradually (Figure 4). Subsequently, beginning about 30 min following depolarization, the levels of both Ser142-phosphorylated CREB and Ser142/Ser143-phosphorylated CREB declined with similar kinetics and were appreciably dephosphorylated by 1 hr after depolarization, while CREB Ser133 phosphorylation was still maintained at a high level.

Ca^{2+} influx induces CREB Ser142 and Ser143 phosphorylation in neurons with kinetics that more closely parallel the kinetics of CREB activation than does Ser133 phosphorylation. This is suggested by the kinetics of CRE-dependent transcription and the transcription of the CREB targets c-*fos* and BDNF in neurons. In a previous study, our laboratory demonstrated that Ca^{2+} influx induces CREB Ser133 phosphorylation more rapidly and for a longer period of time than the time course of c-*fos* and BDNF transcriptional induction in response to membrane depolarization (Tao et al., 1998). By contrast, CREB phosphorylation at Ser142 and Ser143 occurs in neurons after depolarization, specifically at times when c-*fos* and BDNF genes are turned on. Around the time that c-*fos* and BNDF transcription shuts off (Tao et al., 1998), CREB becomes dephosphorylated at Ser142 and Ser143. The kinetics of CREB phosphorylation and dephosphorylation at Ser142 and Ser143 suggest that the coordinated phosphorylation of CREB at these sites might play a role in the Ca^{2+}-specific activation of CREB-regulated genes.

Ca^{2+} influx through L-VSCCs leads to CREB Ser133 phosphorylation via the rapid but transient activation of CaMKIV, followed by the more prolonged activation of the Ras/MAPK/Rsk pathway (Wu et al., 2001). Given the distinct kinetics of Ser142/Ser143 phosphorylation and Ser133 phosphorylation, we asked if these events were mediated by different kinase pathways. The CaMK inhib-

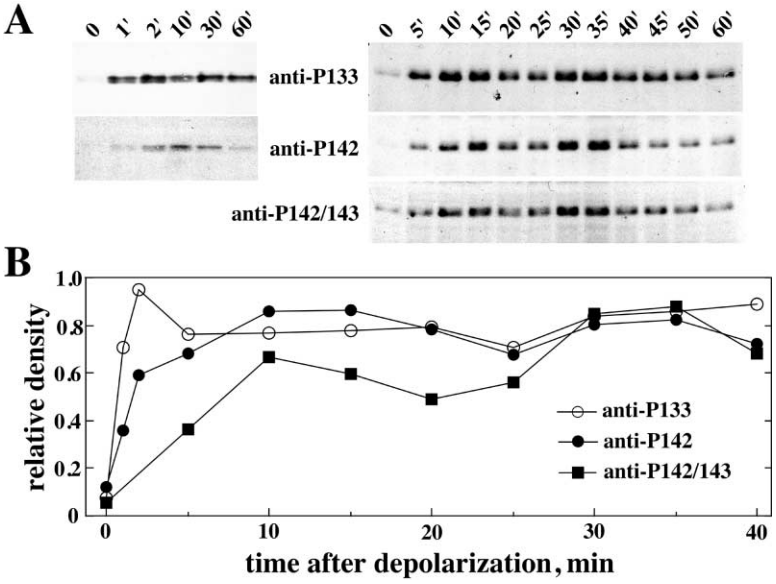

Figure 4. Distinct Kinetics of Depolarization-Induced Phosphorylation of CREB at Ser133, Ser142, and Ser142/Ser143

(A) Cortical neuronal cultures were untreated (con) or depolarized with KCl for the indicated times and then lysed and analyzed by Western blotting with anti-P142, anti-P142/143, and anti-P133.

(B) Data from several Western blot experiments, including that shown in (A), were scanned densitometrically to quantify amounts of immunoreactivity to the antibodies. Values within each experiment were normalized to the highest value (maximum level = one), and mean values across experiments are plotted for anti-P133 (open circles), anti-P142 (closed circles), and anti-P142/143 (closed squares) signals. Each data point represents at least three independent experiments.

itor, KN-62, blocked glutamate induction of Ser142 phosphorylation, but did not significantly reduce Ser133 phosphorylation at 30 min (Figure 5A), a time point when the CaMKIV-dependent phase of Ser133 phosphorylation is over. Because KN-62 is known to interfere with Ca^{2+} influx through L-VSCCs in addition to inhibiting CaMKs, we stimulated neurons with glutamate in the presence of the L-VSCC blocker nimodipine. Thus, the glutamate-stimulated CREB phosphorylation we observe is due to Ca^{2+} influx through glutamate receptor channels without any contribution from L-VSCCs, and the KN-62 inhibition of Ser142 phosphorylation is attributable to the specific inhibition of CaMKs. By contrast, the MEK inhibitor PD98059 did not interfere with glutamate induction of Ser142 phosphorylation, although it partially blocked Ser133 phosphorylation. These results suggest that a CaMK may be required for Ser142 phosphorylation, while the MAPK pathway appears to be dispensable. Given the ability of CaMKII (but not CaMKIV) to phosphorylate CREB Ser142 in vitro, it is possible that a nuclear-localized isoform(s) of CaMKII (reviewed in Soderling et al., 2001) catalyzes Ca^{2+}-stimulated Ser142 phosphorylation in neurons.

To further examine if Ca^{2+}-stimulated Ser142 phosphorylation is mediated by a signaling pathway that is distinct from the pathways that mediate Ser133 phosphorylation, we used a recently described functional knockin technique (Dolmetsch et al., 2001) to determine if the specific features of the L type VSCCs that are required for signaling to CREB Ser133 are also required for Ser142 phosphorylation. Neurons were transfected with a mutated form of the L type channel (DHP-LTC) that is insensitive to dihydropyridine channel blockers, such as nimodipine, but is otherwise normal. When neurons transfected with the DHP-LTC are treated with nimodipine, endogenous L type channels are inhibited, but the DHP-LTC can still mediate Ca^{2+} influx in response to membrane depolarization, so that depolarization leads to Ca^{2+}-dependent signaling events specifically in cells expressing the DHP-LTC. Using this knockin approach, it was possible to define features of the L-VSCC that are critical for CREB Ser133 phosphorylation and transcriptional activation (Dolmetsch et al., 2001). Dolmetsch et al. (2001) showed that calmodulin (CaM) binding to an isoleucine-glutamine (IQ) motif in the carboxy-terminal cytoplasmic domain of the channel is important for Ras/MAPK/Rsk activation and CREB Ser133 phosphorylation when neurons are exposed to elevated levels of KCl.

To assess whether CaM coupling to the Ras/MAPK pathway is critical for membrane depolarization induction of CREB Ser142 phosphorylation (Dolmetsch et al., 2001), we transfected neurons with the wild-type L-VSCC, the DHP-LTC, or various mutants of the DHP-LTC IQ motif that are deficient for CaM binding. We then depolarized the neurons and monitored the level of Ser142 phosphorylation in the transfected cells by immunofluorescence staining. We found that a point mutation (IA) in the IQ region of the DHP-LTC that specifically disrupts the association of CaM with the channel, but does not affect Ca^{2+} influx through the channel, abolished CREB Ser133 phosphorylation (Dolmetsch et al., 2001), but did not lead to a decrease in Ser142 phosphorylation (Figure 5B). Another point mutation (IE) in the DHP-LTC, which generates only a transient rise in intracellular Ca^{2+} and therefore mediates less Ca^{2+} influx than the wild-type DHP-LTC, was less effective at mediating Ser142 phosphorylation. These results suggest that while the amount of Ca^{2+} influx through the L-VSCC affects the level of CREB Ser142 phosphorylation, CaM binding to the channel is not required for depolarization induction of Ser142 phosphorylation. Because CaM binding to the L-VSCC is coupled to the activation of the Ras/MAPK/Rsk pathway, these results suggest that activation of Ras and MAPK are not required for Ser142 phosphorylation, consistent with our findings using pharmacological inhibitors of MEK (Figure 5A). Ca^{2+} influx through the L-VSCC may by itself

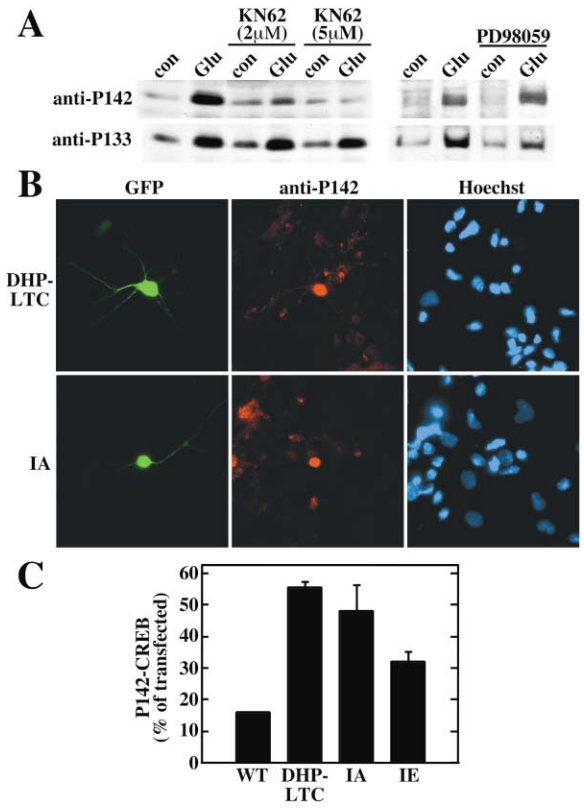

Figure 5. Ca^{2+}-Stimulated CREB Ser142 Phosphorylation Is Mediated by a Signaling Pathway Distinct from Ser133 that Requires a CaM Kinase, but Not MAP Kinase

(A) Cortical neurons were treated with the CaM kinase inhibitor KN62 (2 or 5 μM) or the MEK inhibitor PD98059 (30 μM) for 30 min or left untreated and were then stimulated with glutamate (Glu) (10 μM) for 20 min or left untreated.

(B) Cortical neurons transfected with DHP-LTC or an IA mutant DHP-LTC (IA) together with GFP were depolarized in the presence of nimodipine and APV for 30 min and then stained with anti-P142 and the nuclear stain Hoechst 33342 (Hoechst).

(C) Anti-P142 CREB staining in neurons transfected with wild-type L-VSCC (wt), DHP-LTC, or DHP-LTC mutants (IA or IE) and depolarized in the presence of nimodipine and APV. The IE mutant elevates intracellular Ca^{2+} to a lesser extent than the DHP-LTC and IA mutant (Dolmetsch et al., 2001). The graph shows the percentage of neurons that are both transfected with the DHP-LTC and positive for phospho-Ser142 CREB (at least 50 transfected cells were scored for each LTC; n = 2 ± range of data).

be sufficient to mediate Ser142 phosphorylation, perhaps by triggering the activation of a CaM kinase that functions in the nucleus.

Phosphorylation of Ser142 and Ser143 Contributes to CREB-Mediated Transcription Induced by Depolarization

We tested the effects of converting Ser142 and/or Ser143 to alanine on CREB-mediated transcription in membrane-depolarized cortical neurons. To compare the ability of wild-type and mutant CREBs to activate transcription and distinguish the effects of exogenously introduced CREB from those of endogenous CREB, wild-type and mutant CREBs were fused to the DNA binding domain of the yeast transcription factor Gal4. Plasmids that drive the expression of wild-type or mutant Gal4CREB were cotransfected into neurons together with reporter genes that contain Gal4 binding sites within their regulatory region, and the ability of these Gal4CREB proteins to activate reporter gene expression was examined before and at times after membrane depolarization. For this analysis, we used a reporter gene construct containing five Gal4 binding sites within a minimal promoter controlling expression of a luciferase gene (Gal4-luciferase), and luciferase activity was assayed to measure reporter gene activation. In addition, some experiments were repeated using a second reporter gene that contained five Gal4 binding sites within a minimal c-*fos* promoter driving expression of a human c-*fos* (c-fos^H) gene, and we assessed Gal4CREB induction of the c-fos^H reporter using an RNase protection assay (Sheng et al., 1988).

Membrane depolarization of cortical neurons led to the activation of wild-type Gal4CREB-dependent expression of both the Gal4-luciferase reporter (Figure 6A) and the Gal4-fos^H reporter (Sheng et al., 1991). The induction of CREB-mediated gene expression required phosphorylation of Gal4CREB at Ser133, since the mutation of Ser133 to an alanine (S133A) abolished the induction of these reporter constructs. In contrast to Ser133, the replacement of Ser142 by an alanine (S142A) did not impair the ability of Gal4CREB to mediate activation of either reporter gene. Rather, the ability of Gal4-CREB S142A to drive reporter gene transcription was enhanced relative to wild-type Gal4CREB (Figure 6A), as has previously been documented in depolarized GH_3 cells (Sun et al., 1994). We conclude that one consequence of CREB phosphorylation at Ser142 alone may be to attenuate the level of CREB-dependent transcription.

In contrast to the Ser142 alanine mutation, the replacement of Ser143 by alanine (S143A) caused a significant reduction in the level of Gal4CREB-mediated transcription of the Gal4-luciferase reporter (Figure 6A). Replacement of both Ser142 and Ser143 by alanines (S142A/S143A) caused an even greater impairment of the ability of Gal4CREB to mediate transcription (Figure 6A). The effects of the Ser143 mutation and the Ser142/Ser143 double mutation were significant, but not as severe as the effect of mutating Ser133 to an alanine. These results suggest that phosphorylation of Ser142 together with Ser143, as it occurs on endogenous CREB in membrane-depolarized neurons, leads to the enhancement of CREB-dependent transcription.

The effects of each of these three mutations (S142A, S143A, and S142A/S143A) on transcription of a Gal4-*fos* reporter gene were similar to their effects on the Gal4-luciferase reporter (data not shown). Mutations of Ser142 and/or Ser143 to alanine had no effect on the expression or the stability of Gal4CREB (data not shown) or on the ability of Gal4CREB to mediate transcriptional responses to cAMP and PKA. In HEK 293 cells, Gal4CREB-dependent transcription, in response to cAMP treatment (Figure 6B) or cotransfection with an active form of PKA (not shown), was not impaired by alanine mutations of Ser142 and/or Ser143, while mutation of Ser133 to alanine abolished PKA-stimulated transcription. Furthermore, in cortical neurons, mutation of Ser143 or both Ser142 and Ser143 to alanine had no effect on cAMP-

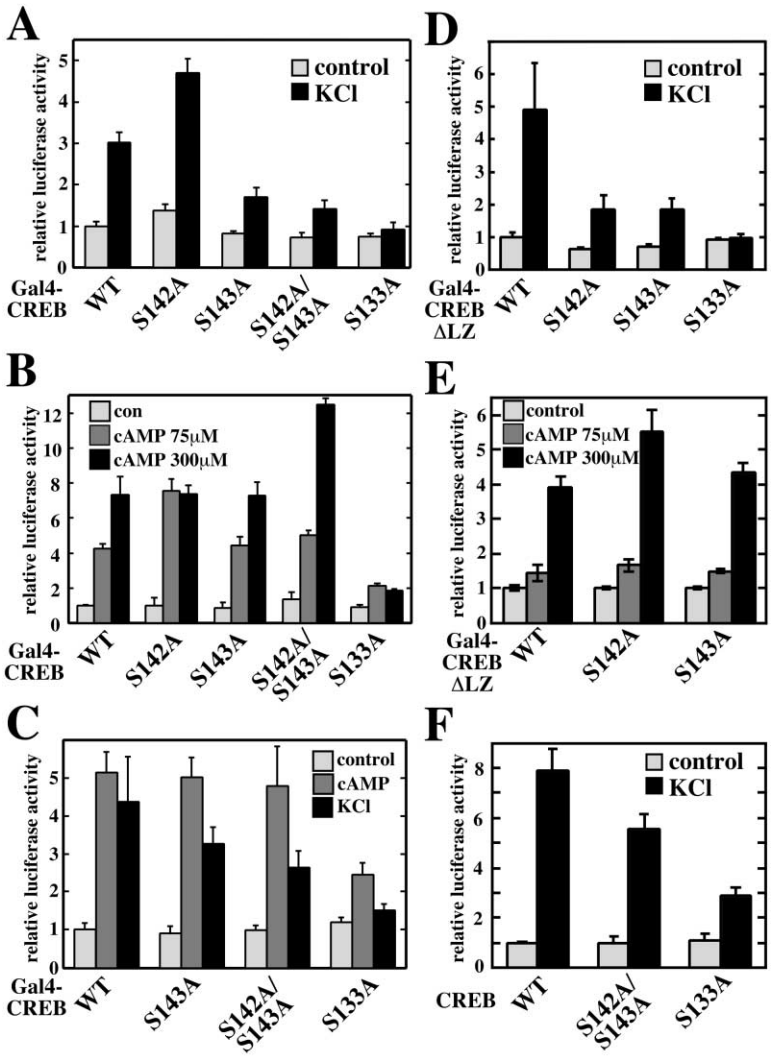

Figure 6. Phosphorylation of Both Ser142 and Ser143 Is Required for Effective CREB-Mediated Transcription Activated by Ca^{2+} Influx

(A) Mutations of Ser142 and Ser143 have distinct effects on Gal4CREB transcriptional activation in response to depolarization. Cortical neuronal cultures (in 12-well plates) were cotransfected with Gal4CREB (1.25 μg), either wild-type or S142A, S143A, S142A/S143A, or S133A mutant, along with the Gal4-luciferase reporter (0.25 μg). Cultures were untreated (control, light bars) or stimulated with 60 mM KCl for 5–7 hr (KCl, dark bars), harvested, and assayed for luciferase activity. In each experiment, each condition was done in triplicate. The mean value of each set of triplicate luciferase measurements was then normalized to the wild-type, unstimulated (wild-type, control) value to allow comparison between experiments. Data are from several independent experiments (n = 4 experiments for S142A and S142A/S143A Gal4CREB; n = 3 experiments for S143A and S133A Gal4CREB). Error bars show standard deviation.

(B) Mutations of Ser142 or Ser143 do not diminish Gal4CREB transcriptional activation by cAMP. HEK 293 cells (in 12-well plates) were cotransfected with Gal4CREB wild-type or mutant constructs (1 μg) together with a Gal4-luciferase reporter (2 μg). Cells were untreated (con, light bars), or stimulated with 75 μM or 300 μM CPT-cAMP (gray and black bars) for 5–7 hr. Each condition was done in triplicate. Error bars show standard deviations.

(C) Mutations of Ser143 or Ser142/Ser143 specifically attenuate depolarization responses in neurons. Cortical cultures (in 12-well plates) were cotransfected with wild-type or mutant Gal4CREB (250 ng) and Gal4-luciferase reporter (50 ng). A lipid-mediated transfection method was used in this experiment to avoid possible nonspecific effects of Ca^{2+} uptake using a Ca^{2+} phosphate transfection protocol. Cells were stimulated and assayed as in (A). Each condition was done in triplicate. Error bars show standard deviation. Data are from four independent experiments.

(D) Ser142 and Ser143 mutations attenuate depolarization-stimulated transcription mediated by Gal4CREBΔLZ. Cortical neuronal cultures were cotransfected with Gal4CREBΔLZ (1.25 μg), either wild-type or mutant, along with Gal4-luciferase (0.25 μg). Cells were stimulated and assayed as in (A). Each condition was done in triplicate. Error bars show standard deviation. Data are from three independent experiments.

(E) Mutations of Ser142 or Ser143 do not attenuate Gal4CREB transcriptional activation by cAMP. HEK 293 cells (in 12-well plates) were cotransfected with Gal4CREBΔLZ WT or mutant constructs (1 μg), together with a Gal4-luciferase reporter (2 μg). Cells were stimulated and assayed as in (B).

(F) CREB S142A/S143A acts as a dominant inhibitor of Ca^{2+}-activated transcription mediated by endogenous CREB. Cortical neurons were transfected with FlagCREB (120 ng), either wild-type, S142A/S143A, or S133A, together with a CRE-luciferase reporter gene (180 ng). A lipid-mediated transfection method was used in this experiment. Cells were stimulated, assayed and quantified as in (A). Each condition was done in triplicate. Error bars show standard deviation. Data are from three independent experiments.

stimulated transcription mediated by Gal4CREB, while these mutations caused a clear reduction in transcription stimulated by depolarization (Figure 6C). These data indicate that the Ser142 and Ser143 alanine mutations produce specific effects upon Ca^{2+}-dependent CREB-mediated transcription rather than nonspecific alterations of CREB function.

It is noteworthy that the Ser142 and Ser143 mutations affected to some extent the level of Gal4CREB-mediated transcription in the absence of any depolarizing stimulus. It may be that these changes reflect a low level of Gal4CREB-dependent transcription in the absence of stimulation due to basal phosphorylation of CREB at Ser142 and Ser143, or they may reflect nonspecific effects of the Ser142 and Ser143 mutations in the context of Gal4CREB. Because the CREB Ser142 and Ser143 mutants are not deficient in transcriptional responses to cAMP, it appears more likely that basal levels of Ser142 and Ser143 phosphorylation could account for the small effects of the mutations that are detected in unstimulated cells.

The observation that the phosphorylation of CREB at

Ser142 has a different effect on CREB activity than does the phosphorylation of CREB at both Ser142 and Ser143 suggests that these phosphorylation events could regulate CREB function at several levels. For example, phosphorylation of CREB at Ser142 by CaMKII has been reported to cause a switch from CREB dimers to monomers bound to DNA in vitro (Wu and McMurray, 2001). This has been proposed to interfere with the recruitment by CREB of CBP to gene promoters and to thereby inhibit transcription. To determine if the changes in Gal4CREB-mediated transcription that we observed with alanine mutations of Ser142 or Ser143 might be due to the inability of Gal4CREB phosphorylated at these sites to form dimers with endogenous CREB family members, we transfected cortical neurons with a version of Gal4CREB in which the leucine-zipper dimerization domain of CREB was deleted (Gal4CREBΔLZ). Because this Gal4CREBΔLZ fusion protein can dimerize only via its Gal4 domain, Gal4CREBΔLZ can not form heterodimeric complexes with endogenous CREB family members. Therefore, the effect of the Ser142 and Ser143 mutations on the ability of CREB to activate transcription could be analyzed independently of effects upon CREB dimerization.

While membrane depolarization of cortical neurons activated transcription mediated by wild-type Gal4CREBΔLZ, the mutation of either Ser142 or Ser143 to alanine led to a significant reduction in transcription mediated by Gal4CREBΔLZ following depolarization (Figure 6D). This indicates that Ser142 and Ser143 phosphorylation have effects on CREB-mediated transcription that are independent of effects on dimer formation, and in this context, Ser142 and Ser143 phosphorylation both contribute to the activation of transcription in response to depolarization. The mutation of either Ser142 or Ser143 to alanine in the context of Gal4CREBΔLZ did not appear to have a nonspecific effect on the ability of CREB to activate transcription, since these mutants, when expressed in HEK 293 cells, showed responses to cAMP stimuli that were indistinguishable from wild-type (Figure 6E).

Surprisingly, while mutation of Ser142 in full-length Gal4CREB led to an enhancement of transcription, this mutation in the context of Gal4CREBΔLZ led to an inhibition of transcription. This suggests that Ser142 phosphorylation may have two distinct effects upon CREB-mediated transcription. Ser142 phosphorylation may, under certain circumstances, attenuate CREB-dependent transcription by inhibiting the ability of CREB to dimerize with particular CREB family members. However, in the context of Gal4CREBΔLZ and, perhaps, under conditions in cells where CREB dimer formation is inhibited, Ser142 phosphorylation (together with Ser143 phosphorylation) may enhance the ability of promoter-bound CREB to activate transcription in response to membrane depolarization.

To further test if the coordinated phosphorylation of Ser142 and Ser143 plays an important role in depolarization-induced transcription mediated by CREB, we examined if CREB in which both Ser142 and Ser143 were converted to alanines could, when expressed at high levels, act as a dominant inhibitor of endogenous CREB function. When cortical neurons were transfected with a CRE-luciferase reporter gene that can be activated by endogenous CREB, the transfection of a plasmid that drives expression of a CREB bearing an alanine mutation of Ser133 (S133A) significantly repressed reporter gene transcription relative to wild-type CREB (Figure 6F). Overexpression of a CREB mutant in which Ser142 and Ser143 were converted to alanines (S142A/S143A) led to a partial inhibition of reporter gene expression, indicating that CREB that cannot be phosphorylated at Ser142 and Ser143, when overexpressed in neurons, can compete with endogenous CREB for binding to CREs and thereby block the ability of endogenous CREB to mediate a Ca^{2+} response.

Phosphorylation of Both Ser142 and Ser143 Disrupts the Interaction of CREB with the CBP KIX Domain

One mechanism by which the phosphorylation of Ser142 and Ser143 might modulate CREB function is by affecting the interaction of CREB with its cofactor CBP. The CREB KID includes Ser142 and Ser143, is required for the interaction of CREB with CBP, and has been shown to be critical for PKA stimulation of CREB-dependent transcription. The solution structure of the Ser133-phosphorylated KID bound to the KIX domain of CBP shows that the residues immediately flanking Ser142 and Ser143 interact directly with the CBP KIX domain (Radhakrishnan et al., 1997). CREB Ser142 is positioned in close apposition to the KIX surface, and the addition of a phosphate to Ser142 is predicted to sterically interfere with binding of the KID to the KIX domain (Parker et al., 1998). Although one study reported that acidic amino acid substitutions at Ser142 do not interfere with KID-KIX binding (Sun and Maurer, 1995), in another report, phosphorylation of a CREB KID polypeptide by CKII disrupted the KID-KIX interaction (Parker et al., 1998). It was not clear from this later study whether it was the phosphorylation of CREB at Ser142, Ser143, or both of these amino acids that was critical for disrupting the KID-KIX interaction.

To determine the effect of CREB phosphorylation at Ser142 and/or Ser143 on the KID/KIX interaction, we employed an in vitro GST binding assay in which Flag epitope-tagged CREB proteins (FlagCREB) were incubated with the KIX domain of CBP fused to glutathione-S-transferase (GST-KIX). Wild-type and mutant forms of FlagCREB were phosphorylated in vitro and then incubated with GST-KIX. The amount of FlagCREB that interacted with GST-KIX was determined by capturing the GST-KIX/CREB complexes with glutathione-agarose beads. The fraction of FlagCREB that bound to the beads and the fraction that failed to bind to GST-KIX was estimated by Western blotting with an anti-Flag antibody. The effect of CREB phosphorylation at Ser133, Ser142, and Ser143 on the KID/KIX interaction was established using the various phosphospecific antibodies to determine whether the different phospho-CREB species were capable of binding to GST-KIX (present in the pellet) or were not capable of binding to GST-KIX (present in the supernatant).

Using this GST pull-down assay, we found that unphosphorylated CREB did not bind to GST-KIX at all, while incubation with PKA, which phosphorylates CREB at Ser133 alone, caused a dramatic increase in CREB

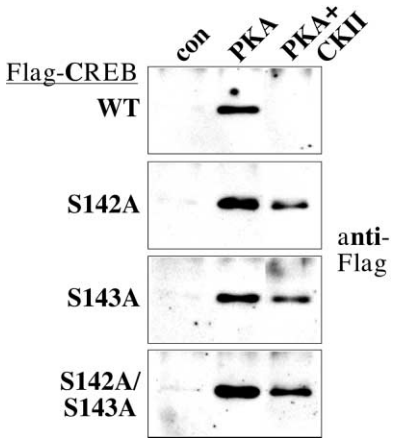

Figure 7. CREB Phosphorylation at Both Ser142 and Ser143 Disrupts CREB Binding to CBP-KIX In Vitro

Recombinant FlagCREB protein, either wild-type or mutant, was either left unphosphorylated (con) or was phosphorylated using PKA (PKA). A portion of the PKA-phosphorylated CREB was then phosphorylated using CKII (CKII). Equal amounts of each CREB sample were incubated with GST-KIX, and glutathione-agarose beads were then added. Supernatants containing CREB that was not bound to the GST-KIX/glutathione bead complex were saved. GST-KIX complexes were precipitated, washed several times, and solubilized in Laemmli buffer. CREB that was either bound to the GST-KIX complex or that remained in the supernatant (not shown) was detected by Western blotting using an anti-Flag antibody (anti-Flag) and by phosphospecific CREB antibodies (not shown).

binding to KIX (Figure 7, top panel). We found that essentially, all of the Ser133-phosphorylated CREB was found in the pellet bound to GST-KIX. This PKA-induced CREB-KIX association was dependent upon Ser133 phosphorylation, as the interaction was completely abolished by the mutation of Ser133 to alanine (data not shown). Thus, as previously shown (Chrivia et al., 1993), the phosphorylation of CREB at Ser133 promotes the interaction of CREB with the CBP KIX domain.

We next asked if the phosphorylation of Ser142 and/or Ser143 affects the ability of Ser133-phosphorylated CREB to interact with the CBP KIX domain. We first phosphorylated CREB at Ser133 with PKA and then incubated the Ser133-phosphorylated CREB with CKII to phosphorylate CREB at Ser142 and Ser143. When CREB was phosphorylated at both Ser142 and Ser143, in addition to Ser133, CREB was completely incapable of binding to GST-KIX (Figure 7, top panel). Western blotting using the phosphospecific CREB antibodies confirmed that CREB that was phosphorylated at Ser133, Ser142, and Ser143 was only found in the unbound fraction (data not shown). This result demonstrates that the phosphorylation of CREB at both Ser142 and Ser143 effectively disrupts the Ser133-dependent interaction between CREB and CBP-KIX (Figure 7, top panel).

We next investigated whether phosphorylation of Ser142, Ser143, or both sites is required to disrupt the CREB-CBP interaction. We employed versions of CREB in which Ser142 and/or Ser143 were mutated to alanine. Under conditions where wild-type or mutant CREBs were phosphorylated at Ser133 by PKA but were left unphosphorylated at Ser142 and Ser143, the wild-type and mutant CREBs bound to CBP KIX equally well, indicating that the mutations of Ser142 and Ser143 did not disrupt the overall structure of the CREB KID. When wild-type CREB was phosphorylated at Ser133, Ser142, and Ser143 by both PKA and CKII, CREB was completely incapable of binding to CBP KIX. By contrast, when CREB Ser142 and/or Ser143 were mutated to alanine and CREB was phosphorylated with PKA, the additional phosphorylation by CKII no longer effectively disrupted the CREB-KIX interaction (Figure 7). This indicates that the phosphorylation of CREB at both Ser142 and Ser143 is required to effectively disrupt the Ser133-dependent CREB-KIX interaction. However, given that this assay does not measure binding affinities, it is possible that the phosphorylation of either Ser142 or Ser143 alone reduces the affinity of CREB binding to CBP KIX to some extent. It should be noted that CREB was effectively phosphorylated at Ser133 by PKA, as determined using the anti-P133 CREB antibody, regardless of whether Ser142 or Ser143 were phosphorylated by CKII or were mutated to alanines.

Discussion

Intracellular signaling pathways that are induced by Ca^{2+} influx into neurons are known to activate CREB by stimulating its phosphorylation at Ser133. However, it has not been clear how Ca^{2+} stimulation of Ser133 phosphorylation selectively induces the transcription of particular genes in neurons, given that some extracellular stimuli that stimulate CREB Ser133 phosphorylation fail to activate Ca^{2+}-specific, CREB-dependent transcriptional programs. We show here that, specifically in response to stimuli that trigger Ca^{2+} influx into neurons, CREB becomes modified by the phosphorylation of Ser133, Ser142, and Ser143 and that these three phosphorylation events together promote CREB-mediated transcription through a mechanism distinct from that activated by other extracellular stimuli. The triple phosphorylation of CREB at Ser133, Ser142, and Ser143 is induced in primary neuronal cultures or PC12 cells by membrane depolarization and also in the cerebral cortex in vivo by seizure activity. The kinetics of Ca^{2+}-stimulated Ser142 and Ser143 phosphorylation is distinct from that of Ser133 phosphorylation; Ser133 phosphorylation occurs first and is followed by the phosphorylation of Ser142 and Ser143.

It has been previously shown that the phosphorylation of Ser133 activates CREB-dependent transcription by allowing recruitment of the coactivator CBP to the promoters of CREB-dependent genes (Chrivia et al., 1993; reviewed in Goldman et al., 1997). We show here that the additional phosphorylation of Ser142 and Ser143, together with Ser133, makes a significant contribution to Ca^{2+}-activated transcription but may cause a disruption of the CREB/CBP interaction. These results suggest that the Ca^{2+}-induced triple phosphorylation of CREB may be a critical step in a Ca^{2+}-specific mechanism of CREB transcriptional activation, one which is not activated by several other stimuli and which may not require the participation of CBP as a cofactor.

While we can readily detect Ser142 and Ser143 phosphorylation in depolarized neurons using phosphospecific antibodies, a more difficult question to address has

been the stoichiometry of these phosphorylation events. It will be important to determine what fraction of CREB molecules in each neuron become inducibly phosphorylated at each site. Experiments using our phosphospecific antibodies in immunoprecipitation and electrophoretic mobility shift assays suggest that these events occur on a significant portion of the CREB in neurons following depolarization. In addition, our finding that alanine mutations of Ser142 or Ser143 significantly reduce Gal4CREB-mediated transcription would not be expected unless Ser142 and Ser143 become phosphorylated to a considerable extent following membrane depolarization. Finally, it should be considered that if a Ca^{2+}-specific activation mechanism involves the Ser142/Ser143 phosphorylation of CREB that is prebound to only a specific subset of CREB target genes, then stoichiometric phosphorylation of these sites on CREB might not be expected.

The identities of the protein kinases that mediate CREB phosphorylation at Ser142 and Ser143 in vivo are not known. Although we have used CaMKII and CKII to phosphorylate these sites in vitro, there is currently no compelling evidence that these are the specific kinases that catalyze Ser142 or Ser143 phosphorylation in neurons. Our results using pharmacological inhibitors, as well as a functional knockin of the L-VSCC, suggest that Ser142 phosphorylation does not depend upon activation of a Ras/MAPK/Rsk pathway, but may instead be mediated by a CaM kinase, possibly one of the nuclear isoform(s) of CaMKII α, δ, or γ (reviewed in Soderling et al., 2001). Interestingly, we have not observed depolarization-induced CREB Ser142/Ser143 phosphorylation in embryonic day (E)18 cortical neurons that were maintained in culture for only 2–3 days, rather than 6–8 days, suggesting that a kinase or other cellular component that mediates signaling to Ser142 and Ser143 may be developmentally regulated during this time period.

In contrast to the phosphorylation of CREB at Ser142 in the absence of Ser143 phosphorylation, CREB phosphorylation at both Ser142 and Ser143 (together with Ser133) appears to activate Ca^{2+}-stimulated transcription (Figure 6), even though the phosphorylation of Ser142 and Ser143 disrupts CREB binding to the KIX domain of CBP (Figure 7). How might these three phosphorylation modifications of CREB function to bring about transcriptional activation? CREB that is triply phosphorylated could interact with a domain of CBP that is distinct from the KIX domain. Alternatively, triply phosphorylated CREB may recruit a different coactivator to CREB-responsive promoters. Whatever the identity of the cofactor that interacts with CREB when it is phosphorylated at serines 133, 142, and 143, this interaction may participate in the assembly of a transcriptional complex at promoters such as the BDNF promoter, which is selectively activated by Ca^{2+} at times when CREB is phosphorylated at all three of these sites.

Our findings suggest the existence of a distinct, stimulus-specific mechanism of CREB activation that is triggered by Ca^{2+} influx into neurons. This type of mechanism may provide a basis for the neural-specific and Ca^{2+}-selective induction of a specific program of gene expression. This Ca^{2+}-specific activation mechanism may allow CREB to function in a unique way to mediate responses to synaptic activity in mature neurons and to selectively activate the transcription of target genes that are critical for adaptive responses in the nervous system.

Experimental Procedures

2D Peptide Mapping

Recombinant FlagCREB (1 μg), either wild-type, S142A, S143A, or S142A/S143A mutant, was incubated at 30°C in kinase buffer (50 mM Pipes [pH 7.3], 10 mM $MgCl_2$, 0.5 mM Na_3VO_4, 1 mM DTT, 0.5 mM $CaCl_2$, 0.1 mM (^{32}P-γ)ATP, 10 μg/ml calmodulin, 0.1 mg/ml BSA, 1 mM PMSF, and 0.5 μg/ml leupeptin) with PKA, CaMKII, or CKII for 1 hr. Phosphorylated CREB proteins were isolated from SDS-PAGE, and 2D tryptic peptide mapping done as described (Boyle et al., 1991) using pH 1.9 electrophoresis and phosphochromatography buffers. To identify the phospho-Ser142- and phospho-Ser143-containing CREB peptides, synthesized phosphopeptides corresponding to the predicted tryptic peptide (ILNDLSSDAPGVPR) were included and their migration visualized by ninhydrin (Sigma) staining.

Antibody Reagents

Peptides encompassing CREB amino acids 134–145 were synthesized with Ser142, Ser143, or both phosphorylated; conjugated to KLH; and injected into rabbits. Antisera were purified by affinity to protein A and peptides (phosphorylated or unphosphorylated). The anti-P133 CREB antibody has been previously described (Ginty et al., 1993). The total CREB antibody was raised against recombinant CREB in rabbits. The anti-Flag (M2) antibody was from Sigma.

Plasmids

FlagCREB (gift of Dr. R. Goodman) was subcloned into pcDNA3 in which the CMV promoter was replaced by an RSV promoter to generate pCD/RSV-FlagCREB for mammalian expression and into pET16b to generate pET16b-FlagCREB for bacterial expression. Active CaMKII (CaMKIIΔCT) (Planas-Silva and Means, 1992) was cloned into pSG5 to generate pSG5-CaMKIIΔCT. The GST-KIX expression construct (KIX 10.4) was a gift of Dr. M. Montminy (Parker et al., 1996). The following have been described: Gal4CREB, Gal4CREBM1, Gal4CREBΔLZ (Sheng et al., 1991) and Gal4-luciferase (pG5E1b/lux) (Abdollah et al., 1997). Mutations were introduced into CREB by PCR. CRE-luciferase contains three copies of a somatostatin CRE in vector pGL3.

Cell Culture, Transfection, and Stimulation

Embyronic day (E)17 and E18 cortical cultures were prepared as described (Xia et al., 1996) and maintained for 7 days except where noted. Cortical cultures were transfected using calcium phosphate (Xia et al., 1996). For experiments using DHP-LTCs, cortical cultures were maintained for 5 days and transfected as described (Dolmetsch et al., 2001). Cultures were membrane-depolarized by addition of 60 mM KCl with 9.2 mM $CaCl_2$. The cAMP analog, 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) was from Sigma; BDNF (Amgen) was used at 100 ng/ml. Nimodipine (5 μM; Sigma-RBI), KN62 (2 μM or 5 μM), or PD98059 (30 μM), when used, were added to cells 30 min before stimulation.

Western Blot and Immunostaining

Western blotting and immunocytochemistry were done as described (Ginty et al., 1993). Treatment of blots with calf alkaline intestinal phosphatase (CIP; NEB) was performed in 50 mM Tris (pH 7.5), 1 mM $MgCl_2$, using 6 U/ml CIP for 15 min at room temperature. For seizure induction, rats were injected intraperitoneally with pentylenetetrazole (Metrazol; 55 mg/kg body weight; Sigma) or a saline control following an IACUC-approved protocol. Cerebral cortices were dissected and solubilized in Laemmli buffer for Western blotting.

In Vitro Phosphorylation and GST-KIX Binding Assays

Wild-type or mutant FlagCREB proteins were expressed and purified as described (Bullock and Habener, 1998) and phosphorylated in vitro with CaMKII, CKII (both recombinant, Calbiochem), or PKA (purified, Sigma) in 50 mM PIPES, 10 mM $MgCl_2$, 10 mM β-glycerol phosphate, 0.4 mM sodium vanadate, 1 mM DTT, 10 μg/ml leupep-

tin, 1 mM PMSF, 0.1 mg/ml BSA, and 0.1 mM ATP for 10–30 min at 30°C. For reactions using CaMKII, 0.5 mM $CaCl_2$ and 10 μg/ml calmodulin were included. GST-KIX pull-down assays were done essentially as described (Parker et al., 1996).

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Note Added in Proof

A report by Gau et al. (2002) in this issue of *Neuron* provides additional evidence that CREB Ser142 phosphorylation contributes to Ca^{2+}-stimulated transcription and physiological responses mediated by CREB. Light was shown to induce CREB Ser142 phosphorylation in the suprachiasmatic nucleus (SCN), and mice in which Ser142 was mutated to alanine showed deficits in both light-induced phase shifts of circadian rhythms and light-stimulated gene expression in the SCN. These results further support an important role for CREB Ser142 phosphorylation in the activation of Ca^{2+}-specific programs of gene expression that mediate adaptive responses.