CREB Phosphorylation and Dephosphorylation: A Ca^{2+}- and Stimulus Duration–Dependent Switch for Hippocampal Gene Expression

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Summary

While changes in gene expression are critical for many brain functions, including long-term memory, little is known about the cellular processes that mediate stimulus–transcription coupling at central synapses. In studying the signaling pathways by which synaptic inputs control the phosphorylation state of cyclic AMP–responsive element binding protein (CREB) and determine expression of CRE-regulated genes, we found two important Ca^{2+}/calmodulin (CaM)–regulated mechanisms in hippocampal neurons: a CaM kinase cascade involving nuclear CaMKIV and a calcineurin-dependent regulation of nuclear protein phosphatase 1 activity. Prolongation of the synaptic input on the time scale of minutes, in part by an activity-induced inactivation of calcineurin, greatly extends the period over which phospho-CREB levels are elevated, thus affecting induction of downstream genes.

Introduction

In all eukaryotic cells, proper communication between extracellular signals and the nucleus is necessary to trigger specific patterns of gene expression (Hunter, 1995). Not only must this signaling transmit information in space, from the surface membrane to the nucleus, but it also must perform a conversion in time, to link external inputs that are often transient to nuclear outputs that are sustained over a much longer period. The linkage between different patterns of cellular activity and gene expression takes on special importance for the understanding of various forms of brain plasticity such as learning and memory. Bursts of synaptic activity that may induce prompt changes in synaptic strength can also control the expression of genes encoding synaptic proteins, ion channels, kinases, and immediate early genes (Bliss and Collingridge, 1993; Curran and Morgan, 1995). Some of these have been shown to be important in memory (Silva et al., 1992; Paylor et al., 1994; Matsuoka et al., 1995). More generally, the production of new RNAs and proteins is required for stable behavioral changes in many animal species (Davis and Squire, 1984; DeZazzo and Tully, 1995; Mayford et al., 1995). Thus, it is likely that the signaling between synapse and nucleus is critical for the generation and persistence of memory. However, it is not yet clear whether postmitotic neurons have evolved specific synapse-

to-nucleus signaling pathways or whether they rely entirely on the cellular machinery found in dividing cells such as fibroblasts.

The cyclic AMP–responsive element binding protein (CREB) is a transcription factor of general importance in both neuronal and other cells (Hunter, 1995; Mayford et al., 1995). CREB phosphorylation on Ser-133 promotes the activation of genes with an upstream CRE element (Brindle and Montminy, 1992). CREB phosphorylation and downstream gene expression can in principle be regulated by protein kinases under the control of cAMP (Gonzalez and Montminy, 1989), Ca^{2+} (Dash et al., 1991; Sheng et al., 1991; Bading et al., 1993; Deisseroth et al., 1996) or both (Impey et al., 1996). Alteration of CREB function specifically affects long-term synaptic changes and long-term memory while sparing short-term changes (Dash et al., 1990; Bourtchuladze et al., 1994; Yin et al., 1994, 1995; Bartsch et al., 1995). Furthermore, CREB-binding protein (CBP), the specific binding protein for phospho-CREB (pCREB) in the active transcriptional complex (Chrivia et al., 1993), displays a mutation in Rubinstein-Taybi syndrome, characterized by congenital malformation and mental retardation (Petrij et al., 1995).

This study focused on the cellular processes that regulate the phosphorylation state of CREB in hippocampal neurons. pCREB was monitored at the single-cell level with an antibody specific for phospho-Ser-133 (Ginty et al., 1993). Synaptic activity simultaneously influenced both CREB phosphorylation (by a phosphorylation cascade involving multiple Ca^{2+}/calmodulin [CaM]–dependent kinases) and pCREB dephosphorylation (through calcineurin [CaN], a Ca^{2+}/CaM–dependent phosphatase). We found that the information contained in the brief bursts of synaptic activity can be stored for a much longer time scale, as embodied in the persistence of pCREB. This occurred in part through activity-induced inactivation of CaN, which affected the efficiency of pCREB dephosphorylation by nuclear protein phosphatase 1 (PP1). Induction of two CRE-dependent genes, c-fos and somatostatin (SS), was found to depend on the same stimulus parameters and signaling pathways that control the persistence of nuclear pCREB.
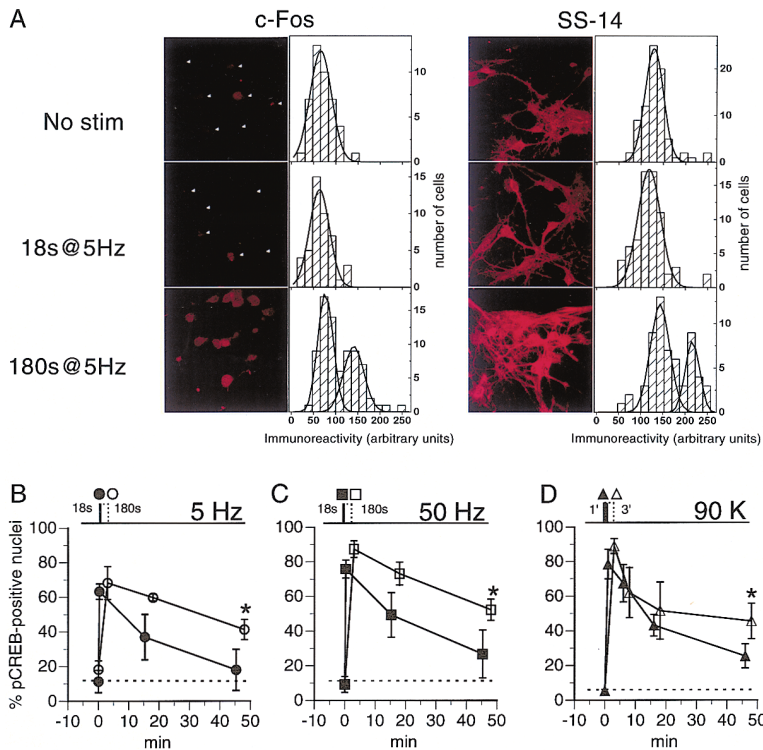
Results

Stimulus Duration Dependence of CRE-Mediated Gene Expression Correlates with Sustained Rather Than Transient pCREB in the Nucleus

We used c-Fos and SS-14 as markers of CRE-mediated gene expression (Brindle and Montminy, 1992; Bading et al., 1993). Ninety minutes after receipt of an electrical stimulus for 18 s at 5 Hz (a frequency within the physiological range of synaptic activity in hippocampal neurons), levels of nuclear c-Fos immunoreactivity (IR) and cytoplasmic SS-14-IR remained unchanged relative to control; however, when the duration of the stimulus train was increased to 180 s, the IR increased in a number of neurons (p < 0.05, Kolmogorov-Smirnov test; Figure 1A).

CREB Ser-133 phosphorylation is critical in CRE-

A

c-Fos   SS-14

No stim

18s@5Hz

180s@5Hz

Immunoreactivity (arbitrary units)   Immunoreactivity (arbitrary units)

B 5 Hz   **C** 50 Hz   **D** 90 K

Figure 1. Stimulus Duration Dependence of CRE-Mediated Gene Expression Correlates with Sustained Rather Than Transient pCREB

(A) (Left) Induction of c-Fos-IR with a stimulus train of 180 s at 5 Hz but not with 18 s at 5 Hz. Corresponding histograms of IR show the appearance of a new peak with higher IR after longer stimulation. (Right) Increase in somatostatin (SS)-14-IR with 180 s at 5 Hz but not with 18 s at 5 Hz. A significant amount of basal cytoplasmic SS-14-IR was present.

(B–D) Stimulus duration controls phospho-CREB decay.

(B) Stimulus trains for either 18 s at 5 Hz or 180 s at 5 Hz induced rapid CREB phosphorylation to a similar extent. However, decay from the phosphorylated state was significantly slowed after the longer stimulus. Mean $\pm$ SD (n = 5).

(C) Similar observations with 50 Hz trains (n = 3).

(D) Similar observations with 90 mM K$^+$ stimulation. Mean $\pm$ SD (n = 4–7).

Arrowheads, nonstained nuclei; asterisks, $p < 0.05$.

dependent gene expression (Brindle and Montminy, 1992). Confocal analysis of nuclear pCREB-IR in hippocampal neurons, by means of an antibody specific for CREB phosphorylated at Ser-133 (Ginty et al., 1993), confirmed its bimodal distribution into IR-positive nuclei or background and indicated that the proportion of the IR-positive nuclei was consistent with that determined by conventional epifluorescence microscopy (Bito et al., unpublished data). We found that the shorter stimulus (18 s) at 5 Hz was as efficient as a longer stimulus (180 s) in triggering an initial rise in pCREB (Figure 1B). Prolongation of the stimulation did not significantly alter the peak level of pCREB-IR or its cellular distribution (data not shown). However, longer stimulation produced greater stability in nuclear pCREB-IR over time, as monitored in neurons kept quiescent with tetrodotoxin (TTX) (Figure 1B). This dependence of pCREB persistence on the duration of stimulation was common to field stimulation at 5 Hz (Figure 1B) or 50 Hz (Figure 1C) and cellular depolarization with 90 mM K$^+$ (Figure 1D).

This series of experiments indicated that the rate of decline of pCREB-IR was consistently regulated by the duration of stimulation. The altered time course of pCREB decay strongly increased its time integral and might thus be expected to exert a substantial effect on CRE-dependent transcription. Indeed, the greater persistence of pCREB after a 3 min stimulus (Figures 1B–1D) was paralleled by increased expression of c-Fos and SS-14 (Figure 1A).

Nuclear CaMKIV May Be Involved in CREB Phosphorylation in Hippocampal Neurons

To clarify the biochemical mechanism underlying the stimulus-duration dependence of pCREB and gene expression, we first sought to identify the nuclear CREB

kinase. Many Ser/Thr kinases can phosphorylate CREB Ser-133. These include protein kinase A (PKA; Gonzalez and Montminy, 1989), protein kinase C (PKC; Xie and Rothstein, 1995), Ca^{2+}/CaM–dependent protein kinases such as CaMKI (Sheng et al., 1991), CaMKII (Dash et al., 1991; Sheng et al., 1991; Yoshida et al., 1995), and CaMKIV (Cruzalegui and Means, 1993; Enslen et al., 1994; Matthews et al., 1994; Sun et al., 1994), as well as a Ras-dependent p105 kinase (Ginty et al., 1994), and a pp90rsk (Bohm et al., 1995).

We used an array of potent kinase inhibitors to help delineate the nature of the kinase (Figures 2A and 2B). Staurosporine, a nonselective kinase inhibitor, largely eliminated pCREB formation at high concentration. A selective CaM kinase inhibitor, KN-93, significantly reduced CREB phosphorylation, whereas its inactive analog, KN-92, had no effect (Figures 2A and 2B). In contrast, pCREB formation was unaffected by saturating doses of selective inhibitors of PKA, PKC, cGMP-dependent protein kinase, mitogen-activated protein kinase, and tyrosine kinases (Figure 2B). Taken together, these data suggest that CaM kinase(s) are the main regulator(s) of CREB phosphorylation in hippocampal neurons.

Figure 2C compares subcellular distributions of CaMKI, CaMKII (α and β isoforms), and CaMKIV, four multifunctional CaM kinase isotypes expressed in hippocampus and capable of phosphorylating CREB in vitro (Braun and Schulman, 1995). The similarity in the pharmacology of the rise in pCREB at two ages—4 days in vitro (div), when synaptic connections had not yet formed, and >9 div, when evoked synaptic transmission could be consistently recorded—suggested that the CREB kinase might be expressed from early on in culture (Figure 2B). At 4 div, CaMKIV was prominently expressed
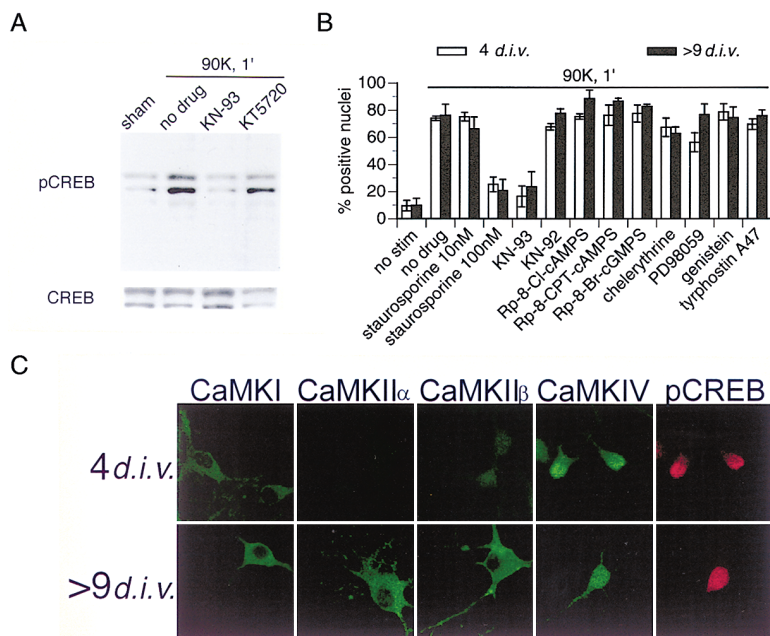
Figure 2. Nuclear CaMKIV May Be Involved in CREB Phosphorylation in Hippocampal Neurons

(A) A KN-93-sensitive kinase is involved in CREB phosphorylation (immunoblot at >9 div).

(B) Similarity of kinase inhibitor responsiveness at 4 div and >9 div. Effects of CaM kinase inhibitors (KN-93, 30 μM; KN-92, an inactive isomer, 30 μM), PKA inhibitors (KT5720, 2 μM; Rp-8-Cl-cAMPS, 100 μM; Rp-8-CPT-cAMPS, 100 μM), a PKG inhibitor (Rp-8-Br-cGMPS, 50 μM), a PKC inhibitor (chelerythrine, 10 μM), an MEK inhibitor (PD98059, 50 μM), and tyrosine kinase inhibitors (genistein, 200 μM; tyrphostin A47, 50 μM). Mean ± SD (n = 4–5).

(C) Subcellular localization of CaM kinases in cultured hippocampal neurons before and after synaptogenesis (4 and >9 div, respectively). Only CaMKIV showed significant nuclear IR at any stage. Activity-dependent translocation was not detected in any case (data not shown).

in the nucleus; there also was a measurable amount of CaMKI-IR and a faint hint of CaMKII-β, whereas CaMKII-α was undetectable (Figure 2C, top row). After synapse formation, at >9 div, all four members of the CaM kinase family appeared in abundance (Figure 2C, bottom row), but only CaMKIV showed a predominant nuclear localization. These findings confirmed previous studies in brain sections (Ito et al., 1994; Brocke et al., 1995; Nakamura et al., 1995; Picciotto et al., 1995). The lack of significant nuclear staining for CaMKII diminishes the likelihood of a role for this kinase in CREB Ser-133 or Ser-142 phosphorylation (Sun et al., 1994). On these grounds, CaMKIV must be regarded as a likely candidate for CREB kinase in hippocampal neurons.

Suppression of Nuclear CaMKIV Disrupts CREB Phosphorylation

We next sought evidence of a linkage between CaMKIV expression and CREB phosphorylation. There was a strong correlation between nuclear CaMKIV expression and the depolarization-induced pCREB-IR at the single-cell level (Figure 3A, no oligos, $p < 0.0001$, χ^2 test). An antisense strategy was used to establish a causal relationship. To manipulate nuclear levels of CaMKIV, two antisense oligonucleotides (HN-1 and HN-3) were used to target the translation initiation sites of the α- and β-splice variants, respectively, of CaMKIV. Corresponding missense oligos (AD-2 and AD-4) served as controls. Application of both antisense oligos together significantly decreased the number of neurons immunopositive for both CaMKIV and pCREB while augmenting the pCREB−/CaMKIV− category (Figures 3A–3C). Thus, the correlation between CaMKIV expression and pCREB remained strong ($p < 0.0001$, χ^2 test). Furthermore, the antisense oligos did not alter total CREB-IR (Figure 3C, right) or Ca^{2+} influx monitored with fura-2 (data not shown). In contrast, missense treatment left the patterns of immunopositivity unchanged relative to untreated

neurons (Figures 3A–3C). With data from the other experiments, the antisense results provide compelling evidence of involvement of CaMKIV as CREB kinase in Ca^{2+}-regulated CREB phosphorylation in hippocampal neurons.

Possibility of a CaM Kinase Kinase Cascade

Studies of recombinant CaMKIV demonstrate a striking activation upon phosphorylation by an upstream kinase (Okuno et al., 1994; Selbert et al., 1995; Tokumitsu et al., 1995). This prompted us to monitor phosphorylation of CaMKIV during stimulation in metabolically ^{32}P-labeled neurons. After 90 mM K^+ depolarization, a >4-fold increase in CaMKIV labeling was found relative to a sham treatment (Figure 4A). This increase was blocked in 0 Ca^{2+}/EGTA or by the CaM kinase inhibitor KN-93, indicating that CaMKIV activation by phosphorylation depended on Ca^{2+} influx and an upstream CaM kinase (Figure 4A). Consistent with the phosphorylation data, the same stimulus increased nuclear Ca^{2+}/CaM–dependent (∼2-fold increase, $p < 0.05$; Figure 4B) as well as Ca^{2+}/CaM-independent (40% increase, $p < 0.05$; data not shown) activities of CaMKIV. If CaMKIV activation were significant for pCREB-dependent transcriptional activation, a kinetic relationship between stimulus-dependent phosphorylation of CaMKIV and formation of the pCREB–CBP complex would be expected. Both CaMKIV phosphorylation and pCREB–CBP complex formation reached a maximal level within 1 min of high K^+ depolarization and did not further increase during the next 2 min of depolarization (Figures 4C and 4D). Furthermore, both pCREB-CBP formation and CaMKIV phosphorylation were sensitive to treatment with KN-93, whereas a PKA inhibitor, KT5720, had little effect. CaMKIV-mediated phosphorylation of CREB thus may be critical for pCREB-CBP association.

Our results indicate that even a short (1 min) depolarization, which induces a rapid and maximal phosphorylation of CREB, also rapidly and maximally activates
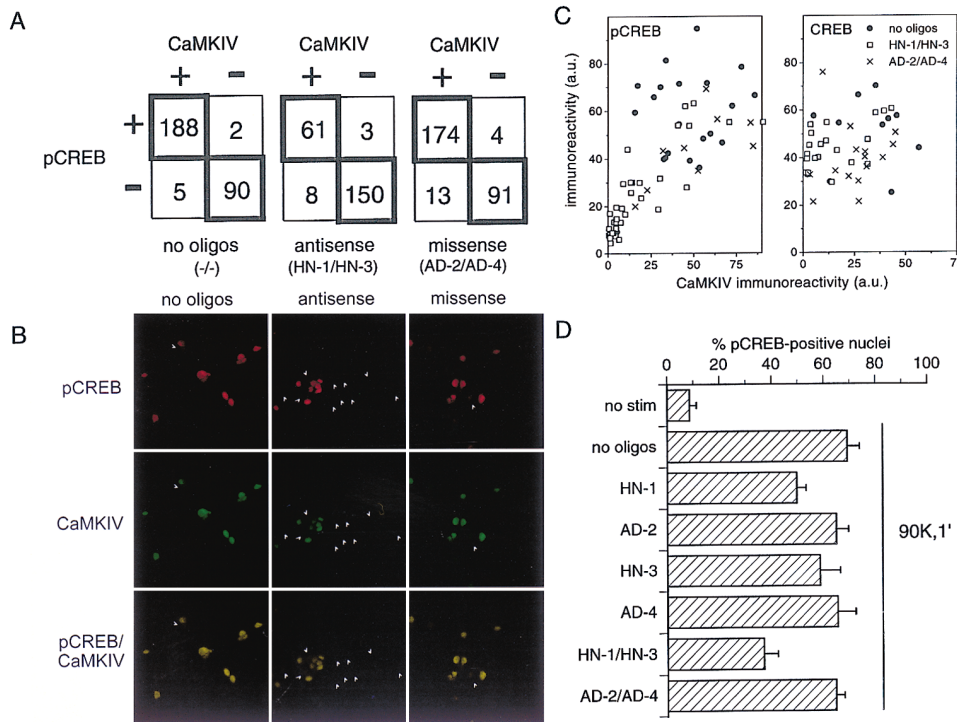
A

B

C

D

Figure 3. Suppression of Nuclear CaMKIV Disrupts Nuclear CREB Phosphorylation

(A) Strong correlation between nuclear CaMKIV expression and depolarization-induced pCREB-IR at the single-cell level. Correlation was preserved even after treatment with antisense or missense oligos.

(B) Example of the combined antisense effect on nuclear CaMKIV-IR and pCREB-IR. Confocal micrographs showing optical sections of nuclei. Arrowheads, IR-negative nuclei.

(C) Reduction of CaMKIV-IR by antisense treatment disrupts pCREB-IR (left) but does not affect CREB-IR (right).

(D) Summary showing that effectiveness of antisense oligos targeted to CaMKIV-α and CaMKIV-β in combination in interfering with pCREB formation, relative to other treatments. Hippocampal neurons at 4 div. Mean $\pm$ SD, n = 6.

CaMKIV and that this activation is mediated in part through phosphorylation of CaMKIV by an upstream CaM kinase kinase. It is thus likely that even brief stimulation facilitates formation of a stable pCREB–CBP complex through CaMKIV-induced CREB phosphorylation. This process was not significantly facilitated by prolongation of the stimulus to 3 min, suggesting that it is not the extent of the initial rise in pCREB that determines its persistence.

Evidence That PP1 Mediates Nuclear CREB Dephosphorylation

We turned next to PPs to determine whether control of CREB dephosphorylation could underlie the stimulus-duration dependence of CRE-mediated gene expression. IRs for both PP1 and PP2A, two major classes of PPs that account for the majority of cellular protein phosphatase activity (Cohen et al., 1990; Shenolikar, 1994), were clearly present in the nuclei of the cultured hippocampal neurons (data not shown; see also Ouimet et al., 1995). In contrast, CaN (PP2B), a Ca^{2+}/CaM–dependent PP, was barely detectable in the nucleus (Figure 5D). Okadaic acid (OA) was used to discriminate between the possible effects of PP1 (block of which requires micromolar OA) and PP2A, PPX/PP4, and PP5 (inhibited by OA in the 10 nM range) (Cohen et al., 1990;

Shenolikar, 1994; Nagao et al., 1995). A low dose (20 nM) of OA failed to alter the decline of pCREB-IR following synaptic stimulation or high K^+ depolarization, whereas a high dose (2 μM) of OA completely inhibited pCREB dephosphorylation (Figures 5A and 5B). An inactive analog, 1-nor-okadaone (2 μM), had no effect, whereas calyculin A (5 nM), which blocks both PP1 and PP2A, completely prevented the CREB dephosphorylation (Figure 5C). None of the phosphatase inhibitors induced CREB phosphorylation by themselves (data not shown). Because the pCREB phosphatase was sensitive to micromolar OA or calyculin A, it could be identified on pharmacological grounds as PP1.

CaN as a Negative Regulator of pCREB

CaN was discounted as the pCREB phosphatase, based on its predominantly cytoplasmic location in the cultured neurons (Figure 5D) and because OA blocked pCREB dephosphorylation completely at 2 μM (Figure 5C), well below the half-maximal inhibitory concentration of OA for CaN (Cohen et al., 1990). The possibility still remained that CaN might serve as a modulator and influence the pCREB dephosphorylation by PP1, acting perhaps from the cytoplasm. Following a brief stimulation, a significantly higher amount of pCREB persisted after 45 min recovery in the presence of 1 μM FK506, a CaN inhibitor,
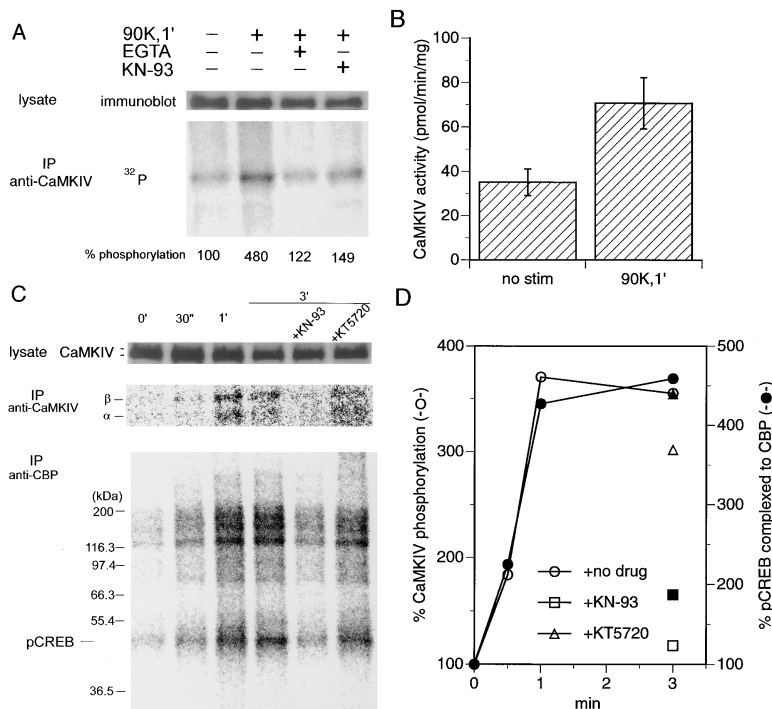
Figure 4. Activation of CaMKIV by a CaM Kinase Kinase Parallels Elevation of pCREB within the CBP Complex

(A) (Top) CaMKIV content in crude cell lysates, monitored by immunoblot. (Bottom) Depolarization-induced phosphorylation of CaMKIV is Ca^{2+}-influx– and CaM kinase–dependent. EGTA (2.5 mM, 0 Ca^{2+}), KN-93 (30 μM, 15 min preincubation). (B) Increase in Ca^{2+}/CaM–dependent activity of CaMKIV in nuclear extracts from hippocampal neurons after a 90 mM K^+ stimulus. Mean $\pm$ SD (n = 6).

(C) (Top) CaMKIV content in crude cell lysates. (Middle) Autoradiograph of time-dependent phosphorylation of CaMKIV-α and CaMKIV-β. (Bottom) Autoradiograph demonstrating time-dependent increase of phosphoproteins in the CBP complex, including a phosphoprotein with a molecular weight consistent with pCREB.

(D) Phosphorylation of CaMKIV correlates with pCREB–CBP complex formation.

than with rapamycin (1 μM), an inactive analog (Figures 5E and 5F). Similar results were obtained after electrical stimulation (50 Hz for 18 s) or after exposure to high K^+ (Figure 5E). Contrary to the effect of OA (Figures 5A and 5B), FK506 did not prevent pCREB dephosphorylation itself but clearly slowed the time course of the return to baseline (Figures 5F, 5G, and 5I). This suggests that CaN may promote pCREB dephosphorylation in the wake of pCREB induction by a brief stimulus train. CaN is probably activated during a field stimulation, even at a frequency of 5 Hz, since the bulk cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) under these conditions (Figure 6A) is adequate for CaN activation (Stemmer and Klee, 1994). We next examined the effect of FK506 following a longer stimulus that was effective in giving rise to CRE-mediated gene expression (Figure 1A). When the same stimulus train was repeatedly applied with a 1 min interstimulus interval or when the stimulus was prolonged 10-fold (from 18 to 180 s), FK506 no longer affected the decline of pCREB (Figures 5H and 5J). Thus, increasing the duration of the stimulus occluded the effect of CaN inhibition. This implies that CaN itself, or signaling molecules downstream of CaN, became inactivated or inaccessible with longer stimulus duration.

Recent work from Klee and colleagues (Wang et al., 1996) has suggested that CaN undergoes inactivation upon prolonged conjunction of elevated levels of $[Ca^{2+}]_i$ and superoxide anions (O_2^-). This prompted us to examine both intracellular Ca^{2+} and redox dynamics during electrical stimulation of the hippocampal neurons. During a 180 s, 5 Hz train, we observed a maintained pedestal of $[Ca^{2+}]_i$ (in 13 of 13 neurons, averaging ~150 nM), whereas $[Ca^{2+}]_i$ fell toward the basal level of ~50 nM following an 18 s, 5 Hz train (Figure 6A). Prolonged electrical stimulation also induced a steadily increasing oxidative conversion of nonfluorescent dihydrorhodamine

6G into fluorescent rhodamine (Figure 6B). The zones with increased oxidation of the probe were seen in both soma and dendrites and tended to be juxtamembranous and contiguous (Figure 6C). These data show that both factors necessary for activity-induced inactivation of CaN (Wang et al., 1996) can in fact be observed in the hippocampal cells. As a further test of the involvement of a reactive oxygen species in the control of pCREB, we studied the effects of agents that interfere with production or inactivation of O_2^-: N-*tert*-butyl-α-(2-sulfophenyl)-nitrone (S-PBN), an electron spin trap and a strong radical scavenger, and diethyldithiocarbamic acid (DDC), a Cu^{2+} chelator that blocks superoxide dismutase (SOD). In the presence of S-PBN, even a long stimulus was incapable of delaying the pCREB decay, whereas in the presence of DDC, even a short stimulus gave rise to a persistent nuclear pCREB (Figures 6D and 6E). These results support the hypothesis that synaptic stimulation increases oxidative activity and thereby promotes the inactivation of CaN (Wang et al., 1996), thus slowing pCREB dephosphorylation.

CaN as a Negative Regulator of CRE-Mediated Gene Expression

If CaN activity spells the difference between short- or long-lived elevation of pCREB, inhibition of CaN might allow even brief stimuli to trigger CRE-dependent transcription successfully. To test this idea, we monitored the effect of CaN inhibition on c-Fos and SS-14 induction (Figure 7) . Indeed, treatment with FK506 enabled short, 18 s stimuli to produce a significant increase in nuclear c-Fos-IR and cytoplasmic SS-14-IR (p < 0.01, Kolmogorov-Smirnov test), whereas the same stimulation had failed to do so in the absence of CaN inhibition (Figure 1). These data underscore the importance of CaN activity in
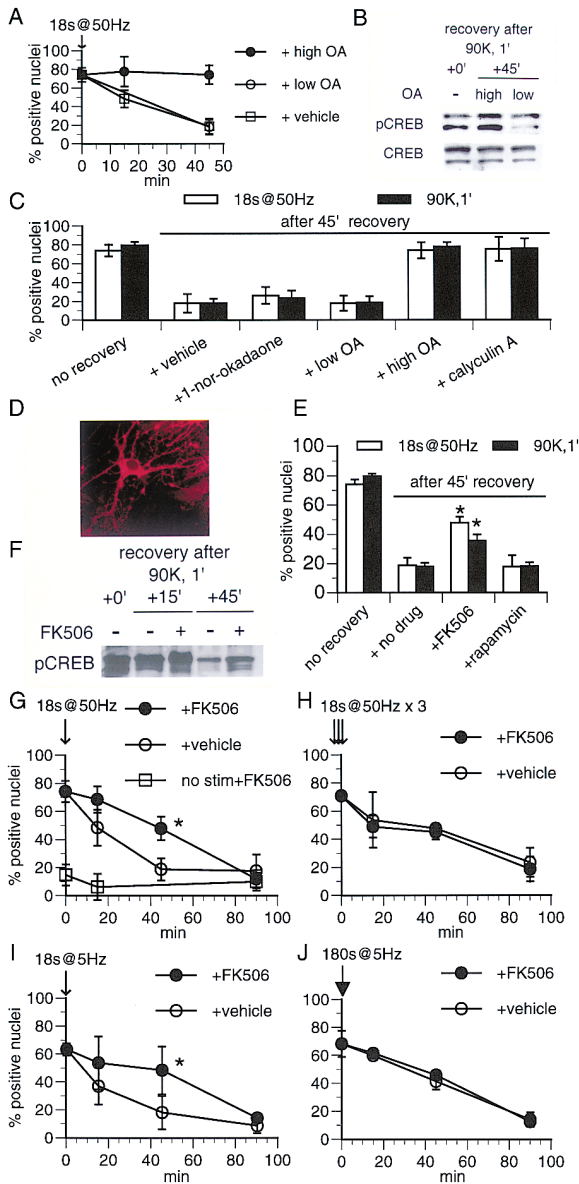
Figure 5. pCREB Dephosphorylation Pathway: PP1 as pCREB Phosphatase and CaN as Regulator

(A) Incubation with a high dose (2 μM) of OA, but not with a low dose (20 nM), completely blocks the decline of pCREB after synaptic stimulation. Mean $\pm$ SD, n = 3–6.

(B) Similar observations after a high K$^+$ stimulus, shown as a Western blot.

(C) Summary of effects of various phosphatase inhibitors. Mean $\pm$ SD (n = 9).

(D) Cytoplasmic localization of regulatory (shown here) and catalytic (not shown) subunits of CaN; both IRs were below detection limit in the nucleus.

(E) Slowing of pCREB dephosphorylation by FK506 (1 μM), a specific CaN inhibitor, but not by rapamycin (1 μM), an inactive analog. Mean $\pm$ SD, n = 9.

(F) Western blot confirms a FK506-sensitive component in pCREB dephosphorylation.

(G and H) Incubation with FK506 during the decay phase significantly slowed the kinetics of decay of pCREB after a short stimulus train (18 s at 50 Hz), but not when train of stimuli was repeated three times with a 1 min interstimulus interval. Mean $\pm$ SD, n = 5.

(I and J) Exposure to FK506 produced a delay in the decline of

controlling the persistence of nuclear pCREB and the occurrence of CRE-dependent nuclear events.

Discussion

A CaM Kinase Cascade and CaN Control CREB Phosphorylation and Dephosphorylation

Our work indicates that activity at hippocampal synapses controls the phosphorylation state of CREB through two opposing mechanisms, apparently both involving Ca^{2+}/CaM–regulated enzymes. A CaM kinase cascade consisting of CaMKIV and a CaMKIV kinase appeared to play a critical role in the formation of pCREB. pCREB formation was rapid, reaching a plateau within 1 min (unpublished data), in accordance with the rapid activation kinetics of CaM kinases (Braun and Schulman, 1995). This rapidity suggests that activation of CaMKIV is achieved as the final step of a CaM kinase cascade rather than by autophosphorylation (Cruzalegui and Means, 1993; Okuno and Fujisawa, 1993; Selbert et al., 1995; Tokumitsu et al., 1995). A kinase cascade would confer noteworthy functional properties in nuclear signaling, including the potential for signal amplification and for convergence of multiple signals (Hill and Treisman, 1995; Hunter, 1995), and may be appropriate as part of a cell switch mechanism (Marshall, 1995; Huang and Ferrell, 1996). It will be relevant to identify upstream CaM kinase kinases and to determine their mode of regulation. A similar CaMKIV cascade has been reported in T cells (Hanissen et al., 1993; Park and Soderling, 1995).

Our experiments suggest that once CREB is phosphorylated, the maintenance of this state is controlled by regulation of a pCREB phosphatase. Our data implicate PP1 as the pCREB phosphatase (Figure 5), consistent with a previous work in PC-12 cells (Hagiwara et al., 1992). We also have identified a novel role for cytoplasmic CaN in regulating the decline in pCREB. Inhibition of CaN retarded the decay of pCREB levels without preventing its ultimate dephosphorylation (Figures 5F, 5G, and 5I). In principle, this effect could be explained if CaN acted by inhibiting CaMKIV or its upstream activators, although no direct biochemical evidence for this possibility is yet available. Another possibility is that CaN acts by potentiating the PP1 pathway, presumably by dephosphorylating a PP1 regulator, such as phospho-inhibitor 1, a PP1 inhibitor. We envision that activation of cytoplasmic CaN, by even brief bouts of synaptic stimuli, would reduce cytoplasmic levels of phospho-inhibitor 1 and eventually diminish nuclear levels as well, thereby potentiating nuclear PP1 activity and accelerating pCREB dephosphorylation. A similar role was attributed to CaN in the regulation of PP1 during the induction of long-term depression at hippocampal synapses (Lisman, 1989; Malenka, 1994).

Because CaN is a Ca^{2+}/CaM–regulated enzyme, in effect we are proposing that signals regulating phosphorylation and dephosphorylation both involve Ca^{2+}/

pCREB dephosphorylation after a stimulus train of 18 s at 5 Hz, but not with 180 s at 5 Hz. Mean $\pm$ SD, n = 5. Asterisk, p $<$ 0.05.
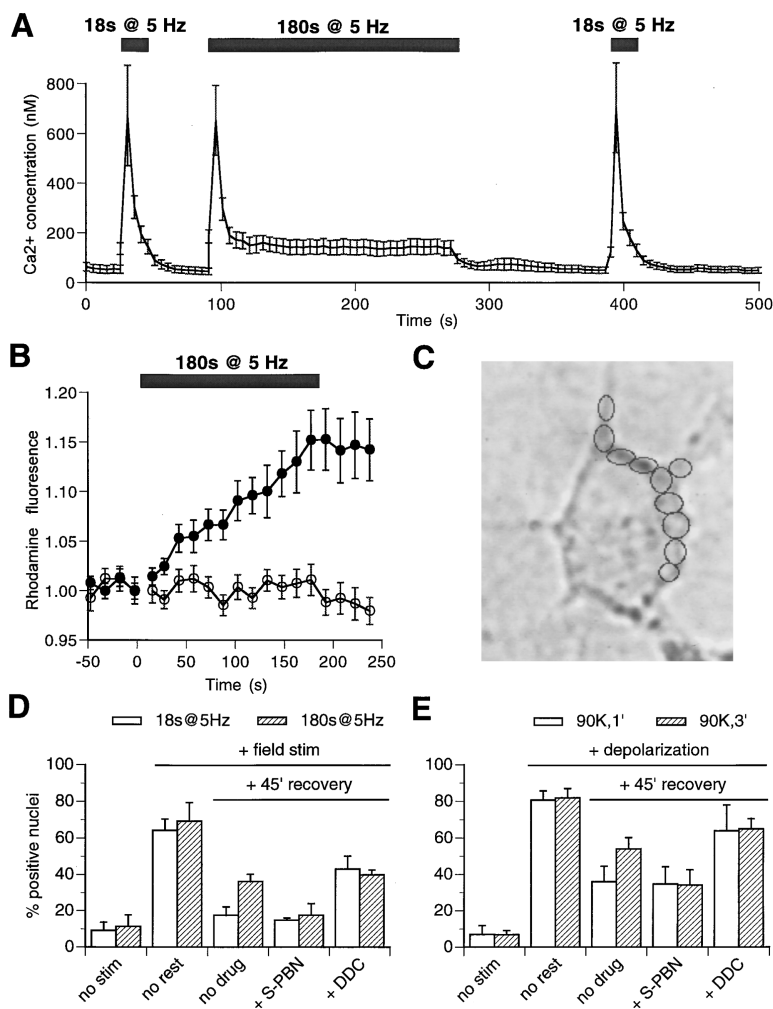
Figure 6. Evidence for Involvement of Synaptic Activity–Induced Oxidative Activity in Regulation of pCREB Dephosphorylation

(A) Changes in bulk $[Ca^{2+}]_i$ during short and prolonged stimulation at 5 Hz. Mean ± SEM, n = 13.

(B) Synaptic stimulation induced a steady increase in dihydrorhodamine oxidation, consistent with a step increase in oxidative activity during stimulation. Closed circles, mean ± SE from 42 subcellular areas with increased fluorescence (6 neurons); open circles, mean ± SE from 52 other areas from the same neurons (including cytoplasmic and nuclear regions), which showed no significant change apart from a slight fading of fluorescence.

(C) Example of the distribution of areas showing oxidative activity.

(D) A spin trap for superoxide (S-PBN, 100 μM) prevented pCREB stabilization after prolonged synaptic stimulation. DDC (5 mM), an SOD inhibitor, rendered a brief stimulus as effective as a prolonged stimulus.

(E) Similar effect of both drugs after 90 mM K^+, 1 min stimulation. The trapping of O_2^- favored pCREB decline, and the prevention of O_2^- clearance delayed it; in either case, duration dependence was eliminated (p > 0.4). Mean ± SD, n = 4–5.

CaM (Figure 8). The bidirectional nature of this synaptic control of CREB seems to be well suited for inducing a transient pulse of CREB phosphorylation. The transience of this CREB peak after brief stimulation appears to be responsible for the inability of brief stimulation to induce CRE-dependent gene expression (Figure 1A).

A Synaptically Controlled, Stimulus Duration–Sensitive Mechanism for Transcription

Our results provide fundamental information about the features of neural inputs that are encoded by CREB phosphorylation. When we compared the time course of pCREB decay following short and long stimulus trains, at frequencies and durations appropriate to induce synaptic potentiation or depression (Deisseroth et al., 1996), we found that it was the duration of the synaptic activity rather than its frequency that determined how long pCREB remained increased above basal levels (Figures 1B and 1D). Lengthening of the stimulus duration from ~1 to 3 min greatly prolonged the residual nuclear pCREB (Figures 1B–1D) and also produced an increase in the expression of CRE-regulated gene products c-Fos and SS-14 (Figure 1A). Thus, the temporal extension of the pCREB signal may serve as a stimulus duration–

sensitive switch for turning on CRE-mediated transcription. The sustained presence of pCREB in the nucleus may ensure stabilization of the pCREB–CBP complex that is necessary for efficient CRE-regulated gene expression; a preinitiation complex must be stabilized to maintain ongoing transcription, as recently demonstrated in vivo by Ho et al. (1996). A similar example of a Ca^{2+}-dependent, duration-sensitive pathway has been noted in gene induction by nuclear factor of activated T cells (NFAT) (reviewed in Crabtree and Clipstone, 1994).

In contrast, what is the significance of the clear but transient rise in pCREB evoked by brief stimuli? It might simply represent the behavior of the switch mechanism as a filter to screen out weak inputs. Another possibility is that the transient pCREB signal may be sufficient to promote transcription in a subset of CRE-regulated genes that are more predisposed to activation than those studied here. Alternatively, the pCREB transient might provide a priming signal, which could act in combination with an otherwise ineffective input to produce a nuclear effect. The potential importance of such a priming action is supported by studies showing that dephosphorylated CREB can act as a dominant negative regulator of CRE-dependent gene expression mediated

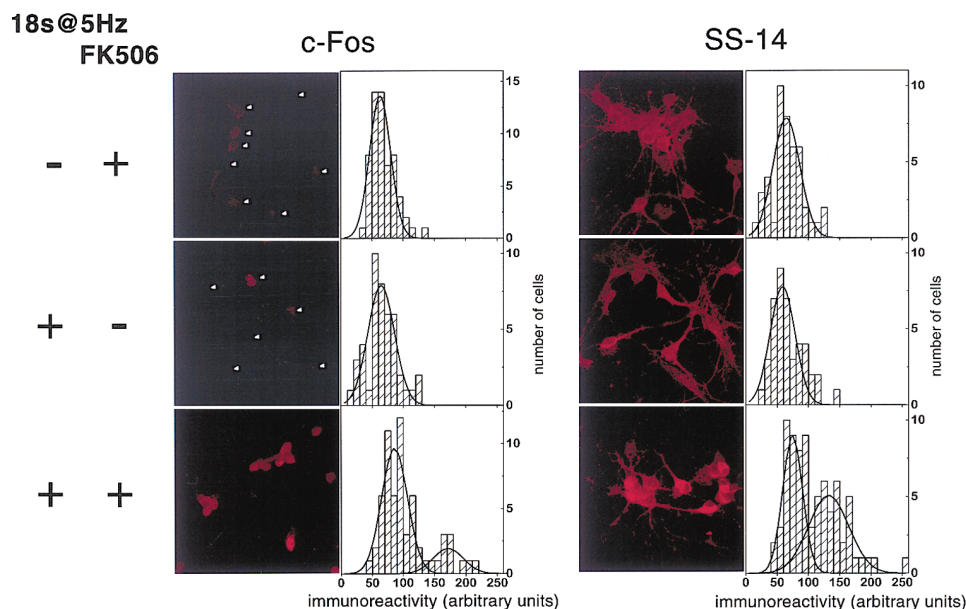**18s@5Hz**
FK506

c-Fos

SS-14

Figure 7. FK506 Reverses the Negative Regulation by CaN and Permits Coupling of Short Stimuli with Downstream Gene Expression, Consistent with Persistence of pCREB

(Left) c-Fos-IR was induced in the presence of FK506 after a short stimulus (18 s at 5 Hz). Arrowheads, IR-negative nuclei. (Right) Similar findings with SS-14-IR.

by other CRE-binding transcription factors (Vallejo et al., 1995).

CaN as a Key Element in the Duration-Sensitive Gene Expression Switch

The prolonged detection of pCREB in the nucleus was not only functionally important; it also was surprising in mechanistic terms because it could not be explained by kinase regulation. Because CREB formation and pCREB–CBP complex formation reached a plateau within the first minute of stimulation (Figure 4C), merely lengthening the stimulus by ∼2 min would be expected only to yield a trivial increase in the persistence of nuclear pCREB and not the prolongation by 20–30 min that actually was observed (Figures 1B–1D). These observations provided early clues that there may be a different kind of regulatory mechanism, one involving control of phosphatase activity.

Our experiments show that CaN is an important player in conditioning the switch mechanism (Figures 5–7). There are three major findings. First, when CaN was blocked by FK506, short stimulus trains became as potent as long stimuli in promoting increases in pCREB, with critical consequences for CRE-dependent gene expression. Of note, FK506 has also been reported to augment pCREB-IR and c-Fos-IR in organotypic slices of the striatum (Liu and Graybiel, 1996). Second, after prolonged stimulation, pCREB-IR could not be further increased by FK506. Third, local, synaptically stimulated generation of O_2^- or a related oxidative species appeared to be critical in regulating the stimulus duration dependence of pCREB dephosphorylation.

The first finding suggests that CaN is involved in accelerating the decay of pCREB, thus uncoupling CRE-regulated gene expression in the wake of a brief stimulus train. The second result implies that the stimulus duration dependence and CaN dependence are mutually occlusive. Finally, the third observation is consistent with a model in which inactivation of CaN is induced in a Ca^{2+}-dependent, activation-specific manner (Stemmer et al., 1995) by a change in its redox state, possibly by the action of O_2^- and regulated by SOD (Wang et al., 1996). Further studies are clearly needed to elucidate fully the role of synaptically induced O_2^- release and its physiological effect on various neuronal signaling pathways.

Neurobiological Perspectives and Significance

Experiments in Aplysia and Drosophila have shown the importance of PKA-dependent signaling both in CRE-induced transcription and in long-term memory (DeZazzo and Tully, 1995; Mayford et al., 1995). However, evidence linking PKA to memory is lacking in mammals; in fact, PKA-deficient mice appeared normal with regard to learning and long-term memory, although late long-term potentiation and long-term depression were perturbed (Huang et al., 1995). Furthermore, our findings have failed to link PKA to CREB phosphorylation, indicating that the mammalian hippocampal CREB pathway may be distinct from those characterized in invertebrates (Mayford et al., 1995).

PKA may be involved in some other aspects of CRE-mediated gene expression in mammals. Indeed, evidence from PC-12 cells has shown that a basal level of PKA activity is required for CRE-mediated gene expression though not essential for CREB phosphorylation itself (Brindle et al., 1995; Thompson et al., 1995). In an acute hippocampal slice preparation, CRE activation induced by a protocol of late long-term potentiation was shown to be mediated at least in part by Ca^{2+}-induced
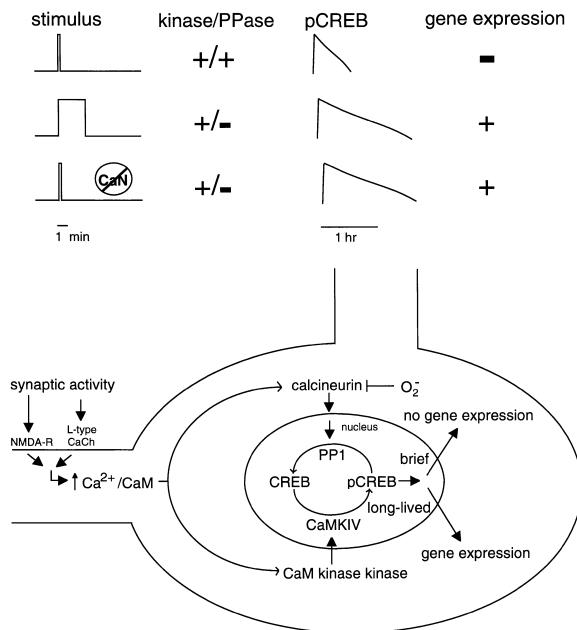
Figure 8. CREB as a Stimulus Duration–Sensitive Switch in Hippocampal Gene Expression

(Top) The persistence of pCREB (hour time scale) can be sharply altered by changes in stimulus duration (minute time scale) by a switch regulated by kinases and phosphatases. Prolonged stimulation induces inactivation of the phosphatase pathway, allowing the temporal persistence of nuclear pCREB, which appears critical in the signaling that leads to activation of CRE-regulated genes.

(Bottom) A working model of Ca^{2+}/CaM–mediated, bidirectional control of pCREB in the nucleus. Synaptic Ca^{2+} entry triggers a submembranous increase in $[Ca^{2+}]_i$, which in turn activates a Ca^{2+}/CaM–dependent process leading to a *trans*-phosphorylation of nuclear CaMKIV, possibly by a CaM kinase kinase. Dephosphorylation of CREB is mediated by a nuclear phosphatase with sensitivity to high OA, presumably PP1. CaN is depicted as causing an acceleration in CREB dephosphorylation rate; a possible target for CaN is phospho-inhibitor-1, acting as an inhibitory subunit of PP1 when phosphorylated. This scheme can account for the results summarized (top). During a brief stimulus, simultaneous activation of both kinase and phosphatase pathways ensures that pCREB elevation will be large but brief. Longer stimuli cause prolongation of nuclear pCREB, possibly by inhibition of CaN via O_2^-, thus hampering the phosphatase pathway. Similarly, when CaN is blocked pharmacologically, even brief stimuli can bring about a prolonged pCREB transient. CaCh, calcium channel.

stimulation of PKA (Impey et al., 1996). PKA could also act as a gate to modify the efficiency of postsynaptic signaling pathways required for CREB phosphorylation, while not being directly responsible for it (Blitzer et al., 1995). Furthermore, phosphorylation of CBP by PKA and mitogen-activated protein kinase may be required to enhance transactivation potential of CBP (Janknecht and Hunter, 1996). Further investigation should clarify the interactions between Ca^{2+}-dependent pathways and other signaling cascades.

An additional layer of transcriptional regulation may be provided by parallel recruitment of CREB activators and inhibitors, the difference or ratio of which is critical for gene expression (Bartsch et al., 1995; Yin et al., 1995). The relative rate of decay of activator and inhibitor after each individual trial is critical in such systems (Yin et

al., 1995). Slowing the decay of the activator relative to the counteracting inhibitor would greatly increase the accumulation of the resultant of these opposing signals. Our results raise the possibility that activity-induced inactivation of CaN might prove important for accumulation of the net differential between activator and inhibitor signals in mammalian hippocampal neurons.

Although we have delineated pathways by which synaptic activity controls CRE-dependent gene expression, we do not know which of the many candidate CRE-regulated genes are vital in memory formation. CREB might be involved in regulating neuronal properties such as action potential firing (e.g., through the K^+ channel Kv1.5), neurotransmitter release (e.g., through synapsin I), or postsynaptic responsiveness (e.g., through CaM-KII-α). We also do not know whether the duration-dependent gene expression switch outlined here participates in memory formation in vivo. However, one implication of our results is that perturbation of CaN function might have noteworthy effects on the protein synthesis–dependent phase of long-term memory. This prediction apparently has been verified recently in a chick learning model (Bennett et al., 1996). Further tests in CaN knock-out mice (Zhang et al., 1996) or in other organisms such as Drosophila are desirable. The identification of novel mechanisms by which synaptic activity controls expression of CRE-dependent genes raises further questions about their role in physiological events (e.g., alteration of synaptic strength, apoptosis, and formation and elimination of synapses), as well as in pathological processes (e.g., epilepsy or ischemic injury).

Experimental Procedures

Electrical Stimulation, Pharmacological Manipulation, and Immunocytochemical Analysis of Hippocampal Neurons

Hippocampal neurons were cultured as described by Deisseroth et al. (1996). After preincubation at room temperature for 2 hr in Tyrode containing 2 mM Ca^{2+}/2 mM Mg^{2+}/10 μM glycine/1 μM TTX, neurons were stimulated either by electrical field stimulation ($\sim$0.5 mA, 1 ms current pulses applied across the coverslip) or by depolarization with 90 mM K^+. Field stimulation was applied after removal of TTX, whereas high K^+ depolarization was done in TTX. Neurons were immediately fixed (30 s after field stimulation or within 5 s after high K^+ stimulation) or returned to Tyrode/TTX.

When used, kinase inhibitors were present during a preincubation period of 20–30 min and also during stimulation. Phosphatase inhibitors were usually applied only after field stimulation, but with high K^+ stimulation, they also were included during the last 45 min of preincubation; in all phosphatase inhibitor experiments, TTX, D-AP5 (50 μM), and CNQX (10 μM) were included to avoid effects due to modification of synaptic transmission. Exposure to the O_2^- modulatory drugs began 30 min prior to stimulation and continued during the 45 min recovery period. Effects of synaptic activity on gene expression were measured by incubation of neurons at 37°C for 90 min in Tyrode/TTX after synaptic stimulation at room temperature as described above.

Immunofluorescent staining was performed as described (Deisseroth et al., 1996). Specimens were examined with a Zeiss Axiophot epifluorescence microscope (courtesy of R. H. Scheller) or with an MDI MultiProbe 2010 confocal laser scanning microscope (CLSM) at the Cell Sciences Imaging Facility, Beckman Center. Scoring of CREB phosphorylation by nuclear pCREB positivity was based on Alberts et al. (1994) and was performed as described (Deisseroth et al., 1996). Photomicrographs were obtained directly on film or as 8-bit TIFF files. The CLSM laser power was adjusted for each series

of experiments to make the best use of the 8-bit scale of the photo-multipliers. Nuclear localization was verified by costaining with DAPI or by scanning z-series sections on a CLSM. Data were analyzed off-line with ImageSpace (MDI); values were mean signal intensity collected over an area of 2.5–5 μm^2 in either a midnuclear section (for pCREB, CREB, and c-Fos-IRs), or in a region of cytoplasm adjacent to the nucleus (for SS-14-IR). Histogram distribution and statistical tests were analyzed with Origin 4.0 (Microcal) and Stat-ViewII (Abacus). Representative images are shown (n = 3–5).

Antibodies were as follows. Polyclonal antibodies were anti-pCREB (UBI, 1:500 dilution); anti-CREB (UBI, 1:1000); anti-CaMKI (a gift from Dr. A. Nairn, 1:50; see Picciotto et al., 1995); anti-CaMKIIβ (Transduction Laboratories, 1:250); anti-PP1 catalytic subunit (UBI, 1:200); anti-PP2A catalytic subunit (UBI, 1:200; Promega, 1:200); anti-c-Fos (Ab-2, Oncogene Science, 1:200); and anti-SS-14 (Chemi-con, 1:200). Monoclonal antibodies were anti-CaMKII-α (clone CBα-2, Life-Technologies, 1:200; clone 6G9, Boehringer-Mannheim, 1:500); anti-CaMKIIβ (clone CBβ-1, Life Technologies); anti-CaMKIV (Transduction Laboratories, 1:200); anti-CaN regulatory subunit (clone VA-1, UBI, 1:250); anti-CaN catalytic subunit (clone CN-A1, Sigma, 1:250); and anti-synaptophysin (clone SY38, Boehringer-Mannheim, 1:250).

Sources of drugs were LC Laboratories (OA, calyculin A, 1-nor-okadaone, and KT5720), Calbiochem (Rp-cAMPS, KT5720, OA, chel-erythrine, TTX, and rapamycin), BIOLOG (Rp-cAMPS, Rp-8-Cl-cAMPS, Rp-8-CPT-cAMPS, and Rp-8-Br-cGMPS), Seikagaku (KN-93 and KN-92), Biomol (genistein, tyrphostin A47, and lavendus-tin A), Kyowa Medix (staurosporine and K252a), RBI (D-AP5 and CNQX), Sigma (DDC), Aldrich (S-PBN), and NEB (PD98059).

18-Mer antisense (HN-1: 5′-GTGACTTTGAGCATCTTC-3′; HN-3: 5′-GCAACTCATGTTTCCTGG-3′; both targeted to sequences around the translation initiation sites for CaMKIV-α and CaMKIV-β, respectively) and missense oligonucleotides (AD-2: 5′-GTGTCTTAGAG CAACTTC-3′; AD-4: 5′-GCAACACAAGTATCCTGG-3′) were based on published rat CaMKIV cDNA sequences (Sakagami and Kondo, 1993) and synthesized by Oligos Etc. Phosphorothioate modification was restricted to the three bases at both ends of the oligos to reduce the nonspecific effects and cytotoxicity observed with fully phosphorothioated oligos. A final 5 μM solution of each oligo, either alone or in combination, was added to the culture medium immedi-ately after plating and until 4 div, when the experiments were per-formed. The medium was changed every 24 hr, with the same con-centration of oligos throughout the experiment.

Phosphorylation Assay and Immunoblot Analysis
To monitor activity-dependent phosphorylation of CaMKIV and CREB, neurons grown on dishes were prelabeled with [^{32}P]ortho-phosphate (1.5 mCi/ml, Amersham) during the last 45 min of TTX pretreatment. After 90 mM K$^+$ stimulation, neurons were washed with ice-cold PBS(−) and immediately frozen on liquid N$_2$. Immuno-precipitation was carried out at 4°C from total cell lysates (200 μl) prepared in a lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, Complete protease inhibitor tablet [Boehringer-Mannheim], 2 mM EGTA, 2 mM EDTA, 10 nM calyculin A, and 1 mM sodium vanadate), using 2.5 μg of the anti-CaMKIV monoclonal antibody (Transduction Laboratories) or 2 μg of anti-CBP polyclonal antibody recognizing the N-terminal end of CBP (A-22, Santa Cruz Biotechnology). Immunoprecipitates were separated with 10% SDS-PAGE, and ^{32}P incorporation was mea-sured with a PhosphorImager (MDI).

For immunoblots, neurons were lysed with boiling SDS-PAGE buffer, electrophoresed, and transferred onto nitrocellulose mem-branes. After a blocking reaction (5% nonfat dry milk in Tris-buffered saline, pH 7.4, 0.05% Tween-20), primary antibodies (anti-pCREB, 1:2500; anti-CREB, 1:5000; and anti-CaMKIV, 1:1000) were incu-bated overnight at 4°C in the blocking buffer. After incubation with a secondary antibody (biotinylated goat anti-rabbit [or anti-mouse] antibody [Cappel], 1:2000]), the membrane was further probed with an HRP-conjugated anti-biotin antibody (NEB, 1:1000); an ECL pro-cedure followed (Amersham).

Nuclear extracts from hippocampal neurons were prepared ac-cording to a published procedure (Andrews and Faller, 1991) and were assayed for CaMKIV activity using peptide-γ (Biomol) as a substrate as described (Okuno et al., 1994).

Measurements of Rise in [Ca^{2+}]$_i$ and Production of Oxygen Radicals
[Ca^{2+}]$_i$ imaging was conducted using a Nikon 40x Fluor objective (N. A. 1.3), a Videoscope ICCD camera, a Sutter filter wheel–shutter assembly, and software from Universal Imaging. Neurons were incu-bated for 1 hr in 1 μM fura-2/AM (Molecular Probes) at room temper-ature. Calibrations were performed by measuring the 340 nm/380 nm ratio at known concentration of [Ca^{2+}]$_i$ in a whole-cell patch pipette, and [Ca^{2+}]$_i$ was calculated as described (Grynkiewicz et al., 1985).

Generation of reactive oxygen species was measured with dihy-drorhodamine 6G (Molecular Probes) as described by Dugan et al. (1995). A 10 μM solution of dihydrorhodamine 6G was prepared in Tyrode subjected to 30 min deoxygenation by sparging with N$_2$. Neurons were loaded for 30 min at room temperature, and rhoda-mine fluorescence was monitored on-line with 240 ms exposures; data were digitized with a Matrox LC board and analyzed off-line. Other dihydrorhodamine derivatives gave similar results; we found dihydrofluorescein derivatives difficult to use owing to rapid photo-conversion.

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