Neuron, Vol. 16, 89–101, January, 1996, Copyright ©1996 by Cell Press

# Signaling from Synapse to Nucleus: Postsynaptic CREB Phosphorylation during Multiple Forms of Hippocampal Synaptic Plasticity

**Karl Deisseroth,*† Haruhiko Bito,*† and Richard W. Tsien***
*Department of Molecular and Cellular Physiology
Beckman Center for Molecular and Genetic Medicine
Stanford University School of Medicine
Stanford, California 94305

## Summary

**Phosphorylation of the transcription factor CREB is thought to be important in processes underlying long-term memory. It is unclear whether CREB phosphorylation can carry information about the sign of changes in synaptic strength, whether CREB pathways are equally activated in neurons receiving or providing synaptic input, or how synapse-to-nucleus communication is mediated. We found that $Ca^{2+}$-dependent nuclear CREB phosphorylation was rapidly evoked by synaptic stimuli including, but not limited to, those that induced potentiation and depression of synaptic strength. In striking contrast, high frequency action potential firing alone failed to trigger CREB phosphorylation. Activation of a submembranous $Ca^{2+}$ sensor, just beneath sites of $Ca^{2+}$ entry, appears critical for triggering nuclear CREB phosphorylation via calmodulin and a $Ca^{2+}$/calmodulin-dependent protein kinase.**

## Introduction

The cAMP responsive element–binding protein (CREB) is an important transcription factor (Sheng and Greenberg, 1990; Brindle and Montminy, 1992; Lee and Masson, 1993; Lalli and Sassone-Corsi, 1994). When CREB is phosphorylated on Ser-133 by either cAMP- or $Ca^{2+}$-dependent protein kinases (Gonzalez and Montminy, 1989; Dash et al., 1991; Sheng et al., 1991), it becomes active in promoting transcription from the cAMP-responsive element (CRE) (Hunter and Karin, 1992; Chrivia et al., 1993). CREs lie upstream of a number of genes, including the immediate early genes (IEGs) *c-fos* (Sassone-Corsi et al., 1988) and *zif/268* (Sakamoto et al., 1991), and genes encoding synapsin I (Sauerwald et al., 1990), a $K^+$ channel (Mori et al., 1993), and the $\alpha$ subunit of $Ca^{2+}$/calmodulin-dependent kinase II (Olson et al., 1995). In hippocampal neurons, the expression of several of these genes is modified by neuronal activity (Morgan and Curran, 1989; Cole et al., 1989; Wisden et al., 1990; Mackler et al., 1992; Worley et al., 1993; Abraham et al., 1994; Thomas et al., 1994).

CREB has been implicated in the formation of long-term memory in three diverse animal species. First, in the mollusc Aplysia, long-term facilitation of synaptic strength is blocked by injection of CRE oligonucleotides into the presynaptic nucleus, although short-term changes do not appear to share this sensitivity (Dash et al., 1990; Kaang et al., 1993; Alberini et al., 1994). Second, in transgenic Drosophila, long-term memory disappears or is augmented upon induction of repressor or activator forms of CREB, while short-term memory appears to be intact (Tully et al., 1994; Yin et al., 1994, 1995). Third, mice in which CREB has been mutated are deficient in both the late phase of long-term potentiation (LTP) of hippocampal synaptic strength and long-term memory, while short-term changes in synaptic strength and short-term memory are largely spared (Bourtchuladze et al., 1994). It seems likely, therefore, that CREB plays a central and highly conserved role in the production of protein synthesis–dependent long-term changes in the brain (Davis and Squire, 1984; Frank and Greenberg, 1994; Stevens, 1994; Mayford et al., 1995a).

Genetic evidence for the importance of CREB in long-term memory far exceeds knowledge of the physiology of its activation. Progress on this front has been hampered by the difficulty of monitoring CREB activation following electrically stimulated synaptic activity or plasticity. Several fundamental questions remain open. Does CREB phosphorylation occur during hippocampal synaptic plasticity? Does it convey information about a particular type of synaptic strength change or more general information about synaptic activity? Are CREB pathways activated in neurons as receivers or as providers of synaptic input? What kinds of signaling molecules are involved? To address these questions, we have employed a combination of electrophysiology and immunocytochemistry to monitor and to manipulate biochemical events leading up to CREB phosphorylation. These approaches allowed us to study synapse-to-nucleus communication in single hippocampal neurons experiencing defined patterns of synaptic activity or spike firing.

## Results

### LTP-Inducing Synaptic Stimuli, but Not Action Potential Firing, Produce Nuclear CREB Phosphorylation

To monitor CREB phosphorylation at Ser-133, we used a polyclonal antibody that recognizes phosphorylated, but not dephosphorylated, recombinant CREB and that has been used to demonstrate activity dependence of nuclear CREB phosphorylation (Ginty et al., 1993; Ghosh et al., 1994). Figure 1A illustrates our immunoblots of nuclear and cytoplasmic fractions of hippocampal cultures (lanes 4–9), with recombinant CREB ($\alpha$ isoform) as a basis for comparison (lanes 1–3). Depolarization with 90 mM $K^+$ in the presence of $Ca^{2+}$ caused the appearance of clear immunoreactivity in the nuclear extract (lane 5), as in previous studies (Ginty et al., 1993). The immunoreactivity appeared in two bands, corresponding to phosphorylated $\alpha$ and $\delta$ isoforms of CREB (Ginty et al., 1993). In contrast, little immunoreactivity was seen in the cytoplasmic supernatant of stimulated neurons (lane 8) in either nuclear or cytoplasmic fractions from unstimulated neurons (lanes 4 and 7), or in extracts from neurons subjected to $K^+$-depolarization in the absence of extracellular $Ca^{2+}$ (lanes 6 and 9).

Having established our ability to detect nuclear CREB

† These authors contributed equally to this work.

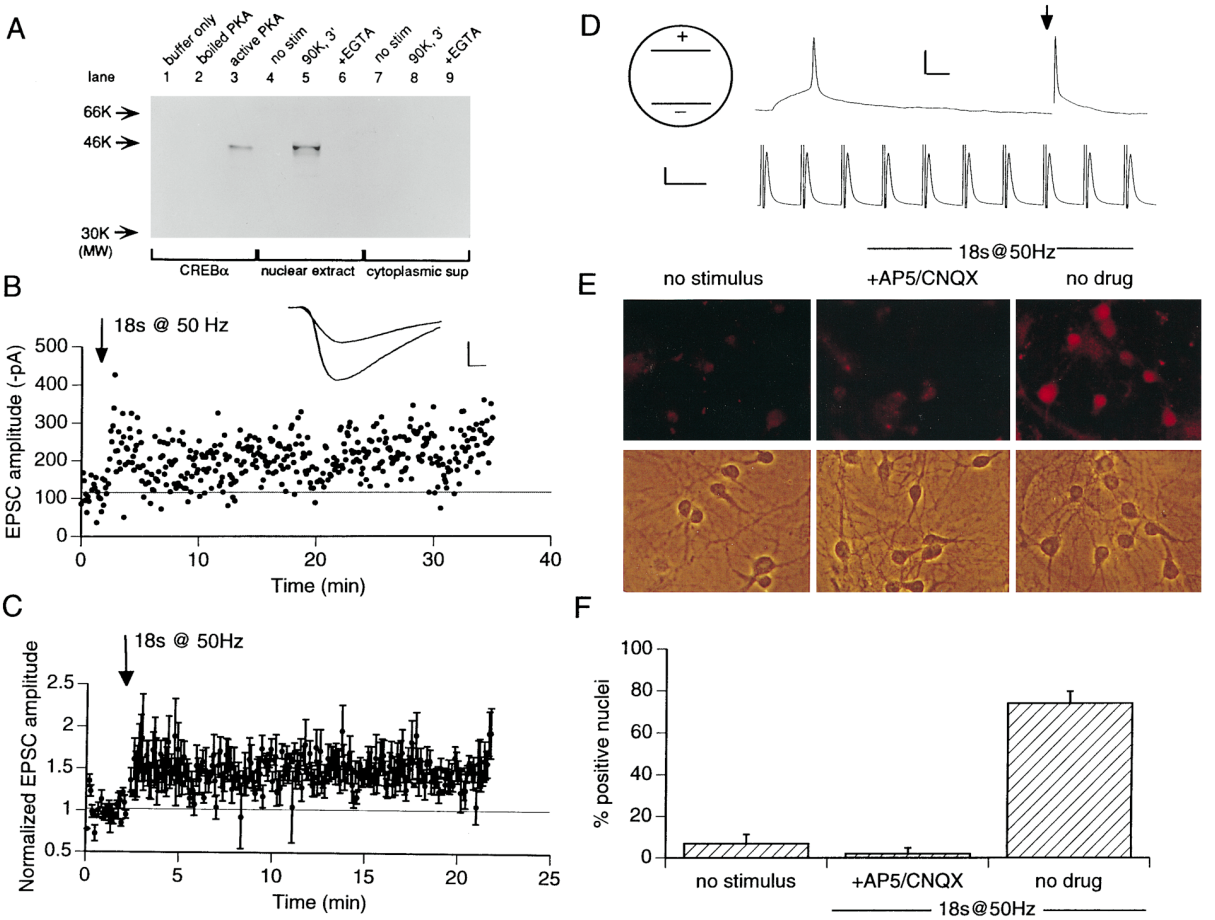


Figure 1. LTP-Inducing Synaptic Stimulation, but Not Spike Activity, Induces CREB Phosphorylation

(A) Western analysis to show specificity of pCREB immunoreactivity in hippocampal neurons. The increase in pCREB was induced by isotonic 90 mM $K^+$ depolarization and was prevented by removal of external $Ca^{2+}$ (+EGTA, 2.5 mM). The pCREB antibody detects phosphorylation of both $\alpha$ and $\delta$ isoforms of CREB (upper and lower bands in lane 5). Bands corresponding to expected sizes of CREM repressor isoforms and ATF1 were not detected.

(B) Stable potentiation of synaptic strength between pairs of cultured neurons could be obtained in a subset of experiments with a stimulus protocol (50 Hz pulses for 18 s) used to induce LTP in hippocampal slices. The postsynaptic cell was held in current clamp during the stimulus. Inset: averaged responses in control (smaller current) and after potentiation (larger inward current). Scale bars, 100 pA and 2 ms.

(C) Potentiation was observed in 4/5 experiments in which recording was conducted for more than 20 minutes (no change in synaptic strength in one case). Ensemble average from all five experiments. Potentiation was never seen in the presence of the NMDA receptor blocker D-AP5 (25 $\mu$M; 10/10 experiments).

(D) Field stimulation in culture. Two silver-chloride electrodes were positioned on opposite sides of the glass coverslip containing cultured neurons (inset). Simultaneous whole-cell recordings monitored stimulated neuronal responses in all experiments. Top trace, synaptically evoked action potential followed by a field-evoked action potential (marked by arrow, 1 ms constant-current pulse, stimulus artifact blanked for clarity in this trace only). Note that the two kinds of action potential were similar in amplitude and time course. Bottom trace, field stimulation–evoked action potentials (50 Hz) in the presence of D-AP5 (25 $\mu$M) and CNQX (10 $\mu$M). Scale bars, 40 mV and 20 ms.

(E) The same stimulus pattern as in (B), applied via field stimulation, triggered CREB phosphorylation. Upper panels, rhodamine immunofluorescence; lower panels, phase contrast; experimental conditions as indicated. CREB phosphorylation required synaptic input, since it was completely blocked by inhibiting glutamatergic synaptic transmission with 25 $\mu$M D-AP5 and 10 $\mu$M CNQX, while high frequency action potential firing was conserved as shown in (D).

(F) Compiled data (mean $\pm$ 1 SD) from experiments similar to those shown in (E) ($n = 5$).

phosphorylation, we proceeded to explore the range of physiological stimuli that trigger such phosphorylation. Synaptic stimulation of individual cultured neurons (18 s @ 50 Hz) produced a stable potentiation (Figures 1B and 1C) that appeared comparable to long-term potentiation recorded over a longer time scale in hippocampal slices. The degree of potentiation was similar to that previously found for LTP in hippocampal cultures (Bekkers and Stevens, 1990; Arancio et al., 1995). The same stimulus pattern was then applied to populations of cultured neurons with field electrodes (Figure 1D, inset). We first examined the effect of high frequency action potential firing in the absence of excitatory synaptic transmission by applying the LTP-inducing stimulus protocol in the presence of the glutamate receptor inhibitors D-2-amino-5-phosphonopentanoic acid (D-AP5) and 6-cyano-7-dinitroquinoxaline-2,3-dione (CNQX). This produced robust repetitive firing, monitored with

direct recording (Figure 1D, bottom). No detectable nuclear CREB phosphorylation was produced (Figures 1E and 1F, D-AP5/CNQX). However, when the same pattern of stimulation was applied in the absence of glutamate receptor blockers, it was highly effective in inducing robust CREB phosphorylation within 30 s (Figures 1E and 1F, no drug). Thus, CREB signaling in hippocampal neurons possesses the ability to distinguish between high frequency synaptic input and high frequency action potential firing. We show later that CREB phosphorylation occurs in postsynaptic neurons under conditions of stimulation of single presynaptic cells (see Figure 4), supporting results of field stimulation shown here. The contrast between effects of synaptic input and spike firing is particularly intriguing since both evoke rises in bulk cytoplasmic and nuclear $Ca^{2+}$ (see Figure 5).

### LTD in Cultured Hippocampal Neurons

It is well established that mild, low frequency (1–5 Hz) synaptic stimulation can give rise to a long-term depression (LTD) of synaptic strength in hippocampal neurons (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Bolshakov and Siegelbaum, 1994, Malenka, 1994; Stevens and Wang, 1994, 1995). However, little is known about the impact of such stimuli on nuclear events. We began by characterizing LTD of evoked transmission in cultured hippocampal neurons (see also Goda and Stevens, 1996). As illustrated in Figures 2A and 2B, sustained depression of transmission between pairs of neurons could be readily induced by 5 Hz stimulation for 3 min with the postsynaptic cell held in current clamp. In contrast, no sustained depression was seen when the postsynaptic cell was voltage-clamped at −70 mV during the 5 Hz stimulation (Figure 2C, open bar). However, when the 5 Hz stimulation pattern was later repeated in the same cell under current clamp (solid bar), long-lasting depression was observed. Thus, postsynaptic depolarization appears to be required to produce the LTD under these conditions.

To determine whether the depolarization acts by promoting $Ca^{2+}$ influx via voltage-gated $Ca^{2+}$ channels, we tested the effects of the specific L-type $Ca^{2+}$ channel blocker nimodipine (10 μM). LTD was prevented by nimodipine (Figures 2D and 2E) but was obtained in the same experiments upon washout of the drug (Figure 2D). The synaptic depression was attenuated but not prevented by the presence of 25 μM D-AP5 (Figure 2F), suggesting that the NMDA receptor contributed to, but is not absolutely required for, LTD. In all of these respects, the LTD we observed in cultured hippocampal neurons is similar to that evoked by the same stimulus paradigm in young hippocampal slices (Bolshakov and Siegelbaum, 1994).

### LTD-Inducing Stimulus Patterns Produce Postsynaptic Nuclear CREB Phosphorylation

As shown in Figure 1, the incidence of CREB phosphorylation depends on the kind of neuronal activity, occurring in response to synaptic input rather than to spike firing alone under the conditions of our experiments. The most obvious question is whether CREB phosphorylation might be even further specific, e.g., for long-lasting potentiation rather than depression, thus carrying information about the type of synaptic plasticity. Figures 3A and 3B summarize the effects of the synaptic stimulation for 180 s @ 5 Hz. The administration of this LTD-inducing synaptic stimulation was consistently effective in increasing the incidence of pCREB-positive nuclei, just as found for the LTP-inducing stimulation in Figure 1. As a control, direct recordings verified that the frequency of neuronal activity during the 5 Hz field stimulation was almost always set by field stimulation itself (Figure 3A). The near absence of secondary action potentials was expected, since synaptic inputs were generally subthreshold for firing action potentials under these culture conditions (Figure 4). The pCREB staining with 5 Hz stimulation was completely prevented by D-AP5 + CNQX (Figure 3B), as in the case of higher frequencies. Thus, CREB phosphorylation, though highly specific to synaptic activity, is not tied specifically to either positive or negative changes in synaptic strength.

Figure 3C shows the effects of varying the stimulation pattern over a wider range of frequencies with the overall stimulation period held constant at 3 min. Whereas little CREB phosphorylation was seen with 0.1 Hz stimulation, stimulation at 1 Hz and above induced robust nuclear pCREB staining. Thus, even mild synaptic inputs were sufficient to trigger events leading to nuclear signaling, in striking contrast to the ineffectiveness of high frequency action potential firing alone (see Figures 1E and 1F). It was interesting that the 10 Hz stimulation pattern produced a rise in CREB phosphorylation that was little different from that found with 5 Hz or 50 Hz (Figure 3C). Stimulation at this frequency failed to produce significant and sustained potentiation or depression of synaptic strength in our hippocampal cultures, as previously reported in rat hippocampal slices (Dudek and Bear, 1992).

We sought to identify the $Ca^{2+}$ influx pathways that supported the synaptic activation of CREB phosphorylation (Figure 3D). A combination of D-AP5 (to prevent $Ca^{2+}$ entry via NMDA receptor channels) and nimodipine (to block L-type $Ca^{2+}$ channels) strongly inhibited nuclear CREB staining. Considerable but less complete inhibition was also seen with D-AP5 alone. The same pattern of results was found in response to 50 Hz stimulation and 5 Hz stimulation. Thus, regardless of the stimulation frequency, NMDA receptor channels and L-type $Ca^{2+}$ channels work together during physiological synaptic activity to provide $Ca^{2+}$ influx to trigger CREB phosphorylation.

### Postsynaptic Action Potentials Are Not Required for Synaptically Induced CREB Phosphorylation

Although action potentials were insufficient by themselves to cause CREB phosphorylation (see Figure 1), it seemed possible that spiking might cooperate with synaptic input to trigger nuclear events. To test this possibility, the effects of synaptic activity were examined in the virtual absence of action potentials (Figures 4A and 4B). Intracellular stimulation of a single pyramidal neuron (stained blue) was carried out in lieu of the much

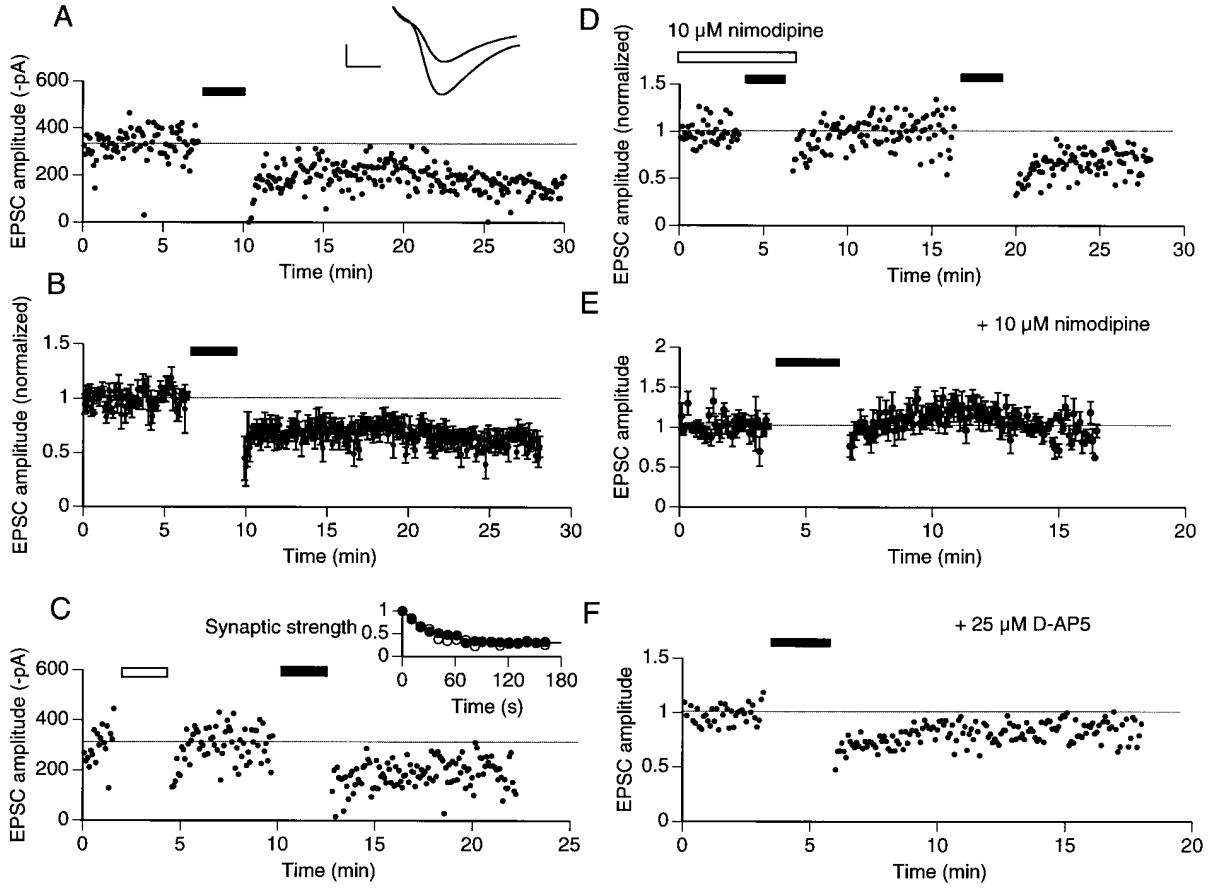


Figure 2. LTD in Hippocampal Culture

(A) Induction of LTD by 3 min of 5 Hz synaptic stimulation with the postsynaptic cell in current clamp (solid bar). Before and after this procedure, synaptic strength was monitored by recording EPSCs under voltage clamp (synaptic stimulation at 0.2 Hz). Inset, averaged responses before (larger trace) and after stabilization of long-term depression. Scale bars, 100 pA and 2 ms.

(B) Averaged results from 4 experiments, same protocol as illustrated in (A) (error bars represent ± SEM).

(C) Postsynaptic depolarization is required for induction of LTD. Synaptic stimulation at 5 Hz for 3 min, while postsynaptic cell was held under voltage clamp at −70 mV (open bar) or while the postsynaptic cell was allowed to depolarize under current clamp (solid bar). Inset: decay of synaptic strength during the 5 Hz stimulation is much the same whether monitored as the EPSC amplitude under voltage clamp (open symbols) or the EPSP amplitude under current clamp (closed symbols); both indices decline exponentially ($\tau$ = 32 s).

(D) Reversible block of LTD by the specific L-type $Ca^{2+}$ channel blocker nimodipine (10 μM; n = 2).

(E) Compiled data from experiments conducted in the presence of nimodipine (n = 7).

(F) Average of 5 experiments in the continuous presence of 25 μM D-AP5.

more general stimulation with extracellular field electrodes. As verified by intracellular recordings, this resulted in subthreshold synaptic potentials in almost all the cells neighboring the stimulated neuron. The evoked EPSPs ranged from 0.5–10 mV in amplitude but hardly ever led to action potentials (Figure 4A). We observed clear immunostaining for nuclear pCREB in cells near the intracellularly stimulated neuron (>90%, 6/6 experiments). Thus, we were able to infer that under these conditions postsynaptic spiking was not required for formation of pCREB. Together, these experiments indicate that synaptic activity in the postsynaptic neurons was not only necessary but also sufficient to trigger CREB phosphorylation, in contrast to action potential firing.

### The Site of $Ca^{2+}$ Action in Synapse-to-Nucleus Signaling

The next stage of this study was to identify the locus of $Ca^{2+}$ action in the communication from synapse to nucleus. Possible mechanisms can be divided into two major categories:

$$\text{synaptic activity} \rightarrow \uparrow\text{bulk cytoplasmic } Ca^{2+} \rightarrow \uparrow\text{nuclear } Ca^{2+} \rightarrow \uparrow\text{pCREB} \quad (1)$$

$$\text{synaptic activity} \rightarrow \uparrow\text{submembrane } Ca^{2+} \rightarrow \uparrow\text{downstream messenger} \rightarrow\rightarrow \uparrow\text{pCREB} \quad (2)$$

The first class of mechanisms (scheme 1) represents the simplest scenario. $Ca^{2+}$ entry generated by synaptic activity would cause free $Ca^{2+}$ to rise in the cytoplasm and, by diffusion, in the nucleus, where the $Ca^{2+}$ signal would lead to CREB phosphorylation. Alternatively, in another class of mechanisms (scheme 2), $Ca^{2+}$ would serve as a local messenger, activating a downstream signal transduction molecule near the plasma membrane, leading to further communication to the nucleus. To distinguish between these broad sets of possibilities, we undertook experiments to observe the intracellular $Ca^{2+}$ signals and to try to interfere with them.

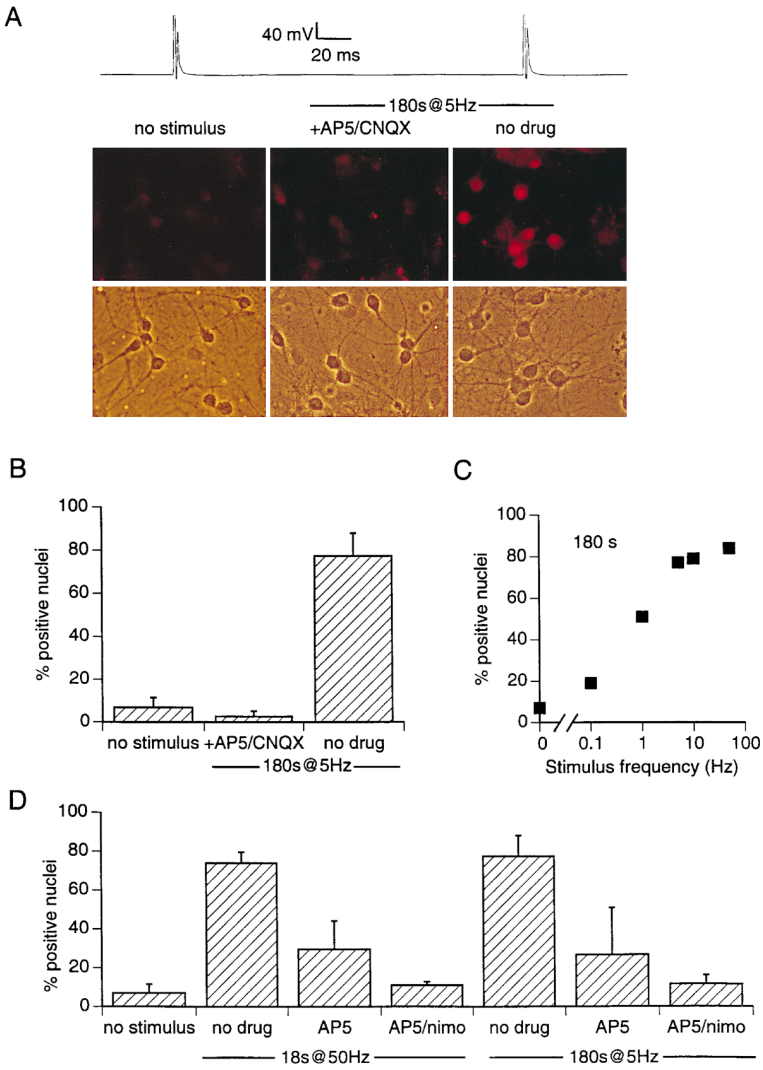


Figure 3. Effect of LTD-Inducing Stimuli on CREB Phosphorylation

(A) Upper trace, 5 Hz spike firing in the presence of D-AP5 (25 μM) and CNQX (10 μM), showing action potential characteristics similar to those in the absence of drugs. Representative fields of cells are shown; 5 Hz synaptic stimulation for 180 s induces nuclear CREB phosphorylation. Upper panels: fluorescent staining for CREB; lower panels: corresponding phase contrast views.

(B) Compiled results from 5 experiments like those illustrated in (A).

(C) Synaptic dose–response relationship for CREB phosphorylation. Field stimuli were presented at the indicated frequency (Hz) while the stimulus duration was held constant at 3 min.

(D) Synaptically induced CREB phosphorylation is largely blocked by 25 μM D-AP5 and completely blocked by the combination of D-AP5 and 10 μM nimodipine. Results were similar for 5 Hz and 50 Hz stimulation (n = 5–6).

The spatial pattern of evoked $Ca^{2+}$ transients was studied with fluo-3 $Ca^{2+}$ imaging on a confocal microscope. Field stimulation reliably induced increases in bulk cytoplasmic $Ca^{2+}$, which extended to the nucleus (Figure 5A). However, similar rises in bulk cytoplasmic and nuclear $Ca^{2+}$ were reliably observed even in the presence of glutamate receptor antagonists (Figure 5B), conditions in which CREB phosphorylation was completely blocked (Figures 1 and 3). These data suggest that, in contradiction to scheme 1, elevation of $Ca^{2+}$ within the nucleus may not be sufficient to determine whether or not CREB phosphorylation takes place.

Is a rise in nuclear $Ca^{2+}$ necessary at all? To test this possibility, we asked whether nuclear CREB phosphorylation could be suppressed by an intracellular chelator. Fluo-3 imaging showed that EGTA was capable of eliminating the bulk $Ca^{2+}$ transient in both cytoplasmic and nuclear regions, when delivered by incubation of neurons with its cell-permeant acetoxymethylester derivative, EGTA-AM (Figure 5C). But EGTA-AM was not able to prevent CREB phosphorylation, even at a higher concentration (Figures 6A–6C). A similar result was also obtained when 10 mM EGTA was included in the whole-cell pipette solution (Figures 6B and 6C).

The results with EGTA may be compared with those obtained using the chemically related $Ca^{2+}$ chelators BAPTA and $Br_2$-BAPTA (Tsien, 1980). Calculations based on the known properties of these buffers predict very different effects on the spatial decay of free [$Ca^{2+}$] near a $Ca^{2+}$ entry site (Figure 6D) (see also Neher, 1986; Stern, 1992; Roberts, 1993). In contrast to EGTA, BAPTA completely abolished CREB phosphorylation, whether it was applied as the AM ester (Figure 6A) or delivered through the patch pipette (Figure 6B). The difference in susceptibility to BAPTA and EGTA is characteristic of a process driven by a rise in submembrane $Ca^{2+}$, without the need for a global $Ca^{2+}$ increase (Neher, 1986; Adler et al., 1991; Swandulla et al., 1991; Roberts, 1993). Additional information was provided by experiments using the low affinity buffer $Br_2$-BAPTA. Even at high concentrations, $Br_2$-BAPTA failed to block CREB phosphorylation (Figures 6A–6C), like EGTA, but unlike BAPTA itself. This rules out a nonspecific side effect of this class of compounds.

The combination of results with the three buffer compounds has not been seen before in other systems, so it is particularly interesting to interpret the data in quantitative terms. Figure 6D shows calculated profiles

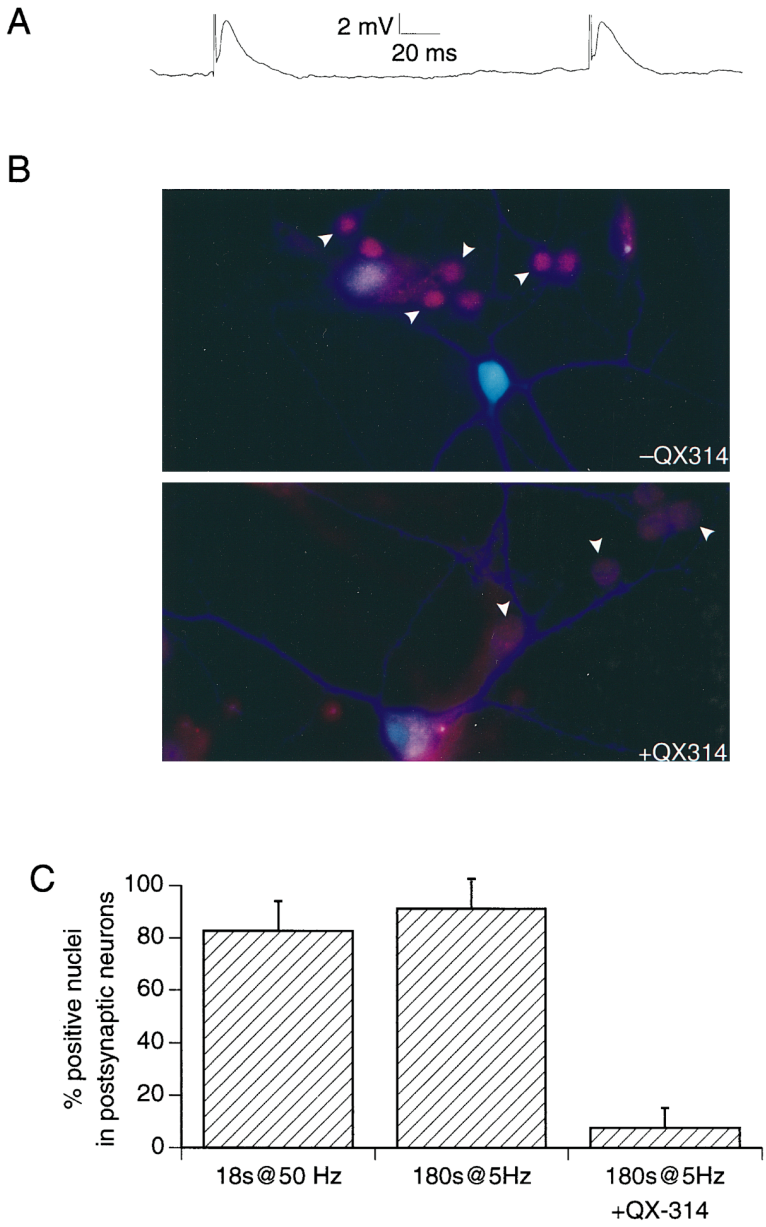


Figure 4. Action Potential Firing Is Not Required for Synaptically Induced CREB Phosphorylation

(A) Excitatory postsynaptic potentials recorded during 5 Hz stimulation of a single presynaptic neuron, showing the typical response characteristics of postsynaptic neurons under these culture conditions, in experiments that paralleled those depicted in (B).

(B) Top panel: postsynaptic CREB phosphorylation driven by stimulation of an individual presynaptic neuron (cell body and processes filled with Neurobiotin stained with Cascade blue dye). Resulting nuclear pCREB staining (red staining of nuclei) can be seen in most of the surrounding cells. Likewise, single neurons stimulated with a bipolar electrode to fire action potentials at 5 Hz for 3 min failed to display a nuclear pCREB signal (data not shown). Bottom panel: inclusion in the patch pipette of 5 mM QX314, an intracellularly effective $Na^+$ channel blocker, completely prevented the rise in pCREB, indicating that spike propagation in the stimulated cell is necessary to activate CREB phosphorylation in the surrounding cells.

(C) Consistency of elevation of nuclear pCREB induced by 5 Hz synaptic stimulation of a single presynaptic cell. Ordinate plots fraction of cells within the zone defined by the processes of the stimulated neuron that displays pCREB-positive nuclei. Error bars represent ± 1 SD.

for free $Ca^{2+}$ as a function of distance from the site of $Ca^{2+}$ influx, based on work of Stern (1992). The calculations take into account the known $Ca^{2+}$-buffering properties of EGTA, BAPTA, and $Br_2$-BAPTA (each at 10 mM bulk concentration); diffusion coefficients for the buffers and for $Ca^{2+}$ are set at 200 $\mu m^2/s$ (Neher, 1986; Roberts, 1993). Because BAPTA and EGTA have similarly high affinity for $Ca^{2+}$ ($K_d \approx 160$ nM), they buffer steady-state $Ca^{2+}$ to the same low level at distances >1.5 $\mu$m from the site of influx. But because EGTA has a much slower on-rate for binding $Ca^{2+}$ compared with BAPTA ($10^6$ versus $10^8$ $M^{-1}s^{-1}$), EGTA is far less effective in reducing $[Ca^{2+}]$ very close to the influx channel. $Br_2$-BAPTA binds $Ca^{2+}$ with the same rapid on-rate as BAPTA and is thus equieffective at close distances, but further away from the influx pathway, its much lower equilibrium affinity ($K_d \approx 3.6$ $\mu$M) results in a higher $[Ca^{2+}]$ in steady state. Thus, the buffers will each produce a different profile of free $Ca^{2+}$ concentration.

The shaded sector in Figure 6D is bounded by the calculated profiles for EGTA, BAPTA, and $Br_2$-BAPTA. It defines ranges of free $Ca^{2+}$ concentration and distances away from the site of $Ca^{2+}$ influx, consistent with the experimental findings, that would be necessary for downstream signaling. The pattern of responses narrows down the spatial and concentration requirements of the putative $Ca^{2+}$ sensor to micromolar $[Ca^{2+}]$ levels within 1–2 $\mu$m of the $Ca^{2+}$ entry site.

### A Test for the Involvement of Intracellular $Ca^{2+}$ Stores

The incoming $Ca^{2+}$ might act on intracellular $Ca^{2+}$ stores immediately below the surface membrane to trigger propagation of a $Ca^{2+}$ wave spreading to the nucleus,

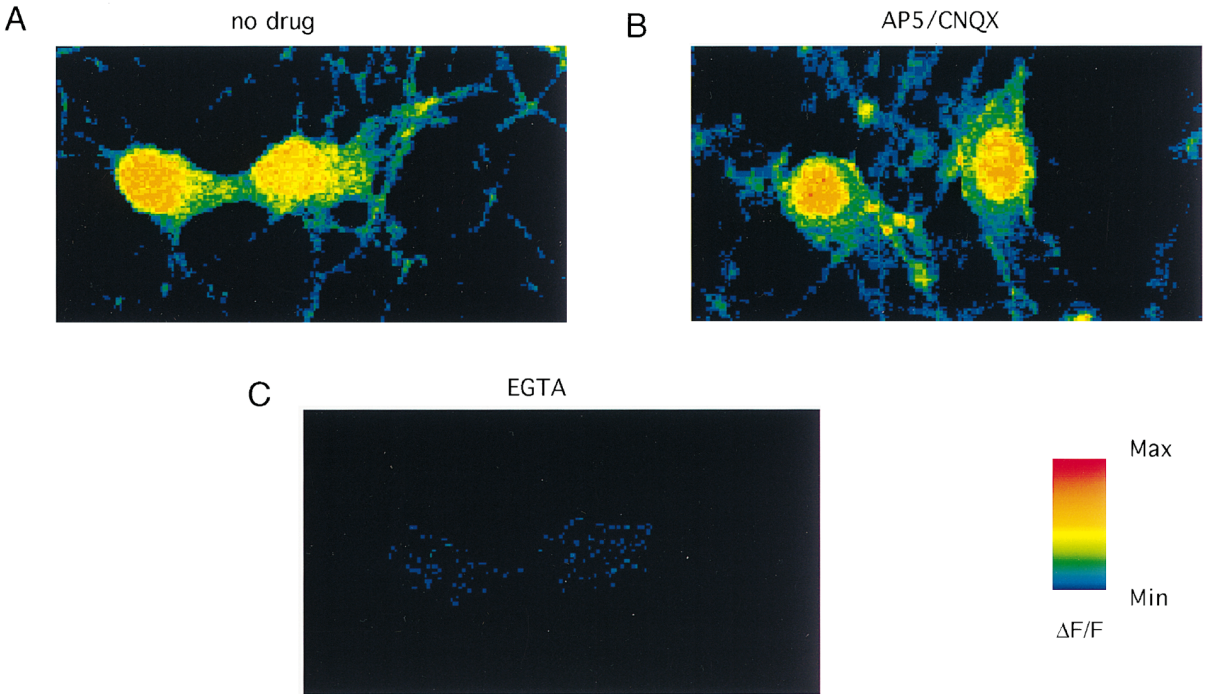


Figure 5. $Ca^{2+}$ Imaging during Action Potential Firing and Synaptic Activity

(A) Changes in fluo-3 fluorescence observed with a confocal microscope. Response of cultured hippocampal neurons to field stimulation in the absence of exogenous buffer or glutamate receptor blockers.

(B) Change in fluorescence in response to field stimulation observed in the presence of the glutamate receptor blockers D-AP5 (25 μM) and CNQX (10 μM).

(C) Change in fluorescence in response to field stimulation observed after loading the dish of cells in (A) with EGTA-AM (1 mM). The same field of cells as in (A) is shown.

as discussed by Berridge (1993). However, we observed no cytoplasmic $Ca^{2+}$ waves with fluo-3 imaging (data not shown). Furthermore, we found that prior depletion of intracellular $Ca^{2+}$ stores by pretreatment with thapsigargin, a potent microsomal $Ca^{2+}$-ATPase inhibitor at the 1 μM dose used (Irving et al., 1992; Ebihara and Akaike, 1993), neither prevented CREB phosphorylation nor altered the inability of EGTA or $Br_2$-BAPTA to block CREB phosphorylation. This was the case whether the CREB phosphorylation was induced by synaptic stimulation or steady depolarization (data not shown).

### Required Downstream Effectors: Calmodulin and a $Ca^{2+}$/CaM Kinase, but Not PKA

Our data indicated that a rise in nuclear $Ca^{2+}$ was neither necessary nor sufficient to induce CREB phosphorylation, in contradiction to scheme 1. This led us to consider various versions of scheme 2. Calmodulin must be considered as a candidate for a downstream effector. With a $K_d$ for $Ca^{2+}$ of ~1 μM, its participation would be consistent with the lack of block of CREB phosphorylation by $Br_2$-BAPTA ($K_d$ for $Ca^{2+}$ ≈ 3.6 μM). We found that the inclusion of a calmodulin antagonist peptide [CaMKII (290–309), 25 μM] in the patch pipette inhibited depolarization-induced CREB phosphorylation (4/5 neurons, Figure 7C). In addition, application of calmidazolium, a membrane-permeable calmodulin inhibitor, completely blocked CREB phosphorylation in response to either LTP- or LTD-inducing synaptic stimulation (Figure 7A). Calmodulin may be the submembrane $Ca^{2+}$ receptor; alternatively, $Ca^{2+}$ might be transferred from some other $Ca^{2+}$-binding molecule to calmodulin.

The binding of $Ca^{2+}$ by calmodulin could lead to phosphorylation of CREB at Ser-133 in various ways. $Ca^{2+}$/calmodulin can activate protein kinases directly (Dash et al., 1991; Sheng et al., 1991; Enslen et al., 1994; Matthews et al.,1994; Sun et al., 1994). It may also act indirectly, through $Ca^{2+}$/calmodulin-dependent adenylyl cyclases (Choi et al., 1993), to raise cAMP and to activate cAMP-dependent protein kinase (PKA) (Frank and Greenberg, 1994; Mayford et al., 1995a). Thus, we considered the scheme:

$$\text{synaptic activity} \rightarrow \uparrow \text{submembrane } Ca^{2+}/\text{CaM} \rightarrow \uparrow \text{cAMP} \rightarrow \uparrow \text{PKA} \rightarrow \uparrow \text{pCREB} \quad (2a)$$

In fact, inclusion of a protein kinase inhibitor (PKI) peptide (25 μM) in the patch pipette failed to block CREB phosphorylation (Figure 7C). Furthermore, two structurally distinct PKA inhibitors, KT5720 (2 μM) and Rp-cAMPS (50 μM), had no significant effect on nuclear CREB phosphorylation in response to either LTD- or LTP-inducing synaptic stimuli (Figure 7A). Control experiments showed that the PKA inhibitors were able to inhibit forskolin-induced CREB phosphorylation. In contrast, KN-62 (10 μM), a $Ca^{2+}$/calmodulin-dependent kinase inhibitor, blocked CREB phosphorylation in response to either LTD- or LTP-inducing stimuli (Figure 7B), without changing the activity of voltage-gated $Ca^{2+}$

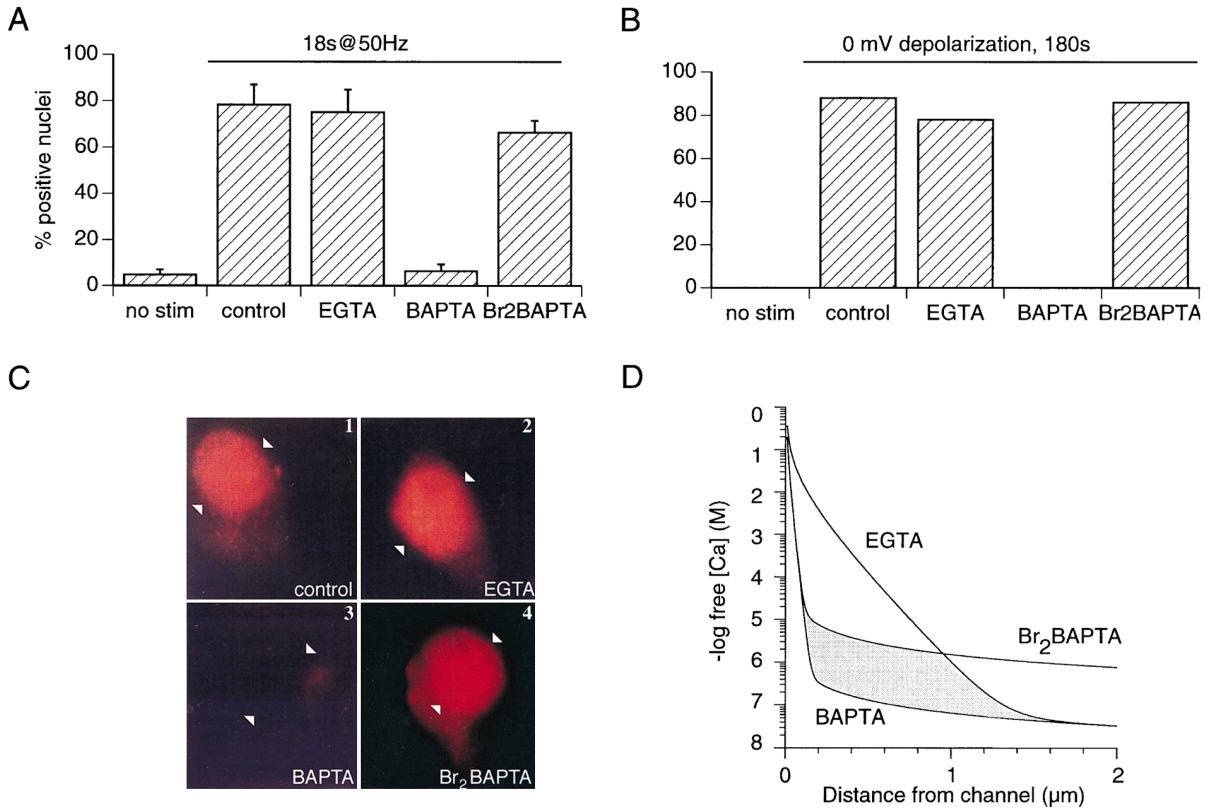


Figure 6. Submembranous $Ca^{2+}$ Mediates Signaling from Synapse to Nucleus

(A) Elevation of nuclear pCREB is prevented by incubation with BAPTA-AM (1 mM), but not by loading with EGTA-AM (3 mM) or $Br_2$-BAPTA-AM (1 mM). Mean ± SD (n = 5–6).

(B and C) Elevation of nuclear pCREB induced in single hippocampal pyramidal neurons by 3 min voltage-clamp depolarization to 0 mV. Cells were fixed immediately after the depolarizing stimulus. Nuclear pCREB elevation was seen despite inclusion in the patch pipette of 10 mM EGTA (7/9 experiments) or 10 mM $Br_2$-BAPTA (5/5 experiments), but not with 10 mM BAPTA (0/6 experiments).

(D) Profiles of free cytoplasmic $Ca^{2+}$ expected in the steady state, plotted as a function of distance from the site of $Ca^{2+}$ influx, in the presence of the three different $Ca^{2+}$ buffers.

channels. These results support the idea that the nuclear CREB phosphorylation involves a $Ca^{2+}$/calmodulin-dependent protein kinase rather than a cAMP/PKA pathway.

$$\text{synaptic activity} \rightarrow \uparrow\text{submembrane } Ca^{2+}/\text{CaM} \rightarrow \uparrow Ca^{2+}/\text{CaM kinase activity} \rightarrow \uparrow\text{pCREB} \quad (2b)$$

## Discussion

### Ability of CREB Phosphorylation to Respond Differently to Synaptic Activity and Rapid Spiking

Phosphorylation of CREB in hippocampal neurons can be readily evoked by synaptic activity but not by rapid action potential firing. One explanation that we considered was that action potentials and synaptic inputs raise global $Ca^{2+}$ to different extents or over different time spans. This cannot be ruled out completely, although the fluo-3 imaging showed that the two types of input resulted in comparable global increases in $Ca^{2+}$. A more likely explanation for the ability to discriminate between spiking or synaptic activity invokes differences in the spatial location of the $Ca^{2+}$ entry. If the consequences of $Ca^{2+}$ entry are local rather than global, as we argue below, the positioning of $Ca^{2+}$ influx pathways relative to $Ca^{2+}$ sensors could be important.

Whatever the explanation, the implication of the data is that alterations in pCREB may arise specifically in neurons acting as receivers of synaptic input rather than as providers of such stimulation. This postsynaptic specificity in hippocampal neurons may be compared with observations at Aplysia sensorimotor synapses, where signaling to the CRE in the presynaptic nucleus may be functionally important for long-term synaptic facilitation (Dash et al., 1990; Kaang et al., 1993). It remains unclear whether there is a fundamental difference between these systems since the specificity of responses to various types of activity (spiking versus synaptic, pre- versus postsynaptic) might not be absolute. Action potentials might be sufficient to cause pCREB elevation in hippocampal neurons if the spike were substantially broadened or voltage-gated $Ca^{2+}$ channels were strongly up-modulated.

### Similar Responsiveness to LTP- and LTD-Inducing Stimulus Patterns

In hippocampal slices from CREB knockout mice, Bourtchuladze et al. (1994) found that early phases of LTP

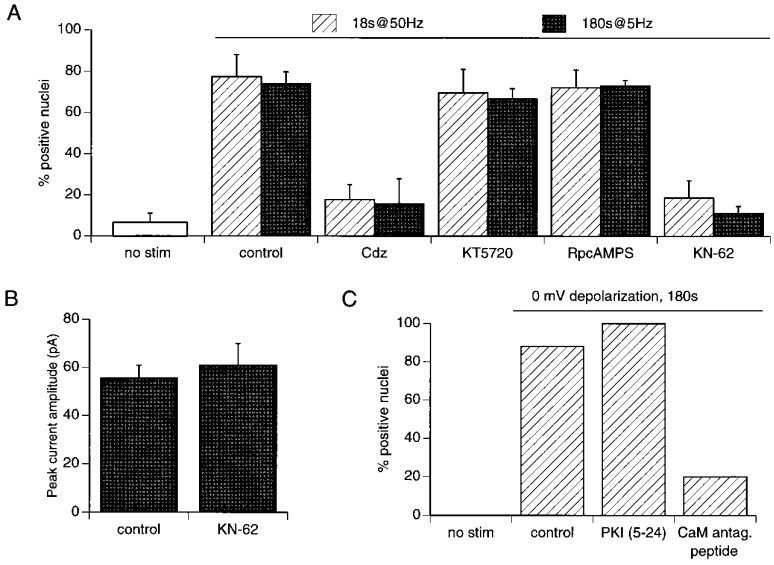


Figure 7. Involvement of Calmodulin and a $Ca^{2+}$/CaM Kinase

(A) Determinants of CREB phosphorylation in synaptically stimulated hippocampal neurons. Nuclear pCREB staining evoked by either LTD-inducing stimulation (180 s @ 5 Hz) or LTP-inducing stimulation (18 s @ 50 Hz) was blocked by calmidazolium (Cdz, 20 μM) or by KN-62 (10 μM), but not by Rp-cAMPS (50 μM) or by KT5720 (2 μM).

(B) Amplitude of voltage-activated $Ca^{2+}$ currents is unaffected by incubation with KN-62 under the conditions of (A). $Ca^{2+}$ currents were evoked by a voltage step from −45 mV to −30 mV; n = 5 for control, n = 6 for KN-62 (see also Wyllie and Nicoll, 1994).

(C) Nuclear CREB phosphorylation induced by steady depolarization of a single neuron was not significantly altered by inclusion in the pipette of PKI(5–24), a PKA inhibitory peptide (5/5 experiments), but was inhibited by a calmodulin antagonist peptide, CaMKII(290–309) (4/5 experiments).

(<30 min) were reduced and that late phases (>2 hr) of LTP were largely eliminated, implicating CREB in hippocampal synaptic plasticity. Our experiments provide a way to test certain hypotheses for the mechanism of CREB action. We found that the cultured hippocampal neurons were capable of exhibiting either LTP or LTD; the sign of the change in synaptic strength could be altered simply by varying the frequency of synaptic stimulation from 50 Hz to 5 Hz. CREB phosphorylation at Ser-133 increased rapidly in response to stimuli at either frequency, and even at intermediate frequencies (10 Hz) that produced no lasting change in synaptic strength (see Figure 3). Our results provide evidence that LTP-inducing stimuli can activate the CREB pathway, but they do not support a scenario whereby this activation is specific to LTP. Thus, the sign of the enduring change in synaptic strength is not likely to be signaled by the CREB phosphorylation event per se.

Other nuclear events might show such a discrimination. Possible points of regulation include other phosphorylation sites on CREB (e.g., Ser-142), where phosphorylation inhibits the activity of Ser-133-phosphorylated CREB (Sun et al., 1994), or Ser-129, where phosphorylation has been found to modulate positively the net effect of Ser-133 phosphorylation (Fiol et al., 1994). Furthermore, additional transcription factors may well be engaged, some capable of forming heterodimers with CREB itself. Finally, information about the sign of the change in synaptic strength might not involve nuclear signaling at all, but might be stored at the synapse itself. One can imagine that common mechanisms may have developed to deal with issues of sign specificity along with location specificity (Silva and Giese, 1994; Lisman, 1995).

### Novel Characteristics of $Ca^{2+}$ Signaling

Because the nucleus represents a target within the interior of the cell, not at its surface, we had initially expected signaling from synapse to nucleus in neurons to behave like signaling from surface membrane to contractile machinery in muscle cells. It came as a surprise to find that phosphorylation of CREB could not be blocked with internal EGTA (see Figure 6), which is very effective at eliminating the myoplasmic $Ca^{2+}$ transient and contraction of muscle cells (e.g., Siegelbaum and Tsien, 1980). But fluo-3 imaging verified that internal EGTA completely suppressed the bulk $Ca^{2+}$ rise (see Figure 5). Furthermore, there was no question of the involvement of intracellular $Ca^{2+}$, given the effectiveness of BAPTA in blocking the rise in pCREB. Thus, we were led to the conclusion that the critical $Ca^{2+}$ signal must be transmitted to a downstream effector within ~1–2 μm of sites of $Ca^{2+}$ entry (see Figure 6D).

The pattern of sensitivity to BAPTA but not EGTA was reminiscent of findings in other systems where the effector is a protein on the surface membrane or closely linked to it. Some well-known mechanisms are $Ca^{2+}$ activation of exocytosis (Adler et al., 1991) and $Ca^{2+}$ inactivation of $Ca^{2+}$ channels (Imredy and Yue, 1992). A recent study focused on the $Ca^{2+}$ requirements of $Ca^{2+}$-activated $K^+$ channels (Roberts, 1993). Their activation by $Ca^{2+}$ entry was prevented by $Br_2$-BAPTA as well as BAPTA (although not by EGTA). The block by $Br_2$-BAPTA in this system made sense because of its rapid on-rate for $Ca^{2+}$ interaction. The lower $Ca^{2+}$ affinity of this buffer did not hamper its effectiveness because $K^+$ channels interact even more weakly with $Ca^{2+}$ ($EC_{50}$ > 10 μM; Roberts et al., 1990). In contrast to the results in $Ca^{2+}$-activated $K^+$ channels, we found that $Br_2$-BAPTA did not prevent CREB phosphorylation. We infer that the putative $Ca^{2+}$ receptor must possess moderately high $Ca^{2+}$ affinity ($K_d$ < 10 μM), consistent with the apparent sensitivity of CREB phosphorylation to synaptic input.

What might the effector be? One intriguing possibility, proposed by Berridge (1993) for cerebellar Purkinje cells, is that $Ca^{2+}$ entry might trigger a propagating wave of $Ca^{2+}$ release from an internal membrane system, extending from sites of $Ca^{2+}$ entry on dendritic spines toward the nucleus. However, thapsigargin did not inhibit CREB phosphorylation, nor did it alter the pattern of effectiveness of BAPTA, EGTA, and $Br_2$BAPTA. Thus, it

seems unlikely that hippocampal neurons rely on internal $Ca^{2+}$ waves to transfer signals from synapse to nucleus (see Huang et al., 1994a, for comparable results in skeletal muscle cells). It would be interesting to carry out similar tests in cerebellar Purkinje neurons, where the internal membrane system is particularly well developed.

### Downstream Effectors in the CREB Signaling Pathway

Calmodulin is an attractive candidate for the submembranous $Ca^{2+}$ sensor for several reasons. First, the $Ca^{2+}$-sensitivity of calmodulin is in the right range. Second, immunostaining with an anti-CaM antibody reveals that calmodulin is particularly abundant at synaptic locations (Caceres et al., 1983; K.D. et al., unpublished data), in good position to receive incoming $Ca^{2+}$ signals. Third, the phosphorylation of CREB is prevented by various inhibitors of calmodulin, either calmidazolium or a CaM inhibitory peptide. The evidence seems consistent so far with the idea that calmodulin acts as both a mobile $Ca^{2+}$ buffer and $Ca^{2+}$ sensor. We imagine that, like other mobile buffers (Stern, 1992; Roberts, 1993), calmodulin will diffuse toward sites of $Ca^{2+}$ entry to compensate for depletion of $Ca^{2+}$-free CaM. Since the $Ca^{2+}$ affinity of CaM is comparable to that of other diffusible buffers, it must see a substantial fraction of the total $Ca^{2+}$ influx. Whether the initial $Ca^{2+}$ sensor is calmodulin or another $Ca^{2+}$ binding protein of moderately high affinity, the operation of a local effector might provide an explanation for the relative specificity of CREB phosphorylation for certain types of activity. This presumes spatial heterogeneities in either the local effector concentration or the rise in $Ca^{2+}$, or both.

Based on studies of CREB signaling in other systems, it has been proposed that PKA could be responsible for Ser-133 phosphorylation of CREB in response to hippocampal synaptic activity (Frank and Greenberg, 1994; Mayford et al., 1995a). Interest in a PKA pathway in mammals has been heightened by the apparent importance of cAMP and PKA in the stabilization of long-term changes in synaptic strength (Frey et al., 1993; Huang et al., 1994b). Since PKA is capable of translocating to the nucleus (Hagiwara et al., 1994) and of phosphorylating CREB at Ser-133 (Gonzalez and Montminy, 1989), rises in $Ca^{2+}$ could cause CREB phosphorylation through $Ca^{2+}$-activated adenylyl cyclase (Xia et al., 1991; Cali et al., 1994) and increases in cAMP and PKA activity. However, other kinases such as $Ca^{2+}$/CaM-dependent kinases I, II, and IV are also activated by $Ca^{2+}$/CaM and can phosphorylate CREB on Ser-133 (Dash et al., 1991; Sheng et al., 1991; Enslen et al., 1994; Matthews et al., 1994; Sun et al., 1994). Our experiments with kinase inhibitors represent the first direct tests of the PKA hypothesis in the context of signaling from synapse to nucleus (see also Thompson et al., 1995, for parallel work in PC-12 cells). We found that the kinase activity required for CREB phosphorylation displays a profile consistent with a $Ca^{2+}$/CaM-dependent kinase, rather than PKA (see Figure 7). The involvement of PKA in long-term changes in the brain might entail regulation of other transcription factors or modulation of effects downstream of CREB phosphorylation.

In light of this data, it is intriguing that in neurons the signaling mechanisms controlling pCREB appear to overlap substantially with those required for induction of both forms of synaptic plasticity (LTP and LTD). Both sets of phenomena involve NMDA receptors (Figures 1–3) (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Goda and Stevens, 1996; but see also Bolshakov and Siegelbaum, 1994), postsynaptic $Ca^{2+}$ (Lynch et al., 1983; Malenka et al., 1992; Mulkey and Malenka, 1992), and a $Ca^{2+}$/CaM-dependent kinase (e.g., Malinow et al., 1989; Silva et al., 1992; Pettit et al., 1994; Stevens et al., 1994; Mayford et al., 1995b). Thus, signaling pathways for CREB signaling and synaptic plasticity might share common steps, even though threshold requirements for activation may be quite different (see Figure 3C).

### Functional Consequences of CREB Activation

It is interesting that the pathway leading to CREB phosphorylation is comparatively sensitive to synaptic activity. If basal frequencies of synaptic activity were substantial (e.g., 5–10 Hz), CREB phosphorylation as such might be hampered in conveying information about further increases in synaptic activity. This sort of biochemical saturation may be avoided because of the low basal activity of the pyramidal cells in the hippocampus (Wilson and McNaughton, 1993). A relatively sensitive trigger for initiation of long-term changes might be useful as part of a stabilizing feedback regulatory mechanism, altering properties of neuronal signal processing to keep them in tune with the ambient level of synaptic input (Bear, 1995; Deisseroth et al., 1995). The ease of engaging the pathway leading to CREB phosphorylation also underscores the importance of understanding dephosphorylation mechanisms, a topic that will be addressed elsewhere (H. B. et al., unpublished data).

Interest in how CREB phosphorylation is regulated in hippocampal neurons is enhanced by our knowledge of the many genes that may depend on it, but the key players and the consequences of their activation are not yet known. A logical direction for future work is to identify CREB-regulated genes in neurons undergoing different patterns of synaptic activity and to sort out biochemical and functional changes resulting from the altered gene expression. Fundamental questions would have to be addressed along the way. Can the effects of CREB be synapse specific or is their impact always global? How many synapses are needed to trigger nuclear CREB phosphorylation? How do synapses cooperate to signal the nucleus? Does dendritic processing influence the decision to trigger CREB phosphorylation? What signaling processes work in parallel with or downstream of the CaM kinase pathway? In seeking answers to these questions, the accessibility of cultured neurons may prove to be of advantage for monitoring synaptic strength, $Ca^{2+}$ signaling, and biochemical changes with appropriate spatiotemporal resolution.

## Experimental Procedures

### Electrophysiology

Large pyramidal cells morphologically similar to CA3/CA1 pyramidal neurons were selected for recording. Whole-cell recordings were

obtained with an Axopatch 1D amplifier (Axon Instruments); the current signal was filtered at 2 kHz, digitized at 10 kHz, and analyzed with programs written in Axobasic (Axon Instruments). Changes in synaptic strength were monitored by measuring monosynaptically evoked excitatory postsynaptic currents (EPSCs), while a single presynaptic neuron was stimulated with a bipolar stimulating electrode made from theta-glass (Clark Electromedical Instruments, UK). Neuron pairs with a response latency greater than 5 ms or with significant polysynaptic connectivity were not studied further. Input resistance and series resistance were continuously monitored. The chamber was perfused at 1.5 ml/min with Tyrode solution containing 129 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM glucose, 25 mM HEPES, 10 μM glycine, and 50 μM picrotoxin (pH 7.3 and osmolarity 313 ± 2 mOsm). Whole-cell patch electrodes (3–8 MΩ resistance) were filled with a solution containing 110 mM KCl, 5 mM $MgCl_2$, 10 mM NaCl, 0.6 mM EGTA, 2 mM ATP, 0.2 mM GTP, and 40 mM HEPES (pH 7.2, 295 ± 2 mOsm), unless otherwise noted. Chemicals were obtained from Fluka (glucose, $CaCl_2$, NaCl, EGTA), Calbiochem (HEPES), Sigma (KCl, $MgCl_2$, picrotoxin, glycine) and Boehringer Mannheim (ATP, GTP). Cells were preincubated for 2–3 hr before stimulation in Tyrode containing 1 μM tetrodotoxin. For patched-cell labeling, the whole-cell pipette solution contained a biotin derivative (Neurobiotin, Vector Laboratories) and in some cases 5 mM QX314 (Alomone Laboratories). Patch clamped neurons were unequivocally identified by staining post hoc with 1:100 Cascade blue–conjugated avidin (Molecular Probes). Field stimulation 1 ms constant current pulses of 5 mA were applied from an Iso-Flex stimulus isolator (A. M. P. I.).

### Cell Culture and Immunocytochemistry

CA3/CA1 hippocampal neurons were cultured by a procedure modified from that previously described (Malgaroli and Tsien, 1992). Dissociated cells from the CA3/CA1 regions of the hippocampi of 2- to 4-day-old Sprague–Dawley rats were maintained in culture for 8–17 days on a Matrigel substrate (1:100 dilution, Collaborative Research) in medium supplemented with B-27 (GIBCO BRL/Life Technologies). Proliferation of nonneuronal cells was prevented by the addition of 2–4 μM cytosine β-D-arabinofuranoside (Sigma) from the second day in culture onward. Functional synapses appear by 8 days in vitro.

Staining materials were as follows: anti-pCREB polyclonal antibody (Upstate Biotechnology Inc., 1:500–1:1000); rhodamine-conjugated goat anti-rabbit antibody (Jackson Immunoresearch, 1:200). Cells were permeabilized with saponin, and antibody incubations were conducted at 4°C overnight. Coverslips were protected from quenching using Citifluor (Citifluor UK Chemical Lab, Canterbury, UK), mounted onto a glass slide and visualized using a Carl Zeiss Axiophot epifluorescence microscope. The nuclear pCREB immunoreactivity in immunocytochemical staining was strongly inhibited by preincubation of the primary antibody reaction with excess amount of phosphoCREB. Nuclear localization of pCREB and CREB immunoreactivity was verified by DAPI costaining or confocal microscopy (data not shown). Control experiments demonstrated that the nuclear immunoreactivity disappeared upon preincubation of the antibody with an excess amount of recombinant pCREB.

For the Western blot, partially purified recombinant human CREBα (Santa Cruz Biotechnology) was incubated at 30°C for 30 min with buffer only, boiled PKA catalytic subunit, or active PKA catalytic subunit (Sigma) to specifically phosphorylate Ser-133 (Gonzalez and Montminy, 1989), and the reaction was stopped by boiling (lanes 1–3). Nuclear extracts and cytoplasmic supernatants (20 μg protein) from E18 hippocampal neurons (11–12 days in vitro) were loaded in lanes 4–9. Immunodetection was carried out using an anti-pCREB polyclonal antibody (1:1000) and was visualized with the enhanced chemiluminescence detection system coupled with a biotin–streptavidin amplification step (Amersham). The specificity of pCREB immunoreactivity toward pCREBα and pCREBδ in the Western blot analysis was ascertained by three criteria: first, the identity of electrophoretic mobility of the upper band with recombinant human pCREBα (see Figure 1A, lane 3); second, the total identity of electrophoretic mobility of pCREB-immunoreactive doublet bands with doublets recognized by anti-CREB antibody (UBI) in cerebellar granule cell samples (data not shown); and third, the absence of immunoreactivity corresponding to expected sizes of CREM repressor isoforms and ATF1, which share in part the sequence surrounding phosphorylated Ser-133 of CREB. Phosphorylated CREMτ activator could comigrate with the pCREB immunoreactive band and could in principle be recognized by anti-pCREB antibody, but this is highly unlikely since CREMτ mRNA was not detected in rat hippocampus (Mellstrom et al., 1993).

### $Ca^{2+}$ Imaging

Fluo-3 $Ca^{2+}$ imaging was carried out in the laboratory of Professor Stephen J Smith, Stanford University, in collaboration with Dr. Noam Ziv. Cells were loaded with fluo-3-AM (Molecular Probes) for 20 minutes before stimulation. EGTA-AM (1 mM) was applied for 30 min before the experiments shown in Figure 5C. Data were collected on a custom-built confocal microscope, and images were analyzed using Adobe Photoshop.

### $Ca^{2+}$ Buffers and Pharmacology

Cells pretreated with 1 μM tetrodotoxin were exposed for 30 min to EGTA-AM (3 mM), $Br_2$-BAPTA-AM(1 mM), or BAPTA-AM (1 mM) in Tyrode/tetrodotoxin (all AM ester compounds from Molecular Probes). Following a subsequent 3 min washout in tetrodotoxin-free Tyrode in the absence or presence of thapsigargin (1 μM, Calbiochem), a 50 Hz, 18 s field stimulation was applied. Although the dimeythyl sulfoxide concentration was as high as 1% during the AM-ester loading, the cell morphology was apparently normal and action potential firing was not altered during stimulation (Figure 6A). Following the end of single-cell depolarization (Figures 6B, 6C, and 7C), cells were rapidly fixed and identified by subsequent staining of Neurobiotin. For peptide inhibitor experiments, an intracellular solution containing Neurobiotin and either 25 μM PKI(5–24) (Calbiochem and Biomol) or the CaM antagonist peptide CaMKII(290–309) (25 μM; Biomol) was used. Whole-cell patched neurons were held at −70 mV for 5 min before application of the 3 min, 0 mV depolarization. For experiments with KT5720 and KN-62 (Figure 7A), cells were preincubated in the drug for at least 20 min; and for Rp-cAMPS, at least 30 min before stimulation. We found that incubation with KN-62 had no effect on the amplitude or kinetics of voltage-gated $Ca^{2+}$ currents in hippocampal neurons (Figure 7B), in agreement with Wyllie and Nicoll (1994).

### Acknowledgments

We thank I. B. Bezprozvanny, D. V. Madison, W. M. Roberts, H. Schulman, T. L. Schwarz , and R. Y. Tsien for comments on the manuscript; members of the laboratory for helpful discussion; Noam Ziv for his collaboration in the investigation of $Ca^{2+}$ buffer effects; and R. H. Scheller for use of the Zeiss microscope. This work was supported by the Silvio Conte-NIMH Center for Neuroscience Research, the Mathers Charitable Trust (R. W. T.), a Medical Scientist Training Program fellowship (K. D.), and a long-term fellowship from the Human Frontiers Science Program (H. B.).

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Received October 17, 1995; revised December 11, 1995.

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