Nuclear calcium signaling controls CREB-mediated gene expression triggered by synaptic activity

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Information storage in the nervous system requires transcription triggered by synaptically evoked calcium signals. It has been suggested that translocation of calmodulin into the nucleus, initiated by submembranous calcium transients, relays synaptic signals to CREB. Here we show that in hippocampal neurons, signaling to CREB can be activated by nuclear calcium alone and does not require import of cytoplasmic proteins into the nucleus. The nucleus is particularly suited to integrate neuronal firing patterns, and specifies the transcriptional outputs through a burst frequency-to-nuclear calcium amplitude conversion. Calcium release from intracellular stores promotes calcium wave propagation into the nucleus, which is critical for CREB-mediated transcription by synaptic NMDA receptors. Pharmacological or genetic modulation of nuclear calcium may directly affect transcription-dependent memory and cognitive functions.

The regulation of gene transcription by neuronal activity is an integral part of the process that underlies learning and memory[1]. Electrical impulse patterns are coded using intracellular calcium signals and linked to specific genomic responses[2,3]. Activity-dependent activation of the transcription factor CREB is heavily implicated in synaptic plasticity, learning and survival[4–8]. CREB is a target for two calcium-regulated signaling pathways: the MAP kinase/extracellular signal-regulated kinase (ERK1/2) cascade[9–13] and the calcium/calmodulin (CaM) dependent protein kinases, in particular, the nuclear CaM kinase IV[13–18]. Either pathway can cause phosphorylation of CREB on its activator site serine 133 (refs. 13,19), which is often regarded as a marker for CREB-mediated transcription. However, CREB phosphorylation on serine 133 does not necessarily indicate that CREB-dependent gene expression is induced. Activation of the ERK1/2 pathway alone, for example, by electrical activity in the presence of inhibitors of CaM kinases or by selective stimulation of ERK1/2 with growth factors or by genetic means, leads to CREB phosphorylation on serine 133 without appreciable induction of CREB-dependent transcription[13,19–21]. These results were explained by the discovery of a second regulatory event that is critical for CREB-mediated transcription. This second event involves stimulation of the activity of the CREB binding protein (CBP)[13,19,22], a transcriptional co-activator that interacts with CREB phosphorylated on serine 133 (ref. 23). The CBP-activating signal is provided by nuclear calcium and CaM kinase IV, but not by the ERK1/2 cascade that consequently fails to induce CREB-dependent transcription[13,22]. Thus, CaM kinase IV (or a closely related nuclear CaM kinase) has emerged as a key regulator of neuronal gene expression. It is necessary and sufficient to activate CREB-dependent gene expression in response to calcium signals, and is likely to control the activity of many other transcription factors that can recruit CBP to a promoter[19,24]. However, it remains uncertain how synaptic signals are conveyed to the nucleus and to nuclear CaM kinases. Studies have suggested that a signal-induced translocation of calmodulin from the cytoplasm to the nucleus relays electrical activity to CREB[25]. Here we show that in hippocampal neurons, synapse-to-CREB signaling can function in the absence of nuclear import of calmodulin. Instead, independent calcium signal processing in neuronal nuclei couples synaptic activity to CREB-mediated gene transcription.

Results

The subcellular localization of calmodulin was analyzed in a hippocampal slice preparation and in cultured hippocampal neurons. Slices from the hippocampus of adult rats showed strong nuclear calmodulin immunoreactivity in most neurons in the dentate gyrus and in the areas CA3 and CA1 (Fig. 1a). In unstimulated hippocampal cultures, virtually all neurons expressed calmodulin in the nucleus (Fig. 1a). Similarly, FITC-labeled calmodulin microinjected into the cytoplasm of cultured rat hippocampal neurons largely accumulated in the nucleus (Fig. 1c). Microinjection *per se* did not change the distribution of the endogenous calmodulin (Fig. 1b). The nuclear localization of the endogenous calmodulin and nuclear accumulation of FITC-labeled calmodulin were unaffected by treatments of the neurons that either increase or decrease their electrical activity and thus seem to be intrinsic properties of hippocampal neurons (Fig. 1d and data not shown). These results indicate that most hippocampal neurons are unlikely to use signal-induced nuclear translocation of calmodulin for the relay of synaptic signals to the transcription-activating machinery.

We next determined whether nuclear import of any protein is important for calcium signaling to CREB. Hippocampal neurons were microinjected into the cytoplasm with wheat germ agglutinin (WGA), a plant lectin that binds to the nuclear pore complex and blocks nuclear import[26]. The inhibition by WGA

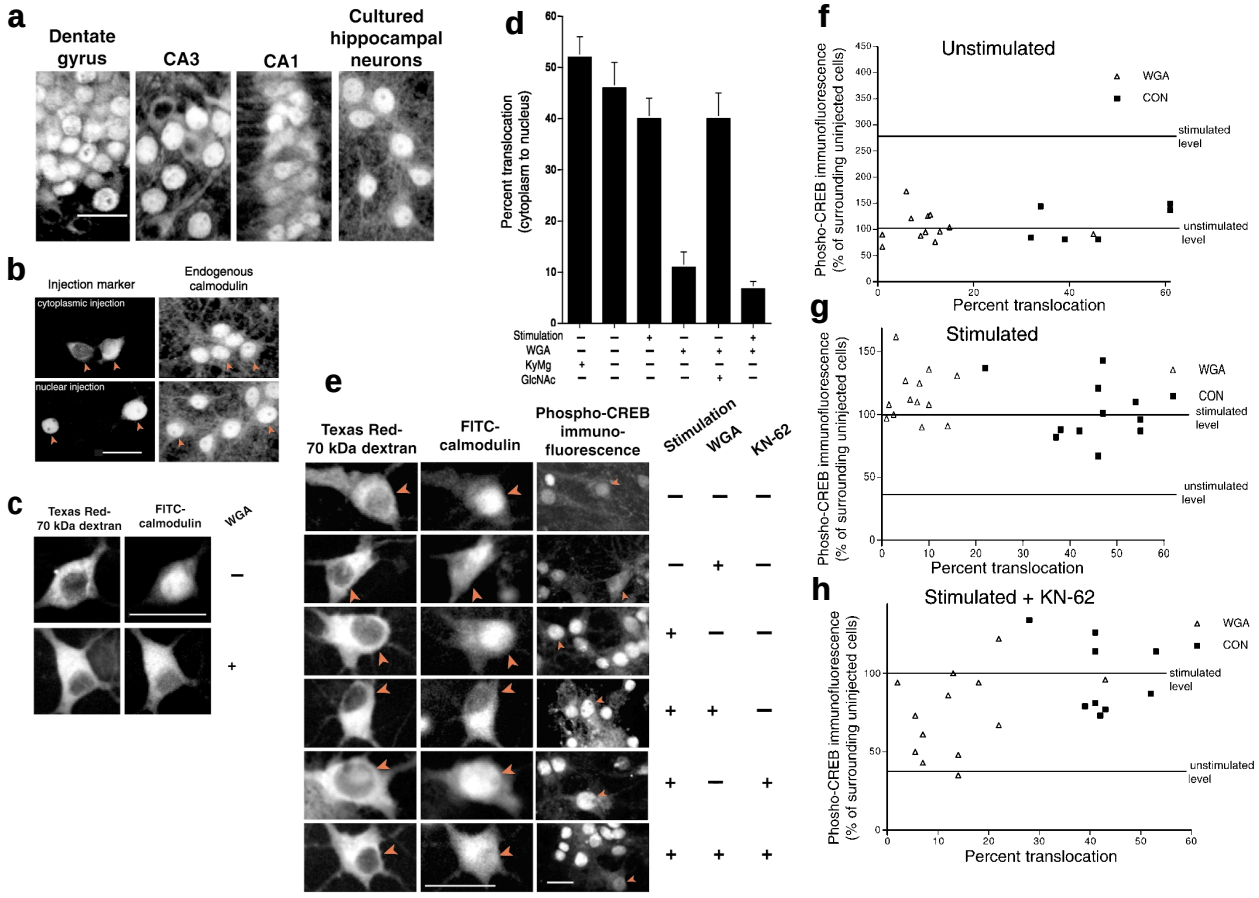

Fig. 1. Dynamics of calmodulin localization in hippocampal neurons: uncoupling of synaptic activity-induced CREB phosphorylation from import of proteins into the nucleus. (**a**) Immunocytochemical analysis of calmodulin expression in hippocampal neurons. Representative confocal images are shown. The percentage of neurons showing strong nuclear calmodulin immunoreactivity was 85 ± 5% (dentate gyrus), 76 ± 9% (CA3), and 64 ± 4% (CA1) (n = 3), > 98% (cultured hippocampal neurons; n = 5). Scale bar, 40 μm. (**b**) Nuclear calmodulin immunoreactivity in cultured hippocampal neurons microinjected with a Texas Red-labeled 70 kDa dextran into the cytoplasm or the nucleus. Arrowheads, microinjected neurons. Left, Texas Red-labeled injection marker; right, calmodulin immunoreactivity. Scale bar, 40μm. (**c**) Wheat germ agglutinin (WGA) blocks nuclear accumulation of FITC-calmodulin microinjected into the cytoplasm of hippocampal neurons. Representative confocal sections are shown. Left, Texas Red-labeled injection marker; right, FITC-calmodulin. Scale bar, 20 μm. (**d**) Quantitative analysis of nuclear translocation of FITC-calmodulin. Hippocampal neurons were microinjected into the cytoplasm with either FITC-calmodulin, FITC-calmodulin/WGA or FITC-calmodulin/WGA/*N*-acetyl glucosamine (GlcNAc). The activity of WGA is quenched by preincubation with GlcNAc. The percentage of cytoplasmic FITC-calmodulin translocated to nucleus was quantified in untreated hippocampal neurons and in hippocampal neurons treated either for 20 to 30 min with 1 mM kynurenate/12 mM $MgCl_2$ (KyMg). Stimulation was with KCl (50 mM) plus APV (100 μM) for 5 min. (**e**) Calcium-induced CREB phosphorylation on serine 133 occurs independently of nuclear translocation of FITC-calmodulin. The Texas Red fluorescence image (left column) identifies injected cells; arrowheads indicate cells microinjected into the cytoplasm. Localization of FITC-CaM (middle column) and CREB phosphorylation on serine 133 (right column) was analyzed in hippocampal neurons, microinjected into the cytoplasm with either FITC-calmodulin or FITC-calmodulin/WGA. Neurons were stimulated with KCl (50 mM) plus APV (100 μM), or were left unstimulated. Pre-treatment with KN-62 (10 μM) was done for 15–20 min. CREB phosphorylation was examined 5 to 10 min or 30 min after stimulation, giving identical results. Scale bar, 40 μm. (**f**, **g**, **h**) Quantitative analysis of calcium-induced CREB phosphorylation as a function of nuclear translocation of FITC-calmodulin in hippocampal neurons microinjected into the cytoplasm with FITC-calmodulin (CON, black squares) or FITC-calmodulin/WGA (WGA, open triangles). Unstimulated neurons (**f**); neurons stimulated with KCl (50 mM) plus APV (100 μM) for 5 minutes without (**g**) or with pre-treatment with 10 μM KN-62 (**h**). Simple regression analysis showed a relationship between inhibition of active protein import (measured by monitoring FITC-calmodulin translocation) and inhibition of CREB phosphorylation when KN-62 was present (that is, only the ERK1/2 pathway was in operation); $p < 0.05$. In addition, comparison of the two data sets (WGA-injected versus non-injected) showed a significant difference only when KN-62 was present; $p < 0.02$, unpaired Student's t-test; $p < 0.05$, Mann–Whitney U test.

of active nuclear transport of FITC-calmodulin microinjected into the cytoplasm (Fig. 1c and d) was used to monitor the efficacy of WGA. Calcium flux through L-type calcium channels, a potent activator of CRE/CREB-dependent transcription[13,14,18–20,27–30], was induced by membrane depolarization, and CREB activation was assayed using an antibody that recognizes CREB in its activated (serine 133-phosphorylated) state[31]. Microinjection of FITC-calmodulin or WGA/FITC-calmodulin *per se* did not induce CREB phosphorylation (Fig. 1e, top two rows and f). Upon stimulation, CREB phosphorylation increased in uninjected and FITC-calmodulin-microinjected neurons but, crucially, also increased in neurons in which import of proteins into the nucleus was blocked by WGA (Fig. 1e, middle two rows and g). WGA did inhibit calcium-induced CREB phosphoryla-

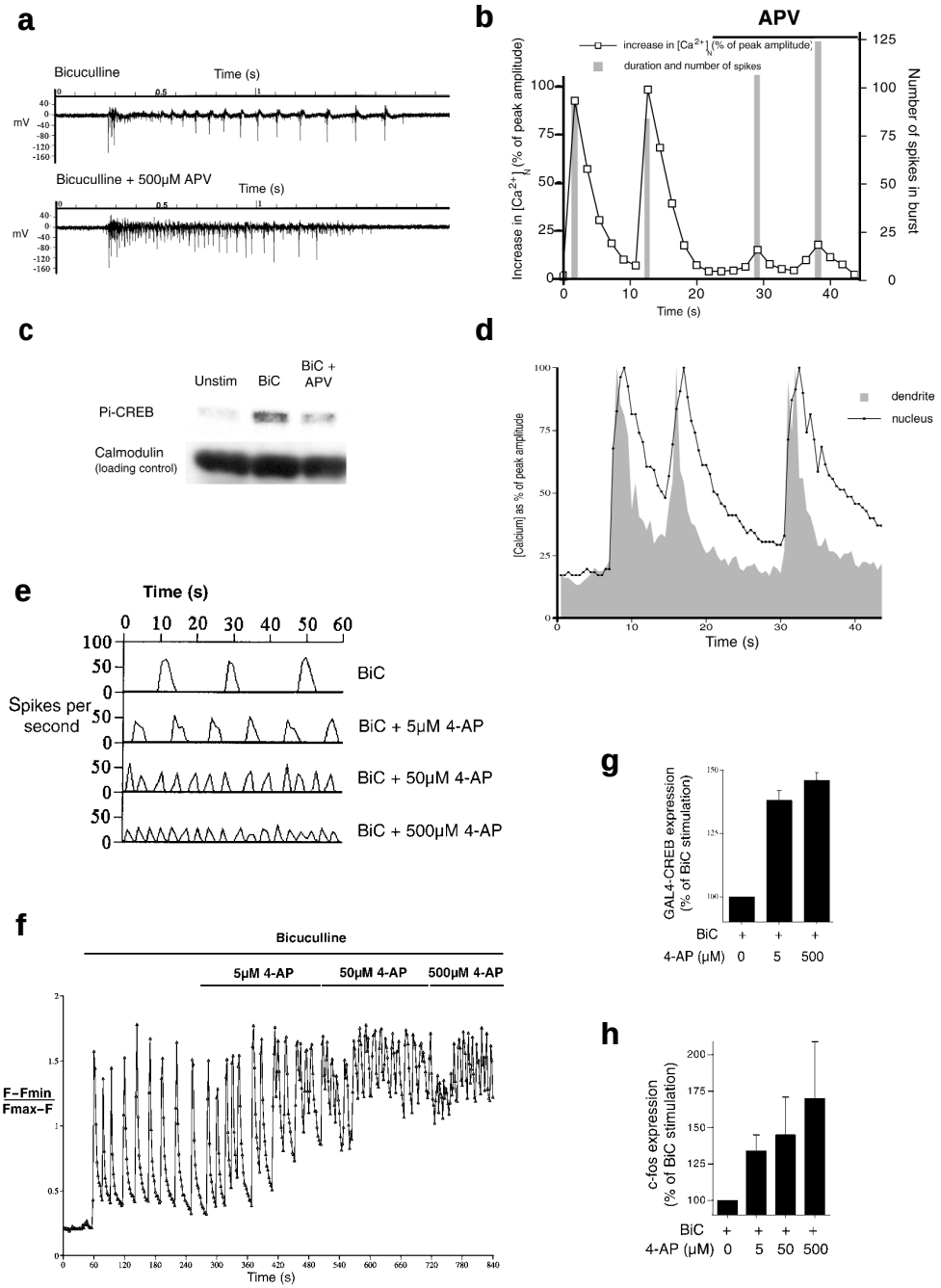

Fig. 2. The nuclear calcium pool specifies the transcriptional response. (**a**) Examples of MEA recordings of bicuculline-induced bursts of action potentials in control and APV-treated hippocampal neurons. (**b**) Bursts of action potentials induce NMDA receptor-dependent nuclear calcium transients that outlast electrical activity. MEA recordings and simultaneous calcium imaging using a CCD camera. Action potential firing was induced by bicuculline; hippocampal neurons were subsequently exposed to bicuculline and APV (500 μM). The width and the height of the bars indicate the duration of the electrical activity and the number of spikes in the burst, respectively. (**c**) Immunoblot analysis of CREB phosphorylation in unstimulated hippocampal neurons and in hippocampal neurons treated for 10 min with bicuculline (50 μM) in the presence and absence of APV (250 μM). (**d**) Examples of nuclear and corresponding dendritic calcium peaks illustrating the temporal delay of nuclear calcium and differences in the decay of the calcium transients. (**e**) MEA recordings illustrating the increase in burst frequency of bicuculline-stimulated hippocampal neurons following treatment with the indicated concentration of 4-AP. The total number of spikes, measured over equal periods of time, did not increase in 4-AP-treated neurons (data not shown). (**f**) Example of nuclear calcium transients illustrating the effects of 4-AP on the frequency of the transients, the peak amplitude and inter-peak calcium levels. (**g**, **h**) Immunocytochemical detection of GAL4-CREB-mediated transcription (**g**) and endogenous c-fos expression (**h**) in hippocampal neurons 2 h after inducing bursts of action potentials for 10 min with bicuculline in the absence or presence of the indicated amount of 4-AP. Hippocampal neurons were microinjected with a GAL4-CREB expression vector and the c-fos-based, myc-tagged GAL4 reporter gene pF222ΔCREmyc[13,29]. Number of cells analyzed, 306, GAL4-CREB; 451, endogenous c-fos. The basal level of GAL4-CREB mediated gene expression was 34 ± 7% of that obtained after bicuculline treatment.

tion when CaM kinases were blocked using KN-62 (Fig. 1e, bottom two rows and h). This was because the remaining pathway to CREB, the Ras-ERK1/2-RSK2 cascade (known to cause CREB phosphorylation[10–13] but not CREB-mediated transcription[13,20,21]) was activated in the cytoplasm and thus depended upon translocation to the nucleus. These results indicate that signaling to CREB following a transmembrane calcium flux into hippocampal neurons does not require nuclear import of proteins. Instead, a nuclear calcium pool, activating an intranuclear CaM kinase, seems to control CREB function.

Analysis of nuclear calcium homeostasis in electrically activated hippocampal neurons revealed a function of the nuclear

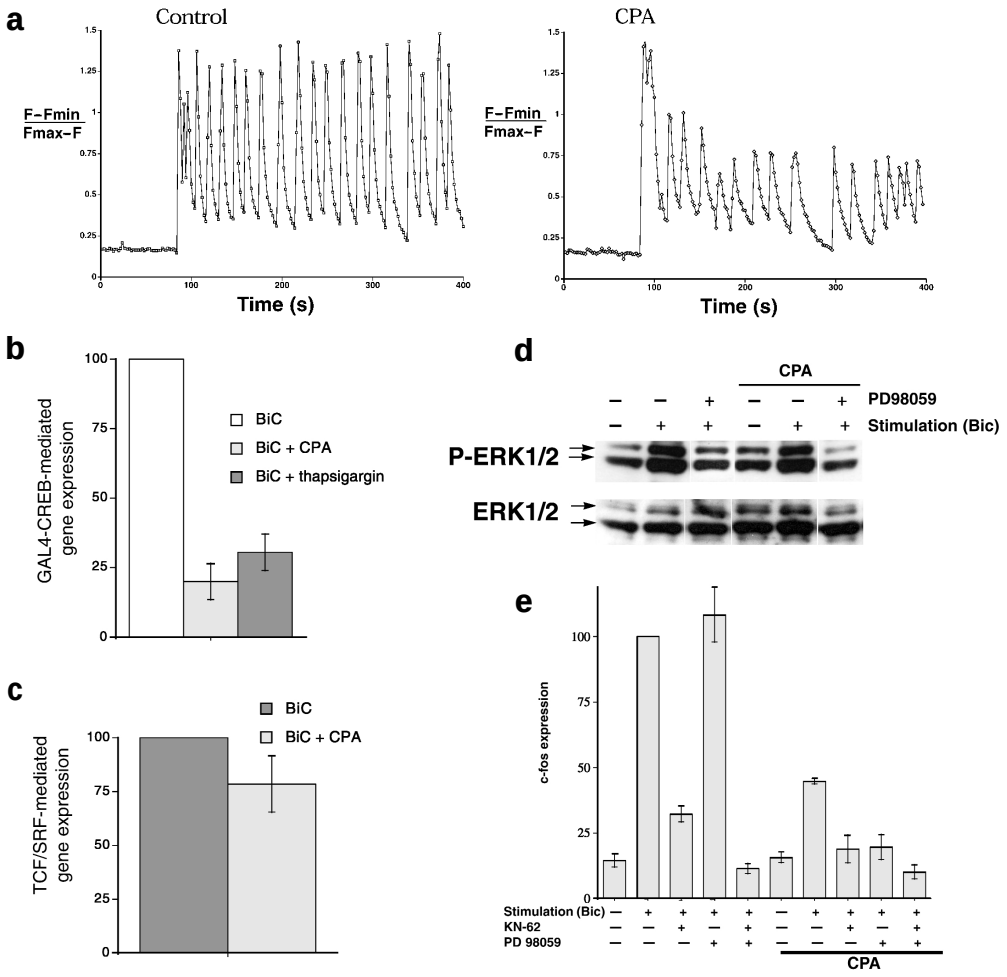

Fig. 3. NMDA receptor-induced calcium release from intracellular stores promotes calcium wave propagation to the nucleus, which is critical for CREB-mediated transcription by synaptic activity. (**a**) Examples of bicuculline-induced nuclear calcium transients in hippocampal neurons with or without CPA-induced depletion of intracellular calcium stores before stimulation. (**b**, **c**) Depletion of intracellular calcium stores blocked burst activity-induced CREB-mediated (**b**) but not TCF/ SRF-mediated (**c**) transcription. Activation of synaptic NMDA receptors following bicuculline treatment was found to be a very potent inducer of CREB-mediated transcription. This is in contrast to the regulation of CREB function by glutamate bath application that only poorly activates CREB-dependent gene expression[19,28] due to activation of extrasynaptic NMDA receptors (unpublished observation). Hippocampal neurons were microinjected either with a GAL4-CREB expression vector and the c-fos-based, myc-tagged GAL4 reporter gene pF222ΔCREmyc or the TCF/SRF-dependent, myc-tagged reporter gene pFosΔCREmyc[13,29]. Transcription activation was assessed immunocytochemically 2 h after inducing bursts of action potentials for 10 min with bicuculline in control hippocampal neurons (**b**, 106 cells analyzed; **c**, 50 cells analyzed), or hippocampal neurons pretreated for 20 min with 10 μM CPA (**b**, 57 cells analyzed; **c**, 56 cells analyzed) or 15 μM thapsigargin (**b**, 53 cells analyzed). Induction by bicuculline fo GAL4-CREB- and TCF/SRF-mediated transcription was 3- to 4-fold. (**d**, **e**) Immunoblot analysis of ERK1/2 activation (**d**) and immunocytochemical analysis of c-fos protein expression (**e**) in hippocampal neurons with or without CPA-induced depletion of intracellular calcium stores before stimulation with bicuculline. Neurons were pre-treated with the indicated inhibitors.

calcium pool as an integrator of neuronal firing patterns. Bursts of action potential firing were induced using the $GABA_A$ receptor blocker bicuculline and monitored using microelectrode array recordings (MEA; Fig. 2a). Action potential bursts occurred with frequencies of 0.05 to 0.15 Hertz, back-propagated into the dendritic tree (unpublished observation), and elicited NMDA receptor-dependent calcium transients that lasted considerably longer than the detectable electrical activity (Fig. 2b). Bicuculline induced rapid phosphorylation of CREB on serine 133 (within 30 s; data not shown; Fig. 2c). This phosphorylation was triggered by activation of NMDA receptors (Fig. 2c) and was mediated by both CaM kinases and the ERK1/2-RSK2 cascade because blockade of CREB phosphorylation required the inhibition of both pathways using KN-62 and the MEK 1 inhibitor PD98059, respectively (data not shown). CREB phosphorylation remained elevated for the duration of synaptic activity, and following its cessation, dephosphorylation occurred with a $t_{1/2}$ of about five minutes (data not shown). These decay kinetics were largely unaffected by inhibition of either the CaM kinases or the ERK1/2-RSK2 pathway (data not shown). This suggests that dephosphorylation is limited by the action of the relevant phosphatase (likely protein phosphatase 1; refs. 18,32) as opposed to any differential shut-down of the two pathways.

Dendritic calcium increases evoked by action potential bursts preceded increases in the nucleus consistent with a calcium wave propagating from the site of calcium entry into the nucleus

(Fig. 2d). The unique property of the nucleus to enable calcium transients to far outlast synaptically evoked dendritic calcium transients (Fig. 2d)[33,34] caused a conversion of a burst frequency-coded electrical signal into an nuclear calcium amplitude-coded signal: by increasing the frequency of action potential bursts following treatment of hippocampal neurons with low doses of the K^+ channel blocker 4-amino pyridine (4-AP; Fig. 2e), nuclear calcium transients failed to return to basal levels between bursts and remain at an elevated plateau (Fig. 2f). This burst frequency-to-nuclear calcium amplitude conversion has implications for gene regulation. The affinity of calmodulin for calcium can increase dramatically following calcium/calmodulin binding to target enzymes[35]. Therefore, sustained elevated nuclear calcium levels may prolong the active state of nuclear calcium/calmodulin-dependent enzymes and facilitate induction of transcription. Indeed, we found that the amplitude of the sustained nuclear calcium signals (a function of the burst frequency) correlates with the transcriptional response: synaptic-activity-induced CREB-mediated transcription and induction of the endogenous c-fos gene (triggered by NMDA receptor activation; data not shown) is enhanced upon increasing the burst frequency with 4-AP (Fig. 2g and h). Thus, the nuclear calcium pool can decode neuronal impulse patterns and may specify the transcriptional response.

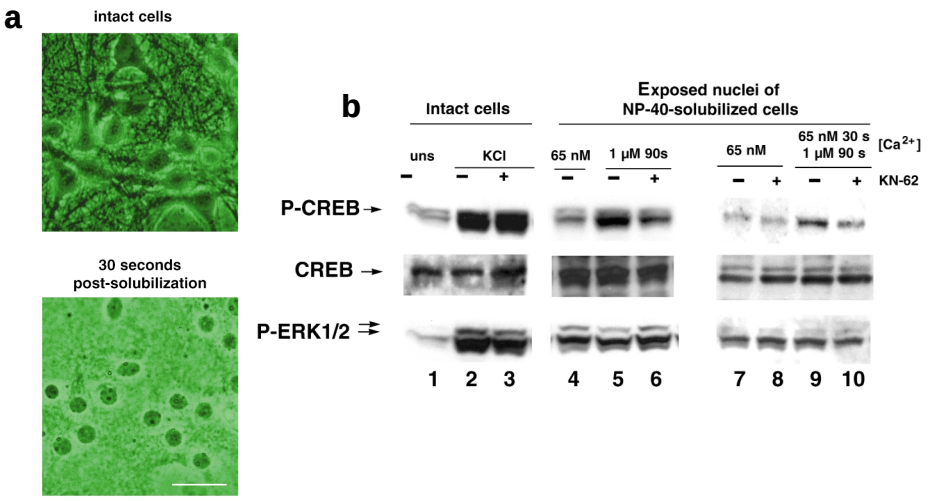

Fig. 4. The nuclear calcium pool is sufficient to signal to CREB. (**a**) Phase contrast photomicrograph of hippocampal neurons before (top) and 30 s after lysis (bottom) in a solution containing 0.5% NP-40. Scale bar, 60 μm. (**b**) Immunoblot analysis of CREB expression, CREB phosphorylation and activation of ERK1/2 in exposed nuclei from detergent-solubilized hippocampal neurons and in intact unstimulated hippocampal neurons (uns) or hippocampal neurons stimulated with KCl (50 mM) plus APV (100 μM) for 5 min. For analysis of intact cells and nuclei, 10 μM KN-62 was added to the cultures 15–20 min before stimulation or lysis, respectively. Inhibition of calcium-induced CREB phosphorylation by KN-62 in isolated nuclei was 89 ± 7% (n = 4).

The source of nuclear calcium is to a large extent provided by intracellular calcium stores. Depletion of intracellular calcium stores with cyclopiazonic acid (CPA) or thapsigargin, although having no effect on action potential firing (data not shown), compromised synaptically evoked nuclear calcium transients (Fig. 3a) and caused a virtually complete block of CREB-mediated gene expression (Fig. 3b). In contrast, transcription mediated by the serum response element (SRE)-interacting complex TCF/SRF[36,37], which is activated by a submembranous calcium pool stimulating the ERK1/2 signaling cascade (unpublished observations), was unaffected by calcium store depletion (Fig. 3c). Thus, calcium release from intracellular stores is critical for calcium wave propagation from synaptic NMDA receptors to the nucleus and, consequently, for CREB-mediated gene expression. In contrast, activation of the ERK1/2 cascade, which is not compromised by CPA-induced store depletion (Fig. 3d), is evidently not sufficient to induce CREB-mediated transcription, but increases transcription mediated by TCF/SRF. These findings suggest that activity-induced expression of a gene such as c-fos that is regulated by both CREB and TCF/SRF[20,28,38] will be compromised, yet not completely blocked, by the depletion of intracellular calcium stores. The remaining induction of CREB/TCF/SRF-regulated genes is predicted to be mediated by the ERK1/2 cascade. This is indeed the case: depletion of intracellular calcium stores with CPA reduced the endogenous c-fos response but left intact a residual induction that was sensitive to inhibition of the ERK1/2 cascade with PD98059 (Fig. 3e). In contrast, PD98059 did not reduce c-fos induction in control neurons (Fig. 3e), indicating that activation of CREB by nuclear calcium is sufficient to fully activate c-fos transcription.

To finally determine whether a nuclear calcium pool is sufficient to signal to CREB, we treated hippocampal neurons with a detergent solution containing calcium/EGTA buffer with a free calcium concentration of 65 nM. This treatment rapidly solubilized the plasma membrane and the cytosol leaving behind isolated nuclei (Fig. 4a). Compared to nuclei that remained at 65 nM free calcium, increasing the free calcium concentration to 1 μM rapidly induced CREB phosphorylation (Fig. 4b; compare lanes 7 and 9). As expected, due to detergent solubilization of cytoplasmic and plasma membrane-associated proteins, ERK1/2 were not activated by increasing the free calcium concentration (Fig. 4b; compare lanes 7 and 9). An increase in CREB phosphorylation and lack of ERK1/2 activation was also observed in nuclei lysed with detergent solution containing 1 μM free calcium (Fig. 4b; compare lanes 4 and 5). Calcium-induced CREB phosphorylation in hippocampal nuclei was blocked by KN-62 (Fig. 4b; compare lanes 5 and 6, and lanes 9 and 10) indicating that the reaction is catalyzed by nuclear CaM kinases. These results demonstrate that nuclei of hippocampal neurons are independent calcium-signal-processing units that contain everything needed to activate CREB.

Discussion

This study indicates that calcium itself, rather than a calcium/calmodulin complex, is the messenger that couples synaptic activity to the nuclear machinery that regulates transcription. Although we fail to observe any activity-dependent calmodulin translocation to the nucleus, we cannot rule out that it does occur in certain cell types and/or experimental condi-

tions. In these cases it would be likely to augment or support nuclear calcium-dependent signaling to CaM kinase IV.

We have shown that calcium influx into hippocampal neurons through synaptic NMDA receptors triggers calcium release from intracellular calcium stores. This amplifies synaptic calcium signals and relays them, perhaps in the form of a regenerative calcium wave, to the nucleus. Synaptically induced nuclear calcium transients may be dependent upon postsynaptic firing and backpropagating dendritic action potentials. Dendritic action potentials (dAPs)[39–44] enhance synaptic calcium transients by depolarization of the dendrites, causing a more efficient relief of the magnesium block of the NMDA receptors. A mechanism involving metabotropic glutamate receptors is also conceivable[42]. In addition, dAPs may cause global calcium transients by facilitating calcium flux through dendritic or somatic voltage-gated calcium channels[43,44]. However, in our experimental system, L-type calcium channels do not seem to contribute to the increases in intracellular calcium; in most hippocampal neurons, inhibition of L-type calcium channels with nifedipine caused only a small decrease in the peak amplitude of bicuculline-induced calcium transients and an unexpected increase in the frequency of the action potential bursts (data not shown).

Our experimental conditions generate vigorous synaptic activity with bursting of neurons and dAPs (unpublished observation). But even weak synaptic inputs can, when coinciding with dAPs, generate large global calcium transients[39–44] that likely trigger nuclear calcium-induced transcription events. Synaptic stimuli coinciding with dAPs can cause robust increases in synaptic efficacy[39,40]. The genomic responses associated with it may help consolidate and maintain changes in potentiated synapses that are perhaps tagged by the local synaptic calcium transients[45].

As information is likely encoded in the firing rates of neurons eliciting distinct genomic responses, impulse patterns must specify activity-dependent gene induction. Our study revealed that the transformation by the nucleus of transient dendritic calcium increases into longer-lasting nuclear calcium transients converts a burst frequency-coded signal into a nuclear calcium amplitude-coded signal. This allows firing patterns to be integrated to generate an elevated nuclear calcium plateau, the amplitude of which is a function of the frequency of the electrical signal. Because the amplitude of calcium signals is a determinant of the type and magnitude of transcription activation[29,46], this frequency-to-amplitude conversion provides a mechanism through which neuronal impulse patterns shape genomic responses.

The importance of CREB in synaptic plasticity and learning[4–8] suggests that its principal regulator, nuclear calcium, may be a key signal for transcription-dependent changes in neuronal function[8]. Enhancement or attenuation of nuclear calcium signals could therefore represent a new strategy for modulating information storage and memory. One possibility involves drugs, such as the dihydropyridines nimodipine or nifedipine, which, similarly to 4-AP (Fig. 2), increase the burst frequency of neuronal firing and enhance gene expression (unpublished observations). Such increases may take place in the intact brain, and thus could explain the observed improvement of cognitive functions with nimodipine in mammals, non-human primates and humans[47–50].

METHODS

Hippocampal cultures, preparation of nuclei and stimulations. Hippocampal neurons were cultured as described[9] except that growth media was supplemented with B27 (Life Technologies, Rockville, Maryland). Bursts of action potential firing were induced by treatment of cultured hippocampal neurons with 50 μM bicuculline. The membrane of hippocampal neurons was depolarized by increasing the extracellular KCl concentration by 50 mM in the presence of 100 μM D(–)-APV[19,28]. Stimulations were terminated by adding 10 mM $MgCl_2$ and 1 mM sodium kynurenate to the cultures[19,28]. Expression of c-fos protein was analyzed 2 hours after stimulation, and CREB phosphorylation on serine 133 and activation of ERK1/2 was analyzed 5 to 10 min after stimulation unless otherwise indicated[19]. PD98059 (50 μM; New England Biolabs, Beverly, Massachusetts) and KN-62 (10 μM; CN Biosciences, Darmstadt, Germany) were added to the cultures 60 min before stimulation and 15 to 20 min before stimulation, respectively. At these concentrations and exposure times, KN-62 or PD98059 did not interfere with bicuculline-induced calcium transients (data not shown). Nuclei were prepared by lysing hippocampal neurons at room temperature in PBS (pH 7.2) containing 0.5% (v/v) NP-40, 7.5 mM K^+2EGTA, 2.5 mM Ca^{2+}EGTA and 2.5 (free calcium concentration is 65 nM). The free calcium concentration was increased to 1 μM by adding 6.2 volumes of 10 mM Ca^{2+}EGTA. After 60 to 90 s, 1.8 volumes of 4× sample buffer was added, nuclei were scraped off the dish, boiled for 7 min, and subjected to SDS-PAGE and immunoblot analyses.

Analysis of calmodulin in hippocampal slices and cultured neurons. Hippocampi were dissected from the brain of adult Long-Evans rats in ice-cold PBS containing 30 mM glucose, 2 μM TTX, 1 mM sodium kynurenate and 10 mM $MgCl_2$. After immediate fixation for 1 to 2 h in PBS containing 4% paraformaldehyde/4 mM EGTA and equilibration for 12 to 36 h at 4°C in PBS containing 30% (w/v) sucrose/0.001% (w/v) sodium azide, slices were cut on a cryostat followed by immunohistochemical staining using an antibody to calmodulin (Upstate Biotechnology, Lake Placid, New York). Cultured hippocampal neurons were fixed for 15 to 20 min in PBS containing 4% paraformaldehyde/3% (w/v) sucrose/4 mM EGTA followed by immunostaining using calmodulin antibodies. Fixation at room temperature and on ice gave identical results.

Microinjection and imaging. Microinjection, Fluo-3 calcium imaging and confocal laser scanning microscopy was done as described[13,19,29]. Calcium concentrations are expressed as a function of the Fluo-3 fluorescence $((F - F_{min})/(F_{max} - F))$. Calmodulin (CN Biosciences) was labeled with FITC using a kit (Sigma). Localization of FITC-calmodulin was assessed 20 to 30 min after microinjection into the cytoplasm of hippocampal neurons. The concentration of WGA (Sigma) in the injection solution was 5.5 μg/μl.

MEA recordings. Hippocampal neurons were plated onto polylysine/laminin-coated chambers containing an array of 60 microelectrodes. MEA recordings (Multichannel Systems, Reutlingen, Germany) and Fluo-3 calcium imaging using a CCD camera (Hamamatsu Photonics UK, UK; Kinetic Imaging Software, Kinetc Imaging, Bromborough, UK) were done at room temperature using 10 to 11 days old hippocampal cultures.

Signaling and gene expression. CREB phosphorylation on serine 133, activation of ERK1/2, and expression of the endogenous c-fos protein and c-fos-based reporter genes was analyzed immunocytochemically or by immunoblotting as described[13,19,29].

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