SINGLE CELL ANALYSIS OF ACTIVITY-DEPENDENT CYCLIC AMP-RESPONSIVE ELEMENT-BINDING PROTEIN PHOSPHORYLATION DURING LONG-LASTING LONG-TERM POTENTIATION IN AREA CA1 OF MATURE RAT HIPPOCAMPAL–ORGANOTYPIC CULTURES

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Abstract—Phosphorylation of the transcription factor cyclic AMP (cAMP)-response element-binding protein (CREB) has been implicated in long-term synaptic plasticity and memory, and its activation has been proposed to be required for the maintenance of long-term potentiation (LTP). The previously described temporal dynamics of CREB phosphorylation during the maintenance of LTP showed differences between experimental models. In the present study the level of CREB phosphorylation was evaluated in organotypic hippocampal slices from young adult rats (P25–30) after long-lasting LTP was induced. Immunohistochemistry and confocal imaging were used to determine the ratio between non-phosphorylated and phosphorylated CREB at a single cell resolution, revealing not only the temporal dynamics but also the extent of CREB phosphorylation. The activation of CREB after LTP induction was compared with cAMP-activation after bath application of forskolin. An increase in cAMP by forskolin resulted in a persistent, uniform increase of the phosphorylated CREB (pCREB/CREB immunofluorescence ratio) in all hippocampal principal neurons. In contrast, the induction of long-lasting LTP in CA1 was accompanied by a local increase in the pCREB/CREB ratio. Both CREB activation and LTP induction in mature cultured slices required N-methyl-D-aspartate (NMDA) receptor activation. CREB phosphorylation continued to increase for 4 h during LTP maintenance. This sustained activation is in contrast to previous observations in acutely prepared slices and suggests the hypothesis that CREB plays an important role during the late phases of LTP.

Key words: hippocampus, synaptic plasticity, L-LTP, cAMP response element-binding protein.

Brief episodes of neuronal activity can result in lasting changes of synaptic efficacy requiring cellular mechanisms responsible for initializing long-lasting synaptic enhancement and permanent changes in neuronal circuitry following brief neuronal activity. It has been shown conclusively that the induction of hippocampal late-long-term potentiation (L-LTP) requires the activation of protein kinase A (PKA) as well as protein synthesis (Krug et al., 1984; Stanton and Sarvey, 1984; Frey et al., 1988, 1996; Nguyen et al., 1994; Huang and Kandel, 1996; Mochida et al., 2001). The cellular mechanisms that initiate gene expression along with long-lasting enhancement of synaptic transmission are uncertain. However, activation of gene expression by phosphorylation of the transcription factor cAMP-response element-binding protein (CREB) has been widely speculated to initiate this process, for considerable evidence has implicated CREB in long-term memory and plasticity (reviewed in Frank and Greenberg, 1994; Silva et al., 1998; Deisseroth et al., 2003; but see Gass et al., 1998; Balschun et al., 2003).

It has been shown, in several forms of LTP, both in vitro and in vivo, that CREB phosphorylation is not just a general marker for neuronal activity, but rather activated in response to synaptic stimulation (Bilo et al., 1996; Deisseroth et al., 1996; Impey et al., 1996). These studies concluded that stimuli sufficient to induce LTP resulted in the phosphorylation of CREB. However, CREB phosphorylation alone was not sufficient to result in gene expression unless associated with the induction of L-LTP (Impey et al., 1996). Revealing the temporal relationship between L-LTP and CREB activation is important in elucidating signals that result in genomic activation. However, previous investigation of the temporal dynamics of CREB phosphorylation in association with L-LTP has revealed different patterns of phosphorylation depending on the experimental model (Matthies et al., 1997; Impey et al., 1998; Schulz et al., 1999). It is crucial to choose an adequate model to investigate CREB-function during LTP for it has been shown that acute hippocampal slices are potentially inappropriate models for such studies (Zhou et al., 1995; Gass et al., 1998; Balschun et al., 2003).

Mature hippocampal–entorhinal cortex slices were used in this study to determine the relationship between the maintenance of long-lasting LTP and CREB activation. It is hypothesized that sustained phosphorylation of the nuclear transcription factor CREB is associated with the expression of L-LTP. The correlation between the duration of LTP and CREB phosphorylation was tested by quantifying changes in CREB phosphorylation throughout the maintenance of long-lasting LTP in pyramidal neurons in mature hippocampal–entorhinal cortex slices (P25–30).
Measuring the extent of CREB phosphorylation at a single cell resolution, using confocal microscopy, revealed the dynamics and extent of CREB phosphorylation in a cell population undergoing long-lasting changes in synaptic strength.

**EXPERIMENTAL PROCEDURES**

**Matric hippocampal–entorhinal cortex slice preparation**

Organotypic slices were prepared from 25 to 30 day old male Wistar rats (Institute breeding stock; SHOE, Magdeburg, Germany) as described previously (Leutgeb et al., 2003). All experiments conformed to institutional, state (Land Sachsen-Anhalt) and international guidelines for the ethical use of animals. Efforts were made to minimize the number of animals used as well as any suffering. Slices were incubated overnight at 34 °C in a humidified carbogen atmosphere (95% O2/5% CO2) and then transferred to ambient O2 and 5% CO2 the following morning. Slices were switched to media with decreased K+ levels (final K+ concentration, 1.6 mM), decreased horse serum concentration (5%), and increased l-glutamine levels (1.0 mM) following 3 days in vitro (DIV). The culture medium was exchanged every 3 days.

**Immunohistochemistry**

Mature hippocampal–entorhinal cortex slice cultures were fixed in 2.5% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 30 min at 4 °C. Forskolin-treated slices and slices in electrophysiological experiments were quickly removed from the incubator or recording chamber and placed directly in PFA solution. Fixed slices were then removed from the anapore membrane and cryoprotected in 30% sucrose in PBS overnight at 4 °C. Forty-five micrometer sections were cut using a cryostat and the free-floating sections were washed two times in chilled PBS. The sections were rocked for 2 h at room temperature in ROTI Immunoblock (Roth GmbH, Karlsruhe, Germany) diluted 1/10 in PBS with 0.5% Triton X-100. Slices were then rocked for 36 h at 4 °C in chilled blocking solution with primary antibodies polyclonal anti-phosphorylated CREB (New England Biolabs, Beverly, MA, USA) and monoclonal anti-CREB IgG (Zymed Laboratory, Inc., San Francisco, CA, USA), diluted 1:300. After incubation at 4 °C, slices were returned to room temperature and rocked for 2 h, then rinsed three times in PBS. Secondary antibodies, Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) were diluted to a working solution of 1:200 in ROTI Immunoblock (Roth) diluted 1/10 in PBS. Following incubation at room temperature for 2 h, slices were washed three times in PBS and mounted on glass coverslips using anti-fade (Molecular Probes) to slow fluorescent decay.

**Confocal microscopy and analysis**

Specimens were examined using a Leica SP1 (Heidelberg, Germany) confocal laser-scanning microscope. Confocal imaging was used to determine the ratio between phosphospecific anti-CREB (specific for Serine [Ser] 133 phosphorylation of CREB; Ginty et al., 1994) and phosphorylation state-independent anti-CREB by detection of labeled immunofluorescent fluorophores. Imaging protocols and settings were kept constant for all specimens examined to minimize differences between experiments (i.e. photomultiplier, pinhole aperture, gain, laser intensity and scanning speed, etc.). Images were recorded using 20×, and 60× oil immersion objective lenses. Only 60× images were used in quantitative CREB analysis. Imaging was performed using an Argon ion laser with two fluorescence channels that scanned the specimen sequentially to eliminate non-specific auto-fluorescent signals. Channel one recorded emission from 488 nm excitation and channel two at 568 nm excitation. Images were collected within the emission detection range. For each channel separate images were acquired, after which a composite overlay image of both channels was created using Leica imaging software. Images were collected from the neuronal cell layer of each hippocampal region (dentate gyrus, CA3, and CA1), through the depth of the entire slice investigated. Following image acquisition, fluorescent staining for phosphospecific anti-CREB (pCREB) and phosphorylation state-independent anti-CREB was quantified blindly, using Leica TCS-NT imaging software. By creating a circle around the circumference of a cell body, the mean fluorescent amplitude was determined from the integrated pixel intensity for each cell on both the 488 nm and 568 nm emission channels separately and simultaneously. Twenty-five adjacent cells were measured from each image.

To determine the level of CREB phosphorylation at Ser 133, a ratio of the mean fluorescent amplitude measured by the excitation of Alexa Fluor 568 bound to phosphorylated CREB was divided by the mean fluorescent amplitude measured by the excitation of Alexa Fluor 488 (CREB), for each individual cell. By creating a relative ratio of pCREB to CREB immunofluorescence variation in staining procedures were compensated for, which allows for a more accurate comparison of phosphorylated CREB levels between slices, and between experiments (modified from Carlson et al., 2000; described a similar method for the quantification of fluorescence DNA probes). A composite overlay image was also created to visualize the immunofluorescent pCREB/CREB ratio visually for each neuron, using Leica imaging software (Fig. 1). Relative pCREB/CREB ratios were reported for each cell (represented as open circles in the representative diagrams), and for each group, as the mean±S.E.M. Differences were tested for statistical significance using the paired Student’s t-test. Probability values of less than 0.05 were considered significantly different.

**Pharmacology**

Analysis of forskolin-induced changes in CREB phosphorylation was performed on slices incubated in either the presence or absence of 20 μM forskolin (RBI Sigma, Deisenhofen, Germany). Slices were incubated in forskolin for various time periods (0, 5, 30, 60, 120, and 240 min; n = 8 for each group), after which they were removed and immediately fixated for immunohistochemical investigation.

**Electrophysiology**

Changes in CREB phosphorylation, as indicated by changes in pCREB/CREB ratios, were assessed in slices left undisturbed in the recording chamber, slices that received continuous low frequency stimulation, as well as slices in which LTP was induced by high-frequency stimulation in the presence or absence of competitive antagonist of the N-methyl-D-aspartate (NMDA) receptor.

Slices cultured for 10–15 DIV were transferred to a carbogen-interface recording chamber (Scientific Systems Design, Inc., Mississauga, Canada) modified to fit tissue culture inserts and optimized for long-term recordings of cultured slices using enhanced humidification (≥90% saturation). One tissue culture insert contained two slices from the same dissection. Slices were allowed to rest in the recording chamber for 30 min, superfused with artificial cerebrospinal fluid (ACSF; 2.5 mM CaCl2, 20 mM glucose, 1.0 mM l-glutamine, 3 mM KCl, 1.24 mM KH2PO4, 110 mM NaCl, 25.6 mM NaHCO3, 1.5 mM MgSO4, osmolality adjusted to levels of culture media) saturated with carbogen at 32 °C. One slice remained undisturbed throughout the experiment. A Teflon-coated bipolar platinum stimulation electrode (5 MΩ; Zak-Medizin Technik, Marktheidenfeld, Germany) altered to result in a final tip
resistance of 400–800 kΩ, was positioned in the CA1-stratum radiatum of the second slice for baseline stimulation. Field excitatory postsynaptic potentials (fEPSPs) were recorded extracellularly in the CA1 region of the stratum radiatum with a glass capillary microelectrode filled with ACSF (tip resistance 1–2 MΩ), amplified by an AxonClamp 2B amplifier (Axon, Union City, CA, USA), and the slope (mV per ms) was measured. Signals were digitized using a CED 1401 A/D converter and analyzed with custom-made software (PWIN, Magdeburg, Germany). The stimulation strength was adjusted to result in 50% of the maximum fEPSP and kept constant throughout the experiment. Responses to test stimuli were measured every 5 min as an average of four individual traces. Only test stimuli were applied in control experiments. Following 5 h of recording, the tissue culture insert was removed and both slices were immediately fixated for immunohistochemical investigation.

For LTP trials, two mature hippocampal slices (10–15 DIV) placed on one tissue culture insert were allowed to rest for 30 min superfused with ACSF in the optimized interface-recording chamber. Recording and stimulating electrodes were positioned on one of the slices for area CA1 recordings, whereas the adjacent slice was left undisturbed for the remainder of the experiment. Responses to Schaffer collateral stimulation in area CA1 were monitored for 1 h with low-frequency stimulation before the delivery of LTP-inducing high-frequency stimulation. High-frequency tetanization consisted of 2 stimulus trains of 100 pulses at 100 Hz with a 10 min intertrain interval. After the tetanus, responses to test stimuli were first measured at 1 min and then every 5 min thereafter as an average of four individual traces. The average slope of the baseline recordings was compared with the slopes after tetanization using the two-tailed Mann-Whitney U test, \( P < 0.05 \) was considered significantly different. The fEPSP amplitudes and slopes are reported as the mean ± S.E.M. LTP was recorded for increasing time intervals (30, 120, 240 min; \( n = 6 \) each group) after which electrodes were removed, and both slices were immediately fixated for immunohistochemical analysis.

Forskolin bath application induces uniform CREB phosphorylation

It was first tested whether changes in CREB phosphorylation could be reliably detected at a single cell resolution. Bath application of forskolin, an activator of the PKA cascade, has been shown to result in an increase in CREB phosphorylation (Kanterewicz et al., 2000). Analysis of forskolin-induced changes in CREB phosphorylation was performed using mature hippocampal–entorhinal cortex slices after 12 DIV. Slices were incubated in forskolin (20 μM) for various time periods (0, 5, 30, 60, 120, and 240 min; \( n = 6 \) each group), after which they were removed and immediately fixated for immunohistochemical investigation. The forskolin induced increases in cAMP resulted in an increase of the pCREB/CREB immunofluorescence ratio in all hippocampal principal neurons, with a small variability between cells (Fig. 2; CA3 and dentate values not shown).

LTP-inducing stimulation is required for postsynaptic nuclear CREB phosphorylation in area CA1

After validating that the ratio imaging detected the extent of nuclear CREB phosphorylation in single cells, it was tested whether LTP induction or, as a control, any of the manipulations required for long-term extracellular recording experiments resulted in CREB phosphorylation. Changes in the pCREB/CREB ratio were assessed in slices left undisturbed in the recording chamber, slices that received continuous low-frequency control stimulation, as well as in slices in which LTP was induced by high-frequency stimulation in the presence or absence of a competitive antagonist of the NMDA receptor site.
Control slices. Mature hippocampal–entorhinal cortex slices (10–15 DIV) were transferred to a carbogen interface recording chamber and subjected to the same experimental conditions and protocol used in LTP experiments. Slices were left in the recording chamber undisturbed for 5 h, after which they were immediately fixed for immunohistochemical investigation (n=4). Handling, chamber conditions, and extended recording duration did not result in a pCREB/CREB immunofluorescence ratio that was different between pyramidal cells in CA1, pyramidal cells in CA3, or dentate granule cells (Fig. 3a). The differences between individual cells remained small as shown by the variability in each subregion. It should be noted that there is a uniform increase in the pCREB/CREB ratio in all principal hippocampal neurons following their removal from the incubator (chamber controls CA1 ratio, 0.30±0.01), compared with slices taken freshly from culture (forskolin incubation CA1 ratio, 0.12±0.002; Fig. 2). A chamber control slice was therefore used along with each LTP recording to directly compare between stimulus-specific processes and changes related to recording conditions.

Low-frequency stimulation. In addition, it was tested whether continued low-frequency stimulation of Schaffer collaterals could activate CREB in CA1 pyramidal cells. To exclude that the routine low-frequency test stimuli further increased cellular CREB phosphorylation, slices were tested for 5 h (i.e. the length of an entire LTP experiment) and then fixed for immunohistochemical analysis. It was shown that sustained baseline synaptic stimulation did not significantly increase CREB phosphorylation beyond chamber control levels (Fig. 3a, b). In addition, CREB phosphorylation in CA1 pyramidal cells remained uniform between cells, as shown by the small variation of the pCREB/CREB ratios of individual neurons. Hippocampal neurons in the CA3 area and dentate gyrus also remained at control CREB phosphorylation levels following low frequency stimulation of the Schaffer collateral fibers in the CA1 area (Fig. 3a).

High-frequency stimulation. After having established that CREB activation was low and uniform in the control experimental paradigm, CREB phosphorylation was assessed following high-frequency stimulation (100 Hz) of the Schaffer collateral input to the CA1 pyramidal neurons in cultured mature hippocampal–entorhinal cortex slices (10–15 DIV). High-frequency tetanization (100 Hz) of the CA1 area resulted in a potentiation of the fEPSP to 165.35±13.73% and a significant approximately two-fold increase of the pCREB/CREB ratio at 4 h after tetanization (Fig. 4). The increase in CREB phosphorylation was observed for the CA1 pyramidal neurons (control, 0.34±0.01; 4 h LTP, 0.73±0.06; n=6, P<0.05), CA3 pyramidal and dentate granule neurons remained at chamber baseline levels (CA3, 0.32±0.02; dentate, 0.30±0.01; n=6). Anti-dromically evoked population spikes (average amplitude of the first population spike, 0.56±0.13 mV; n=4; not shown) were seen in CA3 pyramidal neurons using extracellular field recordings in area CA3 during CA1 tetanization, indicating that antidromic stimulation of CA3 pyramidal neurons was not sufficient to increase nuclear CREB phosphorylation (Fig. 4c).

Specific for CA1 neurons following tetanization was a marked variability of CREB phosphorylation between adjacent cells as shown by the high variance in the pCREB/CREB ratio for CA1 pyramidal neurons following the 4 h maintenance of LTP (Fig. 4c, d). The varied level of CREB phosphorylation between CA1 pyramidal cells following long-lasting LTP is also apparent in the confocal images from slices with LTP (Fig. 4e). The high variation in the proportion of phosphorylated CREB in individual pyramidal neurons following synaptic input is in contrast to the uniform levels after long-term baseline recordings and after...
forskolin bath application (Fig. 4e). These data suggest that prolonged CREB phosphorylation requires high-frequency stimulation that also results in long-lasting hippocampal plasticity.

To determine whether CREB phosphorylation in mature hippocampal–entorhinal cultured slices requires NMDA receptor activation the slices were treated with the NMDA receptor antagonist APV prior to LTP induction and the level of CREB phosphorylation was investigated 2 h after a 100 Hz stimulus. When 100 Hz stimulation was applied to the CA1 area in the presence of APV (50 μM), the potentiation was significantly reduced relative to control LTP. Moreover, APV not only prevented the induction of LTP but also blocked the enhancement of CREB phosphorylation, which remained at control levels (Fig. 5; CA1 control, 0.38±0.01, n=6; LTP, 0.68±0.07, n=6; APV, 0.30±0.03, n=6; P<0.05). Thus, long-lasting LTP as well as the associated increases in CREB phosphorylation in mature hippocampal–entorhinal cortex cultures require NMDA receptor activity.

**Spatio-temporal dynamics of CREB phosphorylation during hippocampal LTP in area CA1**

CREB phosphorylation was assessed at different time intervals (30, 120, 240 min; n=6 each group) following LTP induction by high-frequency stimulation (100 Hz) in the CA1 area of mature slice cultures (10–15 DIV). In addition...
Fig. 4. CREB phosphorylation in individual CA1 pyramidal neurons increases, in varying proportions, along with L-LTP. (A) High-frequency tetanization (HFS; two trains of 100 pulses at 100 Hz, 10 min intertrain interval) induced long-lasting LTP for 4 h. Top: superimposed representative field potentials taken 10 min before and 120 min after HFS. (B) An overlay composite pCREB/CREB image (pCREB, red; CREB, blue) created by confocal imaging of immunostained CA1 pyramidal neurons after 4 h LTP. Following LTP inducing stimuli, CREB is phosphorylated in the synaptically activated neuron population. Positions of the stimulation (S) and recording (R) electrodes are indicated by white arrows. The image is superimposed on a schematic of a proportionally magnified hippocampal trisynaptic circuit. (C) HFS of the CA1 area in mature cultured slices \( n=6 \) resulted in a significant increase of the pCREB/CREB ratio \( P<0.05 \) after 4 h LTP. pCREB/CREB ratios were calculated for individual hippocampal neurons in area.
to investigating the temporal dynamics of the average CREB phosphorylation after LTP in mature cultured slices, it was further investigated to what extent nuclear CREB phosphorylation changed within the activated neuron population. The pCREB/CREB ratio was increased at 30 min after LTP, and continued to increase at longer durations (Fig. 6; 30 min, 0.50 ± 0.05; 2 h, 0.60 ± 0.06; 4 h, 0.73 ± 0.06; n = 6, P < 0.05). The continuing phosphorylation of CREB was specific for CA1 pyramidal cells, whereas CA3 pyramidal cells and dentate granule cells remained at baseline levels for the entire LTP duration (4 h time point shown in Fig. 4c, additional data not shown). Moreover, the variability of CREB phosphorylation between CA1 neurons remained throughout the duration of LTP (Fig. 6), but with the mean level of CREB phosphorylation increasing.

**DISCUSSION**

The acute hippocampal slice has been a preferred model for the investigation of LTP. It has been shown that the routine incubation of acute slices can result in the up-regulation of immediate early-genes, whose transcription...
depends on CREB activation, presumably as a result of hypoxic injury and apoptosis (Zhou et al., 1995). Moreover, CREB can be specifically phosphorylated during the activation of neuronal cell death and apoptosis (Vyas et al., 2002) as well as in the processes of neuronal survival (Finkbeiner et al., 1997; Walton and Dragunow, 2000; Mantamadiotis et al., 2002). These processes are of particular concern for correlating CREB phosphorylation with L-LTP recordings in acute slices, when considering their temporally limited viability and greater potential for necrosis. In contrast, mature hippocampal slice cultures have been shown to be free of cell death and necrosis after 7 DIV (Xiang et al., 2000). They retain intact synaptic connections within the hippocampal trisynaptic cascade and can be maintained at near-physiological temperatures (32 °C) during electrophysiological recordings (Leutgeb et al., 2003), making them a well-suited model for the long-term investigation of gene activation during long-lasting synaptic enhancement.

Controls to distinguish between stimulus-specific processes and changes related to recording conditions showed that even the comparatively minor manipulation of removal from tissue culture incubation resulted in a uniform increase in CREB phosphorylation for all principal hippocampal neurons (comparing incubator control values from Fig. 2 to all other controls). However, the change was of a moderate magnitude and uniform for all principal hippocampal neurons. It occurred presumably due to the low basal activity of quiescent slices in culture and the subsequent activation of normal “housekeeping” functions. Moreover, we showed that repeated baseline stimulation did not evoke additional CREB phosphorylation in comparison to the chamber control experiments. However, high-frequency tetanization (100 Hz) of the CA1 area induced long-lasting LTP and an approximately two-fold increase (compared with recording chamber controls) of the pCREB/CREB ratio at 4 h after tetanization (Fig. 4). This increase in the proportion of cellular CREB phosphorylation can be considered to be a result of synaptically activating the CA1 neuron population, where CREB phosphorylation occurred in response to stimuli that resulted in L-LTP.

Previous investigation of the temporal dynamics of CREB activation has revealed that the multiple phases of LTP are associated with diverse patterns of CREB phosphorylation, differences were seen depending on the experimental model. Matthies et al. (1997) investigated CREB phosphorylation in acute hippocampal slices and observed that high-frequency stimulation (100 Hz) in the CA1 area resulted in L-LTP (4 h). LTP induction was associated with an immediate increase in CREB phosphorylation, which was transient and declined after 30 min despite the continued synaptic enhancement. Transient CREB phosphorylation that returned to baseline after 2 h is observed in vivo only after inducing decremental early LTP with perforant path stimulation (Schulz et al., 1999). Non-decremental L-LTP resulted in biphasic CREB phosphorylation consisting of an initial robust peak at 30 min and sustained phosphorylation of CREB from 2 h up to 24 h after LTP induction (Schulz et al., 1999). The induction of L-LTP in the CA1 area of mature cultured slices resulted in the sustained phosphorylation of nuclear CREB. CREB phosphorylation was observed at 30 min after LTP induction, and remained high after 4 h of LTP maintenance (Fig. 6). The dynamics of CREB phosphorylation in mature cultured slices thus resemble the dynamics of CREB phosphorylation described in vivo rather than the pattern observed during L-LTP in acute slices. Sustained CREB phosphorylation along with L-LTP seems therefore characteristic for

![Graph showing CREB phosphorylation over time](image)
observed in acute slices, CREB activation is maintained at high levels during late phases of LTP. The transcription factor CREB could therefore contribute to translating physiologically expressed late LTP to structural changes at the synapse, a proposed mechanism for the storage of long-term memory.

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