Bidirectional Regulation of Cytoplasmic Polyadenylation Element-Binding Protein Phosphorylation by Ca2+/Calmodulin-Dependent Protein Kinase II and Protein Phosphatase 1 during Hippocampal Long-Term Potentiation

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Induction of hippocampal long-term potentiation (LTP) requires activation of Ca2+/calmodulin-dependent protein kinase II (CaM KII), whereas maintenance of LTP additionally requires protein synthesis. We recently reported that CaM KII stimulates protein synthesis in depolarized hippocampal neurons through phosphorylation of the mRNA translation factor cytoplasmic polyadenylation element-binding protein (CPEB), and this phosphorylation is rapidly reversed by protein phosphatase 1 (PP1). Protein synthesis-dependent late-phase LTP (L-LTP) in the hippocampus requires calcium influx through the NMDA-type glutamate receptor (NMDA-R) to activate CaM KII as well as concomitant inhibition of PP1 mediated by protein kinase A. Therefore, we investigated the regulation of CPEB phosphorylation during L-LTP. Pharmacological stimulation of the NMDA-R in hippocampal slices to produce chemical long-term depression induced a brief dephosphorylation of CPEB. Modest LTP induction (once at 100 Hz), which induces a protein synthesis-independent early-phase LTP (E-LTP), resulted in a transient phosphorylation of CPEB. However, stronger stimulation (four times at 100 Hz), known to induce protein synthesis-dependent L-LTP, elicited a prolonged phosphorylation of CPEB. Furthermore, CPEB phosphorylation correlated with phosphorylation of PP1 inhibitor dopamine- and cAMP-regulated phosphoprotein, a known substrate for protein kinase A. These results evoke the hypothesis that bidirectional regulation of CPEB phosphorylation by CaM KII and protein phosphatases may serve as a mechanism to convert E-LTP into protein synthesis-dependent L-LTP by stimulating protein synthesis and thereby stabilizing synaptic enhancement.

Key words: CaM KII; CPEB; DARPP-32; protein phosphatase; long-term potentiation; protein synthesis

Introduction

Long-lasting changes in synaptic strength, such as long-term potentiation (LTP), require intertwining biochemical cascades for their induction and maintenance. Induction of early-phase LTP (E-LTP) in the CA1 region of the hippocampus requires Ca2+ influx through the NMDA-type glutamate receptor (NMDA-R) to activate Ca2+/calmodulin-dependent protein kinase II (CaM KII) (Lisman, 2003). To convert E-LTP into late-phase LTP (L-LTP), inhibition of protein phosphatase 1 (PP1) is essential to prolong CaM KII activation and phosphorylation of downstream substrates (Blitzer et al., 1995, 1998; Brown et al., 2000; Bradshaw et al., 2003a). PP1 is inhibited during L-LTP by stimulation of Ca2+/calmodulin-dependent adenyl cyclases, raising cAMP levels to activate protein kinase A (PKA) (Roberson and Sweatt, 1996; Nguyen and Kandel, 1997; Wong et al., 1999; Otmakhova et al., 2000). PKA phosphorylates dopamine- and cAMP-regulated phosphoprotein (DARPP-32) and inhibitor-1, potent inhibitors of PP1 when phosphorylated (Foulkes et al., 1983; Hemmings et al., 1984; Blitzer et al., 1995, 1998; Brown et al., 2000). Thus, activation of CaM KII combined with inhibition of PP1 “gates” E-LTP into L-LTP.

To maintain L-LTP, gene transcription and protein synthesis are required (Frey et al., 1988; Nguyen et al., 1994; Woo and Nguyen, 2003). Although mechanisms for initiating gene transcription during LTP are well known (Kandel, 2001), stimulation of protein synthesis is poorly understood. Recent evidence indicates that protein synthesis is stimulated in dendrites near synapses undergoing LTP (Bradshaw et al., 2003b; Klann et al., 2004). Hippocampal dendrites contain mRNAs, including CaM KII (Burgin et al., 1990), NMDA-R subunit 1 (Benson, 1997), activity-regulated cytoskeletal protein (Steward et al., 1998), and the AMPA-type glutamate receptor (AMPA-R) subunit 1 (GluR1) (Ju et al., 2004). All of the components necessary for protein synthesis are present in dendrites (Steward and Schuman, 2001), and after LTP induction, polyribosomes are recruited into spines (Ostoff et al., 2002), as is CaM KII mRNA (Havik et al., 2003). This leads to synthesis of CaM KII protein in dendrites within 5 min of LTP induction (Ouyang et al., 1999).
Regulation of dendritic protein synthesis can be accomplished by phosphorylation of several mRNA translation factors (Klann et al., 2004). For example, the translation factor cytoplasmic polyadenylation element binding protein (CPEB) binds the 3′-untranslated region (UTR) of mRNAs containing the CPE, which includes CaMKII mRNA (Wu et al., 1998). When phosphorylated, CPEB stimulates mRNA polyadenylation and translation initiation (Mendez et al., 2000a,b). Although originally characterized in Xenopus (Mendez et al., 2000b), CPEB is also found in hippocampal dendrites (Huang et al., 2002).

We recently demonstrated that CPEB is phosphorylated by CaMKII, but not by other protein kinases known to be activated during LTP, in cultured hippocampal neurons after depolarization (Atkins et al., 2004). CaMKII robustly phosphorylated CPEB and stimulated CPEB-dependent protein synthesis. To extend our studies, we investigated CPEB phosphorylation in hippocampal slices during LTD and LTP. Our results demonstrate that CPEB phosphorylation is bidirectionally regulated by CaMKII and PP1 in hippocampal slices, and that CPEB phosphorylation is prolonged during protein synthesis-dependent L-LTP.

Materials and Methods

Western blot analysis. Lysates were electrophoresed (10% SDS-PAGE) and Western blotted using the following antibodies: phospho-CPEB (pCPEB) threonine 171 (Thr171; 1:20 of hybridoma supernatant) (Atkins et al., 2004), glutathione S-transferase (GST) (1:10,000; kind gift from Dr. James W. Tracy, University of Wisconsin–Madison, Madison, WI), total CPEB (1:20 of hybridoma supernatant), phospho-GluR1 serine 831 (Ser831; 1:1000; Upstate Cell Signaling Solutions, Charlotteville, VA), GluR1 (1:5000; Upstate Cell Signaling Solutions), phospho-CaMKII threonine 286 (Thr286; 1:1000; Affinity BioReagents, Golden, CO), CaMKII (1:2000; Affinity BioReagents), phospho-DARPP-32 threonine 34 (Thr34; 1:500; Cell Signaling Technology, Beverly, MA), DARPP-32 (1:1000; Cell Signaling Technology), and β-tubulin (1:5000; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Epitopes were visualized with HRP-conjugated secondary antibodies (1:1000 to 1:5000; Amersham Biosciences, Arlington Heights, IL) using ECL or ECL Plus (Amersham Biosciences) and developed on film (Phenix x-ray film BX; Phenix Research Products, Hayward, CA). Film was developed to be in a linear range and densitized using Kodak (Rochester, NY) Digital Science 1D software.

Levels of phospho-protein immunoreactivity (e.g., phospho-CPEB) were normalized to total protein immunoreactivity (e.g., CPEB) and then to β-tubulin immunoreactivity to control for total protein levels. Statistical analyses were one-way ANOVAs with Tukey’s t tests. Data presented are mean ± SEM values.

Hippocampal slice physiology. Transverse hippocampal slices (400 μm) were prepared from male Sprague Dawley rats (3–4 weeks of age) with a vibratome in sucrose–artificial CSF (ACSF) containing the following: 110 mM sucrose, 60 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 2 mM NaHCO3, 5 mM KCl, 1.25 mM MgCl2, and 600 μM ascorbate, saturated with 95% O2/5% CO2. Slices were recovered in standard ACSF (in mM: 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 25 NaHCO3, 10 d-glucose, 2 CaCl2, and 1 MgCl2, saturated with 95% O2/5% CO2) for 30 min at 37°C and then recovered for 1–2 h at room temperature. Slices were equilibrated for 60–90 min in a Fine Science Tools (Foster City, CA) interface chamber at 31°C, except for NMDA-induced chemical long-term depression (Chem-LTD) experiments, in which recordings were done in a submersion chamber. A bipolar Teflon-coated platinum-iridium-stimulating electrode was placed in the Schaffer collateral/commissural pathway, and a borosilicate-recording microelectrode (1–3 MΩ; filled with ACSF) was placed in the stratum radiatum of area CA1 in the hippocampus. Recordings were bandpass filtered (1–10 kHz) and then digitally sampled at 10 kHz with Molecular Devices (Union City, CA) Digidata 1200. Data were collected and analyzed using Axoscope 8.0 software. Field EPSP (fEPSP) strength was quantified by...
measuring the initial slope. Test stimuli were delivered and recorded at 0.0167 Hz, and stimulus intensity (5–50 μA) was adjusted to produce a field response of −1.5 mV, a 30–50% maximal response. Stable baselines were recorded for at least 20 min before LTP or LTD induction. LTP was induced by a single 100 Hz train 1 s long (1 × 100 Hz) with no increase in stimulus strength to ensure L-LTP was not induced or four 100 Hz trains each 1 s long separated by 5 min (4 × 100 Hz). The antagonists 2-[2-amino-5-phosphonovaleric acid (APV) and 2-[(2-hydroxyethyl)]-N-(4-methoxybenzensulfonyl)amino-N-(4-chlorocinnamyl-N-methyl)benzylamine) (KN-93) were added to the ACSF solution for 30 min before baseline recording and were applied continuously throughout the recordings. For biochemical analysis, the slices were frozen in liquid nitrogen, and the CA1 region was microdissected.

**Hippocampal slice biochemistry.** Hippocampal slices were pharmacologically stimulated with NMDA for the indicated times in a submersion chamber containing 10 ml of ACSF at 32°C. To induce chemical LTD, slices were incubated with 25 μM NMDA in ACSF for 3 min at 32°C. Slices were then washed in 10 ml of ACSF and transferred to a new chamber with ACSF to assay CPEB phosphorylation at 20 and 60 min after the initial 3 min of NMDA treatment. After stimulation, the slices were transferred to a nylon screen and frozen on liquid nitrogen. For pharmacological experiments, two slices were pooled and homogenized for each condition. For physiology experiments, the CA1 region was microdissected, and one CA1 region was homogenized and assayed for each condition. Samples were homogenized using a Dounce homogenizer in lysis buffer containing the following: 50 mM HEPES, pH 7.5, 10 mM Na₂PO₄, 100 mM NaCl, 1 mM benzamidine, 20 mg/ml aprotinin, 20 mg/ml leupeptin, 1 μM microcystin-Lr, 1 μM PMSF, 1× phosphatase inhibitor cocktail set I (Calbiochem, La Jolla, CA), and 1× complete protease inhibitor cocktail set II (Roche Applied Science, Indianapolis, IN). Samples were boiled with 2× sample buffer supplemented with 1 μM microcystin-Lr, 100 mM NaCl, 100 mM β-glycerol phosphate, 20 mM EDTA, 20 mM EGTA, 1 mM PMSE, 25 μg/ml aprotinin, and 25 μg/ml leupeptin.

**Materials.** NMDA was obtained from Sigma (St. Louis, MO). KN-93 was obtained from Calbiochem. Tetrodotoxin (TTX) and APV were obtained from Tocris Cookson (Ellisville, MO).

**Results**

**Regulation of CPEB phosphorylation by NMDA treatment of hippocampal slices**

Depolarization of cultured hippocampal neurons produces a robust, but transient (maximal in <1 min), phosphorylation of CPEB at its regulatory site, Thr¹⁷¹, by CaMKII (Atkins et al., 2004). This is accompanied by a significant increase in CPEB-dependent protein synthesis measured 3 h later. Because CaMKII-mediated phosphorylation of CPEB is potently enhanced by inhibition of PP1 in cultured neurons, we speculated that the transient nature of CPEB phosphorylation after neuronal depolarization was a result of strong phosphatase activity. Thus, sustained phosphorylation of CPEB would require simultaneous activation of CaMKII and inhibition of PP1, conditions demonstrated to be essential for induction of protein synthesis-dependent L-LTP (Blitzer et al., 1995, 1998; Brown et al., 2000; Bradshaw et al., 2003a).

As a first step toward understanding the regulation of CPEB phosphorylation in hippocampal slices, we tested whether NMDA-R stimulation, which is required for hippocampal LTP, would modulate CPEB phosphorylation. NMDA-R stimulation triggers the activation of CaMKII or protein phosphatases depending on the level of receptor activation and resulting Ca²⁺ influx intensity and duration (Schulman, 1995). CPEB was modestly and transiently phosphorylated between 10 s and 1 min during a 10 min NMDA stimulation (Fig. 1A, B). This paralleled activation of CaMKII, as measured by autophosphorylation at Thr²⁸⁶ (Fig. 1A, C). However, after 10 min of NMDA-R stimulation, CaMKII activation returned to near basal levels, whereas CPEB was strongly dephosphorylated. To determine whether differential stimulation of the NMDA-R could prolong CPEB phosphorylation, an NMDA dose–response curve was performed. At all concentrations tested, NMDA treatment resulted in significant dephosphorylation of CPEB at 10 min (Fig. 1D, E). This dephosphorylation of CPEB was blocked by the NMDA-R antagonist APV, whereas an AMPA-R antagonist (DNQX) or blockade of action potential firing by the sodium channel blocker TTX was ineffective (Fig. 1F).

In the context of hippocampal slice physiology, brief bath application of NMDA to hippocampal slices induces Chem-LTD. The mechanisms underlying Chem-LTD are not well known, but it cludes induction of low-frequency-induced LTD (LFS-LTD), suggesting that they use common mechanisms. LFS-LTD
phosphorylation and downstream substrate phosphorylation (Blitzer et al., 1995, 1998; Brown et al., 2000; Bradshaw et al., 2003a). If hippocampal slices are tetanized with modest tetanic stimulation (e.g., 1 × 100 Hz), only protein kinase-dependent E-LTP, which does not require protein synthesis, is induced and lasts for 1–3 h (Huang and Kandel, 1994). However, if multiple tetanic stimulation (e.g., 4 × 100 Hz) is used, both protein kinase-dependent E-LTP and protein synthesis-dependent L-LTP that lasts beyond 3 h are induced (Huang and Kandel, 1994; Nguyen et al., 1994; Tang et al., 2002; Kelleher et al., 2004). E-LTP activates CaMKII (Fukunaga et al., 1993; Barria et al., 1997), and L-LTP stimulates dendritic protein synthesis (Ouyang et al., 1999; Miller et al., 2002; Bradshaw et al., 2003b).

Thus, prolonging CaMKII-mediated phosphorylation of CPEB by concomitant PKA-mediated phosphorylation of DARPP-32 to inhibit PP1 would be an attractive model of how protein synthesis is selectively stimulated in L-LTP.

LTP was induced with either a single 100 Hz tetanus, 1 s long, or four 100 Hz tetani, each 1 s long spaced by 5 min (Fig. 3A). E-LTP induction (1 × 100 Hz) produced a modest but transient CPEB phosphorylation that was significant at 5 min and returned to baseline levels at 10 min (Fig. 3B, C). There was no detectable increase in DARPP-32 phosphorylation with this protocol. However, an L-LTP induction protocol (4 × 100 Hz) produced a prolonged CPEB phosphorylation that lasted ≥30 min, and this paralleled phosphorylation of DARPP-32 (Fig. 3B, D).

To test whether the increase in CPEB phosphorylation during L-LTP was mediated by NMDA-R activation, we tetanized hippocampal slices (4 × 100 Hz) in the presence of the NMDA-R antagonist APV and then assayed for CPEB phosphorylation. APV blocked LTP (Fig. 4A) and the increases in both CPEB and DARPP-32 phosphorylation (Fig. 4B). The general CaM kinase inhibitor KN-93 strongly reduced, but did not completely attenuate, the potentiation in EPSP response with tetanic stimulation (Fig. 4A), but it did block the increase in CPEB phosphorylation as well as CaMKII autophosphorylation (Fig. 4B). We have shown previously that KN-93, but not its inactive analog KN-92, attenuated CPEB phosphorylation and CaMKII autophosphorylation in response to neuronal depolarization (Atkins et al., 2004). KN-93 treatment also inhibited phosphorylation of DARPP-32 surprisingly so, because CaMKII does not phosphorylate DARPP-32. However, stimulation of NMDA-R during LTP can also activate CaM-dependent phosphodiesterases (PDEs) to lower cAMP. We have shown previously that activated CaMKII phosphorylates and inhibits CaM-dependent PDEs (Hashimoto et al., 1989), which would result in elevated PKA activity and, therefore, increased phosphorylation of DARPP-32. Inhibition of CaMKII by KN-93 would have the opposite effect, thereby suppressing phosphorylation of DARPP-
Bidirectional CPEB Phosphorylation in LTP

Discussion

Tightly controlling the phosphorylation status of substrates is integral for decoding synaptic inputs and tuning into the appropriate second-messenger signaling cascades. We have shown previously that the dendritic mRNA translation factor CPEB is phosphorylated at its regulatory site, Thr171, by CaMKII and dephosphorylated by PP1 in cultured hippocampal neurons (Atkins et al., 2004). These observations prompted the hypothesis that CaMKII-mediated phosphorylation of CPEB may regulate dendritic protein synthesis during NMDA-R-dependent LTP in the hippocampus. Our current results demonstrate that bath application of NMDA to hippocampal slices under conditions that induce Chem-LTD produced a rapid but transient dephosphorylation of CPEB. Stimulation of hippocampal slices with a paradigm that induced E-LTP (1 × 100 Hz) that is protein synthesis independent only transiently increased CPEB phosphorylation. However, induction of protein synthesis-dependent LTP (4 × 100 Hz) resulted in a prolonged CPEB phosphorylation that correlated with phosphorylation of DARPP-32, a PP1 inhibitor. Thus, our current data support the model that CaMKII-mediated phosphorylation of CPEB, coupled with inhibition of PP1 by PKA phosphorylation of DARPP-32/inhibitor-1, may be involved in regulating dendritic protein synthesis during L-LTP (Fig. 5). In support of this model, downregulation of PP1 activity during LTP has been observed previously (Mulkey et al., 1994).

During hippocampal LTP, CPEB and DARPP-32 phosphorylation were blocked by APV, an inhibitor of the NMDA-R, and by KN-93, a general CaM-kinase inhibitor. Although DARPP-32 is phosphorylated by PKA (Hemmings et al., 1984), its phosphorylation can be indirectly modulated by CaM-kinases. During hippocampal LTP, elevated intracellular Ca2+ can activate CaM-dependent PDEs to lower cAMP levels. However, this negative-feedback loop is countered by activated CaMKII, which phosphorylates and inhibits CaM-dependent PDEs (Hashimoto et al., 1989), thereby elevating PKA activity and increasing phosphorylated DARPP-32 levels. Thus, inhibition of CaMKII by KN-93 would have the opposite effect to suppress pDARPP-32, as we observed during L-LTP.

Bidirectional regulation of CPEB phosphorylation has important physiological implications, in that the ratios of key kinase/phosphatase activities determine downstream cellular responses (Fig. 5). For example, the phosphorylation status of DARPP-32 and inhibitor-1 is determined by the ratio of PKA/PP2B activities, thus gating the expression of L-LTP. L-LTP suppressed by a PKA inhibitor can also be rescued by postsynaptic injection of phosphorylated inhibitor-1 (Blitzer et al., 1998). Furthermore, L-LTP is impaired in mice lacking CaM-dependent adenyl cyclase types 1 and 8 (Wong et al., 1999), whereas in mice overexpressing type 1 adenyl cyclase, a 1 × 100 Hz stimulus induces sustained LTP that is blocked by a PKA inhibitor (Wang et al., 2004). Moreover, in mice expressing a PP2B inhibitor, transient suppression of PP2B activity facilitates LTP in vitro and in vivo (Malleret et al., 2001), showing that PP2B exerts an inhibitory constraint on LTP. Thus, activation of PKA overcomes PP2B activity to gate E-LTP into L-LTP. In our previous study (Atkins et al., 2004), the PKA inhibitor H-89 did not affect pCPEB induced by depolarization, but because CPEB was only transiently phosphorylated, it is unlikely that PKA was activated under those conditions.

Similarly, the ratio of CaMKII/PP1 activities determines the phosphorylation state of CPEB and, thereby, CPEB-dependent protein synthesis. Thus, induction of E-LTP produced only a transient phosphorylation of CPEB (Fig. 5). In contrast, a protocol known to induce protein synthesis-dependent L-LTP gave a prolonged CPEB phosphorylation that was sustained beyond 30 min. Moreover, the phosphorylation of CPEB during L-LTP correlated temporally with increased phosphorylation of DARPP-32 (Fig. 3). Thus, CPEB-dependent protein synthesis during L-LTP may require simultaneous activation of CaMKII and inhibition of PP1 through PKA-mediated phosphorylation of DARPP-32 or inhibitor-1. This suggests that the ratio of CaMKII/PP1 activities in LTP regulates the
magnitude and duration of CPEB phosphorylation, which may in turn control the level of dendritic protein synthesis.

Although it is well established that CPEB phosphorylation stimulates protein synthesis, the role of CPEB in synaptic plasticity is not well defined. In a model of NMDA-dependent synaptic plasticity in the visual cortex, experience-induced synthesis of αCaMKII, the mRNA of which contains two CPEs (Wu et al., 1998), was dependent on mRNA polyadenylation and translation (Wu et al., 1998; Wells et al., 2001), strongly implicating the CPEB pathway. Consistent with this interpretation, translation in hippocampal neurons of transfected reporter constructs containing the 3′-UTR of CaMKII with its two CPEs was stimulated by depolarization with KCl (Atkins et al., 2004). In mice lacking the CPEB-1 gene, which encodes for the only CPEB isoform that is regulated by calcium/calmodulin-dependent protein kinase II (CaMKII) mRNA translation in synapses undergoing potentiation (Scheetz et al., 2000). These pathways are among several that could stimulate translation of dendritic mRNAs and compensate for the lack of CPEB-1 in the knock-out mouse (Klann et al., 2004).

Phosphorylation of CPEB Thr^{171} is one mechanism that regulates CPEB; however, the three other mammalian CPEB isoforms do not contain a homologous regulatory Thr^{171} site, suggesting that they have alternative regulatory mechanisms (Theis et al., 2003). These other mammalian CPEB isoforms do contain several predicted phosphorylation sites. For example, PKA-mediated phosphorylation of Aplysia CPEB, which lacks a homologous Thr^{171} site, increased actin mRNA polyadenylation and protein synthesis during long-term facilitation (Liu and Schwartz, 2003). Additionally, a tantalizing observation is that one of the Aplysia neuronal CPEB isoforms exists in two conformations; one is a prion-like state (Si et al., 2003). When in this state, CPEB-mediated protein translation is activated, possibly generating a self-perpetuating molecular memory to increase dendritic protein synthesis. This has led to the speculation that perhaps this mechanism produces a very stable change in synaptic efficacy (Si et al., 2003). It remains to be determined whether mammalian CPEB isoforms form prion-like states, and whether this occurs during hippocampal synaptic plasticity.

Initiating dendritic protein translation through CPEB phosphorylation allows for rapid, local increases in protein levels at the synapses undergoing potentiation. Although CaMKII is activated during both E-LTP and L-LTP, we found that coincident activation of CaMKII and inhibition of PP1 was necessary to sustain the CaMKII-mediated phosphorylation of CPEB during LTP (Fig. 5). Having CPEB coupled to PP1 may provide the neuron a means to tightly regulate dendritic protein synthesis during different forms of synaptic plasticity.

References


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