
Jin-A Lee, Hyoung Kim, Yong-Seok Lee, Bong-Kiun Kaang*

National Research Laboratory, Institute of Molecular Biology and Genetics, School of Biological Sciences, College of Natural Sciences, Seoul National University, San 56-1 Silim-dong Kwanak-gu, Seoul 151-742, South Korea

Received 24 September 2002; received in revised form 26 October 2002; accepted 28 October 2002

Abstract

Cyclic AMP-response element binding protein-2 (CREB2) is a member of the CREB/transcription factor (CREB/ATF4) family. CREB2 is a transcription factor known to be involved in Aplysia long-term facilitation. To further examine the role of ApCREB2 on long-term synaptic facilitation, we isolated ApCREB2 from Aplysia kurodai in full-length cDNA library, and found that the overexpression of ApCREB2 blocked 5-hydroxytryptamine (5-HT)-induced long-term synaptic facilitation in Aplysia sensory-to-motor synapses. Furthermore, a single pulse of 5-HT, which normally induces only short-term facilitation, in the presence of ApCREB2 inhibition by RNA interference, induced long-term facilitation in Aplysia sensory-to-motor synapses. These results suggest that ApCREB2 is a functional repressor of long-term facilitation in Aplysia sensory-to-motor synapses.

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Keywords: Aplysia; Cyclic AMP-response element binding protein-2; RNA interference; Long-term facilitation; Transcription factor; Sensory-to-motor synapse

Long-term memory storage is known to require RNA and protein synthesis [9,11]. In particular, the transcriptional regulation of genes is critical for the induction of the late phase of long-term memory. The molecular analysis of the transcriptional regulation for long-term memory continues to be explored, but is known to be evolutionarily conserved at the cellular level in various model systems, for example, in sensitization in the marine snail Aplysia, olfactory learning in Drosophila, and contextual learning in mice [9–11]. Studies of long-term memory in each system have shown that cyclic AMP-response element binding proteins (CREBs) plays important roles in long-term memory storage [4–7]. In addition, recent studies in Aplysia have shown that cyclic AMP-response element binding proteins (CREBs) plays important roles in long-term memory storage [4–7]. In this process, CREB1, which is activated by PKA, activates a cascade of genes required for long-term memory storage. On the other hand, CREB2 is thought to be involved in the repression of long-term memory induction [2,3]. Aplysia CREB2 as well as human CREB2 and transcription factor-4 (ATF4) have been found to repress CREB1 transcriptional activity. ApCREB2 is constitutively expressed and its expression level is not changed by 5-HT treatment. Bartsch et al. [2] reported that ApCREB2 represses long-term memory storage, after finding that long-term facilitation was induced by injecting ApCREB2 antibody and treating with one pulse of 5-HT.

In the present study, we examined the hypothesis that ApCREB2 acts as a repressor by both overexpressing ApCREB2 and inhibiting ApCREB2 by RNA interference in sensory-to-motor synapses. For this purpose, we first isolated the CREB2 of Aplysia kurodai from the expressed sequence tag (EST) database of a full-length cDNA library. Sequence analysis showed that ApCREB2 has an open reading frame of 1131 bp encoding a polypeptide of 377 amino acids and that it contains a basic leucine zipper (bZIP) domain at its C-terminal at 303–367. The amino acid sequence of CREB2 from Aplysia kurodai has ca. 95% similarity with that of Aplysia californica. ApCREB2 DNA was
amplified by polymerase chain reaction (PCR) using specific primers 5'-CGCCTCGAGCCACCAGGTGAGCTG-GACCTTG-3' and 5'-CGGATCCCTTTCACTGAAATACCTT-3'. ApCREB2 DNA fragment was inserted into the SalI/BamHI sites of the pNEX expression vector to create pNEXd-ApCREB2. ApCREB2 fragment was digested with BamHI/KpnI in pNEXd-ApCREB2 and subcloned into BamHI/KpnI sites of pEGFP-C1 vector (Clontech) to generate enhanced green fluorescent protein (EGFP) fused to N-terminus of ApCREB2. EGFP-ApCREB2 was amplified from pEGFP-C1-ApCREB2 by specific primers 5'-CGGGATCCGGCGACCAGGTGAGCTG-GACCTTG-3' and 5'-CGGATCCCTTTCACTGAAATACCTT-3' and then subcloned into the SalI/BamHI sites of the pNEXd expression vector to construct the pNEXd-EGFP-ApCREB2. The cell culture method used and the detection of GFP through the confocal fluorescence microscope were performed as described previously by Lee et al. [8].

To examine the expression pattern of ApCREB2, we microinjected pNEXd-EGFP-ApCREB2 into cultured Aplysia neurons. Forty-eight hours after DNA injection, the fusion construct was found to be expressed mainly in the cell nucleus and partially in neurites (Fig. 1), thus implying that ApCREB2 functions as a transcriptional regulator in the nucleus. Expressed fusion proteins were observed to form clusters or puncta within the nucleus, though it was not clear whether clusters were formed by homomeric or heteromeric dimerization of the leucine zipper domain.

We overexpressed ApCREB2 by microinjecting pNEXd-ApCREB2 into sensory neurons that were synaptically connected to motor cells in sensory-to-motor coculture, to determine the effect of its overexpression on synaptic plasticity. Sensory-to-motor coculture and conventional recording to measure synaptic strength were carried out as described previously by Lee et al. [2]. The amount of synaptic facilitation was defined as the percentage change in excitatory postsynaptic potential (EPSP) amplitude caused by the 5-HT treatment. All data are presented as the mean percentage change ± SEM of the EPSP amplitude. Transgenic expression of ApCREB2 did not affect basal synaptic transmission (data not shown). The sensory-to-motor coculture was exposed to five pulses of 5-HT to induce long-term synaptic facilitation, which is known to require new protein, mRNA synthesis and structural changes. Cells overexpressing ApCREB2 did not produce an increase in synaptic potential amplitude 24 h after 5-HT treatment (4.3 ± 4.1%, n = 9) (Fig. 2). However, nonexpressed cells (control cells) showed a normal increase in synaptic strength (97.4 ± 26.4%, n = 10) (Fig. 2). These data show...
that the overexpression of ApCREB2 can inhibit long-term synaptic facilitation.

Finally, to further investigate the role of ApCREB2, we attempted to block ApCREB2 expression by microinjecting double-stranded ApCREB2 RNA into the sensory neuron of a sensory-to-motor synapse, as it was reported that double strand RNA inhibits gene expression specifically in Aplysia neurons [8]. For the in vitro transcription of ApCREB2, the DNA fragment (~450 bp from the initiation codon ATG), encoding ApCREB2, was amplified by PCR using primers 5’-GGAATTCATGGAGCTGACCCTTGG-3’ and 5’-AAGGATCCAGGGTGTTTCCATC-3’ and subcloned into pLITMUS 28i vector (New England BioLabs. Inc.) using EcoRI/BamHI sites. Double strand ApCREB2 RNA was generated using a HiScribe™ RNAI transcription kit (New England BioLabs. Inc.). We microinjected ApCREB2 dsRNA or ApCREB2 sense RNA, and, 24 h later, exposed these cells to a single pulse of 5-HT, which normally induces short-term facilitation. We found that cells injected with ApCREB2 dsRNA showed an increased synaptic potential amplitude (65.4 ± 12.9%, n = 12); however, non-injected control cells and cells injected with ApCREB2 sense RNA failed to produce long-term facilitation (0.6 ± 5.5%, n = 11 and −6.8 ± 7.2%, n = 3, respectively) (Fig. 3). In addition, the basal synaptic strength was not affected by the microinjection of ApCREB2 dsRNA into sensory neurons (percentage change; 0.4 ± 16.6%, n = 4 (dsRNA injection) vs. 1.0 ± 7.1%, n = 4 (noninjection control)). Taken together, these data show that the inhibition of ApCREB2 by dsRNA, when combined with one pulse of 5-HT, induces long-term synaptic facilitation, probably by lowering the threshold for long-term facilitation in Aplysia sensory-to-motor synapses.

In this work, we confirmed the role of ApCREB2 as a transcriptional repressor in long-term facilitation by overexpressing and by inhibiting ApCREB2 expression. As a result, we found that the overexpression of ApCREB2 blocked 5-HT-induced long-term facilitation, which is consistent with the finding that the induced expression of the *Drosophila* CREB2-b repressor disrupted long-term memory [11]. Bartsch et al. [2] showed that CREB2 antibody injection combined with one pulse of 5-HT treatment induced long-term facilitation. In the present study, we attempted to inhibit CREB2 expression at the mRNA level, but not at the protein level, by using RNA interference. In a previous report, RNA interference was shown to effectively block both constitutive and inducible gene expression in *Aplysia* neurons [8]. Our RNAi study showed that the inhibition of ApCREB2 gene expression lowers the threshold of long-term facilitation. Our data, together with that from an antibody experiment [3] strongly suggest that CREB2 represses the transactivation activity (presumably mediated by CREB1) that is required for 5-HT induced long-term facilitation. In conclusion, this study shows that ApCREB2 acts as a gatekeeper or as a repressor of long-term facilitation in *Aplysia*sensory-to-motor synapses.

This work was supported by grants M1-0104-00-0140 under the NRL Program and M1-0108-00-0075 from MOST, and No. R01-1999-000-00147-0 from KOSEF. J.-A.L., Y.-S.L & H.K. were supported by BK21 Fellowship from the Korean Ministry of Education.


