Expressions of CCAAT/Enhancer-binding Proteins β and δ and Their Activities Are Intensified by cAMP Signaling as Well as Ca^{2+}/Calmodulin Kinases Activation in Hippocampal Neurons*

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The transcription factor, *Aplysia* CCAAT enhancer-binding protein (ApC/EBP), plays a crucial role in long term facilitation, a synaptic mechanism of long term memory in *Aplysia*. To gain a clue to whether the mammalian C/EBP family of transcription factors are also involved in long term memory, we examined how C/EBP activities in hippocampal neurons can be modulated in response to cAMP and Ca^{2+}, crucial inductive signals for memory formation. As a result, stimulation of either cAMP or Ca^{2+} signals in hippocampal neurons was found to enhance mRNA expressions and DNA binding activities of C/EBPβ and C/EBPδ. Furthermore, it is indicated that CaM kinases have essential roles for increasing the expression and DNA binding activities of C/EBPβ in hippocampal neurons activated by membrane depolarization. Overexpression of constitutively active calcium/calmodulin-dependent kinase IV was found to directly stimulate either C/EBPβ-dependent or C/EBPδ-dependent transcription, reinforcing the evidence that C/EBP family members contribute to Ca^{2+}-dependent transcription. Thus, these results suggest that C/EBPβ and C/EBPδ may be involved in the transcription-dependent phase of memory formation by increasing the expression of both the DNA binding and the transcriptional activities under the direction of cAMP and/or Ca^{2+} signaling in hippocampal neurons.

Memory has two phases: short term memory and long term memory. A number of pharmacological studies have demonstrated that the stabilization of long term memory requires the synthesis of new proteins and RNAs (1, 2). This requirement suggests that transcription is critical for this process. Among a number of transcription factors expressed in neuronal cell nuclei, the Ca^{2+}/cAMP-responsive element-binding protein (CREB)† has been implicated as being essential to long term memory of *Aplysia*, *Drosophila*, and mice (3–7). In *Aplysia*, long term facilitation is a basic synaptic mechanism for a non-associative learning response. The long term synaptic modification is characterized by a consolidation period during which gene expression is required. During this phase, the transcription factor *Aplysia* CCAAT enhancer-binding protein (ApC/EBP) is found to be increasingly induced in response to cAMP signals (8). There is a CRE site in the 5′-untranslated region of ApC/EBP, suggesting that CREB can control the expression of ApC/EBP (8). Furthermore, blocking the function of ApC/EBP either by anti-sense oligonucleotides or by specific antibody inhibits long term facilitation selectively without affecting the short term processes (8). These data indicate that induction of another transcription factor ApC/EBP by CREB is also essential for long term facilitation, in addition to CREB activation (8).

In mammals, seven members of C/EBP family of transcription factors have been cloned molecularly and their biological roles extensively analyzed in a variety of systems (9–26). However, much less is known about the expression and function of C/EBP family members in the neurons of mammalian brain, except that C/EBPα mRNA was detected in mouse hippocampus, cerebellum, and cortex by *in situ* hybridization (27). It was recently shown that two CRE sites in the core promoter of C/EBPβ gene are mostly recognized by CREB in liver and in several cell lines (28). Transfection experiments with promoter constructs where the CREB sites were mutated further indicated that these sites are important to maintain both basal promoter activity and C/EBPβ inducibility through CREB (28). However, in several brain regions including the hippocampus, little information is available about the induction of C/EBP family members by cAMP and Ca^{2+}, both of which are known to activate CREB in neuronal cells such as hippocampal neurons (29–33).

The hippocampus in mammalian brain is involved in the normal formation of long term declarative memory (34). Here, we have found that a prime C/EBP transcription factor in the hippocampus is C/EBPβ. To clarify whether C/EBPβ activity in hippocampal neurons is further modulated by the stimulation of cAMP or Ca^{2+} signals, which are crucial inducers for memory formation (3–7, 35–39), we used cultured hippocampal neurons for detailed biochemical analyses (40). We have found that the expression and DNA binding activities of C/EBPβ and δ are enhanced by the stimulation of cAMP or Ca^{2+} signals in cultured hippocampal neurons. Our results also suggest that CaMKIV activated by Ca^{2+} signal not only induces expression of C/EBP members, but also directly enhances C/EBP-dependent gene transcriptions. Therefore, our study supports the possibility that both C/EBPβ and C/EBPδ may be involved in long term plasticity in mammalian brain.
EXPERIMENTAL PROCEDURES

Hippocampal Neuron Cultures—Hippocampal neuron cultures were done as described by Baranes et al. (40). To stimulate CAMP signals, water-soluble forskolin (50 μM; Research Biochemicals International) was added to 14-day-old cultures. To induce membrane depolarization of hippocampal neurons in the culture, the cultures were treated as described by Bitto et al. (35). When used, kinase inhibitor KN93 (30 μM, Calbiochem), KN92 (10 μM, Calbiochem), and KN62 (30 μM, Calbiochem) were present during the preincubation period of 30 min prior to depolarization and during depolarization. Effects of cAMP signal stimulation and membrane depolarization on gene expressions or DNA binding activities were measured 3–4 h after stimulation.

Immunofluorescence Analysis—Rat hippocampal neurons were grown on poly-d-lysine- and laminin-coated 12-mm glass coverslips in 35-mm dishes, then washed with PBS three times and permeabilized with 0.25% Triton X-100 in PBS at 37 °C for 5 min, and the nonspecific binding sites were blocked with 10% goat serum, 0.1% Triton X-100, 200 μg/ml glycine in PBS at 37 °C for 30 min. Double immunolabeling was performed by incubating cells overnight with a mouse monoclonal anti-MAP2 antibody (Sigma, 1:100) and rabbit polyclonal anti-C/EBPβ or anti-C/EBPδ antibodies (Santa Cruz, 1 μg/ml). Following three washes with PBS, the cells were incubated with Cy3-conjugated goat anti-mouse IgG (Cedarlane Laboratories Ltd, 1:100) and fluorescein-conjugated goat anti-rabbit IgG (Cedarlane Laboratories Ltd, 1:100) in PBS at room temperature for 1 h. The coverslips were washed five times with PBS and examined by fluoresceoscopy. (Sp)-cAMPs Treatment of Hippocampal Slices—(Sp)-cAMPs stimulation of hippocampal slices was done as described by Huang et al. (41). Hippocampal slices were incubated in perfusion solutions containing 50 μM (Sp)-cAMPs (Sp-cAMPs treatment) or perfusion solutions without (Sp)-cAMPs (control) for 30 min. After 30 min of incubation, slices were maintained in the perfusion solution for 2 h. Then, RNAs were prepared from those slices for RNase protection assay.

RNase Protection Assay—A 220-bp rat C/EBPβ cDNA NcoI fragment, 342-bp mouse C/EBPβ cDNA NcoI-PstI fragment covering the leucine zipper domain, 298-bp mouse C/EBPδ cDNA NcoI-XhoI fragment, and 490-bp rat C/EBPδ cDNA NcoI-NcoI fragment were subcloned into pBluescript SK (Stratagene). Templates for preparing Zif268 cRNA (180 bp is protected), actin cRNA (250 bp), and cyclinH cRNA probe (100 bp) were purchased from Ambion. Antisense cRNA probes were synthesized as described previously (42). Total RNAs were prepared from tissues or cultured hippocampal neurons using the acid phenol extraction method of Chomczynski and Sacchi (43) with RNAzol B solution purchased from Biotecx Laboratories, Inc and performed according to manufacturer’s protocol. RNase protection assays were performed by hybridizing 25 μg of total RNA from the hippocampus or 2.5 μg of total RNA from cultured hippocampal neurons with 1 μg of each labeled cRNA at 68 °C for more than 1 h. RNase digestation and analysis were performed as described previously (42).

Immunoblot Analysis—For Western blot analysis, nuclear extracts prepared from cultured hippocampal neurons for gel shift assay were used. Twenty micrograms of each sample were adjusted to give a final solution of 60 μl Tris- HCl, pH 6.8, 2.5% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol, heated at 100 °C for 5 min, electrophoresed through 10% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membrane (Millipore). C/EBPβ was detected with the ECL Western blotting detection system as instructed by the manufacturer (Amersham). The dilution factor for anti-C/EBPβ antibody was 1:100 (Santa Cruz).

Gel Shift Assay—Nuclear extracts of mice hippocampus were prepared as described by others (30) with some modifications. The tissues were homogenized (10 strokes) in four volumes of a buffer containing 0.25 M sucrose, 15 mM Tris-HCl, pH 7.6, 60 mM KCl, 15 mM NaCl, 5 mM EDTA, 1 mM EGTA, and protease inhibitors (1 × α-Complete™ from Roche Molecular Biochemicals; 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) in a Dounce homogenizer. After centrifugation, the pellet was resuspended in 0.6X PBS, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1× α-Complete, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol and rocked on an Eppendorf shaker for 20 min at 4 °C. The supernatant solution after centrifugation was frozen as nuclear extract. To prepare nuclear extracts from hippocampal neurons in culture, cells were washed with Tyrode’s solution once, then immersed with 300 μl of solution A, scraped, and collected into Eppendorf tubes. The following solution A is identical to the above for preparation of tissue nuclear extracts.

RESULTS

C/EBPβ is a Prime C/EBP in the Mouse Hippocampus—To know whether any C/EBP family member is expressed in the hippocampus, which is a crucial region for explicit memory formation in vertebrates, we examined the mRNA expressions of C/EBP members in hippocampus by performing RNase protection assay. The result indicated that C/EBPβ and δ transcripts are expressed in the mouse hippocampus (Fig. 1A). It is also shown that C/EBPβ mRNA is more highly expressed in the mouse hippocampus than C/EBPδ mRNA (Fig. 1A).

Next, we asked which members of C/EBP family is most active in mouse hippocampus by performing EMISA using high affinity C/EBP binding site as a probe. A retarded band was observed in mouse hippocampus nuclear extracts (Fig. 1B). With the addition of anti-C/EBPβ antibodies to the binding reaction, the result was a prominent supershift of the retarded band, which was removed by the addition of cold C/EBPβ binding sites as a competitor (Fig. 1B). Antibodies to C/EBPδ and α also induced supershifts in EMSA of hippocampus, which are much weaker than the supershift induced by anti-C/EBPβ antibodies (Fig. 1B). Addition of the antibodies to CRP1, another newly identified C/EBP member to the binding reaction could not induce any supershift in EMSA suggesting that CRP1 is not a component of hippocampal C/EBP (Fig. 1B). Thus, we find that C/EBPβ is a prime C/EBP in the mouse hippocampus.
express C/EBPδ. C/EBPδ is found to be rather highly expressed in astrocytes, which are easily distinguished from neurons by remaining unstained by MAP2 (Fig. 2, C and D). This fact is consistent with the previous findings of others (46).

**C/EBPβ Can Be a Downstream Target Gene of CREB in Mouse Hippocampus and Cultured Hippocampal Neurons**—A small region containing two C/EBPβ sites in the C/EBP promoter is found to be important in controlling transcription of C/EBPβ in liver and several cell lines (28). To examine the possibility that CREB can also control the expression of C/EBPβ even in hippocampus and cultured hippocampal neurons, EMSA of nuclear extracts from hippocampus and cultured hippocampal neurons were performed using the region containing first CREB site of C/EBPβ promoter as a probe. Several retarded bands could be observed in this EMSA suggesting that nuclear factors are actually binding to the region of the C/EBPβ promoter. Furthermore, the addition of CREB antibody to the EMSA reaction could induce a supershift of the major retarded band demonstrating that main binding activity to the C/EBPβ promoter region is due to CREB (Fig. 3). Further addition of cold C/EBPβ promoter probes as competitors weakened the intensity of supershifted bands, thereby demonstrating specific binding reactions in EMSA. The addition of antibodies to other ATP/CREB family members, such as activating transcription factor 3 (ATF3) and activating transcription factor 4 (ATF4), into the EMSA binding reaction could not supershift retarded bands. These results suggest that CREB binds to the crucial region of C/EBPβ promoter and controls the expression of C/EBPβ gene in hippocampal neurons.

**Stimulation of cAMP Signaling Pathway in Hippocampal Neurons Increases Both mRNA Expressions and DNA Binding Activities of C/EBPβ and δ**—To know whether CREB activated by cAMP signaling can actually control the expression of C/EBPβ in hippocampal neurons, cultured hippocampal neurons were stimulated by the direct adenylate cyclase activator forskolin and changes of C/EBPβ transcripts were followed by RNase protection assay. After 4 h of exposure to forskolin, C/EBPβ mRNA was found to be prominently induced in cultured hippocampal neurons (Fig. 4A). The increase of C/EBPβ transcript was observed even from 30 min after addition of forskolin to the culture (data not shown). Consistent with the induction of mRNA, immunoblot analysis with anti-C/EBPβ antibodies showed that the amount of both the 39- and 33-kDa form of C/EBPβ are increased 4 h after forskolin treatment of the culture (Fig. 4B).

To examine whether the increase of C/EBPβ mRNA and protein can enhance its DNA binding activity to C/EBPβ sites, EMSA was performed using both nuclear extract from culture treated with forskolin for 4 h and nuclear extract from control culture. Forskolin treatment of the culture was found to increase the binding of C/EBPβ family members to the C/EBP site in EMSA (Fig. 4C, lanes 1 and 2). Anti-C/EBPβ antibodies induced a supershift, and the amount of these supershifted bands were greatly enhanced by forskolin treatment of the culture (Fig. 4C). A supershift by anti-C/EBPβ antibodies was also found to be increased by forskolin treatment of the culture (Fig. 4C). Unrelated antibodies such as anti-Rb antibodies do not induce any supershifts, and the addition of cold competitors almost erased the shifted bands demonstrating specificity of this EMSA. Thus, it is clear that binding of C/EBPβ to C/EBP binding sites is intensified in cultured hippocampal neurons after forskolin treatment. C/EBPβ and δ mRNA Are Induced in Hippocampal Slices by cAMP Signaling—(S)p-cAMPS is known to stimulate cAMP signaling in hippocampal slices (29, 41), which preserve the anatomical relation of neurons in the intact hippocampus. To examine whether stimulation of cAMP signaling can induce the mRNA expression of C/EBP family members even in hippocampal slices, rat and mouse hippocampal slices were treated with (S)p-cAMPS for 30 min. Two hours after the 30 min of (S)p-cAMPS treatment, RNAs were prepared from slices and used for RNase protection assay. Both C/EBPβ and δ mRNAs were induced by (S)p-cAMPS in mouse hippocampal slices (Fig. 5A). In rat slices, both C/EBPβ and δ mRNAs are induced after (S)p-cAMPS treatment (Fig. 5B). Thus, the increase of C/EBPβ and δ mRNA can be induced by the stimulation of cAMP signaling pathway, even in hippocampal slices. This result suggests that cAMP signal can induce mRNA expressions of C/EBP family members in intact neurons of hippocampal slices. Ca2+ Signal through CaM Kinase Activation Enhances C/EBPβ and δ Activities in Hippocampal Neurons, and CaMKIV Can Enhance Gene Transcription Mediated by C/EBPβ and δ—Previous studies indicated that Ca2+/calmodulin (CaM)-regulated systems can control CREB activity in hippocampal neurons (32, 33, 47). Our EMSA, which uses core promoter sequence of C/EBPβ gene as a probe, suggests that CREB can control the expression of C/EBPβ in hippocampal neurons (Fig. 3). Given that the expression of C/EBPβ can be controlled by CREB, C/EBPβ activity is predicted to be enhanced by Ca2+ signals in hippocampal neurons. To examine this possibility, hippocampal neurons in cultures were subjected to membrane depolarization by incubating them with high K+ solution for 5 s (33). Four hours after membrane depolarization, nuclear extracts were prepared from both depolarized neurons and control neurons incubated with normal Tyrode’s solution. EMSA using a C/EBP binding site as a probe showed that the DNA binding activity of C/EBPβ is augmented by membrane depolarization of hippocampal neurons (Fig. 6A). The DNA binding activity of C/EBPδ is also found to be enhanced by membrane depolarization (Fig. 6A). The increase of
C/EBPβ mRNA was observed in hippocampal neurons 4 h after membrane depolarization, suggesting that the enhanced DNA binding activity of C/EBPβ results from the increase of C/EBPβ mRNA (Fig. 6B).

In hippocampal neurons, CaM kinases, probably CaMKIV, can activate CREB after Ca²⁺ signal reaches nuclei of hippocampal neurons with heightened synaptic activities (33). To determine which signaling pathway is involved in the enhancement of C/EBPβ activity by membrane depolarization of hippocampal neurons, we depolarized hippocampal neurons in the presence of CaM kinase inhibitor KN93. Inactive form KN92 did not erase the increase of C/EBPβ activity in neurons that received membrane depolarization treatment (Fig. 7). In contrast, CaM kinase inhibitor KN93 clearly blocked the enhancement of DNA binding activities of C/EBPβ in depolarized neurons, indicating that CaM kinase pathway, probably CaMKIV activation, is involved in the increase of C/EBPβ activity by Ca²⁺ in hippocampal neurons (Fig. 7). Another CaM kinase inhibitor, KN62 also blocked the enhancement of C/EBPβ binding to DNA after membrane depolarization (data not shown). Both CaM kinases inhibitors also blocked the increase of C/EBPβ mRNA expression in hippocampal neurons after membrane depolarization (data not shown). These results indicate that Ca²⁺ signal enhances mRNA expressions of C/EBPβ and δ, and their DNA binding activities in hippocampal neurons through CaM kinases activation.

C/EBPβ has in its b-ZIP domain a consensus sequence for CaM kinases phosphorylation. CaMKII can actually phosphorylate C/EBPβ and enhance C/EBPβ-dependent gene transcription (48). Thus, it may be possible that CaMKIV has a similar positive effect to C/EBPβ-dependent gene transcription. To test this possibility, constructs for expressing the constitutively active form of CaMKIV (45), vectors for expressing C/EBPβ or δ and a reporter construct with C/EBP binding sites were transfected and C/EBP-dependent gene transcriptions were assayed by measuring luciferase activity. As a result, overexpression of active CaMKIV was found to enhance C/EBPβ-dependent gene transcription (Fig. 8). Unexpectedly, overexpression of active CaMKIV is found to significantly stimulate C/EBPδ-dependent transcription (Fig. 8). This result suggests that CaMKIV activated by Ca²⁺ signal can enhance either C/EBPβ- or δ-dependent gene transcription when these C/EBP family members are expressed within nuclei of hippocampal neurons with heightened synaptic activities.

FIG. 2. C/EBPβ and δ are expressed in hippocampal neurons. A and B, double immunofluorescence analysis demonstrating that C/EBPβ is expressed in the nuclei of MAP2 positive hippocampal neurons. C and D, double immunofluorescence analysis showing that C/EBPδ is also expressed in the nuclei of some MAP2 positive neurons.
DISCUSSION

We have found that C/EBPβ transcripts are expressed in mouse hippocampus more than another C/EBP member, C/EBPδ (Fig. 1A). In previous work, C/EBPa transcripts were detected in CA1 to CA4 regions of the mouse hippocampus by in situ hybridization analysis (27). However, we found that C/EBPa mRNA is hardly detectable in rat hippocampus even by RNase protection assay (data not shown). Although it may simply represent the species difference used in both studies, C/EBPa does not seem to constitute a major C/EBP member even in mouse hippocampus. Our experimental ground for this notion is that the antibodies to C/EBPa induce much weaker supershifts in EMSA of mouse hippocampus than the antibodies to C/EBPβ (Fig. 1B). Thus, supershift experiments have clearly demonstrated that C/EBPβ and C/EBPδ are expressed in hippocampal neurons subjected to depolarization. B, RNase protection assay showing induction of C/EBPβ and α mRNAs in rat hippocampal slices treated with (Sp)-cAMPS.
cultured hippocampal neurons by forskolin, a potent adenylate cyclase activator. Within 30 min after forskolin stimulation of the culture, phosphorylation of CREB was significantly induced in the nuclei of hippocampal neurons. It has been recently reported that forskolin increases the endogenous C/EBPβ mRNA expression in a neuronal cell line, Neuro 217 cells suggesting that forskolin might also induce its mRNA expression in primary neurons in culture (28). We actually observed that level of mRNA, protein, and DNA binding activity of C/EBPβ are augmented 4 h after forskolin treatment in hippocampal neurons (Fig. 4, A–C). Thus, it is possible that CREB activated by cAMP signaling pathway can induce the expression of C/EBPβ mRNA in hippocampal neurons. C/EBPδ, another member of C/EBP, was also induced in this culture by forskolin. It was previously demonstrated that C/EBPδ can be induced by the stimulation of cAMP signals in mouse cortical astrocytes in culture (49). Our immunocytochemistry suggests that C/EBPδ is moderately expressed in astrocytes, while it is weakly expressed in some hippocampal neurons (Fig. 2, C and D). Thus, our data suggest that stimulation of cAMP signaling in hippocampal neurons also enhances C/EBPδ expression through CREB activation in hippocampal neurons.

Membrane depolarization of hippocampal neurons induced the increase of mRNA expression and the DNA binding activity of C/EBPβ and δ (Fig. 6). Since CaM kinase inhibitors KN93 and KN62 blocked the enhancement of C/EBPβ mRNA and its activity by membrane depolarization of hippocampal neurons (Fig. 7), this indicates that activation of CaM kinases is involved in the induction of mRNA expression and DNA binding activity of C/EBPβ by membrane depolarization (Fig. 7). Bito et al. (33) recently reported that CaMKIV activated by Ca2+ signal can phosphorylate CREB and enhance its activity in hippocampal neurons in culture. Our data suggest that CREB binds to the essential promoter of C/EBPβ gene in cultured hippocampal neurons, as well as mice hippocampus (Fig. 3). It is possible that CaMKIV through CREB activation is critically involved in the enhancement of C/EBPδ expression and its activity in hippocampal neurons activated by membrane depolarization. We also observed that active CaMKIV can enhance both C/EBPβ- and C/EBPδ-dependent gene transcription (Fig. 8). It has been previously shown that CaMKII can augment C/EBPβ-dependent gene transcription by phosphorylating a consensus sequence within the leucine-zipper of C/EBPβ (48). However, CaMKII is found to be localized in the cytoplasm of the hippocampal neurons, even after synaptic activities of those neurons are intensified by strong direct depolarization (50). In contrast, CaMKIV is constantly localized in nuclei of hippocampal neurons before and after synaptic activation (50).

It may be concluded that CaMKII may not be an upstream kinase for C/EBPβ in hippocampal neurons unless CaMKII phosphorylates newly synthesized C/EBPβ in cytoplasm.

Rather, it is possible that nuclear CaMKIV phosphorylates C/EBPβ to increase C/EBPβ-dependent gene transcription in nuclei of hippocampal neurons. Though C/EBPδ does not have any known phosphorylation consensus sequence for CaM kinases, overexpression of the constitutively active form of CaMKIV significantly stimulated C/EBPδ-dependent gene transcription (Fig. 8). One possibility is that C/EBPδ is making heterodimer with an endogenous bZIP partner or an unknown

Fig. 7. CaM kinase inhibitor KN93 blocks the enhancement of C/EBPβ activity in hippocampal neurons subjected to membrane depolarization. In the presence of inactive analog KN92, membrane depolarization of hippocampal neurons enhances C/EBPβ activity, which is shown by the supershifted band with C/EBPβ antibodies (Antiβ). In contrast, KN93 completely blocks the increase of C/EBPβ activity in depolarized neurons. −, control culture; +, membrane depolarization.

Fig. 8. Overexpression of active form of CaMKIV increases C/EBPβ- and δ-dependent transcriptional gene activations. HeLa cells were cotransfected with 0.4 μg of C/EBP reporter construct along with 0.4 μg of each of the indicated plasmids: expression vectors for C/EBPβ, C/EBPδ, and constitutive active CaMKIV.

Fig. 9. Regulation of C/EBPβ and δ activities by cAMP and Ca2+. Regulation of C/EBPβ and δ activities by cAMP and Ca2+ in hippocampal neurons. Heightened synaptic activities of hippocampal neurons induce Ca2+ influx through glutamate receptor channels of NMDA-type (NMDA-R) and L-type Ca2+ channel (Ca2+). The synaptic activities in hippocampal neurons also induce cAMP signaling through modulatory input such as dopamine (R, dopamine receptor). Ca2+ and cAMP signals converge into the nuclei of hippocampal neurons to activate CREB. Activated CREB in turn induces the expression of C/EBPβ and δ. CaMKIV and cAMP kinase (PKA) with enzymatic activity may stimulate newly synthesized C/EBPδ and δ-dependent gene expressions to fully transactivate effector genes for long term memory (LTM). CaM KK, CaM kinase kinase.
physiological partner in host cell lines used in transfection assay. As synergy between β and δ or α and δ to C/EBP-dependent transcription could not be observed in the presence of the constitutively active form of CaMKIV, it is unlikely that C/EBPδ enhances its transcriptional activity by making heterodimers with endogenous C/EBPα or β. Alternatively, it may be possible that CaMKIV directly phosphorylates some sequences on C/EBPβ. This alternative might be addressed in the future. Thus, in addition to transcriptional control of C/EBPβ and δ by Ca²⁺, C/EBPδ signal might strengthen transcriptional activities of C/EBP family members using CaM kinases in hippocampal neurons.

A number of studies have indicated that both Ca²⁺ and cAMP signaling pathways have crucial roles in the process of learning and memory in many species (3–7, 35–39). Our data suggests that both cAMP and Ca²⁺ signal might enhance the expression of C/EBPβ and δ through CREB activation in hippocampal neurons with heightened synaptic activities during memory formation. Furthermore, CaM kinases may augment the transcriptional activities of C/EBPβ and δ whose expressions are increased in hippocampal neurons during certain phases of long term memory. Even protein kinase A might enhance C/EBPβ and C/EBPδ activity since C/EBPβ-δ-dependent gene transcription can be strengthened by the overexpression of the catalytic subunit of protein kinase A. Dual roles of these kinases in enhancing both expression and function of C/EBP family members might play pivotal roles while acting under the direction of CREB in hippocampal neurons for stabilization of long term memory (Fig. 9). Long term potentiation LTP is believed to be a basic synaptic mechanism for some kinds of learning and memory (51–53). Thomas and Hunt (54) recently observed that C/EBPβ mRNA levels were dramatically induced in the dentate granule cells 2 h after in vivo LTP induction in situ hybridization. Besides this observation, they could hardly detect any C/EBPβ mRNA in intact hippocampus. Their finding accords with our data, which show the induction of C/EBPβ mRNA by cAMP and Ca²⁺ in cultured hippocampal neurons, if we consider that Ca²⁺ and cAMP are crucial signals for induction and maintenance of several kinds of LTP (29, 41, 52, 55). To ask if C/EBP members are critically involved in synaptic plasticities such as hippocampal LTP and long term depression LTD or certain kinds of memory, it may be desirable to analyze mutant mice lacking the C/EBP family of transcription factors (23–26) or conditional knock-out mice (56).

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