Neurobiology of Learning and Memory 135 (2016) 50–56

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

The role of nuclear PKMζ in memory maintenance

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ARTICLE INFO

Article history:
Received 10 May 2016
Revised 8 June 2016
Accepted 13 June 2016
Available online 14 June 2016

Keywords:
PKMζ
Memory maintenance
Histone acetylation
CREB-binding protein
Translocation into nucleus

ABSTRACT

Recently, protein kinase M ζ (PKMζ) has emerged as an important player for maintaining memory. It has been reported that PKMζ regulates the trafficking of GluA2 in postsynaptic membranes to maintain memory. However, there has been no study on PKMζ outside the synaptic region regarding memory maintenance. Here, we found that PKMζ is transported to the nucleus in a neural activity-dependent manner. Moreover, we found that PKMζ phosphorylates CREB-binding protein (CBP) at serine residues and that PKMζ inhibition reduces the acetylation of histone H2B and H3. Finally, we showed that the amnesic effect of PKMζ inhibition can be rescued by enhancing histone acetylation level. These results suggest the possibility that nuclear PKMζ has a crucial role in memory maintenance.

1. Introduction

Learning and memory are unique abilities of higher organisms that aid in their survival. Information about the external world is internally transformed and stored in the neural circuits and can be used to guide future behavior. To date, extensive studies have revealed how memory is formed, consolidated, and extinguished in terms of signaling molecules and neural circuits (Kandel, 2001; Kandel, Dudai, & Mayford, 2014). The mechanism of memory maintenance, however, has not been studied to the extent of other stages of memory.

Recently, PKMζ, an atypical protein kinase C (PKC) isoform, has emerged as a key molecule in memory maintenance (Ling et al., 2002; Pastalkova et al., 2006; Sacktor, 2011; Tsokas et al., 2016). PKMζ is a constitutively active form of PKC because it lacks a regulatory subunit. Active PKMζ regulates N-ethylmaleimide-sensitive factor (NSF)-dependent GluA2 trafficking that ultimately maintains the synaptic strength of specific synaptic connections involved in memory (Migues et al., 2010; Yao et al., 2008). However, synapses are not in a static state, and synaptic molecules, including GluA2, are basally degraded and synthesized. If the synaptic configuration is not properly maintained in condition of continuous protein turnover, memories will not be maintained and will eventually collapse. Sustaining specific receptors such as AMPARs, including GluA2, on the synaptic membrane is effective for a relatively short time window; however, this mechanism cannot explain how some memories are maintained permanently. Thus, it is plausible that a transcriptional and translational mechanism may be involved in maintaining memories such as lifelong memories (Kandel et al., 2014).

To date, studies on PKMζ have focused on the function of PKMζ in synaptic regions. However, it has been recently revealed that PKMζ is located in the nucleus as well as postsynaptic sites (Hernandez, Oxberry, Crary, Mirra, & Sacktor, 2014). The presence of PKMζ in the nucleus raises the possibility that it can regulate the gene expression required for memory maintenance. In this study, we examined how PKMζ moves from cytosol into the nucleus and whether nuclear PKMζ can affect transcriptional and epigenetic regulation. Finally, we determined if epigenetic changes can rescue amnesia induced by PKMζ inhibition in the amygdala.

2. Materials and methods

2.1. Animals

Male C57BL/6NCrljBgi mice aged between 6 and 8 weeks were purchased from Orient Bio (Korea). Animals were housed in standard laboratory cages on a 12-h light-dark cycle and provided

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http://dx.doi.org/10.1016/j.nlm.2016.06.010
1074-7427/

with access to food and water ad libitum. Mice were used for all experiments 1–2 weeks after being housed in laboratory cages. All experiments were approved by the Institute of Laboratory Animal Resources of Seoul National University.

2.2. Primary neuronal cultures

Embryonic hippocampal neurons were prepared as previously described (Cho et al., 2015). Briefly, rat hippocampi were dissected from E17 embryos and dissociated mechanically after trypsin treatment. Approximately 1,200,000 cells/plate or 100,000 cells/coverslip were plated onto poly-D-lysine-coated 100 mm plastic culture dishes (for Fig. 2D) or cover slips (for Fig. 1C and D). After a 3–4 h recovery in MEM-EBBS with 2 mM glutamine, 10% FBS, 0.45% glucose, 0.11 mg/mL sodium pyruvate, and penicillin/streptomycin, cells were maintained in Neurobasal medium supplemented with B27, GlutaMAX, and penicillin/streptomycin.

2.3. Immunoprecipitation

HEK293T cells in 100-mm culture dishes were transfected with 16 μg of FLAG-CBP and 4 μg of pCMV-PKMζ expression plasmid (or 4 μg of EGFP-N1 expression plasmid for control groups). Two days later, transfected cells were harvested and washed with cold PBS. The collected cells were lysed with a buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, benzonase (Sigma), phosphatase-inhibitor cocktail (Roche), and protease-inhibitor cocktail (Roche). After centrifuging at 13,300 rpm for 15 min, the supernatant was diluted 1/10 with a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, and protease-inhibitor cocktail (Roche). After BCA assay for protein quantification, 1 mg of protein was incubated with 3-times washed 50 μl (bead volume) of mouse anti-FLAG M2 antibody-conjugated beads (Sigma) (or mouse IgG-Agarose (Sigma) for control groups) at 4 °C for 2 h. Subsequently, the beads were washed twice with a buffer containing 0.1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 2 mM EDTA and then twice with 1X TBS. Finally, the immunoprecipitate was eluted by adding 3XFLAG peptides (Sigma).

Western blotting was performed as previously described (Li et al., 2010). The samples were separated on 4–12% Bis-Tris Plus Gels (Invitrogen) and then transferred to nitrocellulose blotting membranes (GE healthcare) overnight. After blocking with 5% non-fat milk, the membrane was incubated with the primary antibody (rabbit anti-phosphoserine (Millipore) 1:500 in 5% BSA, rabbit anti-phosphothreonine (Millipore) 1:500 in 5% BSA, mouse anti-PKCζ (Santa Cruz) 1:5000 in 5% milk, mouse anti-EGFP (NeuroMab) 1:5000 in 5% milk, and mouse anti-FLAG (Sigma) 1:10,000 in 5% milk) and horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody. After washing, the membrane was treated with Immobilon western chemiluminescent HRP substrate (Millipore) according to the manufacturer's instructions. Imaging was performed using the ChemiDoc MP system (Bio-Rad).

2.4. Co-immunoprecipitation

Co-immunoprecipitation and western blotting were performed as previously described (Jun et al., 2015; Kim et al., 2014). Briefly, HEK293T cells were transfected with a 3XFLAG-PKMζ (interaction between PKMζ and importin) or 3XFLAG-CBP and pCMV-PKMζ (interaction between PKMζ and CBP) expression plasmid. Immunoprecipitation was conducted as described above. The transferred membranes were incubated with the primary antibody (for PKMζ-importin: mouse anti-importin-α (Sigma) 1:1000, mouse anti-importin-β (Abcam) 1:1000, and mouse anti-FLAG (Sigma) 1:10,000, for PKMζ-CBP: mouse anti-PKC/Mζ (Santa Cruz) 1:5000, and mouse anti-FLAG (Sigma) 1:10,000) and horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody. After washing, the membrane was treated with Immobilon western chemiluminescent HRP substrate (Millipore) according to the manufacturer's instructions. Imaging was performed using the ChemiDoc MP system (Bio-Rad).

2.5. Kinase assay

Protein purification and *in vitro* kinase assay were performed as previously described (Lee et al., 2006). Briefly, GST, GST-CBP (1-668), and GST-CBP(1-668)S436A were overexpressed in the BL21 cell line and purified with glutathione Sepharose 4 Fast Flow (GE Healthcare). PKMζ-3XFLAG was overexpressed in the HEK293T cell line and purified with anti-FLAG M2 affinity gels (Sigma). For the *in vitro* kinase assay, 3.5 μg of substrate was incubated for 30 min at 37 °C in 25 μl of reaction solution (0.2 mM ATP, 1 mCi [γ-^{32}P]ATP, 50 ng of purified PKMζ protein, 50 mM Tris [pH 7.5], 10 mM $MgCl_2$). The zeta inhibitory peptide (ZIP, 10 μM) (Invitrogen) was added to verify the specificity of PKMζ kinase activity. Reactions were stopped by adding SDS sample buffer and heating to 95 °C for 10 min. Samples were separated by SDS-PAGE and analyzed with a Bio-Imaging Analyzer (BAS-2500, Fuji).

2.6. Purification of the nuclear fraction

At 15–17 days *in vitro* (DIV), cultured neurons were treated with 1 mM sodium butyrate (NaB; Sigma-Aldrich) or/and 10 μM ZIP (Invitrogen) for 1 h, followed by washing with PBS. After harvesting using a scrapper, neurons were lysed with TX buffer (50 mM Tris-Cl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) containing a protease inhibitor cocktail (Roche). The homogenate was incubated on ice for 15 min, followed by centrifugation at 500*g* at 4 °C for 10 min to purify the nuclear fraction. The supernatant was removed, and the pellet was lysed with TX buffer containing 0.2 N HC1 and protease inhibitor cocktail. After incubation on ice for 30 min, the nuclear lysate was subjected to centrifugation at 9300*g* at 4 °C for 10 min. The supernatant was used for western blot analysis.

2.7. Activity-dependent translocation of PKMζ

At 7 DIV, embryonic hippocampal cultured neurons were transduced with PKMζ-mCherry expressing adeno-associated virus (AAV) (2×10^9/well, coverslip in 12-well plate). PKMζ was tagged with mCherry at the C-terminus. The viral vector expressing the PKMζ-mCherry fusion protein under the CaMKIIα promoter was packaged into AAV (serotype 2/1) as previously reported (Choi et al., 2014). At 18 DIV, a chemical long-term potentiation (cLTP) stimulation protocol was used for inducing neural activity. Briefly, cultured neurons were incubated with cLTP solution (200 μM glycine, 20 μM bicuculline, 124 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES (pH 7.3), 10 mM glucose) for 5 min, and then the medium was exchanged with cLTP solution without glycine for 30, 60, or 90 min. After completing the cLTP stimulation protocol, cultured neurons were briefly washed with cold PBS and then fixed with 4% paraformaldehyde/4% sucrose in PBS for 15 min on ice. After fixation, cultured neurons onto the coverslip were again briefly washed with PBS and mounted on the slide glass with mounting medium (VECTASHIELD containing DAPI). A confocal laser scanning microscope (LSM700, Zeiss) was used for obtaining images of PKMζ-mCherry signals. To obtain PKMζ-mCherry signals only in the nucleus, the focal plane was set to the region showing the largest DAPI signal. The ImageJ program was used for image analysis. Fluorescence intensity of the nucleus was divided by that of the cytosol (sum ratio, Fig. 1D left panel). To overcome the