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PKM ζ is necessary and sufficient for synaptic clustering of PSD-95

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Abstract

The persistent activity of protein kinase Mzeta (PKM ζ), a brain-specific, constitutively active protein kinase C isoform, maintains synaptic long-term potentiation (LTP). Structural remodeling of the postsynaptic density is believed to contribute to the expression of LTP. We therefore examined the role of PKM ζ in reconfiguring PSD-95, the major postsynaptic scaffolding protein at excitatory synapses. In primary cultures of hippocampal neurons, PKM ζ activity was critical for increasing the size of PSD-95 clusters during chemical LTP (cLTP). Increasing PKM ζ activity by overexpressing the kinase in hippocampal neurons was sufficient to increase PSD-95 cluster size, spine size, and postsynaptic AMPAR subunit GluA2. Overexpression of an inactive mutant of PKM ζ did not increase PSD-95 clustering, and applications of the ζ -pseudosubstrate inhibitor ZIP reversed the PKM ζ -mediated increases in PSD-95 clustering, indicating that the activity of PKM ζ is necessary to induce and maintain the increased size of PSD-95 clusters. Thus the persistent activity of PKM ζ is both necessary and sufficient for maintaining increases of PSD-95 clusters, providing a unified mechanism for long-term functional and structural modifications of synapses.

Keywords

PKM zeta; protein kinase Mzeta; PSD-95; LTP; cLTP; GluA2

Introduction

The persistent increased enzymatic activity of PKM ζ maintains both LTP, a sustained enhancement of synaptic transmission (Bliss and Collingridge, 1993), and several forms of long-term memory storage (Ling et al., 2002; Pastalkova et al., 2006; Shema et al., 2007; Sacktor, 2008; Sacktor, 2011; Shema et al., 2011). Both LTP and long-term memory are associated with persistent changes in the morphology of synapses (Kandel, 2001).

Postsynaptic structural modifications are most commonly observed in LTP, including increases in spine size (Yuste and Bonhoeffer, 2001; Lang et al., 2004; Yang et al., 2008), in the postsynaptic density (PSD) (Desmond and Levy, 1986; Bourne and Harris, 2010), and in clusters of PSD-95 (Antonova et al., 2001; El-Husseini Ael et al., 2002; Stein et al., 2003; Ehrlich and Malinow, 2004; Chen et al., 2007; Ehrlich et al., 2007), the major scaffolding protein of the PSD (Cheng et al., 2006). Whether the persistent activity of PKM ζ that maintains the functional enhancement of synaptic strength in LTP also regulates the structural reorganization of synapses is unknown. To address this question, we examined the role of PKM ζ in the reorganization of PSD-95 during a persistent, protein synthesis-dependent form of chemical LTP (cLTP) induced by forskolin (Gobert et al., 2008), that, like late-LTP induced by afferent tetanic stimulation, activates PKM ζ (Li et al., 2010).

Materials and Methods

Primary hippocampal culture and treatments

Primary hippocampal neurons were prepared from Sprague-Dawley E18/19 rats (Hilltop), according to the method described by Brewer et al. (Brewer et al., 1993). Cultured neurons were used after 12 to 18 days *in vitro*. Cultured neurons were treated with forskolin (50 μ M, Sigma) to induce a chemical form of late-phase LTP (Gobert et al., 2008). All drugs were stored as 100x concentrated stocks (ZIP in water, forskolin and chelerythrine in DMSO), which were added directly to culture media to obtain the final concentrations as indicated.

Preparation of Sindbis virus PKM ζ expression vectors

Sindbis virus vectors were used to express PKM ζ according to methods described in the Invitrogen Sindbis Expression Manual. PKM ζ is ligated into a plasmid vector (pSinRep5) under the control of the Sindbis subgenomic promoter using a PCR-based strategy. An insert corresponding to amino acids 184-592 of PKC ζ was obtained using a plasmid containing the mouse cDNA as a template. The forward primer (5'-AAATCTAGAACATGGCATACCCATACGACGTCC-CAGACTACGCTATGGATTCTGTCATGCCTTCC-3') was flanked by an XbaI restriction site followed by a sequence corresponding to enhanced green fluorescent protein (eGFP). The reverse primer (5'-AATTCTAGATCACACGGACTCC-TCAGC-3') was flanked by an XbaI restriction site. Plasmids containing eGFP alone (eGFP-control), eGFP-PKM ζ , or the kinase inactive mutation eGFP-PKM ζ -K281W (Drier et al., 2002; Ling et al., 2002; Shema et al., 2011) were replicated in Baby Hamster Kidney (BHK) cells and collected in Dulbecco's Modified Eagle Media for infection. Primary hippocampal neuronal cultures were infected with 1-5 μ l (1.2×10^6 – 2.3×10^6 infectious units/ml) of eGFP-control, eGFP-PKM ζ , or eGFP-PKM ζ -K281W. Sixteen to 20 hr after the infection, trypan blue staining of the cultures showed no discernible neuronal death (data not shown). In addition to eGFP expression (Fig. 2A, inserts), overexpression of eGFP-PKM ζ and eGFP-PKM ζ -K281W were verified by immunoblot analysis and phosphotransferase assay (data not shown) (Ling et al., 2002).

Immunofluorescence

Following drug treatments or transfection, cells were washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (PFA) with 4% sucrose in PBS for 15 min, and permeabilized by 0.01% Triton X-100 in PBS. Primary antibodies were incubated in PBS-Triton X-100 solution overnight at 4°C. For PSD-95 labeling, a rabbit polyclonal anti-PSD-95 (Abcam, ab18258) was used at 1:200 dilution. For PKM ζ and PSD-95 double labeling, a rabbit polyclonal anti-PKM ζ (1:500) (Hernandez et al., 2003) and a mouse monoclonal anti-PSD-95 (1:200) (NeuroMab, clone K28/43) were used. For labeling surface GluA2, the mouse monoclonal anti-GluA2 (1:200) (Chemico, Cat# 292) was applied

overnight at 4°C prior to permeation with PBS-Triton X-100 solution, then double-labeled with the rabbit anti-PSD-95 or rabbit anti-synaptophysin (1:1000) (Abcam, ab23754). Following the primary antibody, cells were washed in PBS-Triton X-100 and incubated with appropriate secondary antibodies conjugated to Alexa 488 or Alexa 568 (Molecular Probes/Invitrogen, Eugene, Oregon) at 1:400 dilutions for 1 hr. Cells on coverslips were mounted on glass slides with ProLong Anti-Fade Gold solution (Molecular Probes) for image analysis.

Image analysis

Images were obtained with an Olympus BX51 microscope equipped with epifluorescence lamp and Olympus DS70 digital camera. Images were acquired under a 100X oil objective with 4080 × 3072 pixel resolution and analyzed by ImagePro Software version 4.2 (Media Cybernetics, Inc). Ten to fifteen neurons with a soma diameter greater than 20 μm on each coverslip were randomly selected for image analysis. For each neuron, 300-900 puncta clusters in secondary dendrites were measured. Each dendritic field (~20 μm in length) was automatically analyzed after establishing an intensity threshold above background that was held constant for each experiment. The software measured pixel intensity over individual clusters (cluster intensity) and cluster size in 2 dimensions. The numbers were exported to Windows Excel spreadsheet for further analysis. Filters were set to exclude background noise of 1 pixel/cluster and to exclude overlaps with areas greater than 2 μm² (> 3 standard deviation of average cluster size). For colocalization analysis, cluster intensity for each color (red, green, or yellow) was measured in merged color images, e.g., PKMζ was labeled as red, PSD-95 was green, and their colocalization was yellow (green + red). To examine changes in spine size in primary and secondary dendrites of transfected neurons, we stained membrane with the lipophilic dye 1,1' - Dioctadecyl - 3,3,3',3' - tetramethylindocarbocyanine iodide (DiI, Sigma) applied as crystals adjacent to neurons in the cultures. Spines were defined as protruberances from dendrites with a length-to-width ratio less than 2 in order to exclude long processes such as filopodia. Methods for quantitative immunoblotting used to measure total PSD-95 (PSD-95 antiserum, 1:500) were as previously described (Sacktor et al., 1993).

Statistical analysis

Data are expressed as mean ± SEM of immunostaining puncta intensity or size in a 20 μm dendritic segment for each treatment, i.e., n is number of dendritic segments. Single treatments were compared with control using Student's two tail *t*-test, with 95% confidence. Multiple treatments were compared by one-way ANOVA with a minimum criterion of *p* < 0.05, followed by post hoc Tukey test. Data presented in each figure are from individual experiments, and experiments were repeated 3 or more times with comparable results.

Results

PKMζ activity is required for increases in PSD-95 cluster size during cLTP

Applications of forskolin for 45 min to primary cultures of hippocampal neurons increased the immunostaining intensity of PSD-95 clusters (Fig. 1A, B, *p* < 0.05, *n* = 16), which coincided with increases in colocalization with PKMζ (Fig. 1A-C, *p* < 0.05; *n* = 16). The cLTP did not alter the amount of PSD-95 in the cultures as measured by immunoblot (relative to controls set at 100%, cLTP, 101.5 ± 2.8%; *p* > 0.05; *n* = 6), suggesting that the cLTP-induced increase in cluster immunostaining was due to a change in the organization, rather than an increase in the total amount of PSD-95.

To determine whether PKMζ activity is required for the increases in PSD-95 clustering induced by cLTP, we applied ZIP, the selective ζ-inhibitory peptide (5 μM) (Serrano et al.,

2005), together with forskolin for 1 hr (Fig. 1D-F). Applications of ZIP prevented the cLTP-induced increase in PSD-95 cluster intensity ($p < 0.01$, relative to cLTP; $p > 0.05$, relative to control, $n = 26$). ZIP applied to the cultures alone for 1 hr did not change PSD-95 cluster immunostaining (Fig. 1D-F, $p > 0.05$, $n = 26$) or the total amount of PSD-95 measured by immunoblot (ZIP, $100.4 \pm 4.6\%$ of controls; $p > 0.05$; $n = 6$). Because applications of forskolin for 45 min were sufficient to increase PSD-95 immunostaining (Fig. 1A, B), to determine whether PKM ζ inhibition could disrupt previously clustered PSD-95, cLTP was first induced by applying forskolin alone for 45 min as in Fig. 1A-C, and then ZIP co-applied for an additional 15 min. ZIP reversed the increase in PSD-95 intensity (Fig. 1D-F, $p < 0.01$, relative to cLTP; $p > 0.05$, relative to controls, $n = 26$). A second PKM ζ inhibitor, the PKC catalytic domain inhibitor chelerythrine ($5 \mu\text{M}$) (Ling et al., 2002) also reversed cLTP-induced increases in PSD-95 intensity (relative to controls set at 100%, cLTP 1 h, $120.1 \pm 10.4\%$, $p < 0.05$; cLTP 1 h with chelerythrine for the last 15 min, $92.0 \pm 17.5\%$, $p > 0.05$, relative to controls; chelerythrine alone 1 h, $92.4 \pm 10.4\%$, $p > 0.05$, relative to controls; $n = 9$ for each condition).

PKM ζ overexpression increases PSD-95 cluster size, spine size, and postsynaptic GluA2

PKM ζ activity is thus necessary for increasing PSD-95 cluster size in neurons, but is it sufficient? To address this question, we introduced into hippocampal neurons by transfection PKM ζ fused to eGFP (eGFP-PKM ζ), eGFP alone, or a catalytically inactive fusion protein (eGFP-PKM ζ -K281W) (Fig. 2A-C). Compared with eGFP-transfected controls, neurons expressing eGFP-PKM ζ for 16 hr showed increases in PSD-95 cluster intensity (Fig. 2A, B, $p < 0.01$, $n = 27$) and size (Fig. 2A, C), similar to that observed after cLTP. As in cLTP, the total amount of PSD-95 in the cultures as measured by immunoblot did not change with PKM ζ overexpression (relative to eGFP controls set at 100%, eGFP-PKM ζ , $107.6 \pm 2.4\%$, $n = 9$, $p > 0.05$). In contrast to eGFP-PKM ζ , neurons overexpressing the inactive eGFP-PKM ζ -K281W showed no change in PSD-95 cluster intensity and size (Fig. 2A-C, $p < 0.05$, relative to eGFP-PKM ζ , $p > 0.05$, relative to eGFP controls, $n = 27$). Furthermore, after 16 hr of transfection, applications of ZIP to the eGFP-PKM ζ -transfected neurons for an additional 4 hr (at $10 \mu\text{M}$ to inhibit overexpressed amounts of the kinase) reversed the increase in PSD-95 clustering (Fig. 2A-C, $p < 0.05$, relative to eGFP-PKM ζ transfected for 20 hr; $p > 0.05$, relative to eGFP controls, $n = 27$). Overexpression of eGFP-PKM ζ also increased spine size, as compared to eGFP control ($p < 0.001$, $n = 29$ dendritic branches, Supporting Information Fig. 1A, B; see also eGFP staining of spines in Fig. 2D). In contrast, overexpression of eGFP-K281W did not alter spine size relative to eGFP ($p > 0.05$, $n = 29$, Supporting Information Fig. 1A, B). There were no significant differences in spine number per unit length of dendrite (spine density) among the three groups ($p > 0.05$, $n = 29$, Supporting Information Fig. 1C).

Previous studies have shown that PKM ζ causes a redistribution of AMPARs to synapses, increasing the number of postsynaptic receptors through trafficking of the GluA2 subunit of the receptor (Ling et al., 2006; Yao et al., 2008; Miguels et al., 2010). To test whether PSD-95 clustering coincides with the reorganization of these receptors by PKM ζ , we determined the distribution of surface GluA2 colocalized with PSD-95 ("synaptic GluA2"), as well as surface GluA2 that was not localized with PSD-95 ("extrasynaptic GluA2," Fig. 2D, E). Compared with eGFP controls, eGFP-PKM ζ increased synaptic GluA2 ($p < 0.01$, $n = 29$) and decreased extrasynaptic GluA2 ($p < 0.01$, $n = 29$). To confirm a PKM ζ -mediated redistribution of GluA2 to synapses, in separate experiments we found that PKM ζ overexpression increased GluA2 colocalized with the presynaptic marker synaptophysin ($p < 0.01$, $n = 27$) and decreased GluA2 without synaptophysin colocalization ($p < 0.01$, $n = 27$; Fig. 2F). In contrast to the increase in postsynaptic PSD-95 cluster immunostaining ($p <$

0.01, $n = 29$, Fig. 2E), presynaptic synaptophysin immunostaining intensity did not change after PKM ζ overexpression ($p > 0.05$, $n = 27$, Fig. 2F).

Discussion

PSD-95, the most abundant structural protein of the postsynaptic density, anchors and organizes postsynaptic signaling molecules and neurotransmitter receptors at excitatory synapses (Sheng and Hoogenraad, 2007; Blanpied et al., 2008; Chen et al., 2008). Overexpression of PSD-95 increases the number of postsynaptic AMPA receptors and is believed to contribute to LTP expression (El-Husseini Ael et al., 2002; Ehrlich and Malinow, 2004) (but see (Migaud et al., 1998)). The molecular mechanism causing the persistent reorganization of PSD-95 during LTP, however, was unknown.

Here we show that increases in the size of PSD-95 clusters induced by cLTP are mediated by the activity of PKM ζ . The PKM ζ inhibitors ZIP and chelerythrine, both of which reverse LTP maintenance (Ling et al., 2002; Serrano et al., 2005) and disrupt established long-term memory (Pastalkova et al., 2006; Serrano et al., 2008; Shema et al., 2009), also block increases in PSD-95 clustering during cLTP. Applications of these inhibitors as well as overexpression of the inactive mutation of PKM ζ that acts as a dominant negative (Drier et al., 2002; Ling et al., 2002; Shema et al., 2011) did not affect basal PSD-95 clusters under our conditions. These findings are in agreement with the ability of PKM ζ inhibitors to reverse LTP maintenance without affecting basal hippocampal synaptic transmission in slices (Ling et al., 2002; Serrano et al., 2005) or *in vivo* (Pastalkova et al., 2006; Madronal et al., 2010). Our results are also consistent with an earlier report showing that PKM ζ is important for stabilizing newly formed synapses in the actively remodeling circuit of the developing tadpole tectum (Liu et al., 2009). PI3-kinase, ERK, and mTOR are required for both forskolin-induced cLTP (Gobert et al., 2008) and the formation of PKM ζ in tetanus-induced LTP (Kelly et al., 2007). Thus the action of these kinases may be upstream of the PKM ζ -mediated changes in PSD-95 observed in cLTP.

How persistent PKM ζ activity regulates the aggregation of PSD-95 remains to be elucidated. Although PKM ζ overexpression increased PSD-95 clustering and PKM ζ inhibition decreased cLTP-dependent PSD-95 clustering, no changes in the amount of PSD-95 protein level were detected after these manipulations. This suggests that PKM ζ increases PSD-95 clustering by regulating PSD-95 aggregation through post-translational modifications rather than by new synthesis of PSD-95, although increases in protein levels below our level of detection or counterbalanced synthesis and degradation cannot be excluded. Interestingly, the effect of PKM ζ on PSD-95 appears similar to that of palmitoylation, an important mechanism for PSD-95 clustering at the postsynaptic membrane (El-Husseini Ael et al., 2002; Noritake et al., 2009). Although the functionally relevant sites of phosphorylation that mediate PSD-95 aggregation by PKM ζ in hippocampal neurons are yet not known, a recent paper indicates that in the developing visual cortex PKM ζ regulates PSD-95 transport to synapses through the palmitoylation enzyme ZDHHC8 (Yoshii et al., 2011). Further work will be required to examine whether PKM ζ mediates PSD-95 clustering in hippocampal neurons through a similar mechanism.

PSD-95 clustering by PKM ζ may serve two purposes in LTP and memory maintenance. First, PKM ζ maintains LTP and long-term memory by altering AMPAR trafficking, sustaining a persistent increase in the number of postsynaptic AMPARs (Ling et al., 2006; Yao et al., 2008; Miguez et al., 2010). Our current results suggest this may involve a shift of the receptors from extrasynaptic surface pools, thought to be compartmentalized in lipid rafts (Hering et al., 2003), to postsynaptic sites (Fig. 2D-F). The increased size of the PSD-95 clusters maintained by PKM ζ may thus serve as a postsynaptic “slot” to receive the

receptors that have been transported from extrasynaptic sites. Second, PSD-95 is thought to increase the stability of synapses in the face of synaptic turnover (Gray et al., 2006; Ehrlich et al., 2007). The PKM ζ -mediated increases in PSD-95 cluster size and spine size that we observed may therefore allow those synapses persistently potentiated by PKM ζ after experience (Madronal et al., 2010) to participate in neuronal circuits longer than non-potentiated synapses. Thus PKM ζ -mediated aggregation of PSD-95 may contribute to increased synaptic efficacy and stability, both of which may be crucial for the ability of PKM ζ to maintain long-term memory (Pastalkova et al., 2006; Shema et al., 2007; Sacktor, 2011).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

PKM ζ increases PSD-95 clusters during cLTP. (A-C) cLTP increases immunostaining intensity of PSD-95 and colocalization of PKM ζ with PSD-95. (A) Representative hippocampal neurons in primary culture (upper panels, whole cells; lower panels, dendrites) double-labeled with antisera to PKM ζ and PSD-95, following treatment with vehicle (control) or 50 μ M forskolin (cLTP) for 45 min. As shown in the merged image (right), cLTP increases PKM ζ (red) and PSD-95 (green) colocalization (yellow). Scale bar, 20 μ m for images of the whole neurons and 5 μ m for images of dendrites. (B) Compared to control cultures, cLTP increases the intensity of immunostaining of PSD-95 clusters and the colocalization of PKM ζ and PSD-95 (mean $\pm$ SEM; * indicates significance in all figures). (C) Cumulative frequency plot shows cLTP increases areas of colocalization of PKM ζ and PSD-95. Mean colocalization area is $0.11 \pm 0.01 \mu\text{m}^2$ for control and $0.15 \pm 0.01 \mu\text{m}^2$ for cLTP ($p < 0.05$, $n = 16$). (D-F) PKM ζ activity is required for cLTP-induced increases in PSD-95 clusters. (D) Representative images of dendrites following treatment with vehicle (control), 50 μ M forskolin for 1 hr (cLTP) in the presence or absence of 5 μ M ZIP, 50 μ M forskolin for 1 hr with ZIP applied for the last 15 min, or ZIP alone for 1 hr. Scale bar, 5 μ m. (E) Compared to controls, cLTP for 1 hr increased average PSD-95 cluster intensity. The application of ZIP either for the 1 hr or for the last 15 min blocked the cLTP-induced increase in PSD-95 cluster intensity; ZIP alone produced no change relative to untreated controls. (F) Cumulative frequency plot shows cLTP increases PSD-95 cluster size and blockade of increases by ZIP. Mean size of PSD-95 clusters (in μm^2): control, 0.13 ± 0.01 ; cLTP, 0.16 ± 0.01 ; cLTP (1 h) + ZIP (1 h), 0.11 ± 0.01 ; cLTP (1 h) + ZIP (15 min), 0.12 ± 0.01 ; ZIP alone (1 h), 0.12 ± 0.01 ($p < 0.05$ between cLTP and other conditions, $n = 26$).

FIGURE 2.

PKM ζ overexpression increases PSD-95 clustering and postsynaptic GluA2. (A-C) PKM ζ activity is necessary and sufficient for increased PSD-95 clustering. (A) Hippocampal neurons were transfected with Sindbis viral vector expressing eGFP alone (control), eGFP-PKM ζ , the inactive eGFP-PKM ζ -K281W, or eGFP-PKM ζ followed by ZIP. Sixteen–twenty hours after transfection, the neurons (detected by eGFP fluorescence, insets in upper panels) were fixed and labeled with PSD-95 antiserum (upper panel, whole cells; lower panels, dendrites). Scale bars, 20 μm for images of the whole neurons and 5 μm for images of dendrites. (B) Compared to eGFP controls, PKM ζ overexpression increases PSD-95 cluster intensity; eGFP-PKM ζ -K281W or addition of ZIP to eGFP-PKM ζ for an additional 4 hr prior to fixation are not significantly different from controls. The PSD-95 cluster intensities for control cultures transfected with eGFP for 16 and 20 hr were indistinguishable, and cultures transfected with eGFP-PKM ζ for 16 and 20 hr were indistinguishable; thus, for clarity, only the data for 16 hr are shown. (C) Cumulative frequency plot shows PKM ζ overexpression led to a rightward shift in PSD-95 cluster size, similar to that in cLTP (compare Fig. 1F). Average size of PSD-95 clusters (in μm^2): eGFP controls, 0.14 ± 0.01 ; eGFP-PKM ζ , 0.18 ± 0.01 ; eGFP-PKM ζ -K281W, 0.14 ± 0.01 ; eGFP-PKM ζ + ZIP, 0.14 ± 0.01 ($p < 0.05$ between eGFP-PKM ζ and the other conditions, $n = 27$). (D-F) PKM ζ overexpression increases synaptic GluA2 and decreases extrasynaptic GluA2. (D) Representative dendrites double-labeled for surface GluA2 and PSD-95. Colocalization

of GluA2 and PSD-95 is defined as synaptic GluA2 (yellow), and GluA2 without PSD-95 as extrasynaptic. The fluorescence of eGFP is shown above. Scale bar is 5 μm . (E) Compared to eGFP controls, eGFP-PKM ζ increases synaptic GluA2 and decreases extrasynaptic GluA2. As expected, eGFP-PKM ζ also increases PSD-95 cluster intensity. (F) Colocalization of surface GluA2 and synaptophysin (synaptic) and surface GluA2 without synaptophysin (extrasynaptic) also shows that eGFP-PKM ζ expression increases synaptic GluA2 and decreases extrasynaptic GluA2. In contrast to PSD-95 (E), the presynaptic synaptophysin immunostaining intensity is not increased by eGFP-PKM ζ overexpression.