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Elevated RalA activity in the hippocampus of PI3Kγ knock-out mice lacking NMDAR-dependent long-term depression

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Phosphoinositide 3-kinases (PI3Ks) play key roles in synaptic plasticity and cognitive functions in the brain. We recently found that genetic deletion of PI3Kγ, the only known member of class IB PI3Ks, results in impaired *N*-methyl-D-aspartate receptor-dependent long-term depression (NMDAR-LTD) in the hippocampus. The activity of RalA, a small GTP-binding protein, increases following NMDAR-LTD inducing stimuli, and this increase in RalA activity is essential for inducing NMDAR-LTD. We found that RalA activity increased significantly in PI3Kγ knockout mice. Furthermore, NMDAR-LTD-inducing stimuli did not increase RalA activity in PI3Kγ knockout mice. These results suggest that constitutively increased RalA activity occludes further increases in RalA activity during induction of LTD, causing impaired NMDAR-LTD. We propose that PI3Kγ regulates the activity of RalA, which is one of the molecular mechanisms inducing NMDAR-dependent LTD. [BMB Reports 2013; 46(2): 103-106]

INTRODUCTION

Phosphoinositide 3-kinases (PI3Ks) are a family of enzymes involved in numerous cellular functions such as cell growth, survival, proliferation, migration, and intracellular vesicular transport (1-3). PI3Ks have unique and complex kinase properties, with dual-kinase activity comprised of both lipid kinase activity, transferring phosphate to the 3-position of the inositol ring in phosphoinositide substrates, and protein kinase activity, transferring phosphate to specific Ser/Thr on protein substrates (4, 5). PI3Ks have been classified into three major classes such as class I, class II, and class III based on their structural features and functional similarities. Class I PI3Ks are further subdivided into classes IA and IB according to the composition of the catalytic and regulatory subunits constituting the PI3K heterodimers (1, 2, 6). The roles of PI3Ks in synaptic plasticity of receptor trafficking, fear memory, memory retrieval and memory extinction in the brain have been studied extensively (7-14). In contrast to previous studies that have focused on broad ranges of PI3K activities and used general PI3K inhibitors such as wortmannin and LY294002, our group recently reported the specific roles of PI3Kγ, the only known member of class IB PI3Ks (15). In that study, we reported that genetic deletion or pharmacological inhibition of PI3Kγ impairs *N*-methyl-D-aspartate receptor-dependent long-term depression (NMDAR-LTD) and behavioral flexibility. We also revealed that p38 mitogen-activated protein kinase (MAPK), but not glycogen synthase kinase 3 beta or Akt, is involved in PI3Kγ-mediated NMDAR-LTD.

Ras-like protein A (RalA) is a well-known small GTPase involved in membrane trafficking such as endocytosis and exocytosis and is thought to have a role in NMDAR-LTD (16-18). In particular, a study showed that NMDAR activation by NMDA in cultured neurons increases RalA activity (17). Furthermore, PI3Ks regulate RalA activation via the Rap1/PI3K/RalGEFs pathway (19-23).

As NMDAR-LTD is a complex cellular event involving dozens of molecules, we proposed that other molecules, besides p38 MAPK, might have a role in PI3Kγ-mediated NMDAR-LTD. In this study, we examined the activity of RalA as a putative downstream molecule in PI3Kγ-mediated NMDAR-LTD.

RESULTS AND DISCUSSION

We performed a RalA pull-down assay after applying an NMDAR-LTD-inducing stimulus to hippocampal slices from PI3Kγ knock-out (KO) mice and their wild-type littermates to

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investigate changes in RalA activity (24). We delivered a low-frequency stimulus (LFS; 1 Hz, 900 s) to acute hippocampal slices, submitting only the CA1 region of each slice to the RalA pull-down assay. We reported previously that NMDAR-LTD is robustly induced by LFS under our experiment conditions (15). The GTP-bound active form of RalA specifically binds to GST-RalBD. In accordance with previous studies, the proportion of RalA in its active form increased significantly by LFS treatment in wild-type littermates (17). However, without LFS, RalA activity increased in PI3Kγ KO mice in comparison to that in wild-type littermates, and LFS induced no further increase in RalA activity (Fig. 1). This result suggests that genetic deletion of PI3Kγ causes increased RalA activity and impedes the activity-dependent increase in RalA activity by LFS. We conclude from these results that the impaired NMDAR-LTD in PI3Kγ KO mice is caused by excessive RalA activity.

We found abnormally elevated RalA activity in PI3Kγ KO mice using electrophysiology and biochemistry. Unlike wild-type littermates, the LFS stimulus failed to induce increases in RalA activity in PI3Kγ KO mice. We propose that elevated RalA activity in PI3Kγ KO mice may impair induction of NMDAR-LTD.

The main question arising from our results concerns the mechanism underlying the increase in basal RalA activity in PI3Kγ KO mice. Previous studies have shown that RalA is activated by cAMP-mediated activation of protein kinase A in epithelial cells (25, 26). This implies that increases in cAMP might increase RalA activity. PI3Kγ has been extensively studied in the heart as a key molecule regulating cardiac contractility. PI3Kγ negatively regulates the cAMP level in cardiomyocytes via direct interaction with phosphodiesterase 3B (27-29). Based on these results, we might assume that basal cAMP levels would increase in PI3Kγ KO mice brains, as well as their hearts, leading to increased RalA activity.

Another important question is how the basally occluded RalA activity prevents the induction of NMDAR-LTD in PI3Kγ KO mice. A previous study showed that activated RalA, mainly induced by activating NMDAR, directly binds to Ral-binding protein 1 (RalBP1) and promotes translocation of both proteins to dendritic spines, which is essential for NMDAR-LTD (17). We assumed that the increase in RalA activity via NMDAR activation is important to trigger the translocation of RalBP1 to dendritic spines. The absence of an activity-dependent increase in RalA activity in PI3Kγ KO mice may fail to recruit RalBP1 to synapses, which causes impairment of NMDAR-LTD. Furthermore, previously activated RalA without NMDAR activation may disrupt recruitment of other components critical for LTD induction. Further studies are required to elucidate the precise mechanisms of altered RalA activity in PI3Kγ KO mice.

The occluded RalA activity in PI3K γ KO mice suggests that occlusion of NMDAR-LTD occurs via reduced AMPAR levels in the basal-state. However, our previous study showed that basal synaptic transmission of PI3Kγ KO mice is normal, indicating that occluded RalA activity has no effect on AMPAR levels in the basal state (15). This is also supported by another study showing that overexpression of the constitutive active form of RalA does not change basal AMPAR levels in cultured neurons (17).

Taken together, our study showed that genetic deletion of PI3Kγ causes an elevation in RalA activity, which might be one of the main reasons for NMDAR-LTD impairment in PI3Kγ KO mice. These findings also support a novel functional relationship between PI3Kγ and RalA, two key molecules involved in NMDAR-LTD.

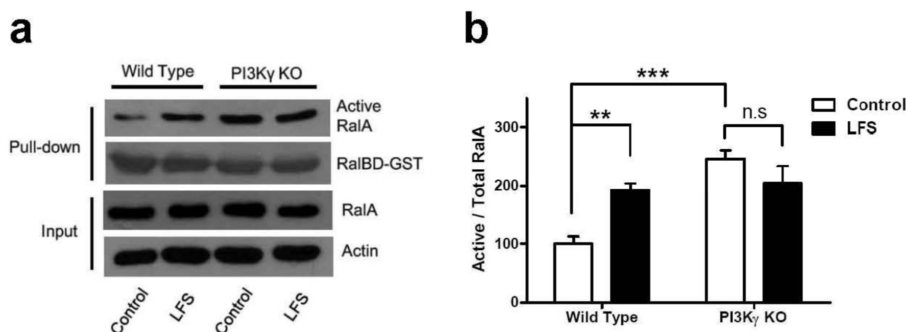

Fig. 1. RalA activation by *N*-methyl-d-aspartate receptor-dependent long-term depression (NMDAR-LTD) stimulus in phosphoinositide 3-kinase (PI3K)γ knockout (KO) and wild-type littermates. (A) A representative Western blotting image. RalA activity changes in CA1 regions after the NMDAR-LTD stimulus in hippocampi of wild-type littermates and PI3Kγ KO mice. (B) Quantification of active/total RalA ratio. The ratio of active/total RalA was normalized to that of the wild-type littermate control group (n = 3). The stimulus-by-genotyping interaction was statistically significant as assessed by two-way analysis of variance ($F_{1,8}$ = 12.92, P < 0.01). Post-hoc analysis using Tukey's test revealed significant differences between the control and low-frequency stimulus (LFS) group within the wild-type (**P < 0.01) and between wild-type littermates and PI3Kγ KO mice within the control group (***P < 0.001).

MATERIALS AND METHODS

Animals

The care and information of *Pik3cg*$^{-/-}$ and wild-type littermates have been described previously (15, 30). Animals were cared for in accordance with the regulations and guidelines of the Animal Care and Use Committee of Seoul National University

Sample preparation

Transverse hippocampal slices (400-μm thick) were prepared from 4-5-week-old PI3Kγ wild-type littermates and KO mice using a manual tissue chopper. Slices were recovered in an interface chamber for at least 2 h before applying LFS-LTD (900 stimuli, 1 Hz). The interface chamber was maintained at 28°C and consistently supplied with oxygenated artificial cerebrospinal fluid containing 124 mM NaCl, 2.5 mM KCl, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 2 mM $CaCl_2$, and 2 mM $MgSO_4$. The intensity of the LTD stimulus was adjusted to approximately 40% of the maximum field excitatory postsynaptic potential slopes. Each slice was incubated for an additional 20 min following delivery of LFS-LTD, and the CA1 regions from each slice were immediately frozen in liquid nitrogen.

RalA pull-down assay with stimulated slices

The CA1 regions of stimulated hippocampal slices from individual mice were pooled to obtain 300-400 μg of protein from each mouse. The RalA pull-down assay was performed as described previously (24). Briefly, 40 μg of GST-RalBD was preincubated with 35 μl of glutathione-Sepharose beads at 4°C for 1 h and washed three times. Sample tissue was lysed in Ral buffer (10% glycerol, 1% Nonidet P-40, 50 mM Tris-HCl, 200 mM NaCl, and 10 mM $MgCl_2$) with protease inhibitor cocktail (Roche, Manheim, Germany) and phosphatase inhibitor cocktails I and II (Sigma, St. Louis, MO, USA), and lysates were centrifuged at 16,000 × g for 20 min at 4°C. The supernatant was added to GST-RalBD pre-coupled beads and incubated for 4 h at 4°C. The incubated beads were washed four times in Ral buffer, and surplus buffer was removed with a 1-ml syringe. Proteins were eluted with sodium dodecyl sulfate (SDS) sample buffer and resolved on SDS-polyacrylamide gel electrophoresis. The amount of proteins was measured by Western blotting using the following antibodies: anti-RalA (1 : 5,000; BD Biosciences, San Diego, CA, USA), anti-actin (1 : 10,000; Sigma), and anti-GST (1 : 10,000; Bethyl Laboratories, Montgomery, TX, USA).

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