ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 10, Issue, 05, pp.69406-69409, May, 2018

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

PKCε ACTIVATOR DCP-LA FACILITATES ASSEMBLY OF NSF/MYOSINE Va/α7 ACH RECEPTOR

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ARTICLE INFO

Article History:
Received 22nd February, 2018
Received in revised form
03rd March, 2018
Accepted 20th April, 2018
Published online 30th May, 2018

Key words:
PKCε,
Myosin Va, NSF, α7 ACh Receptor,
Association.

ABSTRACT

The PKCε activator 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) stimulates vesicular transport of α7 ACh receptor. The present study was conducted to explore the underlying mechanism using rat hippocampal slices. DCP-LA enhanced serine phosphorylation of the motor protein myosin Va, that moves on the actin filament towards the plus-end (the plasma membrane) and carries cargos, and the effect was abrogated by the PKC inhibitor GF109203X. DCP-LA increased an association of *N*-ethylmaleimide-sensitive factor (NSF) and myosin Va, but not myosin VI, in a PKC-dependent manner. Moreover, DCP-LA increased an association of myosin Va and α7 ACh receptor in a PKC-dependent manner. Overall, the results of the present study indicate that PKCε, activated by DCP-LA, phosphorylates myosin Va, to increase assembly of NSF/myosinVa/α7 ACh receptor. This may account for DCP-LA-induced stimulation of vesicular transport and exocytosis of α7 ACh receptor.

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Citation: Tomoyuki Nishizaki. 2018. "PKCε activator DCP-LA facilitates assembly of NSF/myosine Va/α7 ACh receptor", *International Journal of Current Research*, 10, (05), 69406-69409.

INTRODUCTION

N-Ethylmaleimide-sensitive factor (NSF), an ATPase, regulates vesicular transport of neurotransmitter receptors such as AMPA receptor, $GABA_A$ receptor, $GABA_B$ receptor, β_2-adrenergic receptor, D_1 and D_2 dopamine receptors, and muscarinic M_1, M_3, M_4 and M_5 ACh receptors (Chen and Liu, 2010; Collingridge and Isaac, 2003; Collingridge*et al*., 2004; Cong *et al.,* 2001; Haas, 1998;Heydorn*et al*. 2004;Leil*et al*., 2004; Lin and Sheng,1998;Pontier*et al*., 2006; Zhao*et al*., 2007; Zou*et al*., 2005). NSF associates with soluble NSF attachment protein (SNAP), that binds to SNAP receptors (SNAREs) including syntaxin, SNAP25, and synaptobrevin. The vesicular SNARE synaptobrevin, which associates with cargo-containing transport vesicle, assembles the target SNAREs syntaxin and SNAP25, and in turn, SNAP binds to the SNARE assembly, followed by NSF binding. Motor proteins include kinesin, that moves on the microtuble mostly towards the plus-end (the plasma membrane), dynein, that moves on the microtuble towards the minus-end (the cytosol), and myosin, that moves on the actin filament, and carry cargos containing neurotransmitters, hormones, and neurotransmitter receptors. Of myosins myosin V moves towards the plus-end, involving exocytosis (Hammer and Wagner, 2013) and myosin VI moves towards the minus-end, involving clathrin-mediated endocytosis (Buss*et al*., 2001). The small G-protein Rab3 Aserves as an adaptor protein that connects the synaptic vesicle to myosin V by activating the Rab3A effector Rabphilin-3A (Li, 1996; Lindsay*et al*., 2013). The linoleic acid derivative 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) activates PKCε selectively (Kanno *et al*., 2006; Kanno *et al*., 2015) and stimulates vesicular transport of α7 ACh receptor (Kanno *et al*., 2012). The mechanism underlying DCP-LA-induced vesicular transport of α7 ACh receptor, however, is far from understanding. To address this question, the present study investigated the implication of NSF and myosin Va in α7 ACh receptor traffic. The results show that DCP-LA enhances serine phosphorylation of myosin Va in a PKC-dependent manner, causing assembly of NSF/myosin Va/α7 ACh receptor, which could trigger vesicular transport and exocytosis of α7 ACh receptor.

MATERIALS AND METHODS

Immunoprecipitation and Western Blotting: Rat hippocampal slices were treated with dimethyl sulfoxide (DMSO) or DCP-LA (100 nM) in the presence and absence of GF109203X (GF)

(100 nM) for 20 min in a standard artificial cerebrospinal fluid (117 mM NaCl, 3.6 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11.5 mM glucose) oxygenated with 95% O_2 and 5% CO_2 at 34 °C. Then, slices were homogenized by sonication in TBS-T [150 mM NaCl, 0.1% (v/v) Tween-20 and 20 mM Tris, pH 7.5] containing 1% (v/v) phosphatase inhibitor cocktail and subsequently, homogenates were centrifuged at 3,000 rpm for 5 min at 4 °C. The supernatants (200 μg of protein) were incubated with an antibody against NSF (Invitrogen, Waltham, MA USA) or myosin Va (Invitrogen) overnight at 4 °C. Then, 20 μL of protein G sepharose (GE healthcare, Piscataway, NJ, USA) was added to the extracts and incubated for 60 min at 4 °C. Pellets were washed three times with TBS-T and dissolved in 30 μL of a sodium dodecyl sulfate (SDS) sample buffer [0.2 mM Tris, 0.05% (w/v) SDS, and 20% (v/v) glycerol, pH 6.8]. After boiling for 5 min, proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a TGX gel (BioRad, Hercules, CA, USA) and then transferred to polyvinylidene difluoride membranes. Blotting membranes were blocked with TBS-T containing 5% (w/v) bovine serum albumin and subsequently incubated with an antibody against phospho-serine (pSer)(QIAGEN, Hilden, Germany), myosin Va (Invitrogen), myosin VI (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Rab3A (Santa Cruz Biotechnology), Rabphilin-3A (Santa Cruz Biotechnology), or α7 ACh receptor (Sigma, St. Louis, MO, USA). After washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG antibody. Immunoreactivity was detected with an ECL kit (GE Healthcare) and visualized using a chemiluminescence detection system (GE Healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

Statistical analysis: Statistical analysis was carried out using analysis of variance (ANOVA) followed by a Bonferonni correction.

RESULTS

DCP-LA increases serine phosphorylation of myosin Vaandstimulatesan association of NSF/myosin Va in a PKC-dependent manner: DCP-LA significantly enhanced the immunoreactive signal for pSer-myosin Va in the immunoprecipitants with an anti-myosin Va antibody from rat hippocampal slices, which is abrogated by the PKC inhibitor GF109203X (Figure 1). This implies that DCP-LA is implicated in PKC-mediated serine phosphorylation of myosin Va. DCP-LA enhanced the immunoreactive signal for myosin Va in immunoprecipitants with an anti-NSF antibody from rat hippocampal slices, and the effect was suppressed by GF109203X (Figure 2). This indicates that DCP-LA increases an association of NSF/myosin Va in a PKC-dependent manner. In contrast, no immunoreactive signal for myosin VI was found in immunoprecipitants with an anti-NSF antibody, although the signal was actually detected in lysates from rat hippocampal slices (Figure 2). This accounts for no association of NSF/myosin VI. Collectively, these results, in the light of the fact that DCP-LA activates PKCε selectively (Kanno *et al*., 2006; Kanno *et al*., 2015), raise the possibility that PKCε, activated by DCP-LA, phosphorylates myosin Va at the serine residues, to stimulate an association of NSF/myosin Va.

DCP-LA does not affect an association of NSF/Rab3A or NSF/Rabphilin-3A: The immunoreactive signals for Rab3A and its effector Rabphilin-3A were detected in immunoprecipitants with an anti-NSF antibody from rat hippocampal slices (Figure 3), indicating an association of NSF/Rab3A or NSF/Rabphilin-3A. DCP-LA had no significant effect on the immunoreactive signals for Rab3A and Rabphilin-3A in in immunoprecipitants with an anti-NSF antibody in the presence and absence of GF109203X (Figure 3). This indicates that DCP-LA does not affect an association of NSF/Rab3A or NSF/Rabphilin-3A.

DCP-LA stimulates an association of myosin Va/α7 ACh receptor in a PKC-dependent manner: DCP-LA enhanced the immunoreactive signal for α7 ACh receptor in immunoprecipitants with an anti-myosin Va antibody from rat hippocampal slices, which is abolished by GF109203X (Figure 4). This indicates that DCP-LA stimulates an association of myosin Va/α7 ACh receptor in a PKC-dependent manner.

DISCUSSION

NSF plays a critical role in the vesicular transport and exocytosis of not only neurotransmitters but neurotransmitter receptors. The motor protein myosin Va moves on the actin filament towards the plasma membrane and carries cargos (Hammer and Wagner, 2013). In the present study, the selective PKCε activator DCP-LA enhanced serine phosphorylation of myosin Va in rat hippocampal slices, which is inhibited by the PKC inhibitor GF109203X. NSF associated with myosin Va, but not myosin VI, in rat hippocampal slices, and DCP-LA increased the association in a PKC-dependent manner. DCP-LA is recognized to activate PKCε selectively (Kanno *et al*., 2006; Kanno *et al*., 2015). Accordingly, PKCε, activated by DCP-LA, appears to phosphorylate myosin Va at the serine residues, to stimulate an association of NSF/myosin Va. It is presently unknown whether DCP-LA phosphorylates NSF, to recruit myosin Va. To address this question, we are currently carrying out further experiments.

DCP-LA, alternatively, increased an association of myosin Va/α7 ACh receptor in a PKC-dependent manner in rat hippocampal slices. This, in the light of the fact that α7 ACh receptor contains no PKC phosphorylation site, suggests that DCP-LA-induced PKCε-mediated phosphorylation of myosin Va also triggers an association of myosin Va/α7 ACh receptor.
The small G-protein Rab regulates vesicular traffic together with its effector (Stenmark, 2009; Zerial and McBride, 2001). The Rab27 effector Rabphilin is shown to stimulate exocytosis of hormone by interacting with SNAP-25 (Tsuboi and Fukuda, 2005). Rab3A and the effector Rabphilin-3Apromote exocytosis of synaptic vesicles by interacting with myosin Va (Li, 1996; Lindsay*et al*., 2013).

In the present study, NSF associated with Rab-3A and Rabphilin-3A in rat hippocampal slices. This suggests that Rab-3A and Rabphilin-3A may also participate in the regulation of vesicular traffic of α7 ACh receptor, although DCP-LA had no effect on an association of NSF/Rab3A or NSF/Rabphilin-3A. Overall, the results of the present study lead to a conclusion that PKCε, activated by DCP-LA, phosphorylates myosin Va at the serine residues, to facilitate assembly of NSF/myosin Va/α7 ACh receptor, causing vesicular transport and exocytosis of α7 ACh receptor.

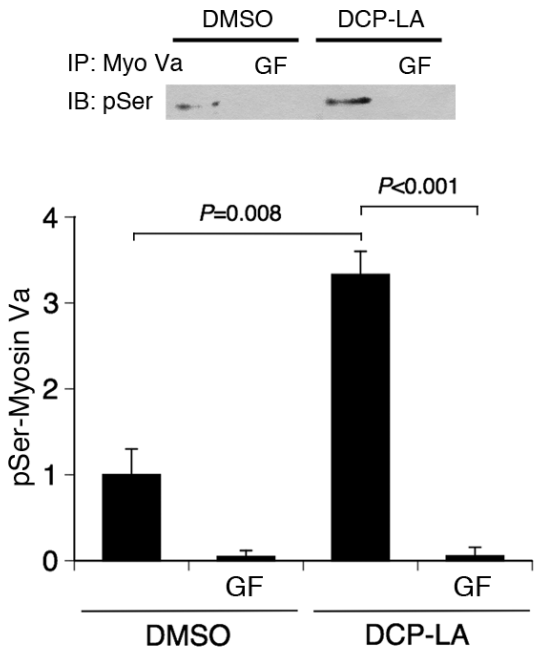

Figure 1. DCP-LA enhances serine phosphorylation of myosin Va in a PKC-dependent manner. Lysates from hippocampal slices treated with DMSO or DCP-LA (100 nM) for 20 min in the presence and absence of GF109203X (GF) (100 nM) were immunoprecipitated with an anti-myosin Va (MyoVa) antibody, followed by Western blotting using an anti-phospho-serine (pSer) antibody. IP, immunoprecipitation; IB, immunoblot. In the graph, each column represents the mean (± SEM) signal intensity for pSer-myosin Varelative to that for slices treated with DMSO in the absence of GF109203X (n=4 independent experiments). *P* values, ANOVA followed by a Bonferroni correction.

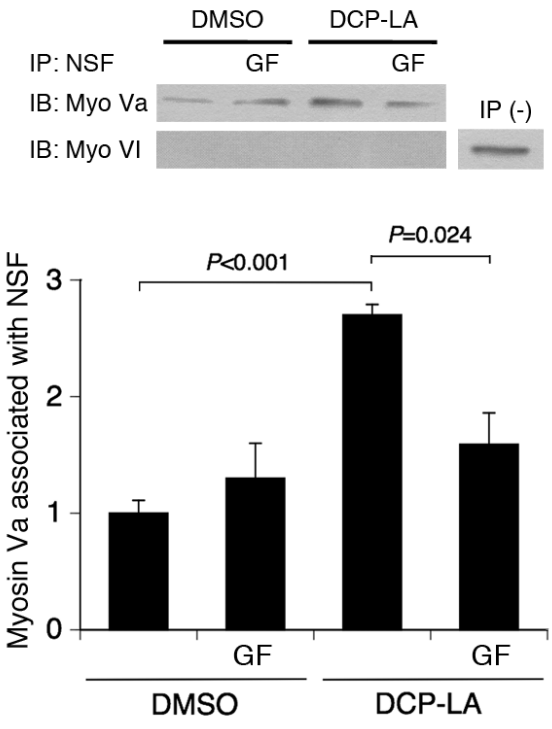

Figure 2. DCP-LA increases an association of NSF/myosin Va in a PKC-dependent manner. Lysates from hippocampal slices treated with DMSO or DCP-LA (100 nM) for 20 min in the presence and absence of GF109203X (GF) (100 nM) were immunoprecipitated with an anti-NSF antibody, followed by Western blotting using antibodies against myosin Va (MyoVa) and myosin VI (Myo VI). In a different set of experiments, Western blotting was carried out in lysates from rat hippocampal slices without immunoprecipitation using an anti-myosin VI antibody. Note that the immunoreactive signal for myosin VI is detected in the samples. IP, immunoprecipitation; IB, immunoblot. In the graph, each column represents the mean (± SEM) signal intensity for myosin Va relative to that for slices treated with DMSO in the absence of GF109203X (n=4 independent experiments). *P* values, ANOVA followed by a Bonferroni correction

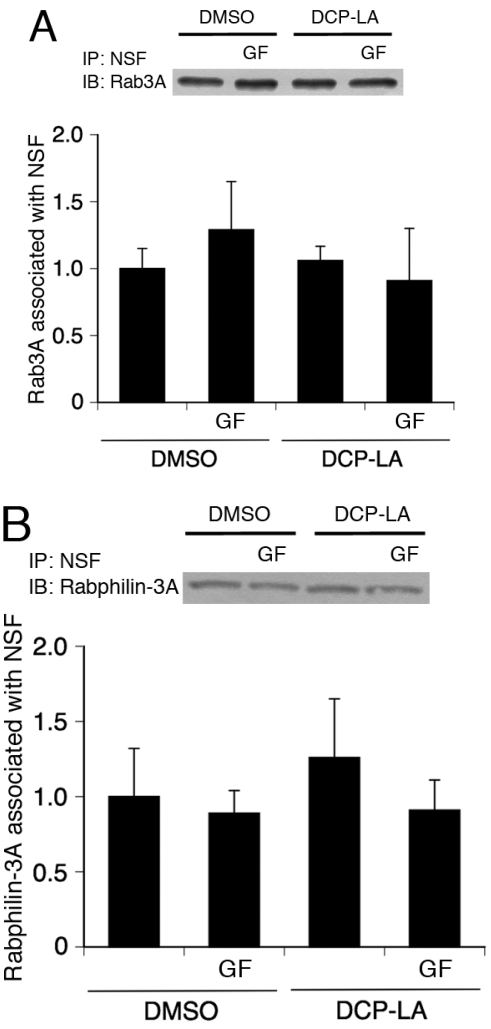

Figure 3. DCP-LA had no effect on an association of NSF/Rab3A or NSF/Rabphilin. Lysates from hippocampal slices treated with DMSO or DCP-LA (100 nM) for 20 min in the presence and absence of GF109203X (GF) (100 nM) were immunoprecipitated with an anti-NSF antibody, followed by Western blotting using antibodies against Rab3A and Rabphilin-3A. IP, immunoprecipitation; IB, immunoblot. In the graphs, each column represents the mean (± SEM) signal intensity for Rab3A or RAbphilin-3A relative to that for slices treated with DMSO in the absence of GF109203X (n=4 independent experiments).

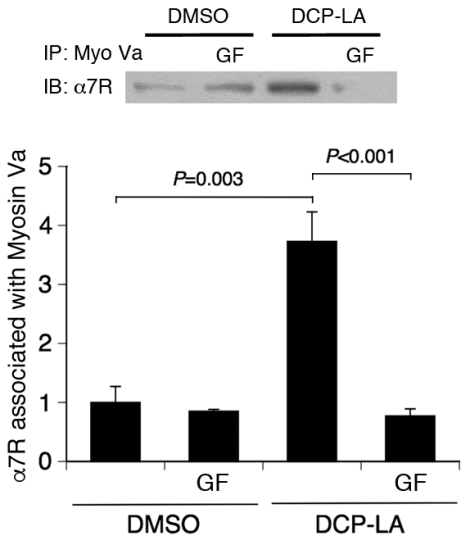

Figure 4. DCP-LA increases an association of myosin Va/α7 ACh receptor in a PKC-dependent manner. Lysates from hippocampal slices treated with DMSO or DCP-LA (100 nM) for 20 min in the presence and absence of GF109203X (GF) (100 nM) were immunoprecipitated with an anti-NSF antibody, followed by Western blotting using an anti-α7 ACh receptor (α7R) antibody. IP, immunoprecipitation; IB, immunoblot. In the graph, each column represents the mean (± SEM) signal intensity for α7 ACh receptor relative to that for slices treated with DMSO in the absence of GF109203X (n=4 independent experiments). *P* values, ANOVA followed by a Bonferroni correction.

Conclusion

DCP-LA activates PKCε, to phosphorylate myosin Va in rat hippocampal slices, which triggers assembly of NSF/myosin Va/α7 ACh receptor. This may provide the novel mechanism underlying regulation of α7 ACh receptor traffic.

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