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RESEARCH ARTICLE

PKCε STIMULATES VESICULAR EXOCYTOSIS OF α7 NICOTINIC ACH RECEPTOR BY TARGETING NSF

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ABSTRACT

The linoleic acid derivative DCP-LA serves as a selective activator of PKCε. The present study aimed at understanding the mechanism underlying PKCε-regulated α7 nicotinic acetylcholine (ACh) receptor trafficking. DCP-LA specifically bound to *N*-ethylmaleimide-sensitive factor (NSF) in lysates from the rat brain. PKCε, activated by DCP-LA, phosphorylated NSF at the serine residues, but not the threonine residues, in differentiated PC-12 cells. DCP-LA enhanced an association of NSF/α7 nicotinic ACh receptor in PC-12 cells, and the effect was cancelled by the PKC inhibitor GF109203X. DCP-LA increased cell surface localization of α7 nicotinic ACh receptor in PC-12 cells, which was abolished by GF1090203X or knocking-down NSF. DCP-LA also increased cell surface localization of α7 nicotinic ACh receptor in rat hippocampal slices, and the effect was suppressed by GF109203X, the vesicular transport inhibitor latrunculin B, or the vesicular exocytosis inhibitor botulinum toxin A. Collectively, the results of the present study show that PKCε, activated by DCP-LA directly binding to NSF, phosphorylates NSF at the serine residues, facilitates an association of NSF/α7 nicotinic ACh receptor, and stimulates NSF-dependent vesicular exocytosis of α7 nicotinic ACh receptor.

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INTRODUCTION

The linoleic acid derivative 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoicacid (DCP-LA) has been developed as a promising and novel anti-dementia drug (Nishizaki *et al*., 2014; Nishizaki, 2017a; Nishizaki, 2017b; Nishizaki, 2018). DCP-LA improves cognitive disorders in a variety of animal models. DCP-LA ameliorates spatial learning and memory decline in 5xFAD mice, an animal model of Alzheimer's disease (Kanno *et al*., 2016), amyloid $\beta_{1\text{-}40}$ ($A\beta_{1\text{-}40}$)- and mutant Aβ-induced spatial learning deficits in rats (Nagata *et al*., 2005; Nagata *et al*., 2010), scopolamine-induced spatial learning and memory impairment in rats (Nagata *et al*., 2005), and spatial learning and memory deterioration in senescence accelerated mice (Yaguchi*et al*., 2006; Kanno *et al*., 2012b). The primary site of action of DCP-LA is PKCε (Kanno *et al*., 2006; Kanno *et al*., 2015). DCP-LA activates PKCε selectively still in the absence of diacylglycerol (DG) and calcium. DCP-LA binds to the phosphatidylserine (PS) binding/associating sites Arg50 and Ile89 in the C2-like domain of PKCε at the carboxyl-terminal end and the cyclopropane rings, respectively, which are distinct from the phorbol 12-myristate 13-acetate (PMA) binding site in the C1 domain (Kanno *et al*., 2015). DCP-LA promotes vesicular transport of α7 nicotinic ACh receptor towards the cell surface in a PKC-dependent manner (Kanno *et al*., 2012a). DCP-LA-induced increase in the α7 nicotinic ACh receptor at presynaptic terminals stimulates presynaptic glutamate release, to facilitate hippocampal synaptic transmission in a PKC-dependent manner (Shimizu *et al*., 2011; Tanaka and Nishizaki, 2003; Yamamoto *et al*., 2005). *N*-ethylmaleimide (NEM)-sensitive factor (NSF) regulates vesicular traffic and exocytosis together with the NSF adaptor soluble NSF attachment protein (SNAP) and SNAP receptors (SNAREs) such as syntaxin, SNAP25 and synaptobrevin (Block*et al*., 1988; Südhof and Rizo, 2011). Accumulating evidence has pointed to the implication of NSF in the vesicular traffic of a variety of neurotransmitter receptors such as AMPA receptor, $GABA_A$ receptor, β_2-adrenergic receptor, D_1 and D_2 dopamine receptors, and M_1, M_3, M_4 and M_5 muscarinic ACh receptors (Chen and Liu, 2010; Chou*et al*., 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; Zhao*et al*., 2007; Zou*et al*., 2005). The effect of NSF on α7 nicotinic ACh receptor trafficking, however, is not fully understood.

The present study was conducted to explore the implication of NSF in the DCP-LA-induced PKCε-regulated vesicular transport ofα7 nicotinic ACh receptor. The results showthat PKCε, activated by DCP-LA binding to NSF, phosphorylates NSF, to enhance an association of NSF/α7 nicotinic ACh receptor, which triggers NSF-dependent vesicular exocytosis of the receptor.

MATERIALS AND METHODS

Animal care: All procedures have been approved by the Animal Care and Use Committee at Hyogo College of Medicine and performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell culture: PC-12 cells, obtained from RIKEN Cell Bank (Tsukuba, Japan), were cultured in DMEM with 10% (v/v) heat-inactivated FBS and 10% (v/v) heat-inactivated horse serum supplemented with penicillin (100 U/ml), and streptomycin (0.1 mg/mL), in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. PC-12 cells were differentiated by treatment with nerve growth factor (100 ng/mL) for 5 days.

Serial affinity chromatography (SAC): The non-specific protein absorption on the commercially available affinity matrix comprising of poly (methacrylate) resins strongly depends on the hydrophobic property. In order to reduce the non-specific protein absorption, we have developed the novel affinity matrix Aqua Firmus^{TM} (IEDA CHEMICALS CO., LTD, Tokyo, Japan) with the hydrophilic property (Takahashi *et al*., 2006), and used it in the present study. The rat brain (male Wistar rat, 6 weeks) was isolated and 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. The affinity resins were mixed with lysates from the rat brain, and binding proteins were eluted by 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 washing with a lysate buffer (0.25 M sucrose, 300 mM N,N-diethylthiocarbamate, 25 mM Tris, pH 7.6), followed by SDS-PAGE. The binding proteins were identified in the MS/MS analysis using LTQ after trypsin digestion. In a different set of experiments, Western blot was carried out in the rat brain lysates using an anti-NSF antibody (Invitrogen, Waltham, MA USA).

Monitoring of α7 nicotinic ACh receptor traffic: Rat hippocampal slices (400 μm in thickness; male Wistar rat, 6 weeks) or PC-12 cells were homogenized by sonication in an ice-cold mitochondrial buffer (210 mM mannitol, 70 mM sucrose, and 1 mM EDTA, 10 mM HEPES, pH 7.5) containing 1% (v/v) protease inhibitor cocktail and centrifuged at 3,000 rpm for 5 min at 4 °C. The supernatants were centrifuged at 11,000 rpm for 15 min at 4 °C and the collected supernatants were further ultracentrifuged at 100,000 *g* for 60 min at 4 °C. The supernatants and pellets were used as the cytosolic and plasma membrane fractions, respectively. Protein concentrations for each fraction were determined using a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Whether the cytosolic and plasma membrane components were successfully separated was confirmed in the Western blot analysis using antibodies against the cytosolic marker LDH (Abcam, Cambridge, MA, USA) and the plasma membrane marker cadherin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After boiling for 5 min, proteins in the cytosolic and plasma membrane fractions were separated by 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 anti-α7 nicotinic ACh receptor antibody (Sigma-Aldrich, St. Louis, MO, USA). After washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Abcam). Immunoreactivity was detected with an ECL kit (GE Healthcare, Piscataway, NJ, USA) and visualized using a chemiluminescence detection system (GE Healthcare).

Immunoprecipitation and Western blotting: PC-12 cells, treated with dimethyl sulfoxide (DMSO) [0.1% (v/v)] or DCP-LA (100 nM) for 10 min, 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, the homogenates were centrifuged at 3,000 rpm for 5 min at 4 °C. The supernatants (200 μg of protein) were incubated with an anti-NSF antibody (Invitrogen) overnight at 4 °C. Then, 20 μL of protein G sepharose (GE healthcare) 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 SDS sample buffer, followed by Western blotting using antibodies against phospho-serine (pSer) (QIAGEN, Hilden, Germany), phospho-threonine (pThr) (QIAGEN), NSF (Invitrogen), and α7 nicotinic ACh receptor (Sigma-Aldrich).

Protein knockdown: The siRNAs to silence the PKCε-targeted gene and NSF-targeted genes were obtained from Santa Cruz Biotechnology and the negative control siRNA (NC siRNA) from Ambion (Carlsbad, CA, USA). siRNAs were transfected into PC-12 cells using Lipofectamine® 3000 Reagent (Invitrogen), and cells were used for experiments 48 h after transfection. To confirm successful knockdown, Western blotting was performed using antibodies against PKCε (BD Biosciences, San Jose, CA, USA), NSF (Invitrogen), and β-actin (Cell Signaling, Beverly, MA, USA).

Chemicals: DCP-LA was synthesized in our laboratory (Shimizu *et al*., 2011). DCP-LA was dissolved with DMSO, which was diluted 1,000 folds with the extracellular solution and used for experiments. GF109203X was purchased from Sigma-Aldrich, latrunculin B from Abcam, botulinum toxin A from Funakoshi Co., Ltd. (Tokyo, Japan), KN-93 from Abcam, and phenylarsine oxide (PAO) from Sigma-Aldrich.

Statistical analysis: Statistical analysis was carried out using unpaired *t*-test and analysis of variance (ANOVA) followed by a Bonferroni correction.

RESULTS

NSF is a binding partner of DCP-LA: To probe DCP-LA-binding proteins, SAC was performed in lysates from the rat brain. Affinity resins bearing DCP-LA were prepared from the synthetic solid materials AquaFirmus^{TM}. In the SAC analysis, a substantial decrease in several binding proteins was found by adding DCP-LA (Figure 1). Each binding protein was eluted and identified in the MS/MS analysis using LTQ after trypsin digestion. The identified proteins included NSF (Figure 1). It was further confirmed in the Western blot analysis that a substantial decrease of NSF was obtained with DCP-LA treatment (Figure 1).

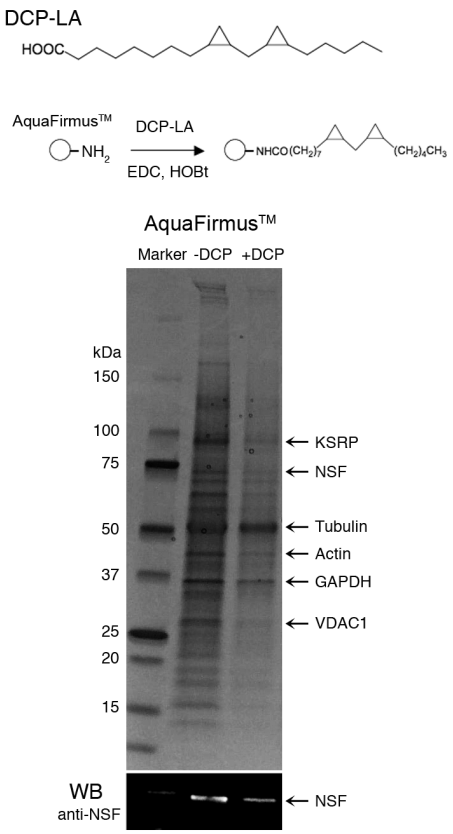

Figure 1. DCP-LA binds to NSF specifically. Affinity resins bearing DCP-LA were constructed using Aqua Firmus™. SAC was performed in a mixture of affinity resins bearing DCP-LA in the absence (-DCP) and presence (+DCP) of DCP-LA (1 μM). Subsequently, DCP-LA binding proteins were identified in the MS/MS analysis, which include KH-type splicing regulatory protein (KSRP), NSF, tubulin, actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and voltage-dependent anion channel 1 (VDAC1). It was further confirmed in the Western blot (WB) analysis using an anti-NSF antibody that a substantial decrease of NSF was obtained with DCP-LA treatment

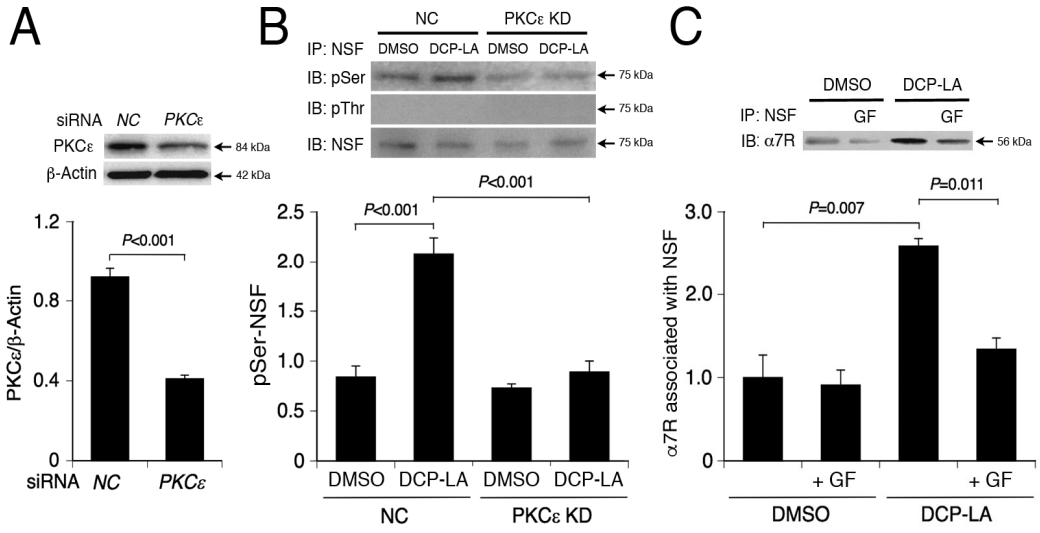

Figure 2. DCP-LA promotes an association of NSF/α7 nicotinic ACh receptor in a PKCε-dependent manner. (A) PKCε knockdown. PC-12 cells were transfected with the negative control (NC) siRNA or the PKCε siRNA, and 48 h later Western blotting was carried out in the cell lysates. In the graph, each column represents the mean (± SEM) signal intensity for PKCε normalized by the signal intensity for β-actin (n=4 independent replicate experiments). P value, unpaired t-test. (B) Cells, transfected with the NC siRNA or the PKCε siRNA, were treated with DMSO or DCP-LA (100 nM) for 10 min, and the cell homogenates were immunoprecipitated with an anti-NSF antibody, followed by Western blotting using antibodies against pSer, pThr, and NSF. KD, knockdown; IP, immunoprecipitation; IB, immunoblot. In the graph, each column represents the mean (± SEM) signal intensity for pSer-NSF (n=4 independent replicate experiments). P values, ANOVA followed by a Bonferroni correction. (C) Cells were treated with DMSO or DCP-LA (100 nM) for 10 min, and the cell homogenates were immunoprecipitated with an anti-NSF antibody, followed by Western blotting using an anti-α7 nicotinic ACh receptor antibody. IP, immunoprecipitation; IB, immunoblot. α7R, α7 nicotinic ACh receptor. In the graph, each column represents the mean (± SEM) signal intensity for α7 nicotinic ACh receptor relative to the signal intensity for the receptor in immunoprecipitants from cells treated with DMSO in the absence of GF109203X (n=4 independent replicate experiments). P values, ANOVA followed by a Bonferroni correction.

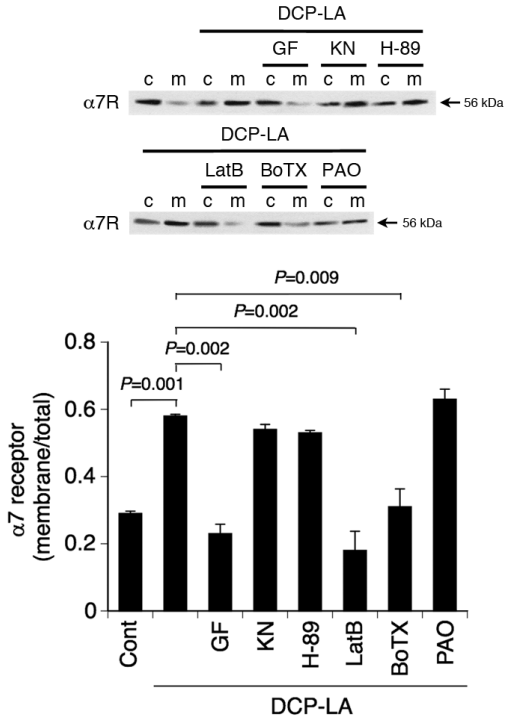

Figure 4. DCP-LA stimulates vesicular exocytosis of α7 nicotinic ACh receptor. Rat hippocampal slices were untreated (Cont) and treated with DCP-LA (100 nM) for 10 min in the absence and presence of GF109203X (GF)(100 nM), KN-93 (KN)(3 μM), H-89 (1 μM), latrunculin B (LatB)(10 μM)), BoTX(0.1 U/mL), or PAO (10 μM). Then, the slice homogenates were separated in the cytosolic (c) and plasma membrane components (m), followed by Western blotting using an anti-α7 nicotinic ACh receptor antibody in each component. α7R, α7 nicotinic ACh receptor. In the graph, each column represents the mean (± SEM) signal intensity for the α7 nicotinic ACh receptor in the plasma membrane components relative to the signal intensity for the receptor in whole cells (n=4 independent replicate experiments). *P* values, ANOVA followed by a Bonferroni correction.

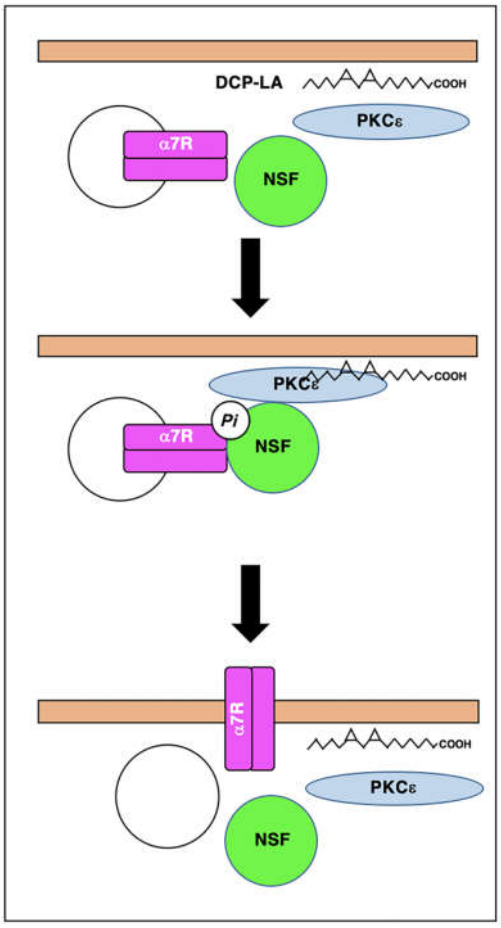

Figure 5. A schematic diagram for DCP-LA-regulated vesicular exocytosis of α7 nicotinic ACh receptor. DCP-LA binds to NSF directly and recruits PKCε, to phosphorylate NSF. Phosphorylated NSF associates with α7 nicotinic ACh receptor, which triggers vesicular exocytosis of the receptor. PM, plasma membrane; α7R, α7 nicotinic ACh receptor.

Taken together, these results indicate that DCP-LA binds to NSF specifically. We have earlier found that DCP-LA stimulates vesicular transport of α7 ACh receptors towards cell surface (Kanno *et al*., 2012a). To understand the mechanism underlying DCP-LA-induced vesicular transport ofα7 ACh receptors, we focused on NSF that regulates vesicular exocytosis.

PKCε, activated by DCP-LA, phosphorylates NSF, to promote an association of NSF/α7 nicotinic ACh receptor: To examine whether PKCε, activated by DCP-LA, is implicated in the association of NSF/α7 nicotinicACh receptor, PKCε was knocked-down. It was confirmed in the Western blot analysis that PKCε is successfully knocked-down in differentiated PC-12 cells (Figure 2A). DCP-LA significantly enhanced the immunoreactive signal for pSer in immunoprecipitants with an anti-NSF antibody from differentiated PC-12 cells, which was abolished by knocking-down PKCε (Figure 2B). In contrast, the immunoreactive signal for pThr was not found under the basal conditions, and DCP-LA produced no signal for pThr (Figure 2B). These results indicate that PKCε, activated by DCP-LA, phosphorylates NSF preferentially at the serine residues. DCP-LA enhanced the immunoreactive signal for α7 nicotinic ACh receptor in immunoprecipitants with an anti-NSF antibody from differentiated PC-12 cells, and the effect significantly inhibited by the PKC inhibitor GF109203X (Figure 2C). Taken together, these results suggest that PKCε, activated by DCP-LA binding to NSF, efficiently phosphorylates NSF at the serine residues, to promote an association of NSF/α7 nicotinic ACh receptor.

PKCε, activated by DCP-LA, increases cell surface localization of α7 nicotinic ACh receptor by targeting NSF: DCP-LA increased cell surface localization of α7 nicotinic ACh receptor in differentiated PC-12 cells, which was cancelled by GF109203X (Figure 3A). This indicates that DCP-LA stimulates translocation of α7 nicotinic ACh receptor towards the cell surface in a PKCε-dependent manner. To examine whether NSF is implicated in α7 nicotinic ACh receptor trafficking, NSF was knocked-down. It was confirmed in the Western blot analysis that NSF is successfully knocked-down in differentiated PC-12 cells (Figure 3B). DCP-LA-induced increase in the cell surface localization of α7 nicotinic ACh receptor was abrogated by knocking-down NSF (Figure 3C). This implies that PKCε, activated by DCP-LA, increases cell surface localization of α7 nicotinic ACh receptor by targeting NSF.

DCP-LA stimulates vesicular exocytosis of α7 nicotinic ACh receptor under the control of PKCε: DCP-LA also increased cell surface localization of α7 nicotinic ACh receptor in rat hippocampal slices (Figure 4). The effect was clearly inhibited by GF109203X, the vesicular transport inhibitor latrunculin B, or the vesicular exocytosis inhibitor botulinum toxin A (BoTX), but otherwise it was not affected by the CaMKII inhibitor KN-93, the PKA inhibitor H-89, or the vesicular endocytosis inhibitor PAO (Figure 4). Overall, these results indicate that DCP-LA increases cell surface localization of α7 nicotinic ACh receptor by stimulating vesicular exocytosis under the control of PKCε.

DISCUSSION

NSF is an ATPase, which is involved in the fusion of synaptic vesicle orchestrated by SNARE proteins (Block *et al*., 1988; Südhof and Rizo, 2011). The vesicular SNARE synaptobrevin, which associates with a cargo-containing transport vesicle, assembles the target SNAREs syntaxin and SNAP25, and in turn, SNAP binds to the SNARE assembly, followed by NSF binding. Then, NSF hydrolyzes ATP into ADP, to produce high-energy phosphate for dissociation of a vesicle/SNAREs/SNAP/NSF complex, allowing fusion and exocytosis of the vesicles. Several lines of evidence have shown that protein kinases and protein phosphatases regulate the NSF-ATPase activity through its phosphorylation and dephosphorylation, respectively. Leucine-rich repeat kinase 2 (LRRK2) phosphorylates NSF at Thr645, causing an enhancement of the NSF-ATPase activity, to increase the rate of SNARE complex disassembling (Belluzzi*et al*., 2016). PKC phosphorylates synaptosomal NSF at Ser237, to refuse recruitment of NSF to a SNAP/SNARE complex and to stimulate neurotransmitter release (Matveeva*et al*., 2001). Pctaire1, a member of the cyclin-dependent replicate kinase-related family, phosphorylates NSF at Ser569. Inhibition of Pctaire1 activity or suppression of NSF-Ser569 phosphorylation enhances the self-association of NSF and increases high K^+-stimulated growth hormone release (Liu *et al*., 2006). Phosphorylation of NSF at Tyr83 elevates the ATPase activity and prevents NSF from αSNAP binding, but the tyrosine phosphatase PTP-MEG2 otherwise promotes secretory vesicle fusion by inhibiting tyrosine phosphorylation of NSF (Huynh *et al*., 2004).

DCP-LA serves as a selective activator of PKCε (Kanno *et al*., 2015; Kanno *et al*., 2006). One of the most striking findings in the present study is that DCP-LA binds to NSF specifically. DCP-LA phosphorylated NSF at the serine residues, but not threonine residues, in a PKC-dependent manner. These results suggest that DCP-LA induces PKCε-mediated serine phosphorylation of NSF, allowing DCP-LA binding to NSF. DCP-LA increased an association of NSF/α7 nicotinic ACh receptor in differentiated PC-12 cells, which was suppressed by the PKC inhibitor GF109203X. This, in the light of the facts that α7 nicotinic ACh receptor has no PKC phosphorylation site and that DCP-LA does not phosphorylate α7 nicotinic ACh receptor (Kanno *et al*., 2012a), suggests that PKCε-mediated serine phosphorylation of NSF causes an increase in the association of NSF/α7 nicotinic ACh receptor. DCP-LA increased cell surface localization of α7 nicotinic ACh receptor in rat hippocampal slices, and the effect was neutralized by GF109203X, the vesicular transport inhibitor latrunculin B, or the vesicular exocytosis inhibitor BoTX. More interestingly, DCP-LA-induced increase in the cell surface localization of α7 nicotinic ACh receptor was abolished by knocking-down NSF in differentiated PC-12 cells. Overall, it appears that DCP-LA induces PKCε-mediated serine phosphorylation of NSF, causing an increase in the association of NSF/α7 nicotinic ACh receptor, to stimulate vesicular transport and exocytosis of α7 nicotinic ACh receptor. In the earlier study, NEM, an inhibitor of NSF, dissociated α7 nicotinic ACh receptor and synaptobrevin 2 from an NSF/α7 nicotinic ACh receptor/β-SNAP/syntaxin 1/synaptobrevin 2 complex and increased cell surface localization of α7 nicotinic ACh receptor at the presynaptic terminals in rat hippocampal slices (Nishizaki, 2016). This implies that inhibition of NSF initiates exocytosis of α7 nicotinic ACh receptor, which seems to be inconsistent with the finding here that DCP-LA increased cell surface localization of α7 nicotinic ACh receptor in an NSF-dependent manner. A plausible explanation for this is that NEM might

promote detachment of α7 nicotinic ACh receptor from an NSF/α7 nicotinic ACh receptor/β-SNAP/syntaxin 1/synaptobrevin 2 complex formed already, possibly by binding to the α7 nicotinic ACh receptor associating site; in contrast, PKCε, activated by DCP-LA, might enhance α7 nicotinic ACh receptor incorporation into and release from the complex by phosphorylating NSF. A study shows that PKCε phosphorylates NSF at Ser460 and Thr461, to increase the NSF-ATPase activity, and decreases cell surface localization of $GABA_A$ receptor (Chou *et al*., 2010), although PKCε here, activated by DCP-LA, phosphorylates NSF only at the serine residues and increases cell surface localization of α7 nicotinic ACh receptor. It is presently unknown why PKCε exerts the bidirectional effects on $GABA_A$ receptor trafficking and α7 nicotinic ACh receptor trafficking by targeting NSF. NSF interacts directly with the $GABA_B$ receptor heterodimer and regulates agonist-promoted recruitment of PKC and receptor phosphorylation (Pontier*et al*., 2006). This raises the possibility that the binding affinity of NSF to the receptor might differ, depending on whether the receptor contains the PKC phosphorylation sites. PKCε-mediated phosphorylation of $GABA_A$ receptor might restrain an association of NSF/$GABA_A$ receptor, resulting in the formation of an NSF/SNAP/SNARE complex lacking $GABA_A$ receptor, to decrease cell surface localization of the receptor, while PKCε might enhance an association of NSF/α7 nicotinic ACh receptor by phosphorylating NSF, but not α7 nicotinic ACh receptor, followed by formation of an NSF/α7 nicotinic ACh receptor/SNAP/SNARE complex, to increase cell surface localization of the receptor. To address this issue, further experiments need to be carried out.

Conclusion

The results of the present study show that DCP-LA binds to NSF specifically and that PKCε, activated by DCP-LA, phosphorylates NSF at the serine residues, facilitates an association of NSF/α7 nicotinic ACh receptor, and stimulates NSF-dependent vesicular exocytosis of α7 nicotinic ACh receptor (Figure 5). This may represent the novel mechanism underlying vesicular transport and exocytosis of α7 nicotinic ACh receptor.

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