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RESEARCH ARTICLE

PROFILIN ENHANCES THE ACTIVATED PKCε ACTVITY AND STIMULATES TRANSLOCATION OF α7 ACH RECEPTOR TOWARDS THE CELL SURFACE

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ABSTRACT

Profilin is an actin-binding protein and engages in the dynamic turnover and restructuring of the actin cytoskeleton. The present study investigated the role of profilin in α7 ACh receptor trafficking. The selective PKCε activator DCP-LA increased cell surface localization of α7 ACh receptor in differentiated PC-12 cells, which was inhibited by knocking down PKCε. The effect of DCP-LA was also cancelled by knocking down profilin. In the cell-free kinase assay, profilin significantly enhanced the activity of PKCε activated by DCP-LA, although no PKCε activation was induced by profilin alone. It is indicated from these results that profilin stimulates translocation of α7 ACh receptor towards the cell surface by enhancing the activated PKCε activity.

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INTRODUCTION

The linoleic acid derivative 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoicacid (DCP-LA) activates PKCε selectivelyand directly by binding to the phosphatidylserine (PS)binding/associating sites in a diacylglycerol (DG)- and calcium-independent manner (Kanno *et al*., 2006; Kanno *et al*., 2015). DCP-LA stimulates vesicular translocation and exocytosis of α7 ACh receptor at the presynaptic terminals in a PKCε-dependent manner (Kanno *et al*., 2012). DCP-LAincreases cell surface localization of α7 ACh receptor in a 4.1N-dependent manner under the control of PKCε, but without phosphorylating 4.1N (Kanno *et al*., 2013). Moreover, DCP-LA enhances PKCε-mediated phosphorylation of the motor protein myosin Va, which triggers an assembly of *N*-ethylmaleimide-sensitive factor (NSF)/myosin Va/α7 ACh receptorrelevant to vesicular exocytosis (Nishizaki, 2018). PKCε, thus, is a critical factor forα7 ACh receptor trafficking. Profilin, an actin-binding protein,has a higher affinity for ATP-actin monomers and promotes actin polymerization (Selden *et al*., 1999).

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Myosin V, that moves on the actin filament towards the plus-end, carries cargos containing neurotransmitters, hormones, and neurotransmitter receptors (Hammer and Wagner, 2013). Then, I wondered whether profilin might play a role in the regulation of α7 ACh receptor trafficking. The present study was conducted to answer this question. The results show that profilin increases cell surface localization of α7 ACh receptor by enhancing the activated PKCε activity.

MATERIALS AND METHODS

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.

Monitoring of α7 ACh receptor trafficking: 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 ultra centrifuged 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 components were separated by SDS-polyacrylamide gel electrophoresis (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 ACh receptor antibody (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 knockdown: The siRNAs for PKCε (Santa Cruz Biotechnology), profiling (Thermo Fisher Scientific), and negative control (NC) (Ambion, Carlsbad, CA, USA) were transfected into PC-12 cells using a Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA), and cells were used for experiments 48 h after transfection. It was confirmed whether each protein is successfully knocked downin theWestern blot analysisusing antibodies against PKCε (BD Biosciences), profilin (Abcam), and β-actin.

Cell-free PKCε assay: The PKCε activity was assayed in the cell-free system by the method as previously described (Kanno *et al*., 2006). Synthetic PKC substrate peptide (Pyr-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu) (Peptide Institute Inc.) (10 μM) was reacted with recombinant human PKCε (Abcam) in a Ca^{2+}-free and phosphatidylserine-free solution containing 10μM DCP-LA, 20 mM Tris-HCl (pH 7.5), 5 mM Mg-acetate, and 100μM ATP in the presence and absence of recombinant human profilin 1 (10 μM) (Abcam) at 30 °C for 5 min. After loading on a reversed phase HPLC (LC-10ATvp, Shimadzu Co.), a substrate peptide peak and a new product peak, corresponding to non-phosphorylated and phosphorylated peptides, respectively, were detected at an absorbance of 214 nm (SPD-10Avp UV-VIS detector, Shimadzu Co.). The quantity of phosphorylated substrate peptide (pmol/min) was calculated and used as an index of PKCε activity.

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

RESULTS

Profilin increases cell surface localization of α7 ACh receptor: DCP-LA significantly increased cell surface localization of α7 ACh receptor in differentiated PC-12 cells, and the effect was significantly inhibited by knocking down PKCε (Figure 1A). This provides evidence that PKCε stimulates translocation of α7 ACh receptor towards the cell surface.

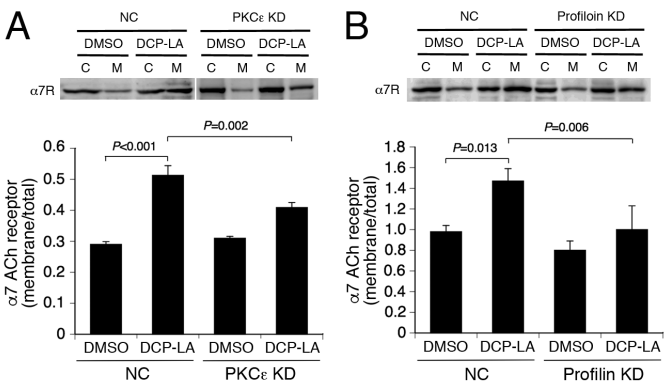

Figure 1.Profilin contributes to DCP-LA-induced increase in the cell surface localization of α7 ACh receptor. (A) PC-12 cells, transfected with the negative controlsi RNA (NC) or the PKCε siRNA (PKCε KD), were treated with DMSO or DCP-LA (100 nM) for 10 min and lysed. (B) Cells, transfected with the negative control siRNA (NC) or the profilin siRNA (Profilin KD), were treated with DMSO or DCP-LA (100 nM) for 10 min and lysed. Then, thoselysates were separated into the cytosolic (C) and plasma membrane components (M), followed by Western blotting using an anti-α7 ACh receptor (α7R) antibody in each component.In the graphs, each column represents the mean (± SEM) signal intensity for α7 ACh receptor in the plasma membrane components relative to the signal intensity in whole cells (n=4 and 6 independent experiments in A and B, respectively). *P* values, ANOVA followed by a Bonferroni correction.

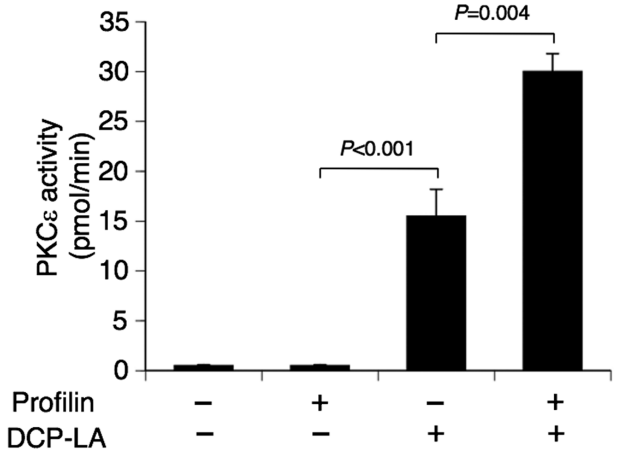

Figure 2. Profilin enhances the activity of PKCε activated by DCP LA. In the cell-free system, synthetic PKC substrate peptide (10μM) was reacted with PKCε in the absence and presence of DCP-LA (10 μM) and/or profilin 1 (10 μM) at 30 °C for 5 min, and the quantity of phosphorylated substrate peptide was calculated. In the graph, each column represents the mean (± SEM) PKCε activity (pmol/min) (n=4 independent experiments). *P* values, ANOVA followed by a Bonferroni correction.

Intriguingly, DCP-LA-induced increase in the cell surface localization of α7 ACh receptor was clearly prevented by knocking down profilin (Figure 1B). This indicates that profilin contributes to PKCε-regulated translocation of α7 ACh receptor towards the cell surface. This also suggests that profilin might be implicated in PKCε activation.

Profilin enhances the activated PKCε activity: To see whether profilin is implicated in PKCε activation, cell-free PKCε assay was carried out.Consistent with the previous studies, DCP-LA activated PKCεin a DG- and calcium-independent manner (Figure 2). Profilin significantly enhanced the activity of PKCε activated by DCP-LA, although no PKCε activation was induced by profilin alone (Figure 2). This implies that profilin modulates the activated PKCε activity.

DISCUSSION

PKC isozymes are classified into conventional PKCs such as PKCα, -βI, -βII, and -γ, novel PKCs such as PKCδ, -ε, -η, -θ, and -μ, and atypical PKCs such as PKCλ/ι for mouse/human, -ζ, and –ν (Steinberg, 2008). The novel PKC isozymes including PKCε are activated by binding DG, PS, or *cis*-unsaturated free fatty acids. DCP-LA activates PKCε by binding tothe 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*., 2006; Kanno *et al*., 2015).In the cell-free system, profilin induced no PKCε activation. This implies that profilin by itself has no potential to activate PKCε directly. Profilin, however, significantly enhanced the activity of PKCε activated by DCP-LA. Collectively, these results indicate that profilin serves as a modulator of the activated PKCε activity. In the present study, DCP-LA increased cell surface localization of α7 ACh receptor in PC-12 cells, and the effect was restrained by knocking-down PKCε. This provides further evidence that PKCε is indispensable for vesicular transport and exocytosis of α7 ACh receptor. Surprisingly, DCP-LA-induced increase in the cell surface localization of α7 ACh receptor was also prevented by knocking-down profilin. Overall, the results of the present study allow drawing a conclusion that profiling enhances the activatedPKCε activity, thereby promotingvesicular transport and exocytosis of α7 ACh receptor. This may provide the novel notion regarding PKCε activation and α7 ACh receptor trafficking under the control of profilin.

Conclusion

The results of the present study demonstrate that profilin enhances the activated PKCε activity and stimulates translocation of α7 ACh receptor towards the cell surface.

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