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The linoleic acid derivative DCP-LA increases membrane surface localization of the α7 ACh receptor in a protein 4.1N-dependent manner

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In yeast two-hybrid screening, protein 4.1N, a scaffolding protein, was identified as a binding partner of the α7 ACh (acetylcholine) receptor. For rat hippocampal slices, the linoleic acid derivative DCP-LA {8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid} increased the association of the α7 ACh receptor with 4.1N, and the effect was inhibited by GF109203X, an inhibitor of PKC (protein kinase C), although DCP-LA did not induce PKC phosphorylation of 4.1N. For PC-12 cells, the presence of the α7 ACh receptor in the plasma membrane fraction was significantly suppressed by knocking down 4.1N. DCP-LA increased the presence of the α7 ACh receptor in the plasma membrane fraction, and the effect was still inhibited by knocking down 4.1N. In the monitoring of α7 ACh receptor mobilization, DCP-LA enhanced signal intensities for the α7 ACh receptor at the membrane surface in PC-12 cells, which was clearly prevented by knocking down 4.1N. Taken together, the results of the present study show that 4.1N interacts with the α7 ACh receptor and participates in the receptor tethering to the plasma membrane. The results also indicate that DCP-LA increases membrane surface localization of the α7 ACh receptor in a 4.1N-dependent manner under the control of PKC, but without phosphorylating 4.1N.

Key words: α7 acetylcholine receptor, 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA), protein 4.1N, scaffolding protein, tethering.

INTRODUCTION

Protein 4.1 is a scaffolding protein and the protein family consists of five members, 4.1B, 4.1G, 4.1N, 4.1O and 4.1R, that are involved in a variety of cellular functions, including regulation of the cytoskeleton, anchorage and localization of transmembrane proteins, control of the cell volume and cell division [1,2]. Protein 4.1 was originally identified in red blood cells (4.1R), and 4.1R interacts with spectrin, actin, band 3 and glycophorin C [3–5]. 4.1B and 4.1N are enriched in the brain and specifically associated with the neuronal plasma membrane, whereas 4.1G is ubiquitously expressed in cells [6,7]. Interestingly, 4.1N directly interacts with the GluR1 subunit of AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors and co-localizes with AMPA receptors at excitatory synapses [8]. 4.1N also regulates expression of D2 and D3 dopamine receptors at the neuronal plasma membrane through its direct interaction [9]. 4.1B, in contrast, serves as an effector molecule of SynCAM1 (synaptic cell adhesion molecule 1) and is implicated in SynCAM1-dependent NMDA (*N*-methyl-D-aspartate) receptor trafficking [10]. Expression of metabotropic glutamate receptor type 8 in the retina may be regulated through interaction with 4.1B [11]. Thus 4.1B/4.1N may play a critical role in the aggregation and immobilization of neurotransmitter receptors.

The α7 ACh (acetylcholine) receptor, a neuronal nicotinic ACh receptor, is abundant in the brain in addition to the α4β2 ACh receptor. The α7 ACh receptor is preferentially localized at presynaptic terminals and stimulates neurotransmitter release [12–15]. DCP-LA {8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid} is a linoleic acid derivative with cyclopropane rings instead of *cis*-double bonds [16]. Intriguingly, DCP-LA selectively activates PKC (protein kinase C) ε in a DAG (diacylglycerol)- and Ca^{2+}-independent manner [17,18], and stimulates vesicular transport of the α7 ACh receptor towards the surface membrane in a PKC-dependent manner [19]. Therefore we were prompted to investigate whether 4.1N is implicated in DCP-LA-regulated membrane surface localization of the α7 ACh receptor.

We show in the present study that 4.1N binds to the α7 ACh receptor and that DCP-LA promotes surface localization of the α7 ACh receptor in a 4.1N-dependent manner under the control of PKC, regardless of PKC phosphorylation of 4.1N.

MATERIALS AND METHODS

Animal care

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

Cell culture

HEK (human embryonic kidney)-293 and PC-12 cells, obtained from the RIKEN Cell Bank (Tsukuba, Japan), were cultured in DMEM (Dulbecco's modified Eagle's medium) with 10 %

Abbreviations used: ACh, acetylcholine; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; BCA, bicinchoninic acid; CTD, C-terminal domain; DCP-LA, 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; GST, glutathione transferase; HA, haemagglutinin; HEK, human embryonic kidney; HRP, horseradish peroxidase; IP_3, inositol 1,4,5-trisphosphate; NC, negative control; NSF, *N*-ethylmaleimide-sensitive factor; PKA, protein kinase A; PKC, protein kinase C; SAB, spectrin–actin binding; siRNA, small interfering RNA; SynCAM1, synaptic cell adhesion molecule 1; TBS, Tris-buffered saline; TBST, TBS containing 1 % Triton X-100; TBS-T, TBS containing 0.1 % Tween 20.

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(v/v) heat-inactivated FBS (fetal bovine serum) and DMEM with 10 % (v/v) heat-inactivated FBS and 10 % (v/v) heat-inactivated horse serum respectively, each supplemented with penicillin (100 units/ml) and streptomycin (0.1 mg/ml), in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C.

Yeast two-hybrid screening

We carried out a yeast two-hybrid screening to search for proteins interacting with the α7 ACh receptor. The intracellular loop (amino acid residues 316–468) of the α7 ACh receptor subunit was subcloned into the pGEM-T vector (Promega). Then, the cytosolic domain was transferred into the yeast vector pGBKT7 as bait (Clontech). The cDNA library was made from the rat brain as a prey using a Matchmaker Library Construction & Screening Kit (Clontech). cDNAs were transferred into the pGADT7 vector (Clontech). Yeast two-hybrid screening was performed in accordance with the manufacturer's protocol (Clontech).

GST (glutathione transferase) pull-down assay

A GST-fused α7 ACh receptor subunit was cloned into the pGEX-6P-3 vector (GE Healthcare). The plasmid was transformed into BL21 *Escherichia coli* cells. *E. coli* was harvested and sonicated after adding TBS (Tris-buffered saline, 20 mM Tris and 150 mM NaCl, pH 7.5) containing a protease inhibitor cocktail (Nacalai Tesque). Then, glutathione–Sepharose 4B (GE Healthcare) was added to the cell lysate or a GST solution, and incubated at 4 °C. After centrifugation (500 ***g*** for 5 min at 4 °C) the resin was washed three times with TBST [TBS containing 1 % (v/v) Triton X-100] to purify the GST-fused α7 ACh receptor subunit or GST. HEK-293 cells expressing HA (haemagglutinin)-tagged 4.1N were lysed and co-incubated with the purified GST-fused α7 ACh receptor subunit or GST overnight at 4 °C. After washing three times with TBST, GST pull-down proteins were resolved and separated by SDS/PAGE, transferred on to a PVDF membrane, and detected by Western blotting using an anti-HA antibody (Covance).

In a similar manner, the GST-fused CTD (C-terminal domain) of 4.1N (GST–4.1N CTD) was cloned into the pGEX-6P-3 vector and transformed into BL21 *E. coli* cells. *E. coli* was harvested and sonicated in TBS containing a protease inhibitor cocktail. Glutathione–Sepharose 4B was added to the cell lysate or a GST solution and incubated at 4 °C. After centrifugation (500 ***g*** for 5 min at 4 °C), the resin was washed three times with TBST to purify GST–4.1N CTD or GST. Whole-cell lysates from the rat brain were co-incubated with purified GST–4.1N CTD or GST overnight at 4 °C. After washing three times with TBST, GST pull-down proteins were resolved and separated by SDS/PAGE, transferred on to a PVDF membrane and detected by Western blotting analysis using an antibody against the α7 ACh receptor subunit (Sigma).

Construction and transfection of siRNAs (small interfering RNAs)

The siRNA to silence the 4.1N-targeted gene (4.1N siRNA) and the negative control siRNA (NC siRNA) were obtained from Ambion. The sequences of the 4.1N siRNA were 5′-GGAGUAACUUCUACAUCAAtt-3′ and 5′-UUGAUGUAGAAGUUACUCCtc-3′. The NC siRNA had a scrambled sequence, and the same GC content and nucleic acid composition. The 4.1N siRNA or the NC siRNA was transfected into PC-12 cells using Lipofectamine™ reagent (Invitrogen). Cells were used for experiments 48 h after transfection.

Immunoprecipitation and Western blotting

The hippocampus was isolated from the rat brain (male Wistar rat, 6 weeks) and hippocampal slices (400 μm in thickness) were prepared. Slices were incubated 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 in the presence and absence of DCP-LA (100 nM) for 20 min at 34 °C, together with and without GF109203X (100 nM) for 20 min before DCP-LA treatment. Then, slices were homogenized by sonication in TBS-T (TBS containing 0.1 % Tween 20) containing 1 % (v/v) phosphatase inhibitor cocktail and, subsequently, homogenates were centrifuged at 1000 ***g*** for 5 min at 4 °C. Supernatants (200 μg of protein) were incubated with antibodies against 4.1N (Protein Express) or NSF (*N*-ethylmaleimide-sensitive factor) (Cell Signaling Technology) 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 [0.2 mM Tris, 0.05 % SDS and 20 % (v/v) glycerol, pH 6.8]. After boiling for 5 min, proteins were separated by SDS/PAGE using a TGX gel (Bio-Rad) and then transferred on to PVDF membranes. Blotting membranes were blocked with TBS-T containing 5 % (w/v) BSA and subsequently incubated with an anti-(α7 ACh receptor subunit) antibody (Sigma), an anti-4.1N antibody (Protein Express), an anti-NSF antibody (Cell Signaling Technology), an anti-phospho-serine antibody (Qiagen) or an anti-phospho-threonine antibody (Qiagen). After washing, membranes were reacted with an HRP (horseradish peroxidase)-conjugated goat anti-rabbit IgG or goat anti-mouse IgG antibody. Immunoreactivity was detected with an ECL (enhanced chemiluminescence) kit (Invitrogen) and visualized using a chemiluminescence detection system (GE Healthcare). Protein concentrations for each sample were determined with a BCA (bicinchoninic acid) protein assay kit (Thermo Fisher Scientific).

Separation into the cytosolic and plasma membrane components

PC-12 cells were homogenized by sonication in an ice-cold mitochondrial buffer [210 mM mannitol, 70 mM sucrose, 1 mM EDTA and 10 mM Hepes (pH 7.5)] containing 1 % (v/v) protease inhibitor cocktail and centrifuged at 1000 ***g*** for 5 min at 4 °C. The supernatants were centrifuged at 15000 ***g*** for 15 min at 4 °C and the collected supernatants were further ultracentrifuged at 52000 rev./min (rotor TLA-120.2) for 60 min at 4 °C. The supernatants and pellets were used as the cytosolic and plasma membrane fractions respectively. Whether the cytosolic and plasma membrane components were successfully separated was confirmed by Western blot analysis using antibodies against the cytosolic marker LDH (lactate dehydrogenase) and the plasma membrane marker cadherin. Protein concentrations for each fraction were determined using a BCA protein assay kit (Thermo Fisher Scientific).

Plasma membrane fraction proteins were resuspended in the mitochondrial buffer containing 1 % (w/v) SDS. Proteins for each fraction were separated by SDS/PAGE and transferred on to PVDF membranes. After blocking with TBS-T containing 5 % (w/v) BSA, blotting membranes were reacted with antibodies against the α7 ACh receptor subunit (Sigma), 4.1N (BD Biosciences) and β-actin (Sigma), followed by an HRP-conjugated goat anti-mouse IgG antibody. Immunoreactivity was detected with an ECL kit (Invitrogen) and visualized using a chemiluminescence detection

system (GE Healthcare). Signal density was measured with Image Gauge software (GE Healthcare).

Immunocytochemistry

PC-12 cells were transfected with NC siRNA or 4.1N siRNA together with a dsRed expression vector and treated with or without DCP-LA (100 nM). Then, cells were fixed with 4 % (w/v) paraformaldehyde and blocked with 10 % (v/v) goat serum in PBS at room temperature (22 °C). Cells were reacted with a mouse monoclonal antibody against the α7 ACh receptor subunit (1:1000; Sigma) overnight at 4 °C followed by a goat anti-mouse IgG conjugated to Alexa Fluor® 488 (Molecular Probes) for 60 min at room temperature. Fluorescence-labelled cells were visualized with a confocal scanning laser microscope (Axiovert/LSM510, Carl Zeiss).

Real-time monitoring of α7 ACh receptor mobilization

PC-12 cells transfected with NC siRNA or 4.1N siRNA together with the dsRed expression vector were reacted with FITC-conjugated α-bungarotoxin (1.5 μg/ml) (Sigma), and α7 ACh receptor mobilization was monitored by detecting fluorescent signals with a laser scanning confocal microscope (Axiovert/LSM510) before and after (20 min) treatment with DCP-LA (100 nM) at 37 °C.

Statistical analysis

Statistical analysis was carried out using Dunnett's test and an unpaired *t* test.

RESULTS

To identify binding partners of the α7 ACh receptor, 316–468 amino acids in the intracellular loop of the α7 ACh receptor subunit were used as bait in a yeast two-hybrid screen against a randomly primed rat brain cDNA library. Among the independent yeast colonies screened, 174 positive colonies were isolated. Plasmids were isolated from the colonies and transformed into *E. coli*. The cloned plasmids were subjected to PCR amplification, and the PCR products were transferred into the pGEM-T vector. Nucleotide sequences of the 26 plasmids were determined, and the DNA sequences were searched using a BLAST program. The amino acid sequence for one of the clones was identical with the open reading frame of protein 4.1N.

We next investigated whether 4.1N actually interacts with the α7 ACh receptor. For cell lysates from HEK-293 cells expressing HA-tagged 4.1N, a single band immunoreactive with an anti-HA antibody, i.e. 4.1N, was identified in the Western blot analysis (Figure 1A). At the same molecular mass a band immunoreactive with the anti-HA antibody was found for GST pull-down samples after co-incubation of cell lysates from HEK-293 cells expressing HA-tagged 4.1N with the purified GST-fused α7 ACh receptor subunit, whereas no band was obtained with GST pull-down samples after co-incubation of cell lysates from HEK-293 cells expressing HA-tagged 4.1N with GST or co-incubation of a lysis buffer with the purified GST-fused α7 ACh receptor subunit (Figure 1A). This accounts for the interaction of 4.1N with the α7 ACh receptor.

For lysates from rat brain, an immunoreactive band was observed with the anti-α7 ACh receptor subunit antibody (Figure 1B), confirming expression of the α7 ACh receptor in the brain. An immunoreactive band using the anti-(α7 ACh receptor subunit) antibody was confirmed at the same molecular mass in the GST pull-down assay using GST–4.1N CTD for lysates from the rat brain, but not in the GST pull-down assay using GST (Figure 1B). This provides further evidence for the interaction of 4.1N with the α7 ACh receptor.

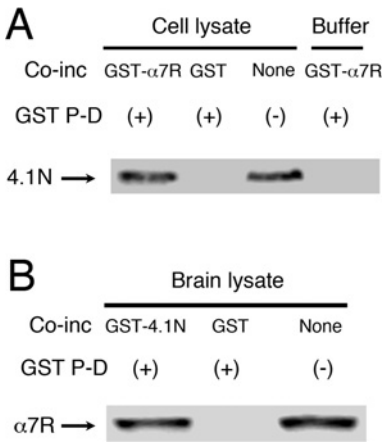

Figure 1 4.1N interacts with the α7 ACh receptor

(**A**) Lysates from HEK-293 cells expressing HA-fused 4.1N or a lysis buffer (Buffer) were co-incubated (Co-inc) without [GST P-D (−)] or with the GST-fused α7 ACh receptor subunit (GST-α7R) or GST [GST P-D (+)].Western blotting using an anti-HA antibody was carried out. Note that similar results were obtained from four independent experiments. GST P-D, GST pull down. (**B**) Lysates from rat brain were co-incubated (Co-inc) without [GST P-D (−)] or with GST–4.1N CTD (GST 4.1N) or GST [GST P-D (+)]. Western blotting using an anti-(α7 ACh receptor subunit) (α7R) antibody was carried out. GST P-D, GST pull down. Note that similar results were obtained from four independent experiments.

Subsequent experiments were conducted to understand the functional role of 4.1N in α7 ACh receptor localization. In our previous study, the linoleic acid derivative DCP-LA stimulated vesicular transport of α7 ACh receptors in a PKC-dependent manner, resulting in increased localization of the receptors at the plasma membrane [19]. For immunoprecipitants with an anti-4.1N antibody using lysates from rat hippocampal slices, DCP-LA (100 nM) significantly increased an association of 4.1N with the α7 ACh receptor, and the DCP-LA effect was partially prevented by GF109203X (100 nM), an inhibitor of PKC (Figure 2). 4.1R, a protein 4.1 family member, is phosphorylated by a variety of kinases such as caseine kinase, tyrosine kinase, PKA (protein kinase A) and PKC [20,21]. Therefore we wanted to investigate whether DCP-LA phosphorylates 4.1N. Unexpectedly, no immunoreactive signal using an anti-phospho-serine antibody or anti-phospho-threonine antibody was detected with immunoprecipitants using an anti-4.1N antibody in lysates from rat hippocampal slices untreated and treated with DCP-LA (100 nM) in the presence and absence of GF109203X (100 nM) (Figure 3A). For a control experiment, a signal band reactive with an anti-phospho-serine antibody was found with immunoprecipitants using an antibody against NSF in lysates from rat hippocampal slices. DCP-LA enhanced the signal intensity, which was reversed by GF109203X (Figure 3B), confirming DCP-LA-induced PKC phosphorylation of NSF. Collectively, these results indicate that DCP-LA increases an association of 4.1N with the α7 ACh receptor in a PKC-dependent manner, but regardless of 4.1N phosphorylation.

To examine whether 4.1N is implicated in α7 ACh receptor trafficking, 4.1N siRNA was constructed and transfected into cultured PC-12 cells. In Western blot analysis expression of 4.1N protein for PC-12 cells transfected with the 4.1N siRNA was

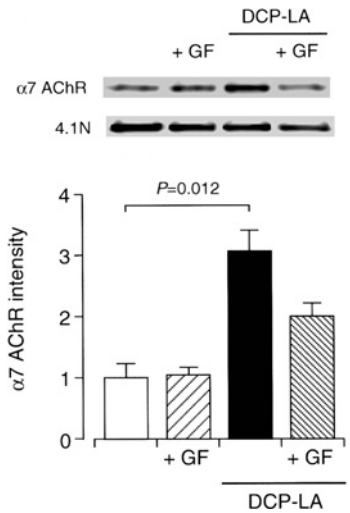

Figure 2 DCP-LA increases an association of the α7 ACh receptor with 4.1N

Lysates from rat hippocampal slices untreated and treated with DCP-LA (100 nM) in the presence and absence of GF109203X (+GF) (100 nM) for 20 min were immunoprecipitated with an anti-4.1N antibody followed by Western blotting with antibodies against the α7 ACh receptor subunit or 4.1N. Signal intensities for the α7 ACh receptor subunit were normalized by the intensities for 4.1N. In the histogram, each column represents the mean (±S.E.M.) normalized α7 ACh receptor subunit intensity ($n = 4$ independent experiments). The P value was calculated using Dunnett's test.

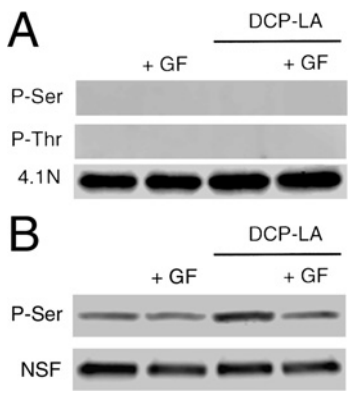

Figure 3 DCP-LA does not phosphorylate 4.1N

Rat hippocampal slices were untreated and treated with DCP-LA (100 nM) in the presence and absence of GF109203X (+GF) (100 nM) for 20 min. (**A**) Lysates from slices were immunoprecipitated with an anti-4.1N antibody followed by Western blotting with an antibody against phospho-serine (P-Ser), phospho-threonine (P-Thr) or 4.1N. Note that similar results were obtained with four independent experiments. (**B**) Lysates from slices were immunoprecipitated with an anti-NSF antibody followed by Western blotting with an antibody against phospho-serine (P-Ser) or NSF. Note that similar results were obtained with four independent experiments.

significantly suppressed as compared with the expression for cells transfected with the NC siRNA (Figure 4A), confirming knock down of 4.1N. To see the cellular distribution of the α7 ACh receptor, PC-12 cells were separated into the cytosolic and plasma membrane fractions. The presence of the α7 ACh receptor in the plasma membrane fraction from cells transfected with 4.1N siRNA was significantly decreased (Figure 4B), suggesting that 4.1N is required for translocation of the α7 ACh receptor towards the plasma membrane. DCP-LA (100 nM) increased the presence of the α7 ACh receptor in the plasma membrane fraction from cells transfected with the NC siRNA, and the DCP-LA effect was still found for cells transfected with 4.1N siRNA (Figure 4B).

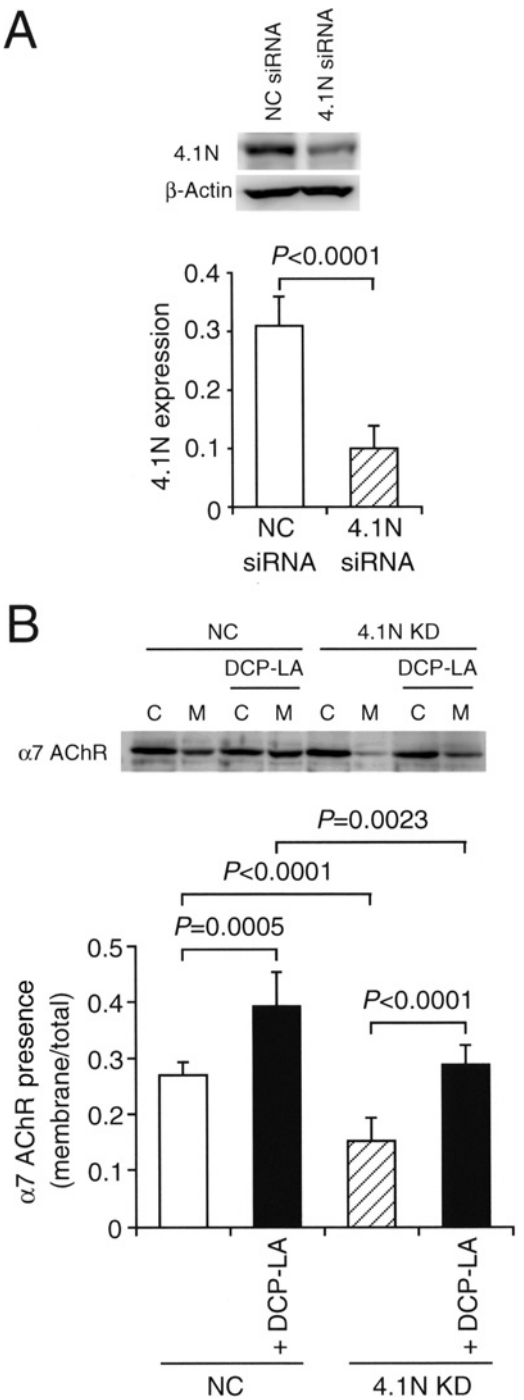

Figure 4 DCP-LA increases the presence of the α7 ACh receptor in the plasma membrane fraction in a 4.1N-dependent manner

(**A**) PC-12 cells were transfected with NC siRNA or 4.1N siRNA and, 48 h after transfection, Western blotting was carried out using antibodies against 4.1N or β-actin. Signal intensities for 4.1N were normalized by those for β-actin. In the histogram, each column represents the mean (±S.E.M.) normalized expression of 4.1N ($n = 4$ independent experiments). The P value was caluclated using an unpaired t test. (**B**) Cells transfected with NC siRNA (NC) or 4.1N siRNA (4.1N KD), were untreated or treated with DCP-LA (100 nM) for 20 min (+DCP-LA). Then, cells were lysed and separated into the cytosolic (C) and plasma membrane (M) fractions, followed by Western blotting using an antibody against the α7 ACh receptor (α7 AChR) subunit. In the histogram, each column represents the mean (±S.E.M.) ratio of the signal intensity for the α7 ACh receptor in the plasma membrane fraction to that in the total plasma membrane and cytosolic fractions ($n = 4$ independent experiments). The P values were calculated using Dunnett's test.

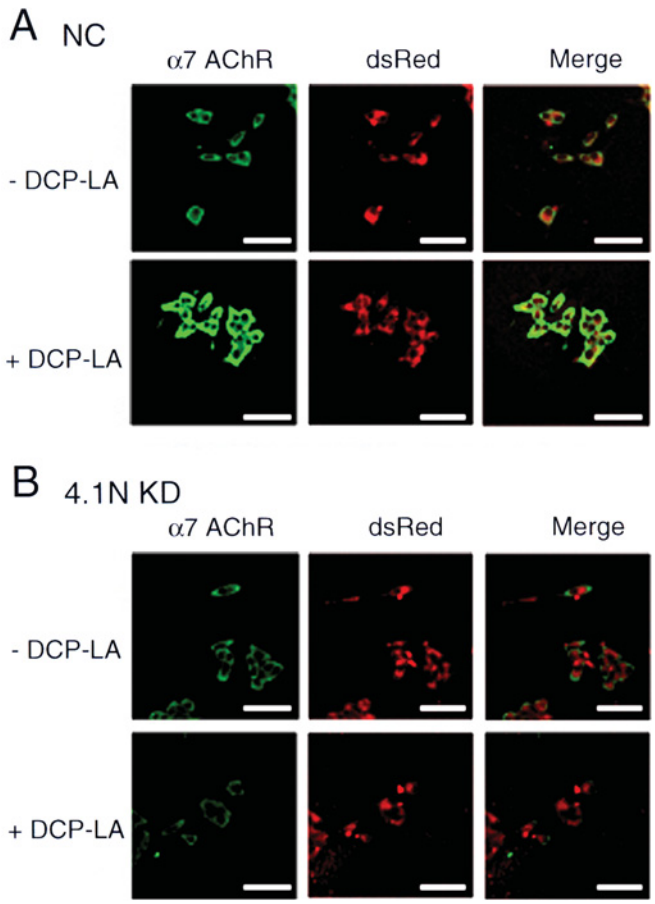

Figure 5 Immunocytochemistry analysis

PC-12 cells transfected with NC siRNA (**A**) or 4.1N siRNA (**B**) together with the dsRed expression vector were untreated (− DCP-LA) or treated with DCP-LA (100 nM) for 20 min (+ DCP-LA) and then immunostained with an anti-(α7 ACh receptor subunit) antibody. Scale bars = 20 μm. Note that a similar result was obtained with four independent experiments.

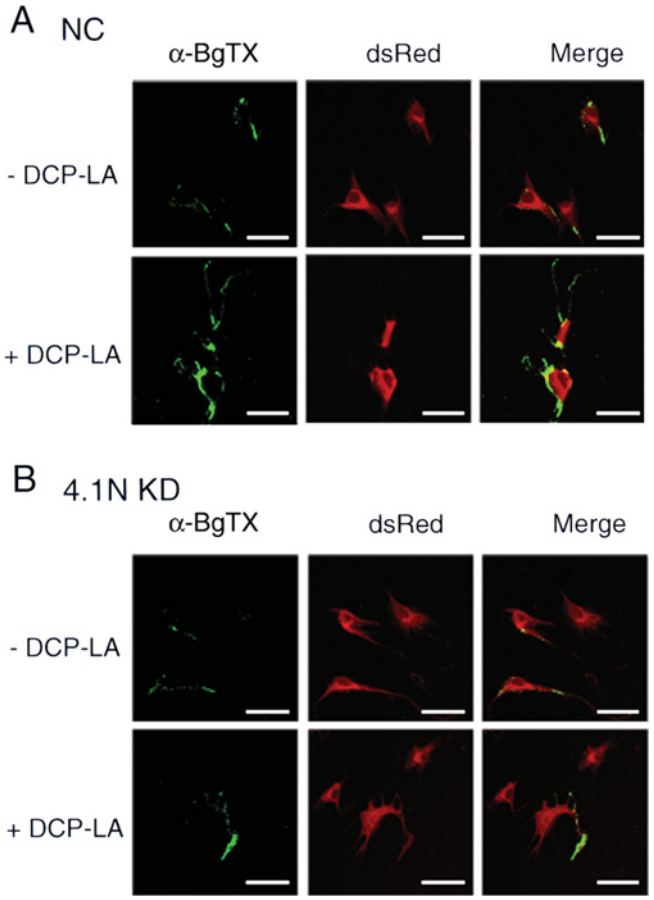

Figure 6 Real-time monitoring of α7 ACh receptor mobilization

PC-12 cells were transfected with NC siRNA (**A**) or 4.1N siRNA (**B**) together with the dsRed expression vector (red). α7 ACh receptor mobilizations were monitored before (− DCP-LA) and after (+ DCP-LA) a 20 min treatment with DCP-LA (100 nM) by detecting FITC-conjugated α-bungarotoxin (α-BgTX) (green). Scale bars = 20 μm. Note that a similar result was obtained with four independent experiments.

DCP-LA induced an increase in the presence of the α7 ACh receptor in the plasma membrane fraction; however, this was significantly inhibited by knocking down 4.1N (Figure 4B). Taken together, these results suggest that DCP-LA increases an association of the α7 ACh receptor with 4.1N, thereby facilitating translocation of the receptor towards the plasma membrane.

To obtain further evidence for this, we carried out immunocytochemistry using PC-12 cells transfected with NC siRNA or 4.1N siRNA. A dsRed expression vector was co-transfected into cells to identify successful transfection with the siRNAs. For cells transfected with NC siRNA, immunoreactive signals for the α7 ACh receptor were found around individual cell membranes and the signals were apparently enhanced after treatment with DCP-LA (100 nM) as compared with those before treatment (Figure 5A). In contrast, for cells transfected with 4.1N siRNA, such enhancement in the immunoreactive signals for the α7 ACh receptor was not obtained with DCP-LA treatment (Figure 5B). This indicates that DCP-LA increases membrane surface localization of the α7 ACh receptor, which is suppressed by knocking down 4.1N.

Finally we monitored α7 ACh receptor mobilization using FITC-conjugated α-bungarotoxin in PC-12 cells transfected with NC siRNA or 4.1N siRNA. α-Bungarotoxin is an antagonist with a high and strong affinity for the α7 ACh receptor, and it binds to the α7 ACh receptor on the plasma membrane under *in situ* conditions. FITC signals, therefore, reflect α-bungarotoxin binding to membrane-surface α7 ACh receptors. For cells transfected with NC siRNA, faint FITC signals were found around individual cell membranes, and DCP-LA (100 nM) apparently enhanced the signals (Figure 6A). For cells transfected with 4.1N siRNA, FITC signals were much weaker and the DCP-LA-induced increase in the signals was much less as compared with those for cells transfected with the NC siRNA (Figure 6B). Overall, DCP-LA appears to stimulate α7 ACh receptor translocation from the cytosol to the plasma membrane under the interaction with 4.1N.

DISCUSSION

4.1N contains 881 amino acid residues and consists of the U1 (unique 1), FERM (4.1/ezrin/radixin/moesin), U2 (unique 2), SAB (spectrin–actin binding), U3 (unique 3) and C-terminal domains [22]. Amazingly, the 4.1N SAB domain does not form a ternary complex with spectrin and actin, whereas 4.1B, 4.1G and 4.1R SAB domains are capable of forming such a complex [23]. 4.1N, however, stabilizes the plasticity of the neuronal membrane through interactions with the spectrin–actin-based skeleton, integral membrane channels and receptors, and

membrane-associated guanylate kinases [6]. 4.1N is abundantly expressed in mammalian central and peripheral neurons, and it is detected in embryonic neurons at the earliest stage of post-mitotic differentiation. 4.1N is enriched at discrete sites of synaptic contact, co-localizing with the postsynaptic density protein of 95 kDa and the GluR1 AMPA receptor subunit [6]. In addition, evidence has pointed to a direct interaction of 4.1N with the GluR1 subunit [8]. The functional role of 4.1N is not fully understood, but studies have suggested requirement of 4.1N for insertion of receptors and ion channels such as AMPA and IP_3 (inositol 1,4,5-trisphosphate) receptors into the membrane [24,25].

So far, no information regarding the interaction of 4.1N with the α7 ACh receptor has been provided. In the present study, yeast two-hybrid screening identified that 4.1N is a binding partner of the α7 ACh receptor. For PC-12 cells, membrane surface localization of the α7 ACh receptor was significantly decreased by knocking down 4.1N. This suggests that 4.1N is required for tethering of the α7 ACh receptor into the plasma membrane, as is the case with AMPA and IP_3 receptors.

We have previously found that the linoleic acid derivative DCP-LA increases the membrane surface presence of the α7 ACh receptor in parallel with a decreased cytosolic presence of the receptor in a PKC-dependent manner [19]. Then, we postulated that 4.1N might participate in DCP-LA-stimulated α7 ACh receptor tethering into the plasma membrane. DCP-LA increased an association of the α7 ACh receptor with 4.1N in rat hippocampal slices which is inhibited by the PKC inhibitor GF109203X. This indicates that DCP-LA enhances 4.1N binding to the α7 ACh receptor in a PKC-dependent manner. 4.1R is recognized to contain phosphorylation sites for a variety of protein kinases including caseine kinase, tyrosine kinase, PKA and PKC [20,21]. 4.1N, accordingly, might also be phosphorylated by PKC. In the present study, serine or threonine phosphorylation of 4.1N was not found with DCP-LA. This suggests that the DCP-LA-induced increase in the association of the α7 ACh receptor with 4.1N is not caused by PKC phosphorylation of 4.1N. PKC phosphorylation of the GluR1 subunit is shown to enhance 4.1N binding to the GluR1 subunit [25]. Therefore one could wonder whether DCP-LA enhances 4.1N binding to the α7 ACh receptor by activating PKC to phosphorylate the receptor. The α7 ACh receptor, however, contains no PKC phosphorylation site [19,26]. It is presently unknown how DCP-LA increases the association of the α7 ACh receptor with 4.1N under the control of PKC. A plausible explanation for this is that DCP-LA phosphorylates an unknown factor or mediator to enhance an association of 4.1N with the α7 ACh receptor.

DCP-LA increased the presence of the α7 ACh receptor in the plasma membrane fraction from PC-12 cell lysates, and the DCP-LA effect was significantly prevented by knocking down 4.1N. In the immunocytochemical study and real-time monitoring of α7 ACh receptor mobilization, membrane surface localization of the α7 ACh receptor for PC-12 cells that were not treated or the DCP-LA-induced increase in the membrane surface localization of the α7 ACh receptor was attenuated by knocking down 4.1N. Overall, these results indicate that DCP-LA promotes membrane surface localization of the α7 ACh receptor in a 4.1N-dependent manner under the control of PKC, but without phosphorylating 4.1N. This may provide a fresh insight into the action of DCP-LA on 4.1N-regulated α7 ACh receptor tethering.

In summary, the results of the present study show that 4.1N, directly binding to the α7 ACh receptor, is required for membrane surface localization of the receptor and that the linoleic acid derivative DCP-LA enhances 4.1N binding to the α7 ACh receptor under the control of PKC to stimulate delivery of the receptor towards the plasma membrane.

AUTHOR CONTRIBUTION

Takeshi Kanno and Ayako Tsuchiya performed the experiments, collected the data and prepared the Figures. Akito Tanaka synthesized DCP-LA. Tomoyuki Nishizaki designed the experiments and wrote the paper. All authors have approved the final paper.

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