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RESEARCH ARTICLE

THE ANTI-DEMENTIA DRUG DCP-LA BINDS TO NSF SPECIFICALLY

[1,*]Tomoyuki Nishizaki, [2]Miyuki Mabuchi and [3]Akito Tanaka

[1]Innovative Bioinformation Research Organization, Kobe 651-1223, Japan
[2]Shanghai University of Traditional Chinese Medicine, Education College of Medicine, Osaka 530-0047, Japan
[3]Laboratory of Chemical Biology, Advanced Medicinal Research Center, Hyogo University of Health Sciences, Kobe 650-8530, Japan

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ABSTRACT

The linoleic acid derivative 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoicacid (DCP-LA) has been developed as a promising and novel anti-dementia drug. The present study probed the binding partners of DCP-LA. In the DCP-LA binding assay using rat hippocampal lysates, fluorescein-conjugated DCP-LA produced a fluorescent signal band at 75 kDa. In the matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOFMS) analysis, the signal band corresponded to *N*-ethylmaleimide-sensitive factor (NSF). In the immunofluorescent cytochemistry using acutely dissociated rat hippocampal neurons, intracellular localization of NSF was well consistent with DCP-LA distribution. The Biacore assay equipped with a sensor chip immobilizing DCP-LA was carried out and the equilibrium dissociation constant (Kd) value between DCP-LA and NSF was measured by applying a variety of concentrations of recombinant NSF. The results of the present study show that DCP-LA binds to NSF specifically, with the Kd value of $3.3x10^{-7}$ M.

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INTRODUCTION

Accumulating evidence has pointed to the anti-dementia effects of 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoicacid (DCP-LA) in a variety of animal models (Kanno *et al*., 2012b; Kanno *et al*., 2016; Nagata *et al*., 2005; Nagata *et al*., 2010; Yaguchi *et al*., 2006). The primary site of action of DCP-LA is PKCε (Kanno *et al*., 2006; Kanno *et al*., 2015). DCP-LA activates PKCε selectively in a diacylglycerol (DG)- and calcium-independent manner. 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 presynaptic terminals in a PKC-dependent manner, causing an increase in glutamate release to facilitate hippocampal synaptic transmission (Kanno *et al*., 2012a; Shimizu *et al*., 2011; Tanaka and Nishizaki, 2003; Yamamoto *et al*., 2005). How DCP-LA regulates vesicular transport of α7 nicotinic ACh receptor, however, remained to be explored. To address this question, the present study aimed at identifying the binding partners of DCP-LA linked to vesicular transport.

***Corresponding author:* Tomoyuki Nishizaki,**
Innovative Bio information Research Organization, Kobe 651-1223, Japan.

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.

Screening of DCP-LA binding proteins: Rat hippocampal slices (400 μm in thickness; male Wistar rat, 6 weeks) were treated with fluorescein-conjugated DCP-LA for 20 min at 37 °C in a standard artificial cerebrospinal fluid (ACSF) (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. 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 lysed in a lysate buffer (0.25 M sucrose, 300 mM N,N-diethylthiocarbamate, 25 mM Tris, pH 7.6), followed by SDS-PAGE. Fluorescent signals were visualized using FluoroPhoreStar3000 (Anatech, Tokyo, Japan).

Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOFMS) analysis: The fluorescent signal band was excised and its amino acid sequence was analyzed with MALDI-TOFMS.

Immunofluorescent cytochemistry: Neurons were mechanically dissociated from rat hippocampal slices (400 μm in thickness; male Wistar rat, 6 weeks). Acutely dissociated neurons were reacted with fluorescein isothiocyanate (FITC)-conjugated DCP-LA for 20 min at 37 °C in ACSF oxygenated with 95% O_2 and 5% CO_2, and intracellular distribution of DCP-LA was monitored in a single neuron by detecting fluorescent signals with a laser scanning confocal microscope (LSM 510, Carl Zeiss Co., Ltd., Oberkochen, Germany). Then, neurons were fixed with 4% (w/v) paraformaldehyde, permeabilized with 0.3% (v/v) Triton X-100, and blocked with 10% (v/v) goat serum at room temperature, and reacted with an anti-NSF antibody (Invitrogen, Waltham, MA USA) overnight at 4 °C, followed by a goat anti-mouse IgG antibody conjugated with Alexa 633 (Molecular Probes, Eugene, OR, USA) for 60 min at room temperature. Fluorescence-labeled cells were visualized with a laser scanning confocal microscope. The nucleus was stained with DAPI.

Biacore assay: NSF was produced in E. Coli in the Gateway system. DCP-LA was immobilized to the sensor chip CM5 via the carboxylic acid. The equilibrium dissociation constant (Kd) value between DCP-LA and NSF was measured with Biacore3000 (GE Health Care Japan, Tokyo, Japan) equipped with CM5 by applying a variety of concentrations of NSF.

RESULTS

DCP-LA binds to NSF specifically: Our first attempt was to probe the DCP-LA-binding proteins. Fluorescein-conjugated DCP-LA produced a single fluorescent signal band at 75 kDa in lysates from rat hippocampal slices (Figure 1A).

The MALDI-TOFMS analysis confirmed that the signal band corresponded to NSF (Figure 1B). The results indicate that DCP-LA binds to NSF specifically.

DCP-LA co-localizes with NSF: We subsequently carried out immunofluorescent cytochemistry using FITC-conjugated DCP-LA in acutely dissociated rat hippocampal neurons. Intracellular localization of NSF was well consistent with DCP-LA distribution (Figure 2).

DCP-LA binds to NSF, with the Kd value of $3.3x10^{-7}$ M: To obtain further evidence for DCP-LA binding to NSF, the Kd value between DCP-LA and NSF was measured using Biacore3000 equipped with the sensor chip CM5 immobilizing DCP-LA by applying a variety of concentrations of recombinant NSF. The result showed that the Kd value is $3.3x10^{-7}$ M (Figure 3).

DISCUSSION

NSF, an ATPase, 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). Notably, NSF regulates not only vesicular exocytosis of neurotransmitters but 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 *et al*., 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).

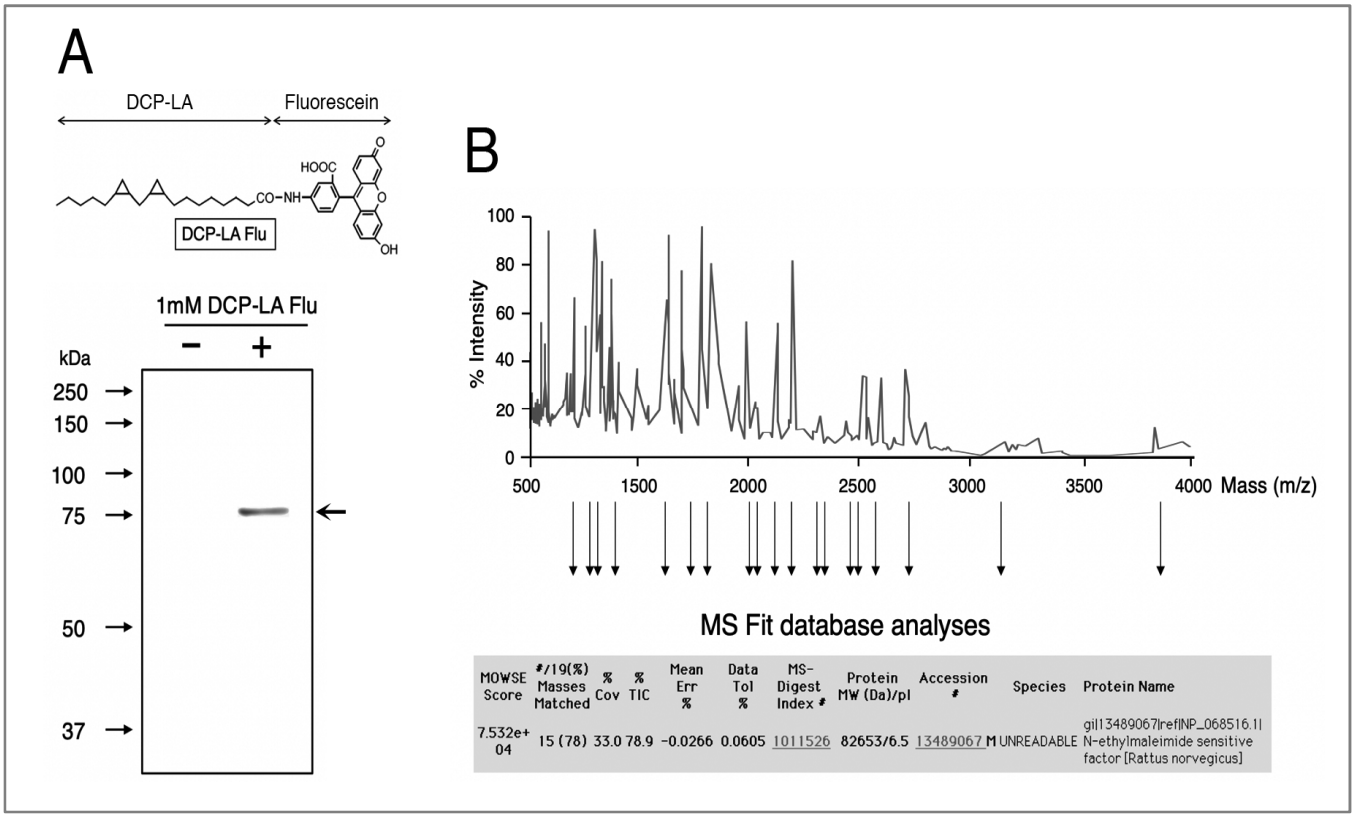

Figure 1. DCP-LA binds to NSF. (A) Chemical structure for fluorescein-conjugated DCP-LA (DCP-LA Flu) is shown in the upper column. Lysates from rat hippocampal slices were treated with DCP-LA Flu (1 mM) for 20 min. A fluorescent signal band is indicated by the arrow. (B) The signal band was excised and the amino acid sequence was analyzed with MALDI-TOFMS. The signal band was consistent with NSF. Note that similar results were obtained with 4 independent experiments

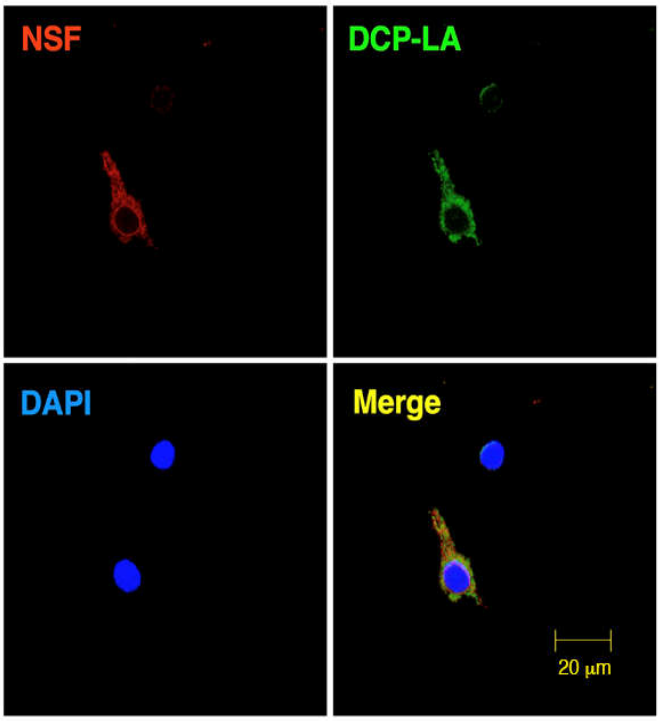

Figure 2. DCP-LA co-localizes with NSF. Acutely dissociated neurons from rat hippocampal slices were reacted with FITC-conjugated DCP-LA for 20 min, followed by immunofluorescent cytochemistry. The nucleus was stained with DAPI. Immnostaining for NSF, red; FITC image for DCP-LA, green; Merged image, yellow; Nucleus, blue

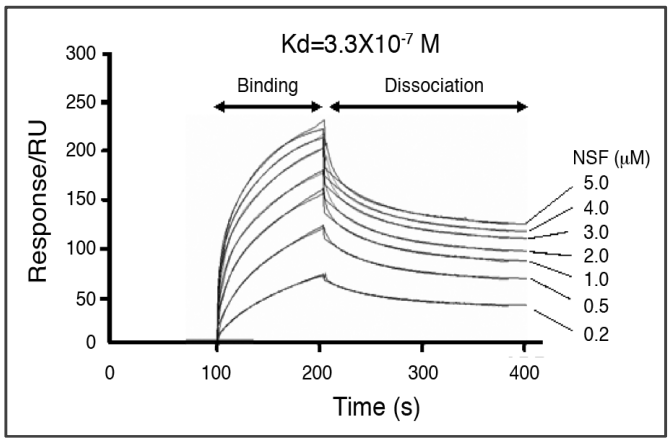

Figure 3. DCP-LA binds to NSF, with the Kd value of $3.3x10^{-7}$ M. The Kd value between DCP-LA and NSF was measured with the Biacore systems by applying recombinant NSF at concentrations as indicated

We have earlier found that DCP-LA stimulates vesicular exocytosis of α7 nicotinic ACh receptor (Kanno *et al*., 2012a). In the present study, DCP-LA bound to NSF in lysates from rat hippocampal slices; intracellular localization of NSF was well consistent with DCP-LA distribution; and the Kd value between DCP-LA and NSF was $3.3x10^{-7}$ M. This raises the possibility that DCP-LA may enhance formation of an α7 nicotinic ACh receptor/SNAP/SNARE complex by targeting NSF, which triggers vesicular exocytosis of the receptor.

Conclusion

The results of the present study provide evidence that DCP-LA binds to NSF specifically, with the Kd value of $3.3x10^{-7}$ M. This may gain further insight into the DCP-LA actions.

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