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α,β-DCP-LA Selectively Activates PKC-ε and Stimulates Neurotransmitter Release with the Highest Potency among 4 Diastereomers

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Key Words

DCP-LA • Diastereomer • Selective optical resolution • PKC-ε • Transmitter release

Abstract

Background/Aims: We have been probing bioactivities of 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA), a linoleic acid derivative with cyclopropane rings instead of *cis*-double bonds, using a racemic modification. Racemic DCP-LA contains possible 4 diastereomers. We, therefore, separately synthesized DCP-LA diastereomers such as α,α-, α,β-, β,α-, and β,β-DCP-LA and assessed the effects of each diastereomer on protein kinase C (PKC) activity and transmitter release. Methods: PKC activity under the cell-free conditions and in PC-12 cells, and glutamate, dopamine, and serotonin released from rat brain slices were assayed with a high performance liquid chromatography (HPLC) system. Results: Of 4 diastereomers α,β-DCP-LA selectively and directly activated PKC-ε, with the highest potency. α,β-DCP-LA stimulated release of glutamate, dopamine, and serotonin from rat hippocampal, striatal, and hypothalamic slices, respectively, under the control of PKC, possibly PKC-ε, and α7 nicotinic ACh receptors, with the highest potency among 4 diastereomers. Conclusion: α,β-DCP-LA serves as a selective and direct activator of PKC-ε, to stimulate transmitter release by targeting α7 nicotinic ACh receptors. This suggests that α,β-DCP-LA could be developed as a promising drug for treatment of not only dementia but neurodegenerative diseases and psychiatric disorders due to reduction/deficiency of neurotransmitters.

Introduction

Accumulating evidence has shown that 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA), an originally synthesized linoleic acid derivative with cyclopropane rings instead of *cis*-double bonds [1], could ameliorate a variety of cognitive disorders including Alzheimer disease. DCP-LA enhances activity of presynaptic α7 acetylcholine (ACh) receptors by activating protein kinase C (PKC), causing a marked increase in glutamate release, and then leading to a long-lasting facilitation of hippocampal synaptic transmission

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[2]. In addition, DCP-LA stimulates release of γ-aminobutyric acid (GABA) from hippocampal interneurons as mediated via presynaptic α7 ACh receptors under the influence of PKC [3]. DCP-LA, alternatively, activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) by inhibiting protein phosphatase-1 (PP-1), to promote exocytosis of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor subunits GluR1 and GluR2, resulting in the increased number of AMPA receptors on the postsynaptic plasma membrane in the hippocampus, which also contributes to facilitation of hippocampal synaptic transmission [4]. The facilitatory action of DCP-LA on hippocampal synaptic transmission accounts for improvement of spatial learning and memory impairment induced by intraperitoneal injection with scopolamine or intraventricular injection with amyloid $\beta_{1\text{-}40}$ peptide [5] or improvement of age-related cognitive decline [6]. Furthermore, DCP-LA neutralizes impairment of long-term potentiation (LTP), a cellular model of learning and memory, in the *in vivo* hippocampus and spatial learning deficits, that are induced by a considerably low dose of mutant amyloid $\beta_{1\text{-}42}$ peptide lacking glutamate-22 [7], suggesting the beneficial effect of DCP-LA against Alzheimer dementia.

DCP-LA, on the other hand, is capable of protecting neurons from oxidative stress-induced apoptosis by inhibiting caspase-3/-9 activation [8]. This, in the light of the fact that oxidative stress-induced neuronal apoptosis is a critical factor for pathogenesis of neurodegenerative diseases such as Alzheimer disease and Parkinson disease [9-11], further supports the possibility that DCP-LA could be developed as a promising drug for treatment of neurodegenerative diseases including Alzheimer disease.

Our previous studies indicate that PKC is the primary action site of DCP-LA. PKC isozymes include conventional PKCs such as PKC-α, -βI, -βII, and -γ, novel PKCs such as PKC-δ, -ε, -η, -θ, and -μ, and atypical PKCs such as PKC-λ/ι for mouse/human, -ζ and -ν. PKCs are activated through several pathways linked to phospholipase C (PLC), phospholipase A_2 (PLA_2), phospholipase D (PLD), and phosphatidylcholine-specific PLC [12-14]. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate (IP_3), the latter activating IP_3 receptors to release Ca^{2+} from intracellular calcium stores, and conventional PKCs are activated by diacylglycerol and Ca^{2+} [13, 14]. Phosphatidylcholine-specific PLC produces diacylglycerol by hydrolysis of phosphatidylcholine, thereby activating PKC [12]. *Cis*-unsaturated free fatty acids such as arachidonic, oleic, linoleic, linolenic, and docosahexaenoic acid, that are produced by PLA_2-catalyzed hydrolysis of phosphatidylcholine, activate novel PKCs in a Ca^{2+}-independent manner [13, 14]. Moreover, the free fatty acids synergistically activate conventional PKCs or sustain activity of conventional PKCs activated [13, 14]. We have earlier found that DCP-LA serves as a selective and direct activator of PKC-ε, possibly by binding to the phosphatidylserine binding site, independently of diacylglycerol and Ca^{2+} [15]. So far we have carried out experiments using racemic modification of DCP-LA. Racemic DCP-LA is thought to consist of 4 diastereomers such as aa (α,α)-, ab (α,β)-, ba (β,α)-, and bb (β,β)-DCP-LA (Fig. 1). Which DCP-LA diastereomer exhibits an ideal effect remains to be explored.

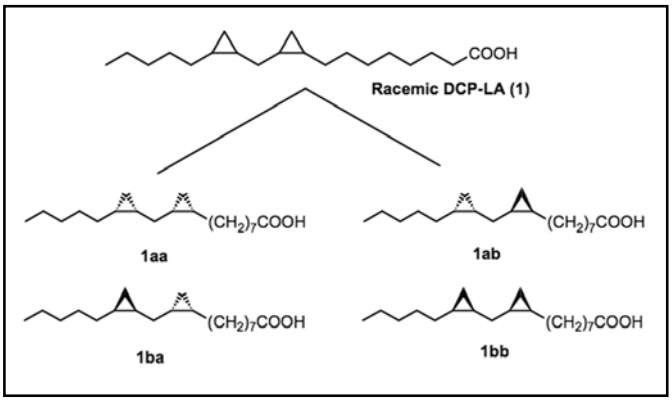

Fig. 1. Structures of racemic DCP-LA and possible diastereomers.

To address this question, we synthesized α,α-, α,β-, β,α-, and β,β-DCP-LA diastereomers, and assayed the effect of each diastereomer on PKC activation and neurotransmitter release. We show here that of 4 diastereomers α,β-DCP-LA selectively and directly activates PKC-ε, and stimulates release of transmitters such as glutamate, dopamine, and serotonin, with the highest potency.

Materials and Methods

Animal care

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

Cell culture

Rat PC-12 cells, that were obtained from RIKEN Cell Bank (Tsukuba, Japan), were cultured in Dulbecco's modified Ea-

gle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 10% (v/v) heat-inactivated horse serum, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

In situ PKC assay

PKC activity in rat PC-12 cells was assayed by the method as previously described [15]. Cells were treated with DCP-LA diastereomers at 37°C for 10 min in an extracellular solution [137 mM NaCl, 5.4 mM KCl, 10 mM $MgCl_2$, 5 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.3 mM Na_2HPO_4, 0.4 mM K_2HPO_4, and 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, pH 7.2]. Then, cells were rinsed with 100 μl of Ca^{2+}-free phosphate-buffered saline (PBS) and incubated at 30°C for 15 min in 50 μl of the extracellular solution containing 50 μg/ml digitonin, 25 mM glycerol 2-phosphate, 200 μM ATP, and 100 μM synthetic PKC substrate peptide (Pyr-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu; MW, 1,374) (Peptide Institute Inc., Osaka, Japan). The supernatants were collected and boiled at 100°C for 5 min to terminate the reaction. Aliquot of the solution (20 μl) was loaded onto a reversed phase high performance liquid chromatography (HPLC) (LC-10ATvp, Shimadzu Co., Kyoto, Japan). A substrate peptide peak and a new product peak were detected at an absorbance of 214 nm (SPD-10Avp UV-VIS detector, Shimadzu Co., Kyoto, Japan). It was confirmed that each peak corresponds to non-phosphorylated and phosphorylated substrate peptide in the analysis of matrix-assisted laser desorption ionization time of flight mass spectrometry (Voyager ST-DER, PE Biosystems Inc., Foster City, USA). Molecular weights were calibrated from the two standard spectrums, bradykinin (MW 1060.2) and neurotensin (MW 1672.9). Areas for non-phosphorylated and phosphorylated PKC substrate peptide were measured (total area corresponds to concentration of PKC substrate peptide used here), and the amount of phosphorylated substrate peptide was calculated. Phosphorylated substrate peptide (pmol/1 min/cell protein weight) was used as an index of PKC activity.

Knocking-down PKC-ε

The sequence for the small, interfering RNA (siRNA) to silence the PKC-ε-targeted gene used here was as follows: 5'-CAC AUC AGU GAC GAA CUC AUT T-3' and 5'-AUG AGU UCG UCA CUG AUG UGT T-3'. The siRNA containing scrambled sequences with the GC content and nucleic acid composition same as those for the PKC-ε siRNA was used as a negative control (NC) siRNA. PC-12 cells were transfected with the PKC-ε siRNA or the NC siRNA, and 24 h later after transfection in situ PKC assay was carried out.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs of PC-12 cells transfected with the PKC-ε siRNA or the NC siRNA were purified by an acid/guanidine/thiocyanate/chloroform extraction method using the Sepasol-RNA I Super kit (Nacalai Tesque, Kyoto, Japan). After purification, total RNAs were treated with RNase-free DNase I (2 units) at 37°C for 30 min to remove genomic DNAs, and 10 μg of RNAs was resuspended in water. Then, random primers, dNTP, 10x RT buffer, and Multiscribe Reverse Transcriptase (Applied Biosystems, CA, USA) were added to an RNA solution and incubated at 25°C for 10 min followed by 37°C for 120 min to synthesize the first-strand cDNA. Real-time PCR was performed using a SYBR Premix Ex Taq (Takara Bio, Otsu, Japan) and the Applied Biosystems 7900 real-time PCR detection system (ABI, Foster City, CA). Thermal cycling conditions were as follows: first step, 94°C for 4 min; the ensuing 40 cycles, 94°C for 1 s, 65°C for 15 s, and 72°C for 30 s for PKC-ε. The PKC-ε mRNA expression level was normalized by the GAPDH mRNA. Primers used for RT-PCR were as follows: 5'-GTT GTG GAT CCC GAA CTT GTG GG-3' and 5'-CTG AAA GCT TTC ATG ACC AAG AAC CC-3' for PKC-ε (accession number NM_017171) and 5'-CCT TCC GTG TTC CTA CCC CCA AT-3' and 5'-CCT CTC TCT TGC TCT CAG TAT CCT TGC T-3' for GAPDH (accession number BC059110).

Western blotting

PC-12 cells transfected with the PKC-ε siRNA or the NC siRNA were homogenized with a sonicator in an ice-cold PBS containing 1% (v/v) protease inhibitor cocktail (Nacalai, Kyoto, Japan), and then centrifuged at 3,000 rpm for 5 min at 4°C. The supernatants (20 μg of protein) were loaded on 10% (v/v) SDS-polyacrylamide gel and electrophoresed. Separated proteins were transferred onto polyvinylidene difluoride membrane. Blotting membranes were blocked with TTBS (150 mM NaCl, 0.1% Tween20 and 20 mM Tris, pH7.5) containing 5% (v/v) bovine serum albumin (Wako, Osaka, Japan) and reacted with an anti-PKC-ε antibody (Chemicon, Temecula, CA, USA) or an anti-β-actin antibody (Sigma, St. Louis, MO, USA), followed by a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody or goat anti-mouse IgG antibody, respectively. Immunoreactivity was detected with an ECL kit (GE Healthcare, Piscataway, NJ, USA) and visualized using a chemiluminescence detection system (FUJIFILM, Tokyo, Japan).

Cell-free PKC assay

PKC activity in the cell-free systems was quantified by the method as previously described [15]. Briefly, synthetic PKC substrate peptide (10 μM) was reacted with a variety of PKC isozymes in a medium containing 20 mM Tris-HCl (pH 7.5), 5 mM Mg-acetate, 10 μM ATP, and DCP-LA diastereomers in the absence of phosphatidylserine and diacylglycerol at 30°C for 5 min. Activity for novel PKCs such as PKC-δ, –ε, –η, and -μ was assayed in Ca^{2+}-free medium and activity for the other PKC isozymes in the medium containing 100 μM $CaCl_2$. After loading on a reversed phase HPLC (LC-10ATvp, Shimadzu Co., Kyoto, Japan), a substrate peptide peak and a new product peak were detected at an absorbance of 214 nm. Areas for non-phosphorylated and phosphorylated PKC substrate peptide were measured (total area corresponds to concentration of PKC substrate peptide used here), and the amount of phosphorylated substrate peptide was calculated. Phosphorylated substrate peptide (pmol/1 min) was used as an index of PKC activity.

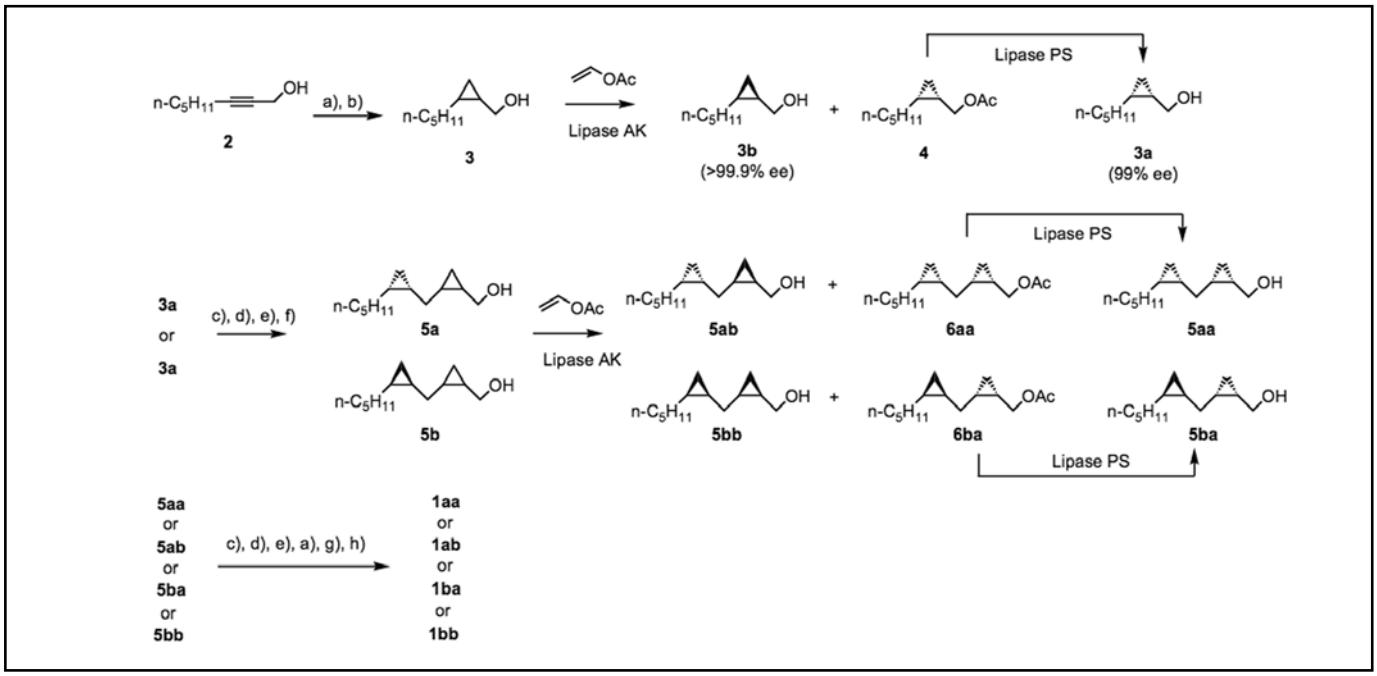

Fig. 2. Synthesis of possible diastereomers. Cyclopropyl methanol derivative (3) was obtained from commercial available compound (2). Treatment of (3) with commercial available Lipase AK and vinyl acetate afforded high enatio-purity of β-cyclopropyl derivative (3b) [>99.9% ee, 38% yield from (3)] with acetyl product (4) (76% ee). Hydrolysis of (4) using Lipase PS provided α-cyclopropyl derivative (3a) [99.0% ee, 28% yield from (3)]. (3a) and (3b) were converted to bicyclopropyl derivatives (5a) and (5b) in 4 steps, respectively. Then, (5ab) (98.0% de) and (5bb) (>99.9% de) were produced from (5a) and (5b), respectively, together with (6aa) and (6ba) in the presence of Lipase AK and vinyl acetate, and (6aa) and (6ba) were converted to (5aa) (98.0% de) and (5ba) (98.0% de), respectively, by Lipase PS. Finally, (5aa), (5ab), (5ba), and (5bb) were converted to desired high enatiopure DCP-LA derivatives (1aa) (α,α-), (1ab) (α,β-), (1ba) (β,α-), and (1bb) (β,β-) in 6 steps, respectively. The [α]D23 values of them were +0.47 (c = 10), -9.9 (c = 1.0), +9.9 (c = 0.90), and -0.45 (c = 10). Conditions: a) H_2/Pd-$CaCO_3$, quinoline, b) Et_2Zn, CH_2I_2, c) Ph_3P, I_2, imidazole, d) HC=CCH_2OTHP, nBuLi, e)TsOH, f) H_2/Pd-$CaCO_3$, ethylenediamine, g) KO_2CN=NCO_2K, AcOH, and h) Joness oxidation.

Assay of glutamate, dopamine, and serotonin

The hippocampus, striatum, and hypothalamus were isolated from the rat brain (male Wistar rat, 6 weeks) and sliced at 400 μm in thickness for assay of glutamate, dopamine, and serotonin, respectively. Slices were incubated 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 $NaHCO_3$, 11.5 mM glucose) oxygenated with 95% O_2 and 5% CO_2 at room temperature for 1 h followed by at 34°C for 50 min. Then, slices were transferred to a chamber filled with 1 ml of ACSF oxygenated with 95% O_2 and 5% CO_2 containing tetrodotoxin (TTX) (0.5 μM) in the presence and absence of nicotine (1 μM) and DCP-LA diastereomers together with and without GF109203X (100 nM) or α-bungarotoxin (α-BgTX) (100 nM) at 34°C for 20 min. After treatment, external solution was collected and glutamate released was labeled with 4-fluoro-7-nitrobenzofurazan (NBD-F). Then, 20 μl of NBD-F-labeled solution was injected onto the column (150 X 4.6 mm), and loaded onto the HPLC system. NBD-F was detected at an excitation of wavelength of 350 nm and an emission wavelength of 450 nm using a fluorescence detector.

Results

Selective optical resolution from racemic DCP-LA

Neither chiral column chromatography nor chiral resolving agents were available for selective optical resolution from racemic DCP-LA. We, therefore, separately synthesized DCP-LA diastereomers through roots as described in Fig. 2, and successfully obtained 4 diastereomers such as α,α-, α,β-, β,α-, and β,β-DCP-LA.

α,β-DCP-LA selectively and directly activates PKC-ε, with the highest potency among 4 diastereomers

To examine the effect of DCP-LA diastereomers on PKC activation, we initially carried out *in situ* PKC assay using PC-12 cells. In the reversed phase HPLC

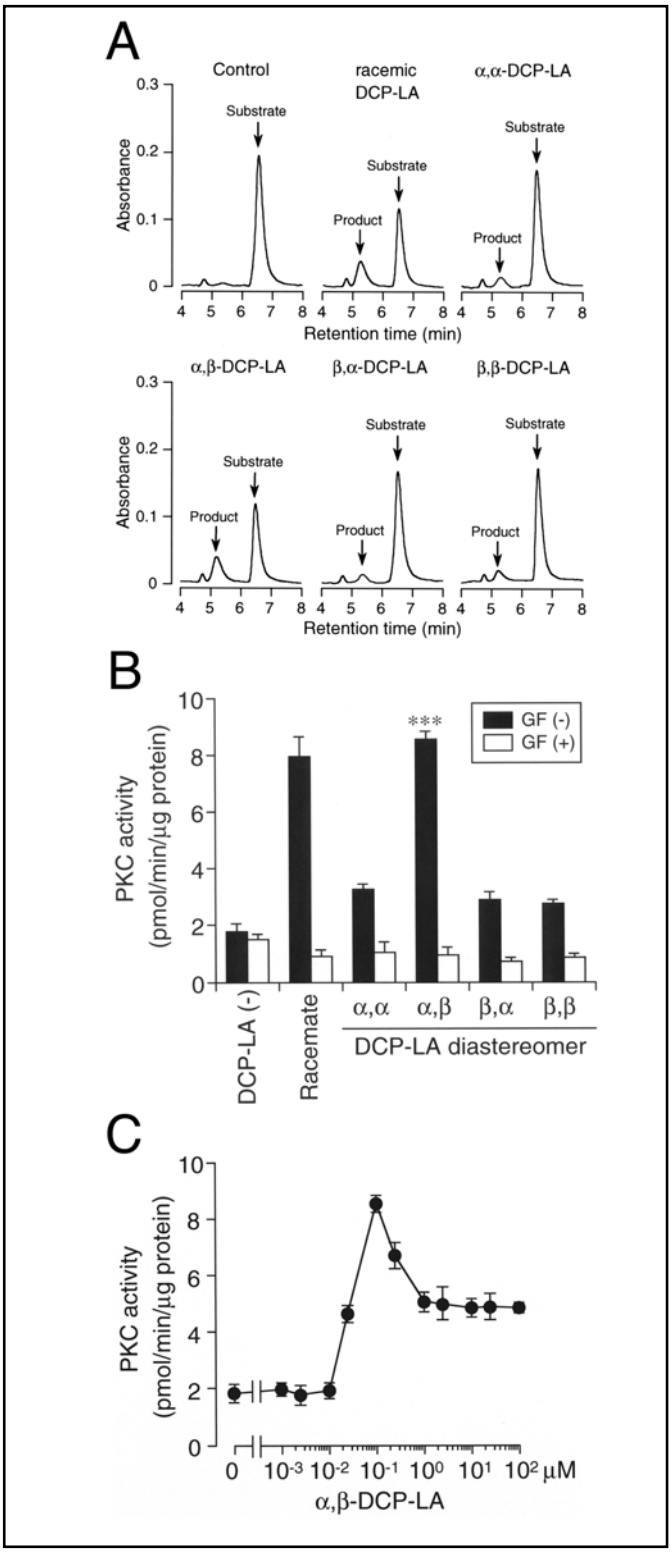

Fig. 3. α,β-DCP-LA activates PKC in PC-12 cells with the highest potency. PC-12 cells were untreated and treated with racemic DCP-LA or DCP-LA diastereomers at a concentration of 100 nM in the presence and absence of GF109203X (GF) (100 nM), and PKC activity was assayed. (A) Reversed phase HPLC profiles. A new peak (Product) is found after treatment with racemic DCP-LA and DCP-LA diastereomers. Substrate, substrate peptide peak. (B) In the graph, each column represents the mean (± SEM) PKC activity (pmol/min/μg protein) (n=6). ***P<0.001 as compared with each PKC activation induced by α,α-, β,α-, or β,β-DCP-LA in the absence of GF109203X, Dunnett's test. (C) Cells were treated with α,β-DCP-LA at concentrations as indicated, and PKC activity was assayed. Each point represents the mean (± SEM) PKC activity (pmol/min/μg protein) (n=6).

profiles, racemic DCP-LA and all the DCP-LA diastereomers at a concentration of 100 nM produced a new peak, that corresponds to phosphorylated substrate peptide (Fig. 3A) and the effect was abolished by GF109203X (100 nM), a PKC inhibitor (Fig. 3B), indicating PKC activation due to racemic DCP-LA and DCP-LA diastereomers. α,β-DCP-LA activated PKC to an extent similar to racemic DCP-LA, and notably, the activation was significantly higher than other DCP-LA diastereomers (Fig. 3B). α,β-DCP-LA activated PKC in a bell-shaped concentration (1 nM-100 μM)-dependent manner, with the maximal effect at 100 nM (Fig. 3C). These results indicate that α,β-DCP-LA is capable of activating PKC in PC-12 cells, with the highest potency among 4 diastereomers.

In our earlier study, racemic DCP-LA selectively and directly activated PKC-ε [15]. We next examined whether α,β-DCP-LA selectively activates PKC-ε. To address this question, we constructed the siRNA to silence the PKC-ε-targeted gene. In the real-time RT-PCR analysis and Western blot analysis, expression of the PKC-ε mRNA and protein for PC-12 cells transfected with the PKC-ε siRNA was significantly reduced as compared with the expression for cells transfected with the NC siRNA (Fig. 4A, B), which confirms PKC-ε knockdown. For PC-12 cells transfected with the NC siRNA, α,β-DCP-LA significantly activated PKC, but for cells transfected with the PKC-ε siRNA no activation of PKC was found (Fig. 4C), supporting the note for selective PKC-ε activation by α,β-DCP-LA.

To obtain further evidence for this, we assayed PKC in the cell-free systems. Of the 9 PKC isozymes examined here (α, βI, βII, γ, δ, ε, μ, η, and ζ), α,β-DCP-LA (10 μM) activated PKC-ε in the absence of 1,2-dioleoyl-*sn*-glycerol, a diacylglycerol, dioleoyl-phosphatidylserine, and Ca^{2+}, with the potency strikingly greater than that for other PKC isozymes (Fig. 5A, B). Like the result from *in situ* PKC assay, α,β-DCP-LA (10 μM) activated PKC to an extent similar to racemic DCP-LA

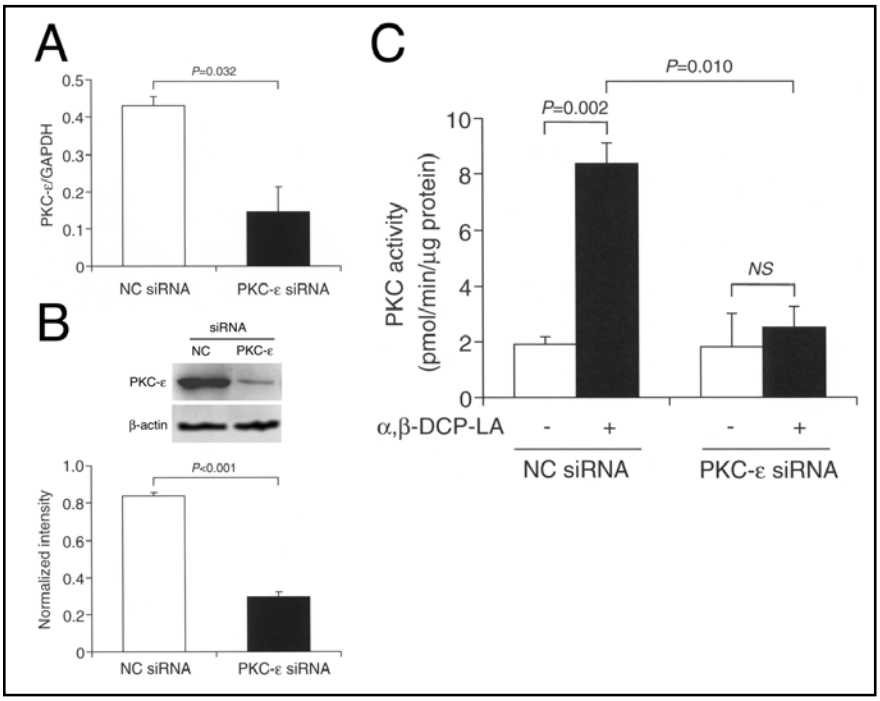

Fig. 4. α,β-DCP-LA activates PKC-ε in PC-12 cells. PC-12 cells were transfected with the NC siRNA or the PKC-ε siRNA, and 24 h later after transfection real-time RT-PCR and Western blotting were carried out. (A) Real-time RT-PCR analysis. Each column represents the mean (± SEM) expression of PKC-ε mRNA normalized by expression of the GAPDH mRNA (n=6). *P* value, unpaired *t*-test. (B) Western blot analysis. Each column represents the mean (± SEM) intensity of PKC-ε protein normalized by β-actin intensity (n=4). *P* value, unpaired *t*-test. (C) Cells transfected with the NC siRNA or the PKC-ε siRNA were untreated and treated with α,β-DCP-LA at a concentration of 100 nM, and PKC activity was assayed. Each column represents the mean (± SEM) PKC activity (pmol/min/μg protein) (n=6). *P* values, unpaired *t*-test. *NS*, not significant.

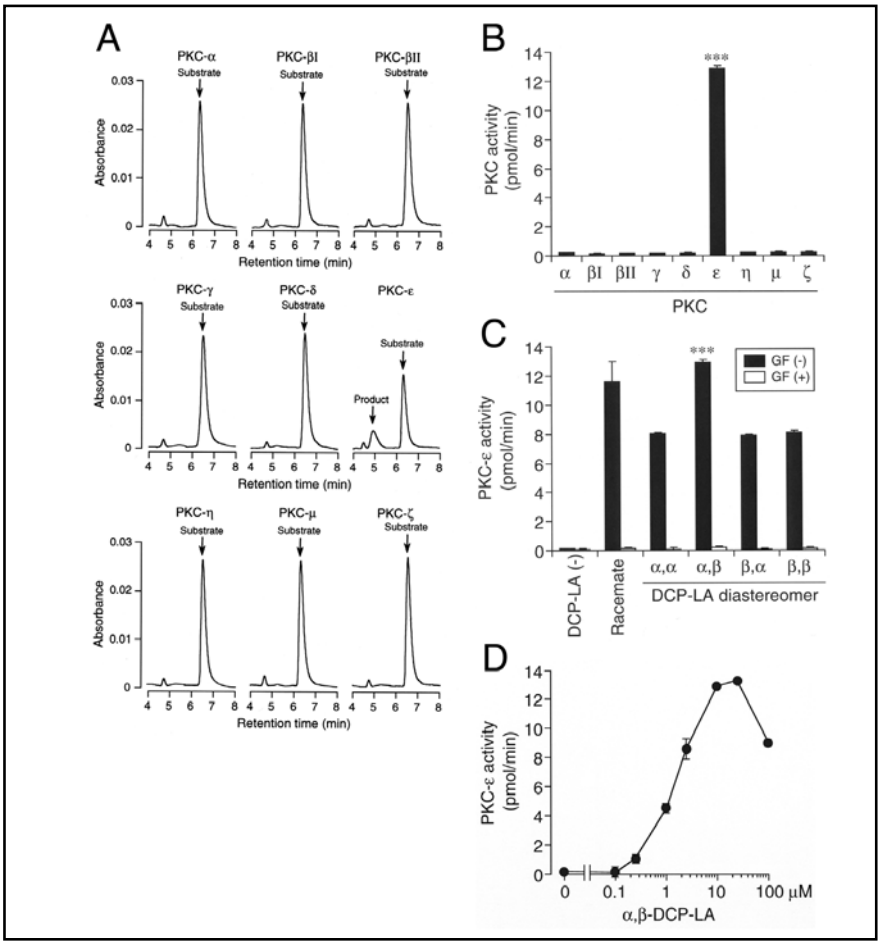

Fig. 5. α,β-DCP-LA selectively activates PKC-ε with the highest potency. In the cell-free systems, PKC activity was monitored. PKC isozymes as indicated were assayed in the presence of α,β-DCP-LA (10 μM). (A) Reversed phase HPLC profiles. (B) In the graph, each column represents the mean (± SEM) PKC activity (pmol/min) (n=6). ***$P<0.001$ as compared with each PKC isozyme activation, Dunnett's test. (C) PKC-ε activity was assayed in the presence and absence of racemic DCP-LA or DCP-LA diastereomers at a concentration of 10 μM together with and without GF109203X (GF) (100 nM). Each column represents the mean (± SEM) PKC activity (pmol/min) (n=6). ***$P<0.001$ as compared with each PKC-activation induced by α,α-, β,α-, or β,β-DCP-LA in the absence of GF109203X, Dunnett's test. (D) PKC-ε activity was measured in the presence of α,β-DCP-LA at concentrations as indicated. Each point represents the mean (± SEM) PKC-ε activity (pmol/min) (n=6).

(10 μM)(Fig. 5C). Other DCP-LA diastereomers (10 μM) also activated PKC-ε, but to lesser extent as compared with α,β-DCP-LA-engaged activation (Fig. 5C). PKC-ε activation induced by all the DCP-LA diastereomers was abolished by GF109203X (100 nM) (Fig. 5C). α,β-DCP-LA activated PKC-ε in a bell-shaped concentration (0.1-100 μM)-dependent manner, with the maximal effect at 25 μM (Fig. 5D). Taken together, these results

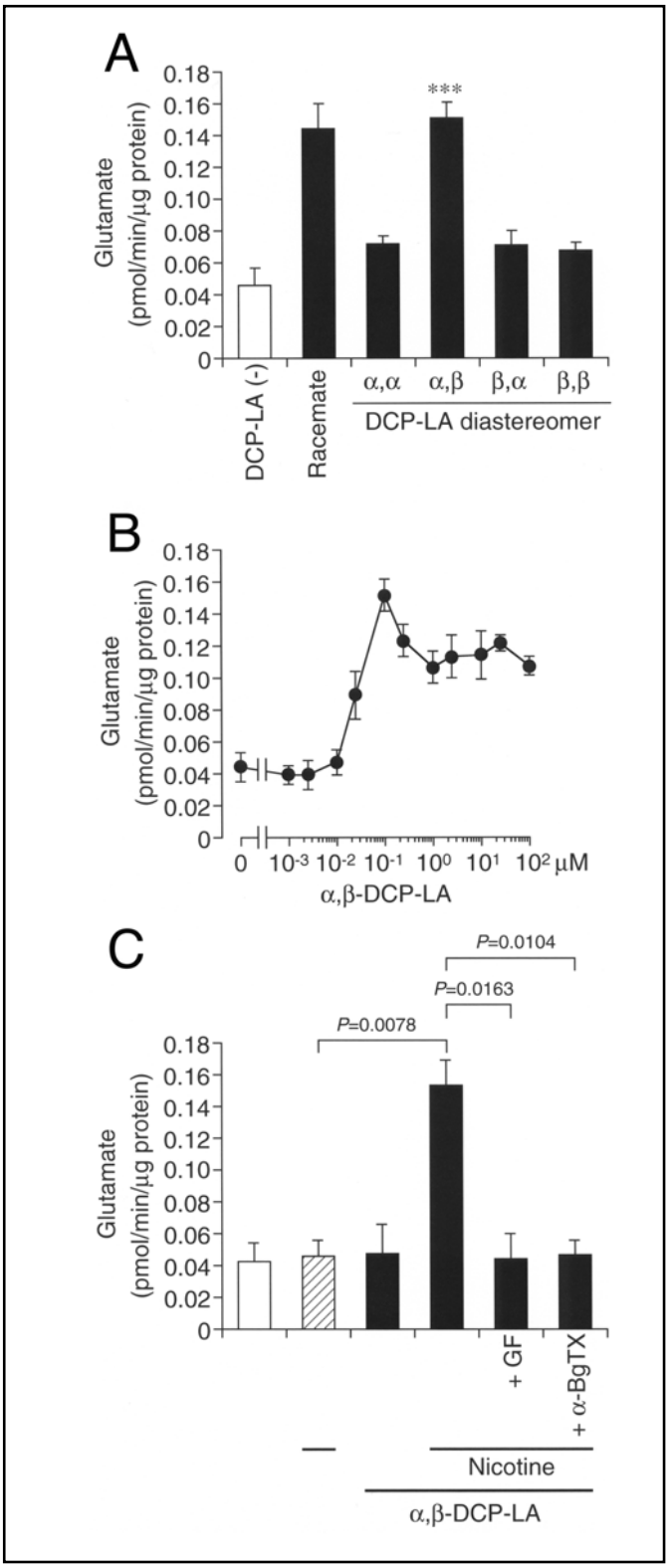

Fig. 6. α,β-DCP-LA stimulates hippocampal glutamate release under the control of PKC and α7 ACh receptors with the highest potency. (A) Rat hippocampal slices were untreated and treated with racemic DCP-LA or DCP-LA diastereomers at a concentration of 100 nM in the presence of nicotine (1 μM), and glutamate released was assayed. Each point represents the mean (± SEM) glutamate concentrations (n=6). ***P<0.001 as compared with each glutamate release induced by α,α-, β,α-, or β,β-DCP-LA, Dunnett's test. (B) Slices were treated with α,β-DCP-LA at concentrations as indicated in the presence of nicotine (1 μM), and glutamate released was assayed. Each point represents the mean (± SEM) glutamate concentrations (n=6). (C) Slices were treated with α,β-DCP-LA (100 nM) in the presence and absence of nicotine (1 μM) together with and without GF109203X (GF) (100 nM) or α-BgTX (100 nM), and glutamate released was assayed. Each point represents the mean (± SEM) glutamate concentrations (n=6). P values, Dunnett's test.

indicate that α,β-DCP-LA serves as a selective and direct activator of PKC-ε, with the highest potency among 4 diastereomers.

α,β-DCP-LA stimulates glutamate release in a PKC- and α7 ACh receptor-dependent manner, with the highest potency among 4 diastereomers

We earlier found that racemic DCP-LA activates PKC, to enhance activity of presynaptic α7 ACh receptors, thereby increasing presynaptic glutamate release, responsible for facilitation of hippocampal synaptic transmission [2]. This prompted to assess the effect of α,β-DCP-LA on glutamate release from neurons. Nicotine (1 μM) alone, to activate nicotinic ACh receptors, or each DCP-LA diastereomer alone had no effect on glutamate release from rat hippocampal slices (Fig. 6A,C). Each diastereomer (100 nM) together with nicotine (1 μM) increased glutamate release, and the most marked increase was obtained with α,β-DCP-LA (Fig. 6A). The extent for α,β-DCP-LA was similar to that for racemic DCP-LA (Fig. 6A). This, in the light of the fact that glutamate released was assayed in the presence of TTX (0.5 μM), an inhibitor of voltage-dependent Na^+ channels, interprets that of 4 diastereomers α,β-DCP-LA has the highest potency to stimulate glutamate release from hippocampal neurons.

α,β-DCP-LA increased glutamate release from hippocampal slices in a bell-shaped concentration (1 nM-100 μM)-dependent manner, with the maximal effect at 100 nM (Fig. 6B). Amazingly, the concentration-dependent effect was in well agreement with the effect of α,β-DCP-LA on PKC activation. The increase in glutamate release induced by α,β-DCP-LA (100 nM) was clearly inhibited by GF109203X (100 nM) or α-BgTX (100 nM), an antagonist of α7 ACh receptors (Fig. 6C). Overall, these results lead to a conclusion that α,β-DCP-LA stimulates hippocampal glutamate release under the control of PKC, possibly PKC-ε, and α7 ACh receptors,

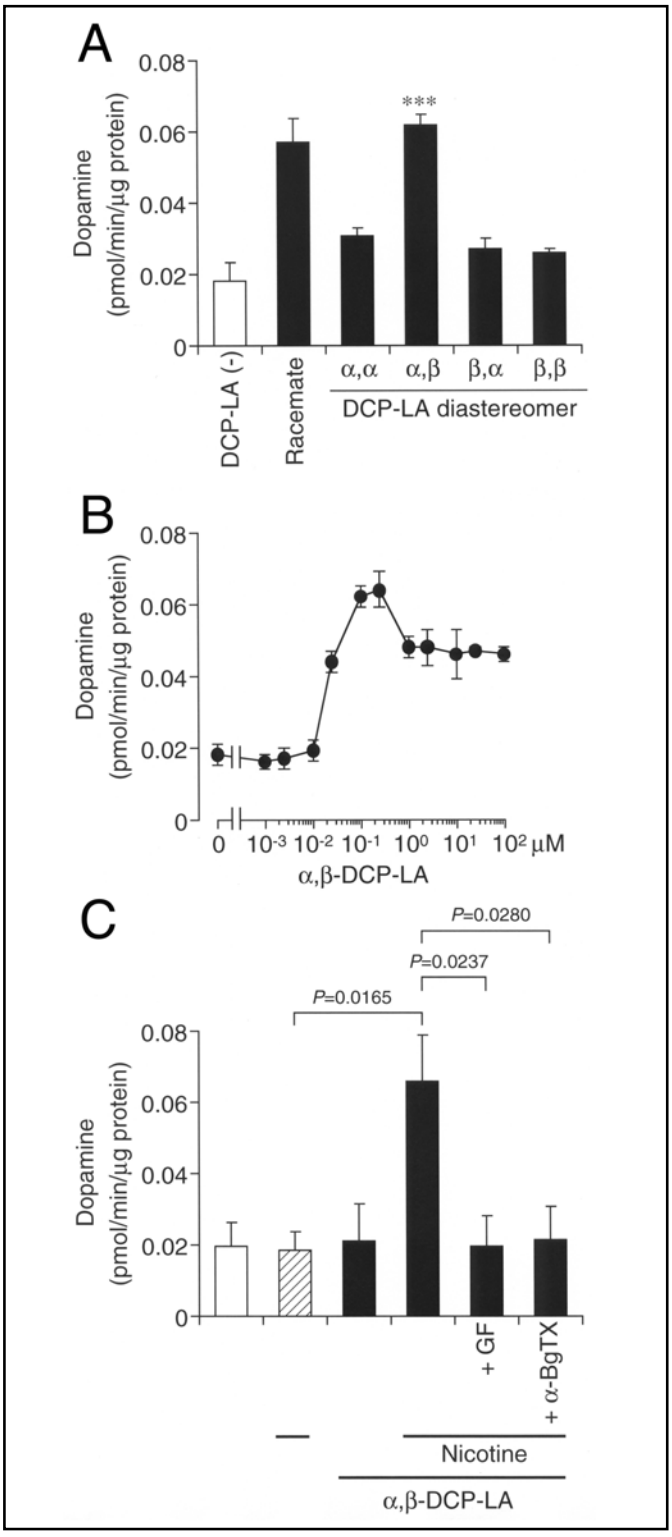

Fig. 7. α,β-DCP-LA stimulates striatal dopamine release under the control of PKC and α7 ACh receptors with the highest potency. (A) Rat striatal slices were untreated and treated with racemic DCP-LA or DCP-LA diastereomers at a concentration of 100 nM in the presence of nicotine (1 μM), and dopamine released was assayed. Each point represents the mean (± SEM) dopamine concentrations (n=6). ***P<0.001 as compared with each dopamine release induced by α,α-, β,α-, or β,β-DCP-LA, Dunnett's test. (B) Slices were treated with α,β-DCP-LA at concentrations as indicated in the presence of nicotine (1 μM), and dopamine released was assayed. Each point represents the mean (± SEM) dopamine concentrations (n=6). (C) Slices were treated with α,β-DCP-LA (100 nM) in the presence and absence of nicotine (1 μM) together with and without GF109203X (GF) (100 nM) or α-BgTX (100 nM), and dopamine released was assayed. Each point represents the mean (± SEM) dopamine concentrations (n=6). P values, Dunnett's test.

with the highest potency among 4 diastereomers. This suggests that α,β-DCP-LA could ameliorate a variety of dementia such as Alzheimer disease, age-related cognitive decline, and vascular dementia by facilitating hippocampal synaptic transmission as a results of increased glutamate release, with a more beneficial efficacy than other diastereomers.

α,β-DCP-LA stimulates dopamine release in a PKC- and α7 ACh receptor-dependent manner, with the highest potency among 4 diastereomers

We have found that racemic DCP-LA stimulates presynaptic release of not only glutamate but GABA [3]. Then, we thought that DCP-LA might still stimulate release of other neurotransmitters such as dopamine and serotonin. To answer this question, we assayed dopamine released from rat striatal slices in a TTX (0.5 μM)-containing extracellular solution. Nicotine (1 μM) alone or each DCP-LA diastereomer alone had no effect on dopamine release (Fig. 7A, C). In contrast, all the DCP-LA diastereomers (100 nM) increased dopamine release in the presence of nicotine (1 μM), with the most prominent effect for α,β-DCP-LA (Fig. 7A). The extent for α,β-DCP-LA was similar to that for racemic DCP-LA (Fig. 7A).

As is the case with glutamate release, α,β-DCP-LA increased dopamine release in a bell-shaped concentration (1 nM-100 μM)-dependent manner, with the maximal effect at 250 nM (Fig. 7B). The increase in dopamine release induced by α,β-DCP-LA (100 nM) was prevented by GF109203X (100 nM) or α-BgTX (100 nM) (Fig. 7C). Collectively, these results indicate that α,β-DCP-LA stimulates striatal dopamine release under the control of PKC, possibly PKC-ε, and α7 ACh receptors, with the highest potency among 4 diastereomers. This raises the possibility that α,β-DCP-LA could exert its beneficial action on Parkinson disease, diffuse Lewy body disease, and extrapyramidal disorders due to reduction/deficiency of dopamine.

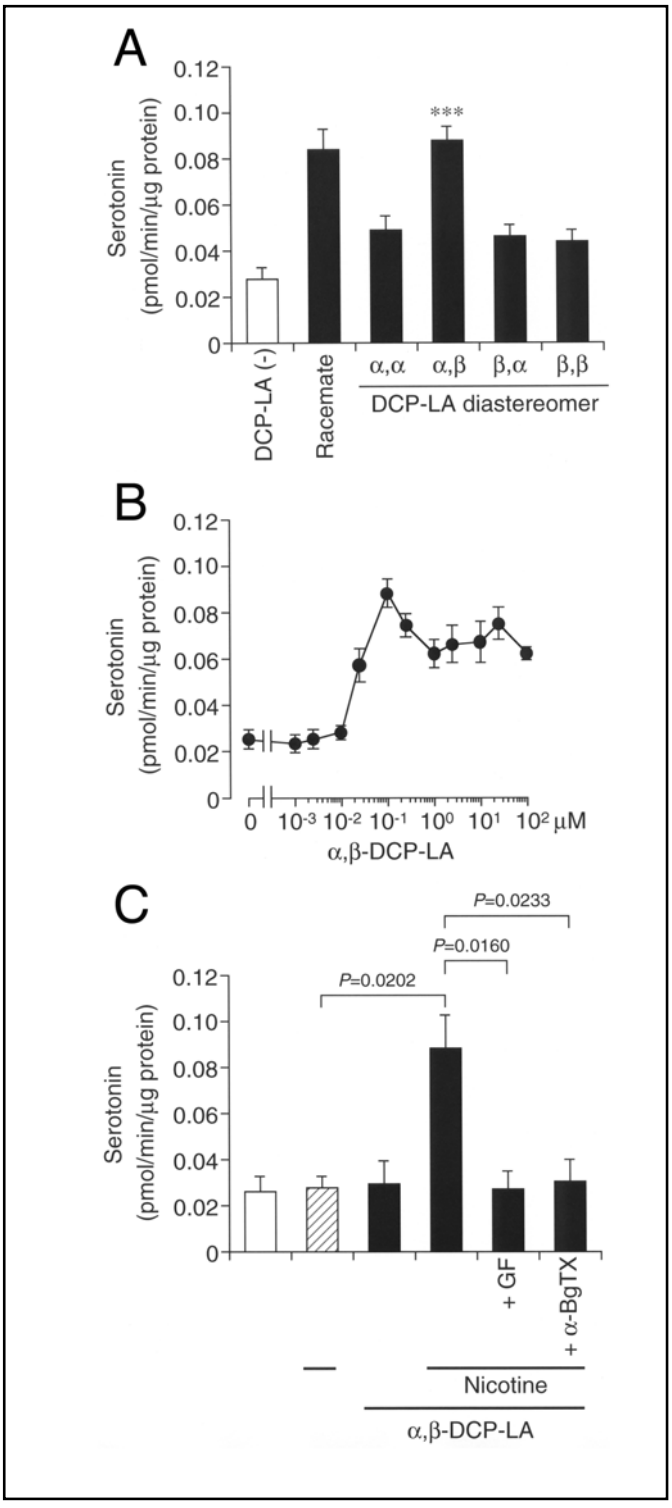

Fig. 8. α,β-DCP-LA stimulates hypothalamic serotonin release under the control of PKC and α7 ACh receptors with the highest potency. (A) Rat hypothalamic slices were untreated and treated with racemic DCP-LA or DCP-LA diastereomers at a concentration of 100 nM in the presence of nicotine (1 μM), and serotonin released was assayed. Each point represents the mean (± SEM) serotonin concentrations (n=6). ***P<0.001 as compared with each serotonin release induced by α,α-, β, α-, or β,β-DCP-LA, Dunnett's test. (B) Slices were treated with α,β-DCP-LA at concentrations as indicated in the presence of nicotine (1 μM), and serotonin released was assayed. Each point represents the mean (± SEM) serotonin concentrations (n=6). (C) Slices were treated with α,β-DCP-LA (100 nM) in the presence and absence of nicotine (1 μM) together with and without GF109203X (GF) (100 nM) or α-BgTX (100 nM), and serotonin released was assayed. Each point represents the mean (± SEM) serotonin concentrations (n=6). P values, Dunnett's test.

α,β-DCP-LA stimulates serotonin release in a PKC- and α7 ACh receptor-dependent manner, with the highest potency among 4 diastereomers

We finally examined the effect of DCP-LA on serotonin release. Nicotine (1 μM) alone or each DCP-LA diastereomer alone had no effect on serotonin released from rat hypothalamic slices in a TTX (0.5 μM)-containing extracellular solution (Fig. 8A, C), but all the DCP-LA diastereomers (100 nM) increased serotonin release in the presence of nicotine (1 μM), with the most greatest effect for α,β-DCP-LA (Fig. 8A). The extent for α,β-DCP-LA was similar to that for racemic DCP-LA (Fig. 8A).

α,β-DCP-LA increased serotonin release in a bell-shaped concentration (1 nM-100 μM)-dependent manner, with the maximal effect at 100 nM (Fig. 8B). The increase in serotonin release induced by α,β-DCP-LA (100 nM) was blocked by GF109203X (100 nM) or α-BgTX (100 nM) (Fig. 8C). Taken together, these results show that α,β-DCP-LA stimulates hypothalamic serotonin release under the control of PKC, possibly PKC-ε, and α7 ACh receptors, with the highest potency among 4 diastereomers. This suggests that α,β-DCP-LA could improve depression, panic disorder, sleep disturbance, and mental irritation, which is caused by reduction/deficiency of serotonin as the major factor.

Discussion

Enantiomers or diastereomers contained in a racemic modification of drugs might exert different actions each other, and therefore, a racemate could exhibit an unexpected effect. For example, α-methyl-N(α)-phthalimidoglutarimide (thalidomide), containing 2 enantiomers such as (S)- and (R)-form, was clinically used as a hypnotic, but (S)-form thalidomide caused the serious side-effect teratogenesis [16]. Racemic DCP-LA contains 4 possible diastereomers. In the present study, we have established roots for selective optical reso-

lution from racemic DCP-LA and succeeded to synthesize 4 diastereomers such as α,α-, α,β-, β,α-, and β,β-DCP-LA. This is of great value and indispensable to assess the bioactivities and side-effects of each DCP-LA diastereomer.

The results of the present study demonstrate that bioactive potentials of DCP-LA vary, depending upon its diastereomers. Of 4 diastereomers α,β-DCP-LA selectively and directly activated PKC-ε, with the highest potency. Notably, α,β-DCP-LA increased release of neurotransmitters such as glutamate, dopamine, and serotonin in a similar fashion, i.e., in a PKC- and -α7 ACh receptor-dependent manner. This suggests that α,β-DCP-LA stimulates transmitter release by targeting presynaptic α7 ACh receptors through PKC-ε activation. α,β-DCP-LA, thus, could be developed as a promising drug for treatment of not only a variety of dementia including Alzheimer disease but neurodegenerative diseases such as Parkinson disease, diffuse Lewy body disease, and extrapyramidal disorders, and psychiatric impairments such as depression, panic disorder, and mental irritation due to reduction/deficiency of neurotransmitters. This may shed a bright light upon huge numbers of patients with those disastrous diseases.

In summary, the results of the present study show that α,β-DCP-LA selectively and directly activates PKC-ε and stimulates release of glutamate, dopamine, and serotonin in the hippocampus, striatum, and hypothalamus under the control of PKC-ε and α7 ACh receptors, with the highest potency among 4 diastereomers.

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