

Original Paper

Effects of Newly Synthesized DCP-LA-Phospholipids on Protein Kinase C and Protein Phosphatases

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Key Words

DCP-LA-phospholipid • Protein kinase C • Protein phosphatase 1 • Protein phosphatase 2A • Protein tyrosine phosphatase 1B

Abstract

Background/Aims: The linoleic acid derivative DCP-LA selectively activates PKC ϵ and inhibits protein phosphatase 1 (PP1). In the present study, we have newly synthesized phosphatidyl-ethanolamine, -serine, -choline, and -inositol containing DCP-LA at the α and β position (diDCP-LA-PE, -PS, PC, and -PI, respectively), and examined the effects of these compounds on activities of PKC isozymes and protein phosphatases. **Methods:** Activities of PKC isozymes PKC α , - β I, - β II, - γ , - δ , - ϵ , ι , and - ζ and protein phosphatases PP1, PP2A, and protein tyrosine phosphatase 1B (PTP1B) were assayed under the cell-free conditions. **Results:** All the compounds activated PKC, with the different potential, but only PKC γ inhibition was obtained with diDCP-LA-PC. Of compounds diDCP-LA-PE alone significantly activated PKC ι and - ζ . diDCP-LA-PE and diDCP-LA-PI suppressed PP1 activity, but otherwise diDCP-LA-PI enhanced PP2A activity. diDCP-LA-PE, diDCP-LA-PS, and diDCP-LA-PI strongly reduced PTP1B activity, while diDCP-LA-PC enhanced the activity. **Conclusion:** All the newly synthesized DCP-LA-phospholipids serve as a PKC activator and of them diDCP-LA-PE alone has the potential to activate the atypical PKC isozymes PKC ι and - ζ . diDCP-LA-PE and diDCP-LA-PI serve as an inhibitor for PP1 and PTP1B, diDCP-LA-PS as a PTP1B inhibitor, diDCP-LA-PI as a PP2A enhancer, and diDCP-LA-PC as a PTP1B enhancer.

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Introduction

8-[2-(2-Pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA), our originally synthesized linoleic acid derivative, serves as a selective activator of protein kinase C ϵ (PKC ϵ) [1, 2]. DCP-LA enhances activity of presynaptic $\alpha 7$ acetylcholine (ACh) receptors by PKC ϵ , causing a marked increase in glutamate release, and then leading to a long-lasting facilitation of hippocampal synaptic transmission [3, 4]. DCP-LA ameliorates spatial learning and memory deficits induced by intraperitoneal injection with scopolamine or intraventricular injection with amyloid β_{1-40} peptide [5] or it improves age-related cognitive decline [6]. Furthermore, DCP-LA neutralizes impairment of long-term potentiation (LTP), that is induced by a considerably low dose of mutant amyloid β_{1-42} peptide lacking glutamate-22 [7], suggesting the beneficial effect of DCP-LA against Alzheimer dementia. DCP-LA, alternatively, activates Ca $^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) by inhibiting protein phosphatase 1 (PP1), 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 localization of AMPA receptors on the postsynaptic plasma membrane in the hippocampus [8]. This DCP-LA action could also contribute to facilitation of hippocampal synaptic transmission. DCP-LA, on the other hand, exerts its protective action against oxidative stress-induced apoptosis in neurons by inhibiting caspase-3/-9 activation [9]. DCP-LA, thus, is implicated in diverse signaling pathways.

We have earlier found that 1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine (DL-PE) suppresses endoplasmic reticulum (ER) stress-induced neuronal death and ameliorates age-related spatial memory deterioration [10]. Then, we were prompted to assess the bioactivities of phosphatidylethanolamine containing DCP-LA instead of linoleic acid at the α and β position.

To address this point, we have newly synthesized DCP-LA-phospholipids such as 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidylethanolamine (diDCP-LA-PE), 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidyl-L-serine (diDCP-LA-PS), 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidylcholine (diDCP-LA-PC), and 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidyl-D-1-inositol (diDCP-LA-PI) (Fig. 1). The present study investigated the effects of these DCP-LA-phospholipids on activities of PKC isozymes and protein phosphatases. We show here that DCP-LA-phospholipids activate PKC isozymes such as PKC α , - β I, - β II, - γ , - δ , - ϵ , - ι , and - ζ , and inhibit protein phosphatases such as protein phosphatase 1, protein phosphatase 2A (PP2A), and protein tyrosine phosphatase 1B (PTP1B), in a different pattern and with the different potential, depending upon the phospholipids.

Materials and Methods

NMR analysis

^1H -NMR spectra were recorded on a JEOL JNM-ECX400 spectrometer, operating at 400 MHz. Chemical shifts were reported downfield from TMS (δ = 0.00) or CHCl_3 (δ = 7.26) for ^1H -NMR. ESI-MS spectra were taken on Bruker micrOTOF-Q mass spectrometer. Column chromatography was performed with silica gel 60 (40-50 μm and 40-100 μm) purchased from KANTO CHEMICAL Co. (Tokyo, Japan). All reaction were monitored by thin-layer chromatography carried out on a 0.25 mm silica gel plates 60 F254 (Merck, Darmstadt, Germany) using UV light, iodine, *m*-bromo cresol green, or 5 % (w/v) ethanolic phosphomolybdic acid solution and heat as developing agents.

Synthesis of (R)-3-benzyloxy-1,2-propanediol

To a solution of (S)-(+)-2,2-dimethyl-1,3-dioxolane-4-methanol (1.0 g, 7.6 mmol) in dimethylformamide (10 ml) were added 60% NaH (0.61 g, 15.1 mmol) and benzyl bromide (1.1 ml, 9.1 mmol) under ice-cooling. After stirring for 15 min at room temperature, water was added to the reaction mixture. The aqueous layer

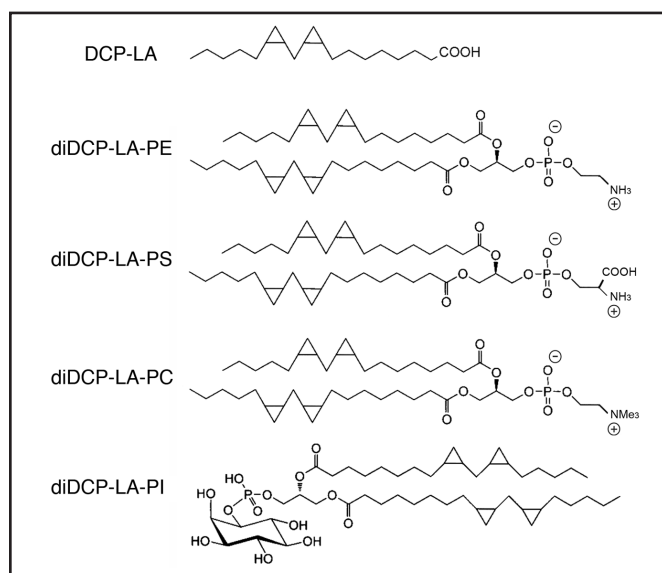


Fig. 1. Chemical structure of DCP-LA and DCP-LA-phospholipids.

was extracted with hexane, and the organic layer was washed with H_2O and brine. Then it was dried over anhydrous MgSO_4 , and concentrated under reduced pressure to give 4-benzyloxymethyl-2,2-dimethyl-1,3-dioxolane, which was used without further purification.

To a solution of 4-benzyloxymethyl-2,2-dimethyl-1,3-dioxolane in methanol (17.4 ml) was added concentrated HCl (1.93 ml) under ice-cooling. After stirring for 30 min at 60°C , a saturated aqueous solution of NaHCO_3 was added to the reaction mixture under ice-cooling. The aqueous layer was washed with hexane, and then the aqueous layer was extracted with ethyl acetate. The combined organic layers were dried over MgSO_4 , and concentrated under reduced pressure to give (*R*)-3-benzyloxy-1,2-propanediol (1.22 g, 88%) as oil. $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 3.53-3.61 (m, 2H), 3.65 (dd, $J = 11.4$ and 5.5 Hz, 1H), 3.72 (dd, $J = 11.4$ and 3.6 Hz, 1H), 3.88-3.93 (m, 1H), 4.56 (s, 2H), 7.30-7.39 (m, 5H).

Synthesis of 3-O-benzyl-1,2-O-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-sn-glycerol

DCP-LA (2.03 g, 6.59 mmol) was added to a solution of (*R*)-3-benzyloxy-1,2-propanediol (0.5 g, 2.74 mmol) in toluene, and then concentrated under reduced pressure, and the residue was dissolved in dichloromethane (15 ml). Then, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.58 g, 8.23 mmol) and *N,N*-dimethylaminopyridine (0.30 g, 2.47 mmol) was added to the solution under ice-cooling. After stirring for 3 h at room temperature under N_2 , 2 N HCl was added to the reaction mixture. The aqueous layer was extracted with ethyl acetate three times, and the combined organic layers were dried over anhydrous MgSO_4 . The solution was concentrated under reduced pressure, and purified by a silica gel column chromatography (hexane:ethyl acetate = 20:1) to give 3-O-benzyl-1,2-O-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-sn-glycerol (2.06 g, 99%) as oil. $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ -0.31- -0.24 (m, 4H), 0.58-0.91 (m, 18H), 0.99-1.20 (m, 5H), 1.25-1.52 (m, 37H), 1.58-1.69 (m, 2H), 2.28 (t, $J = 7.3$ Hz, 2H), 2.33 (t, $J = 7.4$ Hz, 2H), 3.59 (d, $J = 5.0$ Hz, 2H), 4.19 (dd, $J = 11.7$ and 6.9 Hz, 1H), 4.34 (dd, $J = 11.7$ and 3.6 Hz, 1H), 4.52 (d, $J = 11.9$ Hz, 1H), 4.57 (d, $J = 11.9$ Hz, 1H), 5.22-5.27 (m, 1H), 7.29-7.37 (m, 5H).

Synthesis of 1,2-O-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-sn-glycerol

3-O-Benzy-1,2-O-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-sn-glycerol (1.20 g, 1.57 mmol) and 10% (w/v) palladium on activated carbon (480 mg) in ethanol (80 ml) was stirred under hydrogen (1 atm) for 15 min at room temperature. The catalyst was removed through a pad of Celite, rinsed with ethyl acetate. The combined organic layers were concentrated under reduced pressure to give 1,2-O-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-sn-glycerol (1.05 g, 100%) as oil. $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ -0.33- -0.23 (m, 4H), 0.58-0.89 (m, 12H), 0.99-1.19 (m, 6H), 1.24-1.69 (m, 44H), 2.03 (t, $J = 6.4$ Hz, 1H), 2.32 (t, $J = 7.3$ Hz, 2H), 2.35 (t, $J = 7.3$ Hz, 2H), 3.73 (dd, $J = 6.3$ and 6.4 Hz, 2H), 4.24 (dd, $J = 11.9$ and 5.5 Hz, 1H), 4.31 (dd, $J = 11.9$ and 4.9 Hz, 1H), 5.08 (ddd, $J = 6.3$, 5.5 and 4.4 Hz, 1H).

Synthesis of diDCP-LA-PE

N,N-Diisopropylmethylphosphonamidic chloride (460 μ l, 2.37 mmol) was added to a solution of 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycerol (1.05 g, 1.57 mmol) and triethylamine (552 μ l, 3.96 mmol) in CH_2Cl_2 (40 ml) under ice-cooling. After stirring for 10 min at room temperature, 2-(carbobenzoylamino)-1-ethanol (0.58 g, 2.97 mmol) and 1*H*-tetrazole (0.56 g, 7.92 mmol) was added to the mixture, and 70% (v/v) aqueous solution of *tert*-butyl hydroperoxide (2.6 ml, 19.8 mmol) was further added and stirred for 20 min at the same temperature. After adding 10% (w/v) aqueous solution of $\text{Na}_2\text{S}_2\text{O}_3$, the resulting aqueous layer was extracted with ethyl acetate and the organic layer was dried over anhydrous MgSO_4 . The solution was concentrated under reduced pressure, and was purified by a silica gel column chromatography (hexane:ethyl acetate = 4:1) to give *O*-[2-*N*-(benzyloxycarbonyl)aminoethyl] *O*-(1',2'-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-3'-glyceryl) *O*-methyl phosphate (1.13 g, 68 %) as oil. $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ -0.33- -0.23 (m, 4H), 0.58-0.89 (m, 18H), 0.99-1.19 (m, 6H), 1.24-1.54 (m, 37H), 1.54-1.69 (br s, 2H), 2.32 (t, J = 7.4 Hz, 2H), 2.33 (t, J = 7.4 Hz, 2H), 3.42-3.56 (m, 2H), 3.75 (dd, J = 11.4 and 4.6 Hz, 3H), 4.05-4.25 (m, 5H), 4.29-4.33 (m, 1H), 5.11 (s, 2H), 5.20-5.26 (m, 1H), 5.38-5.42 (m, 1H), 7.31-7.40 (m, 5H); ESI-HRMS (negative ion, sodium formate) calculated for $\text{C}_{54}\text{H}_{90}\text{NO}_{10}\text{PNa}$ ($[\text{M}+\text{Na}]^+$) 966.6191; found 966.6089.

NaI (899 mg, 6.0 mmol) was added to a solution of *O*-[2-*N*-(benzyloxycarbonyl)aminoethyl] *O*-(1',2'-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-3'-glyceryl) *O*-methyl phosphate (1.2 g, 1.6 mmol) in 2-butanone (23 ml). After stirring for 1 h at 80 $^\circ\text{C}$, 2 N HCl was added to the reaction mixture, and the aqueous layer was extracted with chloroform. The organic layer was washed with H_2O and brine, and the combined organic layer was dried over anhydrous MgSO_4 and concentrated under reduced pressure to give *O*-[2-*N*-(benzyloxycarbonyl)aminoethyl] *O*-(1',2'-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-3'-glyceryl) phosphate.

To a solution of *O*-[2-*N*-(benzyloxycarbonyl)aminoethyl] *O*-(1',2'-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-3'-glyceryl) phosphate in methanol (120 ml) was added 10% (w/v) palladium on activated carbon (500 mg). The resulting suspension was stirred under hydrogen (1 atm) for 24 h at room temperature. The catalyst was removed through a pad of Celite, rinsed with ethyl acetate, and concentrated under reduced pressure. The resulting crude product was purified by a silica gel column chromatography (chloroform: methanol = 10:1) to give 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidylethanolamine (0.60 g, 70%) as a yellow oil. $^1\text{H-NMR}$ (400 MHz, $\text{C}_6\text{D}_5\text{N}$): δ -0.16- -0.08 (m, 4H), 0.69-0.99 (m, 18H), 1.16-1.59 (m, 42H), 1.64-1.80 (m, 5H), 2.43 (t, J = 7.8 Hz, 2H), 2.48 (t, J = 6.4 Hz, 2H), 3.56-3.64 (br s, 2H), 4.33-4.45 (br s, 2H), 4.53 (dd, J = 11.9 Hz, 1H), 4.56-4.69 (br s, 2H), 4.73 (dd, J = 9.6 and 2.7 Hz, 1H), 6.87 (d, J = 9.2 Hz, 1H), 6.91 (dd, J = 8.2 and 1.8 Hz, 1H), 7.00 (d, J = 1.8 Hz, 1H), 7.03 (d, J = 2.3 Hz, 1H), 7.16 (d, J = 1.8 Hz, 1H), 7.47 (d, J = 8.3 Hz, 2H), 7.67 (d, J = 8.3 Hz, 2H); ESI-HRMS (negative ion, sodium formate) calculated for $\text{C}_{45}\text{H}_{82}\text{NO}_8\text{PNa}^+$ ($[\text{M}+\text{Na}]^+$) 818.5670; found 818.5652.

Synthesis of diDCP-LA-PS

N,N-Diisopropylmethylphosphonamidic chloride (0.15 ml, 0.79 mmol) was added to a solution of 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycerol (0.44 g, 0.66 mmol) and triethylamine (0.18 ml, 1.3 mmol) in CH_2Cl_2 (15 ml) under ice-cooling. After stirring for 10 min at room temperature, *N*-carbobenzoyl-serine benzyl ester (0.33 g, 0.99 mmol) and 1*H*-tetrazole (0.19 g, 2.6 mmol) were added to the mixture, and 70% (v/v) aqueous solution of *tert*-butyl hydroperoxide (0.87 ml, 6.6 mmol) was further added and stirred for 20 min at the same temperature. After adding 10% (w/v) aqueous solution of $\text{Na}_2\text{S}_2\text{O}_3$, the resulting aqueous layer was extracted with ethyl acetate and the organic layer was dried over anhydrous MgSO_4 . The solution was concentrated under reduced pressure and purified by a silica gel column chromatography (hexane:ethyl acetate = 3:1) to give *O*-[*N*-(benzyloxycarbonyl)-L-serine benzyl ester] *O*-(1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-3'-glyceryl) phosphate (490 mg, 68%) as a colorless oil. $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ -0.33- -0.20 (m, 4H), 0.55-0.85 (m, 12H), 0.87-0.95 (m, 6H), 0.96-1.70 (m, 44H), 2.30 (t, J = 7.3 Hz, 2H), 2.31 (t, J = 7.3 Hz, 2H), 3.65 (dd, J = 11.5 and 2.8 Hz, 3H), 4.00-4.20 (m, 3H), 4.21-4.41 (m, 2H), 4.42-4.49 (m, 1H), 4.59-4.65 (m, 1H), 5.13 (s, 2H), 5.14-5.20 (m, 1H), 5.21 (s, 2H), 5.93 (dd, J = 8.2 and 8.2 Hz, 1H), 7.20-7.44 (m, 10H); ESI-HRMS (positive ion, sodium formate) calculated for $\text{C}_{62}\text{H}_{96}\text{NO}_{12}\text{PNa}$ ($[\text{M}+\text{H}]^+$) 1100.6562; found 1100.6307.

NaI (139 mg, 0.93 mmol) was added to a solution of *O*-[*N*-(benzyloxycarbonyl)-L-serine benzyl ester] *O*-(1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-3-glycerol) *O*-methyl phosphate (200 mg, 0.19 mmol) in 2-butanone (3 ml). After stirring for 1 h at 80 °C, 2 N HCl was added to the reaction mixture, and the aqueous layer was extracted with chloroform. The organic layer was washed with H₂O and brine, and the combined organic layer was dried over anhydrous MgSO₄ and concentrated under reduced pressure to give *O*-[*N*-(benzyloxycarbonyl)-L-serine benzyl ester] *O*-(1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-3-glycerol) *O*-methyl phosphate.

To a solution of *O*-[*N*-(benzyloxycarbonyl)-L-serine benzyl ester] *O*-(1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-3-glycerol) *O*-methyl phosphate in ethanol (30 ml) was added 10% (w/v) palladium on activated carbon (72 mg). The resulting suspension was stirred under hydrogen (1 atm) for 2 h at room temperature, and the catalyst was removed through a pad of Celite, rinsed with ethyl acetate, and concentrated under reduced pressure. The resulting crude product was purified by a silica gel column chromatography (chloroform:methanol = 10:1) to give 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidyl-L-serine (50 mg, 31%) as a white solid. ¹H-NMR (400 MHz, CDCl₃): δ -0.30- -0.20 (m, 4H), 0.55-0.85 (m, 12H), 0.87-0.95 (m, 6H), 0.96-1.70 (m, 44H), 2.25-2.43 (m, 4H), 3.80-4.30 (m, 4H), 4.32-4.51 (m, 2H), 5.20-5.32 (m, 1H); ESI-HRMS (negative ion, sodium formate) calculated for C₄₆H₈₁NO₁₀P ([M-H]⁻) 838.5604; found 838.5599.

Synthesis diDCP-LA-PC

N,N-Diisopropylmethylphosphonamidic chloride (0.048 ml, 0.25 mmol) was added to a solution of 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycerol (0.14 g, 0.21 mmol) and triethylamine (0.058 ml, 0.42 mmol) in CH₂Cl₂ (5 ml) under ice-cooling. After stirring for 10 min at room temperature, 2-chloroethanol (0.025 g, 0.31 mmol) and 1*H*-tetrazole (0.058 g, 0.42 mmol) were added, and 70% (v/v) aqueous solution of *tert*-butyl hydroperoxide (0.27 ml, 2.1 mmol) was added to the reaction mixture and stirred for 20 min at the same temperature. After adding 10% (w/v) aqueous solution of Na₂S₂O₃, the aqueous layer was extracted with CH₂Cl₂. The combined organic layer was dried over anhydrous MgSO₄ and concentrated under reduced pressure. The crude product was purified by a silica gel column chromatography (hexane:ethyl acetate = 3:1) to give *O*-(1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-3-glycerol) *O*-(2'-chloroethyl) *O*-methyl phosphate, (107 mg, 60%) as a colorless oil. ¹H-NMR (400 MHz, CDCl₃): δ -0.33- -0.20 (m, 4H), 0.55-0.85 (m, 12H), 0.87-0.95 (m, 6H), 0.96-1.72 (m, 44H), 2.32 (t, *J* = 7.8 Hz, 2H), 2.34 (t, *J* = 7.8 Hz, 2H), 3.70 (t, *J* = 6.5 Hz, 2H), (dd, *J* = 11.5 and 2.8 Hz, 3H), 4.10-4.40 (m, 6H), 5.25 (ddd *J* = 5.0, 5.0 and 5.0 Hz, 1H); ESI-HRMS (positive ion, sodium formate) calculated for C₄₆H₈₂ClO₈PNa ([M+Na]⁺) 851.5334; found 851.5283.

Trimethylamine (5 ml) was added to a solution of *O*-(1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-3-glycerol) *O*-(2'-chloroethyl) *O*-methyl phosphate (100 mg, 0.19 mmol) in CH₃CN (3 ml) under dry-ice-cooling. After stirring for 7 h at 70 °C, the reaction mixture was cooled to room temperature and concentrated under reduced pressure. The resulting crude product was purified by a thin layer column chromatography (chloroform:ethanol = 20:1) to give 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidylcholine (15 mg, 18%) as a white solid. ¹H-NMR (400 MHz, CDCl₃): δ -0.33- -0.20 (m, 4H), 0.55-0.84 (m, 12H), 0.87-0.95 (m, 6H), 0.96-1.72 (m, 44H), 2.32 (t, *J* = 7.8 Hz, 2H), 2.34 (t, *J* = 7.8 Hz, 2H), 3.23 (s, 9H), 3.60-3.70 (m, 4H), 4.00 (m, 2H), 4.20 (m, 1H), 4.25-4.40 (m, 2H), 4.45 (m, 1H), 5.26 (m, 1H); ESI-HRMS (negative ion, sodium formate) calculated for C₄₈H₈₉NO₈P ([M+H]⁺) 838.6320; found 838.6310.

Synthesis of diDCP-LA-D-PI

N,N-Diisopropylmethylphosphonamidic chloride (0.028 ml, 0.14 mmol) was added to a solution of 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycerol (0.080 g, 0.12 mmol) and triethylamine (0.033 ml, 0.24 mmol) in CH₂Cl₂ (2 ml) under ice-cooling. After stirring for 10 min at room temperature, (-)-2,3,4,5,6-penta-*O*-benzyl-D-1- inositol (0.11 g, 0.18 mmol) and 1*H*-tetrazole (0.033 g, 0.48 mmol) were added, and 70% (v/v) aqueous solution of *tert*-butyl peroxide (0.16 ml, 1.2 mmol) was added to the reaction mixture and stirred for 20 min at the same temperature. After adding 10% (w/v) aqueous solution of Na₂S₂O₃, the aqueous layer was extracted with CH₂Cl₂. The combined organic layer was dried over anhydrous MgSO₄ and concentrated under reduced pressure. The crude product was purified by a silica gel column chromatography (hexane: diethyl ether = 1:1) to give *O*-(1,2-*O*-bis-[8-{2-(2-pentyl-

cyclopropylmethyl)-cyclopropyl]-octanoyl]-sn-3-glycerol) *O*-methyl *O*-(2',3',4',5',6'-penta-*O*-benzyl-D-1'-inositol) phosphate (30 mg, 17%) as a white solid. ¹H-NMR (400 MHz, CDCl₃): δ -0.33- -0.21 (m, 4H), 0.52-0.85 (m, 12H), 0.87-0.95 (m, 6H), 0.96-1.70 (m, 44H), 2.24 (t, *J* = 7.3 Hz, 2H), 2.26 (t, *J* = 7.3 Hz, 2H), 3.46-3.54 (m, 2H), 3.67 (d, *J* = 11.4 Hz, 3H), 3.88 (dd, *J* = 11.9 and 6.0 Hz, 1H), 3.94 (ddd, *J* = 6.8, 6.4 and 5.0 Hz, 1H), 4.00-4.15 (m, 4H), 4.24 (ddd, *J* = 7.7, 7.4 and 2.1 Hz, 1H), 4.34 (t, *J* = 2.1 Hz, 1H), 4.67 (d, *J* = 11.4 Hz, 1H), 4.73 (d, *J* = 11.4 Hz, 1H), 4.75-4.85 (m, 4H), 4.85-4.95 (m, 3H), 4.95 (d, *J* = 11.4 Hz, 1H), 5.00-5.07 (m, 1H).

NaI (0.017 g, 0.11 mmol) was added to a solution of *O*-(1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoyl]-sn-3-glycerol) *O*-methyl *O*-(2',3',4',5',6'-penta-*O*-benzyl-D-1'-inositol) phosphate (30 mg, 0.020 mmol) in 2-butanone (2 ml). After stirring for 2 h at 80 °C, 2 N HCl was added to the reaction mixture, and the aqueous layer was extracted with chloroform. The organic layer was washed with H₂O and brine, and the combined organic layer was dried over anhydrous MgSO₄ and concentrated under reduced pressure to give *O*-(1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoyl]-sn-3'-glycerol) *O*-(2',3',4',5',6'-penta-*O*-benzyl-D-1'-inositol) phosphate.

To a solution of *O*-(1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoyl]-sn-3'-glycerol) *O*-(2',3',4',5',6'-penta-*O*-benzyl-D-1'-inositol) phosphate in ethanol (3 ml) was added 10% (w/v) palladium on activated carbon (21 mg). The resulting suspension was placed under hydrogen (1 atm) and stirred for 2 h at room temperature. The catalyst was removed through a pad of Celite, rinsed with ethyl acetate, and concentrated under reduced pressure. The resulting crude product was purified by a silica gel column chromatography (chloroform:methanol = 10:1) to give 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoyl]-sn-glycero-3-phosphatidyl-D-1-inositol (10 mg, 55%) as a white solid. ¹H-NMR (400 MHz, CDCl₃): δ -0.33- -0.20 (m, 4H), 0.55-0.85 (m, 12H), 0.87-0.95 (m, 6H), 0.96-1.70 (m, 44H), 2.20-2.43 (m, 4H), 3.80-4.51 (m, 5H), 5.18-5.32 (m, 1H); ESI-HRMS (negative ion, sodium formate) calculated for C₄₉H₈₆O₁₃P ([M-H]⁻) 913.5811; found 913.5806.

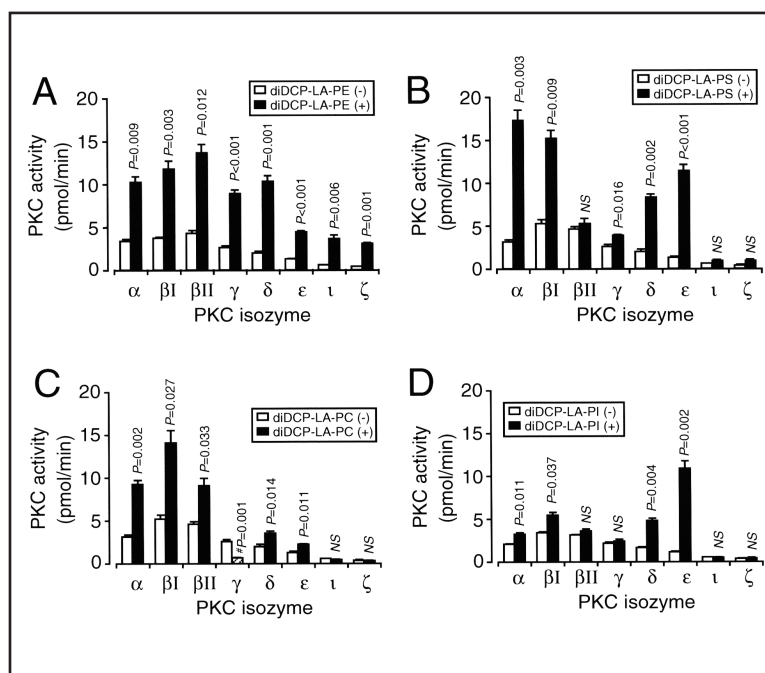
Cell-free PKC assay

PKC activity in the cell-free systems was quantified by the method as previously described [1]. 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 synthesized DCP-LA phospholipids in the absence of phosphatidylserine and diacylglycerol at 30 °C for 5 min. Activity for novel PKCs such as PKC-δ and -ε was assayed in Ca²⁺-free medium and activity for the other PKC isozymes in the medium containing 100 μM CaCl₂. 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.

Assay of PP1, PP2A, and PTP1B activities under the cell-free conditions

Activities of protein phosphatases under the cell-free conditions were assayed by the partially modified method as previously described [11]. The human recombinant PP1 was purchased from New England Biolabs Inc. (Ipswich, MA, USA) and the human recombinant PP2A from Millipore (Billerica, MA, USA). The human PTP1B was cloned into pGEX-6P-3 vector with a GST tag at the NH₂ terminus, and expressed in competent *E. coli* BL21 (DE3), suitable for transformation and protein expression. GST-fusion PTP1B was affinity-purified using Glutathione Sepharose 4B (GE Healthcare Bio-Science KK, Tokyo, Japan). Each phosphatase activity was assayed by reacting with *p*-nitrophenyl phosphate (*p*-NPP) (Sigma, St. Louis, MO, USA) as a substrate. Enzyme was preincubated at 30 °C (for PP1) or 37 °C (for PP2A and PTP1B) for 30 min in a reaction medium [50 mM HEPES, 100 mM NaCl, 2 mM dithiothreitol, 0.01% (v/v) Brij-35, 1 mM MnCl₂, pH 7.5 for PP1; 50 mM Tris-HCl, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, pH 7.0 for PP2A; and 50 mM HEPES, 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, pH 7.2 for PTP1B] in the presence and absence of phosphatase inhibitors, diDCP-LA-PE, diDCP-LA-PS, diDCP-LA-PC, or diDCP-LA-PI. Then, *p*-NPP at a concentration of 5 mM for PP1, 500 μM for PP2A, and 10 mM for PTP1B was added to the reaction medium followed by 60-min incubation, and the reaction was terminated by adding 0.1 N NaOH. Dephosphorylated *p*-NPP, i.e., *p*-NP, was quantified at an absorbance of 405 nm with a SpectraMax PLUS384 (Molecular Devices, Sunnyvale, CA, USA).

Fig. 2. Effects of DCP-LA-phospholipids on PKC activity. In the cell-free systems, PKC activity was monitored. Activity of PKC isozymes as indicated was assayed in the absence (open column) and presence (closed column) of diDCP-LA-PE (100 μ M)(A), diDCP-LA-PS (100 μ M)(B), diDCP-LA-PC (100 μ M)(C), or diDCP-LA-PI (100 μ M)(D). Each value represents the mean (\pm SEM) PKC activity (pmol/min)(n=4 independent experiments). *P* values as compared with each PKC isozyme activity in the absence of DCP-LA-phospholipids, unpaired *t*-test. NS, not significant.



Statistical analysis

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

Results

Effects of DCP-LA-phospholipids on PKC activity

In the cell-free PKC assay, diDCP-LA-PE (100 μ M) activated PKC α , - β I, - β II, - γ , - δ , and - ϵ to an similar extent (Fig. 2A). Amazingly, diDCP-LA-PE also activated atypical PKC isozymes such as PKC ζ and - ζ , and this effect was not found with other DCP-LA-phospholipids examined here (Fig. 2A).

diDCP-LA-PS (100 μ M) significantly activated PKC α , - β I, - γ , - δ , and - ϵ , with the relatively higher potential for PKC α and - β I activation (Fig. 2B).

diDCP-LA-PC (100 μ M) significantly activated PKC α , - β I, - β II, - δ , and - ϵ , with the relatively higher potential for PKC α , - β I, and - β II activation (Fig. 2C). Notably, PKC γ activation was conversely inhibited by diDCP-LA-PC (Fig. 2C).

diDCP-LA-PI (100 μ M) activated PKC α , - β I, and - δ to a small extent and highly activated PKC ϵ (Fig. 2D).

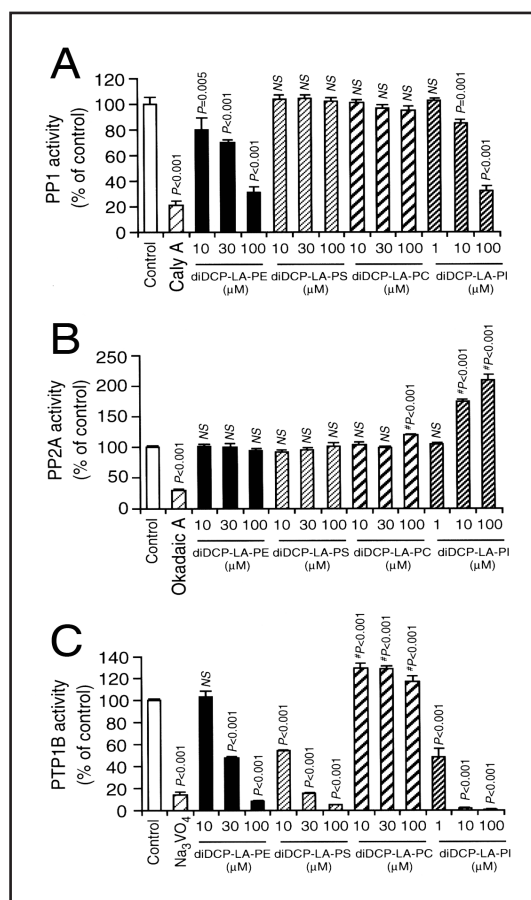
Effects of DCP-LA-phospholipids on protein phosphatase activity

In the cell-free protein phosphatase assay, calyculin A (10 nM), an inhibitor of PP1, okadaic acid (1 nM), an inhibitor of PP2A, or sodium orthovanadate (Na_3VO_4) (1 μ M), an inhibitor of PTP1B, actually inhibited PP1, PP2A, or PTP1B, respectively (Fig. 3A,B,C), confirming that the assay used here is reliable for assessment of protein phosphatase activity.

diDCP-LA-PE (100 μ M) and diDCP-LA-PI (100 μ M) suppressed PP1 activity to approximately 30% of control levels, but no significant inhibition was obtained with diDCP-LA-PS (100 μ M) or diDCP-LA-PC (100 μ M)(Fig. 3A).

None of diDCP-LA-PE (100 μ M), diDCP-LA-PS (100 μ M), and diDCP-LA-PC (100 μ M) affected PP2A activity, but surprisingly, diDCP-LA-PI (100 μ M) enhanced PP2A activity to nearly 180% of control levels (Fig. 3B).

Fig. 3. Effects of DCP-LA-phospholipids on protein phosphatase activity. PP1 (A), PP2A (B), or PTP1B (C) was reacted with *p*-NPP and dephosphorylated *p*-NPP was quantified. Caly A, calyculin A (10 nM); Okadaic acid (1 nM); and Na₃VO₄, sodium orthovanadate (1 μM). In the graphs, each value represents the mean (± SD) percentage of basal phosphatase activity (Control)(n=4 independent experiments). *P* values as compared with each control protein phosphatase activity, Dunnett's test. NS, not significant.



A marked inhibition of PTP1B activity was found with diDCP-LA-PE, diDCP-LA-PS, and diDCP-LA-PI (Fig. 3C). diDCP-LA-PE reduced PTP1B activity in a concentration (10-100 μM)-dependent manner, reaching 8% of control levels at 100 μM (Fig. 3C). Likewise, diDCP-LA-PS reduced PTP1B activity in a concentration (10-100 μM)-dependent manner, reaching 5% of control levels at 100 μM (Fig. 3C). diDCP-LA-PI the most strongly suppressed PTP1B activity, reaching 1 and 0.6% of control levels at 10 and 100 μM, respectively (Fig. 3C). In contrast, diDCP-LA-PC conversely enhanced PTP1B activity to around 120-130% of control levels at concentrations ranging 10 to 100 μM (Fig. 3C).

Discussion

PKC isozymes include conventional PKCs such as PKCα, -βI, -βII, and -γ, novel PKCs such as PKCδ, -ε, -η, -θ, and -μ, and atypical PKCs such as PKCι, -ζ and -ν [12, 13]. Established pathways for PKC activation are mediated by phospholipase C (PLC), phospholipase A₂ (PLA₂), phospholipase D (PLD), and phosphatidylcholine-specific PLC [12-14]. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate (IP₃), the latter activating IP₃ receptors to release Ca²⁺ from intracellular calcium stores, and conventional PKCs are activated by diacylglycerol and Ca²⁺ [12, 13]. Phosphatidylcholine-specific PLC produces diacylglycerol by hydrolysis of phosphatidylcholine, thereby activating PKC [14]. *Cis*-unsaturated free fatty acids such as arachidonic, oleic, linoleic, linolenic, and docosahexaenoic acid, that are produced by PLA₂-catalyzed hydrolysis of phosphatidylcholine, activate novel PKCs in a Ca²⁺-independent manner [12, 13]. In the present study, all the investigated DCP-LA-phospholipids diDCP-LA-PE, diDCP-LA-PS, diDCP-LA-PC, and diDCP-LA-PI activated PKC isozymes, although the PKC

isozymes activated and the degree of PKC activation differed among the phospholipids. The assay used here was carried out under the cell-free conditions, which rules out the pathways linked to PLC, PLA₂, PLD, and phosphatidylcholine-specific PLC. diDCP-LA-PE, diDCP-LA-PS, diDCP-LA-PC, and diDCP-LA-PI, accordingly, would activate PKC by directly binding to PKC, regardless of their metabolites.

Very interestingly, of investigated DCP-LA-phospholipids diDCP-LA-PE alone activated the atypical PKC isozymes PKC ι and - ζ . PKC ι is activated through its phosphorylation at Tyr325 due to receptor tyrosine kinase (RTK) or Src [15]. PKC ζ , on the other hand, is thought to be activated through its phosphorylation at Thr410/Thr560 due to phosphoinositide-dependent kinase-1 (PDK-1)/Akt downstream RTK [15]. The results of the present study suggest that diDCP-LA-PE might be a binding partner for PKC ι and - ζ . This may represent a novel pathway for activation of PKC ι and - ζ .

The most complicated finding is that whereas diDCP-LA-PC activated PKC α , - β I, - β II, - δ , and - ϵ , the compound drastically suppressed PKC γ activity. There is presently no plausible explanation for the reason why only PKC γ is inhibited by diDCP-LA-PC.

We have earlier found that DCP-LA has the potential to inhibit PP1 [8]. Our next attempt was to understand the effect of DCP-LA-phospholipids on protein phosphatases. diDCP-LA-PE and diDCP-LA-PI clearly inhibited PP1, while diDCP-LA-PS and diDCP-LA-PC had no effect. As is the case with DCP-LA [8], diDCP-LA-PE and diDCP-LA-PI could indirectly activate CaMKII by inhibiting PP1.

Amazingly, diDCP-LA-PI significantly enhanced PP2A activity, reaching 200% of control levels, but no effect was obtained with diDCP-LA-PE, diDCP-LA-PS, or diDCP-LA-PC. This, in the light of the fact that PP2A dephosphorylates MAP kinase cascades [16], suggests that diDCP-LA-PI could attenuate activity of MAP kinase cascades.

diDCP-LA-PE, diDCP-LA-PS, and diDCP-LA-PI suppressed PTP1B activity in a concentration-dependent manner, with the order of the potency: diDCP-LA-PI > diDCP-LA-PS > diDCP-LA-PE. In contrast, diDCP-LA-PC significantly enhanced PTP1B activity. PTP1B could inhibit insulin receptor signaling by dephosphorylating the receptor [17-19]. PTP1B, alternatively, might suppress proliferation or differentiation of tumor cells by inhibiting RTK [20]. To see the physiological role of DCP-LA-phospholipids, we are currently attempting further experiments.

In summary, the results of the present study demonstrate that all the newly synthesized DCP-LA-phospholipids diDCP-LA-PE, diDCP-LA-PS, diDCP-LA-PC, and diDCP-LA-PI serve as a PKC activator, except for diDCP-LA-PC-induced PKC γ inhibition; particularly, diDCP-LA-PE has the potential to activate the atypical PKC isozymes PKC ι and - ζ . The results also indicate diDCP-LA-PE and diDCP-LA-PI as an inhibitor for PP1 and PTP1B, diDCP-LA-PS as a PTP1B inhibitor, diDCP-LA-PI as a PP2A enhancer, and diDCP-LA-PC as a PTP1B enhancer.

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