Cellular Physiology and Biochemistry

Cell Physiol Biochem 2014;33:300-309
DOI: 10.1159/000356670
Published online: January 31, 2014
Accepted: January 07, 2014

Karger Open access

1421-9778/14/0332-0300$39.50/0

Original Paper

DCP-LA-Phosphatidylinositol and Its Enantiomer Exhibit Different Bioactivities

Ayako Tsuchiya[a] Takeshi Kanno[a] Tadashi Shimizu[b] Syuhei Nakao[b] Akito Tanaka[b] Tomoyuki Nishizaki[a]

[a]Division of Bioinformation, Department of Physiology, Hyogo College of Medicine, Nishinomiya, [b]Laboratory of Chemical Biology, Advanced Medicinal Research Center, Hyogo University of Health Sciences, Chuo-ku, Kobe, Japan

Key Words
DCP-LA-phospholipid • Enantiomer • Protein kinase C • Protein phosphatase • Akt

Abstract
Background/Aims: The present study was conducted to understand biochemical and biological characteristics of the phosphatidylinositol (PI) derivative 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidyl-D-1-inositol (diDCP-LA-PI) and its enantiomer 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidyl-L-1-inositol (diDCP-LA-PIe), with 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) on the α and β position. ***Methods:*** Activities of protein kinase C (PKC) and protein phosphatases such as protein phosphatase 1 (PP1), PP2A, and protein tyrosine phosphatase 1B (PTP1B) were assayed under the cell-free conditions and in PC-12 cells. Akt1/2 activity was monitored by quantifying phosphorylation at Thr308/309 and Ser473/474 in PC-12 cells. ***Results:*** diDCP-LA-PI significantly activated PKCα, -βI, -δ, and -ε, to an extent greater than that for diDCP-LA-PIe. diDCP-LA-PI still activated PKC in PC-12 cells, with the potential higher than that for diDCP-LA-PIe. Both diDCP-LA-PI and diDCP-LA-PIe reduced PP1 activity to a similar extent (30% of basal levels). diDCP-LA-PI enhanced PP2A activity to 180% of basal levels, while diDCP-LA-PIe had no effect. Drastic inhibition of PTP1B was obtained with diDCP-LA-PI and diDCP-LA-PIe, the extent reaching nearly 0% of basal levels. diDCP-LA-PI and diDCP-LA-PIe increased phosphorylation of Akt1/2 at Thr308/309 and Ser473/474 in PC-12 cells in the presence and absence of the PP2A inhibitor okadaic acid, respectively. ***Conclusion:*** The results of the present study show that diDCP-LA-PI and diDCP-LA-PIe exhibit different bioactivities with the different potentials each other.

Prof. Tomoyuki Nishizaki

Division of Bioinformation, Department of Physiology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya 663-8501 (Japan)
Tel. +81-798-45-6397, Fax +81-798-45-6649, E-Mail tomoyuki@hyo-med.ac.jp

Introduction

The phospholipid phosphatidylinositol (PI) is a member of plasma membrane components and plays a critical role in cell proliferation, differentiation, migration, chemotaxis, phagocytosis, and survival [1]. Furthermore, emerging evidence has pointed to the implication of PI in the control of vesicular trafficking, membrane dynamics, actin cytoskeleton organization, activation of ion channels, and transporters [2, 3]. Phosphatidylinositol 4,5-biphosphate (PIP_2) is hydrolyzed into diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) by phospholipase C [4, 5]. IP_3 releases Ca^{2+} through IP_3-gated calcium channel on the endoplasmic reticulum, an intracellular calcium store, and classical PKCs PKCα, -βI, -βII, and -γ are activated by intracellularly released Ca^{2+} and diacylglycerol [4, 5]. PIP_2, on the other hand, serves as a substrate for phosphatidylinositol 3-kinase (PI3K) to produce phosphatidylinositol 3,4,5-trisphosphate (PIP_3). PIP_3 recruits and activates 3-phosphoinositide-dependent protein kinase 1 (PDK1), which in turn, activates Akt followed by Rac1/Cdc42. PI, thus, is linked to diverse signaling cascades.

To understand the bioactivities of PI by itself, but not metabolites, we have newly synthesized the PI derivative 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidyl-D-1-inositol (diDCP-LA-PI) and its enantiomer 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidyl-L-1-inositol (diDCP-LA-PIe), with 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) on the α and β position (Fig. 1), which would not be easily degraded in cells. We have earlier found that the linoleic acid derivative DCP-LA selectively and directly activates protein kinase Cε (PKCε)[6, 7] or indirectly activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) by inhibiting protein phosphatase 1 (PP1) [8].

In the present study, we examined the effects of diDCP-LA-PI, diDCP-LA-PIe, the natural types of PIs 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-myo-inositol)(DOPI) and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-myo-inositol)(DPPI)(Fig. 1) on activities of PKC, protein phosphatases such as PP1, PP2A, and protein tyrosine phosphatase 1B (PTP1B) and Akt1/2. We show here that PIs by themselves, but not metabolites, serve as an intracellular signaling cascade and that diDCP-LA-PI and diDCP-LA-PIe exhibit different bioactivities with different potentials.

Materials and Methods

NMR analysis

^{1}H-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 ^{1}H-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 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,

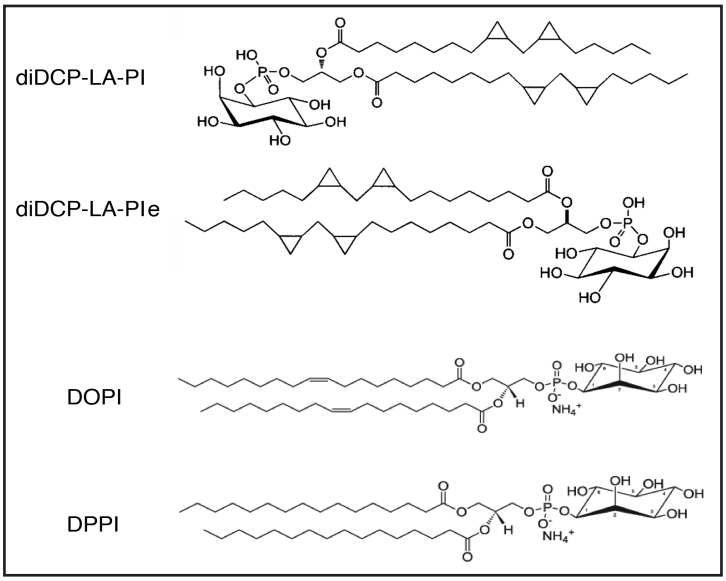

Fig. 1. Chemical structures of diDCP-LA-PI, diDCP-LA-PIe, DOPI, and DPPI.

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}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}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}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-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 CH_2Cl_2 (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 $Na_2S_2O_3$, the aqueous layer was extracted with CH_2Cl_2. The combined organic layer was dried over anhydrous $MgSO_4$ 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-glyceryl) *O*-methyl *O*-(2',3',4',5',6'-penta-*O*-benzyl-D-1'-inositol) phosphate (30 mg, 17%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): 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-glyceryl) *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 H_2O and brine, and the combined organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give *O*-(1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-3'-glyceryl) *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'-glyceryl) *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. ^{1}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), 2.20-2.43 (m, 4H), 3.80-4.51 (m, 5H), 5.18-5.32 (m, 1H); ESI-HRMS (negative ion, sodium formate) calculated for $C_{49}H_{86}O_{13}P$ ([M-H]$^-$) 913.5811; found 913.5806.

Synthesis of diDCP-LA-L-PIe

N,N-Diisopropylmethylphosphonamidic chloride (0.061 ml, 0.31 mmol) was added to a solution of 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycerol (0.175 g, 0.26 mmol) and triethylamine (0.072 ml, 0.52 mmol) in CH_2Cl_2 (5 ml) under ice-cooling. After stirring for 10 min at room temperature, (+)-2,3,4,5,6-penta-*O*-benzyl-L-1-inositol (0.25 g, 0.39 mmol) and 1*H*-tetrazole (0.073 g, 1.04 mmol) were added, and 70% (v/v) aqueous solution of *tert*-butyl peroxide (0.34 ml, 2.6 mmol) was added to the reaction mixture and stirred for 20 min at the same temperature. After adding 10% (w/v) aqueous solution of $Na_2S_2O_3$, the aqueous layer was extracted with CH_2Cl_2. The combined organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by a silica gel column chromatography (toluene:ethyl acetate = 6:1) to give *O*-(1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-3-glyceryl) *O*-methyl *O*-(2',3',4',5',6'-penta-*O*-benzyl-L-1'-inositol) phosphate (90 mg, 25%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): 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), 2.25 (t, *J* = 7.3 Hz, 2H), 2.26 (t, *J* = 7.3 Hz, 2H), 3.46-3.54 (m, 2H), 3.61 (d, *J* = 11.4 Hz, 3H), 3.94 (dd, *J* = 11.9 and 6.0 Hz, 1H), 4.00-4.32 (m, 7H), 4.68 (d, *J* = 11.9 Hz, 1H), 4.72 (d, *J* = 11.9 Hz, 1H), 4.73-4.85 (m, 8H), 5.15-5.25 (m, 1H).

NaI (50 mg, 0.33 mmol) was added to a solution of *O*-(1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-3-glyceryl) *O*-methyl *O*-(2',3',4',5',6'-penta-*O*-benzyl-L-1-inositol) phosphate (90 mg, 0.070 mmol) in 2-butanone (2 ml). After stirring for 3 h at 80 °C, 2 N HCl was added to the reaction mixture. 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*-(1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-3-glyceryl) *O*-(2',3',4',5',6'-penta-*O*-benzyl-L-1-inositol) phosphate.

To a solution of *O*-(1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-3-glyceryl) *O*-(2',3',4',5',6'-penta-*O*-benzyl-L-1-inositol) phosphate in ethanol (5 ml) was added 10% (w/v) palladium on activated carbon (70 mg). The resulting suspension was stirred under hydrogen (1 atm) 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-L-1-inositol (20 mg, 43%) as a white solid. ^{1}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), 2.20-2.43 (m, 4H), 3.50-4.51 (m, 5H), 5.10-5.42 (m, 1H); ESI-HRMS (negative ion, sodium formate) calculated for $C_{49}H_{86}O_{13}P$ ($[M-H]^-$) 913.5811; found 913.5811.

Cell culture

PC-12 cells, obtained from RIKEN Cell Bank (Tsukuba, Japan), were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 10% (v/v) heat-inactivated horse serum, supplemented with penicillin (100 U/ml), and streptomycin (0.1 mg/ml), in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

Cell-free PKC assay

PKC activity in the cell-free systems was quantified by the method previously described [6]. 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, and 10 μM ATP without phosphatidylserine and diacylglycerol in the presence and absence of diDCP-LA-PI, diDCP-LA-PIe, DOPI, or DPPI at 30 °C for 5 min. Activity for novel PKCs such as PKC-δ and -ε 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.

In situ PKC assay

PKC activity in PC-12 cells was assayed by the method previously described [6]. Cells were treated with diDCP-LA-PI or diDCP-LA-PIe in the presence and absence of GF109203X at 37 °C for 10 min in an extracellular solution (137 mM NaCl, 5.4 mM KCl, 10 mM $MgCl_2$, 5 mM EGTA, 0.3 mM Na_2HPO_4, 0.4 mM K_2HPO_4, and 20 mM HEPES, pH 7.2). Then, cells were rinsed with 100 μl of Ca^{2+}-free phosphate-buffered saline 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. 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 HPLC (LC-10ATvp). A substrate peptide peak and a new product peak were detected at an absorbance of 214 nm. Areas for non-phosphorylated and phosphorylated substrate peptide were measured (total area corresponds to concentration of substrate peptide used here), and the amount of phosphorylated substrate peptide was calculated. Phosphorylated substrate peptide (pmol/min/cell protein weight) was used as an index of PKC activity.

Assay of PP1, PP2A, and PTP1B activities

Activities of protein phosphatases under the cell-free conditions were assayed by the method previously described [9]. The human recombinant PP1 was purchased from New England BioLabs Inc. (Ipswich, MA, USA) and the human recombinant PP2A from Wako Pure Chemical Industries (Osaka, Japan). The human PTP1B was cloned into pGEX-6P-3 vector with a GST tag at the NH_2 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 (pNPP)(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 $MnCl_2$, 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-PI, diDCP-LA-PIe, DOPI, or DPPI. Then, pNPP 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 pNPP was quantified at an absorbance of 405 nm with a SpectraMax PLUS384 (Molecular Devices, Sunnyvale, CA, USA).

Western blotting

PC-12 cells were treated with diDCP-LA-PI or diDCP-LA-PIe in the presence and absence of okadaic acid, and then lysed in a lysate solution [150 mM NaCl, 20 mM Tris, 0.1% (v/v) Tween-20 and 0.1% (w/v) sodium dodecyl sulfate (SDS), pH 7.5] containing 1% (v/v) protease inhibitor cocktail and 1% (v/v) phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The lysates were centrifuged at 3,000 rpm for 5 min at 4 °C. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) using a TGX gel (BioRad) and then transferred to polyvinylidene difluoride (PVDF) membranes. Blotting membranes were blocked with TBS-T [150 mM NaCl, 0.1% (v/v) Tween-20 and 20 mM Tris, pH 7.5] containing 5% (w/v) bovine serum albumin and subsequently reacted with antibodies against phospho-Thr308/309-Akt1/2 (pT308/309)(Cell Signaling Technology, Inc., Danvers, MA, USA), phospho-Ser473/474-Akt1/2 (pS473/474)(Cell Signaling Technology), and Akt1/2 (Cell Signaling Technology). After washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody. Immunoreactivity was detected with an ECL kit (Invitrogen, Carlsbad, CA, USA) and visualized using a chemiluminescence LAS-4000mini detection system (GE healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

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

Results

Effects of diDCP-LA-PI and diDCP-LA-PIe on PKC activities

We initially examined the effect of diDCP-LA-PI and diDCP-LA-PIe on PKC activity. Of the PKC isozymes PKCα, –βI, –βII, –γ, –δ, –ε, –ι, and -ζ diDCP-LA-PI (100 μM) significantly activated PKCα, –βI, –δ, and -ε, and PKCε was the most highly activated (Fig. 2A). Likewise, diDCP-LA-PIe also activated PKCα, –βI, –δ, and -ε, but to a lesser extent as compared with that for diDCP-LA-PI (Fig. 2B).

DPPI (100 μM) also significantly activated PKCα, –βI, –δ, and -ε, with the highest activation of PKCβI (Fig. 2D), although DOPI (100 μM) activated PKCα, –βI, and -ε except for PKCδ (Fig. 2C).

diDCP-LA-PI (1 μM) significantly activated PKC in PC-12 cells in a concentration (0.1-10 μM)-dependent manner, and the effect of diDCP-LA-PI was abrogated by the PKC inhibitor GF109203X (100 nM) (Fig. 3A). diDCP-LA-PIe also activated PKC in PC-12 cells, but the potential was lesser than that for diDCP-LA-PI (Fig. 3B). These results indicate that diDCP-LA-PI serves as a PKC activator, with the potential greater than that for diDCP-LA-PIe.

Effects of diDCP-LA-PI and diDCP-LA-PIe on protein phosphatase activities

We next examined the effect of diDCP-LA-PI and diDCP-LA-PIe on protein phosphatases PP1, PP2A, and PTP1B. In the cell-free 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 μM), an inhibitor of PTP1B, actually inhibited PP1, PP2A, or PTP1B, respectively (Fig. 4A-F), confirming that the assay used here is reliable for assessment of protein phosphatase activity.

Both diDCP-LA-PI and diDCP-LA-PIe suppressed PP1 activity in a concentration (1-100 μM)-dependent manner, reaching approximately 30% of basal levels at 100 μM, with no significant difference in the potential between diDCP-LA-PI and diDCP-LA-PIe (Fig. 4A). DOPI (100 μM) and DPPI (100 μM) still attenuated PP1 activity, but to a lesser extent as compared with that for diDCP-LA-PI and diDCP-LA-PIe (Fig. 4D).

Notably, diDCP-LA-PI (100 μM) enhanced PP2A activity to about 180% of basal levels, but no effect was found with diDCP-LA-PIe (100 μM) (Fig. 4B). In contrast, DOPI and DPPI

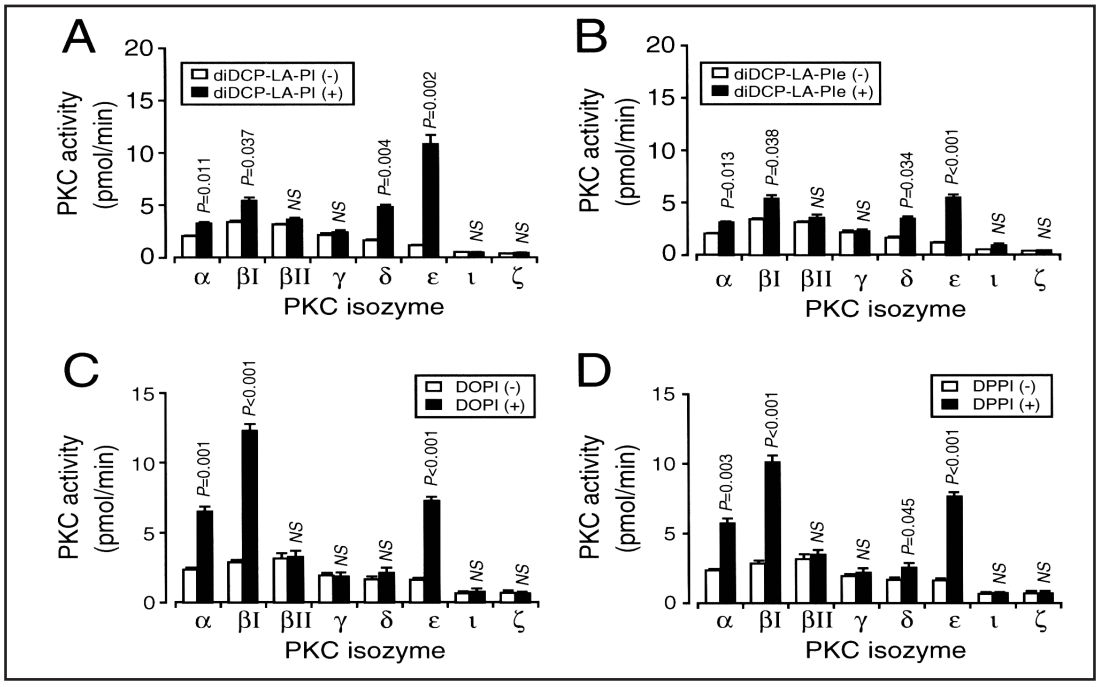

Fig. 2. Effect of PIs on PKC activity. In the cell-free systems, PKC activity was monitored. PKC isozymes as indicated were assayed in the absence and presence of diDCP-LA-PI (100 μM)(A), diDCP-LA-PIe (100 μM) (B), DOPI (100 μM)(C), and DPPI (100 μM)(D). In the graphs, each value represents the mean (± SEM) PKC activity (pmol/min) (n=4 independent experiments). *P* values as compared with each PKC isozyme activity in the absence of PIs, unpaired *t*-test. *NS*, not significant.

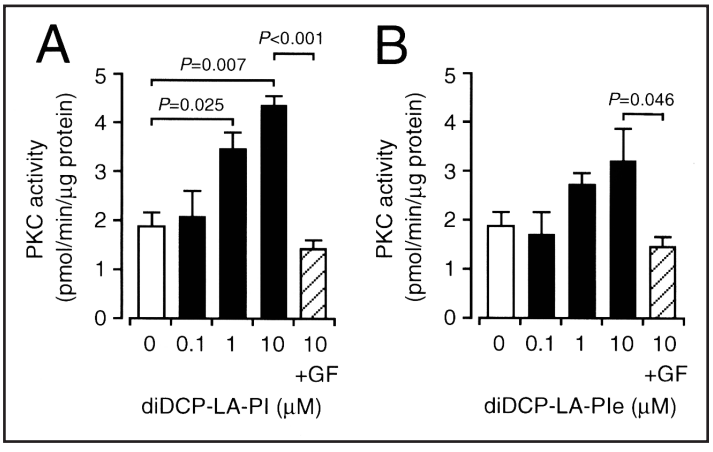

Fig. 3. Effects of diDCP-LA-PI or diDCP-LA-PIe on PKC activity in PC-12 cells. Cells were untreated and treated with diDCP-LA-PI (A) or diDCP-LA-PIe (B) at concentrations as indicated in the presence and absence of GF109203X (GF)(100 nM). Phosphorylated substrate peptide (pmol/min/μg cell protein) was used as an index of PKC activity. In the graphs, each column represents the mean (± SEM) PKC activity (n=4 independent experiments). *P* value, Dunnett's test.

inhibited PP2A activity in a concentration (1-100 μM)-dependent manner, reaching 71 and 36% of basal levels at 100 μM, respectively (Fig. 4E).

All the investigated PIs inhibited PTP1B activity in a concentration (1-100 μM)-dependent manner, reaching almost 0% of basal levels at 10 μM for diDCP-LA-PI and diDCP-LA-PIe (Fig. 4C) and at 100 μM for DOPI and DPPI (Fig. 4F).

Effects of diDCP-LA-PI and diDCP-LA-PIe on Akt1/2 activity

If diDCP-LA-PI and diDCP-LA-PIe reduce PTP1B activity, then these lipids might upregulate Akt activity through a pathway along receptor tyrosine kinase (RTK)/insulin receptor substrate 1 (IRS-1)/PI3K/PDK1/Akt(1/2) axis as a result of persistent tyrosine phosphorylation of RTK and IRS-1 due to PTP1B inhibition. Amazingly, diDCP-LA-PI (1 μM)

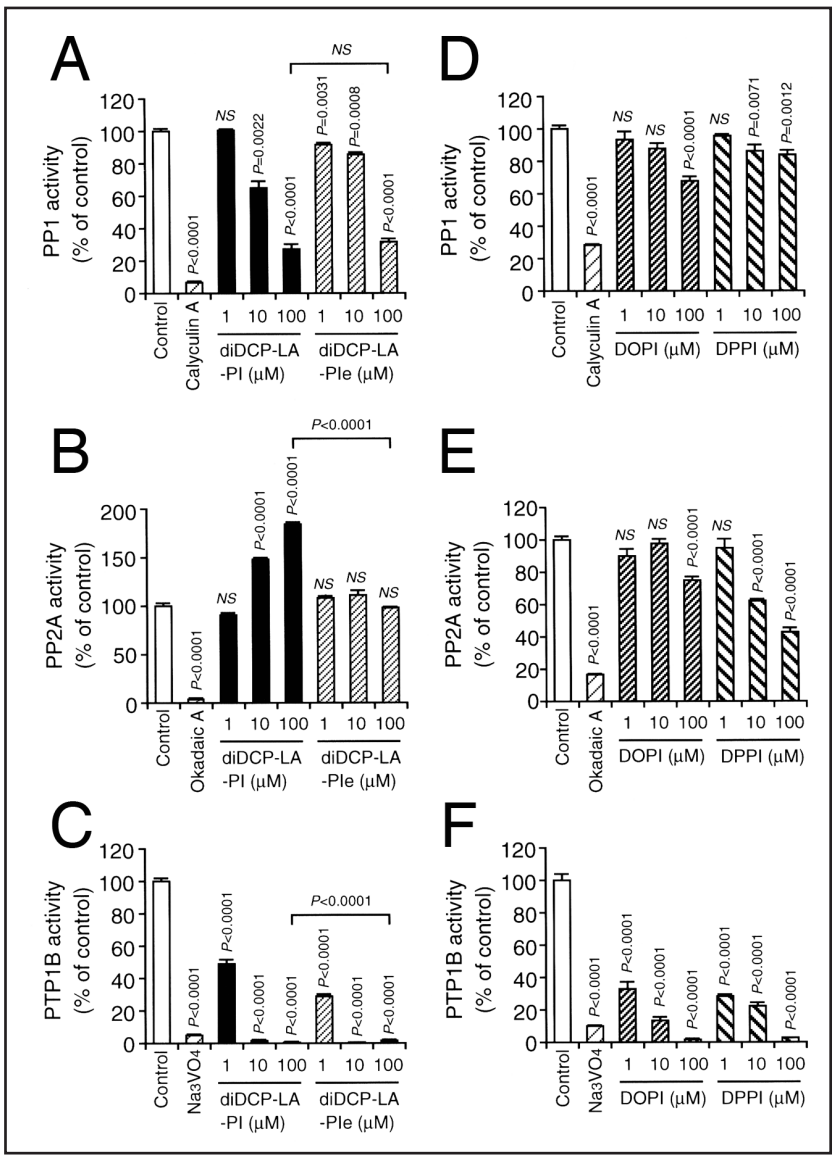

Fig. 4. Effect of PIs on protein phosphatase activity. PP1 (A,D), PP2A (B,E), or PTP1B (C,F) was reacted with pNPP in the presence and absence of diDCP-LA-PI, diDCP-LA-PIe, DOPI, or DPPI at concentrations as indicated and dephosphorylated pNPP was quantified. Calyculin A, 10 nM; Okadaic A (okadaic acid), 1 nM; and Na_3VO_4 (sodium orthovanadate), 1 μM. In the graphs, each value represents the mean (± SEM) 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.

had no effect on phosphorylation of Akt1/2 at Thr308/309 and Ser473/474 in PC-12 cells, while diDCP-LA-PIe (1 μM) significantly increased phosphorylation of Akt1/2 at Thr308/309 and Ser473/474 (Fig. 5A). diDCP-LA-PI had the potential to activate PP2A, a Ser/Thr protein phosphatase that could dephosphorylate Ser/Thr phosphorylation of Akt. Then, we postulated that diDCP-LA-PI might promote dephosphorylation of Akt1/2 in association with PP2A activation as well as phosphorylation of Akt1/2 in association with PTP1B inhibition. Expectedly, diDCP-LA-PI significantly increased phosphorylation of Akt1/2 at Thr308/309 and Ser473/474 in the presence of okadaic acid (100 nM), an inhibitor of PP2A (Fig. 5B).

Discussion

diDCP-LA-PI is a D-form of PI derivative with DCP-LA on the α and β position and its enantiomer diDCP-LA-PIe is a L-form of PI derivative. Enantiomers or diastereomers contained in a racemic modification of drugs might exert their different actions. α-methyl-N(α)-phthalimidoglutarimide (thalidomide), containing 2 enantiomers (S)- and (R)-form, was developed as a hypnotic, but (S)-form thalidomide otherwise caused the serious side-effect teratogenesis [10]. Racemic DCP-LA contains 4 possible diastereomers α,α-, α,β-,

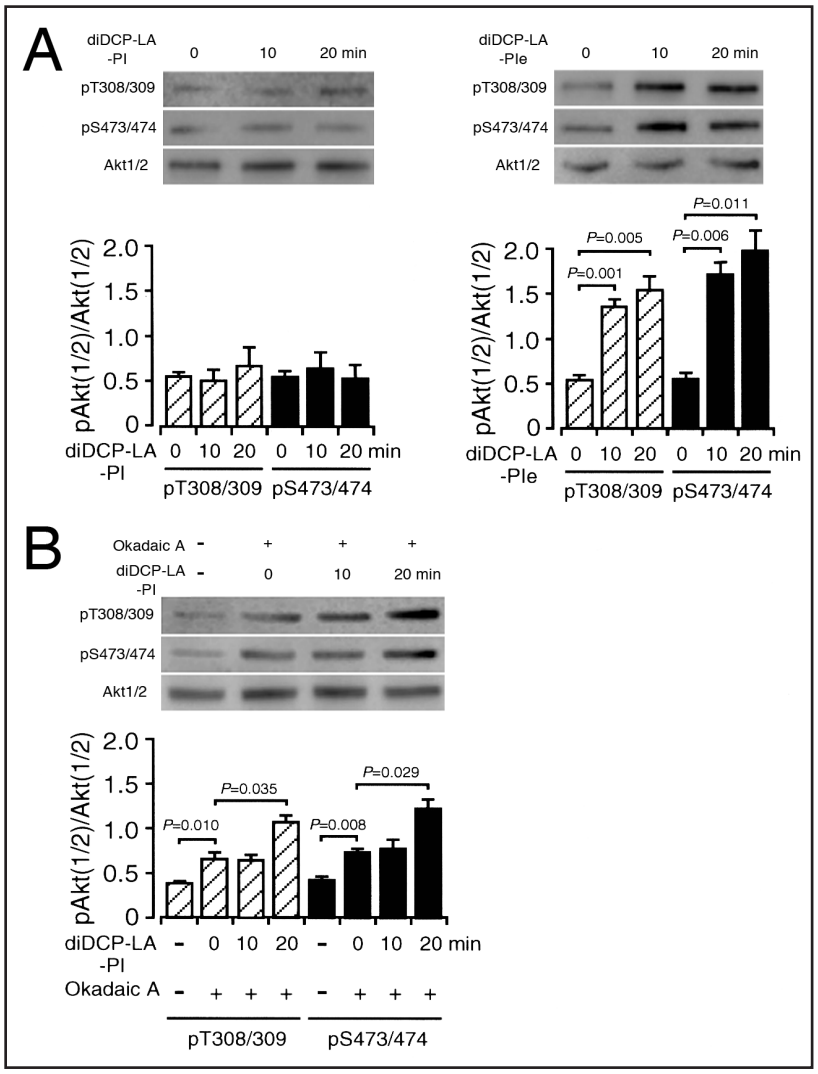

Fig. 5. Effect of diDCP-LA-PI and diDCP-LA-PIe on phosphorylation of Akt1/2. (A) PC-12 cells were treated with diDCP-LA-PI (1 μM) or diDCP-LA-PIe (1 μM) for the periods of time as indicated followed by Western blotting using antibodies against pT308/309, pS473/474, and Akt1/2. (B) Cells were untreated (-) and treated with diDCP-LA-PI (1 μM) for the periods of time as indicated in the absence (-) and presence of okadaic acid (Okadaic A)(100 nM) followed by Western blotting using antibodies against pT308/309, pS473/474, and Akt1/2. The signal intensity for pT308 and pS473 was normalized by that for total Akt1/2. In the graphs, each column represents the mean (± SEM) normalized intensity for pT308/309 and pS473/474 (n=4 independent experiments). *P* values, Dunnett's test.

β,α-, and β,β-DCP-LA, and all the diastereomers consistently serve as a selective activator of PKCε and stimulates release of glutamate, dopamine, and serotonin from rat hippocampal, striatal, and hypothalamic slices, respectively, under the control of PKCε and α7 nicotinic ACh receptors [7]. Of 4 diastereomers α,β-DCP-LA has the highest potential for PKCε activation and transmitter release [7]. This raises the possibility that diDCP-LA-PI and diDCP-LA-PIe might exhibit different bioactivities with different potentials.

Expectedly, diDCP-LA-PI activated PKCα, –βI, –δ, and -ε more potentially than diDCP-LA-PIe, especially for PKCε activation. Of particular interest is the finding that diDCP-LA-PI and diDCP-LA-PIe, whereas DCP-LA selectively activates PKCε alone, activated PKCα, –βI, and -δ in addition to PKCε. The natural types of PIs DOPI and DPPI still activated PKCα, –βI, and -ε, and DPPI activated PKCδ as well. In the present study, PKC activity was assayed under the cell-free conditions in the absence of phospholipase A_2, phospholipase C, and phospholipase D. This indicates that PIs by themselves, but not their metabolites such as unsaturated free fatty acids, diacylglycerol and IP_3, have the potential to activate PKC. To our knowledge, this is the first evidence for PI-induced PKC activation. diDCP-LA-PI activated PKC in PC-12 cells, with the potential than that for diDCP-LA-PIe. This provides further evidence for PI as a PKC activator.

Both diDCP-LA-PI and diDCP-LA-PIe suppressed PP1 activity in a concentration (1-100 μM)-dependent manner, to an extent much greater than that for the natural types of PIs DOPI and DPPI, reaching about 30% of basal levels. We have found that DCP-LA interacts with PP1,

to indirectly activate CaMKII [8]. This suggests that the inhibitory effect of PIs on PP1 activity might be due to direct binding of DCP-LA or free fatty acids to PP1.

One of the most striking findings in the present study is that diDCP-LA-PI strongly enhanced PP2A activity, while no effect was obtained with diDCP-LA-PIe. In contrast, DOPI and DPPI attenuated PP2A activity. The mechanism underlying diDCP-LA-PI-induced enhancement and DOPI-/DPPI-induced suppression in the PP2A activity remains to be explored.

Drastic inhibition of PTP1B was found with all the investigated PIs diDCP-LA-PI, diDCP-LA-PIe, DOPI, and DPPI. PTP1B is recognized to function as a negative regulator of RTK signaling pathways along a RTK/IRS/PI3K/PDK1/Akt axis and a RTK/Shc2/Grb2/SOS/Ras/Raf/MEK/ERK axis by dephosphorylating the receptor. Conversely, inhibition of PTP1B should enhance RTK signaling pathways. To address this point, we highlighted the former pathway and assayed Akt1/2 activity. diDCP-LA-PI had no effect on phosphorylation of Akt1/2 at Thr308/309 and Ser473/474 in PC-12 cells, but otherwise diDCP-LA-PIe significantly increased phosphorylation of Akt1/2 at Thr308/309 and Ser473/474. This implies that diDCP-LA-PIe stimulates phosphorylation of Akt1/2 at Thr308/309 and Ser473/474 by preventing tyrosine dephosphorylation of RTK and IRS-1 in association with PTP1B inhibition, allowing upregulation of RTK signaling. The question addressing is why diDCP-LA-PI did not increase Akt1/2 phosphorylation. Akt1/2 is dephosphorylated by PP2A and diDCP-LA-PI, but not diDCP-LA-PIe, served as a strong PP2A activator. diDCP-LA-PI significantly increased phosphorylation of Akt1/2 at Thr308/309 and Ser473/474 in the presence of okadaic acid, a PP2A inhibitor. Overall, these results indicate that diDCP-LA-PI concomitantly proceeds phosphorylation and dephosphorylation of Akt1/2 in association with PTP1B inhibition and PP2A activation, respectively.

In summary, the results of the present study show that diDCP-LA-PI, diDCP-LA-PIe, and DPPI activated PKCα, –βI, –δ, and -ε and DOPI activated PKCα, –βI, and -ε except for PKCδ; all the investigated PIs suppress activity of PP1; diDCP-LA-PI enhanced PP2A activity, while diDCP-LA-PIe had no effect or DOPI and DPPI reduced PP2A activity; A drastic PTP1B inhibition was obtained with all the investigated PIs; and diDCP-LA-PI and diDCP-LA-PIe stimulated Akt1/2 activation in the presence and absence of the PP2A inhibitor okadaic acid, respectively. Taken together, these results indicate that PIs by themselves, but not metabolites, serve as an intracellular signaling messenger and that diDCP-LA-PI and its enantiomer diDCP-LA-PIe exhibit different bioactivities with different potentials.

References

1 Cantley LC: The phosphoinositide 3-kinase pathway. Science 2002;296:1655-1657.
2 Di Paolo G, De Camilli P: Phosphoinositides in cell regulation and membrane dynamics. Nat Rev 2006;443:651-657.
3 Balla T, Szentpetery Z, Kim YJ: Phosphoinositide signaling: new tools and insights. Physiol 2009;24:231-244.
4 Nishizuka Y: Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 1992;258:607-614.
5 Nishizuka Y: Protein kinase C and lipid signaling for sustained cellular responses. FASEB J 1995;9:484-496.
6 Kanno T, Yamamoto H, Yaguchi T, Hi R, Mukasa T, Fujikawa H, Nagata T, Yamamoto S, Tanaka A, Nishizaki T: The linoleic acid derivative DCP-LA selectively activates PKC-ε, possibly binding to the phosphatidylserine binding site. J Lipid Res 2006;47:1146-1156.
7 Shimizu T, Kanno T, Tanaka A, Nishizaki T: α,β-DCP-LA selectively activates PKC-ε and stimulates neurotransmitter release with the highest potency among 4 diastereomers. Cell Physiol Biochem 2011;27:149-158.
8 Kanno T, Yaguchi T, Nagata T, Tanaka A, Nishizaki T: DCP-LA stimulates AMPA receptor exocytosis through CaMKII activation due to PP-1 inhibition. J Cell Physiol 2009;221:183-188.
9 Kanno T, Tsuchiya A, Shimizu T, Tanaka A, Nishizaki T: Indomethacin serves as a potential inhibitor of protein phosphatases. Cell Physiol Biochem 2012;30:1014-1022.
10 Blaschke G, Kraft HP, Fickentscher K, Köhler F: Chromatographische racemattrennung von thalidomid und teratogene wirkung der enantiomere. Arzneimittelforschung 1979;29:1640-1642.