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Molecules and Cells

Gintonin, Newly Identified Compounds from Ginseng, Is Novel Lysophosphatidic Acids-Protein Complexes and Activates G Protein-Coupled Lysophosphatidic Acid Receptors with High Affinity

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Recently, we isolated a subset of glycolipoproteins from *Panax ginseng*, that we designated gintonin, and demonstrated that it induced $[Ca^{2+}]_i$ transients in cells *via* G-protein-coupled receptor (GPCR) signaling pathway(s). However, active components responsible for Ca^{2+} mobilization and the corresponding receptor(s) were unknown. Active component(s) for $[Ca^{2+}]_i$ transients of gintonin were analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry and ion-mobility mass spectrometry, respectively. The corresponding receptor(s) were investigated through gene expression assays. We found that gintonin contains LPA $C_{18:2}$ and other LPAs. Proteomic analysis showed that ginseng major latex-like protein and ribonuclease-like storage proteins are protein components of gintonin. Gintonin induced $[Ca^{2+}]_i$ transients in B103 rat neuroblastoma cells transfected with human LPA receptors with high affinity in order of LPA2 > LPA5 > LPA1 > LPA3 > LPA4. The LPA1/LPA3 receptor antagonist Ki16425 blocked gintonin action in cells expressing LPA1 or LPA3. Mutations of binding sites in the LPA3 receptor attenuated gintonin action. Gintonin acted via pertussis toxin (PTX)-sensitive and -insensitive G protein-phospholipase C (PLC)-inositol 1,4,5-trisphosphate (IP_3)-Ca^{2+} pathways. However, gintonin had no effects on other receptors examined. In human umbilical vein endothelial cells (HUVECs) gintonin stimulated cell proliferation and migration. Gintonin stimulated ERK1/2 phosphorylation. PTX blocked gintonin-mediated migration and ERK1/2 phosphorylation. In PC12 cells gintonin induced morphological changes, which were blocked by Rho kinase inhibitor Y-27632. Gintonin contains GPCR ligand LPAs in complexes with ginseng proteins and could be useful in the development of drugs targeting LPA receptors.

INTRODUCTION

Ginseng, the root of *Panax ginseng* C.A. MEYER, is a popular herbal medicine consumed as a functional health food throughout the world. As the oldest Chinese herbal medicine book, Sheng-nong Ben-cao Jing, states, ginseng has various beneficial effects (e.g., replenishment of vital energy, mood elevation, and longevity), and recent studies have revealed that it exhibits diverse physiological and pharmacological effects on nervous and non-nervous systems (Nah et al., 2007). However, knowledge of the molecular basis of the multiple pharmacological effects of ginseng is rudimentary.

Lysophosphatidic acid (LPA; 1-acyl-2-sn-glycerol-3-phosphate) is a simple but potent lysophospholipid that evokes multiple physiological responses *via* G proteins in most cell types (Moolenaar, 1994). The LPA receptor was first cloned from the developing brain and proved to be a G protein-coupled receptor (GPCR) (Hecht et al., 1996). Currently, at least six different subtypes of LPA receptor are known in nervous and non-nervous tissues (Chun et al., 2010; Noguchi et al., 2009). Activation of LPA receptors affects cell survival and proliferation, migration, morphological changes, and cell cycle progression in neuronal and non-neuronal cells by linking LPA receptors to G proteins such as $G\alpha_{i/o}$, $G\alpha_{12/13}$, $G\alpha_{q/11}$, and $G\alpha_s$ (Chun et al., 2010). The LPA receptors are coupled to diverse downstream events including stimulation of phospholipase C and D, inhibition of adenylyl cyclase, and stimulation of small GTPases, mitogen-activated protein kinases, and phosphoinositide 3-kinase (Chun et al., 2010; Gardell et al., 2006).

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Previously, we demonstrated that a crude ginseng total saponin (cGTS) fraction activated the endogenous Ca^{2+}-activated Cl^- channel (CaCC) in *Xenopus* oocytes *via* a $G\alpha_{q/11}$-phospholipase C (PLC) β-inositol 1,4,5-trisphosphate (IP_3)-Ca^{2+} pathway and that repeated treatment with the cGTS fraction caused rapid desensitization of the CaCC via GRK2 and β-arrestin I (Choi et al., 2001a; 2001b; Lee et al., 2004). Recently, we isolated a subset glycolipoproteins from ginseng and named it gintonin (Pyo et al., 2011a; 2011b). Gintonin contains proteins with a high abundance of hydrophobic and acidic amino acids, and glucose is the main carbohydrate component. It also contains several fatty acids in ester form (Pyo et al., 2011a). Interestingly, gintonin but not ginseng saponin (also called ginsenoside) induced transient Ca^{2+} mobilization in mouse Ehrlich ascites tumor (EAT) cells and activated CaCC in *Xenopus* oocytes at very low concentration via the same pathway as the cGTS fraction (Pyo et al., 2011a). However, the active element(s) of gintonin responsible for the transient Ca^{2+} mobilization and the protein(s) with which gintonin might interact were not identified.

Here, we report that gintonin contains LPAs, especially LPA $C_{18:2}$ and that they activate LPA receptor subtypes with high affinity. Methanol extraction of gintonin permitted the LPAs to be separated from the protein components of gintonin. We discuss the possible reasons why the LPA complexes making up gintonin have different affinities for the various LPA receptor subtypes, and the roles of the protein components in gintonin-mediated activation. We speculate that co-existence of LPAs with ginseng proteins found in gintonin may be useful for developing novel drugs targeting LPA receptors.

MATERIALS AND METHODS

Materials

Gintonin was prepared according to Pyo et al. (2011a), and we used the crude gintonin fraction in the present studies without isolating the individual glycolipoproteins because of their scarcity and because of the similar ED_{50} values of the crude gintonin fraction (Pyo et al., 2011a). Lysophosphatidic acids (LPA $C_{16:0}$, LPA $C_{18:0}$, LPA $C_{18:1}$, and LPA $C_{18:2}$) were purchased from Avanti Polar Lipids (USA). Pertussis toxin (PTX) was purchased from Biomol Laboratories (USA). pcDNA3.1+ (control vector), LPA receptors (LPA1, LPA2, LPA3, LPA4, LPA5, LPA6), Hemagglutinin (HA)-tagged LPA receptors (LPA1, LPA2, LPA3), GPR35, GPR87, S1P receptors (S1P2, S1P3), GPR40, GPR41, GPR43, GPR120, GPR45, GPR63, 5-HT_{1C}, M1, M3, M5 (all in pcDNA 3.1) were purchased from Missouri S&T cDNA Resource Center (USA). The endogenous GPCRs in *Xenopus* oocytes such as GPR4, GPR56, GPR89, GPR125, and GPR143 were purchased from Openbiosystems (USA). Ki16425 was from Cayman Chemicals (USA). 5-Bromo-2′-deoxyuridine (BrDU) cell proliferation assay kit (enzyme-linked immunosorbent assay, ELISA) and phosphatase inhibitor cocktail (PhosSTOP) were purchased from Roche (Germany). Recombinant human vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were purchased from R&D systems (USA). Ginsenoside Rb_1, Rg_1, and Rg_3 were purchased from the LKT Institute (USA). Phospholipase A_1, phospholipase A_2, phospholipase C, phospholipase D, penicillin, streptomycin, Dulbecco's modified Eagle's medium (DMEM), RPMI1640 and fetal bovine serum (FBS) were purchased from Invitrogen (USA). Medium 199 (M199) was purchased from WelGene (Korea). All other reagents were from Sigma-Aldrich (USA).

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis of LPAs in gintonin

Stock solutions of each LPA were prepared and diluted in high pressure liquid chromatography (HPLC) grade methanol. All solutions were stored at 4°C. LPAs were quantified by liquid chromatography mass spectrometry using an API 2000 (triple-quadrupole) LC-MS/MS system (Applied Biosystems, USA) equipped with an electrospray ionization interface to generate positive ions $[M-H]^-$ (Yoon et al., 2003). The HPLC columns (Agilent series 1100, USA) were equipped with a quart pump (G1311A), degasser (G1322A), an autosampler (G1313A) containing a 100-μl-sample loop, Colcom (G1316A). Samples were separated on C_{18} stainless columns (ZORBAX Eclipse XDB, 2.1 mm I.D × 100 mm, particle size: 3.5 μm, Agilent, USA), and eluted with a mobile phase consisting of a mixture of acetonitrile and 5 mM ammonium acetate in double-distilled H_2O (80:20, v/v, pH 2.3). The isocratic mode was run at a flow-rate of 0.2 ml/min and aliquots of 10 μl were injected into the column. The column temperature was maintained at 25°C.

The ion spray interface was operated in negative ion mode at 5500 V and 350°C. The operating conditions, optimized by a flow injection of a mixture of all analytes, were as follows: nebulizing (GS1), auxiliary (GS2), and curtain gas flows of 30.0, 70.0, and 20.0 PSI, respectively; collision gas (nitrogen) pressure, 6 × 10^{-5} Torr; declustering potentials (DP), -26 to -101 V; focusing potentials (FP), -290 to -300 V; entrance potentials (EP), -8.5 to -12 V; collision cell entrance potentials (CEP), -16 to -36 V; collision energies (CE), -36 to -86 V; collision cell exit potentials (CXP), -4 V. Quantitation was performed *via* multiple reaction monitoring (MRM) of the precursor ions and the related product ions for acyl-LPAs, using an internal standard method with peak area ratios. The mass transitions (precursor ion/product ion) used for LPA $C_{18:2}$, LPA $C_{18:1}$, LPA $C_{16:0}$, and LPA $C_{18:0}$ (internal standard, I.S.) were *m/z* 433 → *m/z* 79, *m/z* 435 → *m/z* 79, *m/z* 409 → *m/z* 79 and *m/z* 437 → *m/z* 153, respectively. Quadrupoles Q1 and Q3 were set at unit resolution. The analytical data were processed with Analyst 1.4.2 software (ABI Inc., USA).

Oocyte preparation

Xenopus laevis care and handling were in accordance with the Guidelines for the *Care and Use of Laboratory Animals,* published by NIH, USA. *Xenopus* oocytes were isolated, treated with collagenase and maintained as previously described (Pyo et al*.,* 2011a).

Oocyte recording

A single oocyte was placed in a small Plexiglas (0.5 ml) constantly superfused with ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.5) in the absence or presence of gintonin. Two-electrode voltage-clamp recordings were made at room temperature using an Oocyte Clamp (OC-725C, Warner Instrument) with Digidata 1200A (Molecular Devices, USA) (Pyo et al., 2011a).

Preparation of LPA3 receptor cRNA, microinjection, and site-directed mutation of LPA

A recombinant plasmid containing the human LPA3 receptor insert was linearized by digestion with appropriate restriction enzymes. cRNA was obtained with an *in vitro* transcription kit (mMessage mMachine; Ambion, USA) with T3, T7, or SP6 polymerase. The RNA was dissolved in RNase-free water at 1 μg/μl, divided into aliquots, and stored at -80°C. Oocytes were injected with H_2O, LPA3, or other receptor cRNAs (5-10 ng) using a Nanoject Automatic Oocyte Injector (Drummond Scien-

tific, USA) (Lee et al., 2004). Site-directed mutagenesis was performed with a Pfu DNA polymerase (QuikChange™ XL Site-Directed Mutagenesis Kit, STRATAGENE, USA) and appropriate sense and antisense primers. Overlap extension of the target domain by sequential polymerase chain reaction was performed in accordance with the supplier's instructions with some modifications. Final PCR products were transformed into *E. coli* DH5α, and screened by PCR. The mutations were confirmed by sequencing the target region (Lee et al., 2004).

Cell culture and transfection

Rat B103 neuroblastoma cells were grown in DMEM supplemented with 10% (v/v) FBS, 100 units/ml penicillin, 100 μg/ml streptomycin. B103 cells were transfected transiently with plasmids, namely pcDNA3.1+ (empty control vector), or pcDNA3.1 containing LPA receptors, mutant LPA3, HA-tagged LPA receptors, or other GPCRs using Lipofectamine 2000 (Invitrogen), as previously described (Lee et al., 2006; 2007). Human umbilical vein endothelial cells (HUVECs) were gifted from Dr. Hwan Myung Lee (Hoseo University, Korea) and cultured in M199 supplemented with 20% (v/v) FBS, 5 units/ml heparin, 3 ng/ml bFGF, 100 units/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37°C in humidified conditions under 5% CO_2. The cells at passages 2-7 were used in all the experiment. PC12 cells, a rat pheochromocytoma cell line, obtained from Korean cell line bank (KCLB, Korea) and cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin.

Western blotting of membrane fractions

To obtain membrane fractions, HA-LPA receptor-expressing B103 cells were washed with cold PBS, collected and lysed with buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM dithiothreitol, 0.32 M sucrose, and protease inhibitor cocktail. The lysates were centrifuged at 12,000 × *g* for 30 min at 4°C and the pellets resuspended in the same lysis buffer containing 1% Triton X-100, incubated on ice for 45 min, and centrifuged again at 12,000 × *g* for 30 min at 4°C. The supernatants were collected and used as membrane fractions. Total cell protein was measured by the BCA method (Thermo Scientific, USA). Membrane fractions were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels, and transferred to polyvinylidene difluoride membranes. HA-tagged LPA receptor expression was detected using mouse anti-HA.11 Clone 16B12 monoclonal antibody (Millipore, USA) and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Millipore), and visualized by enhanced chemiluminescence (GE Healthcare, UK).

Confocal laser imaging of LPA receptor-expressing cells

B103 cells (4×10^4/well) were cultured on cover slips in 12-well plates for 24 h then transfected with HA-tagged LPA receptor-pcDNA3.1 in serum free medium using Lipofectamine 2000 (Invitrogen). Four hours after transfection, the medium was changed to growth medium and the cells were incubated for 24 h. After overnight serum-starvation in serum-free medium, they were fixed with 4% paraformaldehyde in PBS for 1 h and incubated with permeabilization solution (0.1% Triton X-100, 3% BSA in PBS) for 15 min. HA-tagged LPA receptors were detected by immunostaining with mouse anti-HA.11 Clone 16B12 monoclonal antibody (Millipore) and Cy3-conjugated anti-mouse IgG secondary antibody (Jackson Immunol Research Laboratories, Inc., USA) (Lee et al., 2006; 2007). Images were obtained by confocal microscopy (Olympus, FV-1000 laser scanning confocal system).

Cyclic AMP (cAMP) measurement

Cells were pretreated with 0.5 mM of 3-isobutyl-methylxanthine and 10 μM of forskolin in serum-free medium for 20 min. They were then stimulated for 30 min with gintonin or LPA and lysed with 0.1 N HCl. cAMP was measured with a cAMP assay kit (Cayman Chemicals, USA) (Lee et al., 2006; 2007).

Fura 2-acetoxymethyl ester (Fura 2-AM) loading of B103 cells and measurement of $[Ca^{2+}]_i$ in cell suspensions

Cells transfected with empty vectors or vectors encoding LPA receptors were detached with trypsin/EDTA, washed with HEPES-buffered saline solution (HBS, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose, 25 mM HEPES, pH 7.4) and loaded with Fura 2-AM (2.5 μM). Intracellular free calcium concentrations were measured by dual excitation spectrofluorometric analysis of cell suspensions loaded with Fura-2 AM (Ex: 340 nm and 380 nm, Em: 510 nm) as previously described (Jørgensen et al., 1999; Pyo et al., 2011a). Cells were assayed in HBS or Ca^{2+} free HBS (120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 0.2 mM EGTA, 25 mM HEPES, pH 7.4). Intracellular calcium concentrations were calculated by the method of Grynkiewicz et al. (1985).

Extracellular signal-regulated kinase (ERK)1/2 phosphorylation assay

HUVECs were plated in 150 mm dishes and serum-starved in M199 containing 1% FBS for 6 h and treated with M199 containing 1% FBS in the presence of gintonin, VEGF, or LPA. The cells washed with cold PBS (pH 7.4), collected and lysed with the modified RIPA buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EGTA, 2 mM sodium orthovanadate, protease inhibitor cocktail and phosphatase inhibitor cocktail (PhosSTOP)]. The cell lysates were analyzed by SDS-PAGE and immunoblotting using rabbit anti-phospho-ERK polyclonal antibody (1:1000) (Cell signaling). Blot was stripped and re-probed with rabbit anti-ERK polyclonal antibody (1:1000) (Cell signaling). Data collection and processing were performed with the luminescent image analyzer LAS-3000 and Multi Gauge software (Fujifilm, Japan).

BrDU incorporation ELISA assay

Proliferation of HUVECs was determined by BrDU incorporation assay, which measures DNA synthesis. Cells were seeded at 3×10^3 cells per well into 96-well plates coated with 0.1% gelatin solution. After 24 h, cells were washed with M199 and incubated for 6 h with M199 containing 1% FBS. The cells were washed with fresh M199 (1% FBS) again and incubated with gintonin at indicated concentrations. After indicated incubation time, the cell proliferation was assessed by means of BrDU cell proliferation ELISA assay, according to supplier's instructions (Roche). Briefly, after 18 h incubation, the cells were added with BrDU labeling reagent in M199 (1% FBS), followed by additional 24 h incubation. The culture medium containing labeling solution was removed carefully. The cells in each well were washed with 200 μl of M199 containing 10% FBS and incubated with 150 μl of precooled fixative for 30 min at room temperature. Relative BrDU incorporation of each well was measured using anti-BrDU-peroxidase and luminometer with photomultiplier technology (Chen et al., 2008; Won et al., 2008).

Migration assay

Cell migration assay were performed as previously described (Kim et al., 2007; Lee et al., 2000). Briefly, the chemotactic motility of HUVECs was measured using modified Boyden chambers with 48-well (Neuro Probe Inc., USA). Polycarbonate mem-

brane with 8-μm pores was coated with 0.1 mg/ml collagen type I (BD biosciences, USA) for 30 min, followed by drying for more than 1 h. Gintonin in M199 (0.1% bovine serum albumin) were added to the lower wells of the chambers. The chambers were assembled with the membrane coated with collagen. Cells (5×10^4 cells/well) were added to the upper wells of the chambers and incubated for 70-80 min at 37°C. The cells on the membrane was fixed and stained with Diff Quik (Sysmex, Japan), and mounted on the slide glass. Non-migrated cells were removed with Kipewipes. The migrated cells in 4 fields were counted under microscope (light microscopy) at magnification of × 200.

Morphological change assay

PC12 cells (2.5×10^5 cells/well) were plated on 6-well plates coated with poly-L-lysine. After 24 h, the culture medium was changed to modified N2 medium (DMEM, 5 μg/ml insulin, 5 μg/ml apotransferrin, 20 nM progesterone, 100 μM putrescine, 30 nM selenium), as described previously (Sato et al., 1997). For differentiation, the cells were incubated with 0.5 ng/ml NGF for 24 h. The differentiated cells were pretreated with vehicle or 10 μM Y-27632 for 30 min, and then incubated overnight at 37˚C in the presence of gintonin (30 μg/ml). Cell morphology was monitored and photographed using Real-Time Cell Observer (Cal Zeiss) (200× magnification).

Data analysis

To obtain concentration-response curves in the presence of gintonin, peak amplitudes were normalized and plotted using Origin software (OriginLab Corp., USA). All values are presented as means ± S.E.M. Differences between the means of control and treated cells were assessed using unpaired Student's *t*-tests or one-way ANOVA. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effects of trypsin, glycosidases, and phospholipases on gintonin-mediated CaCC activation in *Xenopus* oocytes

In a previous report, we showed that gintonin consists of carbohydrates, fatty acids in ester form, and proteins (Pyo et al., 2011a). In the present study, we first investigated which component of gintonin plays an important role in CaCC activation in *Xenopus* oocytes. For this, we examined the effects of trypsin, glycosidases, and phospholipase on CaCC activation. As shown in Supplementary Table S1, trypsin digestion had no significant effect on CaCC activation by gintonin. We used mixture of PNGase F, sialidase, β-galactosidase, glucosaminidase, and O-glycosidase to remove N-linked and many O-linked oligosaccharides from gintonin. Deglycosylation of gintonin abolished CaCC activation (Supplementary Table S1). When we examined the effects of various phospholipases such as PLA_1, PLA_2, PLC, or PLD, we found that only PLA_1 substantially reduced CaCC activation (Supplementary Table S1), pointing to the importance of esterification at position 1 of the fatty acid components of gintonin.

Gintonin contains a large amount of LPA $C_{18:2}$ as the major LPA

Tigyi and Miledi (1992) demonstrated that LPAs bound to serum albumin activated CaCC in *Xenopus* oocytes and activation was prevented by digestion with PLA_1. They further showed that the LPAs could be dissociated from serum albumin by methanol extraction. Following this lead, we found that methanol extracts of gintonin strongly induced CaCC activation in *Xenopus* oocytes (data not shown) and this prompted us to use LC-ESI-MS/MS analysis (Yoon et al., 2003) to examine whether methanol extracts of gintonin contained LPAs. We were surprised to find that methanol extracts contained 394 ± 7.1 and 44.2 ± 2.9 nmole/g weight ginseng of LPA $C_{18:2}$ and LPA $C_{16:0}$,

Table 1. Amounts of LPAs in gintonin and gintonin methanol extract (nmole/g weight ginseng)

	LPA $C_{16:0}$	LPA $C_{18:1}$	LPA $C_{18:2}$
Gintonin*	48.9 ± 5.9	BSL	438 ± 8.8
Gintonin MeOH extract	44.2 ± 2.9	BSL	394 ± 7.1

The amounts of LPAs in gintonin were calculated by measuring the height of each LPA relative to that of the internal standard (LPA $C_{18:0}$). Data are means ± SD of four determinations. *Ginseng contains about 0.2% gintonin (Pyo et al., 2011b) and these amounts of LPAs correspond to about 9.5% of gintonin by weight. BSL: below the sensitivity limit.

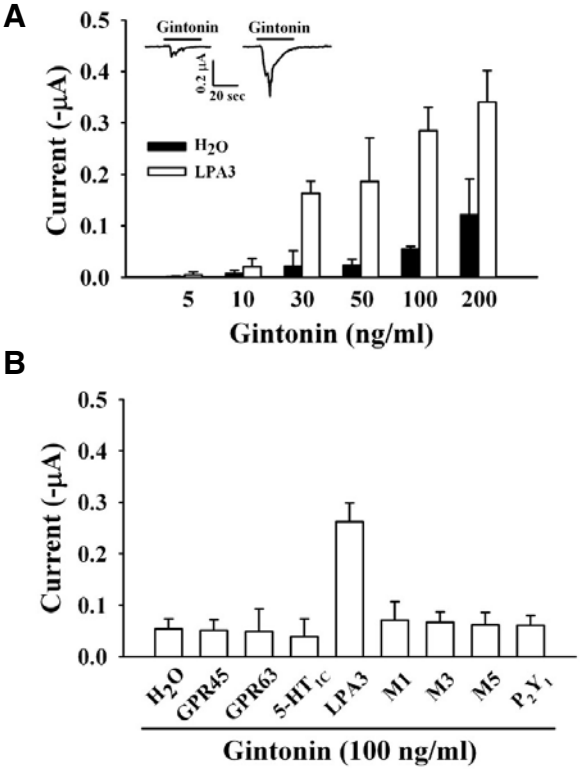

Fig. 1. Effects of gintonin on Ca^{2+}-activated Cl^- channel (CaCC) currents in *Xenopus* oocytes injected with H_2O or cRNA encoding various receptors. (A) Effects of gintonin on Ca^{2+}-activated Cl^- channel (CaCC) currents in *Xenopus* oocytes injected with H_2O or cRNA encoding the LPA3 receptor. *Inset*; Representative traces showing enhancement of CaCC currents by gintonin (100 ng/ml) in oocytes expressing the LPA3 receptor compared with H_2O-injected oocytes. Gintonin enhanced CaCC currents in oocytes expressing the LPA3 receptor with concentration-dependent manner. (B) Effects of gintonin on CaCC currents in *Xenopus* oocytes injected with H_2O or cRNAs encoding the various receptors. Enhancement of CaCC currents by gintonin (100 ng/ml) was only observed in oocytes expressing the LPA3 receptor compared with other receptors. Details of the methods of analysis of CaCC currents are given in the "Materials and Methods".

Table 2. IM-MS spectrometry of gintonin proteins

MW(kDa) /PI	Amino acid sequences	Protein	Accession no.
16.87/4.9	RDIEAHHLPK KDPTSYLDFLLSVTRD KEEIVAIDEEDKS KLNESVKD	Ginseng major latex-like protein (MLP151)	EU939308
27.3/5.5	RSDYPWAM KAFDIVGLLNQEGIYPN NDLYRPKM KSLLNTFTIHGLYPYNAKG KHLNAVPEIDFTKN RTALAFRK	Ginseng ribonuclease-like storage protein	AAR88098

respectively (Supplementary Fig. S1 and Table 1). Recovery of the LPAs after methanol extraction was about 90% (Table 1).

Identification of the protein components of gintonin

We analyzed the proteins present by ion-mobility mass spectrometry (IM-MS) (Gao et al., 2008; Ono et al., 2006). This analysis showed that gintonin contains two main types of protein: ginseng major latex-like protein (MW: 16.8 kDa) and ribonucle-ase-like storage proteins (MW: 27.3 kDa) (Kim et al., 2004; Sun et al., 2010) (Table 2).

Activation of CaCC in *Xenopus* oocytes by gintonin is increased by expressing LPA receptor subtypes

Since the above results suggested that the active components of gintonin in CaCC activation are LPAs, we tested whether CaCC activation was enhanced by overexpressing the LPA3 receptor. This was indeed the case (Fig. 1A), thus supporting the view that activation by gintonin is due to LPAs acting on LPA receptors on the oocyte membrane. However, gintonin failed to further increase CaCC currents in oocytes expressing 5-HT_{1C} receptor (Dascal et al., 1986), muscarinic acetylcholine receptor subtypes (M1, M3, and M5), which are endogenously expressed in *Xenopus* oocytes (Herrera et al., 1994), brain GPR45 and GPR63, which are homologous with endogenous *Xenopus* oocyte PSP24 (Guo et al., 1996; Kawasawa et al., 2000), or P_2Y_1 receptor (Buchholz et al., 2004) (Fig. 1B). In addition, gintonin did not enhance CaCC currents in oocytes expressing endogenous orphan GPCRs of *Xenopus* oocytes such as GPR4, GPR89, GPR125, and GPR143 (data not shown).

Gintonin induces transient Ca^{2+} mobilization in B103 cells transfected with LPA1, LPA2, LPA3, LPA4, or LPA5 receptors

To confirm this interpretation we transiently expressed several human LPA receptors in B103 cells, which do not contain any endogenous LPA receptors (Ishii et al., 2000). Western blotting identified protein bands of the expected molecular mass for each LPA receptor in cell membrane fractions (Supplementary Fig. S2A). We also detected HA-tagged LPA receptors on

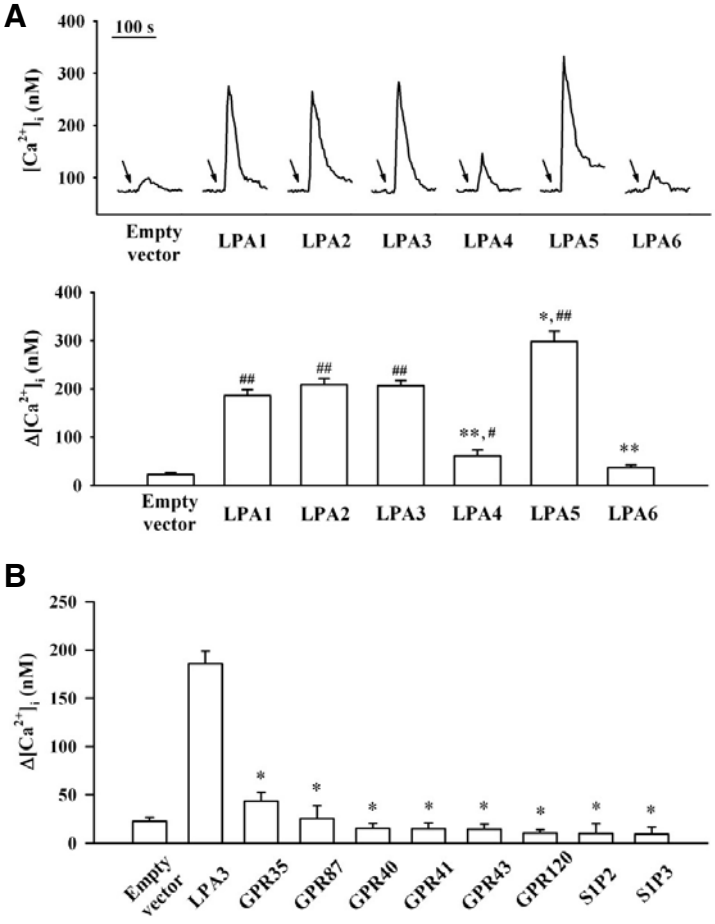

Fig. 2. Effects of gintonin on transient Ca^{2+} mobilization in B103 cells expressing LPA receptor subtypes and other G protein-coupled receptors. (A) Representative traces of Ca^{2+} mobilization in B103 cells expressing empty vector and LPAs 1-6. The histograms present the net stimulation of Ca^{2+} mobilization ($^{\#}p < 0.05$, $^{\#\#}p < 0.001$, compared to empty vector; $^{*}p < 0.01$, $^{**}p < 0.001$, compared to LPA3 receptor). Ca^{2+} mobilization was measured with Fura-2AM as described in the "Materials and Methods". Arrows indicate application of gintonin (1 μg/ml). Data are means ± S.E.M. of three or four determinations. (B) Net Ca^{2+} mobilization in B103 cells expressing other receptors.

transfected B103 cell membrane by labeling with Cy3-anti HA (Supplementary Fig. S2B).

As shown in Fig. 2A, gintonin treatment (1 μg/ml) had minimal effect on B103 cells transfected with empty vector. However, it strongly induced transient Ca^{2+} mobilization in B103 cells transfected with LPA1, LPA2, LPA3, and LPA5 receptors (Fig. 2A, *lower panel*). It also caused some mobilization in cells expressing LPA4 but not in those expressing LPA6, and it had no effect in cells expressing other GPCRs (Fig. 2B). As a positive control, we showed that LPA $C_{18:1}$ induced concentration-dependent Ca^{2+} mobilization in B103 cells expressing the LPA3 receptor, with an ED_{50} of 58.9 ± 5.5 nM (Fig. 3A), which is consistent with the results of a previous study (Ishii et al., 2000). The mobilization of Ca^{2+} by gintonin was concentration-dependent with EC_{50} values of 0.10 ± 0.02, 0.004 ± 0.0004, 0.12 ± 0.01, 0.69 ± 0.09, and 0.046 ± 0.003 μg/ml for LPA1, LPA2, LPA3, LPA4, and LPA5, respectively (Fig. 3B). Since activation of LPA receptors can also affect cAMP formation (Yanagida et al., 2009), we examined the effect of gintonin on cAMP formation in B103 cells transfected with the LPA3 or LPA6 receptor and found no significant effect (Fig. 3C). The ginsenosides Rb_1, Rg_1, and Rg_3 (10 μM each) also had no effect on transient Ca^{2+} mobilization in B103 cells expressing the LPA3 receptor (data not shown).

An LPA1 and LPA3 receptor antagonist blocks gintonin action, and site-directed mutations of LPA binding sites of an LPA receptor attenuate gintonin action

As shown in Fig. 6, gintonin-mediated transient Ca^{2+} mobilization was abolished in the presence of the LPA1 and LPA3 receptor antagonist Ki16425 (Ohta et al., 2003), In addition, we examined whether site-directed mutations of LPA binding sites in the LPA3 receptor affected Ca^{2+} mobilization. We constructed an R3.28A mutant of the LPA3 receptor, since R3.28 is thought to be an important site in LPA receptors 1-3 (Valentine et al., 2008). We also constructed a W4.64 LPA3 receptor mutant, since this amino acid residue may be a binding site for the LPA3 receptor (Valentine et al*.,* 2008). As shown in Fig. 4, gintonin treatment induced significantly less mobilization of Ca^{2+} in B103 cells transfected with mutant R3.28A and W4.64A LPA3 receptors, than in cells transfected with wild-type receptor. These finding strongly support the view that the $[Ca^{2+}]_i$ transients induced by gintonin are due to its content of LPAs.

Gintonin acts *via* PTX-sensitive and -insensitive G proteins/PLC/IP_3 receptor/Ca^{2+} pathways

We examined the signaling pathways by means of which gintonin induces transient Ca^{2+} mobilization. We first showed that PTX treatment (200 ng/ml, 16 h) significantly attenuated Ca^{2+} mobilization in B103 cells expressing LPA1 or LPA3 receptors but had no effect on B103 cells expressing the LPA2 and LPA5 receptors (Fig. 5), indicating that gintonin acts through PTX-sensitive and -insensitive G proteins, depending on the LPA receptor subtype (Ishii et al., 2000). In addition, the PLC inhibitor, U-73122 inhibited gintonin-induced transient mobilization of Ca^{2+} (Fig. 6) as did the membrane permeable IP_3 receptor antagonist, 2-aminoethoxydiphenyl borate (2-APB) (Dobrydneva et al., 2001). Moreover buffering intracellular free Ca^{2+} with membrane permeable 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA-AM) (50 μM final) also abolished Ca^{2+} mobilization, and incubation in Ca^{2+}-free buffer solution (in the presence of 0.2 mM EGTA) significantly reduced Ca^{2+} mobilization, indicating that gintonin-induced $[Ca^{2+}]_i$ transients are due to a combination of Ca^{2+} entry and Ca^{2+} release from intracellular stores. These results imply that gintonin-mediated $[Ca^{2+}]_i$ transients arise *via* PTX-sensitive and -insensitive G proteins-PLC-IP_3-Ca^{2+} pathways.

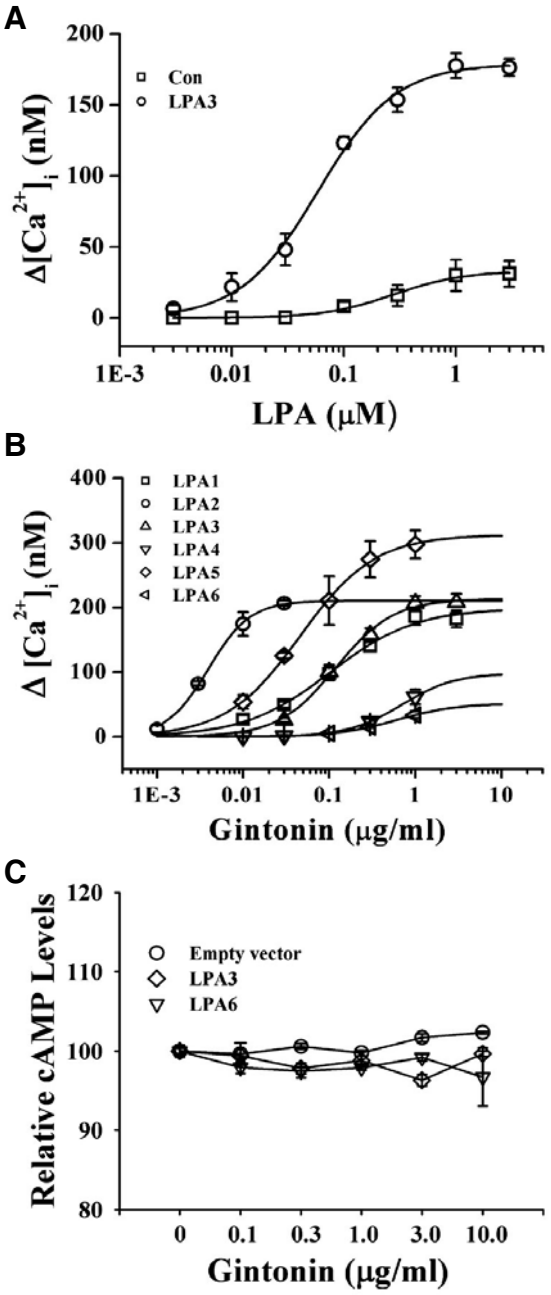

Fig. 3. Concentration-dependence of the effects of LPA or gintonin on transient Ca^{2+} mobilization and cAMP levels in B103 cells expressing LPA receptor subtypes. (A) In B103 cells expressing LPA3 receptor, LPA treatment increased Ca^{2+} mobilization in a concentration-dependent manner, but, it had almost no effects on B103 cells expressing empty vector (Con). (B) In B103 cells expressing LPA1, LPA2, LPA3, or LPA5 receptors, gintonin induced Ca^{2+} mobilization in a concentration-dependent manner, whereas it caused only a slight increase of $[Ca^{2+}]_i$ in B103 cells expressing the LPA4 receptor and had no effect *via* the LPA6 receptor. (C) Gintonin did not affect cAMP levels in B103 cells expressing the LPA3 or LPA6 receptors. Data are means ± S.E.M. of four determinations.

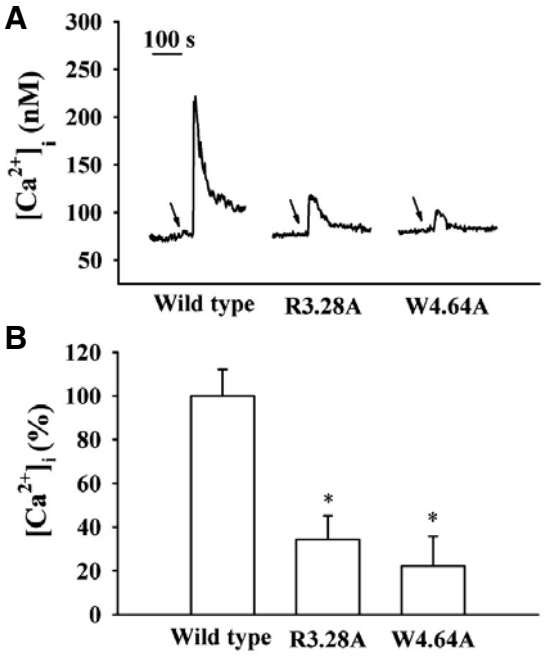

Fig. 4. Effects of gintonin on Ca^{2+} mobilization in B103 cells expressing mutant LPA3 receptors. The representative traces of Ca^{2+} mobilization in B103 cells expressing wild-type, R3.28A, or W4.64A LPA3 receptors. The histograms show net increases of Ca^{2+} mobilization calculated from the tracings. The arrows indicate application of gintonin (1 μg/ml). (*$p < 0.001$, compared to wild-type receptor). Data are means ± S.E.M. of three-four determinations.

Effects of gintonin on ERK1/2 phosphorylation in cells that endogenously express LPA receptors

It is known that phosphorylation of ERK1/2 is primary signaling events for the proliferation and migration of HUVECs (Lee et al., 2000; Sengupta et al., 2004). Next, we examined whether gintonin regulates the ERK1/2 phosphorylation in cells that endogenously express LPA receptors. We chose HUVECs, since previous reports have shown that HUVECs endogenously express LPA1 and LPA3 receptors (Lee et al., 2000) and that ginseng water extract stimulates proliferation and migration of HUVECs (Kim et al., 2007). Treatment of HUVECs with gintonin stimulated ERK1/2 phosphorylation in concentration- and time-dependent manners (Figs. 7A and 7B). Gintonin-mediated ERK1/2 phosphorylation was saturated at 30 μg/ml and maximal ERK1/2 phosphorylation was observed after 10 min of gintonin treatment and declined after 30 min (Figs. 7A and 7B). However, the stimulatory effects of gintonin on ERK1/2 phosphorylation were blocked by PTX (15 ng/ml, 6 h) (Fig. 7C). These results indicate that gintonin-mediated ERK1/2 phosphorylation of HUVECs is achieved through PTX-sensitive $G\alpha_{i/o}$ proteins coupled to LPA receptors.

Effects of gintonin on proliferation, migration, or morphological changes in cells that endogenously express LPA receptors

As next step, we examined gintonin effects on proliferation, migration, or morphological changes in HUVECs and PC12 cells. Treatment of HUVECs with gintonin stimulated cell proliferation and migration in a concentration-dependent manner. The ED_{50} on cell proliferation was 13.1 ± 2.2 μg/ml (Fig. 8A). Interestingly, the migrating effect of gintonin was saturated at 10 μg/ml (Fig. 8B). The stimulatory effects of gintonin on cell

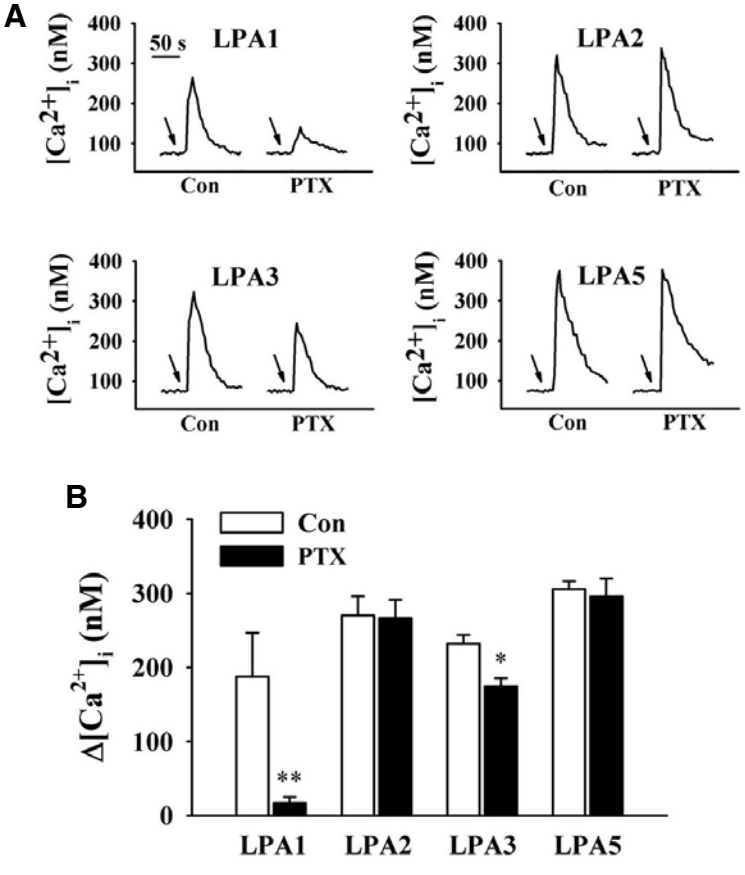

Fig. 5. Effects of pertussis toxin (PTX) on gintonin-mediated Ca^{2+} mobilization in B103 cells expressing LPA1, LPA2, LPA3, or LPA5 receptors. (A) Representative traces of Ca^{2+} mobilization in B103 cells expressing LPA1, LPA2, LPA3, or LPA5 receptors without (Con) or with PTX-pretreatment (200 ng/ml, 16 h). Arrows indicate the application of gintonin (1 μg/ml). (B) The histograms present net increases of Ca^{2+} mobilization calculated from the tracings without PTX or with PTX (*$p < 0.05$, **$p < 0.001$, compared to no PTX). Data represent the mean ± S.E.M. from three-four determinations.

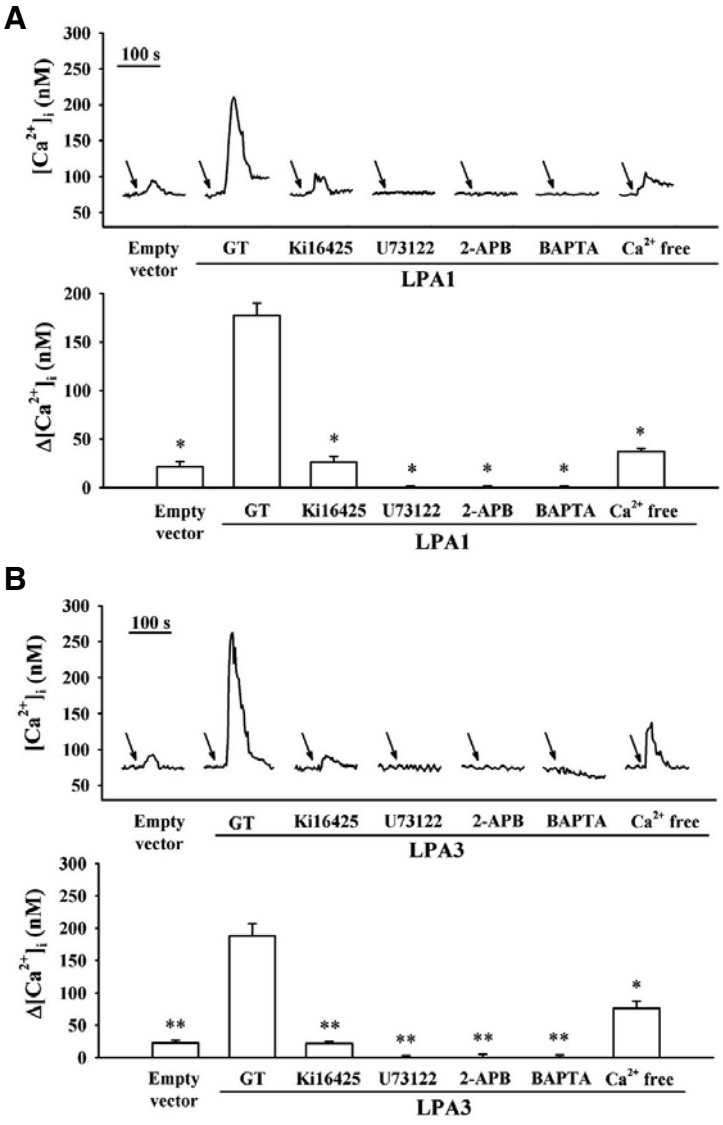

Fig. 6. Effects of an LPA1 and LPA3 receptor antagonist (Ki16425), PLC inhibitor (U73122), IP_3 receptor antagonist (2-APB), Ca^{2+} chelator (BAPTA-AM), and Ca^{2+}-free buffer. Representative traces of gintonin-mediated Ca^{2+} mobilization in B103 cells expressing the LPA1 (A) or LPA3 (B) receptor in the absence or presence of various agents. The arrows indicate application of gintonin (1 μg/ml). Ki16425 (10 μM), U73122 (5 μM), 2-APB (100 μM), and BAPTA-AM (50 μM) were added before the gintonin. Ca^{2+} free buffer was prepared as described in the "Materials and Methods". The histograms present net increases of gintonin-mediated Ca^{2+} mobilization calculated from the tracings in the absence or presence of various agents (*$p < 0.01$; **$p < 0.001$, compared to gintonin only treatment). Data are means ± S.E.M. of three-four determinations.

migration of HUVECs were blocked by PTX treatment (15 ng/ml, 16 h) (Lee et al., 2000), indicating that gintonin-mediated HUVECs migration is achieved *via* PTX-sensitive G proteins (Fig. 7D). Next, we examined whether gintonin affects cell morphology. For this, we used PC12 cells, since differentiated PC12 cells endogenously express LPA1, LPA2, and LPA3 receptors (Park et al., 2006) and were well-characterized in neurite retraction and cell rounding after LPA treatment (Jalink et al., 1994; Sato et al., 2005). Treatment of PC12 cells with gintonin induced transient Ca^{2+} mobilization, which was attenuated by Ki16425 (data not shown). As shown in Fig. 8C, treatment of differentiated PC12 cells with gintonin first induced neurite retraction and caused cell rounding after 1 h. However, gintonin-mediated neurite retraction and cell rounding of PC12 cells was blocked by Rho kinase inhibitor Y-27632, indicating that gintonin-induced morphological changes of PC12 cells is mediated by $G\alpha_{12/13}$ proteins coupled to Rho kinase of LPA receptor.

DISCUSSION

Some herbal medicines are GPCR ligands. For example, the opium poppy contains morphine, which interacts with opioid receptors (Pert and Snyder, 1973). The *Cannabis genus* contains cannabinoids, which interact with cannabinoid receptors (Matsuda et al., 1990). The fungus *Isaria sinclairii* is a widely-used Chinese medicine. Recent work has revealed that it contains ISP-1, which has a sphingosine-like structure. It was ultimately modified as the S1P receptor agonist FTY720 for clinical use (Fujita et al., 1994). It was not known whether ginseng contains active ligand(s) acting on GPCRs, but recently we isolated a subset of glycolipoproteins from ginseng, named gintonin, which we showed transient Ca^{2+} mobilization through an unidentified $G\alpha_{q/11}$-coupled receptor signaling pathway (Pyo et al., 2011a).

In the present study, we have made four principal observations indicating that gintonin mobilizes Ca^{2+} by activating LPA receptors. First, it contains a high concentration of LPAs, especially LPA $C_{18:2}$ (Table 1), and mobilized Ca^{2+} in B103 cells transfected with vectors harboring the LPA1, LPA2, LPA3, or LPA5 receptor but not empty vector (Fig. 2). Second, mobilization in B103 cells expressing the LPA1 or LPA3 receptor was blocked by the LPA1 and LPA3 receptor antagonist Ki16425

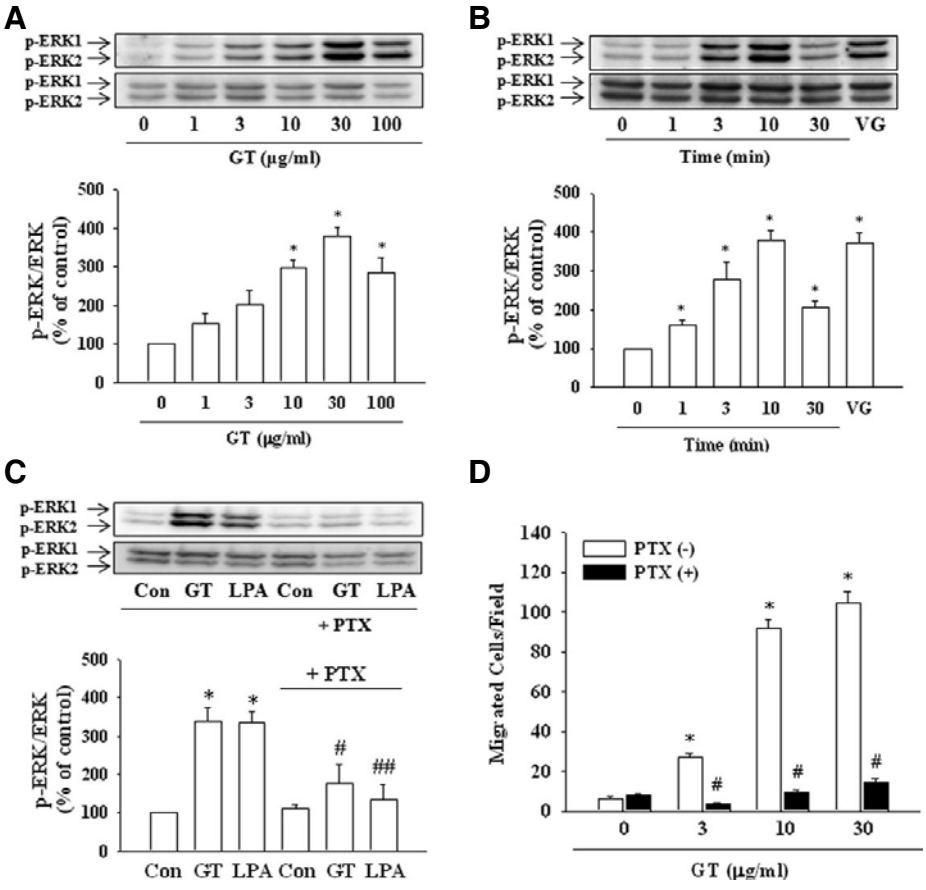

Fig. 7. Effects of PTX on gintonin-mediated cell migration and ERK 1/2 phosphorylation in HUVEC. (A) Gintonin stimulated ERK1/2 phosphorylation in a concentration-dependent manner but with slight decrease of ERK1/2 phosphorylation at high concentration of gintonin (100 μg/ml). Cells were stimulated for 10 min with vehicle (0) or indicated concentration of gintonin and ERK1/2 phosphorylation was determined as described in the "Materials and Methods". The upper panels represent basal and different concentration of gintonin-stimulated ERK1/2 phosphorylation by immunoblotting from a representative experiment performed with HUVECs. Data are presented as fold increase of basal ERK1/2 phosphorylation, where the basal amount of ERK1/2 phosphorylation in untreated cells is assigned a value of 100% (*p < 0.01, compared to gintonin untreated control). (B) Gintonin-mediated ERK1/2 phosphorylation was time-dependent and peaked at 10 min after gintonin treatment. VG, VEGF (20 ng/ml) (*p < 0.01, compared to gintonin untreated control). Cells were stimulated for indicated time with gintonin (30 μg/ml). (C) Gintonin- and LPA-mediated stimulation of ERK1/2 phosphorylation was blocked by PTX pretreatment. Cells were pre-treated with PTX (200 ng/ml, 6 h) and stimulated with gintonin (30 μg/ml) or LPA (10 μM) for 10 min (*p < 0.01, compared to gintonin untreated control; $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, compared to without PTX treatment). (D) Gintonin-mediated HUVECs migration is achieved *via* PTX-sensitive G proteins. In the absence of PTX, gintonin stimulates cell migration in a concentration-dependent manner, but PTX pretreatment (15 ng/ml, 6 h) abolished gintonin action (*p < 0.05, compared to gintonin untreated control; $^{\#}p$ < 0.001, compared to PTX untreatment). Data shown represent the mean ± S.E.M. values of duplicate determinations from each of three separate experiments.

(Fig. 6). Third, amino-acid substitutions in the LPA binding site in the LPA3 receptor attenuated gintonin action (Fig. 4). Fourth, the identified protein components of gintonin do not function as LPA receptor ligands. These results indicate that the LPAs in gintonin play a key role in Ca^{2+} mobilization. Interestingly, gintonin was unable to mobilize Ca^{2+} *via* GPR35 and GPR87 or the S1P2 and S1P3 receptors (Oka et al., 2010; Tabata et al., 2007), whose ligand has a similar chemical structure to LPA (Chun et al., 2010), or *via* free fatty acid receptors (Ichimura et al., 2009; Wellendorph et al., 2009) (Fig. 2B). In addition, gintonin did not enhance CaCC currents in oocytes expressing other GPCRs examined (Fig. 1B).

LPAs are found in animal tissues and plants such as soybean and various seeds (Liu et al., 2007; Tanaka et al., 2010; Tokumura et al., 1978). In plants LPAs exist in the free form or together with lecithin in the range of 2-6 nmole/g weight (Liu et al., 2007; Tanaka et al., 2010), whereas in animal plasma LPAs are usually bound to serum albumin (Tigyi and Miledi, 1992). The LPAs in gintonin have three characteristics distinguishing them from those of other plants. First, ginseng contains 80-240 times as much LPAs as other plants (Table 1). Second, gintonin contains predominantly LPA $C_{18:2}$ unlike other plants. Third, the LPAs in gintonin are present in complexes with protein components, unlike conventional plant LPAs (Liu et al., 2007; Tanaka et al., 2010). The active LPAs could be dissociated from other components of gintonin by methanol extraction as in the case of LPA complexes with serum albumin (Tigyi and Miledi, 1992), and methanol extracts of ginseng contained most of the activity responsible for CaCC activation in *Xenopus* oocytes (data not shown). In addition, since deglycosylation of gintonin also caused a loss of gintonin action (Supplementary Table S1), the gintonin LPAs may exist in complexes with the carbohydrates, ginseng major latex-like protein, and ribonuclease-like storage protein (Table 2) (Kim et al., 2004; Sun et al., 2010). Although carbohydrate and protein components of gintonin might contribute LPA stability, further studies will be required to elucidate the exact roles of carbohydrate and/or protein components in gintonin.

Interestingly, Ca^{2+} mobilization by gintonin was most pronounced in B103 cells transfected with the LPA5 receptor (Fig. 2A). Gintonin displayed affinities for the various LPA receptor subtypes for Ca^{2+} mobilization in the order LPA2 > LPA5 > LPA1 > LPA3 > LPA4 (Fig. 3B). The EC_{50} value of gintonin was in the range of 0.45 to 18 nM if we take the native molecular weight of gintonin as 67 kDa (Pyo et al., 2011), whereas the EC_{50} of LPA $C_{18:1}$ on LPA3 receptor, dissolved in fatty acid-free

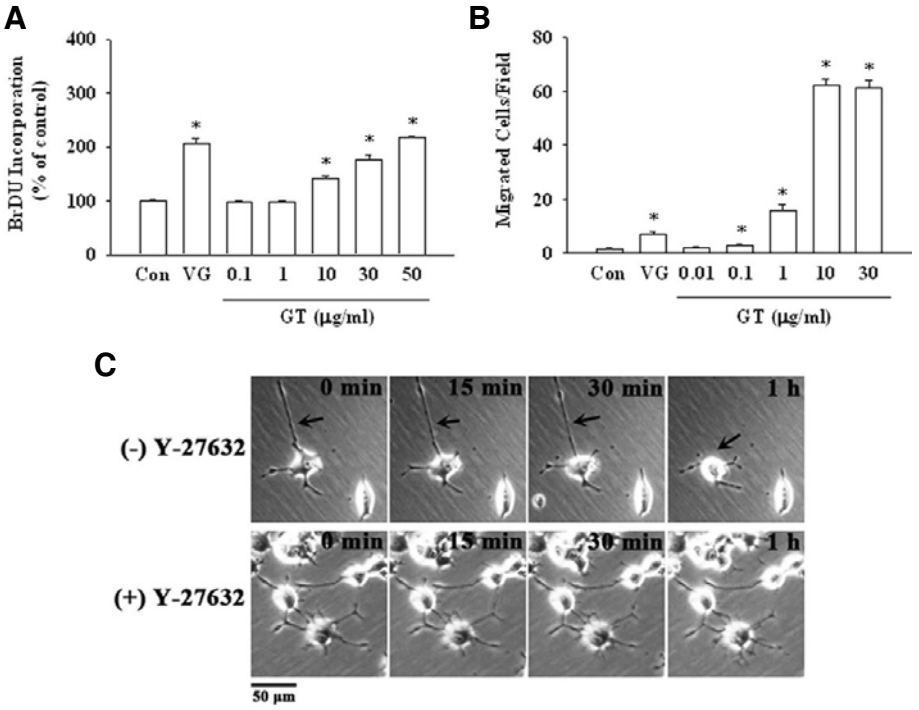

Fig. 8. Effects of gintonin on cell proliferation, migration, or morphological changes in HUVECs and PC12 cells that are endogenously expressing LPA receptors. (A) Gintonin stimulated HUVEC proliferation in a concentration-dependent manner. BrDU assay was described in the "Materials and Methods". VG, VEGF (20 ng/ml). (*$p < 0.001$, compared to gintonin untreated control). (B) Gintonin stimulated cell migration in a concentration-dependent manner in HUVECs. Cell migration assay was described in the "Materials and Methods". (*$p < 0.001$, compared to gintonin untreated control). (C) Gintonin (30 μg/ml) also induced morphological changes in PC12 cells in a time-dependent manner but gintonin actions on cell morphological changes was blocked by Rho kinase inhibitor Y-27632.

albumin solution, was 58.9 ± 5.5 nM (Fig. 3A). Thus, the EC_{50} value of gintonin for the various LPA receptors was 3-130 fold lower than that of LPA in LPA3 receptor-mediated Ca^{2+} mobilization. One possible contribution of high affinity of gintonin to LPA receptors might be derived from complexes of LPAs with carbohydrates and proteins, which helps to make an easy access to its receptor. However, further studies will be necessary to explain how the carbohydrate and protein components of gintonin help to have higher affinity of gintonin to LPA receptors than free LPA.

LPA receptors are coupled to $G\alpha_i$, $G\alpha_{q/11}$, $G\alpha_{12/13}$, and $G\alpha_s$ proteins to exert their pleiotropic effects such as cell survival, proliferation, migration, and morphological changes (Chun et al., 2010). When we examined PTX effects on gintonin-mediated transient Ca^{2+} mobilization, we found that PTX treatment showed differential effects on gintonin actions, depending on LPA receptor subtypes. Thus, preincubation of PTX almost blocked gintonin-mediated Ca^{2+} mobilization in B103 cells expressing LPA1 receptor and partially but significantly blocked gintonin actions in B103 cells expressing LPA3 receptor. In addition, gintonin-mediated cell migrations and ERK1/2 phosphorylation in HUVECs endogenously expressing LPA1 and LPA3 were blocked by PTX (Figs. 7C and 7D). However, PTX had no effects on gintonin actions on transient Ca^{2+} mobilization in B103 cells expressing LPA2 or LPA5 receptors. These results show that PTX-sensitive and -insensitive G proteins are involved in gintonin-mediated Ca^{2+} mobilization, -cell migration, or -ERK1/2 phosphorylation. In addition, gintonin treatment to PC12 cells expressing LPA1, LPA2, and LPA3 receptors also induced morphological changes, which are known to be achieved through the activation $G\alpha_{12/13}$ proteins and blocked by Rho kinase inhibitor Y-27632 (Yanagida et al., 2009). Altogether, these results indicate that gintonin is coupled to diverse G proteins such as $G\alpha_{i/o}$, $G\alpha_{q/11}$, or $G\alpha_{12/13}$ proteins through activation of LPA receptors.

In summary, the herbal medicine-derived ingredients acting on GPCRs have been targets for novel GPCR-related drug development for prevention or therapy of a variety of animal and human diseases (Im, 2003). We found that gintonin, a novel component of ginseng, contains LPAs in complexes with ginseng proteins. We also found that LPAs bound to gintonin are the main active ingredient for gintonin-mediated LPA receptor activations using *Xenopus* oocyte gene expression systems and mammalian cell gene expression system. Finally, gintonin isolated from ginseng might be novel bioactive LPA complexes and could be applied for novel drug development targeting LPA receptors.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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REFERENCES

Buchholz, B., Tauber, R., Steffl, D., Walz, G., and Köttgen, M. (2004) An inwardly rectifying whole cell current induced by Gq-coupled receptors. Biochem. Biophys. Res. Commun. *322*, 177-185.

Chen, Z., Liu, F., Ren, Q., Zhao, Q., Ren, H., Lu, S., Zhang, L., and Han, Z. (2008). Hemangiopoietin promotes endothelial cell proliferation through PI-3K/Akt pathway. Cell Physiol. Biochem. *22*, 307-314.

Choi, S., Kim, H.J., Ko, Y.S., Jeong, S.W., Kim, Y.I., Simonds, W.F., Oh, J.W., and Nah, S.Y. (2001a). $G\alpha_{q/11}$ coupled to mammalian phospholipase C beta3-like enzyme mediates the ginsenoside effect on Ca^{2+}-activated Cl^- current in the Xenopus oocyte. J. Biol. Chem. *276*, 48797-48802.

Choi, S., Rho, S.H., Jung, S.Y., Kim, S.C., Park, C.S., and Nah, S.Y. (2001b). A novel activation of Ca^{2+}-activated Cl^- channel in

Xenopus oocytes by Ginseng saponins: evidence for the involvement of phospholipase C and intracellular Ca^{2+} mobilization. Br. J. Pharmacol. *132*, 641-648.
Chun, J., Hla, T., Lynch, K.R., Spiegel, S., and Moolenaar, W.H. (2010). International union of basic and clinical pharmacology. LXXVIII. Lysophospholipid receptor nomenclature. Pharmacol. Rev. *62*, 579-587.
Dascal, N., Ifune, C., Hopkins, R., Snutch, T.P., Lübbert, H., Davidson, N., Simon, M.I., and Lester, H.A. (1986) Involvement of a GTP-binding protein in mediation of serotonin and acetylcholine responses in Xenopus oocytes injected with rat brain messenger RNA. Brain Res. *387*, 201-209.
Dobrydneva, Y., and Blackmore, P. (2001). 2-Aminoethoxydiphenyl borate directly inhibits store-operated calcium entry channels in human platelets. Mol. Pharmacol. *60,* 541-552.
Fujita, T., Inoue, K., Yamamoto, S., Ikumoto, T., Sasaki, S., Toyama, R., Chiba, K., Hoshino, Y., and Okumoto, T. (1994). Fungal metabolites. Part 11. A potent immunosuppressive activity found in Isaria sinclairii metabolite. J. Antibiot. (Tokyo) *47*, 208-215.
Gao, B.B., Stuart, L., and Feener, E.P. (2008). Label-free quantitative analysis of one-dimensional PAGE LC/MS/MS proteome: application on angiotensin II-stimulated smooth muscle cells secretome. Mol. Cell Proteomics *7*, 2399-2409.
Gardell, S.E., Dubin, A.E., and Chun, J. (2006). Emerging medicinal roles for lysophospholipid signaling. Trends Mol. Med. *12*, 65-75.
Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. *260*, 3440-3450.
Guo, Z., Liliom, K., Fischer, D.J., Bathurst, I.C., Tomei, L.D., Kiefer, M.C., and Tigyi, G. (1996). Molecular cloning of a high-affinity receptor for the growth factor-like lipid mediator lysophosphatidic acid from Xenopus oocytes. Proc. Natl. Acad. Sci. USA *93*, 14367-14372
Hecht, J.H., Weiner, J.A., Post, S.R., and Chun, J. (1996). Ventricular zone gene-1 (vzg-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. J. Cell Biol. *135*, 1071-1083.
Herrera, L., Carvallo, P., Antonelli, M., and Olate, J. (1994). Cloning of a Xenopus laevis muscarinic receptor encoded by an intronless gene. FEBS Lett. *352*, 175-179.
Ichimura, A., Hirasawa, A., Hara, T., and Tsujimoto, G. (2009). Free fatty acid receptors act as nutrient sensors to regulate energy homeostasis. Prostaglandins Other Lipid Mediat. *89*, 82-88.
Im, D.S. (2003). Linking Chinese medicine and G-protein-coupled receptors. Trends Pharmacol. Sci. *24*, 2-4.
Ishii, I., Contos, J.J., Fukushima, N., and Chun, J. (2000). Functional comparisons of the lysophosphatidic acid receptors, LPA1/VZG-1/EDG-2, LPA2/EDG-4, and LPA3/EDG-7 in neuronal cell lines using a retrovirus expression system. Mol. Pharmacol. *58*, 895-902.
Jalink, K., van Corven, E.J., Hengeveld, T., Morii, N., Narumiya, S., and Moolenaar, W.H. (1994). Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. J. Cell Biol. *126*, 801-810.
Jørgensen, N.K., Pedersen, S.F., and Hoffmann, E.K. (1999). Thrombin-, bradykinin-, and arachidonic acid-induced Ca^{2+} signaling in Ehrlich ascites tumor cells. Am. J. Physiol. *276*, C26-C37.
Kawasawa, Y., Kume, K., Nakade, S., Haga, H., Izumi, T., and Shimizu, T. (2000). Brain-specific expression of novel G-protein-coupled receptors, with homologies to Xenopus PSP24 and human GPR45. Biochem. Biophys. Res. Commun. *276*, 952-956.
Kim, S.I., Kweon, S.M., Kim, E.A., Kim, J.Y., Kim, S., Yoo, J.S., and Park, Y.M. (2004). Characterization of RNase-like major storage protein from the ginseng root by proteomic approach. J. Plant Physiol. *161*, 837-845.
Kim, Y.M., Namkoong, S., Yun, Y.G., Hong, H.D., Lee, Y.C., Ha, K.S., Lee, H., Kwon, H.J., Kwon, Y.G., and Kim, Y.M. (2007). Water extract of Korean red ginseng stimulates angiogenesis by activating the PI3K/Akt-dependent ERK1/2 and eNOS pathways in human umbilical vein endothelial cells. Biol. Pharm. Bull. *30*, 1674-1679.
Lee, H., Goetzl, E.J., and An, S. (2000). Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing. Am. J. Physiol. Cell Physiol. *278*, C612-C618.
Lee, J.H., Jeong, S.M., Lee, B.H., Noh, H.S., Kim, B.K., Kim, J.I., Rhim, H., Kim, H.C., Kim, K.M., and Nah, S.Y. (2004). Prevention of ginsenoside-induced desensitization of Ca^{2+}-activated Cl^- current by microinjection of inositol hexakisphosphate in *Xenopus laevis* oocytes: involvement of GRK2 and beta-arrestin I. J. Biol. Chem. *279*, 9912-9921.
Lee, C.W., Rivera, R., Gardell, S., Dubin, A.E., and Chun, J. (2006). GPR92 as a new $G_{12/13}$- and G_q-coupled lysophosphatidic acid receptor that increases cAMP, LPA5. J. Biol. Chem. *281*, 23589-23597.
Lee, C.W., Rivera, R., Dubin, A.E., and Chun, J. (2007). LPA(4)/GPR23 is a lysophosphatidic acid (LPA) receptor utilizing G_s-, G_q/G_i-mediated calcium signaling and $G_{12/13}$-mediated Rho activation. J. Biol. Chem. *282*, 4310-4317.
Liu, X.W., Sok, D.E., Yook, H.S., Sohn, C.B., Chung, Y.J., and Kim, M.R. (2007). Inhibition of lysophospholipase D activity by unsaturated lysophosphatidic acids or seed extracts containing 1-linoleoyl and 1-oleoyl lysophosphatidic acid. J. Agric. Food Chem. *55*, 8717-8722.
Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., and Bonner, T.I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature *346*, 561-564.
Nah, S.Y., Kim, D.H., and Rhim, H. (2007). Ginsenosides: are any of them candidates for drugs acting on the central nervous system? CNS Drug Rev. *13*, 381-404.
Moolenaar, W.H. (1994). LPA: a novel lipid mediator with diverse biological actions. Trends Cell Biol. *4*, 213-219.
Noguchi, K., Herr, D., Mutoh, T., and Chun, J. (2009). Lysophosphatidic acid (LPA) and its receptors. Curr. Opin. Pharmacol. *9*, 15-23.
Ohta, H., Sato, K., Murata, N., Damirin, A., Malchinkhuu, E., Kon, J., Kimura, T., Tobo, M., Yamazaki, Y., Watanabe, T., et al. (2003). Ki16425, a subtype-selective antagonist for EDG-family lysophosphatidic acid receptors. Mol. Pharmacol. *64*, 994-1005.
Oka, S., Ota, R., Shima, M., Yamashita, A., and Sugiura, T. (2010). GPR35 is a novel lysophosphatidic acid receptor. Biochem. Biophys. Res. Commun. *395*, 232-237.
Ono, M., Shitashige, M., Honda, K., Isobe, T., Kuwabara, H., Matsuzuki, H., Hirohashi, S., and Yamada, T. (2006). Label-free quantitative proteomics using large peptide data sets generated by nanoflow liquid chromatography and mass spectrometry. Mol. Cell Proteomics *5*, 1338-1347.
Park, S.Y., Schinkmann, K.A., and Avraham, S. (2006). RAFTK/Pyk2 mediates LPA-induced PC12 cell migration. Cell Signal. *18*, 1063-1071.
Pert, C.B., and Snyder, S.H. (1973). Opiate receptor: demonstration in nervous tissue. Science *179*, 1011-1014.
Pyo, M.K., Choi, S.H., Hwang, S.H., Shin, T.J., Lee, B.H., Lee, S.M., Lim, Y.H., Kim, D.H., and Nah, S.Y. (2011a). Novel glycoproteins from ginseng. J. Ginseng Res. *35*, 92-103.
Pyo, M.K., Choi, S.H., Shin, T.J., Hwang, S.H., Lee, B.H., Kang, J., Kim, H.J., Lee, S.H., and Nah, S.Y. (2011b). A simple method for the preparation of crude gintonin from ginseng root, stem, and leaf. J. Ginseng Res. *35*, 209-218.
Sato, K., Tomura, H., Igarashi, Y., Ui, M., and Okajima, F. (1997). Exogenous sphingosine 1-phosphate induces neurite retraction possibly through a cell surface receptor in PC12 cells. Biochem. Biophys. Res. Commun. *240*, 329-334.
Sato, K., Malchinkhuu, E., Muraki, T., Ishikawa, K., Hayashi, K., Tosaka, M., Mochiduki, A., Inoue, K., Tomura, H., Mogi, C., et al. (2005). Identification of autotaxin as a neurite retraction-inducing factor of PC12 cells in cerebrospinal fluid and its possible sources. J. Neurochem. *92*, 904-914.
Sengupta, S., Toh, S.A., Sellers, L.A., Skepper, J.N., Koolwijk, P., Leung, H.W., Yeung, H.W., Wong, R.N., Sasisekharan, R., and Fan, T.P. (2004). Modulating angiogenesis: the yin and the yang in ginseng. Circulation *110*, 1219-1225.
Sun, H., Kim, M.K., Pulla, R.K., Kim, Y.J., and Yang, D.C. (2010). Isolation and expression analysis of a novel major latex-like protein (MLP151) gene from *Panax ginseng*. Mol. Biol. Rep. *37*, 2215-2222.
Tabata, K., Baba, K., Shiraishi, A., Ito, M., and Fujita, N. (2007). The orphan GPCR GPR87 was deorphanized and shown to be a lysophosphatidic acid receptor. Biochem. Biophys. Res. Commun. *363*, 861-866.
Tanaka, T., Horiuchi, G., Matsuoka, M., Hirano, K., Tokumura, A., Koike, T., and Satouchi, K. (2009). Formation of lysophosphatidic acid, a wound-healing lipid, during digestion of cabbage

leaves. Biosci. Biotechnol. Biochem. *73*, 1293-1300.
Tigyi, G., and Miledi, R. (1992). Lysophosphatidates bound to serum albumin activate membrane currents in Xenopus oocytes and neurite retraction in PC12 pheochromocytoma cells. J. Biol. Chem. *267*, 21360-21367.
Tokumura, A., Fukuzawa, K., Akamatsu, Y., Yamada, S., Suzuki, T., and Tsukatani, H. (1978). Identification of vasopressor phospholipid in crude soybean lecithin. Lipids *13*, 468-472.
Valentine, W.J., Fells, J.I., Perygin, D.H., Mujahid, S., Yokoyama, K., Fujiwara, Y., Tsukahara, R., Van Brocklyn, J.R., Parrill, A.L., and Tigyi, G. (2008). Subtype-specific residues involved in ligand activation of the endothelial differentiation gene family lysophosphatidic acid receptors. J. Biol. Chem. *283*, 12175-12187.
Wellendorph, P., Johansen, L.D., and Bräuner-Osborne, H. (2009). Molecular pharmacology of promiscuous seven transmembrane receptors sensing organic nutrients. Mol. Pharmacol. *76*, 453-465.
Won, K.J., Park, S.H., Park, T., Lee, C.K., Lee, H.M., Choi, W.S., Kim, S.J., Park, P.J., Jang, H.K., Kim, S.H., et al. (2008). Cofilin phosphorylation mediates proliferation in response to platelet-derived growth factor-BB in rat aortic smooth muscle cells. J. Pharmacol. Sci. *108*, 372-379.
Yanagida, K., Masago, K., Nakanishi, H., Kihara, Y., Hamano, F., Tajima, Y., Taguchi, R., Shimizu, T., and Ishii, S. (2009) Identification and characterization of a novel lysophosphatidic acid receptor, p2y5/LPA6. J. Biol. Chem. *284*, 17731-17741.
Yoon, H.R., Kim, H., and Cho, S.H. (2003). Quantitative analysis of acyl-lysophosphatidic acid in plasma using negative ionization tandem mass spectrometry. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. *788*, 85-92.