

Identification and Characterization of a Novel Lysophosphatidic Acid Receptor, p2y5/LPA₆^{*S}

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p2y5 is an orphan G protein-coupled receptor that is closely related to the fourth lysophosphatidic acid (LPA) receptor, LPA₄. Here we report that p2y5 is a novel LPA receptor coupling to the G₁₃-Rho signaling pathway. “LPA receptor-null” RH7777 and B103 cells exogenously expressing p2y5 showed [³H]LPA binding, LPA-induced [³⁵S]guanosine 5′-3-O-(thio)triphosphate binding, Rho-dependent alternation of cellular morphology, and G_{s/13} chimeric protein-mediated cAMP accumulation. LPA-induced contraction of human umbilical vein endothelial cells was suppressed by small interfering RNA knockdown of endogenously expressed p2y5. We also found that 2-acyl-LPA had higher activity to p2y5 than 1-acyl-LPA. A recent study has suggested that p2y5 is an LPA receptor essential for human hair growth. We confirmed that p2y5 is a functional LPA receptor and propose to designate this receptor LPA₆.

Lysophosphatidic acid (LPA³; 1- or 2-acyl-*sn*-glycero-3-phosphate) is a naturally occurring lipid mediator with diverse biological activities (1, 2). LPA plays important roles in many biological processes, such as the nervous system (3), tumor metastasis (4), wound healing (5), cardiovascular functions (6), and reproduction (7), through its specific G protein-coupled receptors (GPCRs). At least five subtypes of LPA receptors have been identified. Three receptors (LPA₁ (8), LPA₂ (9), and LPA₃ (10, 11)) share about 50% amino acid sequence identities, and form the Edg (endothelial differentiation gene) family together

with the GPCRs for sphingosine 1-phosphate. Two additional LPA receptors, p2y9/LPA₄ (12) and GPR92/LPA₅ (13, 14), which show small similarities with the Edg family GPCRs, were recently identified. These LPA receptors, by coupling with different sets of G proteins, transduce various responses in many cell types. Depending on the functional coupling of a given LPA receptor to the G proteins, LPA activates diverse signaling cascades involving phosphoinositide 3-kinase, phospholipase C, mitogen-activated protein kinase, Rho-family GTPase, and adenyl cyclase (2).

The fact that p2y5 shares the highest sequence homology with p2y9/LPA₄ among all GPCRs (12) strongly suggested that LPA is a ligand for p2y5. However, we could not detect LPA-induced Ca²⁺ mobilization or cAMP level changes in p2y5-overexpressing cells at the time of the identification of p2y9/LPA₄ as the fourth LPA receptor in our laboratory (12). In the course of the further analysis of p2y5-overexpressing cells, we found that p2y5 actually responded to LPA with activation of the G₁₃-Rho signaling pathway. Our results confirm the identification of p2y5 as an LPA receptor and extend the knowledge of the functional roles of LPA.

EXPERIMENTAL PROCEDURES

Lipids—1-Oleoyl-LPA, 1-palmitoyl-LPA, 1-stearoyl-LPA, 1-myristoyl-LPA, and 1-arachidonoyl-LPA were purchased from Avanti Polar Lipids (Alabaster, AL). 1-Linoleoyl-LPA was from Echelon Biosciences (Salt Lake City, UT). These lipids were stored at −30 °C (10 mM stock in 50% ethanol). Alkyl-OMPT (10 mM stock in dimethyl sulfoxide stored at −30 °C) was from Echelon Biosciences. 2-Acyl-LPA was prepared from phosphatidic acid (PA) using a 1,3-specific lipase derived from fungi (15). Briefly, dioleoyl-PA, dilinoleoyl-PA, and diarachidonoyl-PA (Avanti Polar Lipids) were incubated with *Rhizomucor miehei* lipase (Sigma) in the reaction buffer (100 mM sodium acetate (pH 7.8), 150 mM NaCl, and 2 mM CaCl₂) at 37 °C for 1 h in the presence of an equal volume of diethyl ether. The aqueous phase was then applied to an Oasis HLB cartridge (60 mg/3 ml; Waters, Milford, MA). 2-Acyl-LPA was eluted with methanol after the washing with water. Since 2-acyl-LPA is unstable (16), we used the fraction within 2 h after the elution. The amount of LPA in the eluted fraction was determined by phosphoric analysis after the separation of LPA by thin layer chromatography.

Cell Culture—RH7777 rat hepatoma cells and B103 rat neuroblastoma cells were kindly provided by Dr. J. Chun (Scripps

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³ The abbreviations used are: LPA, lysophosphatidic acid; GPCR, G protein-coupled receptor; alkyl-OMPT, 1-O-oleyl-2-O-methyl-*sn*-glycero-3-phosphothionate; PA, phosphatidic acid; HUVEC, human umbilical vein endothelial cell; HA, hemagglutinin; BSA, bovine serum albumin; PTX, pertussis toxin; mPA-PLA₁α, membrane-bound PA-selective phospholipase A₁ α; GTPγS, guanosine 5′-3-O-(thio)triphosphate; CRE, cAMP-response element.

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Research Institute, La Jolla, CA). These cells were maintained on collagen-coated 100-mm dishes (Iwaki, Tokyo, Japan) in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen). B103 cells and RH7777 cells stably expressing p2y5, LPA₁, or LPA₃ (see below) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 0.3 mg/ml G418 (Invitrogen). Human umbilical vein endothelial cells (HUVECs) were purchased from Cell Applications (San Diego, CA) and cultured in collagen-coated 100-mm dishes with endothelial cell growth medium (Cell Applications). The cells were used for assays from passages 4–7. In some experiments, cells were pretreated with 5 μ M Y27632 (Calbiochem; from a 5 mM stock in water stored at –30 °C) for 10 min.

Cloning of Human p2y5—A DNA fragment containing the entire open reading frame of human p2y5 (GenBank™ accession number NM_005767) was first amplified from human genomic DNA by PCR using *Pfu* turbo (Stratagene, La Jolla, CA) and oligonucleotides (sense primer, 5'-AAAGTGCTTCAAAGTAAATG-3'; antisense primer, 5'-CCCAGTAGTCTAATGGTTTTAT-3'). The entire open reading frame of p2y5 with an additional sequence of hemagglutinin (HA) epitope at the 5'-end was subsequently amplified from the resultant PCR products using KOD-Plus (Toyobo, Osaka, Japan) and oligonucleotides (sense primer containing KpnI and HA tag sequences, 5'-GGGGTACCGCCATGTACCCCTACGACGTGCCCGACTACGCCGTAAGCGTTAACAGCTCC-3'; antisense primer containing XbaI sequence, 5'-GCTCTAGATCAGGCAGCAGATTCATT-3'). The resultant DNA fragment was digested with KpnI and XbaI and subsequently cloned into the mammalian expression vector pCXN2.1, a slightly modified version of pCXN2 (17) with multiple cloning sites, between the KpnI and NheI sites.

Cloning of Human LPA Receptors—HA-tagged human LPA₁ and LPA₄ DNA were constructed and cloned into pCXN2.1, as described previously (18). The entire open reading frame of human LPA₂ (GenBank™ accession number NM_004720) with an additional sequence of HA epitope at the 5'-end was amplified from LPA₂ cDNA in pCDNA3.1 (kindly provided by Dr. Izumi, Gunma University, Japan) using KOD-Plus and oligonucleotides (sense primer containing KpnI and HA tag sequences, 5'-GGGGTACCGCCATGTACCCCTACGACGTGCCCGACTACGCCGTCATCATGGGCCAGTGC-3'; antisense primer containing XbaI sequence, 5'-GCTCTAGACTAAAGGGTGGAGTCCATCAG-3'). A DNA fragment containing the entire open reading frame of human LPA₃ (GenBank™ accession number NM_012152) was first amplified from a cDNA prepared from human kidney poly(A)⁺ RNA (Clontech, Palo Alto, CA) by PCR using *Pfu* turbo and oligonucleotides (sense primer, 5'-TCTAGGATGTTCACTTCTTCTCCAC-3'; antisense primer, 5'-GAGAGGCATCCAGAGTTAGGAAGT-3'). The entire open reading frame of LPA₃ with an additional sequence of HA epitope at the 5'-end was subsequently amplified from the resultant PCR products using KOD-Plus and oligonucleotides (sense primer containing KpnI and HA tag sequences, 5'-GGGGTACCGCCATGTACCCCTACGACGTGCCCGACTACGCCAATGAGTGTCTACTATGAC-3'; antisense primer containing XbaI sequence, 5'-

GCTCTAGATTAGGAAGTGCTTTTATTGCA-3'). A DNA fragment containing the entire open reading frame of human LPA₅ (GenBank™ accession number NM_020400) was first amplified from human genomic DNA by PCR using *Pfu* turbo and oligonucleotides (sense primer, 5'-ACTTGAGGCTGAGAAAAGATTCAG-3'; antisense primer, 5'-ACTTTGTACTCTTCTGCGTTGCTAA-3'). The entire open reading frame of LPA₅ with an additional sequence of HA epitope at the 5'-end was subsequently amplified from the resultant PCR products using KOD-Plus and oligonucleotides (sense primer containing KpnI and HA tag sequences, 5'-GGGGTACCGCCATGTACCCCTACGACGTGCCCGACTACGCCCTTAGCCAACAGCTCCTCA-3'; antisense primer containing XbaI sequence, 5'-GCTCTAGATCAGAGGGCGGAATCCTG-3'). All resultant DNA fragments were digested with KpnI and XbaI and subsequently cloned into pCXN2.1.

Transient Expression and Membrane Preparation—RH7777 cells (2 × 10⁶) were seeded in collagen-coated 100-mm dishes and transfected with each receptor expression plasmid using the Lipofectamine 2000 reagent (Invitrogen). After 24-h serum starvation with Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin (BSA; fatty acid-free; Sigma), the cells were washed with phosphate-buffered saline twice and scraped off. After further washing with binding buffer (25 mM HEPES-NaOH (pH 7.4), 10 mM MgCl₂, and 0.25 M sucrose), the cells were suspended in the buffer containing an additional protease inhibitor mixture (Complete; Roche Applied Science), sonicated three times at 15 watts for 30 s, and centrifuged at 8,000 × g for 10 min at 4 °C. The supernatant was further centrifuged at 100,000 × g for 60 min at 4 °C, and the resultant pellet was homogenized in ice-cold binding buffer. The protein concentration of the homogenate was determined with a Bradford assay (Bio-Rad) using BSA as a standard.

Western Blotting—The membrane fraction (4 μ g of protein) from RH7777 cells was digested with 2 units of *N*-glycosidase F (Roche Applied Science) to remove asparagine-bound *N*-glycans according to the manufacturer's instructions. The protein sample (with 5% 2-mercaptoethanol) was analyzed by 10% SDS-PAGE without heat denaturation, followed by transfer to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membrane was blocked with 5% skim milk (Difco) and probed with a 3F10 rat monoclonal anti-HA antibody (Roche Applied Science). The bands were visualized with an ECL chemiluminescence detection system (GE Healthcare) using horseradish peroxidase-conjugated anti-rat IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Radioligand Binding Assay—A total of 20 μ g each of the membrane fractions was incubated in 200 μ l of binding buffer containing 0.25% BSA with the indicated concentrations of [³H]LPA (1-oleoyl[oleoyl-9,10-³H]LPA, 57 Ci/mmol; PerkinElmer Life Sciences) for 70 min at 4 °C. Membrane-bound [³H]LPA was collected onto a Unifilter-96-GF/C (PerkinElmer Life Sciences) using a MicroMate 196 harvester (PerkinElmer Life Sciences). The filter was then rinsed 10 times with ice-cold binding buffer and dried for 12 h at 50 °C. A volume of 25 μ l of MicroScint-0 scintillation mixture (PerkinElmer Life Sciences) was added per well. The radioactivity that remained on the filter was measured using a Top-

Count microplate scintillation counter (PerkinElmer Life Sciences). Total and nonspecific bindings were evaluated in the absence and presence of 10 μM unlabeled 1-oleoyl-LPA, respectively.

Stable Expression—B103 cells and RH7777 cells stably expressing p2y5 (referred to hereafter as B103-p2y5 cells and RH7777-p2y5 cells, respectively) were established as follows. B103 cells and RH7777 cells were transfected with the p2y5-expression plasmid (see above). Expression of the HA epitope on the cell surface was confirmed by flow cytometric analysis (EPICS XL, Beckman Coulter, Fullerton, CA) using a 3F10 anti-HA antibody and phycoerythrin-labeled anti-rat IgG (Beckman Coulter) as the secondary antibody. Stable transfectants were selected with 1 mg/ml G418 for 20 days. After labeling the drug-resistant cells with phycoerythrin, as described above, a group of HA-positive cells was sorted using an automated magnetic cell sorter (autoMACS; Miltenyi Biotec, Auburn, CA) with the use of anti-phycoerythrin MicroBeads (Miltenyi Biotec). B103 cells and RH7777 cells stably expressing LPA₁ or LPA₃ were established essentially as described previously (18).

[³⁵S]GTP γ S Binding Assay—The membrane fraction (5 μg of protein) from RH7777 cells was incubated in 100 μl of GTP γ S binding buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.3 μM GDP, and 0.1% BSA) containing 0.5 nM [³⁵S]GTP γ S (46.2 MBq/mmol; PerkinElmer Life Sciences) with or without various concentrations of ligands for 30 min at 30 °C. Membrane-bound [³⁵S]GTP γ S was separated from free by rapid filtration through a Unifilter-96-GF/C, which was rinsed 10 times with ice-cold TMN buffer (10 mM Tris-HCl (pH 7.5), 25 mM MgCl₂, and 100 mM NaCl). Radioactivity was measured as described above.

[³⁵S]GTP γ S Binding to the G α_{13} Protein—The membrane fraction (40 μg of protein) from RH7777 cells transiently expressing p2y5 was incubated in 100 μl of GTP γ S binding buffer containing 0.5 nM [³⁵S]GTP γ S with or without 10 μM 1-oleoyl-LPA for 30 min at 30 °C. Then membrane protein was resuspended in 900 μl of solubilization buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1.25% Nonidet P-40, 0.2% SDS, 100 μM GDP, 100 μM GTP, and Complete). 50 μl of protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) were added to the extracts and mixed by rotation for 3 h at 4 °C to preclear nonspecific binding. Agarose beads were then pelleted, and the supernatant was incubated with 1:100 anti-G α_{13} antibody (A-20; Santa Cruz Biotechnology) for 60 min at 4 °C, and then 50 μl of protein A/G PLUS-agarose was added. Following rotation for 12–16 h at 4 °C, the immune complexes were collected by centrifugation and washed three times with wash buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 100 μM GDP, and 100 μM GTP). [³⁵S]GTP γ S binding in the immunoprecipitates was quantified by liquid scintillation counting. Nonspecific binding was determined by the immunoprecipitation without antibody.

Construction of G_{s/13} Chimeric Protein Expression Plasmid—Human G_s-encoding cDNA in pcDNA3.1 (G α_s long subunit; cDNA Resource Center, Rolla, MO) was used as a template to replace its C-terminal five amino acids (QYELL) with those of G₁₃ (QLMLQ) (19) using PCR-based mutagenesis with two oli-

gonucleotides (sense primer, 5'-ATTAATACGACTCACTA-TAGG-3'; antisense primer, 5'-GGTCTAGATTACTGT-AGCATAAGCTGACGAAGGTGCATGCGCTGAA-3'). The resultant DNA fragment was digested with KpnI and XbaI and subsequently cloned into the pcDNA3.1 vector. The NheI-XbaI DNA fragment (containing the G_{s/13} sequence) in the resultant plasmid replaced the *Renilla* luciferase sequence of the pRL-CMV vector (Promega, Madison, WI). Intact pRL-CMV vector served as the control for the G_{s/13} expression vector.

cAMP Measurement—To determine whether LPA receptors affect the activity of adenylyl cyclases, an AlphaScreen cAMP assay kit (PerkinElmer Life Sciences) was used as recommended by the manufacturer. Cells (4 \times 10⁴) were seeded in collagen-coated 96-well plates (Iwaki), followed by a 12-h serum starvation in the presence or absence of 100 ng/ml pertussis toxin (PTX; List Biological Laboratories, Campbell, CA; from a 400 $\mu\text{g}/\text{ml}$ stock in 10 mM Tris-HCl (pH 7.4) and 2 M urea stored at 4 °C). The cells were washed with buffer A (Hanks' balanced salt solution containing 25 mM HEPES-NaOH (pH 7.4) and 0.1% BSA) and incubated in 100 μl of buffer A containing 0.5 mM 3-isobutyl-1-methylxanthine (Sigma; from a 50 mM stock in dimethyl sulfoxide stored at -30 °C) for 20 min at room temperature. The reaction was initiated by adding 50 μl of various concentrations of LPA in buffer A with 10 μM forskolin (Sigma; from a 10 mM stock in dimethyl sulfoxide stored at -30 °C). After 30 min of incubation at room temperature, the reaction was terminated by adding 15 μl of 10% Tween 20, followed by overnight storage at 4 °C. The cAMP concentration in the cell lysate was measured in triplicate with the Fusion system (PerkinElmer Life Sciences). In some experiments, the cells were pretreated with 10 μM Ki16425 (Sigma; from a 10 mM stock in dimethyl sulfoxide stored at -30 °C) for 20 min at room temperature.

mRNA Expression Profile of LPA Receptors in HUVECs—Total RNA was extracted from HUVECs using an RNeasy minikit (Qiagen, Tokyo, Japan) and reverse-transcribed using Superscript II reverse transcriptase (Invitrogen). An equal aliquot of the cDNA solution was used to amplify the different receptor transcripts using *ExTaq* DNA polymerase (Takara, Tokyo, Japan). The PCR program was as follows: denaturation at 94 °C for 2.5 min and 40 cycles of amplification consisting of denaturation at 96 °C for 10 s, annealing at 57 °C for 10 s, and extension at 72 °C for 20 s. The sequences of primers used for LPA₁, LPA₂, LPA₃, LPA₄, and LPA₅ are the same as those used by Kotarsky *et al.* (13). The primers for p2y5 were designed to amplify a 358-bp fragment (sense primer, 5'-AGAATTGTGA-GAAAGCGACCTC-3'; antisense primer, 5'-TCTGTGACC-AGAATGAAACCAC-3'). The PCR products were separated by electrophoresis on a 2% agarose gel.

LPA-induced Morphological Changes of HUVECs—To evaluate LPA-induced cell morphological changes of HUVECs, we utilized the xCELLigence system (Roche Applied Science), which measures cell-substrate impedance (20). Briefly, HUVECs were seeded at 8,000 cells/well in an E-plate (Roche Applied Science) coated with poly-D-lysine, followed by a 12-h culture. The cells were serum-starved with 100 μl of endothelial cell basal medium (Cell Applications) containing 0.1% BSA for 4 h and stimulated with 5 μM of 1-oleoyl-LPA (by adding 5 μl of

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100 μ M stock in phosphate-buffered saline containing 3% BSA). Phosphate-buffered saline containing 3% BSA served as the control for LPA stimulation. The cell index was continuously monitored every 15 s for the duration of the experiments. For the experiments with siRNAs, the cells (60,000 cells/well) cultured in collagen-coated 12-well plates (Iwaki) were transfected with 5 nM human p2y5 siRNAs (Silencer Select siRNA, ID numbers s19796 and s19798; Ambion, Austin, TX) and control siRNA (Silencer Select negative control 1; Ambion) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. After a 24-h incubation, the cells were reseeded at 8,000 cells/well in a poly-D-lysine-coated E-plate and used for the assay as described above.

Quantitative Real Time PCR—In the siRNA experiments, the mRNA levels of LPA₁, p2y5, and β -actin were quantified using the LightCycler system (Roche Applied Science). The PCRs were set up in microcapillary tubes in a volume of 20 μ l, consisting of 2 μ l of cDNA solution, 1 \times FastStart DNA Master SYBR Green I (Roche Applied Science), and 0.5 μ M each sense and antisense primers. The PCR program was as follows: denaturation at 95 $^{\circ}$ C for 3 min and 45 cycles of amplification consisting of denaturation at 95 $^{\circ}$ C for 15 s, annealing at 65 $^{\circ}$ C for 5 s, and extension at 72 $^{\circ}$ C for 7 s. The primers for LPA₁ (sense primer, 5'-GGCTATGTTCCGCCAGAGGACTAT-3'; antisense primer, 5'-TCCAGGAGTCCAGCAGATGATAA-3') were designed to amplify a 135-bp fragment. The primers for p2y5 (sense primer, 5'-GGTAAGCGTAAACAGCTCCCACT-3'; antisense primer, 5'-TTTGAGGACGCAGATGAAAATGT-3') were designed to amplify a 139-bp fragment. The primers for β -actin (sense primer, 5'-CAGGATGCAGAAGGAGATC-3'; antisense primer, 5'-TACTCCTGCTTGCTGATC-CACAT-3') were designed to amplify a 153-bp fragment. The cDNA level of each sample was quantified using the second derivative maximum method in LightCycler analysis software.

Statistical Analysis—To determine statistical significance, the values were compared by unpaired two-tailed *t* test, analysis of variance followed by Tukey-Kramer test, or two-way analysis of variance using Prism 4 software (GraphPad Software, San Diego, CA). The differences were considered significant if *p* values were less than 0.05.

RESULTS

Cell Aggregation of B103-p2y5 Cells in Serum-containing Medium—An orphan receptor p2y5 shares the highest amino acid sequence homology with LPA₄ among all GPCRs (12), which led us to hypothesize that p2y5 is an additional LPA receptor. At first, we tested this hypothesis by detecting Ca²⁺ mobilization or the changes of intracellular cAMP level using RH7777 cells and B103 cells stably overexpressing p2y5 (RH7777-p2y5 cells and B103-p2y5 cells, respectively). These cell lines were selected because they lack endogenous responses to LPA in these assays (18, 21). Although flow cytometry analysis proved the high expression levels of p2y5 in these cells (Fig. 1A), we could not observe any p2y5-dependent signaling by 1-oleoyl-LPA (data not shown). Reporter gene assays detected no p2y5-dependent activation of the *zif268* or the CRE promoter (data not shown).

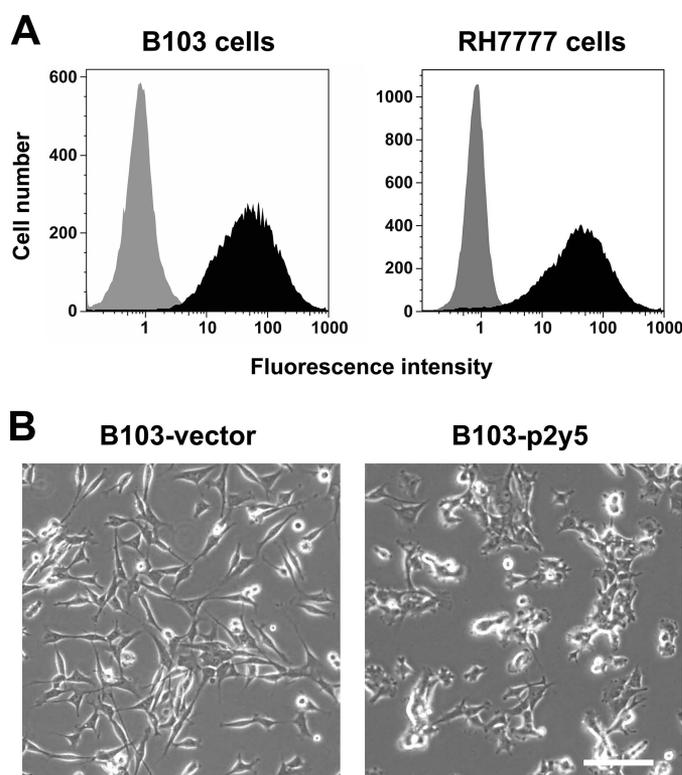


FIGURE 1. Stable expression of p2y5 in B103 and RH7777 cells. A, flow cytometry analysis. B103 and RH7777 cells were stably transfected with the expression vector for p2y5 tagged with the HA epitope at the N terminus. After staining with an anti-HA antibody and a phycoerythrin-conjugated secondary antibody, HA-positive cells were sorted by the automated magnetic cell sorter and then subcultured. Data shown are the surface expression levels of HA epitope in subcultured polyclonal cells (closed area). Empty vector-transfected polyclonal cells served as a negative control (gray area). B, morphologies of B103-vector and B103-p2y5 cells in serum-containing medium. The cells were photographed 24 h after seeding. Bar, 50 μ m.

In the course of the study of p2y5, we noticed that B103-p2y5 cells formed aggregates in serum-containing medium (Fig. 1B). We previously showed that B103 cells expressing LPA₄ formed aggregates through Rho in serum-containing medium, which is abundant in LPA (18). Thus, the similar cell morphology observed in B103-p2y5 cells implied the activation of the LPA-p2y5-Rho signaling axis in these cells.

Neurite Retraction in B103-p2y5 Cells and Membrane Blebbing in RH7777-p2y5 Cells by LPA—Since Rho activation in B103 cells results in neurite retraction (14, 18, 21, 22), we examined whether LPA can induce neurite retraction in B103-p2y5 cells. Indeed, 1-oleoyl-LPA at a concentration of 1 μ M induced rapid neurite retraction in B103-p2y5 cells but not in B103-vector cells (Fig. 2A, left and middle). Other related lysophospholipids with glycerol, choline, serine, inositol, or ethanolamine as headgroup (at 10 μ M) did not induce neurite retraction at all (data not shown). This morphological change in B103-p2y5 cells was Rho-associated kinase-dependent, because the LPA effect was inhibited by the pretreatment with the Rho-associated kinase inhibitor Y27632 (Fig. 2A, right). To confirm the Rho-activating property of p2y5 in another cell line, RH7777-p2y5 cells were stimulated with 1 μ M LPA. LPA induced membrane blebbing in RH7777-p2y5 cells much more potently than in RH7777-vector cells (Fig. 2B, left and middle). It has been reported that membrane blebbing is regulated by

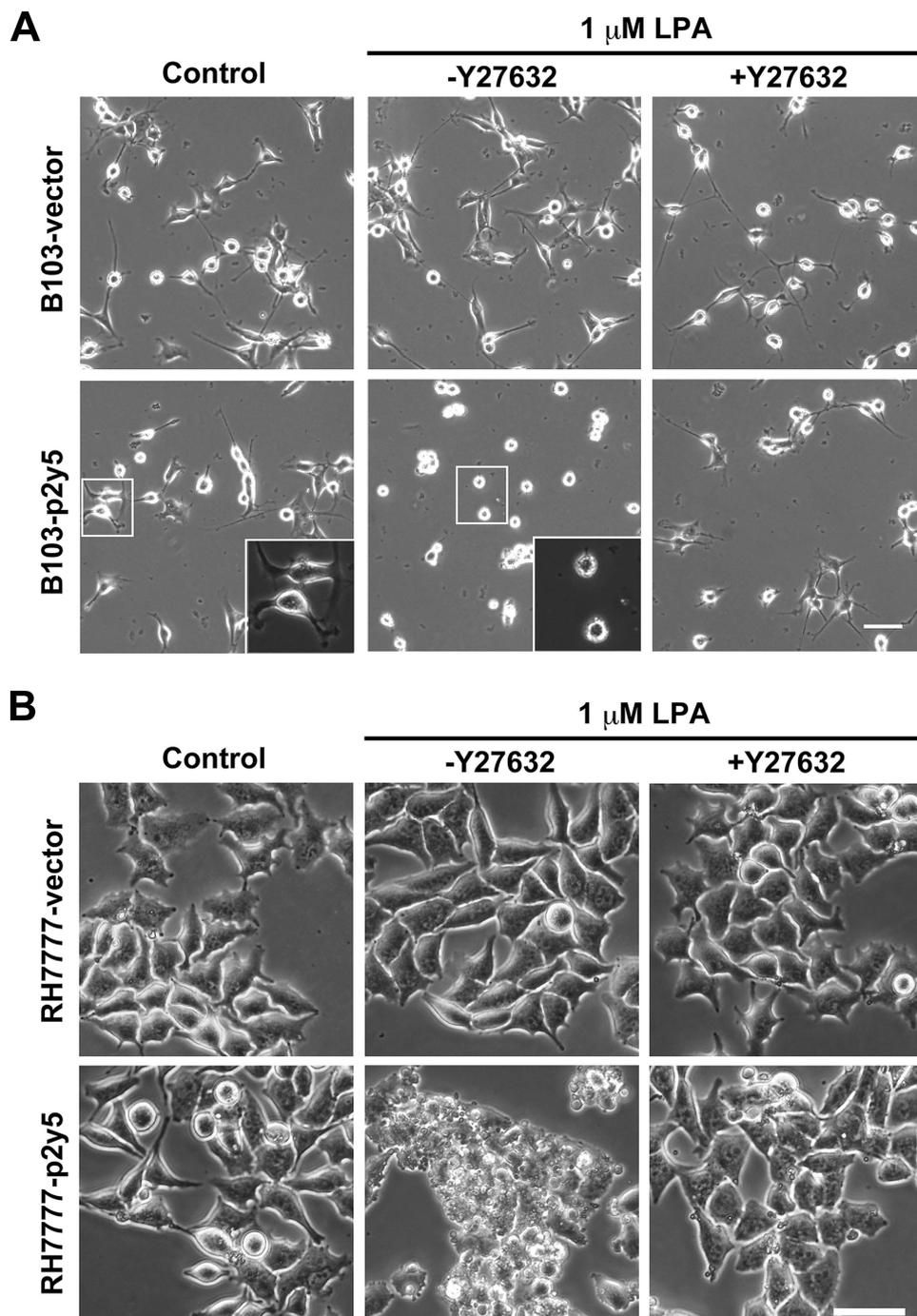


FIGURE 2. LPA-induced neurite retraction in B103-p2y5 cells and membrane blebbing in RH7777-p2y5 cells. *A*, induction of neurite retraction in B103-p2y5 cells. The cells cultured in collagen-coated 24-well plates were serum-starved for 12 h, stimulated with 1 μM 1-oleoyl-LPA for 15 min, and fixed with 2% paraformaldehyde. Where indicated, the cells were pretreated with 5 μM Y27632 for 10 min (*middle panels*). Bar, 50 μm . The boxed areas are magnified in the inset in the same panels. *B*, induction of membrane blebbing in RH7777-p2y5 cells. Cell culture, stimulation, and fixation were performed in the same manner as in *A*. Bar, 50 μm .

Rho (23–25). Consistently, the membrane blebbing in RH7777-p2y5 cells was severely attenuated by Y27632 (Fig. 2*B*, right).

[³H]LPA Binding to RH7777-p2y5 Cell Membrane—Next, we performed the LPA binding assay using membrane fractions from RH7777 cells transiently expressing p2y5 or each of the known five LPA receptors (LPA₁–LPA₅). Protein expression of these receptors was confirmed by Western blotting analysis (Fig. 3*A*, top). Under our experimental conditions, we could

not detect any specific binding of [³H]1-oleoyl-LPA to transiently expressed p2y5 or LPA₃ (Fig. 3*A*, bottom). Other four LPA receptors displayed specific binding to [³H]1-oleoyl-LPA (Fig. 3, *A* (bottom) and *B*). Functional LPA₃ receptors were actually expressed at the cell surface, because treatment with LPA induced Ca²⁺ mobilization in the cells expressing LPA₃ (data not shown). The failure to detect the specific binding to p2y5 and LPA₃ might be due to the low affinities or the rapid off rates of LPA (see “Discussion”).

In contrast to the transient expression system, small but significant specific binding was successfully detected when we used membranes from RH7777 cells stably expressing p2y5 (Fig. 3*C*). The specific binding did not reach saturation even at 200 nM [³H]LPA (data not shown), suggesting that the affinity of 1-oleoyl-LPA to the receptor is too low for Scatchard analysis under our experimental conditions. The pretreatment with 100 μM GTP γ S severely decreased the [³H]LPA binding of p2y5-expressing membranes (Fig. 3*D*), suggesting that the specific binding to [³H]LPA involves GPCRs.

LPA-induced GTP γ S Binding to Cell Membrane via p2y5—To demonstrate that LPA signals directly through p2y5, we performed a [³⁵S]GTP γ S binding assay using membrane fractions from RH7777 cells expressing p2y5. After the optimization of GDP concentration (0.3 μM), we found that 1-oleoyl-LPA increased [³⁵S]GTP γ S binding in a dose-dependent manner both in transient and stable p2y5-expressing cells (Fig. 4*A*).

As described earlier, p2y5 induced striking morphological changes (*i.e.* neurite retraction and membrane

blebbing) in a Rho-associated kinase-dependent manner (Fig. 2, *A* and *B*). This strongly suggested that p2y5 coupled to G_{12/13} protein. To verify the G₁₃ coupling of p2y5, we examined [³⁵S]GTP γ S incorporation into G α ₁₃ protein by immunoprecipitation with anti-G α ₁₃ protein antibody. With membranes from RH7777 cells transiently expressing p2y5, 10 μM 1-oleoyl-LPA induced an ~4-fold increase in [³⁵S]GTP γ S binding to G₁₃ proteins, whereas LPA had no effect on vector transfectants (Fig. 4*B*).

A Novel LPA Receptor, p2y5/LPA₆

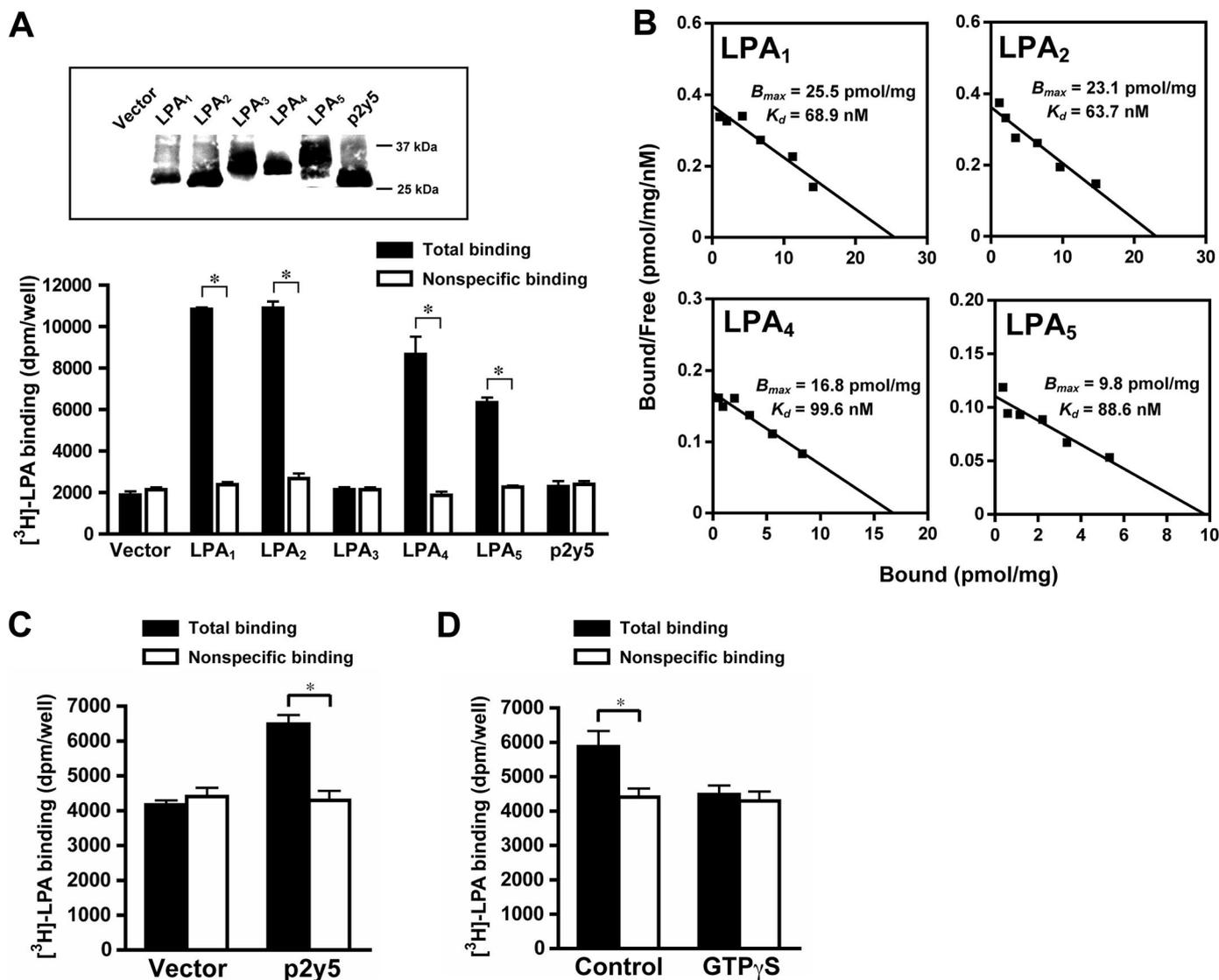


FIGURE 3. [³H]LPA binding to RH7777-p2y5 cell membrane. A, Western blotting of HA-tagged LPA receptors (top) and [³H]-1-oleoyl-LPA binding to transiently expressed LPA receptors (bottom). Membrane fractions of RH7777 cells transiently expressing each receptor were used to confirm the receptor expression by anti-HA antibody (top). Membrane fractions were incubated with 10 nM [³H]LPA in the presence (for detecting nonspecific binding) or absence (for total binding) of 10 μM unlabeled 1-oleoyl-LPA for 70 min at 4 °C (bottom). Data are means ± S.E. (n = 3) of a representative of three independent experiments with similar results. *, p < 0.01 (using unpaired two-tailed t test). B, Scatchard analysis data of the specific binding of [³H]LPA to LPA₁, LPA₂, LPA₄, and LPA₅. Data shown are representative of at least two independent experiments with similar results. C, [³H]-1-oleoyl-LPA binding to the membrane fractions from RH7777 cells stably expressing p2y5. Membrane fractions of RH7777-p2y5 cells were incubated with 30 nM [³H]LPA in the presence (for detecting nonspecific binding) or absence (for total binding) of 10 μM unlabeled 1-oleoyl-LPA for 70 min at 4 °C. Data are means ± S.E. (n = 3) of a representative of three independent experiments with similar results. *, p < 0.05 (using unpaired two-tailed t test). D, sensitivity to GTPγS. Membrane fractions of RH7777-p2y5 cells were incubated with or without 100 μM GTPγS for 30 min at room temperature, and a [³H]LPA binding assay was performed in the same manner as in A. Data are means ± S.E. (n = 3) of a representative of three independent experiments with similar results. *, p < 0.05 (using unpaired two-tailed t test).

Adenylyl Cyclase Activation by p2y5 through a G_{s/13} Chimeric Protein—For further confirmation of G_{12/13} proteins coupling of p2y5, we applied G_{s/13}, a chimeric α subunit of G_s and G₁₃ proteins that enables G₁₃-coupling GPCRs to activate adenylyl cyclases (19). 1-Oleoyl-LPA again failed to induce any significant change in the intercellular cAMP level in B103-p2y5 cells with or without transfection of a G_{s/13} expression vector. However, when the cells transfected with G_{s/13} were treated with PTX, LPA significantly increased the cAMP level in a dose-dependent manner (Fig. 5). In B103-vector cells, there was no significant change in cAMP levels even after the transfection of G_{s/13} and treatment with PTX (Fig. 5).

Ligand Selectivity of p2y5—We next examined the ligand selectivity of p2y5 by detecting increases in intercellular cAMP levels in the G_{s/13}-transfected B103-p2y5 cells after PTX treatment. LPA with saturated fatty acid (such as stearic acid, palmitic acid, and myristic acid) at the sn-1-position and 1-arachidonoyl-LPA was less potent than 1-oleoyl-LPA (Fig. 6A). 1-Linoleoyl-LPA showed higher activity than 1-oleoyl-LPA (Fig. 6A, right). We next examined the potencies of nine synthetic LPA analogs as p2y5 agonists and found that the LPA₃-selective agonist alkyl-OMPT (26, 27) had agonistic activity almost equal to or slightly higher than 1-oleoyl-LPA (Fig. 6B). Pretreatment with Ki16425, an antagonist for the Edg family

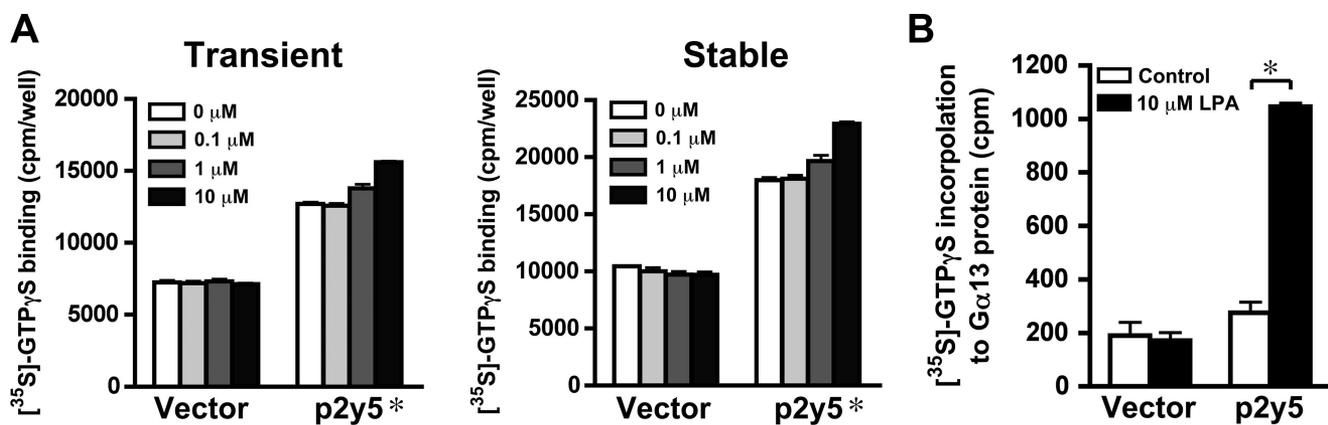


FIGURE 4. LPA-induced [³⁵S]GTP γ S binding via p2y5. *A*, membrane fractions from RH7777 cells transiently (*left*) or stably (*right*) expressing p2y5 were incubated with 0.5 nM [³⁵S]GTP γ S and the indicated concentrations of LPA for 30 min at 30 °C in the presence of 0.3 μ M GDP. Data are means \pm S.E. ($n = 3$) of a representative of three independent experiments with similar results; *, $p < 0.001$ for vector versus p2y5 using two-way analysis of variance. *B*, LPA-induced [³⁵S]GTP γ S incorporation to G α_{13} protein. The membrane fraction from RH7777 cells transiently expressing p2y5 was incubated with 0.5 nM [³⁵S]GTP γ S and 10 μ M 1-oleoyl-LPA for 30 min at 30 °C in the presence of 0.3 μ M GDP. Then membrane protein was solubilized, and G α_{13} protein was immunoprecipitated to detect [³⁵S]GTP γ S incorporated. Data are means \pm S.E. ($n = 3$) of a representative of two independent experiments with similar results. *, $p < 0.001$ (using unpaired two-tailed *t* test).

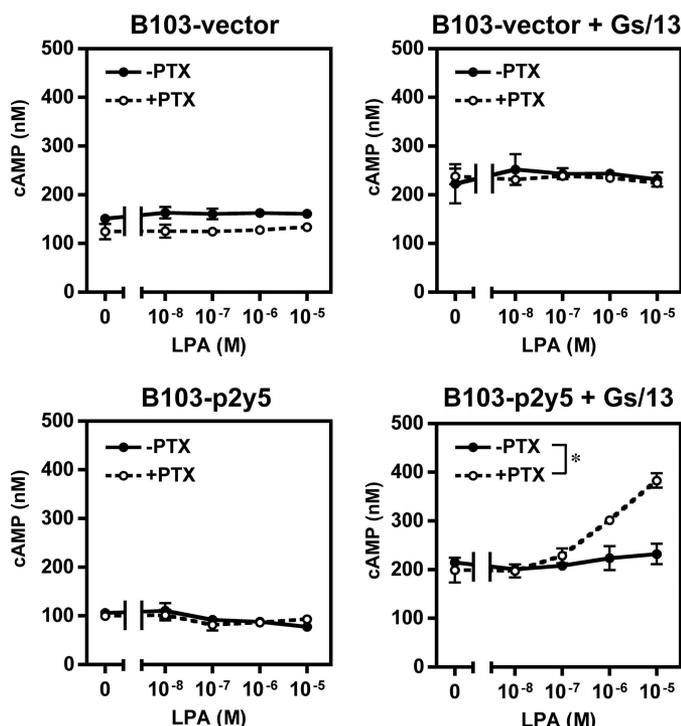


FIGURE 5. Adenylyl cyclase activation by p2y5 through a G_{s/13} chimeric protein. B103-vector and B103-p2y5 cells were transfected with G_{s/13} expression plasmid (*right*) or control plasmid (*left*). Serum-starved cells were pre-treated with or without 100 ng/ml PTX for 12 h and stimulated with the indicated concentrations of 1-oleoyl-LPA in the presence of 0.5 mM 3-isobutyl-1-methylxanthine and 10 μ M forskolin. After a 30-min incubation at room temperature, the cells were solubilized. cAMP concentrations in the cell lysates are shown. Data are means \pm S.E. ($n = 3$) of a representative of three independent experiments with similar results. *, $p < 0.001$ (using two-way analysis of variance).

LPA receptors, LPA₁, LPA₂, and LPA₃ (28), did not alter the response of B103-p2y5 cells to LPA (Fig. S1), indicating that K116425 is not an antagonist for p2y5.

Recently, p2y5 was identified as a causative gene for human hair growth deficiency (29–33). Of note, the 2-acyl-LPA-producing enzyme, membrane-bound PA-selective phospholipase A₁ α (mPA-PLA₁ α or LIPH), was also encoded by a gene

responsible for a similar hair disease (34). Thus, it was strongly suggested that the endogenous and authentic ligand for p2y5 is 2-acyl-LPA. We next examined whether p2y5 shows a preference for 2-acyl-LPA over 1-acyl-LPA. Commercially unavailable 2-acyl-LPA species were prepared using the *R. miehei* lipase (15) and various PA species as described under “Experimental Procedures.” When 1-palmitoyl-2-oleoyl PA was digested with this enzyme, mass spectrometry analysis demonstrated that the products mainly consisted of oleoyl-LPA, whereas a very small amount of palmitoyl-LPA was produced (data not shown), indicating the selective digestion at the *sn*-1-position of PA. Next, 2-acyl-LPA species from dioleoyl-PA, dilinoleoyl-PA, and diarachidonoyl-PA were prepared. Contrary to LPA₁, LPA₃ showed a preference for 2-acyl-LPA (35). We consistently observed that the 2-oleoyl-LPA prepared by our method showed higher activity than 1-oleoyl-LPA in the Ca²⁺ mobilization of RH7777 cells stably expressing LPA₃ but not in the cells expressing LPA₁ (Fig. S2). cAMP assays gave consistent results (Fig. 6C, *left* and *middle*), thus we examined the effect of the position of fatty acid chain on p2y5 activation in the cAMP assay with G_{s/13}-transfected B103 cells after PTX treatment. As expected, 2-oleoyl-LPA showed higher activity in forming cAMP via G_{s/13} than 1-oleoyl-LPA (Fig. 6C, *right*). LPA species containing linoleic acid and arachidonic acid at the *sn*-2-position also showed higher activity than those at the *sn*-1 position (Fig. 6D). Like 1-acyl LPA species, 2-linoleoyl-LPA was more potent than 2-oleoyl-LPA, whereas 2-arachidonoyl-LPA was less potent than 2-oleoyl-LPA (data not shown). The preference of p2y5 for 2-acyl-LPA was consistently observed in the [³⁵S]GTP γ S binding assay using membrane fractions from RH7777-p2y5 cells (Fig. 6E). Taken together, these results suggest that 2-acyl-LPA is a potent ligand for p2y5.

LPA-induced Cell Contraction via Endogenous p2y5 in HUVECs—Finally, we examined the function of the endogenously expressed p2y5. For this purpose, we used HUVECs, which are shown to express p2y5 mRNA at a high level by a comprehensive data base of gene expression profiles (available on the World Wide Web). We also confirmed the high

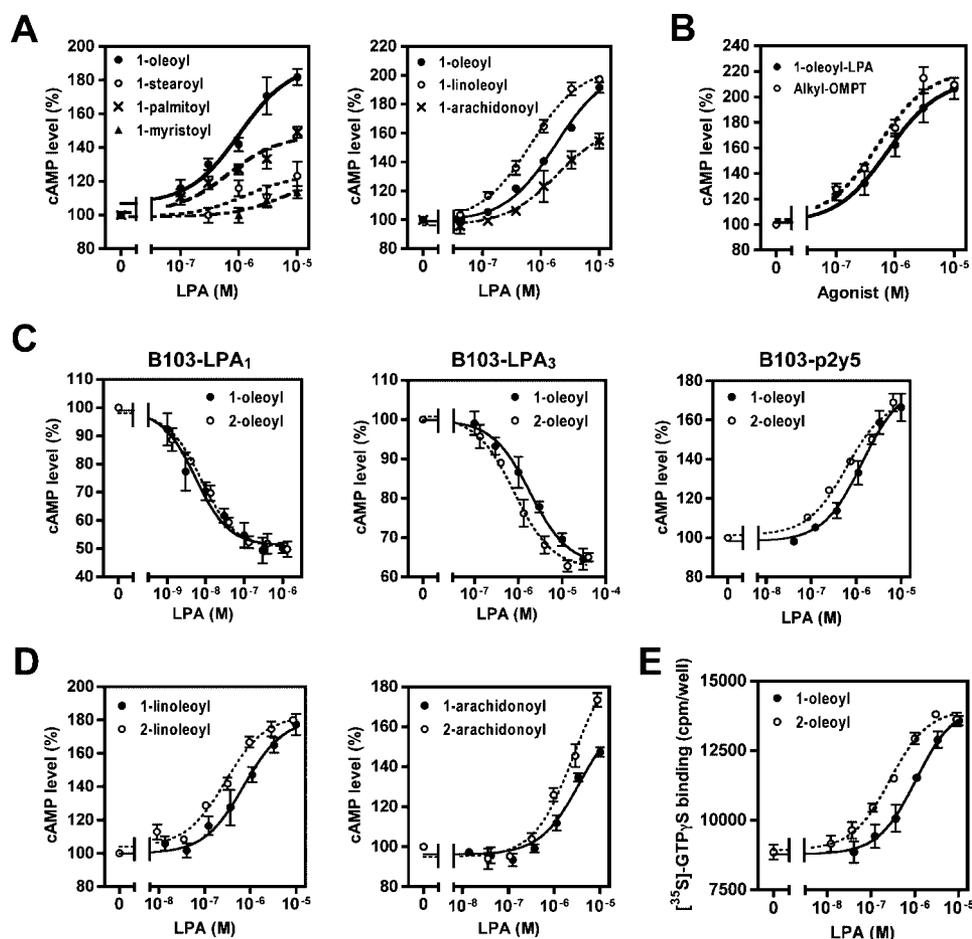
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FIGURE 6. **Ligand selectivity of p2y5.** Activities of various LPA species and alkyl-OMPT were examined by the cAMP assay using B103-p2y5 cells transfected with $G_{s/13}$ after pretreatment with PTX (A–D). Agonistic activities of 1-acyl-LPA species with saturated fatty acid (A, left) or unsaturated fatty acid (A, right), alkyl-OMPT (B), and 2-acyl-LPA species (C and D) are presented as a percentage of forskolin-induced cAMP accumulation in the absence of LPA. 2-Acyl-LPA species (C–E) were prepared as described under “Experimental Procedures.” The preference of p2y5 for 2-acyl-LPA was consistently observed in the GTP γ S binding assay using membrane fractions from RH7777-p2y5 cells (E). Data are means \pm S.E. ($n = 3$) of a representative of three independent experiments with similar results.

expression of p2y5 mRNA in HUVECs by reverse transcription-PCR (Fig. 7A). Consistent with previous reports (36, 37), LPA₁ mRNA was also highly expressed in these cells. No mRNA expression of LPA₂, LPA₃, LPA₄, and LPA₅ was detected under our experimental conditions. It has been reported that HUVECs show Rho-dependent actin reorganization in response to LPA (38). When these cells were seeded on poly-D-lysine-coated glass bottom dishes and serum-starved, 1-oleoyl-LPA induced rapid contraction and rounding of these cells (Fig. 7B). Because similar morphological changes were observed in LPA-stimulated B103-p2y5 cells (Fig. 2A), it was probable that the contraction of HUVECs was modulated by p2y5.

For the objective evaluation and quantification of the morphological changes, we utilized the xCELLigence system from Roche Applied Science, which detects the cell-substrate impedance (20). For example, a decrease in cell adhesion or cell contraction, *i.e.*, loss of the cell-electrode contact area, leads to a decrease in the impedance (displayed as “cell index”). Consistent with the rapid contraction and rounding of HUVECs as shown in Fig. 7B, a decrease of cell index was

observed upon LPA-stimulation (Fig. 7C) in a dose-dependent manner (data not shown). We next examined the role of p2y5 in this effect by utilizing siRNA-mediated gene silencing. The selective and effective suppression of p2y5 gene expression by siRNAs was confirmed by quantitative real time PCR (Fig. 7D). The cells treated with p2y5 siRNA showed severe defects in the LPA-induced cell index decrease compared with control siRNA-treated cells (Fig. 7E, left). Another p2y5-siRNA with a different target sequence gave consistent results (Fig. S3). On the other hand, thrombin decreased the cell index equally in control siRNA-treated and p2y5 siRNA-treated cells (Fig. 7E, right). These results demonstrate that not only overexpressed p2y5 but also endogenously expressed p2y5 can function as an LPA receptor that changes cell morphologies.

DISCUSSION

In the current study, we have identified LPA as a ligand for p2y5, which shares the highest sequence homology with LPA₄ (12). The morphological characteristics of p2y5-overexpressing cells led us to hypothesize that p2y5 is an LPA receptor coupling with $G_{12/13}$ proteins.

This hypothesis was validated by the following experiments: [³H]LPA binding assay, [³⁵S]GTP γ S binding assay, observation of cellular morphology, cAMP assay with $G_{s/13}$ proteins, and gene knockdown study using HUVECs. Furthermore, we have shown that 2-acyl-LPA has higher potency to p2y5 than 1-acyl-LPA.

Ligand binding has been recognized to be most important for the identification of receptor ligands (39). We could not detect specific binding of [³H]1-oleoyl-LPA to RH7777 cells transiently expressing p2y5. However, RH7777 cells stably expressing p2y5 showed small but significant specific binding (Fig. 3C). Considering the sensitivity to GTP γ S (Fig. 3D), the specific binding of LPA can be ascribed to p2y5. The reasons for the discrepancy between the transient and stable expression systems are currently unknown. With the use of the HA epitope and a magnetic cell sorting system, the expression level of p2y5 was found to be extremely high in the stable cell line (Fig. 1A), which might enable us to detect the low specific [³H]1-oleoyl-LPA binding. Indeed, the specific binding of p2y5 was lower compared with other LPA receptors (*i.e.* LPA₁, LPA₂, LPA₄, and LPA₅). Even in the stable expression system, we failed to detect

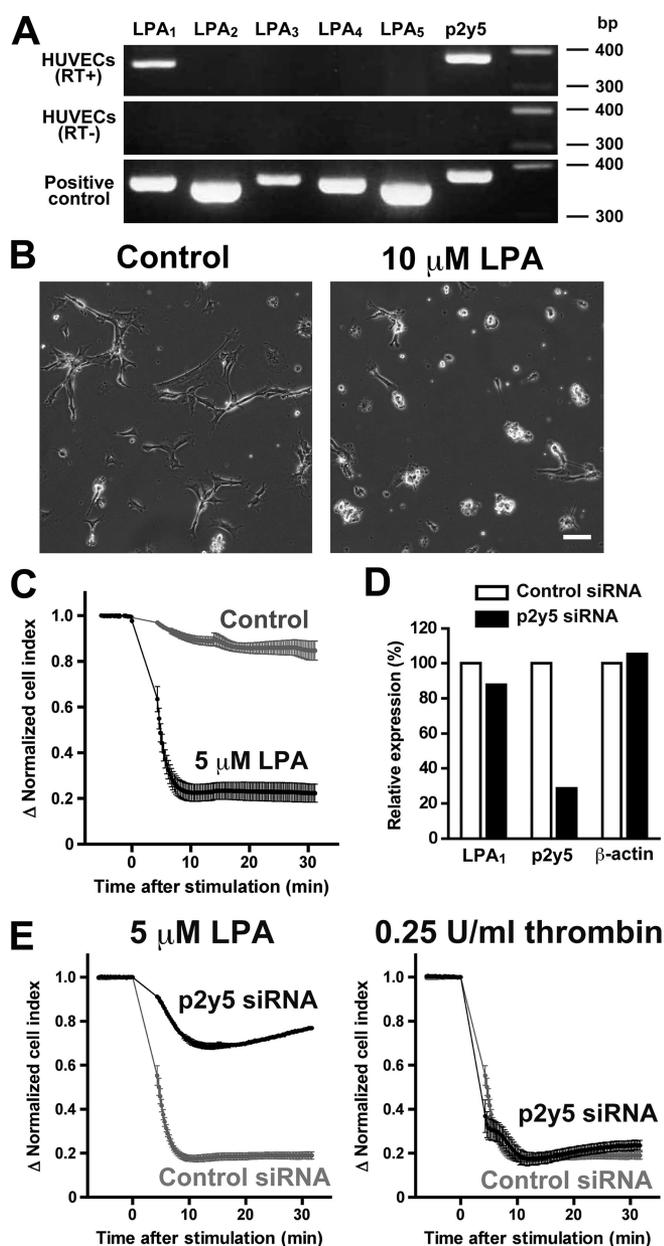


FIGURE 7. p2y5-dependent contraction of HUVECs. *A*, mRNA expression profile of LPA receptors in HUVECs. RNA was isolated from HUVECs and reverse-transcribed. cDNA was amplified using primers described under "Experimental Procedures." PCR templates for "positive controls" were cDNA from HEK293 cells (for LPA₁ and LPA₃), cDNA from HL-60 cells (for LPA₄ and p2y5), and expression plasmids for LPA₂ and LPA₅ in pCXN2.1. *B*, contraction of HUVECs by LPA stimulation. HUVECs cultured in poly-L-lysine-coated glass-bottomed 35-mm dishes were serum-starved, stimulated with 10 μ M 1-oleoyl-LPA for 30 min, and fixed with 2% paraformaldehyde. *Bar*, 50 μ m. *C*, quantification of HUVEC contraction using the xCELLigence system. The serum-starved cells were stimulated with 5 μ M LPA. A decrease in cell index was observed upon LPA stimulation. Data are means \pm S.D. ($n = 6$) of a representative of two independent experiments with similar results. *D*, quantitative real time PCR analysis in HUVECs transfected with siRNAs. Expression levels of LPA₁, p2y5, and β -actin mRNA in the cells transfected with p2y5 siRNA (Silencer Select siRNA, ID numbers; s19798) are presented as a percentage of those with control siRNA. Data are means ($n = 2$) of a representative of four independent experiments with similar results. *E*, effect of p2y5 on contraction of HUVECs. The cells transfected with siRNAs were serum-starved. The morphological changes upon the stimulation with 5 μ M LPA (*left panel*) or 0.25 units/ml thrombin (*right panel*) were evaluated in the same manner as *C*. Data are means \pm S.D. ($n = 6$) of a representative of four (LPA) and two (thrombin) independent experiments with similar results. Another p2y5 siRNA with different target sequence gave consistent results (Fig. S3).

the saturation of the binding at 200 nM [³H]1-oleoyl-LPA (data not shown). In the [³⁵S]GTP γ S binding assay and cAMP assay, the EC₅₀ values of 1-oleoyl-LPA were about 1 μ M, which was consistent with the specific binding too low to detect the saturation of the binding under our experimental conditions. Thus, radiolabeled ligands with much higher affinities to p2y5 than 1-oleoyl-LPA will be required to determine an accurate K_d value for this receptor. Another possible reason for the poor detection of the specific binding might be the rapid off-rate of the receptor. In our assay system, filter-trapped membrane proteins were washed 10 times with a buffer. The receptor-bound ligand might be released from the receptors if the off-rate is much faster than the time spent in washing. We note that an assay with three-time washing resulted in nonspecific binding too high to allow detection of the specific binding (data not shown). The receptor purification may overcome the problem of high nonspecific binding, as recently reported in the binding assay for free fatty acid receptor (40). Alternatively, a binding assay without washing steps, such as the binding assay with scintillation proximity assay technology and surface plasmon resonance biosensor (41), may be needed for more reliable LPA binding assays. The [³⁵S]GTP γ S binding assay detects direct receptor-G protein interactions (42) and is another important assay for the demonstration of the direct receptor activation by ligands. 1-Oleoyl-LPA increased the incorporation of [³⁵S]GTP γ S into the membrane fractions both from the cells transiently and stably expressing p2y5 in a dose-dependent manner (Fig. 4A). Although the signal-to-noise ratio is relatively low in the standard [³⁵S]GTP γ S binding assay (about a 1.3-fold increase), we could detect an \sim 4-fold increase of [³⁵S]GTP γ S incorporation to G α_{13} protein (Fig. 4B) by another binding assay employing immunoprecipitation steps (43). Taken together, these results demonstrated that p2y5 is directly activated by LPA.

In the first course of our screening of lipid ligands for p2y5 by the Ca²⁺ assay and cAMP assay, no LPA-dependent signaling of p2y5 was observed in "LPA receptor-null" RH7777 and B103 cells (data not shown). Besides this, neither the *zif268* nor the CRE promoter was activated by LPA in reporter gene assays using p2y5-expressing PC12h rat pheochromocytoma cells (data not shown). Pasternack *et al.* (30) showed LPA-induced CRE-directed luciferase activation in Chinese hamster ovary cells expressing p2y5. This inconsistency might be due to the fact that different cells, Chinese hamster ovary cells, were used. Under our experimental conditions, LPA strongly enhanced luciferase activity in PC12h cells transiently expressing LPA₄ in the CRE-driven reporter gene assay (data not shown). Thus, at least the potency of p2y5 to couple with G_s protein might be much lower than that of LPA₄ in this cell line. G_q family proteins did not seem to be involved in p2y5 signaling, because B103 cells and RH7777 cells overexpressing p2y5 did not show any Ca²⁺ mobilization in response to LPA. In accordance with this observation, in Chinese hamster ovary cells that have endogenous LPA receptors, p2y5-overexpression had no effect on the Ca²⁺ signaling by LPA (12). Even in the presence of the promiscuous G protein, G₁₆ (44), p2y5-dependent Ca²⁺ signaling was not observed in RH7777 cells (data not shown).

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In contrast to the Ca²⁺ assay and cAMP assay, LPA induced drastic responses in the morphology of B103 cells and RH7777 cells overexpressing p2y5 (Fig. 2, A and B). Given the susceptibility to Y27632, the morphological changes were Rho-associated kinase-dependent. Furthermore, because the morphological changes were resistant to PTX or the G_{q/11} inhibitor YM254890 (data not shown), G_{12/13} protein seemed to be involved, as previously reported in other LPA receptors (14, 18, 45). The G_{12/13} protein coupling of p2y5 was consistently demonstrated not only by G₁₃-specific [³⁵S]GTPγS binding assay (Fig. 4B) (see above) but also by the increase in intracellular cAMP levels through a G_{s/13} chimeric G protein (Fig. 5). However, the cAMP elevation was observed only after pretreatment with PTX. Pasternack *et al.* (30) also showed a similar enhancing effect of PTX in reporter gene assays of p2y5. PTX-sensitive G proteins (possibly G_{i/o} family G proteins) might modulate the signaling of p2y5, although the precise mechanism is unclear and awaits further signaling analysis of this receptor. We also demonstrated that LPA regulates cell morphology via endogenous p2y5 using siRNAs (Fig. 7 and Fig. S3). It has been reported that LPA induces Rho-dependent morphological changes in endothelial cells, which are supposed to regulate endothelial permeability (46, 47). Taken together, our present data of overexpression and knockdown experiments indicate that the G_{12/13}-Rho axis is involved in p2y5 signaling.

Autotaxin is an LPA-producing enzyme responsible for the generation of LPA in plasma and serum (48, 49). Autotaxin knock-out mice show embryonic lethality mainly because of severe blood vessel malformation (50, 51). Of note, G₁₃ knock-out (52) and G_{12/13} double knock-out mice (53) had similar phenotypes, which can be rescued by re-expression of G₁₃ protein in the endothelial cells (54). These facts suggest that the LPA-G₁₃-Rho signaling pathway in endothelial cells is deeply involved in vascular development. However, the responsible LPA receptor(s) has not yet been identified (49). Since p2y5 played a main role in the Rho-involved morphological changes induced by LPA in HUVECs (Fig. 7 and Fig. S3), p2y5 might be the missing link between the functions of autotaxin and G₁₃.

mPA-PLA₁α is another enzyme that produces LPA (55). Recent studies showed that human hair growth deficiencies are linked to genetic defects in mPA-PLA₁α (34, 56–59) and p2y5 (29–33). Both p2y5 and mPA-PLA₁α are highly expressed in the inner root sheaths of hair follicles and are supposed to participate in the development of hair follicles (30, 33, 34). In this study, we showed that 2-acyl-LPA activated p2y5 more efficiently than 1-acyl-LPA (Fig. 6). Since mPA-PLA₁α produces 2-acyl-LPA by digesting PA (55), our results are consistent with the similar phenotypes due to defects of mPA-PLA₁α and p2y5 (48). Unfortunately, no detection method for discrimination between 1-acyl- and 2-acyl-LPA has been established (60). However, it is acceptable to assume that LPA prepared by our method was rich in 2-acyl-LPA for the following reasons. First, we used *R. miehei* lipase that has 1,3-selective lipase activities (15) to avoid the digestion at the sn-2-position of PA. Actually, when 1-palmitoyl-2-oleoyl PA was digested with the lipase, mass spectrometry analysis demonstrated that the products mainly consisted of oleoyl-LPA (data not shown). Second, to

reduce the effect of rapid acyl migration, we used the fraction within 2 h after the column elution. Finally, using the products, we reconfirmed the preference of LPA₃ for 2-oleoyl-LPA over 1-oleoyl-LPA (Fig. 6C and Fig. S2), which was reported by Bando *et al.* (35). Further analysis, such as molecular species profiling of LPA in the inner root sheaths of hair follicles, will be needed to reveal the role of the mPA-PLA₁α-p2y5 axis proposed in human hair growth.

In conclusion, we report here the identification of p2y5 as a novel sixth LPA receptor (LPA₆), which activates the G_{12/13}-Rho signaling pathways. 2-Acyl-LPA had higher activity to p2y5 than 1-acyl-LPA, suggesting the functional coupling of mPA-PLA₁α with p2y5. We also showed the endogenous p2y5 function that affects the morphology of HUVECs. Our present study will help to better understand the physiological and pathological roles of p2y5 as well as detail mechanisms of human hair growth.

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