LPA₄/GPR23 Is a Lysophosphatidic Acid (LPA) Receptor Utilizing Gₛ, Gq/G₁, G₁₂/1₃-mediated Calcium Signaling and G₁₂/1₃-mediated Rho Activation

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Lysophosphatidic acid (LPA) is a bioactive lysophospholipid that signals through G protein-coupled receptors (GPCRs) to produce a range of biological responses. A recently reported fourth receptor, LPA₄/GPR23, was notable for its low homology to the previously identified receptors LPA₁–₃, and for its ability to increase intracellular concentrations of cAMP and calcium. However, the signaling pathways leading to LPA₄-mediated induction of cAMP and calcium levels have not been reported. Using epitope-tagged LPA₄ pharmacological intervention, and G protein mini-genes, we provide independent confirmatory evidence that supports LPA₄ as a fourth LPA receptor, including LPA concentration-dependent responses and specific membrane binding. Importantly, we further demonstrate new LPA-dependent activities of LPA₄ that include the following: receptor internalization; G₁₂/₁₃- and Rho-mediated neurite retraction and stress fiber formation; G₉ protein and pertussis toxin-sensitive calcium mobilization and activation of a nonselctive cation conductance; and cAMP increases mediated by Gₛ. The receptor is broadly expressed in embryonic tissues, including brain, as determined by Northern blot and reverse transcription-PCR analysis. Adult tissues have increased expression in skin, heart, and to a lesser extent, thymus. These data confirm the identification and extend the functionality of LPA₄ as an LPA receptor, bringing the number of independently verified LPA receptors to five, with both overlapping and distinct signaling properties and tissue expression.

Lysophosphatidic acid (LPA, 1-acyl-sn-glycerol-3-phosphate) is a water-soluble bioactive phospholipid that can be generated by many cell types and has been shown to influence multiple intracellular signaling pathways, including stimulation of phospholipase C and D, activation of small GTPases, MAPK (mitogen-activated protein kinase), and phosphoinositide 3-kinase (1, 2), and inhibition of adenyl cyclase (3, 4). LPA signaling through G proteins mediates a variety of biological functions, including cell proliferation, cell survival, cytoskeletal remodeling, cell migration, and alterations in differentiation (3, 5–9). In mice, gene deletion studies of the LPA receptors (10, 11) have shown that LPA receptor-mediated signaling contributes to many other functions in normal and pathological states (12), including vascular and nervous system development (10, 13, 14), female fertility and implantation (15), and the initiation of neuropathic pain (16).

Five LPA-specific GPCRs have thus far been identified, termed LPA₁–₅ (17–22). LPA₄ is the only receptor that has yet to receive independent confirmation as a bona fide LPA receptor since its initial report (20). This putative LPA receptor was remarkable for its relatively low predicted amino acid sequence homology compared with the well studied LPA₁–₃. Using high affinity binding and calcium mobilization assays, we first confirmed the finding that GPR23 is a LPA₄ receptor. Five LPA₄ constructs were expressed in CHO cells and tested for LPA-mediated calcium responses. In CHO cells, LPA₄ showed a Kᵦ~ of 45 nM for LPA₄ and an ability to mobilize calcium and increase cAMP production (20). Here we confirm the finding that GPR23 is indeed a biologically relevant receptor for LPA and report several novel aspects of LPA₄ signaling that extend its functional roles.

MATERIALS AND METHODS

Cell Culture and Stable Transfection—B103 neuroblastoma cells and RH7777 hepatoma cells (23) were cultured in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and antibiotics (Invitrogen). LPA₄-expressing stable cell lines were generated by transfecting B103 cells with linearized HA-tagged mouse LPA₄-pcDNA3.1 (Invitrogen) using Effectene transfection reagent (Qiagen, Valencia, CA). Stable transfectants were selected for by adding 1 mg/ml geneticin (Invitrogen) to the culture media for 2 weeks.

Production of LPA₄ Retrovirus and G Protein Minigens—HA-tagged mouse and human LPA₄ cDNAs were amplified using the Expand High Fidelity PCR system (Roche Applied Science) using the following primers: 5’-ATGTACCACGATGTTCAGATTTACGCTATGGGTAAGAGATT-ATTG-3’ (forward) and 5’-CTAGAAGGTGATTCCAGCAG-ATT-3’ (reverse). PCR products were subcloned into the pGEM-T Easy T vector (Promega, Madison, WI), and the cDNA insert was sequenced at the The Scripps Research Institute (TSRI) sequencing core facility. The HA-tagged LPA₄ cDNA was subsequently cloned into the NotI site of the LZRS-
EGFP Moloney murine leukemia retroviral vector. The Phoenix ecotropic packaging cell line (24) was transfected with the retroviral construct using FuGENE 6 transfection reagent (Roche Applied Science). Retrovirus expression vector (LZRS-EGFP) and Phoenix retrovirus packaging cell lines were provided by Dr. Garry P. Nolan (Stanford University, Stanford, CA). At 48 h post-transfection, retroviral supernatant was filtered through a 0.45-μm filter and frozen in aliquots. Construction of \( \gamma_{11}, \gamma_4, \gamma_{12}, \) and \( \gamma_{13} \) minigene retroviruses was described previously (22, 25).

**Western Blotting of Membrane Protein Fraction—**HA-LPA\(_4\)-expressing cells were homogenized in 20 mM Tris buffer, pH 7.5, containing 1 mM EGTA, 1 mM EDTA, and protease inhibitor mixture (Roche Applied Science) using a Dounce homogenizer. The sample was pre-cleared by centrifugation at 2,000 rpm for 5 min at 4 °C. The supernatant was then spun at 15,000 rpm for 90 min. Pellets were resuspended in ice-cold homogenization buffer containing 1% Triton X-100 and then centrifuged at 15,000 rpm for 20 min. The supernatant containing the membrane fraction was separated on a 4–12% SDS-polyacrylamide gel (Invitrogen) under reducing, denaturing conditions and transferred to polyvinylidene difluoride membrane (Millipore, Woburn, MA). HA-tagged LPA\(_4\) receptor expression was detected using an anti-HA antibody (Covance, Berkeley, CA) and horseradish peroxidase-conjugated anti-mouse secondary antibody and visualized with ECL Plus (Amersham Biosciences).

**F-actin Detection and Receptor Internalization Assay—**Cells were grown overnight on poly-l-lysine-coated 12-mm glass coverslips. The following night, cells were switched to serum-starved conditions and loaded with Fura-2 acetoxymethyl ester (Fura2-AM) (2.5 μM) for 30 min at 37 °C in Opti-MEM (Invitrogen) containing Fura2-AM. Images of Fura-2-loaded cells with the excitation wavelength of 340 M and 380 M were captured using a cooled CCD camera (Carl Zeiss). The ratio of fluorescence intensity at the two wavelengths was calculated after subtraction of background fluorescence. Ratio levels were determined from groups of 20–40 individual cells and analyzed using MetaFluor (Universal Imaging Corp., West Chester, PA).

**[\( ^{3} \)H]LPA Binding to Isolated Membranes—**The LPA-binding assay has been described previously by Fukushima et al. (23). Briefly, membranes were isolated from transfected RH7777 cells harvested in ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.5) containing 1 mM EDTA and protease inhibitors (Roche Applied Science) and centrifuged at 2,000 rpm for 10 min at 4 °C. The supernatant was then centrifuged at 15,000 rpm for 30 min at 4 °C. 40 μg of membrane fraction was incubated with \( [\text{3H}] \text{LPA} \) (1-oleoyl-9,10-\( ^{3} \)H)LPA, 47 Ci/mmol; PerkinElmer Life Sciences) in LPA-binding buffer containing 0.1% fatty acid-free BSA (Sigma) and 0.5 mM CuSO\(_4\) for 30 min at room temperature. The \( [\text{3H}] \text{LPA} \) membrane fraction mixture was collected onto a Unifilter 96-GF/B (PerkinElmer Life Sciences). The filter was washed 10 times with binding buffer containing 1% BSA and dried for 30 min at 50 °C. Thirty microliters of MicroScint-O was added to each well of the filter, and radioactivity was measured using a microplate liquid scintillation counter (PerkinElmer Life Sciences). Total and nonspecific binding were evaluated in the absence and presence of 10 μM unlabeled LPA, respectively.

**G Proteins and Rho Inhibition in Cultured Cells—**To investigate G protein coupling with LPA\(_4\), stable LPA\(_4\)-expressing B103 cells were infected with several G protein minigenes and tested 2 days later or treated with PTX (200 ng/ml; List Biological Laboratories, Campbell, CA) for 12 h. To inhibit the Rho pathway, LPA\(_4\)-expressing cells were treated with either the Rho inhibitor C3 transferase (10 μg/ml; Cytoskeleton Denver, CO) for 24 h or the ROCK inhibitor Y27632 (10 μM; Calbiochem) for 45 min. Rounded cells were counted following treatment with 1 μM LPA for 30 min in serum-free conditions.

**cAMP Measurements—**Both acutely infected and stable transfectants expressing LPA\(_4\) were used in these experiments. LPA\(_4\)-expressing and control B103 cells were serum-starved overnight in 24-well plates with or without PTX (200 ng/ml; List Biological Laboratories). Following treatment with 0.5 mM 3-isobutyl-1-methylxanthine for 20 min, cells were exposed to LPA (0, 1, 10, and 100 nm and 1 μM) for 30 min with or without forskolin (5 μM). Cells were then lysed in 0.1 N HCl, and cellular cAMP levels were quantified using an enzyme-linked immunosorbent assay-based detection kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer’s directions.

**Determination of Intracellular Calcium Mobilization—**B103 cells stably expressing HA-tagged LPA\(_4\) were infected with G protein minigenes 2 days prior to testing and/or exposed to PTX overnight, and control cells were plated on glass coverslips and loaded with Fura-2 acetoxyethyl ester (Fura2-AM) (2.5 μM) for ratiometric calcium imaging studies. Cells lacking LPA\(_4\) expression served as controls. Cells were incubated for 30–60 min at 37 °C in Opti-MEM (Invitrogen) containing Fura2-AM (2.5 μM) and 1.5 μM of pluronic acid (Molecular Probes, Eugene, OR) and then briefly washed with Opti-MEM. Coverslips were perfused with Opti-MEM in a laminar flow perfusion chamber (Warner Instrument Corp., Hamden, CT). LPA (1 μM) was bath-applied by gravity perfusion when indicated. Images of Fura-2-loaded cells with the excitation wavelength alternating between 340 and 380 nm were captured with a cooled CCD camera (Carl Zeiss). The ratio of fluorescence intensity at the two wavelengths was calculated after subtraction of background fluorescence. Ratio levels were determined from groups of 20–40 individual cells and analyzed using MetaFluor (Universal Imaging Corp., West Chester, PA).

**Electrophysiology—**The whole cell patch clamp technique was used to record and measure LPA-induced effects on whole cell currents of B103 cells stably expressing LPA\(_4\) receptor. The involvement of \( \gamma_q \) in the modulations of cellular conductance was determined with stable LPA\(_4\)-B103 cells after infection with virus expressing the \( \gamma_q \) minigene or empty vector that served as the control for minigene infection. Some LPA\(_4\)-B103
cells were treated overnight with PTX as described above. The extracellular solution (pH 7.4 with NaOH) contained the following: NaCl 145 mM, KCl 2.5 mM, CaCl₂ 1.5 mM, MgSO₄ 1.5 mM, HEPES 10 mM, dextrose 10 mM. LPA and vehicle were added to the bath by gravity perfusion at room temperature. Recording electrodes were fabricated and coated with dental periphery wax as described previously (22). Intracellular solution (pH 7.4) contained potassium gluconate 100 mM, KCl 25 mM, MgCl₂ 3 mM, CaCl₂ 0.483 mM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N''-tetraacetic acid-K₄ 1.0 mM, hemi-Na-HEPES 10 mM. The resistive whole cell configuration and data acquisition were achieved as described in Ref. 22. Cells were chosen for study if depolarization-activated peak inward current density was less than −30 pA/pF. During application of LPA (1 μM) or washout, cells were held at −50 mV, and the \( V_m \) value was stepped to −120 mV for 60 ms and ramped to +120 mV (at a rate of 1 mV/ms) every 2 s. All data are expressed in terms of the \( C_m \) value during stimulus application (current density).

Reverse Transcription-PCR—TRIzol reagent (Invitrogen) was used to extract RNA from cultured cells or tissues as described by the manufacturer. Five micrograms of total RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen). An equal quantity of cDNA was used to amplify LPA₄ and β-actin transcripts using the following conditions: 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 45 s for a total of 30–35 cycles. The primers were used as follows: LPA₄, 5'-AGGCCATGAGCACATTCTC-3' (forward) and 5'-CAACCTGGGTCTGAGACTTG-3' (reverse); β-actin, 5'-TGGAATCCTGTGGCATCCATGAAAC-3' (forward) and 5'-TAAAACGCAGCTCAGTAACAGTCCG-3' (reverse). The PCR products were analyzed by electrophoresis on a 1.2% agarose gel.

Northern Blot Analysis—A Northern blot (OriGene Technologies, Rockville, MD) containing 2 μg/lane of adult mouse poly(A)⁺ RNA from several mouse tissues was probed with a random primed full-length ³²P-labeled mouse LPA₄ and human β-actin cDNA. DNA labeling was performed with the high prime DNA labeling kit (Roche Applied Science), and unincorporated nucleotides were separated out by passing the reaction mixture over a Sephadex G-50 quick spin column (Roche Applied Science). The Northern blot was then probed overnight in ULTRAhyb hybridization solution (OriGen), washed several times, and analyzed using a PhosphorImager detection system.

Statistical Analysis of Data—Each data point was calculated from triplicate samples unless otherwise indicated. The data are presented ± S.D. Statistical analysis was performed by one-way analysis of variance and Dunnett’s method, or Student’s \( t \) test.

RESULTS

Mouse LPA₄ cDNA was epitope-tagged with hemagglutinin (HA) sequence at the 5’-end of the extracellular domain, and the construct was introduced into a murine leukemia, replication-deficient, bicistronic retroviral vector (Fig. 1A). This construct co-expresses tagged LPA₄ and EGFP, thus allowing for the identification of receptor expression in living and fixed cells by fluorescence microscopy. To demonstrate cell surface receptor gene expression, LPA₄-infected B103 cells were labeled with an anti-HA primary antibody, and receptor was visualized using Cy3-conjugated secondary antibody (Cy3), and these cells also expressed EGFP. C. Western blot assay of the membrane fraction isolated from B103 cells infected with the empty vector or HA-tagged LPA₄ retroviruses. D. LPA-mediated LPA₄ internalization. Infected B103 cells were treated with BSA, 1 μM LPA, or 1 μM sphingosine 1-phosphate (SIP) for 15 min after overnight serum starvation, and then immunostained as in C. An arrowhead shows surface expression of HA-LPA₄, and an arrow indicates internalization of the receptor. E, specific [³²H]LPA binding to cell membranes isolated from stable LPA₄-expressing RH7777 cells. Forty μg of membrane fraction from empty vector- or LPA₄-expressing cells was incubated with [³²H]LPA (4,042 dpm) for 30 min. Data are the mean ± S.D. (n = 3). **, \( p < 0.01 \) (using Student’s \( t \) test) versus empty vector-expressing cells.
Diverse Signaling Pathways Activated by LPA<sub>4</sub>

LPA the cells retracted their processes, a response observed for activation of heterologously expressed LPA<sub>1,2,5</sub> in B103 cells (see below). LPA<sub>4</sub> internalization was not a consequence of cell rounding because sphingosine 1-phosphate-induced retraction did not cause LPA<sub>4</sub> internalization.

We next sought to determine the signaling pathways mediating LPA<sub>4</sub>-induced morphological changes in LPA<sub>4</sub>-expressing B103 cells. A strong cell rounding response occurred within 30 min of LPA treatment (Fig. 2A). However, control vector-infected cells were unresponsive to LPA (data not shown). F-actin staining with rhodamine-phalloidin demonstrated stress fiber formation in LPA<sub>4</sub>-expressing RH7777 cells (Fig. 2B) but not control cells. In B103 cells expressing LPA<sub>4</sub>, LPA produced a concentration-dependent increase in the proportion of rounded cells (Fig. 2C), with ~70% of LPA<sub>4</sub>-infected cells rounding after exposure to 1 μM LPA.

PTX and G-protein minigenes were used to determine which members of the heterotrimeric G-protein family are responsible for mediating the LPA-induced cell rounding of LPA<sub>4</sub>-expressing cells (Fig. 3A). G<sub>12</sub>/<sup>13</sup> interacts with p115 RhoGEF, the Rho guanine nucleotide exchange factor (29, 30), activating the Rho signaling pathway to produce actin cytoskeleton rearrangement (31, 32). Using G<sub>12</sub> and G<sub>13</sub> minigenes to inhibit G<sub>12</sub>/<sup>13</sup> signaling, LPA-induced cell rounding was significantly reduced (Fig. 3A).

revealed statistically significant specific [³H]LPA binding compared with membranes isolated from control cells (Fig. 1E).

G protein-coupled receptors typically undergo internalization during prolonged agonist exposure (28). We reasoned that if GPR23 is a physiologically relevant receptor for LPA but not other lysophospholipids, then LPA exposure would likely produce agonist-induced receptor internalization, whereas other ligands would not. We performed a standard internalization assay using B103 cells expressing HA-tagged LPA<sub>4</sub> and visualized LPA<sub>4</sub> localization with anti-HA immunolabeling and confocal microscopy. We found that receptor internalization occurred following LPA treatment but not with another lysophospholipid, sphingosine 1-phosphate (Fig. 1D). We noticed that during exposure to Recently, G<sub>q/11</sub> has also been shown to activate a Rho-dependent pathway in G<sub>12</sub>/<sup>13</sup>-deficient cells (33). However, blocking this pathway using a pan-G<sub>q/11</sub> minigene did not inhibit cell rounding in response to LPA (Fig. 3A). Blocking G<sub>q</sub> signaling with PTX pretreatment (Fig. 3A) or G<sub>q</sub> signaling using a G<sub>q</sub> minigene (data not shown) also failed to inhibit LPA-induced cell rounding.

To test the involvement of Rho signaling in LPA receptor-mediated cell rounding, we used C3 toxin and Y27632 to inhibit Rho and Rho kinase, respectively. Similar to LPA<sub>1</sub>-and LPA<sub>2</sub>-induced Rho signaling-dependent cell rounding in infected cells (26), the cell rounding response in LPA<sub>4</sub>-expressing B103 cells was inhibited by both C3 and Y27632 (Fig. 3B). Furthermore, LPA<sub>4</sub>-mediated stress fiber formation in
Diverse Signaling Pathways Activated by LPA₄

Because all known lysophospholipid GPCRs can influence cAMP levels (14), we analyzed cAMP levels in LPA₄-expressing B103 cells after LPA exposure. LPA increased intracellular cAMP levels in LPA₄-expressing B103 cells. It is well established that Gβγ subunits of PTX-sensitive G proteins activate phospholipase Cβ (36).

To further investigate intracellular signaling pathways modulated by LPA₄ receptor activation, we used a “resistive” whole cell patch clamp technique (22) to determine whether LPA could influence ion channel function through LPA₄ signaling. LPA (100 nM (not shown) and 1 µM) activated a transient conductance in stably transfected LPA₄-expressing cells that was not observed in cells transfected with empty vector (Fig. 6A). Whole cell currents induced by LPA₄ receptor activation had a latency of 31 ± 2 ms (n = 18), consistent with a GPCR-mediated effect and a reversal potential of −7 ± 2 mV (n = 17), suggesting enhancement of nonselective cation channel activity (Fig. 6B). LPA-CI µM induced current density was −1.8 ± 0.3 pA/pF and +2.5 ± 0.4 pA/pF at −120 and +120 mV, respectively (n = 16). Stable LPA₄-B103 transfectants were used to identify G protein signaling pathways involved in activation of the putative nonselective cation conductance (Fig. 6C). PTX pretreatment decreased ramp-induced currents at −120 and +120 mV by 42 and 45%, respectively (Fig. 6C), and PTX together with expression of a Gq minigene substantially reduced LPA-induced currents even further to 3 and 6%, respectively (Fig. 6C). Thus, activation of LPA-induced nonselective cation conductance by G proteins had a similar pharmacological profile as the LPA-induced intracellular calcium response.

We next performed Northern blot and RT-PCR analysis to assess the tissue expression of LPA₄ (Fig. 7). LPA₄ mRNA expression was detected in heart, skin, thymus, bone marrow, mouse embryonic fibroblast, embryonic brain, and embryonic stem cells (Fig. 7).

DISCUSSION

A critical aspect of understanding receptor-mediated lysophospholipid signaling has been the identification of receptors that meet clear, unambiguous criteria for receptor function, combined with independent confirmation of the proposed identity. In the lysophospholipid receptor field, multiple instances of initial receptor mis-identification have occurred, most notably the following: OGR1 as a sphingosylphosphorylcholine receptor (38), GPR4 as a lysophosphatidylcholine and sphingosylphosphorylcholine receptor...
respect to adenyl cyclase activation. This activity is consistent with previous reports in which phosphorylation of the βγ-adrenergic receptor by cAMP-dependent protein kinase is proposed to switch its coupling specificity from Gi to Gs (43). Receptor-dependent activation of Gi could thus release sufficient Gβγ to activate phospholipase Cβ (36). Hence, the Gs/Gi switching model is a potential mechanism to explain the ability of LPA₄ to activate both Gs and Gi.

Second, activation of LPA₄ evoked a nonselective cation conductance through similar G protein-mediated pathways as those that modulate calcium signaling. This activity may contribute to the nonselective cation conductance seen in physiological responses of primary neuroprogenitor cells from the embryonic cerebral cortex (46).

Third, LPA₄ stimulation induced G₁₂/₁₃-mediated Rho activation producing neurite retraction and stress fiber formation. These functions are shared with all other LPA receptors except LPA₃ (26). This may have relevance to embryonic brain development, where LPA signaling alters the shapes and positions of young neuroblasts (8, 44, 45).

Fourth, Gs produced increased cAMP levels during activation of LPA₄ in B103 cells. Other potential mechanisms for increasing cAMP have been reported, including Gβγ subunit activity that is independent of Gαs subunit (34, 35). It is likely that this mechanism, if involved, plays only a minor role because blocking Gs activity with the minigene approach virtually abolished LPA-induced cAMP production. Thus, receptor-mediated LPA signaling can raise levels of intracellular cAMP via two different GPCRs, LPA₄ and LPA₅ (22), complementing the cAMP-attenuating activities of LPA₁-₃ (14, 26).

Fifth, this is the first demonstration of LPA₄ gene expression in the mouse that reveals the highest levels in heart, skin, and thymus and contrasts somewhat with the lower expression levels observed in human tissues (20). In addition, gene expression was identified in multiple new tissues, including embryonic brain, bone marrow, and embryonic stem cells.

In a recent publication it has been reported that deletion of the autotaxin gene in mice leads to early embryonic lethality because of a defect in blood vessel formation (47). This demonstrates an essential function for autotaxin in normal develop-
ment that most likely involves LPA signaling mechanisms (although nucleotide pyrophosphatase/phosphodiesterase functions cannot be excluded) (37, 48, 49). The existence of five confirmed LPA receptors makes it probable that most if not all LPA receptors will need to be deleted to recapitulate the auto-taxin-null phenotype, consistent with the partially viable phenotype of mice deficient for one or two LPA receptors (10, 11). The biological roles of LPA4 in concert with the other four LPA receptors remain to be determined.

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REFERENCES