LPA$_4$/p2y$_9$/GPR23 Mediates Rho-dependent Morphological Changes in a Rat Neuronal Cell Line*

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Lyosphosphatidic acid (LPA) is a potent lipid mediator that evokes a variety of biological responses in many cell types via its specific G protein-coupled receptors. In particular, LPA affects cell morphology, cell survival, and cell cycle progression in neuronal cells. Recently, we identified p2y$_9$/GPR23 as a novel fourth LPA receptor, LPA$_4$ (Noguchi, K., Ishii, S., and Shimizu, T. (2003) J. Biol. Chem. 278, 25600–25606). To assess the functions of LPA$_4$ in neuronal cells, we used rat neuroblastoma B103 cells that lack endogenous responses to LPA. In B103 cells stably expressing LPA$_4$, we observed G$_{q/11}$-dependent calcium mobilization, but LPA did not affect adenylyl cyclase activity. In LPA$_4$ transfectedants, LPA induced dramatic morphological changes, i.e. neurite retraction, cell aggregation, and cadherin-dependent cell adhesion, which involved Rho-mediated signaling pathways. Thus, our results demonstrated that LPA$_4$ is as well as LPA$_1$ coupler to G$_{q/11}$ and G$_{12/13}$ whereas LPA$_4$ differs from LPA$_1$ in that it does not couple to G$_{i/o}$. Through neurite retraction and cell aggregation, LPA$_4$ may play a role in neuronal development such as neurogenesis and neuronal migration.

Both LPA$_1$ and LPA$_2$ couple to at least three types of G proteins, G$_{i/o}$, G$_q$, and G$_{12/13}$ whereas LPA$_3$ couples to G$_{i/o}$ but not G$_{12/13}$ (6). Depending on the functional coupling of a given LPA receptor to G proteins, LPA activates diverse signaling cascades involving phosphoinositide 3-kinase, phospholipase C, mitogen-activated protein kinase, and Rho family GTPases, and adenylyl cyclase (2, 7).

LPA is present in the brain at relatively high levels compared with other organs (8, 9). LPA influences the cell morphology of several neuronal cells, neuronal progenitors, and primary neurons (10). It has also been reported that LPA affects electrophysiology, cell survival, and cell cycle progression in neuronal cells (10, 11). Targeted deletion of LPA$_1$ in mice produces olfactory deficits (12) and a behavioral abnormality (13). Furthermore, the use of LPA$_1$ knockouts revealed that LPA$_1$ is involved in the initiation of neuropathic pain (14). Exposure of the developing cerebral cortex to LPA produces dramatic changes in the folding of the brain, which do not occur in LPA$_1$ and LPA$_2$ double knockouts (15). However, the LPA receptor subtypes responsible for some neuronal effects have not been identified (16–18).

Recently, we identified p2y$_9$/GPR23 as a fourth LPA receptor (LPA$_4$) that is structurally distinct from the three LPA receptors of the EDG family (19). The expressed sequence tag cDNA encoding LPA$_4$ was originally isolated from human brain (20, 21), and LPA$_4$ expression has been detected in rat embryonic hippocampal neurons (22) and immortalized hippocampal progenitor cells (18). These facts suggest that LPA$_4$ may have important roles in neurodevelopmental processes such as neurogenesis and neuronal migration. However, only very limited information is available regarding its physiological and biological functions. To assess the functional roles of LPA$_4$ in neuronal cells, we generated B103 cells stably expressing LPA$_4$. This study demonstrates that treatment of the LPA$_4$-expressing cells with LPA leads to morphological changes, including cell rounding and cadherin-dependent cell adhesion following cell aggregation, both of which are mediated by the Rho/Rho-associated kinase (ROCK) pathway. The effects of LPA$_4$ on the morphology of the neuronal cells were clearly distinct from those of LPA$_1$, probably because LPA$_4$ does not couple to G$_{i/o}$.

EXPERIMENTAL PROCEDURES

Cell Culture—B103 rat neuroblastoma cells were kindly provided by Dr. J. Chun (The Scripps Research Institute, La Jolla, CA). B103 cells expressing each of the LPA receptors were maintained on poly-1-lysine-coated 100-mm dishes (Iwaki,
times at 15 watts for 30 s, and centrifuged at 800 × g for 10 min at 4 °C. The supernatant was further centrifuged at 10^5 × g for 60 min at 4 °C, and resultant pellet was homogenized in ice-cold binding buffer. Binding assays were performed in 96-well plates in triplicate. 20 μg each of the membrane fractions from the twice-immunopurified cells was incubated in binding buffer containing 0.25% bovine serum albumin (BSA) (fatty acid-free, very low endotoxin grade; Serologicals Proteins, Kankakee, IL) with 2-fold serial dilutions (50–3.125 nM) of [3H]LPA (1-oleoyl)[oleoyl-9,10-3H]LPA, 57 Ci/mmol; PerkinElmer Life Sciences) for 60 min at 4 °C. The bound [3H]LPA was collected onto a Unifilter-96-GF/C (PerkinElmer Life Sciences) using a MicroMate 196 harvester (Packard Instrument Co.). The filter was then rinsed 10 times with ice-cold binding buffer and dried for 12 h at 50 °C. 25 μl of MicroScint-0 scintillation mixture (PerkinElmer Life Sciences) was added per well. The radioactivity that remained on the filter was measured with TopCount microplate scintillation counter (Packard Instrument Co.).

Total and nonspecific bindings were evaluated in the absence and presence of 10 μM unlabeled LPA [1-oleoyl (18:1)-LPA; Cayman Chemical, Ann Arbor, MI), respectively. The specific binding value (disintegrations/min) was calculated by subtracting the nonspecific binding value (disintegrations/min) from the total binding value (disintegrations/min). A dissociation constant (K_d) and a maximum binding capacity (B_max) were calculated by Scatchard analysis. B_max and K_d values for B103-LPA_1 cells were 0.8 pmol/mg protein and 18 nM, respectively. Those for B103-LPA_4 cells were 6.0 pmol/mg protein and 58 nM. No specific binding was observed in vector-transfected B103 cells (B103-vector cells).

**cAMP Measurement**—Cells (3.2 × 10^5) were seeded in collagen-coated 96-well plates (Iwaki), followed by 24 h of serum starvation. To determine whether LPA receptors mediate the inhibition of adenyl cyclase, an AlphaScreen cAMP assay kit (PerkinElmer Life Sciences) was used as recommended in the manufacturer’s instructions. The cells were washed twice with buffer A (Hanks’ balanced salt solution (HBSS) containing 25 mM HEPES-NaOH (pH 7.4) and 0.1% BSA (Serologicals Proteins)) and incubated in 100 μl of buffer A containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX)(from a 20 mM stock in dimethyl sulfoxide stored at −30 °C ( Sigma) for 15 min at room temperature. The reaction was initiated by adding 50 μl of various concentrations of LPA in buffer A with 50 μM forskolin (Wako; 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 16.6 μl of 10% Tween 20, followed by overnight storage at 4 °C. After centrifugation at 800 × g for 5 min, the cAMP concentration in the supernatant was measured in quadruplicate with a fusion system (PerkinElmer Life Sciences). To determine whether LPA receptors mediate the stimulation of adenyl cyclase, the cAMP Biotak EIA system (Amersham Biosciences) was used as recommended in the manufacturer’s instructions. The cells were washed twice with HEPES-Tyrode’s buffer (25 mM HEPES-NaOH (pH 7.4), 140 mM NaCl, 2.7 mM KCl, 1 mM CaCl_2, 0.49 mM MgCl_2, 12 mM NaHCO_3, 0.37 mM Na_2HPO_4, and 5.6 mM d-glucose) containing 0.1% BSA (HEPES-Tyrode’s BSA buffer) and incubated in 100 μl of HEPES-Tyrode’s BSA buffer con-
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taining 0.5 mM IBMX for 15 min at 37 °C. The reaction was initiated by adding 100 μl of various concentrations of LPA in HEPES-Tyrode's BSA buffer. After 30 min of incubation at 37 °C, the reaction was terminated by adding 25 μl of lysis buffer. Cell lysates in a volume of 100 μl were used to determine the cAMP concentration using an enzyme immunoassay method.

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\text{Ca}^{2+} \text{ Measurement—Cells serum-starved for 24 h were detached with PBS containing 2 mM EDTA, washed with HEPES-Tyrode's buffer, and then loaded with 3 μM Fura-2 AM (Dojindo, Kumamoto, Japan) in HEPES-Tyrode's BSA buffer for 1 h at 37 °C. The cells were washed twice and resuspended in HEPES-Tyrode's BSA buffer at a density of } 1 \times 10^6 \text{ cells/ml. The cell suspension (0.5 ml) was applied to a CAF-100 spectrofluorometer (Jasco, Tokyo, Japan), and 5 μl of 100 μM LPA in HEPES-Tyrode's BSA buffer was added. The intracellular Ca}^{2+} \text{ concentration ([Ca}^{2+}]_i) \text{ was measured as the ratio of emission fluorescence at 500 nm in response to excitation at 340 and 380 nm.}
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\text{Cell Rounding Assay—Cells (1} \times 10^6) \text{ were seeded in poly-d-lysine-coated 12-well plates (BD Biosciences). After 24 h of incubation, the cells were washed three times with DMEM containing 0.1% BSA and serum-starved for 24 h. Three hours after a medium change, the cells were treated with 1 μM LPA for 15 min. The cells were examined for a round cell morphology lacking any neurite extensions or filopodia. Extended neurites were defined as having a length greater than the cell body. The number of rounded cells was expressed as a percentage of the observed cells (>200 cells/well).}
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\text{Rho Inhibition Study—Cells (5} \times 10^5) \text{ were seeded in poly-l-lysine-coated 35-mm dishes (Iwaki) in DMEM supplemented with 10% fetal bovine serum. After 24 h, either the Clostridium botulinum C3 exoenzyme expression vector (pEF-C3) (25) (a kind gift from Dr. S. Narumiya, Kyoto University, Kyoto, Japan) or the corresponding control vector (pEF-BOS) (26) (a kind gift from Dr. S. Nagata, Osaka University, Osaka, Japan) was cotransfected with an enhanced green fluorescent protein (EGFP) expression vector (pEGFP-C1; Clontech) at a 4:1 weight ratio, with 3 μg of total DNA, using the Lipofectamine 2000 reagent (Invitrogen). After 24 h, the cells were seeded in poly-l-lysine-coated 12-well plates and cultured for 24 h. The cells were then serum-starved for 12 h and treated with 1 μM LPA for 15 min. Following fixation with 1% paraformaldehyde for 15 min at 4 °C, EGFP images were obtained using a fluorescence microscope (Diaphot, Nikon, Tokyo, Japan). EGFP-positive cells were examined for a round morphology without any neurite extensions or filopodia. At least 20 different fields were observed with a minimum of 100 EGFP-positive cells. The number of rounded cells was expressed as a percentage of the EGFP-positive cells.}
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\text{Quantification of Cell Clustering—The degree of cell clustering was quantified by observing the distribution of the cell nuclei. Cells (1.5} \times 10^5) \text{ were seeded in poly-d-lysine-coated 24-well plates (BD Biosciences). After 24 h of incubation, the cells were washed three times with DMEM containing 0.1% BSA and serum-starved for 24 h. Three hours after a medium change, the cells were treated with 1 μM LPA for 3 h, followed by fixation and staining with a Diff-Quik kit (Kokusai Shiyaku, Kobe, Japan). The distribution of the cells was mapped in rectangular areas (1710 × 1290 μm) by photographing the cultures (Cool Pix 990, Nikon). Each map was overlaid with grids at equal intervals (30 μm) and divided into 2451 unit squares. The randomness in spatial distribution was tested by counting the number of unit squares containing at least one nucleus. The intensity of the cell clustering was expressed as the percentage of the unit squares without any nuclei.}
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\text{Cell Dissociation Assay—The Ca}^{2+} \text{ sensitivity of cell-cell adhesion was estimated using trypsin treatment in the presence of either CaCl}_2 (TC treatment) or EDTA (TE treatment) as described (27, 28), with minor modifications. Briefly, 5} \times 10^5 \text{ cells were seeded in poly-d-lysine-coated 35-mm dishes (BD Biosciences) and cultured overnight. After 24 h of serum starvation, the cells were stimulated with 1 μM LPA for 2 h and washed with HBSS containing either 2 mM CaCl}_2 or 2 mM EDTA. The washed cells were treated with 0.01% trypsin for 30 min at 37 °C and then dissociated by pipetting 10 times gently in 1 ml of HBSS with 0.01% trypsin. The number of cell clusters was counted with a particle counter (Beckman Coulter). The degree of cell-cell adhesion was expressed as the ratio of particles in the TC condition to particles in the TE condition (TC/TE). Negative control experiments without LPA treatment were also performed.}
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\text{Western Blotting—Cells (4} \times 10^6) \text{ were seeded in poly-l-lysine-coated 100-mm dishes. Following 24 h of serum starvation, the cells were treated with 1 μM LPA for 3 h, washed twice with PBS, and harvested in buffer B (25 mM HEPES-NaOH (pH 7.4), 10 mM MgCl}_2, and 0.25 M sucrose). The cells were centrifuged at 800} \times \text{g for 10 min at 4 °C, suspended in ice-cold buffer B containing 20 μM 4-aminophenylmethylsulfonyl fluoride (Sigma) and a protease inhibitor mixture (Complete, Roche Applied Science), and sonicated three times for 30 s each at 4 °C. The cell debris was removed by centrifugation at 800} \times \text{g for 10 min at 4 °C. The protein concentration of the homogenate was determined with a Bradford assay (Bio-Rad) using BSA as a standard. Five micrograms of protein sample containing 5% 2-mercaptoethanol was analyzed by 7.5% SDS-PAGE 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 mouse monoclonal antibody against N-cadherin or E-cadherin (BD Biosciences). The bands were visualized with an ECL chemiluminescence detection system (Amersham Biosciences) using horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences).}
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\text{Immunofluorescence—Cells (3} \times 10^5) \text{ were seeded into poly-l-lysine-coated glass-bottomed 35-mm dishes (Matsunami, Tokyo, Japan) and serum-starved for 24 h. Following stimulation with 1 μM LPA for 3 h at 37 °C, the cells were fixed with 4% paraformaldehyde for 20 min at 4 °C and rinsed twice with ice-cold PBS. Subsequently, the cells were incubated with a mouse monoclonal antibody against N-cadherin in PBS containing 1/4× permeabilization reagent (Beckman Coulter) for 1 h at room temperature. The primary antibody staining was visualized with an Alexa 488-conjugated goat anti-mouse IgG (Invitrogen). Images were obtained using an LSM510 laser-scanning confocal microscope (Carl Zeiss, Jena, Germany) equipped with an argon laser as the light source.}
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Stable Expression of LPA₁ and LPA₄ in B103 Cells Results in Distinct Morphologies—To address the functional roles of LPA₄ in neuronal cells, B103 rat neuroblastoma cells were stably transfected with the expression vectors for LPA₁ or LPA₄, each tagged with an HA epitope at the N terminus. After staining with an anti-HA antibody and a phycocyanin-conjugated secondary antibody, HA-positive cells were sorted with a cell sorter and then subcultured. Data shown are the surface expression levels of the HA epitope in subcultured polyclonal cells obtained by the second round of cell sorting. Empty vector-transfected polyclonal cells served as a negative control. B, morphology of B103-vector, B103-LPA₁, and B103-LPA₄ cells in serum-containing medium. The cells were photographed 24 h after seeding. Each stable cell line showed similar growth rate. Bar, 40 μm.

Statistical Analysis—All values in the figures are expressed as means ± S.E. To determine statistical significance, the values were compared by analysis of variance followed by Tukey-Kramer test using Prism 4 software (GraphPad Software, San Diego, CA). The differences were considered significant if p values were less than 0.05.

RESULTS

Stable Expression of LPA₁ and LPA₄ in B103 Cells Results in Different Morphologies in Serum-containing Medium—To address the functional roles of LPA₄ in neuronal cells, B103 rat neuroblastoma cells were stably transfected with the expression vector for either LPA₁ or LPA₄. B103 cells were selected because they lack endogenous responses to LPA (29, 30). Consistently, no specific binding was observed in B103-vector cells in the radioligand binding assays (see “Experimental Procedures”). To determine the intrinsic gene expression profiles of LPA receptors in B103 cells, we performed a reverse transcription-PCR analysis of total cellular RNA from the cells. Although LPA₄ mRNA expression was slightly detected, no mRNA expression of the other three receptors, LPA₁, LPA₂, and LPA₃, was observed (data not shown). This finding is consistent with a recent report by Tsukahara et al. (31). The apparent discrepancy between the expression of LPA₄ and the lack of response to LPA might occur because the expression of LPA₄ is too low to respond to LPA. Alternatively, post-transcriptional/translational modifications (32) may produce discordance between mRNA and protein expression. Thus, we discounted the low expression of LPA₄ in B103 cells and took advantage of their unresponsiveness to LPA and their neuronal nature for the purpose of examining the functional roles of LPA₄ in neuronal cells.

For the construction of stably transfected cell lines, LPA₁ and LPA₄ were tagged with an HA epitope at the N terminus to enable us to determine the levels of expression on the cell surfaces. Fluorescence-activated cell sorting enriched a polyclonal population of the drug-resistant cells that expressed each LPA receptor. These populations of stable clones are free of any clonal deviation that could cause functional variations. Following two rounds of cell sorting, we observed that the fluorescence intensity of B103-LPA₁ cells was higher than that of B103-LPA₄ cells (Fig. 1A), although the Bₘₐₓ value for B103-LPA₁ cells (0.8 pmol/mg of protein) was lower than that for B103-LPA₄ cells (6.0 pmol/mg of protein). The apparent discrepancy might be because of two possibilities as follows: the usage of organelar membrane-rich microsome fractions and the difference in HA antibody immunoreactivity to the HA epitope tagged to two receptors. To confirm that no expression of the other subtypes of LPA receptors was enhanced secondary to the transfection, reverse transcription-PCR was performed with specific primers for LPA₁, LPA₂, LPA₃, and LPA₄ in B103-vector, B103-LPA₁, and B103-LPA₄ cells. As in the parental B103 cells, we observed only a low expression of LPA₄ and virtually no expression of the other LPA receptors in all of the transfected cell lines (data not shown).

Although these stably transfected cell lines showed similar growth rates (data not shown), they showed distinctly different morphologies in serum-containing medium (Fig. 1B). As reported previously (33), B103-LPA₁ cells displayed a flattened and more migratory morphology compared with B103-vector cells. Interestingly, B103-LPA₄ cells had an epithelial like morphology and appeared to adhere more tightly to each other than B103-vector cells. These observations suggest that LPA₁ and LPA₄ have distinct signaling pathways that produce different cell morphologies.

LPA₄ Does Not Affect Adenylyl Cyclase Activity in B103 Cells—We examined whether LPA₄ mediates the inhibition of adenylyl cyclase activity in B103 cells, as the other three LPA receptors do (30) (Fig. 2A). In B103-LPA₁ cells, LPA caused a dose-dependent inhibition of adenylyl cyclase activity with IC₅₀ values below 10 nM (Fig. 2A). This inhibition was completely
blocked by PTX treatment, indicating the primary role of G\textsubscript{i/o} proteins. However, LPA at concentrations up to 10 \textmu M did not blunt the forskolin-driven rises in cAMP accumulation in either B103-vector or B103-LPA\textsubscript{4} cells, suggesting that LPA\textsubscript{4} does not couple to G\textsubscript{i/o} proteins.

Previously, we reported that LPA induces cAMP accumulation in LPA\textsubscript{4}-expressing Chinese hamster ovary cells (19). However, LPA at concentrations up to 10 \textmu M did not blunt the forskolin-driven rises in cAMP accumulation in either B103-vector or B103-LPA\textsubscript{4} cells, suggesting that LPA\textsubscript{4} does not couple to G\textsubscript{i/o} proteins.

LPA\textsubscript{1} and LPA\textsubscript{4} Mediate Ca\textsuperscript{2+} Mobilization via Distinct Signaling Pathways—LPA has been shown to induce intracellular Ca\textsuperscript{2+} mobilization in many cell types (34), and all EDG family LPA receptor subtypes mediate Ca\textsuperscript{2+} mobilization when expressed in B103 cells (30). We therefore examined whether LPA\textsubscript{4} mediates Ca\textsuperscript{2+} mobilization in B103 cells. Although B103-vector cells displayed no response to 1 \textmu M LPA, increases in [Ca\textsuperscript{2+}], were observed both in B103-LPA\textsubscript{1} and B103-LPA\textsubscript{4} cells (Fig. 3). LPA induces phospholipase C-mediated Ca\textsuperscript{2+} mobilization via the PTX-sensitive G\textsubscript{i/o} and/or PTX-insensitive G\textsubscript{q/11}-mediated pathways (34). To examine the signaling pathways leading to Ca\textsuperscript{2+} mobilization in B103-LPA\textsubscript{1} and B103-LPA\textsubscript{4} cells, we treated the cells with a G\textsubscript{q/11}-selective inhibitor, YM-254890 (23) (Fig. 3). ATP was used as a positive control, because ATP evokes Ca\textsuperscript{2+} mobilization via P2Y receptors predominantly through G\textsubscript{q/11} (35). The LPA-induced Ca\textsuperscript{2+} response in B103-LPA\textsubscript{4} cells and the ATP-induced Ca\textsuperscript{2+} response in both transfected cell lines were completely abolished by pretreatment with 5 \textmu M YM-254890 (Fig. 3). In B103-LPA\textsubscript{1} cells, YM-254890 only partially inhibited the LPA-induced response (Fig. 3), but the combination of PTX and YM-254890 produced complete inhibition (data not shown). The degree of inhibition with YM-254890 in B103-LPA\textsubscript{1} cells was not altered at higher concentrations (up to 20 \textmu M; data not shown), indicating that 5 \textmu M YM-254890 was sufficient to inhibit the activation of G\textsubscript{q/11} proteins. These results suggest that both G\textsubscript{i/o} and G\textsubscript{q/11} proteins mediate Ca\textsuperscript{2+} mobilization in B103-LPA\textsubscript{1} cells, whereas G\textsubscript{q/11} is the dominant mediator of the response in B103-LPA\textsubscript{4} cells.

Both LPA\textsubscript{1} and LPA\textsubscript{4} Mediate Cell Rounding via Rho-dependent and G\textsubscript{i/o}- and G\textsubscript{q/11}-independent Pathways—LPA induces rapid growth cone collapse, neurite retraction, and neuronal cell rounding in several neuronal cell types...
Mouse LPA₁ and LPA₂ and human LPA₁ have been reported to mediate LPA-induced cell rounding in B103 cells (29, 30, 33); we examined whether human LPA₄ also mediates cell rounding in B103 cells by seeding cells at a low cell density (Fig. 4, A and B). Overexpression of LPA₁ and LPA₄ slightly increased the percentages of rounded cells even before LPA application. Within 15 min of LPA stimulation, about 80% of B103-LPA₄ cells became rounded and underwent neurite retraction (Fig. 4A). Cell rounding was observed in B103-LPA₁ cells as reported previously (33), but to a lesser degree than in B103-LPA₄ cells. LPA-induced cell rounding was not observed in B103-vector cells.

The role of Rho in LPA-induced cell rounding is now well established (36), and the G₁₂/₁₃ types of heterotrimeric G proteins are known to be upstream activators of Rho proteins (1, 2, 7). On the other hand, there are reports that G₉/₁₁ activation induces cell rounding through Rho-dependent (37) and -independent (38) pathways. To determine which G proteins and signaling molecules are involved in LPA-induced cell rounding, we pretreated the cells with PTX, YM-254890, and a ROCK inhibitor, Y-27632 (Fig. 4B). In B103-LPA₄ cells, neither PTX nor YM-254890 inhibited LPA-induced cell rounding; in contrast, Y-27632 completely inhibited this morphological change. Y-27632 also hampered LPA-induced cell rounding in B103-LPA₁ cells, whereas YM-254890 did not affect the number of rounded cells. Interestingly, pretreatment with PTX increased the degree of LPA-induced cell rounding in B103-LPA₁ cells. To confirm the involvement of Rho, B103-LPA₄ cells were transfected with C3 exoenzyme, which inactivates Rho by ADP-ribosylation. The transfected cells were identified by cotransfection of an EGFP expression construct. C3 exoenzyme transfection blunted LPA-induced cell rounding in B103-LPA₄ cells, again indicating the involvement of Rho (Fig. 4C).

**LPA₄ Mediates ROCK-dependent Cell Aggregation**—As described earlier, B103-LPA₄ cells appeared to form aggregates in serum-containing medium to a greater extent than B103-vector cells (Fig. 1B). To determine whether the binding of LPA to LPA₄ mediates the induction of cell-cell adhesion, B103-LPA₄ cells at a medium cell density were stimulated with 1 μM LPA after 24 h of serum starvation. Although the rapid cell rounding after LPA application was difficult to evaluate at this cell density because of the formation of cell-cell contacts, LPA caused a slow but dramatic aggregation in B103-LPA₄ cells (Fig. 5A, panel f). The morphological change observed in B103-LPA₄ cells was transient, reaching a maximum 2–3 h after the treatment and then returning to the base line 24 h after the treatment (data not shown).

To investigate the signaling pathways downstream of LPA₄ that are involved in the cell aggregation, we treated B103-LPA₄ cells with several inhibitors. The LPA-induced morphological changes in B103-LPA₄ cells were completely prevented by Y-27632 (Fig. 5A, panel o). In contrast, neither PTX nor YM-254890 inhibited the cell aggregation (Fig. 5A, panels i and l). We quantified the degree of cell aggregation by examining the randomness in the spatial distribution of the cells (see the “Experimental Procedures”; Fig. 5B). These results suggest that...
Rho mediates LPA-induced cell aggregation in B103-LPA$_4$ cells in a G$_{q/11}$- and G$_{i/o}$-independent manner. Rho regulates the reorganization of the actin cytoskeleton, which can modify the intensity of adhesion (28, 39). To examine whether the reorganization of the actin cytoskeleton was involved in this effect, B103-LPA$_4$ cells were pretreated with cytochalasin D (an inhibitor of actin polymerization). In these cells, morphological changes were not observed after LPA stimulation, indicating that actin reorganization is involved in the LPA-induced cell aggregation (data not shown). Like B103-LPA$_4$ cells, PTX-
treated B103-LPA\(_4\) cells became aggregated after LPA stimulation (Fig. 5A, panel h).

**LPA\(_4\) Mediates N-cadherin-dependent Cell-Cell Adherence**—Through LPA-induced cell aggregation, B103-LPA\(_4\) cells formed tightly compact aggregates (Fig. 5A, panel f), which dissociated very little after pipetting (data not shown). Cell-cell adhesion mechanisms can be Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent, and cadherins are the major components of the Ca\(^{2+}\)-dependent system. Cadherin-dependent adhesion was originally defined as being trypsin-resistant in the presence of Ca\(^{2+}\) and trypsin-sensitive in the absence of Ca\(^{2+}\) (40). We examined whether LPA-induced cell adhesion was mediated by cadherins using a cell dissociation assay, one of the adhesion assays for the evaluation of the cadherin activity (27, 28). We defined the TC/TE index as a ratio of the cell particle number after trypsin treatment in the presence of Ca\(^{2+}\) (TC) to the number after trypsin treatment with EDTA (TE). Cadherin-dependent adhesion remains after trypsin-Ca\(^{2+}\) treatment, whereas trypsin-EDTA treatment disrupts cell adhesion nearly completely. In either treatment, an increase in particles would occur when a large aggregate breaks into small particles by pipetting; the higher the number of particles, the lower the aggregation (adhesion). Thus, the TC/TE index is negatively correlated with cadherin-mediated adhesion. The aggregation level of B103-LPA\(_4\) cells increased after LPA stimulation (Fig. 6A; note that the index inversely reflects cadherin activity). LPA treatment did not significantly affect the TC/TE index in either B103-vector or B103-LPA\(_4\) cells. These results suggest that LPA increased the cadherin-mediated adhesive activity in B103-LPA\(_4\) cells. Even LPA-untreated B103-LPA\(_4\) cells had significantly more cadherin-dependent adhesion activity, i.e. a lower TC/TE index, than LPA-untreated B103-vector and B103-LPA\(_4\) cells (Fig. 6A).

The cadherins constitute a large superfamily of molecules that includes the classic cadherins, the desmosomal cadherins, the protocadherins, and the cadherin-like signaling receptors (41). The levels of the two classic cadherins most commonly expressed in the nervous system, N- and E-cadherin, were determined by Western blotting of LPA-treated or -untreated B103 cells. Consistent with a previous report (42), these cells abundantly expressed N-cadherin (Fig. 6B), whereas E-cadherin was undetectable (data not shown). The expression level of N-cadherin was not up-regulated by LPA treatment in any of the transfected cell lines (Fig. 6B), and N-cadherin was intact in the cells undergoing TC treatment. In contrast, TE treatment resulted in complete digestion of N-cadherin (data not shown), as reported previously (43). We next examined whether LPA increases N-cadherin-mediated cell-cell contacts. LPA promoted the assembly of N-cadherin in the form of a thick, bright band at the cell-cell contact area in B103-LPA\(_4\) cells but not in B103-vector cells (Fig. 6C).

**DISCUSSION**

A number of studies have shown that LPA mediates morphological changes in neuronal cells through the Rho-ROCK pathway (10, 11, 44). It has been proposed that these effects are mediated by LPA\(_1\) and/or LPA\(_2\) (10, 11, 44). Recently, we identified p2\(_y_9\)/GPR23 as a fourth LPA receptor (LPA\(_4\)) that is structurally distinct from the EDG family of LPA receptors (19). The expression of LPA\(_4\) in neuronal cells implies a significant role for this receptor in the nervous system (18). The results in this study demonstrate that LPA\(_4\) caused morphological changes in B103 neuronal cells, including cell rounding and N-cadherin-associated cell aggregation, both of which were mediated by the Rho-ROCK pathway.

Ca\(^{2+}\) mobilization and adenylyl cyclase inhibition are the major cellular responses to LPA (45). When expressed in B103
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neuronal cells, each of the EDG family LPA receptors, LPA₁, LPA₂, and LPA₃, mediates both of these reactions (30). LPA₁ is likely to mediate the Ca²⁺ response through Gq/11 proteins (Fig. 3). The PTX-sensitive inhibition of adenyl cyclase (Fig. 2A) suggests that LPA₁ also couples to G_i/o. We showed that LPA₄ mediates the Ca²⁺ response in B103 cells (Fig. 3). This is consistent with our previous report (19) that the stable expression of LPA₄ in Chinese hamster ovary cells significantly enhanced the LPA-induced Ca²⁺ mobilization. From our results using YM-254890, LPA₄ probably mediates Ca²⁺ mobilization through Gq/11 proteins (Fig. 3). In contrast, LPA did not inhibit adenyl cyclase in B103-LPA₄ cells (Fig. 2A). These results indicate that unlike the other LPA receptor subtypes, LPA₄ does not couple to G_i/o proteins.

Neurite retraction and neurite formation play a role in the remodeling of neurons for guidance and synaptic plasticity (46). Neurite retraction in neuronal cells is induced by lysophospholipids, including LPA and sphingosine 1-phosphate (S1P), in addition to semaphorins, netrins, and ephrins (10, 11, 47). LPA induces neurite retraction through LPA₁ or LPA₂ when expressed in B103 cells (30). In this study, we showed that 1 μM LPA induced cell rounding in B103-LPA₄ cells (Fig. 4A). Sugiuira et al. (48) reported that rat brain contains 3.73 nmol of LPA/g of tissue. These results suggest a role for LPA₄ in LPA-induced neurite retraction. Neurite initiation and formation involve actin cytoskeletal changes, and as a regulator of actin reorganization, Rho GTPase has a profound effect on neuritogenesis (47). For example, S1P induces Rho-dependent neurite retraction through the S1P₂ (49, 50), S1P₃ (49), and S1P₅ receptors (50, 51). Several studies have also revealed a critical role for Rho and ROCK in LPA-induced neurite retraction (36, 52, 53), although some studies have reported Rho-independent neurite retraction (38, 54). Judging from its complete inhibition by C3 exoenzyme and Y-27632, the cell rounding induced by LPA₄ depended on Rho and ROCK in B103-LPA₄ cells (Fig. 4, B and C).

In general, activation of G₁₂/₁₃ proteins leads to an increase in RhO guanine nucleotide exchange, activation of ROCK, and actin polymerization (55, 56), although some studies have implied that Gq/11 proteins can also activate Rho (57, 58). Based on our results, it is conceivable that the G₁₂/₁₃ proteins are upstream regulators of Rho in B103-LPA₁ cells and B103-LPA₄ cells, because YM-254890 inhibited cell rounding in both cell types (Fig. 4B). In many studies reporting the induction of neurite retraction by LPA (10), the LPA receptor subtypes responsible have not been identified, or LPA₁ and LPA₂ were suggested as candidate subtypes based on mRNA expression in the neuronal cells examined (59–62). This study suggests the possibility that LPA₄ was involved in the neurite retraction in some of these studies.

LPA has been shown to stimulate cell motility and to modulate tumor cell invasion, both of which are mediated mainly by LPA₁ and G_i/o proteins (33, 63, 64). In the presence of serum, B103-LPA₄ cells exhibited a flattened morphology and were widely dispersed throughout the dish (Fig. 1B) (33). This morphological phenotype was probably evoked by LPA through the LPA₁-G_i/o-Rac signaling axis (33). In sharp contrast to B103-LPA₁ cells, B103-LPA₄ cells formed cell aggregates in serum-containing medium (Fig. 1B), apparently through activation of the G₁₂/₁₃-Rho-ROCK signaling axis (Fig. 5). This cell-cell adhesion involved N-cadherin without de novo synthesis (Fig. 6). Because Rho affects cadherin-dependent adhesion through actin cytoskeleton reorganization (28), we presume that LPA-induced cytoskeletal changes affect the subcellular distribution of N-cadherin, as shown in Fig. 6C, leading to strong cell-cell adhesion in B103-LPA₄ cells. This is supported by the current results that treatment with Y-27632 and cytochalasin D abolished the LPA-induced cell aggregation (Fig. 5 and data not shown). N-cadherin is widely expressed in the nervous system and has critical roles in neural development and functions, including synapse formation and myelination. Weiner et al. (65) previously reported that LPA induced cell-cell junctions containing N-cadherin in rat Schwann cells. Furthermore, LPA was reported to induce cell clustering in neural progenitor cells prepared from embryonic rat hippocampus (66) and in mouse postmitotic cortical neurons (16). Our results raise the possibility that in addition to LPA₁ and LPA₂, LPA₄ might also be involved in these effects in neural cells and have critical roles in the development and function of the nervous system. In contrast to LPA₁ and LPA₂, which activate Rac through G_i/o (17, 64), LPA₄ is unlikely to activate the G_i/o-Rac pathway because LPA₄ did not inhibit adenyl cyclase activity (Fig. 3A). Therefore, LPA₄ might have a unique role in keeping a proper balance between Rho and Rac activation, which is important for neuronal development and function (47).

We observed that PTX significantly enhanced the intensity of LPA₁-mediated cell rounding (Fig. 4B). This "permissive effect" is consistent with a previous report that PTX enables LPA-induced cell rounding in 1321N1 astrocytoma cells (61). It is known that Rho activity is inhibited by Rac activation through G_i/o proteins (67). Indeed, Rac activation functionally antagonizes Rh-mediated neurite retraction in 1321N1 astrocytoma cells (61). LPA₁ was shown to couple to G_i/o and activate Rac strongly in B103 cells (33) and other cells, including mouse embryonic meningeal fibroblast (MEMF) and mouse skin fibroblast (MSF) cells (17, 64). Taken together, we suggest that PTX treatment of B103-LPA₄ cells suppresses G_i/o proteins and subsequently suppresses Rac activation by LPA, which in turn permits Rho-mediated cell rounding. This mechanism probably also accounts for the LPA-induced aggregation of PTX-treated B103-LPA₄ cells (Fig. 5, A, panel h, and B).

We showed here that LPA₄ has Rho-dependent morphological effects. It has been reported that LPA-induced Rho activation is mediated by LPA₁ and/or LPA₄. However, pathways independent of LPA₁ and LPA₂ have also been proposed. Conitos et al. (17) showed that MEMF cells from LPA₁ and LPA₂ double knockouts remained capable of forming stress fibers in response to LPA. This study proposed the presence of unknown LPA receptors in MEMF cells because of the absence of LPA₃ mRNA. Consistent with this, Hama et al. (64) reported that LPA activates Rho in MSF cells from LPA₁ and LPA₂ double knockouts. Our results, together with the abundant expression of LPA₄ in MSF cells (64), suggest that LPA₄ may also be involved in LPA-induced Rho activation in these cells. Furthermore, Hama et al. (64) observed that Rac activation was totally
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In summary, as shown in Fig. 7, we demonstrate for the first time that the novel LPA receptor subtype LPA<sub>4</sub> is coupled to the activation of Rho in a rat neuronal cell line. The activation of Rho through LPA<sub>4</sub> leads to morphological changes, including cell rounding and cell aggregation. LPA<sub>4</sub> is well known to induce neurite retraction and cell clustering in neural cells. The identification of Rho as an effector of LPA<sub>4</sub> will give insight into some of the physiological and morphological effects of LPA that could not be explained by the EDG family LPA receptors. A full understanding of the potential roles of the endogenous LPA<sub>4</sub> receptor in the development and function of the nervous system awaits future studies.

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