

Functional Comparisons of the Lysophosphatidic Acid Receptors, LP_{A1}/VZG-1/EDG-2, LP_{A2}/EDG-4, and LP_{A3}/EDG-7 in Neuronal Cell Lines Using a Retrovirus Expression System

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ABSTRACT

Lysophosphatidic acid (LPA) is a potent lipid mediator with diverse physiological actions on a wide variety of cells and tissues. Three cognate G-protein-coupled receptors have been identified as mammalian LPA receptors: LP_{A1}/VZG-1/EDG-2, LP_{A2}/EDG-4, and LP_{A3}/EDG-7. The mouse forms of these genes were analyzed in rodent cell lines derived from nervous system cells that can express these receptors functionally. An efficient retrovirus expression system was used, and each receptor was heterologously expressed in B103 rat neuroblastoma cells that neither express these receptors nor respond to LPA in all assays tested. Comparative analyses of signaling pathways that are activated within minutes of ligand delivery were carried out. LPA induced cell rounding in LP_{A1}- and LP_{A2}-

expressing cells. By contrast, LP_{A3} expression resulted in neurite elongation in B103 cells and inhibited LPA-dependent cell rounding in TR mouse neuroblast cells that endogenously express LP_{A1} and LP_{A2} but not LP_{A3}. Each of the receptors could couple to multiple G-proteins and induced LPA-dependent inositol phosphate production, mitogen-activated protein kinase activation, and arachidonic acid release while inhibiting forskolin-induced cAMP accumulation, although the efficacy and potency of LPA varied from receptor to receptor. These results indicate both shared and distinct functions among the three mammalian LPA receptors. The retroviruses developed in this study should provide tools for addressing these functions in vivo.

Lysophosphatidic acid (LPA; 1-acyl-2-*sn*-glycerol-3-phosphate) is a simple, yet potent, lipid mediator that exerts diverse biological effects on many types of cells and tissues. It influences fundamental cellular processes, which include proliferation, differentiation, survival, and actin-based cytoskeletal alterations, and it has been proposed to be involved in several clinical disorders, including ovarian cancer and atherosclerosis (Moolenaar, 1995, 1997, 1999). On a biochemical level, LPA activates various signaling cascades, including activation of the small GTPase Rho, phospholipase C (PLC), mitogen-activated protein kinase (MAP kinase), and phosphoinositide 3-kinase (Moolenaar, 1995, 1997). In addition, LPA treatment can result in inhibition of adenylyl cyclase activation. Recent progress in understanding the mechanisms through which LPA exerts its effects has been accelerated by the identification of several G-protein-coupled receptors that can account for these LPA-dependent effects (Chun, 1999; Chun et al., 1999).

The identification of mouse and human LP_{A1}/VZG-1/EDG-2 as the first LPA receptor (Hecht et al., 1996; An et al., 1997) has been complemented by the identification of two related human LPA receptors: LP_{A2}/EDG-4 (An et al., 1998a) and LP_{A3}/EDG-7 (Bandoh et al., 1999; Im et al., 2000). We have independently characterized mouse genomic DNA for *lp*_{A2} (Contos and Chun, 2000) and *lp*_{A3} (J. J. A. Contos and J. Chun, in preparation). Genomic analyses of these three genes in mice demonstrate high amino acid identity (47.7–51.9%) and similarity (57.6–60.9%) among the three, as well as conserved intron-exon boundaries in their genomic structures, all of which are consistent with a common evolutionary origin. Contrasting with the three highly related LP_A receptors, a dissimilar receptor cloned from *Xenopus*, PSP24, was reported to be a high-affinity LPA receptor (Guo et al., 1996). However, the functional and physiological roles of this protein in mediating LPA signals in mammals requires clarification and thus was not examined in this study.

When one considers that a single LP_{A1} receptor can mediate multiple cellular responses (Fukushima et al., 1998), the existence of LP_{A2} and LP_{A3} receptors raises the question of

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ABBREVIATIONS: LPA, lysophosphatidic acid; EGFP, enhanced green fluorescent protein; IBMX, 3-isobutyl-1-methylxanthine; MAP kinase, mitogen-activated protein kinase; ORF, open reading frame; PBS, phosphate-buffered saline; PLC, phospholipase C; PMA, phorbol-12-myristate-13-acetate; PTX, pertussis toxin; BSA, bovine serum albumin; PHAS-I, phosphorylated heat- and acid-stable protein-I.

how these receptors compare functionally to one another. We have cloned cDNAs for LP_{A2} and LP_{A3} from a mouse testis cDNA library (J. J. A. Contos and J. Chun, in preparation). In the current study, each of the mouse receptor genes was assayed using heterologous and overexpression approaches. We selected murine neuronal cell lines to characterize the receptors because previous work indicated that the nervous system is one of the major loci of LP_{A1} receptor expression and function (Chun, 1999), and both LP_{A2} and LP_{A3} receptor genes are also expressed in the developing brain (J. J. A. Contos and J. Chun, in preparation). This was made possible by the prior identification of the B103 rat neuroblastoma cell line that does not express LP_{A1} (Fukushima et al., 1998), LP_{A2} (Chun et al., 1999), or LP_{A3} receptors (J. J. A. Contos and J. Chun, unpublished observation) and it does not respond to LPA in guanosine 5'-[γ -thio]triphosphate binding, cell rounding, serum-responsive element activation, and bromodeoxyuridine incorporation (Fukushima et al., 1998). This cell line does express several heterotrimeric G-protein α -subunits thought to couple to these LPA receptors, including $G_{\alpha_{i/o}}$, $G_{\alpha_{q/11}}$, and $G_{\alpha_{13}}$ (Fukushima et al., 1998). Importantly, we report the use of a retroviral expression system that provides several advantages over transfection strategies: very high efficiency expression (approaching a 10-fold increase over previously used transfection efficiencies; Fukushima et al., 1998), single-copy/cell infection (Kang, 1995) driven by a common promoter, the ability to infect tissues in vivo, and minimal cell death and damage compared with that encountered during standard transfection approaches. Here we show comparative similarities and differences in signaling responses for all three mammalian LPA receptors.

Experimental Procedures

Materials. [γ - ^{32}P]ATP, [α - ^{32}P]deoxy-CTP, myo-[2- 3H (N)]inositol, [3H]arachidonic acid, and ^{125}I -cAMP were purchased from NEN Life Science Products (Boston, MA). LPA (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate) was purchased from Avanti Polar-Lipids (Alabaster, AL). Pertussis toxin (PTX), phorbol-12-myristate-13-acetate (PMA),

U-73122, U-73433, and anti-cAMP polyclonal antibody were purchased from Calbiochem (La Jolla, CA). B103 rat noncortical neuroblastoma cells (Schubert et al., 1974) were gifts from Dr. David Schubert (The Salk Institute, La Jolla, CA). Retrovirus expression vector (LZRS-EGFP) and Phoenix ecotropic retrovirus producer cell lines were gifts from Dr. Garry P. Nolan (Stanford University, Stanford, CA). Y-27632 was a gift from Yoshitomi Pharmaceutical Industries (Saitama, Japan). The MAP kinase assay kit was purchased from Stratagene (La Jolla, CA). Trizol and all cell culture reagents were purchased from Life Technologies (Rockville, MD). Forskolin, 3-isobutyl-1-methylxanthine (IBMX), puromycin, anti-FLAG M2 monoclonal antibody, and other reagents were purchased from Sigma (St. Louis, MO), unless otherwise noted.

Construction of Retrovirus Vectors and Production of Retrovirus Supernatants. Mouse cDNA for lp_{A1} was cloned as described (Hecht et al., 1996). Mouse cDNAs for lp_{A2} (Contos and Chun, 2000) and lp_{A3} (the deposited GenBank accession no. AF293845; J. J. A. Contos and J. Chun, in preparation) were cloned from Mouse Marathon-Ready testis cDNA from Clontech (Palo Alto, CA). The entire open reading frame (ORF) of each receptor was subcloned into the pFLAG-CMV-1 mammalian expression vector (Eastman Kodak Co., Rochester, NY) for introducing preprotrypsin-leader and FLAG-tag sequences into an extracellular amino terminus of each receptor. These sequences enabled higher expression levels of the receptors in the plasma membrane (data not shown) and immunohistochemical detection of the receptors, respectively. Next, all coding sequences were cloned into a Moloney murine leukemia retrovirus vector, LZRS-EGFP (Dardalhon et al., 1999), and complete inserts of the construct were confirmed by sequencing. The sequences of all constructs are the same except for the ORFs. Proviral organization of each construct is shown in Fig. 1A. The internal ribosome entry site sequence permitted concomitant expression of the target gene and enhanced green fluorescent protein (EGFP) gene from a single transcript driven by a single 5'-long terminal repeat promoter. Retroviral supernatants were prepared following the Nolan laboratory protocols (Pear et al., 1997). Each construct was transfected into ecotropic Phoenix producer cells with DNA-calcium phosphate coprecipitation methods. After 2 weeks in culture under puromycin (2 μ g/ml) selection, high-titer and helper-free retrovirus supernatants were obtained and stored at $-80^{\circ}C$ until use.

Cell Culture and Retrovirus Infection. B103 cells were maintained as a monolayer culture on tissue culture dishes in Dulbecco's

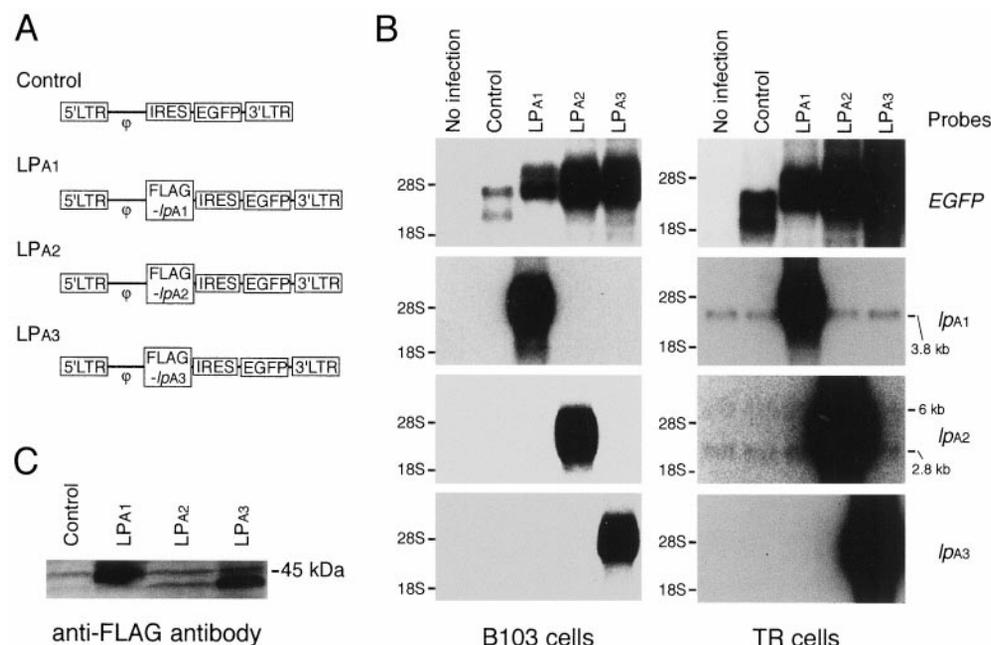


Fig. 1. A retrovirus expression system. A, schematic proviral organization of retroviruses used. LTR, long terminal repeat; ψ , retrovirus-packaging signal; IRES, internal ribosome entry site. B, Northern blot analysis of total RNA isolated from B103 or TR cells infected with retroviruses. Total RNA (15 μ g/lane) on nitrocellulose membranes was hybridized with specific probes for EGFP, lp_{A1} , lp_{A2} , or lp_{A3} (28S and 18S indicate the positions of rRNAs, 4.7 kb and 1.9 kb, respectively). Endogenous transcripts of lp_{A1} (3.8 kb) and lp_{A2} (6 and 2.8 kb) are indicated. C, Western blot analysis with anti-FLAG antibody of the cell extracts from the infected TR cells.

modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT) and antibiotics. TR mouse neocortical neuroblast cells (Chun and Jaenisch, 1996) were maintained as a monolayer culture in Opti-MEM I reduced-serum medium supplemented with 2.5% heat-inactivated fetal calf serum, 20 mM glucose, 55 μ M 2-mercaptoethanol, and antibiotics. The viral supernatant supplemented with 5 μ g/ml hexadimethrine bromide was added to the media of the cells on multiwell dishes, and the dishes were centrifuged (700g) at 32°C for 1 h (referred to later as the centrifugation method). The cells were cultured for 24 h, serum-starved for another day, and then used for each experiment. Effective infection was confirmed by EGFP fluorescence of the infected cells. For the immunostaining shown in Fig. 2A, cells were seeded onto glass coverslips (12-mm diameter) coated with 1.5 μ g of Cell-Tak (Becton Dickinson Labware, Bedford, MA) according to manufacturer's protocols.

Northern Blot Analysis. Cells on six-well dishes were washed once with phosphate-buffered saline (PBS) and solubilized in 1 ml of Trizol. Total RNA was isolated following the instructions by Life Technologies, and 15 μ g of each RNA were separated on 6% formaldehyde/1% agarose gels. After transferring to a GeneScreen Plus Hybridization Transfer membrane (NEN), hybridization was performed at 55°C for 16 h in the hybridization buffer [25% (v/v) formamide, 5% SDS, 1% bovine serum albumin (BSA), 0.5 M Na₂HPO₄, 1

mM EDTA, and 100 μ g/ml salmon sperm DNA]. The specific probes used were the *NheI-XhoI* fragments of the pEGFP-Tub vector (Clontech) for EGFP and the entire ORF cDNA for *lp_{A1}*, *lp_{A2}*, and *lp_{A3}*. The probes were radiolabeled with [³²P]-deoxy-CTP by conventional random primer-labeling methods.

Western Blot Analysis. TR cells on six-well dishes were washed once with PBS and then solubilized in the sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 6% (w/v) 2-mercaptoethanol, and 10% (w/v) glycerol]. The sample was sonicated three times for 5 s each with 10-s intervals on ice, using the Micro-ultrasonic cell disrupter (KONTES, Vineland, NJ) with the following settings: power 4, tune 4. The sonicated sample was separated on a 10% SDS-polyacrylamide gel and transferred to the Protran nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The FLAG-tagged proteins were detected with anti-FLAG antibody using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and ECL Plus detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunostaining. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% (w/v) Triton X-100/10% (v/v) normal goat serum (Vector) in PBS. EGFP protein was detected with anti-GFP polyclonal antibody (Clontech) and fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody (Vector). FLAG-tagged receptor was detected with anti-FLAG antibody and Cy3-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West

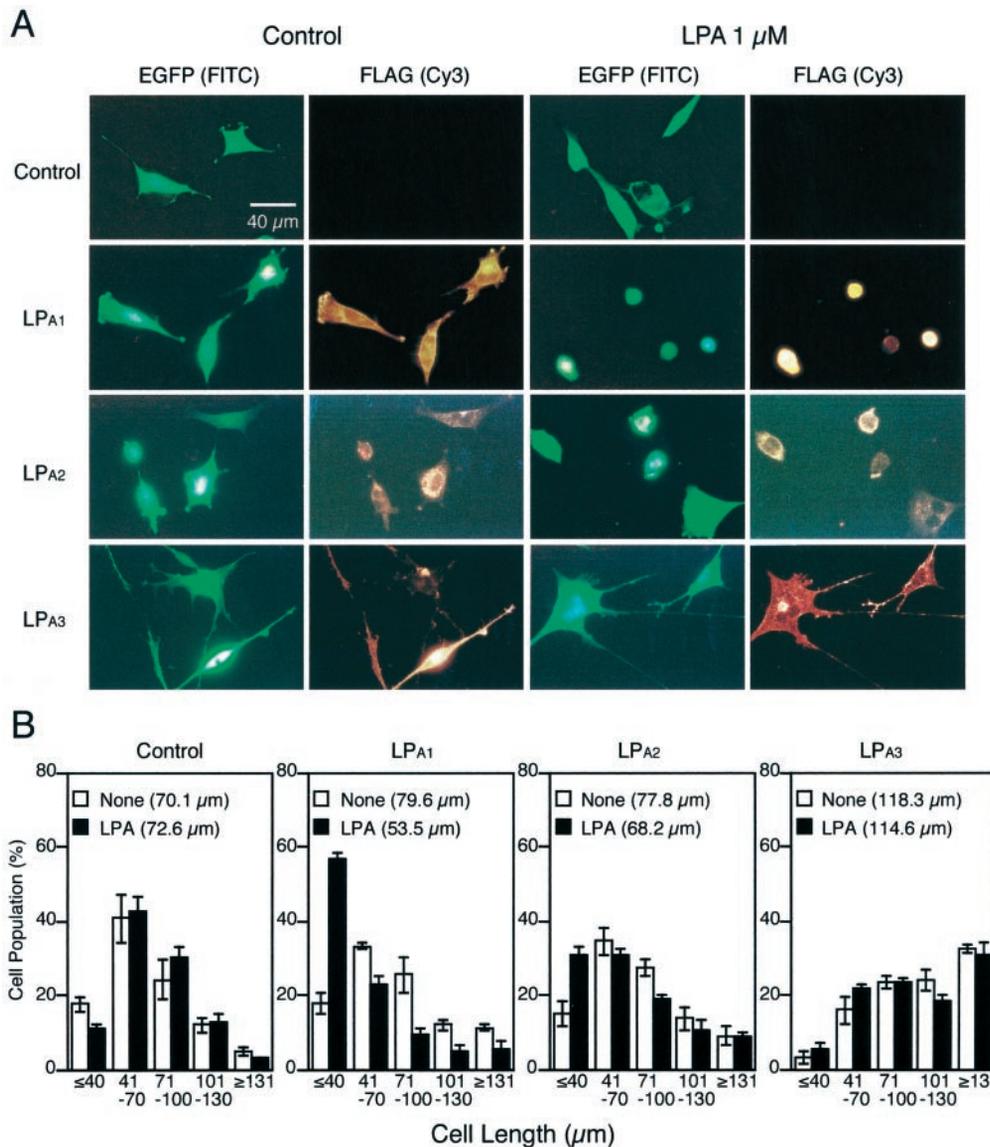


Fig. 2. Microscopic analysis of B103 cells infected with retroviruses. A, immunostaining for detection of EGFP and FLAG-tagged receptor expression. Cells on coated coverslips were treated with 1 μ M LPA for 15 min, fixed, and stained with anti-GFP antibody (fluorescein isothiocyanate) and anti-FLAG antibody (Cy3). B, measurement of cell length. Cells on 24-well dishes were stimulated with 1 μ M LPA for 15 min, fixed, and stained with anti-GFP antibody. Cell lengths of >200 EGFP-positive cells were measured on each coverslip and percentages in five size pools (≤ 40 , 41–70, 71–100, 101–130, and ≥ 131 μ m) were determined; 100% = total cells counted. Bar graph data are the means \pm S.E. of triplicate samples from the representative experiment. Average cell lengths are shown in parentheses.

Grove, PA). The cells were observed under a Zeiss Axiovert S100 or Axioplan 2 microscope with Zeiss Fluor 20× or Plan-Apochromat 63× oil-immersion objective lens and the fluorescent images were taken with MC100 microscope cameras (Carl Zeiss, Thornwood, NY). To measure cell length, the infected cells on 24-well dishes were stimulated with LPA, fixed, and stained with anti-GFP antibody. The fluorescent images of the cells were collected into a Apple Power Macintosh G4 computer with DEI-47 cooled charge-coupled device color camera (Carl Zeiss) and the Scion Image software (Scion Corp., Frederick, MD). The cell lengths were measured using the Scion Image, and percentages in five size pools (≤ 40 , 41–70, 71–100, 101–130, and ≥ 131 μm) were determined with 100% as total cells counted (>200 EGFP-positive cells on each well). The cells with ≤ 40 - μm cell lengths are not necessarily the rounded cells.

PLC Assay. B103 cells on 12-well dishes were prelabeled with [^3H]inositol (2 $\mu\text{Ci}/\text{well}$) for 24 h in inositol-free Dulbecco's modified Eagle's medium. The cells were then incubated for 30 min in Hepes/Tyrode's/BSA buffer (Ishii et al., 1997) containing 10 mM LiCl and stimulated with LPA. After a 15-min incubation, the reaction was terminated by aspirating the buffer and adding 500 μl of ice-cold 0.4 M HClO_4 . After standing on ice for 20 min, the 400- μl supernatant was neutralized with 200 μl of 0.72 N KOH/0.6 M KHCO_3 . The precipitated material was removed by centrifugation, and the supernatant was applied to an AG anion exchange column (1-X8, 100–200 mesh, formate form from Bio-Rad Laboratories, Hercules, CA; Ishii et al., 1997). Inositol phosphate fractions ($\text{IP}_1 + \text{IP}_2 + \text{IP}_3$) of the samples were eluted with the stepwise gradients of ammonium formate (Berridge et al., 1983).

Measurement of Intracellular cAMP Contents. B103 cells on 24-well dishes were incubated in Hepes/Tyrode's/BSA buffer containing 0.5 mM IBMX for 20 min. The cells were stimulated for 20 min with or without 1 μM forskolin in the presence or absence of various concentrations of LPA. The reaction was terminated by aspirating the buffer and adding 250 μl of 0.1 N HCl. After a 20-min extraction, supernatant was collected for the determination of cAMP contents with a radioimmunoassay using anti-cAMP antibody and [^{125}I]cAMP, following Calbiochem protocols.

MAP Kinase Assay. B103 cells on 12-well dishes were stimulated by LPA or PMA. After a 10-min stimulation, the cells were lysed in the lysis buffer [20 mM Tris-HCl (pH 8.0), 20 mM β -glycerophosphate, 1 mM sodium orthovanadate, 2 mM EGTA (pH 8.0), 2 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride] and quickly frozen with liquid nitrogen. The cell extract was assayed for its activity to phosphorylate-specific MAP kinase substrate, PHAS-I protein (Haystead et al., 1994), following the protocols of Stratagene. The radioactivity in PHAS-I 21-kDa proteins on SDS-polyacrylamide gels was measured using the AMBIS radioanalytic imaging system (AMBIS Systems, San Diego, CA).

Arachidonic Acid Release. B103 cells on 12-well dishes were prelabeled for 24 h with [^3H]arachidonic acid (0.2 $\mu\text{Ci}/\text{well}$) in Dulbecco's modified Eagle's medium. The cells were subjected to a 20-min arachidonic acid release assay as described previously (Ishii et al., 1997, 1998).

Statistical Analysis. Results shown are representative of at least three experiments. Data are the means \pm S.E. of the triplicate samples. Statistical analysis was performed by ANOVA and a post hoc analysis was done with the Fisher's protected least significant difference test using StatView software (Abacus Concepts, Berkeley, CA).

Results

Establishment of a Retrovirus Expression System. Mouse lp_{A1} , lp_{A2} , and lp_{A3} cDNAs were epitope tagged with FLAG sequences at the extracellular amino terminus, and the constructs were introduced into the retroviral vector. Ecotropic Phoenix packaging cell lines were used to obtain

high-titer, helper-free retroviral supernatants that coexpressed a given LPA receptor with EGFP (but not a LPA receptor-EGFP fusion protein), providing identification of receptor expression in living and fixed cells using fluorescence microscopy. These retroviruses were used to express each of the three LP_A receptors in two neuronal cell lines: B103 rat neuroblastoma cells that were derived from noncortical regions (Schubert et al., 1974) and TR mouse neuroblast cells that were derived from the cerebral cortex (Chun and Jaenisch, 1996).

To ascertain receptor gene expression, total RNA was isolated from infected B103 or TR cells and analyzed by Northern blot (Fig. 1B). B103 cells do not endogenously express any of the lp_A genes, whereas TR cells express low levels of lp_{A1} and lp_{A2} but not lp_{A3} [single 3.8-kb lp_{A1} transcript and two lp_{A2} transcripts (2.8 and 6 kb) in uninfected TR cells]. Reverse transcription-polymerase chain reaction confirmed the results obtained with TR cells (data not shown). Infection of both cells with each retrovirus led to the overexpression of the desired LPA receptor gene with the EGFP gene (Fig. 1B).

To ascertain receptor protein expression, Western blot analysis demonstrated bands approximating the expected sizes (41–44 kDa) containing FLAG epitopes from extracts of cells infected with each receptor virus but not in cells infected with control virus (a minor band near 45 kDa is nonspecific; Fig. 1C). Molecular masses predicted from amino acid sequences were 43.5, 41.3, and 43.8 kDa for FLAG- LP_{A1} , FLAG- LP_{A2} , and FLAG- LP_{A3} , respectively. Differences between the predicted and observed molecular masses of each receptor may derive from post-translational modification of the receptors [e.g., glycosylation, because each of the LPA receptors has one or two N-glycosylation consensus sequences (N-X-S/T) in their amino-terminal extracellular domains]. Protein expression was further ascertained by double immunolabeling with anti-GFP and anti-FLAG antibodies (e.g., Fig. 2A). In both assays (Figs. 1C and 2A), expression levels of LP_{A2} protein seemed to be lower than those of LP_{A1} or LP_{A3} , but their expression was enough to mediate various LPA responses (Figs. 3–6). EGFP expression was observed within the entire cell bodies and processes. Infection using the centrifugation method for retroviral delivery (see *Experimental Procedures*) routinely produced expression in 70 to 90% of the total cells in culture based on detection of coexpressed EGFP and FLAG-tagged receptor.

LP_{A1} and LP_{A2} , but Not LP_{A3} , Mediate LPA-Induced Cell Rounding. B103 cells heterologously expressing each receptor were treated with 1 μM LPA for 15 min, stained for EGFP and FLAG epitope, and then observed for morphological changes compared with controls (Fig. 2A). Percentages of infected cells that had rounded morphology were determined in both untreated and LPA-treated samples. Infection with LP_{A1} or LP_{A2} virus slightly increased the population of the rounded cells before exogenous LPA application ($\leq 10\%$; Fig. 3A). LPA application induced cell rounding in cells expressing LP_{A1} or LP_{A2} in a concentration-dependent manner (EC $_{50}$ approximately 10 nM; Fig. 3A, left). However, infection of B103 cells with LP_{A3} virus resulted in neurite elongation (Fig. 2B), and LPA-induced cell rounding was not observed (Fig. 3A). Expression of LP_{A3} significantly increased both the number of cells with elongated neurites and the average cell length (Fig. 2B). The population of LP_{A3} -expressing cells with elongated (≥ 131 μm) neurites increased more than

6-fold compared with controls, whereas the average length of these cells increased more than 60% (Fig. 2B). Neither parameter was affected by LPA application in LP_{A3}-expressing cells.

To determine the relationship of LP_{A3}-dependent neurite elongation to LPA-induced cell rounding, TR cells that endogenously express *lp_{A1}* and *lp_{A2}* genes were examined. TR cells were known from previous studies to respond to LPA with cell rounding, an effect that could be increased by LP_{A1} receptor overexpression (Hecht et al., 1996; Fukushima et al., 1998). Overexpression of LP_{A1} or LP_{A2} significantly increased the population of rounded cells after LPA exposure and also increased the baseline responses in the absence of LPA (Fig. 3A, right). In contrast, expression of LP_{A3} inhibited the endogenous LPA-dependent responses of cell rounding (Fig. 3A, right). This inhibition was accompanied by a second phenomenon in which neurites actually increased in length, as had been observed in B103 cells (data not shown).

To examine the involvement of Rho and PTX-sensitive G_{i/o} proteins in LPA-induced cell rounding, B103 cells infected with LP_{A1} or LP_{A2} virus were pretreated with a Rho kinase inhibitor, Y-27632 (Uehata et al., 1997), or PTX before LPA application. With both LP_{A1} and LP_{A2}, Y-27632 completely inhibited LPA-induced cell rounding, but PTX did not (Fig. 3B). LP_{A3} expression did not mediate cell rounding but did promote neurite elongation. Use of PTX treatment had no effect on LP_{A3}-dependent neurite elongation (data not shown). By contrast, Y-27632 produced some degree of neurite elongation independent of LP_{A3} expression (data not shown), and therefore, this compound could not be used to assess receptor-dependent elongation.

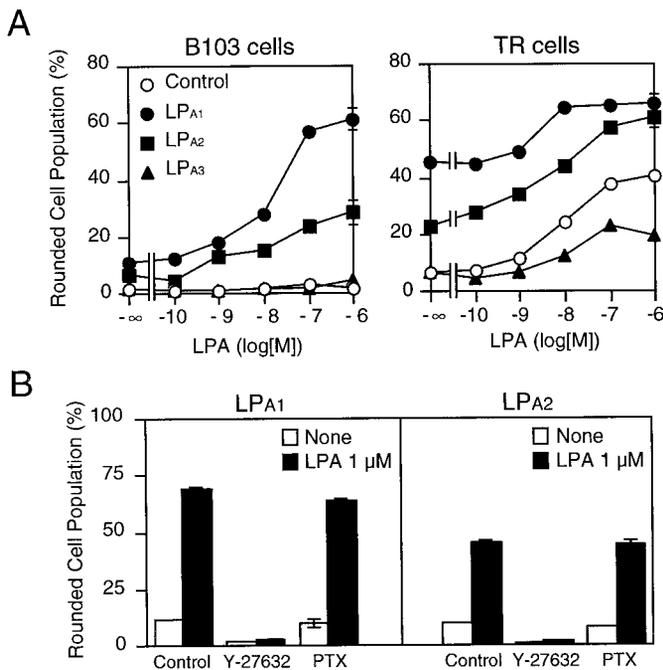


Fig. 3. LPA-induced cell rounding in B103 and TR cells. A, concentration-dependent effects of LPA on cell rounding in both cell types. Cells on 24-well dishes were treated with LPA for 15 min, and numbers of rounded cells were counted from >200 EGFP-positive cells in each well. The population of rounded cells is shown. B, effects of Y-27632 and PTX on LPA-induced cell rounding in B103 cells expressing LP_{A1} or LP_{A2}. Cells were pretreated with 5 μM Y-27632 for 10 min or 100 ng/ml PTX for 24 h and then treated with 1 μM LPA for 15 min. In all panels, data are the means ± S.E. of triplicate samples from the representative experiment.

All LPA Receptors Mediate PLC Activation. Next, we examined the effectiveness of each of the receptors in mediating PLC activation, an LPA response previously shown in fibroblasts (van Corven et al., 1989; Plevin et al., 1991) and human LP_{A1}- or LP_{A2}-expressing HTC4 rat hepatoma cells (An et al., 1998b). PLC activation leads to the production of two second messengers, diacylglycerol and inositol triphosphate, the latter of which can be followed using radioisotope labeling. B103 cells were labeled with [³H]inositol for 24 h in inositol-deficient medium and then stimulated with LPA, and the radioactivity in the inositol phosphate fractions was measured (Fig. 4). LPA activated inositol phosphate production in LP_{A1}-, LP_{A2}- and LP_{A3}-expressing cells, but not in control B103 cells, in a concentration-dependent manner. In all cases, this activation was significantly inhibited by pretreatment with a PLC-specific inhibitor (U-73122) but not with its structurally related inactive analog (U-73343). This activation was not inhibited by pretreatment with Y-27632 or PTX.

All LPA Receptors Mediate Inhibition of Adenylyl Cyclase. LPA has been shown to inhibit adenylyl cyclase activity in fibroblasts (van Corven et al., 1989), TR cells (Hecht et al., 1996), human LP_{A1}- or LP_{A2}-expressing HTC4 cells (An et al., 1998b), and mouse LP_{A1}-expressing RH7777 rat hepatoma cells (Im et al., 2000) via a PTX-sensitive pathway. In contrast, LPA increased forskolin-induced cAMP accumulation in human LP_{A2}- or LP_{A3}-expressing Sf9 insect cells in a PTX-insensitive manner (Bandoh et al., 1999). We therefore examined which of the mouse LPA receptors mediated inhibition or activation of adenylyl cyclase activity (Fig. 5). In LP_{A1}-expressing B103 cells, LPA potently inhibited forskolin-induced cAMP accumulation (maximum 80% inhibition at 10 μM). In LP_{A2}- and LP_{A3}-expressing cells, LPA

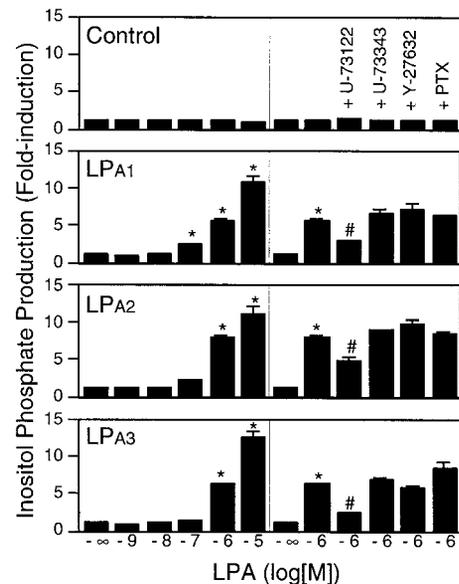


Fig. 4. Effects of LPA on inositol phosphate production in B103 cells. Cells labeled with [³H]inositol were pretreated with 10 μM U-73122, 10 μM U-73343, or 5 μM Y-27632 for 10 min, or 100 ng/ml PTX for 24 h, and stimulated with LPA for 15 min, and the radioactivity in the inositol phosphate fraction of the cell extract was determined. The activity is expressed as fold induction above basal levels (800–1200 cpm for PTX-nontreated cells and 500–700 cpm for PTX-treated cells). Bar graph data are the means ± S.E. of triplicate samples from the representative experiment. Effects of LPA and U-73122 are significant (**P* < .01 versus basal; #*P* < .01 versus 1 μM LPA treated).

also inhibited forskolin-induced cAMP accumulation, but both potency and efficacy were lower than those in LP_{A1}-expressing cells. These effects were completely abolished by PTX pretreatment but were not affected by Y-27632 pretreatment (data not shown). LPA did not increase basal cAMP content in the cells expressing any of the three LPA receptors (Fig. 5B).

All LPA Receptors Mediate MAP Kinase Activation and Arachidonic Acid Release. LPA has been shown to stimulate MAP kinase activation in fibroblasts (Kumagai et al., 1993; Hordijk et al., 1994) and LP_{A2}-expressing PC12 rat pheochromocytoma cells (Bandoh et al., 1999). LPA-induced MAP kinase activation via each LP_A receptor was examined in B103 cells. After 10-min of LPA stimulation, the cells were quickly lysed and MAP kinase activity in the lysate was determined via PHAS-I phosphorylation activity. LPA induced MAP kinase activation in B103 cells expressing any of the three LP_A receptors but not in the control cells (Fig. 6A). LPA effects in each of the samples were small but significant ($P < .05$) and were comparable with those of PMA, a potent activator of protein kinase C and MAP kinase. These LPA effects were completely abolished with the PTX pretreatment. In PTX-pretreated cells expressing any of the LP_A receptors, LPA induced inhibition of MAP kinase by unknown mechanisms.

Finally, we examined whether LP_A receptor activation induced arachidonic acid release, which indicates phospholipase A₂ activation. LPA has been shown to induce arachidonic acid release in fibroblasts (van Corven et al., 1989). The cells were prelabeled with [³H]arachidonic acid, and release of radiolabeled arachidonic acid into the medium after a 20-min LPA stimulation was measured. LPA significantly induced arachidonic acid release in cells expressing any of

the three LP_A receptors but not in the control cells (Fig. 6B). In LP_{A1}- and LP_{A2}-expressing cells, PTX pretreatment significantly, but not completely, inhibited LPA-induced release. By comparison, in LP_{A3}-expressing cells, PTX pretreatment completely abolished this LPA effect.

Discussion

The molecular cloning of mammalian LPA receptors (Hecht et al., 1996; An et al., 1997, 1998a; Bandoh et al., 1999; Im et al., 2000) has furthered our understanding of the versatility of this simple lipid. The existence of multiple receptors suggests distinct receptor functions in vivo. In this study, we used a retroviral system to express these receptors heterologously within cells from the same or similar species as the assayed receptor gene. Unlike prior studies, including our own, that relied on either small percentages (<5–10%) of transfected cells in transient assays, or possible clonal variations associated with stable cell lines, this approach provides both improved expression efficiency and reduced physical insult to cells. These benefits should provide a better approximation of how these receptors can signal, and this retroviral system should be suitable for future studies in vivo. In the present study, we focused only on short-term responses (all within 10–20 min after LPA application) in an effort to eliminate indirect effects potentially encountered with prolonged assays such as those used for cell proliferation.

Five signaling pathways were examined, of which three were activated similarly by each LP_A receptor. Each receptor

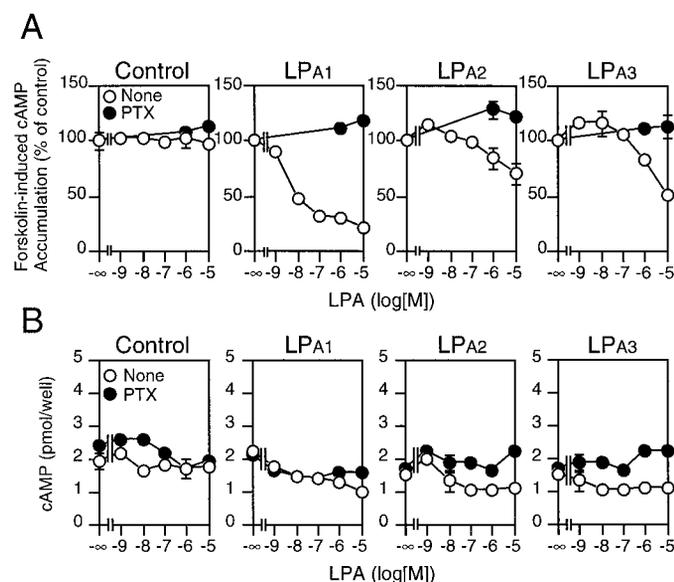


Fig. 5. Effects of LPA on cAMP accumulation in B103 cells. A, effects of LPA on forskolin (1 μ M)-induced cAMP accumulation for 20 min in the presence of 0.5 mM IBMX. Forskolin-induced cAMP accumulation (97.8–158.7 pmol/well in all experiments) was expressed as 100%. B, effects of LPA on basal cAMP contents in a 20-min incubation. In all panels, closed circles represent the data from cells pretreated with 100 ng/ml PTX for 24 h, and open circles represent data from nontreated controls. Data are the means \pm S.E. of triplicate samples from the representative experiment.

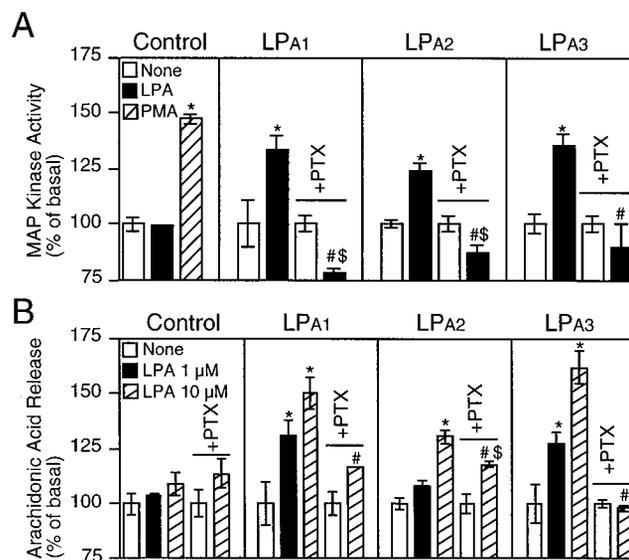


Fig. 6. Effects of LPA on MAP kinase activation and arachidonic acid release in B103 cells. A, LPA (1 μ M)-induced MAP kinase activation within 10 min. Cells infected with control virus were also treated with 100 nM PMA. The basal activities of the control cells (70–100 arbitrary units for PTX-nontreated cells and 60–100 arbitrary units for PTX-treated cells) are expressed as 100%. B, LPA (1 or 10 μ M)-induced arachidonic acid release in 20 min. The basal activities of the control cells (1000–1100 cpm for PTX-nontreated cells and 650–750 cpm for PTX-treated cells) are expressed as 100%. In both experiments, cells were pretreated with or without 100 ng/ml PTX for 24 h. Bar graph data are the means \pm S.E. of triplicate samples from the representative experiment. The effects of PMA and LPA are significant (* $P < .05$ versus basal in control cells; # $P < .05$ versus basal in PTX-treated cells). The effects of PTX are significant (* $P < .05$ versus 1 μ M LPA-treated cells in MAP kinase activation, 10 μ M LPA-treated cells in arachidonic acid release).

mediates inositol phosphate production in a Rho-independent and $G_{i/o}$ -independent fashion (Fig. 4), consistent with G_q activation of PLC. Activation of all three receptors also inhibited forskolin-induced cAMP accumulation (Fig. 5A), consistent with the expected interaction with $G_{i/o}$. LP_A receptors also activated MAP kinase (Fig. 6A), and this also appears to be mediated through $G_{i/o}$, based on results from PTX pretreatment that completely abolished MAP kinase activation. In addition, LP_{A1} and LP_{A2} both similarly activated arachidonic acid release and cell rounding. Arachidonic acid release for these two receptors was decreased but not abolished by PTX pretreatment, suggesting activation of a PTX-insensitive G-protein(s). The involvement of $G_{i/o}$ was expected, because MAP kinase is suggested to be involved in agonist-induced cytosolic phospholipase A_2 activation that leads to arachidonic acid release (Kramer and Sharp, 1997; Gijaon and Leslie, 1999), and MAP kinase activation was PTX sensitive (Fig. 6A). The mechanism by which LP_{A1} and LP_{A2} produce arachidonic acid release may also use the G_q /PLC pathway, because LPA-induced arachidonic acid release was not completely PTX sensitive (Fig. 6B).

Cell rounding constituted a second response that was shared by LP_{A1} and LP_{A2} . We previously showed that LP_{A1} mediates cell rounding in B103 cells in a Rho-dependent manner (Fukushima et al., 1998), and here we found that LP_{A2} similarly mediates this response (Fig. 3A, left). The G-proteins that likely mediate these Rho-activating effects are $G_{12/13}$ -type proteins. $G\alpha_{12}$ and $G\alpha_{13}$ have been shown to interact with p115 RhoGEF, the Rho guanine nucleotide exchange factor (Hart et al., 1998; Kozasa et al., 1998). We observed that, even without LPA application, LP_{A1} or LP_{A2} expression significantly increased the numbers of rounded cells in the TR cell lines (Fig. 3A, right). This is not surprising because overexpression of G-protein-coupled receptors can lead to a constitutive activation in the absence of agonists (Bond et al., 1995; Ishii et al., 1997). The presence of endogenous LPA could also result in receptor activation in some cells.

In contrast to the similarities among LP_{A1} , LP_{A2} , and LP_{A3} that we have reported thus far, differences were also observed. In several assays, quantitative differences could be identified. These data must be interpreted with caution, because it was not possible to express identical amounts of each receptor protein, despite similar levels of mRNA transcription (Fig. 1B). Nevertheless, reduced levels of LP_{A2} expression compared with the other two receptors (Figs. 1C and 2A) may be what occurs normally in vivo, because a similar reduction in protein expression was previously observed (Bandoh et al., 1999). For two of the five examined signaling responses, qualitative differences were observed for LP_{A3} . Arachidonic acid release was differentially affected by PTX pretreatment. It completely abolished LPA-induced release in LP_{A3} -expressing cells but not in LP_{A1} - and LP_{A2} -expressing cells (Fig. 6B). This result suggests that LP_{A3} preferentially utilizes $G_{i/o}$ in arachidonic acid release, whereas LP_{A1} and LP_{A2} also utilize a PTX-insensitive G-protein(s).

Most striking, however, was the effect of LP_{A3} on cell morphology compared with LP_{A1} and LP_{A2} . LP_{A3} expression in B103 cells did not induce cell rounding. This lack of effect was not due to nonfunctional receptors, because LP_{A3} stimulated other examined pathways in an LPA-dependent manner. In addition, LP_{A3} partially inhibited LPA-induced cell

rounding when expressed in TR cells that endogenously express LP_{A1} and LP_{A2} (Fig. 3A, right). Furthermore, LP_{A3} expression produced marked neurite elongation in both B103 (Fig. 2) and TR cells (data not shown). Neurite elongation was independent of LPA concentration. A hypothetical mechanism that could explain these results is inhibition of Rho activity, because neurite elongation was observed in B103 and TR cells exposed to only the Rho kinase inhibitor, Y-27632, alone (data not shown). If this were true, then neurite elongation could be considered a default state augmented by comparatively low Rho activity. The biological role of LP_{A3} in such a process is currently unclear.

Our results are in general agreement with prior work on LP_{A1} and LP_{A2} receptors (An et al., 1998b) and complement recent reports on LP_{A1} , LP_{A2} , and LP_{A3} receptors (Bandoh et al., 1999; Im et al., 2000), although there are several differences in technical approach and results. First, a potentially important detail is that there are isoforms of LPA receptors. The second LPA receptor encoded by the gene *edg-4* (An et al., 1998a,b) varies significantly in the carboxyl terminus compared with mouse and human *lp_{A2}* clones (Contos and Chun, 2000). This initially characterized gene contains a frame-shift mutation associated with an ovarian tumor, the source of cDNA for their expression constructs. Direct comparisons of the normal and mutant forms of LP_{A2} were not examined here. Our data indicate a close functional similarity between LP_{A1} and normal LP_{A2} , including similar responses in all of the assays examined.

Second, prior studies obtained results in Sf9 insect cells infected with baculovirus to express mouse LP_{A1} (Zondag et al., 1998) or human LP_{A1} , LP_{A2} , and LP_{A3} (Bandoh et al., 1999). In these systems, both mouse and human LP_{A1} receptors were nonfunctional, underscoring probable incompatibilities with the available insect G-proteins and LP_{A1} receptors. Differences in such downstream signaling components may also explain the observation of increased forskolin-induced cAMP accumulation in LP_{A2} - and LP_{A3} -expressing Sf9 cells, compared with our data in which all LP_A receptors mediate LPA-dependent inhibition of forskolin-induced cAMP accumulation. Interestingly, mouse LP_{A1} , but not human LP_{A2} and LP_{A3} , receptors mediated inhibition in forskolin-induced cAMP accumulation in RH7777 cells (Im et al., 2000), suggesting the different signaling properties in neuronal cells and hepatic cells.

A third difference was observed in examining PC12 cells in which human LP_{A2} , but not LP_{A1} and LP_{A3} , induced MAP kinase-mediated Elk1 (Marais et al., 1993) activation (Bandoh et al., 1999), whereas we found that all LPA receptors activate MAP kinase. There are likely biological differences between PC12 cells that are derived from the adrenal medulla and the central nervous system cells utilized here. For example, PC12 cells undergo cell rounding via both a Rho-dependent and G_q -dependent mechanism (Katoh et al., 1998), whereas in B103 cells, G_q activation was not sufficient for cell rounding based on data using LP_{A3} receptor (Figs. 3A, left, and 4). These differences in receptor responses might be explained by amino acid sequence variation between mouse and human LP_{A1} , LP_{A2} , and LP_{A3} (97.3, 90.8, and 90.8% identity, respectively).

Multiple LPA receptors with shared signaling properties could provide cells with overlapping properties for essential functions. Additionally, distinct signals, as observed here for

LP_{A3}, could underscore more specialized functions. One possible specialized function we observed was the morphological effects of LP_{A3}, which, in contrast to LP_{A1} and LP_{A2}, did not produce cell rounding and actually promoted neurite elongation. Because the developing nervous system is one of the major loci for LP_A receptor expression (Chun, 1999; J. J. A. Contos and J. Chun, in preparation), the functional antagonism for cell rounding could have consequences for neural development. All of the receptors are expressed in the brain, but there is a variation of both spatial and temporal expression patterns. For example, lp_{A1} is expressed in neuroblasts during embryonic development (Hecht et al., 1996) but in oligodendrocytes and Schwann cells at later ages (Weiner et al., 1998; Weiner and Chun, 1999). Other developmental and spatial patterns of expression appear to occur for both lp_{A2} and lp_{A3} (J. J. A. Contos and J. Chun, in preparation). The recent demonstration that LPA increases Alzheimer's disease-like Tau phosphorylation accompanied by neurite retraction in SY-SH5Y human neuroblastoma cells (Sayas et al., 1999) suggests that LP_{A1} or LP_{A2} receptor signaling may be involved in some neuronal disorders. Information concerning their expression in a single cell, or single cell type, is only just emerging, and there exists the additional possibility of receptor combinatorial functions or synergism in cells expressing more than one receptor that could further modify cellular responses to LPA.

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