Identification of Residues Responsible for Ligand Recognition and Regioisomeric Selectivity of Lysophosphatidic Acid Receptors Expressed in Mammalian Cells*

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The endothelial differentiation gene family encodes three highly homologous G protein-coupled receptors for lysophosphatidic acid (LPA). Based on baculoviral overexpression studies, differences have been proposed in the structure–activity relationship (SAR) of these receptors. We have compared the SAR of the individual receptors either overexpressed transiently at high or at lower levels following stable transfection in LPA-nonresponsive RH7777 cells. The SAR in transfected RH7777 cells was markedly different from that described in insect cells. The LPA1 receptor has been proposed to be selectively activated by unsaturated LPA species and shows a strong preference for sn-2 versus the sn-1 acyl-LPA regioisomer. Because of the short half-life of sn-2 LPA due to acyl migration under some conditions, we have synthesized acyl migration-resistant analogs using an acetyl group in place of the free hydroxyl group in order to evaluate LPA receptor SAR. Only LPA1 and LPA3 showed regioisomeric preference and only for the 18:2 fatty acyl-stabilized LPA sn-1 regioisomer. To identify residues involved in ligand recognition of LPA3, we developed and validated computational models of LPA3 to identify residues involved in ligand recognition of LPA3. The models identified Leu-2.60 and Val-7.39 of LPA3 as critical interactions with the polar headgroup. This biochemical pathway involves the nascent production of an unstable sn-2 LPA intermediate by phospholipase A1, followed by lysophospholipase D cleavage of the headgroup. This biochemical pathway involves the nascent production of an unstable sn-2 LPA intermediate that rapidly undergoes acyl migration and yields the more stable sn-1 analog. In the pH range 7–8, the half-life of sn-2 lysophosphatidylcholine is only a few minutes, and binding to serum albumin does not prolong its stability (13).

Bandoh et al. (14) published the most comprehensive analysis to date of the LPA structure–activity relationship (SAR) using recombinant LPA receptors expressed through baculovirus transduction of Sf9 insect cells. They noted important differences in ligand preferences between the three receptors. These authors reported that LPA2 was unique in that it could only be activated by unsaturated acyl LPA and showed an enhanced response to unsaturated acyl LPA with respect to LPA1. The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

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3 The abbreviations used are: LPA, lysophosphatidic acid; AGP, alkyl-ether glycerophosphate; BSA, bovine serum albumin; cPA, cyclic phosphatidic acid; EC50, effective concentration for half population; EDG, endothelial differentiation gene; E_max, maximal effect; GPCR, G protein-coupled receptor; PA, phosphatidic acid; PBS, phosphate-buffered saline; RH7777, rat hepatoma cell; SAR, structure-activity relationship; S1P, sphingosine 1-phosphate; TM, transmembrane domain; FACS, fluorescence-activated cell sorting; GTPγS, guanosine 5'-3'-O-(thiotriphosphate); PPARγ, peroxisome proliferator-activated receptor γ.
order of magnitude preference for sn-2 over sn-1-substituted LPA with acyl chain lengths between 16 and 20 carbons. The specificity for unsaturated fatty acyl LPA species has not been confirmed by Im and colleagues (16), who, using a GTPγS binding assay, found that saturated LPA species also activated LPA₃ expressed in HEK293T cells. However, HEK293 cells are endogenously responsive to LPA (15) and require heterologous co-expression of G₁₁αC351F (16). Based on the regiosmERIC selectivity, Bandoh et al. (14) proposed that nascent sn-2 LPA may function as a selective activator of LPA₃. This hypothesis reinforced an earlier observation by Xu et al. (17) that sn-2 LPA was generated by ovarian cancer cells and functioned as a modulator of cancer growth and invasion. These observations that endow sn-2 LPA with unique ligand properties carry inherent pitfalls. First, heterologous expression of mammalian GPCR in Sf9 cells might not represent the pharmacological properties of the same receptor expressed at the endogenous level in mammalian cells. Many factors, including a lack of higher order glycosylation, sulfation, and the impact of spare receptors due to high levels of overexpression, may all play roles in potential discrepancies. Second, insect G proteins do not couple to several mammalianGPCRs, including LPA₁ (14), unless mammalian G protein subunits are co-expressed. To overcome this problem Bandoh et al. (14) did not co-express mammalian G protein subunits but instead produced a receptor chimera by replacing the C-terminal 30 amino acids of LPA₁ with the last 53 amino acids of LPA₂. The impact of this protein engineering on ligand binding and receptor activation has not been evaluated and may introduce changes in ligand recognition. Thus far, not a single cell type has been found that endogenously expresses LPA₂ alone, preventing a verification of its unique SAR. Third, although pH was adjusted to 6.2, which slows acyl migration between the sn-2- and sn-1-positions, time between lipid solution preparation and use was not defined; therefore, regiosmERIC composition of the ligand at the time of the assay is unclear. Finally, pKᵦ values of amino acid residues in proteins can have strong environmental dependence, and the effect of pH on ligand recognition in the LPA receptor family has not previously been studied.

Our groups have previously examined S1P and LPA recognition of the S1P₁, S1P₄, and LPA₁ EDG family receptors using computational modeling and site-directed mutagenesis (18–21). These studies have identified two conserved positively charged residues, Arg-3.28 and Arg/Lys-5.38 in the third and fifth transmembrane domain, respectively, that are required for ligand binding and receptor activation. Additionally, a cationic residue in the seventh transmembrane domain at position 7.34 in S1P₁₃₋₄ but 7.33 in S1P₄, is required for ligand binding and activation of S1P₁ but not S1P₄. Our previous studies succeeded in pinpointing position 3.29, conserved as glutamine in LPA-specific and glutamate in S1P-specific members of the EDG family, as the single locus that determines ligand specificity for S1P versus LPA (19, 20). Although residues involved in phosphate recognition are conserved between LPA₁ and LPA₃ receptors, differences in the SAR between the two receptors (14) led us to hypothesize that residues positioned lower in the transmembrane domains might be involved in ligand recognition and selectivity.

To elucidate the structural foundations of proposed differences in ligand specificity between LPA receptor subtypes, we examined multiple objectives in the present study. First, we compared the SAR of the three LPA receptors expressed in RH7777 rat hepatoma cells transiently (high levels) or stably (low levels). RH7777 cells are devoid of EDG family LPA receptors (5) and do not produce intracellular Ca²⁺ transients in response to LPA. Transfection with individual LPA receptors confers robust Ca²⁺ responses to nanomolar concentrations of LPA in these cells (12). Second, we synthesized acyl migration-resistant sn-1 and sn-2 regioisomers of LPA with saturated and unsaturated fatty acyl chains by blocking the free hydroxyl with an acetyl group and evaluated their ligand properties. Third, we validated a previously developed computational model of LPA₁ (23), which revealed distinct residues responsible for the polar headgroup interactions and for selective interaction with the acetyl groups of the stabilized sn-1 and sn-2 regioisomers of LPA. Our results obtained from transient and stable transfection of LPA₁ in RH7777 cells expressing each of the three LPA GPCRs in solutions buffered at pH 7.4 revealed substantial differences compared with results obtained in Sf9 cells in solutions buffered at pH 6.2 (4, 14) in that we could not establish an exquisite requirement for unsaturated fatty acid-containing LPA, and we found no regiosmERIC preference for stabilized sn-2 over sn-1 analogs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Palmitoyl-LPA 16:0, stearoyl-LPA 18:0, oleoyl-LPA 18:1, and arachidoyl-LPA 20:0 were purchased from Avanti Polar Lipids (Alabaster, AL). Linoleoyl-LPA 18:2, linolenoyl-LPA 18:3, and arachidonoyl-LPA 20:4 were obtained from Echelon Bioscience, Inc. (Salt Lake City, UT). 1-Acyl-2-acetyl-sn-glycerol-3-phosphates (PA 18:2/18:0) and 1-acetyl-2-acyl-sn-glycerol-3-phosphates (PA 20:0/18:0) with 18.0 (x = 0), 18.1 (x = 1), and 18.2 (x = 3) fatty acids were synthesized as previously described (8). 2,3-Cyclophosphatic acid (cPA) analogs were provided by Dr. Susumu Kobayashi (University of Tokyo, Tokyo, Japan) (24). Alkyl-ether glycerophosphate (AGP) analogs of LPA were purchased from Avanti Polar Lipids. Lipid samples were prepared fresh by dissolution in Krebs buffer containing 1 mM charcoal-stripped BSA powder or chloroform stocks kept under argon gas. Lipids were routinely monitored with electrospray ionization mass spectrometry (LCQ Advantage; Thermo Finnigan) prior to assay. Dulebco’s modified Eagle’s medium was from Cellgro (Herndon, VA) and G418 was purchased from Invitrogen. Fura-2/AM and Alexa 488 donkey anti-mouse IgG conjugate were obtained from Molecular Probes, Inc. (Eugene, OR). Bovine serum albumin (BSA; fraction V, fatty acid-free) was from Sigma.

**Computational Studies**—The detailed methods used to develop computational models of LPA₁, LPA₂, and LPA₃ have been published previously (19, 21, 23). Briefly, our validated model of the S1P₁ receptor (18, 19, 21) was used as a template for the generation of LPA receptor models. Homology model development was performed using the automated algorithm implemented in the MOE software program (26). The best model was geometry-optimized using the MMFF94 (27) force field to a root mean square gradient of 0.1 kcal/molÅ. The individual receptor models were used in docking studies of the LPA, AGP, cPA, and acetyl-LPA species studied experimentally. LPA, AGP, and acetyl-LPA species were modeled with phosphate groups carrying a −2 charge, whereas cPA was modeled with a charge of −1. Docking calculations were performed using the AutoDock 3.0 software (28) with default values for all parameters except the number of runs (5), energy evaluations (2.5 × 10⁶), generations (30,000), and local search iterations (3000). The complex chosen as the best geometry from each docking calculation was that with the lowest final docked energy value. Complexes used to select mutations to validate the interactions between receptor and ligand (LPA 18:1; PA 18:1/2:0, and PA 20:0/18:1 in LPA₃) were subjected to molecular dynamics simulations using 1-fs time steps for 500 ps. The lowest energy structure sampled at 1-ps intervals during the simulation was geometry-optimized and visually evaluated to predict residues involved in ligand recognition and selectivity.

**Site-directed Mutagenesis**—cDNA encoding human LPA₃ (GenBankTM accession number NM012152) subcloned into the pcDNA3 expression plasmid (Invitrogen) was a generous gift from Dr. Junken.
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Aoki (University of Tokyo, Tokyo, Japan). An amino-terminally FLAG epitope-tagged LPA3 construct was generated by PCR. Site-specific mutations were generated as described previously (18, 19) using two complementary oligonucleotide primers containing the desired mutation and a silent mutation constituting a new restriction site for selection purposes. The mutations introduced into the LPA3 wild type sequence are listed in Table One. The mutated strands were extended during temperature cycling using Pfu ultra high fidelity DNA polymerase (Stratagene, La Jolla, CA). Following temperature cycling, parental DNA was removed via digestion with DpnI. Mutated DNA was transformed into TOP10-competent cells (Invitrogen), and clones were verified by complete sequencing of the inserts.

Cell Culture and Transfection—LPA nonresponsive RH7777 cells (ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells plated in 10-cm dishes were transfected with 2 μg of DNA using Effectene according to the manufacturer’s protocol (Qiagen, Valencia, CA). RH7777 cells stably expressing the LPA1 and LPA3 receptors have been characterized elsewhere (12). RH7777 cells stably expressing LPA2 receptors were a generous gift from Dr. Fumikazu Okajima (Gunma University, Gunma, Japan) and were characterized previously (29). Stable transfectants were maintained in Dulbecco’s modified Eagle’s medium, containing 10% fetal bovine serum and supplemented with 250 μg/ml G418.

Flow Cytometry—Cell surface expression of LPA3 mutants was evaluated by flow cytometry. Cells plated in 35-mm dishes were transfected with 4 μg of DNA using PolyFect (Qiagen). After a 24-h incubation, the cells were trypsinized and washed twice with ice-cold FACS buffer consisting of phosphate-buffered saline (PBS) supplemented with 3% (w/v) BSA. Cells were then incubated in blocking buffer (PBS, 5% BSA, 5% normal donkey serum) for 30 min. The cells were subsequently washed with FACS buffer three times, incubated with anti-FLAG M2 monoclonal antibody in blocking buffer (1:100 dilution) for 1 h, rinsed in FACS buffer three times, and incubated in the dark at 4 °C with Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:1600 dilution) for 30 min. The cells were rinsed three times with FACS buffer and analyzed using an LSR II flow cytometer (Becton Dickinson, San Jose, CA).

Measurement of [Ca2+]i—RH7777 cells transiently transfected with either 2 μg of wild type LPA3 or its site-directed mutants were cultured for 24 h and replated onto poly-l-lysine (0.1 mg/ml)-coated 96-well plates. Cells were then incubated in blocking buffer (PBS, 5% BSA, 5% normal donkey serum) for 30 min. The cells were subsequently washed with FACS buffer three times, incubated with anti-FLAG M2 monoclonal antibody in blocking buffer (1:100 dilution) for 1 h, rinsed in FACS buffer three times, and incubated in the dark at 4 °C with Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:1600 dilution) for 30 min. The cells were rinsed three times with FACS buffer and analyzed using an LSR II flow cytometer (Becton Dickinson, San Jose, CA).

RESULTS

Our first objective in this study was to evaluate the SAR of the three individual LPA receptors expressed either transiently or stably in RH7777 cells at the typical physiological pH of 7.4. Structures of the LPA analogs used in the present study are shown in Fig. 1. The LPA, AGP, and cPA analogs applied to wild type RH7777 cells did not elicit detectable changes in [Ca2+]i, up to 10 μM (supplemental Fig. 1). Initially, we compared the effect of acyl-chain lengths and the degree of unsaturation on ligand properties for seven 1-acyl-LPA analogs using intracellular Ca2+ mobilization for a read-out (Fig. 2). LPA 200 failed to activate LPA2 after either transient or stable expression (Fig. 2, A and D). The rank order of the six remaining analogs in cells transiently expressing LPA2 was 18:2 > 18:3 > 20:4 > 18:1 > 16:0 > 18:0 (Fig. 2A). Unexpectedly, the stably expressed LPA1 cells showed a different response when exposed to the seven LPA analogs. We did not find significant differences in the EC50 between 18:2, 18:3, 18:1, and 20:4, whereas 16:0 and 18:0 were less potent than in the transient expression experiments (Fig. 2D). The rank order of potency for the same set of LPA analogs in cells transiently transfected with LPA1 was 18:3 > 20:4 > 18:2 = 18:1 > 18:0 > 16:0 ≈ 20:0 (Fig. 2B). RH7777 cells stably expressing LPA2 showed a rank order that was essentially the same as that in the cells with transient expression (Fig. 2E). It is of note that the separation of the individual dose-response curves was not as clear as in the transient expression experiments, and the apparent EC50 values shifted to considerably higher values. Of note, the stable clones used in the present study did not acquire expression of any other EDG family LPA receptor in addition to the subtype originally transfected (supplemental Fig. 1).

In contrast to the report by Bandoh et al. (4) but in agreement with the report of Im et al. (16), in our mammalian expression experiments, saturated LPA species were also active, albeit with distinctly lower potency (LPA 18:0 EC50 = 573 ± 41 nM, LPA 16:0 > 1 μM, in contrast to LPA 18:1 EC50 = 87 ± 25 nM; Fig. 2, C and F). In cells stably expressing LPA3, the difference in potency (EC50) was not as distinct as in cells transiently expressing LPA2 (Fig. 2, compare C and F). The present results obtained with either transient or stable expression of EDG family LPA receptors indicate that unsaturated LPA species uniformly display higher potency when compared with saturated species of the same length hydrocarbon chain for all LPA GPCRs examined. Furthermore, saturated LPA species were capable of activating LPA3 expressed in the RH7777 cells, and the maximal responses (Emax) showed no significant difference between the saturated and unsaturated analogs.

Modeled complexes of individual LPA species with LPA1, LPA2, and LPA3 have been overlaid and are shown in Fig. 2, G, H, and I, respectively. LPA 20:0 is colored cyan to emphasize differences compared with the more potent analogs. In LPA1, the best complex identified with LPA 20:0 shows somewhat better overlap with other LPA species, an observation that is consistent with the experimental finding that LPA 20:0 activates LPA2. The phosphate group of LPA 20:0 is offset relative to the remaining LPA species in LPA2, thus weakening its interactions with cationic residues in the receptor. This poor ionic interaction is consistent with the high concentrations needed to activate the receptor. In LPA3, the phosphate group...
of LPA 20:0 is able to interact well with the cationic residues of the receptor, but the hydrophobic tail diverges from the site occupied by the tails of the other LPA species, suggesting a poor fit in the lower end of the agonist binding pocket. Thus, computational modeling results show a common binding orientation and occupied volume for effective agonists. Conversely, the models of LPA1, LPA2, and LPA3 highlight differ-

FIGURE 1. Chemical structure of LPA analogs used in the study. A, structures of 1-acyl-LPA, 1-acyl-2-acetyl-PA (PA 18:0/2:0, PA 18:1/2:1, and PA 18:2/2:0) and 1-acetyl-2-acyl-PA (PA 2:0/18:0, PA 2:0/18:1, and PA 2:0/18:2). Also shown are structures of AGP (B) and cPA (C).
ences when comparing weak ligands with more effective agonists. The modeling results fail to provide evidence of favorable interactions targeting \( /H_9266 \) bonds of the unsaturated analogs that would explain their greater potency at all three receptors. The preference for unsaturated analogs appears instead to be due to the cis geometry of the \( /H_9266 \) bond that more naturally follows the curve of all three hydrophobic binding pockets.

We next examined the ligand properties of AGP at the three LPA receptors. AGP analogs were less potent than their corresponding LPA forms (compare Figs. 2 and 3). Nonetheless, AGP 16:0, AGP 18:0, and AGP 18:1 were capable of inducing \( \text{Ca}^{2+} \) transients (Fig. 3). AGP 16:0 was more potent (EC\(_{50} = 715 \pm 205 \text{nM}\)) than AGP 18:1 (EC\(_{50} = 1.5 \pm 0.5 \text{mM}\)) at LPA\(_{1}\). Thus, the rank order of potency for AGP at LPA\(_{1}\) was 16:0 > 18:1 >> 18:0. This is markedly different from the corresponding LPA set, where the rank order was 18:1 > 16:0 >> 18:0. Similar results were obtained for the LPA\(_{2}\) receptor, although the AGP isoforms were active at lower concentrations (AGP 16:0 EC\(_{50} = 76 \pm 16 \text{nM}\); AGP 18:1 EC\(_{50} = 101 \pm 23 \text{nM}\)) (Fig. 3B). In contrast to LPA\(_{1}\) and LPA\(_{3}\), the three AGP analogs we tested were weak agonists of LPA\(_{3}\), particularly in stable transfectants (Fig. 3F). Moreover, at LPA\(_{3}\), AGP 18:0 and 18:1 were equipotent, whereas AGP 16:0 showed 5–6 times higher potency than the C18 analogs (Fig. 3C). The model complexes shown in Fig. 3, G–I, provide an explanation for much of the observed SAR. In particular, the AGP species shown in cyan are those with the lowest potency at each receptor. These species either show differences in the position of their phosphate head group (AGP 18:0 in LPA\(_{2}\) (Fig. 3H) and AGP 18:1 in LPA\(_{3}\) (Fig. 3I)) or hydrophobic tail (AGP 18:1 in LPA\(_{1}\) (Fig. 3G) and AGP 18:0 in LPA\(_{3}\) (Fig. 3I)) as compared with the more potent AGP analogs or the corresponding LPA species (Fig. 2, G–I). These differences indicate poor electrostatic or shape complementarity to the agonist-binding pocket, respectively.

cPA has been shown to evoke partial cross-desensitization with LPA, suggesting that the two lipids activate overlapping receptor populations (30, 31). For this reason, we also tested the ligand properties of cPA at the three LPA receptors (Fig. 4, A–F). Although both cPA analogs activated all three LPA receptor subtypes, cPA 16:0 and cPA 18:1 were less potent than LPA 16:0 or LPA 18:1. In cells expressing LPA\(_{1}\), the EC\(_{50}\) values for cPA 16:0 and 18:1 were in the high micromolar range (Fig.
In cells transiently or stably expressing LPA2, cPA 16:0 was more efficacious than LPA 18:1 (Fig. 4, B and E, respectively). Cells stably expressing LPA3 failed to respond to cPA 16:0 up to 10 μM, the highest concentration tested (Fig. 4F). In contrast, cPA 18:1 was an agonist with an apparent EC50 in the low micromolar range (Fig. 4, C and F). These results suggest that LPA1–3 receptors show a strong preference for unsaturated cPA over saturated cPA. In all cases, the same cPA species showed higher potency in the transient expression paradigm than in the stable transfectants. Models of cPA species in the LPA receptors are shown in Fig. 4, G–I. These models demonstrate that in LPA1 (Fig. 4G) and LPA3 (Fig. 4I), the phosphate groups and hydrophobic tails of the cPA species overlap with that of the corresponding LPA. The reduced potency can be attributed to the inability of the disubstituted phosphate to adopt a −2 charge, thus reducing its electrostatic interaction with the surrounding cationic residues from transmembrane domains 3, 5, and 7. The models of cPA species in LPA2 (Fig. 4H) show differences in phosphate group position (cPA 18:1) or acyl chain position (cPA 16:0) relative to the more potent LPA species (Fig. 2H). These results indicate poor electrostatic or shape complementarity to the agonist-binding site, respectively. We have examined the stability of cPA in aqueous buffer and found that during a 24-h incubation, over 75% of the cPA remained intact, and the degradation of the lipid produced no significant generation of LPA at 37 °C (Fig. 5). It is of note that during the first 2 h of incubation, no detectable cPA loss was detectable, excluding the possibility that a rapid degradation of cPA or the formation of LPA could explain the weak potency of cPA (supplemental Fig. 3).

Our previous studies of the S1P1, S1P4, and LPA1 receptors identified a cluster of four conserved amino acids with essential roles in ligand recognition by the EDG family of receptors (18–21). The original report by Bandoh et al. (4, 14) noted profound differences in the ligand selectivity of LPA3 compared with LPA1 and LPA2. These proposed differences in the ligand recognition of LPA3 prompted us to generate a computational model and investigate residues that are predicted to interact with LPA ligands (Fig. 6). The residues predicted by the model...
FIGURE 4. SAR for three cPA analogs at LPA GPCR and models of the receptors ligand complexes. Intracellular Ca\(^{2+}\) transients (mean ± S.D.) were measured in response to the application of increasing concentrations of various analogs of cPA. RH7777 cells transiently expressing LPA\(_1\) (A), LPA\(_2\) (B), and LPA\(_3\) (C) or stably expressing LPA\(_1\) (D), LPA\(_2\) (E), and LPA\(_3\) (F) were used. 100% represents the maximal Ca\(^{2+}\) mobilization elicited by LPA 18:1 at the individual LPA receptors. Samples were run in triplicate, and the data are representative of at least three independent experiments. Models of the LPA\(_1\) (G), LPA\(_2\) (H), and LPA\(_3\) (I) receptors docked with cPA are shown as a ribbon model shaded from red at the amino terminus to blue at the carboxyl terminus. Ligands are shown as stick models, with cPA 16:0 colored cyan.

FIGURE 5. Stability of cPA in aqueous buffer. Hydrolysis of cPA to LPA in aqueous buffer is shown. PBS solutions of cPA 18:1 (10 mM) were incubated at 37 °C for up to 24 h. At each time point, samples were taken and supplemented with equal molar LPA 18:0 as an internal standard. Following extraction with a modified Bligh-Dyer protocol, the relative amounts of cPA 18:1 (white bars) and its hydrolysis product LPA 18:1 (black bars) were normalized to internal standard LPA 18:0 using liquid chromatography-mass spectrometry.
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Figure 6. Model of the LPA3 receptor docked with LPA 18:1 and ligand-induced activation of LPA3 and its mutants. Close-up view of critical interactions between LPA3 residues and the phosphate group of the ligand (A). Computed distances (Å) from the residues to the ligand are shown in the inset. B–E, normalized calcium transient (mean ± S.D.) elicited by increasing concentrations of LPA 18:1 in RH7777 cells transiently expressing LPA3 or its mutants. 100% represents the maximal response to LPA 18:1 at wild type LPA3. Samples were run in triplicate, and the mean ± S.D. was plotted. The data are representative of at least three independent experiments.

as well as other residues selected for control purposes were mutated to alanine, and their expression at the cell surface was tested by fluorescence-activated cell sorting (TABLE ONE). All mutants, with the exception of R5.38A, which did not express (data not shown), showed comparable cell surface expression. Arg-3.28 is conserved in all EDG family members, and modeling predicted that it forms a critical salt bridge with the LPA phosphate (Fig. 6A). In agreement with this hypothesis, the R3.28A mutant showed no dose-dependent Ca2+ response upon LPA 18:1 stimulation (Fig. 6B). The model predicted that Arg-3.24, a residue one helical turn away, is more distant from the phosphate, and its
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TABLE ONE

| Mutation | Wild type codon | Mutated codon | Silent mutation | Introduced restriction site | Cell surface expression level* % |
|----------|-----------------|---------------|-----------------|----------------------------|---------------------------------|
| R3.24Q   | 303CGC          | CAA           | 295GTAAAC       | Hpal                       | 39.8                            |
| R3.28A   | 315CGT          | GCT           | 318AGGCCCT      | Stal                       | 39.8                            |
| Q3.29A   | 346CAG          | GCA           | 348AGGCCCT      | Stal                       | 30.5                            |
| S5.37A   | 525AGC          | GCC           | 525AGATCT       | BgIII                      | 33.4                            |
| R5.28N   | 553AGG          | ATT           | 553GAATTC       | EcoRI                      | 30.1                            |
| R7.35A   | 823AAA          | GCC           | 823TCGCAG       | NruI                       | 31.6                            |
| R7.36A   | 825AGG          | GCT           | 825AAGCTT       | HimdIII                    | 37.3                            |
| L3.40V   | 340CTC          | GTG           | 343GCTAGCC      | Nhel                       | 35.2                            |
| L2.60A   | 256CTG          | GCG           | 254TCGCAG       | NruI                       | 28.0                            |
| L7.39A   | 835CTG          | GCT           | 836GCTAGG       | Nhel                       | 33.4                            |

*a Vector, 2.0%; LPA3 wild type, 38.7%.

replacement would not have an effect on LPA activation (Fig. 6A). Indeed, R3.24Q showed no attenuation in $E_{50}$ and showed a slightly lower $E_{50}$ compared with wild type LPA3 (Fig. 6C). In the LPA3/SIP1 receptor pair and in S1P1, the Gln-3.29 residue determines selectivity to LPA over S1P and the Q3.29A mutant showed no ligand binding or activation (19, 20). The Q3.29A point mutant of LPA3 showed a profound 40% reduction in $E_{50}$ and an order of magnitude shift to higher $E_{50}$ for LPA 18:1 compared with the wild type LPA3 (Fig. 6C).

Recent computational and mutational analysis of the SIP1 receptor identified Arg-5.38 as a residue critical for ligand binding and activation (21). In the LPA-specific members of the EDG family, residue 5.38 is not conserved, since it is aspartic acid in LPA1, lysine in LPA2, and arginine in LPA3. Interestingly, LPA 18:1 docked into the LPA3 model showed favorable interactions between Arg-5.38 and ligand phosphate headgroup, whereas Ser-5.37 points away from the phosphate and is not likely to form a hydrogen bond (Fig. 6A). To test these hypotheses, R5.38A, R5.38N, and S5.37A point mutants were generated. Unfortunately, the R5.38A mutant did not express in RH7777 cells (data not shown); thus, we were limited to the analysis of R5.38N and S5.37A. R5.38N showed a ~5-fold increase in $E_{50}$ and a modest reduction in $E_{50}$ in response to LPA 18:1 (Fig. 6D). The S5.37A construct showed an identical dose response to that of the wild type LPA3 (Fig. 6D). Thus, Arg-5.38 appears to play an important role in ligand recognition.

Previous mutagenesis studies of the SIP1 and SIP3 receptors confirmed the importance of a positively charged residue within transmembrane helix 7 in SIP1 but not SIP3. This arginine forms a salt bridge with the SIP7 phosphate group in SIP1 but not in SIP3. Unique to LPA3, there are two adjacent positively charged residues, Lys-7.35 and Arg-7.36, either of which could form ion-pairing interactions with the ligand. The computational model of LPA3 assigned this salt bridge to Lys-7.35 rather than the misoriented Arg-7.36 (Fig. 6A). Alanine replacement mutagenesis of these two residues confirmed this prediction, since the ligand interaction properties of R7.36A were indistinguishable from those of the wild type LPA3, whereas K7.35A showed a drastically increased $E_{50}$ of ~8000 nM (Fig. 6E). Thus, the present data reinforce the essential role of three charged and one polar residue in ligand headgroup recognition by LPA3.

The sn-2 LPA regioisomers have been proposed to be more potent ligands of LPA3 as compared with the sn-1 analogs. If proven correct, this pharmacology would distinguish LPA3 from the other two subtypes (14). Due to use of a low pH of 6.2 to slow acyl migration of the sn-2 LPA analogs in the previous study and the unknown effect of pH on ligand recognition by LPA receptors, we synthesized analogs with 18-carbon hydrocarbon chains with various degrees of saturation and with a short chain acetyl group esterified to the free hydroxyl for analysis at pH 7.4. Acetylated analogs of LPA have long been known to mimic the biological effects of LPA (8, 32). Cells transiently expressing LPA3 showed no difference in the apparent $E_{50}$ for PA 18:0/2:0 and PA 2:0/18:0, although the former was less efficacious than LPA 18:0 (Fig. 7A). Introduction of a double bond in the $D_9$-position caused a slight separation between the $E_{50}$ values of the two regioisomers, with PA 2:0/18:1 being slightly more potent than PA 18:1/2:0. Both isomers were equally efficacious compared with LPA 18:1 (Fig. 7B). When two double bonds were introduced at positions $D_9$ and $D_{12}$, the $E_{50}$ values of both regioisomers increased into the high micromolar range (Fig. 7C). This is in stark contrast to LPA 18:2, which had an $E_{50}$ of ~20 nM and was the most potent LPA analog tested at this receptor (Fig. 2A). Nonetheless, in cells stably expressing LPA3, no differences were found between the potency and efficacy of these compounds (data not shown).

The same regioisomers were tested in RH7777 cells transiently expressing LPA3. No differences in the apparent $E_{50}$ of PA 18:0/2:0 and PA 2:0/18:0 were detected. Both were full agonists although with lower potency than LPA 18:0 (Fig. 7D). Similar findings were observed for the two regioisomers containing an 18:1 hydrocarbon chain (Fig. 7E). The 18:2 regioisomers were significantly less potent than LPA 18:2 with $E_{50}$ values in the hundred nanomolar range (Fig. 7F). Nevertheless, PA 18:2/2:0 was ~3 times more potent than PA 2:0/18:2 ($E_{50}$ values were 98 ± 19 and 330 ± 32 nM, respectively).

In the case of LPA3, the regioisomers showed essentially identical $E_{50}$ values as compared with the corresponding LPA species carrying 18:0 or 18:1 hydrocarbon chains (Fig. 7, G and H). Unlike in LPA1 and LPA2, the two 18:2 regioisomers showed no difference in $E_{50}$ or $E_{50}$ (Fig. 7I). PA 18:2/2:0 was equally potent as LPA 18:2 or PA 2:0/18:2, with $E_{50}$ values of 77 ± 18, 50 ± 11, and 75 ± 28 nM, respectively. The lack of difference between these three analogs indicates that the acetyl group did not influence the recognition by LPA3. Altogether, these experiments revealed that LPA1 and LPA2, but not LPA3, recognize differences in chain substitution at the sn-1- and sn-2-positions, and these differences are strongly influenced by the number and position of double bonds.

Our original hypothesis, based on the report by Bandoh et al. (14), proposed a preference for the sn-2 regioisomer of LPA at LPA3. However, in the mammalian expression system and using migration-resistant analogs at physiological pH, we were unable to validate such a preference. Therefore, we had to modify our hypothesis. We focused on LPA3, because in our hands it showed no preferential recognition of the LPA regioisomers (Fig. 7F). Our goal was to identify the residue(s) capable of influencing sn-1 versus sn-2 selectivity. The computational model
of the LPA$_3$-PA 2:0/18:1 complex indicates that the methyl group of the acetyl moiety in the $sn$-1-position makes favorable van der Waals contact with residues Leu-2.60 and Leu-7.39, as shown in Fig. 8. We reasoned that mutation of leucine 2.60 and 7.39 to residues with smaller side chains would diminish the van der Waals interactions. As a result, PA 2:0/18:1 should be less potent at both LPA$_3$ mutants. On the other hand, the PA 18:1/2:0 complex predicts favorable van der Waals interactions with only the Leu-7.39 residue, suggesting that only the L7.39A mutant should show less potent activation by both PA 18:1/2:0 and PA 2:0/18:1 (Fig. 8). The lack of interaction between PA 18:1/2:0 and Leu-2.60 suggests that selectivity would be introduced in the L2.60A mutant that would not be present in the wild type LPA$_3$ receptor. To examine these hypotheses, we tested the alanine replacement mutants of these two residues. The EC$_{50}$ value for PA 2:0/18:1 of the L2.60A mutant was reduced 6-fold to 315 ± 110 nM compared with 55 ± 24 nM of the wild type LPA$_3$. In contrast, the EC$_{50}$ for PA 18:1/2:0 of the L2.60A mutant was not altered relative to that of the wild type, 100 ± 64 and 62 ± 49 nM, respectively. These results confirm the importance of Leu-2.60 to selectively interact with the $sn$-2 acetyl moiety.

In further support of the model, in the case of the L7.39A mutant, both the EC$_{50}$ and the efficacy of PA 18:1/2:0 and PA 2:0/18:1 were significantly elevated to >1 μM ($E_{\text{max}} = 55\%$) and 868 ± 544 nM ($E_{\text{max}} = 68\%$), respectively (TABLE TWO). Alanine replacement at Leu-7.39 diminished receptor activation by both PA 18:1/2:0 and PA 2:0/18:2. These results are in complete agreement with the prediction of the LPA$_3$ computational model and suggest that Leu-2.60 is one of the key residues involved in the selective interactions with LPA regioisomers.

**DISCUSSION**

Differences in ligand preferences between the three EDG family LPA receptors are important for at least two reasons. First, these differences could invoke selectivity in the physiological responses elicited by different LPA species generated in biological fluids. Second, such differences could be explored in developing receptor-selective ligands for therapeu-
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Our results are in agreement with those of Im et al. (16) obtained using a GTPγS assay in HEK293T cells. However, HEK293T cells are endogenously responsive to LPA (15), which confounds the interpretation of the structure-activity relationship derived from expression studies with this cell line. Thus, our data go beyond this report, since for the first time here we showed that without the heterologous co-expression of Gaα1cC351F in the endogenously LPA-nonresponsive RH7777 cells, LPA3 displayed no specificity for unsaturated fatty acyl species.

When expressed in RH7777 cells, all three LPA receptors showed preferential activation with unsaturated LPA, AGP, or cPA analogs relative to saturated analogs with the same hydrocarbon chain length. Furthermore, we found that differences between the potency, measured by the EC50 values of the ligands, varied between the cells transiently or stably transfected with the same receptor construct. We can only speculate that high level overexpression may lead to a higher degree of receptor cooperativity, which in turn affects the potency of a given ligand. This speculation suggests that greater consistency of the modeling results with the assays in stably transfected cells is due to consideration of only a single receptor molecule in all of the modeling studies. Hetero- and homodimerization has been reported for a number of GPCRs (35), including the S1P receptors (36). However, at the present time, homo- or heterotopic receptor cooperativity between LPA receptors remains unexplored.

Alternatively, based on the spare receptor hypothesis, higher level expression tends to shift EC50 values left of the Kd, which appears to be the case in our study (37). Since many groups, including ours, have used cells stably or transiently transfected with LPA receptors to evaluate the pharmacological properties of novel synthetic agonists and antagonists, the present findings caution that potency values derived from such experiments may not be representative for cells that endogenously express one or multiple LPA receptor subtypes at much lower levels.

**FIGURE 8.** Model of the LPA3 complex docked with PA 2:0/18:1 and PA 18:1/2:0. Close-up top down (A) and side (B) views of critical interactions between LPA3 residues (Leu-2.60 and Leu-7.39) and the acetyl moiety of acetylated LPAs is shown.

### TABLE TWO

Properties of LPA3 and its mutants designed to alter regioisomeric selectivity

| Acyl group | EC<sub>50</sub> | EC<sub>100</sub> | Sn-1 Wild type | Sn-2 L2.60A | Sn-2 1.2.60A | Sn-2 1.7.39A | Sn-2 2.7.39A |
|------------|---------------|---------------|----------------|-------------|-------------|-------------|-------------|
| 18:1       | 62 ± 49, 85   | 55 ± 24, 100  | 100 ± 64, 93   | 315 ± 107, 115 | >1 μM, 55% at 10 μM | 868 ± 544, 68 |
| 18:2       | 114 ± 45, 90  | 142 ± 29, 96  | 197 ± 80, 96   | 601 ± 173, 92  | No activation | No activation |

Divalent cations have been known to form complexes with high level overexpression in insect or mammalian cells and concerns about the potential influence of pH on ligand selectivity. Differences in assay conditions for the insect (14) versus the mammalian cells (see Im et al. (16) and the present study) are likely to affect ligand selectivity. Although we used 1.8 mM Ca<sup>2+</sup> at a neutral pH 7.4 and LPA was complexed with a 1:1 molar ratio of albumin, the insect cell assay utilized 10 mM Ca<sup>2+</sup>, 17 mM Mg<sup>2+</sup> at pH 6.2 in the presence of 0.1% bovine serum albumin. Divalent cations have been known to form complexes with LPA and quench its activity (33); thus, the 27 mM combined Ca<sup>2+</sup> and Mg<sup>2+</sup> might have a confounding effect on the ionization state of the ligand, which in turn could affect its biological activity. Furthermore, the pH in the insect assay is below the pK<sub>a</sub> of the phosphate moiety and is likely to affect the salt bridge formation between the two arginines and the lysine moiety we have identified as critical to ligand activation of LPA3 (Fig. 6). Last, Hama et al. (34) has shown that high albumin concentrations inhibit the activation of LPA3 but not LPA1 and LPA2; thus, the 0.1% albumin in the insect assay medium might have had an impact on the results.

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note is the important novel finding that LPA 20:0 failed to activate any of the three LPA receptors up to 300 nM, regardless of whether they were expressed transiently or stably (Fig. 2, open circles). Thus, this LPA analog could be applied as an "inactive control" for the EDG family receptors. This notion is well supported by computational modeling that identified LPA 20:0 as the analog that is least effective in making the critical interactions identified by site-directed mutagenesis (Fig. 6).

Alkyl-ether forms of LPA, particularly AGP 16:0, have long been known to be analogs with very high potency in activating platelet responses (8, 38, 39). AGP is unique in that its potency far exceeds that of LPA in activating peroxisome proliferator-activated receptor γ (PPARγ) (40). The reason why AGP and unsaturated acyl LPA species are the best activators of PPARγ in reporter gene assays remains unclear and may also reflect the differential delivery of these LPA analogs to PPARγ versus cPA and saturated LPA species, which are inactive. Hence, we evaluated three AGP analogs for their ligand properties at the three LPA receptors expressed transiently or stably (Fig. 3). Whereas AGP 18:1, 18:0, and 16:0 were less potent ligands at each receptor than LPA 18:1, surprisingly the AGP 16:0 analog was equipotent or even more potent than AGP 18:1. This is a feature that distinguishes AGP from LPA, since LPA 16:0 is always considerably less potent than LPA 18:1. Therefore, the carbonyl group (LPA) is the favored variant of the pharmacophore compared with the methylene moiety (AGP). Our data recapitulate the rank order of potency found among AGP analogs in platelets, but they do not duplicate the higher potency of the AGP analogs over LPA, reinforcing the notion that platelets express unique LPA receptor(s). Consistent with the very low activity of AGP 18:0 observed in cells stably expressing AGP1, and LPAα, the models indicate that the phosphate group of this analog fails to form the conserved ion pairs with cationic residues in the TM3, TM5, and TM7 seen with LPA. On the other hand, AGP 18:1 showed the highest potency for PPARγ activation, yet it was less potent than AGP 16:0 for LPA GPCR. This observation lends further support to a distinct SAR for LPA GPCR and PPARγ.

The biological properties of cPA are distinct from that of LPA. Whereas LPA has been linked to tumor progression and metastasis (41), cPA inhibits these responses (10). Furthermore, cPA inhibits LPA-induced Ca2+ mobilization in platelets (42). We reported earlier that cPA elicits partial desensitization of the LPA response in fibroblasts and oocytes, suggesting that the two lipids worked on partially overlapping receptor types (31). The present data show that cPA 18:1 and 18:0 activate all three LPA receptors expressed transiently or stably, whereas cPA 16:0 fails to activate transiently expressed LPAα (Fig. 4). The LPAα receptor also stands out in that cPAs are not full agonists of this receptor (Fig. 4, C and F). In contrast, at LPAα, the maximal response to cPA 18:1 surpasses that of LPA 18:1, although this was most prominent in the stably transfected cells (Fig. 2F). Altogether, the present results reinforce the hypothesis that cPA 18:1 is an agonist for all three EDG family receptors and that activation of these receptors cannot explain the potent anti-tumor and anti-metastatic effects of cPA analogs (10, 43). The modeling studies suggest that the reduced potency of cPA species relative to LPA counterparts is due to the reduced negative charge on the cyclic phosphodiester relative to the phosphomonoester. This change in electrostatic properties reduces affinity for the receptors, resulting in a rightward shift of the activation dose-response curves. A change in phosphate group charge can be induced by lowered pH as well as by cyclization and may have influenced the SAR previously reported (14).

We set rigorous criteria for validating the LPAα model, which predicted headgroup interactions with four residues in TM3, TM5, and TM7, of which only the residues in TM3 are rigorously conserved throughout the LPA and S1P EDG family. Another challenge we faced with the model was the presence of two adjacent positively charged residues, Lys-7.35 and Arg-7.36, a feature unique to LPAα. Only Arg-7.36 corresponds to the cationic lysine residues present in LPA1 and LPA2. The model predicted that Lys-7.35 and Arg-7.36 were both near the lipid headgroup headgroup, 3.7 and 5.7 Å away, respectively. Nonetheless, the model placed Lys-7.35 in a more favorable geometry with respect to the phosphate moiety, which suggests a difference between LPAα and the other two LPA receptors, both of which have an acidic residue at this position. The experimental findings are in complete agreement with these predictions. Thus, the two strictly conserved residues in the LPA-specific members of the EDG receptor family, Arg-3.28 and Glu-3.29, appear to play an identical role in ligand recognition to the corresponding residues in S1Pα, S1Pβ, LPAα, and LPAα. Additionally, the R538N mutation exhibited a dose-response curve shifted to higher concentrations relative to that of the wild type receptor. Reduced potency is consistent with the ability of asparagine to hydrogen-bond with the phosphate but not form the stronger ion pair possible for lysine or arginine. This residue is basic in all S1P and LPA receptors in the EDG family except LPAα, in which it is acidic. A comparison of S1Pα and S1Pβ mutagenesis studies has already shown a variable role for charged residues in TM7. The current findings extend this variability from the S1P to LPA receptor subfamily, since only LPAα has a basic residue at position 7.35.

Our previous success with site-specific mutants derived from computational models of lysophospholipid receptors led us to seek an answer for the proposed sn-2 regioisomeric preference of LPA. In order to examine regioisomeric selectivity, we elected to synthesize and apply acetylated analogs to prevent rapid acyl migration that occurs within minutes in aqueous solvents at neutral pH (13). We are obligated to note that this structural change from a free hydroxyl group to an acetyl group not only eliminates acyl migration but also converts a bifunctional hydrogen bond donor and acceptor into a multifunctional hydrogen bond acceptor. However, such acyl/acytel regiosomers gave us the necessary confidence that at the time of the assay, the ligand was in the correct regioisomeric configuration without the need to lower pH to slow migration. The choice of the acyl/acytel LPA analogs was based on earlier reports that established that these analogs possessed comparable ligand properties in cells endogenously expressing various LPA receptor subtypes (8, 32) and was reinforced by our experiments performed in cells with either transient (Fig. 7) or stable expression of the individual LPA receptors (data not shown). Unexpectedly, in cells with transient or stable expression (data not shown) of LPAα, we could not find such regioisomeric preference using sn-1/sn-2 regioisomeric pairs of acetyl LPA with 18:0, 18:1, and 18:2 fatty acids. In contrast, we found that the LPAα and LPAβ subtypes showed regioisomeric selectivity, but only when the compounds contained an 18:2 fatty acyl moiety. Furthermore, the regioisomeric selectivity for LPAα was only present in cells with transient expression (Fig. 7C) and was absent in stable cells (not shown). This unexpected finding has forced us to modify our hypothesis and ask what residues have the ability to confer a regioisomeric preference on LPAα. In PA 18:1/2:0 over PA 2:0/18:1. The model of the LPAα PA 2:0/18:1 complex predicted that the methyl group of the acetyl moiety at the sn-1-position makes good van der Waals contact with residues Leu-7.30 and Leu-7.39 (Fig. 8). In contrast, the acetyl group at the sn-2-position interacts with Leu-7.39 but not Leu-2.60. Thus, we hypothesized that regioisomeric preference would be strongest in the L2.60A mutant. Replacement of these two residues with alanine in LPAα introduced regioisomeric selectivity, endowing the Leu-2.60 mutant with a 3-fold preference for PA 18:1/2:0 over PA 18:2/2:0 compared with the
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wild type receptor (TABLE TWO). Examination of the models suggests that a free hydroxyl group at the sn-1-position in sn-2 LPA might be able to donate a hydrogen bond to the backbone carboxyl group of Arg-3.28, an interaction not possible for the acetyl group. No potential hydrogen bonds are noted for the free hydroxyl group that would occur at the sn-2-position of sn-1 LPA. This property difference between an acetyl and a hydroxyl group may also contribute to the differences in SAR between this and previous reports. Although the model was successfully able to identify mutations in LPA1 that confer selectivity for acetylated sn-1 LPA analogs, these amino acid positions do not explain the observed sn-2 selectivity with 18:2 acyl groups at the LPA1 and LPA2 receptors. Analysis of the PA 18:2/2:0 and PA 2:0/18:2 complexes in LPA1 suggests several contributions to the preference that include Asp-333, Leu-5.41, and Ala-5.45. The complex interactions asserted by these residues will have to be evaluated in a future study. The only other report in the literature that investigated regioisomeric selectivity of LPA receptors is by Heise et al. (25). However, these investigators used N-oleyl ethanolamide phosphoric acid and not LPA in their analysis, which observed sn-2 selectivity with 18:2 acyl groups at the LPA1 and LPA2 receptors. Although a direct comparison of their data with the logs were more potent agonists than the 2-substituted ones at all three LPA1 receptors. These investigators noted that the 1-substituted analogs used LPA in their analysis, which observed sn-2 LPA might be able to donate a hydrogen bond to the backbone carbonyl group of Arg-3.28, an interaction not possible for the acetyl group. No potential hydrogen bonds are noted for the free hydroxyl group that would occur at the sn-2-position of sn-1 LPA. This property difference between an acetyl and a hydroxyl group may also contribute to the differences in SAR between this and previous reports. Although the model was successfully able to identify mutations in LPA1 that confer selectivity for acetylated sn-1 LPA analogs, these amino acid positions do not explain the observed sn-2 selectivity with 18:2 acyl groups at the LPA1 and LPA2 receptors. Analysis of the PA 18:2/2:0 and PA 2:0/18:2 complexes in LPA1 suggests several contributions to the preference that include Asp-333, Leu-5.41, and Ala-5.45. The complex interactions asserted by these residues will have to be evaluated in a future study. The only other report in the literature that investigated regioisomeric selectivity of LPA receptors is by Heise et al. (25). However, these investigators used N-oleyl ethanolamide phosphoric acid and not LPA in their analysis, which limits the comparison with our study that utilized migrationally stabilized LPA analogs. These investigators noted that the 1-substituted analogs were more potent agonists than the 2-substituted ones at all three LPA receptors. Although a direct comparison of their data with the present data is not feasible, nonetheless their results on the regioisomeric preference for 1-substituted N-oleyl ethanolamide phosphoric acid analogs at LPA1 and LPA2 receptors support our assessment that regioisomeric selectivity of the LPA receptors is influenced by multiple factors, which include the degree of unsaturation, the length and type of the side chain, and the backbone itself.

In summary, the present results underscore the difficulties in establishing authentic ligand preferences for lysophospholipid mediators from heterologous expression systems, insect or mammalian, transient or stable. In our opinion, more emphasis should be given to cells with endogenous expression of a single LPA receptor subtype as well as to computationally guided mutagenesis studies that might identify the residues determining ligand recognition. Last, we propose that regioisomeric ligand selectivity originally assigned only to LPA1 be reconsidered and redefined as a property that depends on the receptor subtype, the fatty acyl composition of the ligand, and potentially the structure.

REFERENCES

1. Ishii, I., Fukushima, N., Ye, X., and Chun, J. (2004) Annu. Rev. Biochem. 73, 321–354
2. Moolenaar, W. H., van Meeteren, L. A., and Giepmans, B. N. (2004) BioEssays 26, 870 – 881
3. Tigyi, G., and Parrill, A. L. (2003) Prog. Lipid Res. 42, 498–526
4. Bandoh, K., Aoki, J., Hosono, H., Kobayashi, S., Kobayashi, T., Murakami-Murofushi, K., Tsujimoto, M., Arai, H., and Inoue, K. (1999) J. Biol. Chem. 274, 27776–27785
5. Ishii, I., Kontos, J. J., Fukushima, N., and Chun, J. (2000) Mol. Pharmacol. 58, 895–902
6. Baker, D. L., Desiderio, D. M., Miller, D. D., Tolley, B., and Tigyi, G. J. (2001) Anal. Biochem. 292, 287–295
7. Baker, D. L., Morrison, P., Miller, B., Riely, C. A., Tolley, B., Westermann, A. M., Bonfrer, J. M., Bais, E., Moolenaar, W. H., and Tigyi, G. (2002) J. Am. Med. Assoc. 287, 3081–3082
8. Tokumura, A., Sinomiya, J., Kishimoto, S., Tanaka, T., Kogure, K., Sugita, T., Satouchi, K., Waku, K., and Fukushima, K. (2002) Biochem. J. 365, 617–628
9. Liliom, K., Fischer, D. J., Virag, T., Sun, G., Miller, D. D., Tseng, I. L., Desiderio, D. M., Seidel, M. C., Erickson, J. R., and Tigyi, G. (1998) J. Biol. Chem. 273, 13461–13468
10. Murakami-Murofushi, K., Uchiyama, A., Fujiwara, Y., Kobayashi, T., Kobayashi, S., Mukai, M., Murofushi, H., and Tigyi, G. (2002) Biochim. Biophys. Acta 1582, 1–7
11. Sano, T., Baker, D., Virag, T., Wada, A., Yatomi, Y., Kobayashi, T., Igarashi, Y., and Tigyi, G. (2002) J. Biol. Chem. 277, 21197–21206
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